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Why Does Enamel in *Klk4*-Null Mice Break above the Dentino-Enamel Junction?

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Key Words
Amelogenesis · Proteases · Teeth · Ameloblasts · Scanning electron microscopy · *lacZ* histostaining

Abstract
Background: The enamel layer of kallikrein 4 (Klk4)-null mice has a normal thickness and a decussating pattern of enamel rods, but it contains residual enamel proteins, is less highly mineralized, and fractures in its deepest part just above the dentino-enamel junction (DEJ). The plane of fracture is puzzling because the deepest enamel is deposited earliest and, through the action of the secretory stage enamel protease (Mmp20), is the most mature part of the enamel layer at the time of the onset of *Klk4* expression. ***Objectives:*** To characterize the planes of fracture in *Mmp20*- and *Klk4*-null mice and to localize *Klk4* expression in developing teeth. ***Methods:*** *Klk4*- and *Mmp20*-null mice were sacrificed at 7 weeks and their mandibular incisors were characterized by scanning electron microscopy. $Klk4^{+/lacZ}$ mice were mated with $Klk4^{+/lacZ}$ mice. Offspring were genotyped by polymerase chain reaction. $Klk4^{+/+}$, $Klk4^{+/lacZ}$, and $Klk4^{lacZ/lacZ}$ (null) littermates on postnatal days 5, 8, 11, and 14 were processed for β-galactosidase histochemistry. ***Results:*** The enamel layer fractures at the DEJ in *Mmp20*-null mice, and fractures occur in enamel above the DEJ in *Klk4*-null mice. Klk4 is not expressed by secretory-stage ameloblasts, murine odontoblasts beneath the secretory stage, or maturation-stage ameloblasts. Klk4 is specifically expressed by transition and maturation-stage ameloblasts. ***Conclusions:*** The breakage of enamel near the DEJ in *Klk4*-null mice is not due to a failure of odontoblasts to express Klk4, but it relates to a progressive hypomineralization of enamel with depth.

Introduction

Matrix metalloproteinase 20 (Mmp20) and kallikrein 4 (Klk4) are secreted proteases that degrade enamel proteins [Bartlett and Simmer, 1999]. Mmp20 generates the enamel protein cleavage products that accumulate in secretory-stage enamel [Ryu et al., 1999; Iwata et al., 2007; Nagano et al., 2009; Chun et al., 2010]. Klk4 degrades enamel proteins during the transition and maturation

Abbreviations used in this paper

DEJ	dentino-enamel junction
Klk4	kallikrein 4
Mmp20	matrix metalloproteinase 20
SEM	scanning electron microscopy

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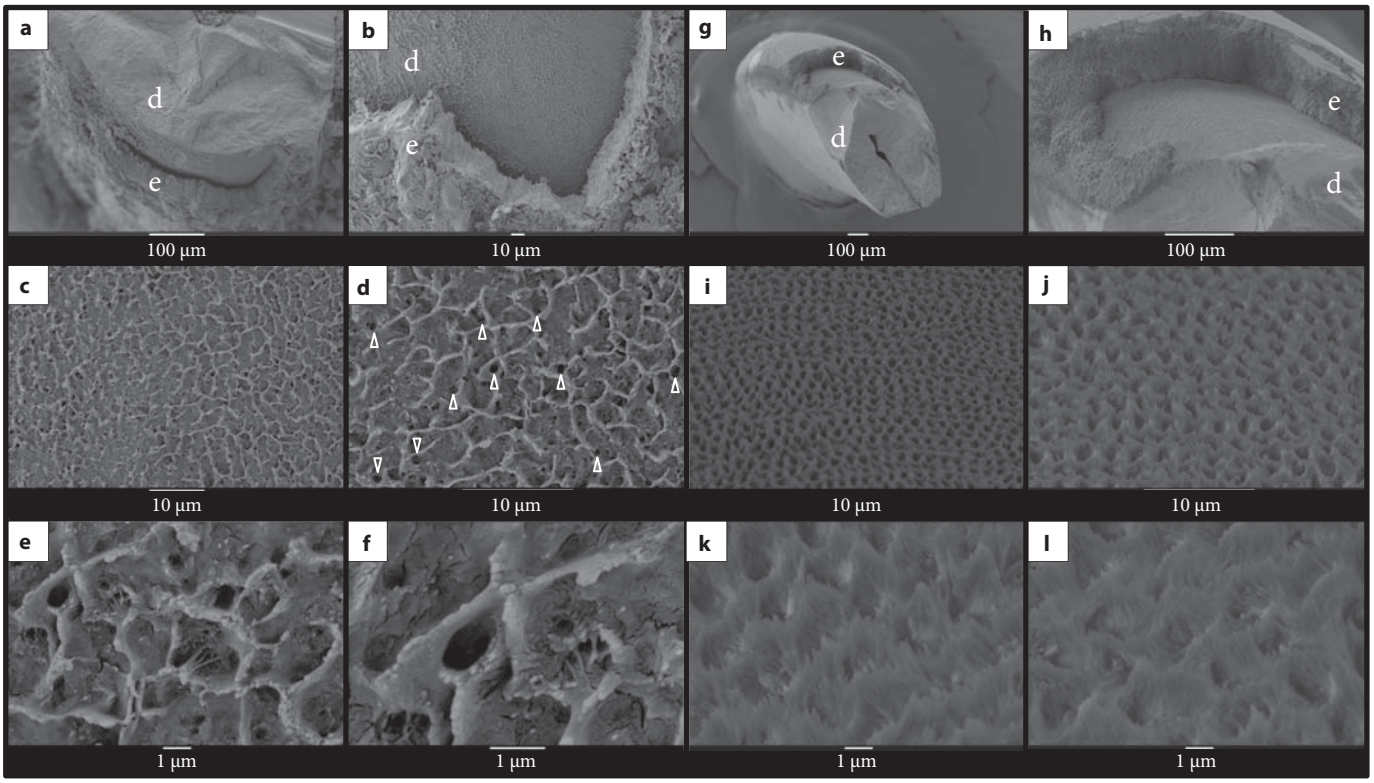

Fig. 1. SEM of mandibular incisors broken at the level of the alveolar bone crest. The Mmp20-null (**a–f**) and Klk4-null (**g–l**) incisors both make enamel that tends to fracture at or near the DEJ. **a–h** At a low magnification it is shown that the enamel nearest to the split dentin surface has entirely or nearly entirely broken away. **c–l** Broken surfaces at a higher magnification. The level of the split is clearly different in the 2 mice. Mmp20-null incisors break at the DEJ. The floor of the cleaved surface is clearly dentin, with an occasional dentinal tubule exposed (arrowheads). Klk4-null incisors break in the deepest layer of enamel just above the DEJ, with rows of circular vacancies left by the lost enamel rods. Jagged edges along the crests of the interrod enamel are the outlines of needle-like enamel crystals. d = Dentin; e = enamel.

stages of amelogenesis [Ryu et al., 2002]. Mutations in *MMP20* [Kim et al., 2005; Ozdemir et al., 2005; Papagerakis et al., 2008; Lee et al., 2010] and *KLK4* [Hart et al., 2004] cause autosomal recessive amelogenesis imperfecta (MIM ID No. 612529 and No. 204700). Only the enamel layer is defective in *Mmp20*- [Caterina et al., 2002] and *Klk4*- [Simmer et al., 2009] null mice and in persons with mutations in these genes, suggesting both enzymes are specialized for dental enamel formation. This conclusion is supported by the finding that whales that have lost the ability to make dental enamel during evolution lack a functional copy of *Mmp20* [Meredith et al., 2010].

When Mmp20 or Klk4 are absent during tooth development, the enamel chips off following tooth eruption. Our objectives in this study were (1) to better characterize the level that shears and (2) to determine if Klk4 is normally expressed by mouse odontoblasts.

Materials and Methods

Scanning Electron Microscopy

Mandibular incisors (3 each) from 7-week-old *Mmp20*- and *Klk4*-null mice were fractured at the level of the labial alveolar crest and mounted on metallic stubs using conductive carbon cement, degassed in a vacuum desiccator overnight, and sputter-coated with an Au-Pd film. The samples were imaged at the University of Michigan Microscopy and Image Analysis Laboratory (Ann Arbor, Mich., USA) using an Amray EF 1910 scanning electron microscope (SEM) operating at an accelerating voltage of 5 kV.

Mouse Breeding

$Klk4^{+/lacZ}$ mice were mated with $Klk4^{+/lacZ}$ mice. Offspring were genotyped by polymerase chain reaction [Simmer et al., 2009]. $Klk4^{+/+}$, $Klk4^{+/lacZ}$, and $Klk4^{lacZ/lacZ}$ (null) littermates on postnatal days 5, 8, 11, and 14 were processed for β-galactosidase histochemistry.

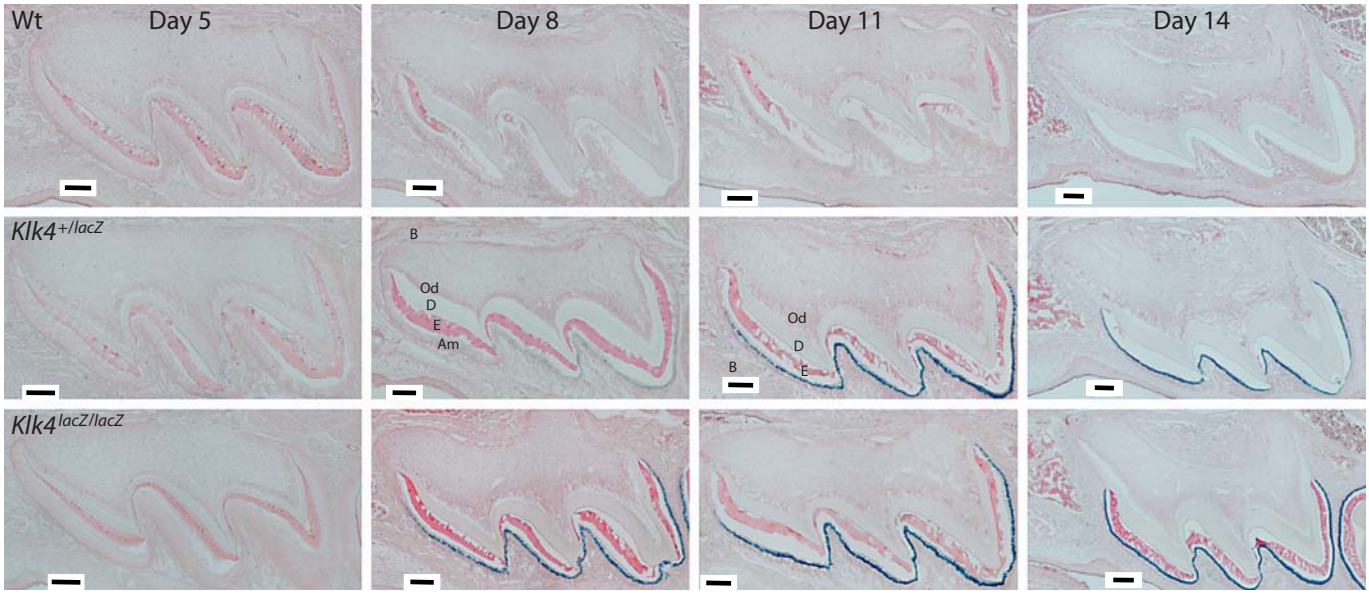

Fig. 2. *LacZ* histochemistry showing nuclear localized β-galactosidase activity where Klk4 is normally expressed. At the 5-hour incubation used, no endogenous (lysosomal) β-gal activity was observed and the wild-type (Wt) mice were negative. In mouse molars, a positive signal was only observed in transition and maturation ameloblasts. No expression was observed in odontoblasts. B = Bone; Od = odontoblasts; D = dentin; E = enamel; Am = ameloblasts. Scale bars = 100 μm.

Tissue Processing for Histochemistry

Mouse heads were quickly dissected of skin, cut in half, and fixed by immersion in 4% paraformaldehyde (pH 7.3) in 1× PBS overnight at 4°C; they were then washed in 1× PBS 4–5 times (every 0.5–1 h) at 4°C. The tissues were decalcified by immersion in 1 liter of 4.13% EDTA (pH 7.3) with agitation, changed every other day at 4°C (day 5: 8–9 days of decalcification, day 8: 19–21 days of decalcification, day 11: 21 days of decalcification, and day 14: 30 days of decalcification), and then washed in 1× PBS 4–5 times (every 0.5–1 h) with 1 overnight wash at 4°C. The tissues were immersed in 15% sucrose (1–2 h) followed by 30% sucrose (3–4 h) at 4°C for cryoprotection and then embedded in optimal cutting temperature media and stored at –80°C. The blocks were cryosectioned at 8 μm at –20 to –22°C on a Leica cryostat. Slides were stored at –80°C until staining.

X-Gal Staining

Slides were removed from –80°C, immediately treated with glutaraldehyde fixative (0.1 M HEPES, 1.25 mM EGTA, 2 mM $MgCl_2$, and 0.5% glutaraldehyde, pH 7.3), and then washed with 0.1 M HEPES and 2 mM $MgCl_2$ (pH 7.3) 3 times for 5 min. The slides were stained with X-gal solution [0.1 M HEPES, 1 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2% Triton X-100, and 1 mg/ml X-gal substrate (Roche), pH 8.0] for 5 h or overnight at 45°C and then washed in 1× PBS several times and counterstained with 0.1% (w/v) Nuclear Fast Red, coverslipped with Aquamount, and imaged using a Nikon Eclipse TE300 inverted microscope.

Results and Discussion

We fractured mandibular incisors from *Mmp20*- and *Klk4*-null mice at the level of the alveolar crest to remove the erupted portion of the tooth that had been altered by masticatory forces and to create a freshly fractured surface in the most mature portion of the incisor that was about to erupt. The incisors were notched on the concave (dentin) side with a rotating disc and the 2 ends of the incisor were bent outward, causing the dentin to fracture roughly in a plane perpendicular to the tangent of the tooth surface for the full thickness of the dentin. The fracture spread along the dentino-enamel junction (DEJ) or near it for a short distance and then broke through the full thickness of the enamel on a path perpendicular to the tangent of the tooth surface. SEMs were used to make a visual interpretation of where the tooth surface fractured (fig. 1).

At low magnification, it appeared that the enamel and dentin of the *Mmp20*- (fig. 1a, b) and *Klk4-null* (fig. 1g, h) mice had delaminated at the level of the DEJ, but closer examination of the *Mmp20* (fig. 1c–f) and *Klk4* (fig. 1i–l) fractured incisor surfaces showed important and revealing differences.

The fractured surface of the *Mmp20*-null mouse incisor displayed thin, plate-like mineral projecting upwards,

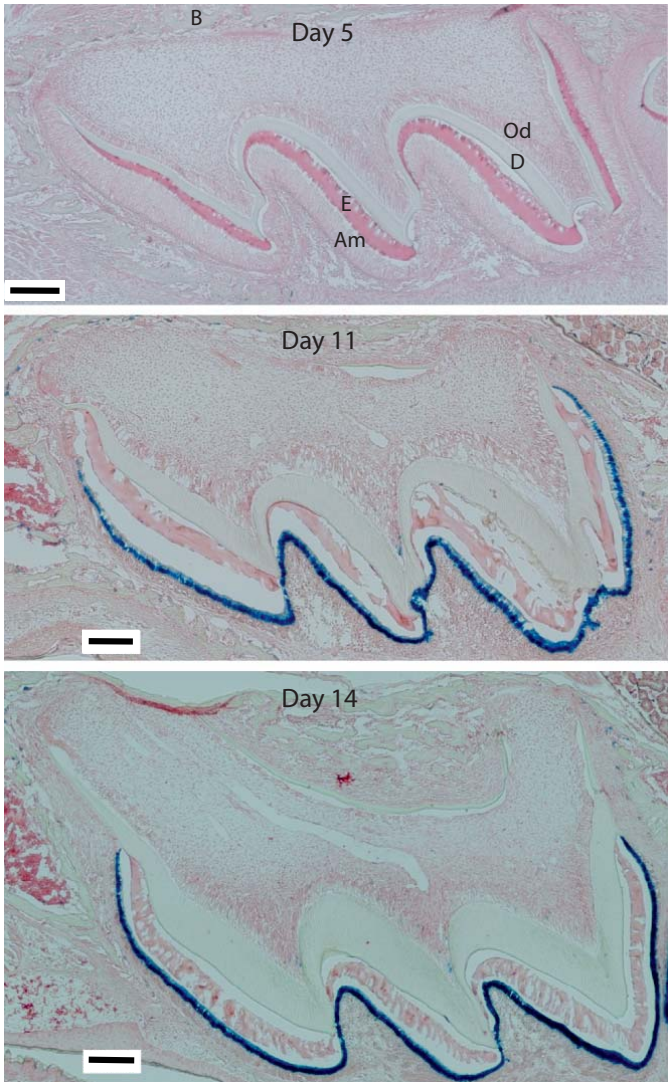

Fig. 3. *LacZ* histochemistry of Klk4-null mice molars after overnight incubation with the X-gal substrate. This experiment shows the limits of sensitivity of the β-galactosidase knockin strategy for Klk4. The staining in maturation ameloblasts is very strong and extends beyond the nuclei. A few sporadic nonameloblasts show staining probably due to detection of endogenous β-galactosidase activity. At no stage of crown formation are odontoblasts positive for Klk4 expression. B = Bone; Od = odontoblasts; D = dentin; E = enamel; Am = ameloblasts. Scale bars = 100 μm.

perpendicular to the surface that resembled the ruined walls of an archeological site (fig. 1c–f). The individual rooms or cells made by these projections were not aligned in rows and seemed to be relatively uniform in size. The floor was occasionally perforated by holes less than 1 μm in diameter which were likely the ends of dentinal tubules. The overall impression was similar to SEMs of the villous surface of mantle dentin just prior to the onset of enamel deposition [Fejerskov and Thylstrup, 1986].

The fractured surface in the *Klk4*-null mouse incisor showed a field of interrod enamel perforated by linear arrays of holes ~1 μm in diameter that were vacancies left by loss of enamel rods that pulled out and fractured away with the rest of the enamel layer. The position of the fracture was the same as what we had previously observed in Klk4-null mouse molars at 7 weeks, which had fractured naturally following their eruption into function [Simmer et al., 2009]. The outline of individual, elongated enamel crystals is apparent along the crests of the interrod material projecting upward from the mineral walls separating the rod vacancies.

Separation of enamel from dentin is not surprising in the *Mmp20*-null mouse because Mmp20 is normally expressed and secreted during DEJ formation [Begue-Kirn et al., 1998; Hu et al., 2002], and formation of the DEJ is disturbed in *Mmp20*-null mice [Beniash et al., 2006]. Klk4, however, is not expressed until the enamel layer has reached full thickness, long after the DEJ has formed [Hu et al., 2000a, b; Simmer et al., 2004]. PCR analyses of developing pig molars detected Klk4 mRNA in odontoblasts, suggesting that Klk4 might be delivered to the DEJ by odontoblastic processes and account for the highly mineralized enamel just above the DEJ [Fukae et al., 2002]. Klk4 mRNA in mouse odontoblasts, however, has not been consistently observed by in situ hybridization analyses. In this study we used a Klk4 knockout/NLS-*lacZ* knockin reporter to specifically monitor *Klk4* expression in developing teeth [Simmer et al., 2009].

Bacterial β-galactosidase activity from the knockin gene is favored over lysosomal β-gal by incubating the tissue sections with substrate (X-gal) at pH 8. When overincubation is performed to detect trace expression from the knockin, the mouse nuclear localization signal fused to the bacterial β-galactosidase helps distinguish expression from the knockin gene in the nucleus from endogenous activity in the lysosomes [Dymecki, 1996; Hu et al., 2008].

Developing first molars at days 5, 8, 11, and 14 were analyzed by *lacZ* histochemistry. The X-gal-to-indigo reaction catalyzed by β-galactosidase (the lacZ product) was specifically detected in maturation-stage ameloblasts in the *Klk4*-null mice at days 8, 11, and 14 (fig. 2). No positive staining was detected in wild-type first molars at any time point (negative control), and no staining was detected in day-5 first molars as amelogenesis had not yet advanced to the transition or maturation stages. Positive staining was observed in maturation stage ameloblasts in $Klk4^{+/lacZ}$ and $Klk4^{lacZ/lacZ}$ first molars at days 8, 11, and 14, although

the signal was barely discernable in the *Klk4*$^{+/lacZ}$ mice at day 8. No staining was observed in odontoblasts at any time point. All signals were restricted to the nucleus of positive cells affirming that the indigo staining was specifically the result of NLS-*lacZ* knockin expression and not endogenous (lysosomal) β-galactosidase.

The sensitivity in this study appeared equal to those of our previous in situ hybridization analyses of *Klk4* mRNA in mouse first molars but with better specificity (less background) [Hu et al., 2002]. We show 5-hour incubations without background that limit staining to the ameloblast nuclei. Overnight incubation showed light staining of sporadic odontoblast nuclei (fig. 3) but stained beyond the nuclei in ameloblasts. Odontoblast expression of Klk4 in mouse is trace and unlikely to be physiologically relevant.

We conclude that breakage of enamel near the DEJ in *Klk4*-null mice is not due to a failure of odontoblasts to express Klk4, but it relates to a progressive hypo-mineralization of enamel with depth [Smith et al., 2011].

Acknowledgements

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