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Expression of kallikrein 4 (Klk4) in dental and non-dental tissues

James P. Simmer¹, Amelia S. Richardson¹, Charles E. Smith^{1,2}, Yuanyuan Hu¹, and Jan C-C. Hu¹

¹Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, 1210 Eisenhower Place, Ann Arbor, MI, USA 48108.

²Facility for Electron Microscopy Research, Department of Anatomy & Cell Biology, and Faculty of Dentistry, McGill University, Montreal, QC

Abstract

Kallikrein 4 (Klk4) is critical for proper dental enamel formation. *Klk4* null mice and persons with two defective *KLK4* alleles have obvious enamel defects, with no other apparent phenotype. Klk4 mRNA or protein is reported to be in tissues besides teeth, including prostate, ovary, kidney, liver, and salivary gland. In this study we used the Klk4 knockout/NLS-lacZ knockin mouse to assay *Klk4* expression using β -galactosidase histochemistry. Incubations for 5 h were used to detect Klk4 expression with minimal endogenous background, while overnight incubations susceptible to false positives were used to look for trace Klk4 expression. Developing maxillary molars at postnatal days 5, 6, 7, 8, and 14, developing mandibular incisors at postnatal day 14, and selected non-dental tissues from adult wild-type and *Klk4*^{lacZ/lacZ} mice were examined by X-gal histochemistry. After 5 h incubation, Xgal staining was observed specifically in the nuclei of maturation stage ameloblasts in molars and incisors from *Klk4*^{lacZ/lacZ} mice and was detected weakly in nuclei of salivary gland ducts and patches of prostate epithelia. We conclude that Klk4 is predominantly a tooth-specific protease with low expression in submandibular salivary gland and prostate, and with no detectable expression in liver, kidney, testis, ovary, oviduct, epididymis, and vas deferens.

Keywords

enamel; kallikrein; proteases; *amelogenesis imperfecta*; submandibular salivary gland

Secretory stage ameloblasts export three enamel matrix proteins: amelogenin, ameloblastin, and enamelin (1). These proteins are cleaved extracellularly, and their digested products accumulate in the enamel matrix (2-4). The cleavage sites that generate many secretory stage enamel components have been characterized (5-9). Matrix metalloproteinase 20 (Mmp20) is expressed during the secretory stage (10), and *in vitro* analyses have demonstrated that Mmp20 is uniquely capable of catalyzing all of these cleavages (11-14). This proteolytic activity is necessary for proper enamel formation as *Mmp20* null mice (15) and persons with *MMP20* mutations produce defective enamel (16-19). During the maturation stage, the enamel layer hardens by widening and thickening hydroxyapatite crystals deposited during the secretory stage (20, 21). Kallikrein 4 (Klk4) is a glycosylated,

Corresponding Author: James P. Simmer DDS, PhD Professor, Dept. of Biologic and Materials Sciences, University of Michigan Dental Research Lab 1210 Eisenhower Pl Ann Arbor, MI 48108 Tel: 734-975-9318 Fax: 734-975-9329 jsimmer@umich.edu .

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Supporting information Additional Supporting Information may be found in the online version of this article:

Fig. S1: Overnight lacZ histostaining of wild-type and *Klk4*^{lacZ/lacZ} null mouse tissues.

Fig. S2: Lack of Klk4 expression in liver, kidney, testis, ovary, and oviduct with overnight incubation.

chymotrypsin-like serine protease that is expressed and secreted by maturation stage ameloblasts (22-25). Klk4 degrades enamel proteins (26), which facilitates their reabsorption by maturation ameloblasts (27). In the absence of Klk4, accumulated enamel proteins are retained in the matrix and the crystals don't fully mature (28). *Klk4* null mice (28) and persons with *KLK4* mutations (29) show enamel defects with no noticeable abnormalities elsewhere in the body. These findings suggest that *Mmp20* and Klk4 both serve tooth-specific functions (30). In the case of *Mmp20*, this specificity is supported by data showing that *Mmp20* has degenerated into a pseudogene in whales that have lost the ability to make teeth or enamel (31).

Klk4 is routinely isolated from developing teeth (12, 26), but has not been isolated from any other tissue. Klk4 mRNA and Klk4 antigen have been detected in tissues besides teeth, but the findings are inconsistent. Immunoassays of 38 healthy adult tissues detected KLK4 primarily in prostate, with no expression in liver, colon, or pituitary (32). Enzyme-linked immunosorbent (ELISA) assays of 37 tissues from healthy adults found that KLK4 was not abundant in any adult tissue, but was highest in pituitary, cervix, and muscle (33). Immunohistochemistry of tissue microarrays were positive for KLK4 in healthy adult kidney, liver, and prostate tissue, but negative for colon, lung, skin or skeletal muscle (34). The results of these surveys depended upon the specificity of the Klk4 antibodies employed, and none of the studies compared *KLK4* expression to levels in developing teeth. Human *KLK4* expression was also surveyed by quantitative polymerase chain reaction (PCR), which showed highest expression in prostate and low, but detectable levels in adrenal, salivary and thyroid glandular tissues (35). In prostate, KLK4 has been proposed to be the enzyme that activates prostate specific antigen (PSA) (36, 37), but recent evidence suggests KLK2 activates PSA (38).

We developed a gene-targeted mouse strain that has a *lacZ* reporter gene with a mouse nuclear localization signal (NLS-βgal) inserted at the natural *Klk4* translation initiation site, which is able to assay *Klk4* expression using β-galactosidase histochemistry (28). We have used these *Klk4* knockout/*lacZ* knockin mice to assay for *Klk4* expression in developing teeth, adult prostate, liver, kidneys, submandibular salivary glands, prostate, ovaries, testis, vas deferens and epididymis.

Materials and methods

Breeding and genotyping

Klk4^{lacZ/lacZ} mice were mated with *Klk4^{lacZ/lacZ}* mice. Genotyping was by polymerase chain reaction (PCR) using genomic DNA obtained by tail biopsy (28). To detect wild-type *Klk4*, we used PCR primers that annealed to intron 3 and exon 5 (5'-AACCTAAGGGACAGGGCAGT and 5'-TGAGGTGGTACACAGGGTCA; 550-bp amplicon). To detect the knockin gene (*Klk4^{lacZ}*), we used a PCR primer pair that annealed to the *Klk4* upstream region and to the NLS-*lacZ* coding region (5'-TGCCTCCAACCAGATAGGTC and 5'-GACAGTATCGGCCTCAGGAA; 595-bp amplicon). The wild-type mice were strain C57BL/6.

Tissue processing for histochemistry

Mice (1-yr-old) were anesthetized with isoflurane and fixed by cardiac perfusion. Blood was cleared from the vasculature using lactated ringer's solution (30-45 s) followed by 4% paraformaldehyde in phosphate buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM Na₂H₂PO₄; pH 7.3) for 20 min. Following perfusion, the liver, kidneys, submandibular salivary glands, prostate, ovaries, testis, vas deferens and epididymis were dissected, immersed in paraformaldehyde fixative (4% paraformaldehyde in PBS; pH 7.3)

for 2-3 h at 4 °C, and washed in PBS 4-5 times (every 0.5-1 h) with one 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, embedded in optimal cutting temperature (OCT) 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.

Processing of dental tissues for histochemistry

Day 5, 6, 7, 8, and 14 mouse heads were quickly dissected of skin, cut in half, and immersed in 4% paraformaldehyde fixative overnight at 4 °C, washed in PBS 4 to 5 times (every 0.5-1 h) at 4 °C, and decalcified at 4 °C by immersion in 1 L of 4.13% disodium ethylenediaminetetraacetic acid (EDTA; pH 7.3) with agitation. The EDTA solution was changed every other day for 8 to 9 d for day 5 mice, 19 to 21 d for day 6, 7, and 8 mice, and for 30 d for day 14 mice. The samples were washed in PBS at 4 °C 4 to 5 times (every 0.5-1 h) followed by one overnight wash. 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 OCT and stored at -80 °C. The blocks were cryosectioned at 8 µm thickness 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 and immediately treated with glutaraldehyde fixative (0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.25 mM ethylene glycol tetraacetic acid (EGTA), 2 mM MgCl₂, 0.5% glutaraldehyde, pH 7.3) and then washed with 0.1 M HEPES, 2 mM MgCl₂ (pH 7.3) 3X for 5 min. The slides were stained with X-gal solution (0.1 M HEPES, 1 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2% Triton X-100, 1 mg/mL X-gal substrate; pH 8.0) for 5 h or overnight at 45 °C and then washed several times in PBS, and counterstained with 0.1% (w/v) Nuclear Fast Red, coverslipped with Aquamount, and imaged using a Nikon Eclipse TE300 inverted microscope.

Results

Klk4 expression in developing teeth

LacZ histostaining of wild-type and *Klk4^{lacZ/lacZ}* maxillary first molars was carried out on sections from days 5, 6, 7, 8, and 14 (Fig. 1), which covers the period when secretory ameloblasts first transition into maturation stage ameloblasts (39). No *lacZ* histostaining was observed in any of the wild-type molar sections or in the day 5 *Klk4^{lacZ/lacZ}* maxillary first molars. The earliest positive staining in maxillary first molars was in ameloblasts at the cusp tips (enamel-free zone) and the cusp slopes in day 6 and day 7 *Klk4^{lacZ/lacZ}* mice. By day 8 positive staining had spread along the ameloblast layer nearly to the cervical margin of the developing crown. By day 14, strong positive *lacZ* histostaining was observed throughout the ameloblast layer. No cells besides ameloblasts were positive. Notably there was no positive staining in secretory stage ameloblasts, odontoblasts, bone, or along the developing roots. At all time points, the *Klk4^{lacZ/lacZ}* maxillary first molars showed organic material (counterstained red) within the enamel layer. The wild-type maxillary first molars exhibited counterstained enamel proteins similar to that of the *Klk4^{lacZ/lacZ}* maxillary first molars for days 5 through 7. By day 8 the residual enamel proteins near the cusp tips were reduced and by day 14 had disappeared. The continued presence of enamel proteins in the maturation stage enamel matrix past day 8 is due to the absence of *Klk4* expression. *LacZ* histostaining of the day 14 *Klk4^{lacZ/lacZ}* continuously growing incisor was negative for secretory stage ameloblasts and positive for maturation stage ameloblasts and some aged odontoblasts near the incisal tip of the tooth (Fig. 2).

Klk4 expression in adult salivary gland, prostate, liver, kidney, testis, ovary, ovarian duct, epididymis and vas deferens

LacZ histostaining of day 14 *Klk4^{lacZ/lacZ}* maxillary first molars was carried out in parallel with the histostaining of selected non-dental tissues obtained from 1-yr-old wild-type and *Klk4^{lacZ/lacZ}* mice (Fig 3). Positive *lacZ* nuclear histostaining was observed in the striated ducts of the submandibular salivary gland (Figs. 3C-3D) and in localized areas of prostate epithelia (Figs. 3F-3H). Nuclear staining was stronger in the salivary gland than in the prostate and clearly above background. Localized endogenous *lacZ* staining (background) was evident in prostate epithelia in wild-type mice when the incubation was allowed to run overnight (Supporting Fig. S1). Nuclei in adult liver (Figs. 4A-C), kidney (Figs. 4D-F), testis (Figs. 4G-I), ovary (Figs. 4J,K), and ovarian duct (Figs. 4J,L) were negative. The wild-type and *Klk4*-null mice were also negative for these tissues in the overnight incubation (Supporting Fig. S2). Cytoplasmic *lacZ* histostaining was detected in the epididymis (Fig. 5A) and vas deferens (Fig. 5B), but this clearly arose from endogenous galactosidase activity, as these tissues gave the same level of staining in the same locations as wild-type controls.

Discussion

This is the first survey that compares *Klk4* expression in developing teeth to that of healthy adult tissues. We confirmed the results of previous *in situ* hybridization studies that showed *Klk4* is expressed by transition and maturation stage ameloblasts (22-25). *Klk4* expression by maturation ameloblasts was far stronger than that of any of the soft tissues examined. We focused on healthy adult tissues, as all previous reports of non-dental *Klk4* expression (excluding cancers) were from adults and some organs, such as prostate and ovaries, develop late and are best examined in adults. In the adult organs surveyed, the striated ducts of the submandibular salivary gland and small patches of prostate epithelia were the only sites that showed unambiguous *Klk4* expression. No *Klk4* expression at all could be detected in kidney, testis, ovary, ovarian duct, epididymis and vas deferens. There were no obvious morphological abnormalities in the non-dental tissues examined in the *Klk4* null mice, suggesting that their normal development is not *Klk4* dependent.

Our findings of only trace *Klk4* expression in mouse non-dental tissues may appear to conflict with the results of human studies, but this is not necessarily the case. Human studies cannot include developing teeth as a reference. *KLK4* expression in prostate is highly elevated *relative to other tissues*, which is consistent with our findings—if developing teeth and submandibular salivary glands are not tested. The frequency of occurrence of expressed sequence tags (EST) is one way to assess levels of protein expression in various tissues. The human expressed sequence tag (EST) profile for *KLK4* (Hs.218366), which does not include developing teeth, lists only 24 *KLK4* transcripts out of 189,345 characterized from healthy prostate, and only 6 additional *KLK4* transcripts out of the more than 4 million characterized from all other tissues combined. By comparison, the EST profile for prostate specific antigen (*KLK3*; Hs.171995) lists 1095 transcripts from prostate and 307 from other tissues, and the EST profile for *KLK2* (Hs.515560), the enzyme thought to activate PSA, lists 578 transcript from prostate and 44 from other tissues.

Even though the number of *KLK4* mRNA transcripts in non-dental tissues is very low, polymerase chain reaction methods are able to amplify them and give the impression of positive *KLK4* expression. Normal human ovaries were positive for *KLK4* expression when assayed by RT-PCR, but negative by *in situ* hybridization and immunohistochemistry (40). Prostate was positive for *KLK4* by RT-PCR, but the *KLK4* protein was barely detected in prostate extracts (10^{-4} times the PSA concentration) and in seminal plasma (10^{-6} times the PSA concentration and 10^{-4} times the *KLK2* concentration) (41). Our findings of only trace

Klk4 expression in mouse prostate are consistent with the low occurrence of *KLK4* transcripts in the human EST database and the trace levels of KLK4 protein detected in prostate extracts and seminal plasma.

The low expression of Klk4 in mouse submandibular ducts is potentially interesting. The short-tailed shrew is the only poisonous mammal in North America. Its toxin (blarinasin; BLTX) is a glycosylated kallikrein-like serine protease (most closely related to KLK1) that is secreted into the saliva by the submandibular salivary gland (42, 43). A role for Klk4 in innate immunity, either directly or by processing salivary proteins like histatins, is easy to imagine. However, *lacZ* histostaining shows only low Klk4 expression and no *KLK4* ESTs were found among the 20,155 sequenced from salivary glands.

Klk4 is able to activate protease activated receptors (PARs), particularly PAR₁ and PAR₂ (44, 45), which are G-protein coupled receptors. Cleavage within the extracellular amino-terminus exposes a tethered ligand domain, which binds to and activates the receptor to initiate multiple signaling cascades. Despite this irreversible mechanism of activation, signaling by PARs is efficiently terminated by receptor desensitization (receptor phosphorylation and uncoupling from G proteins) and downregulation (receptor degradation by cell-surface and lysosomal proteases) (46). While PAR-mediated signaling may not be a part of the normal Klk4 physiological activity, aberrantly expressed KLK4 is able to signal via aberrantly expressed PAR₁ in colon tumorigenesis (47). The expression of protease activated receptors (PAR₁ through PAR₄) in developing teeth has never been explored.

In this study we found that mouse *Klk4* expression is vastly higher in maturation stage ameloblasts than any of the adult tissues examined. As the number of *KLK4* transcripts listed in the human and mouse EST databases is very low, all current evidence supports the conclusion that Klk4 expression is predominantly enamel-specific. As enamel malformations are the only phenotype detected in persons with both *KLK4* alleles mutated and in *Klk4* null mice, current evidence also supports the conclusion Klk4 functions as a tooth-specific protease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *Klk4* expression in day 5-14 maxillary first molars

Wild-type (WT) sections from post-natal days, 5, 6, 7, 8, and 14 are shown in the left column. Comparable sections from *Klk4* null mice are shown on the right column. No lacZ histostaining was observed in the wild-type sections demonstrating an absence of background staining in these sections. *Klk4* (lacZ) expression was first observed at the cusp tips of day 6 and day 7 maxillary first molars. By day 14 *Klk4* expression extended throughout the ameloblast layer. Note the red counterstaining of enamel proteins diminished in day 8 and was gone in day 14 wild-type enamel, but persisted in the *Klk4* null mice through day 14. **Key:** Am, ameloblasts; E, enamel; D, dentin; Od, odontoblasts; P, pulp. Bars = 100 μ m.

Figure 2. Klk4 expression in day 14 mandibular first and second molars and continuously erupting incisor

A: Day 14 mandibular longitudinal section through the incisor and three molars of a $Klk4^{lacZ/lacZ}$ null mouse. Arrowheads indicate positions of the higher magnification views shown below. **A:** *LacZ* histostaining was observed throughout the ameloblast layer in the first and second molar and in the incisor starting approximately at the level of the mesial surface of the first molar. **B-D:** Positive staining for Klk4 expression in maturation stage ameloblasts. **E-F:** Absence of Klk4 expression in secretory stage ameloblasts. Scale bars: A, 200 μm ; B-F, 50 μm .

Figure 3. Klk4 expression in submandibular salivary gland and prostate
A-B: Wild-type submandibular salivary gland shows no endogenous (background) staining for *lacZ* in 5 h incubations. **C-D:** Intralobular (striated) duct cells show positive nuclei for Klk4 expression in the $Klk4^{lacZ/lacZ}$ null mouse. **E:** Day 14 maxillary molars histostained along with non-dental tissues as a positive control. **F-H:** Prostate epithelia showed small patches of weakly positive nuclei indicative of Klk4 expression. Scale bars: A/C/E/G, 200 μ m; B/D/F, 50 μ m; H, 50 μ m.

Figure 4. Lack of Klk4 expression (no nuclear staining) in liver, kidney, testis, ovary, and oviduct

Wild-type sections for these tissues were negative (data not shown). All panels are from *Klk4^{lacZ/lacZ}* null mice. **A-C:** liver; **D-F:** kidney; **G-I:** testis; **J-K:** ovary; **L:** oviduct. Bars on left: 200 μ m; middle: 100 μ m; right: 50 μ m.

Figure 5. Lack of Klk4 expression (no nuclear staining) in epididymis and vas deferens
Positive *lacZ* staining was observed in the epididymis and vas deferens but in cytoplasm rather than nuclei and at similar locations in *Klk4^{lacZ/lacZ}* null and wild-type mice. **A:** Strong endogenous *lacZ* staining was observed in epithelial tissues in the head of the epididymis (left) whereas the tail of the epididymis (right) was more weakly stained. Bars: top row = 200 μ m; second row = 50 μ m. **B:** Weak endogenous *lacZ* staining in epithelium of the vas deferens. Bars: left pair = 100 μ m; right pair = 50 μ m.