Expression of kallikrein-related peptidase 4 in dental and non-dental tissues


Kallikrein-related peptidase 4 (KLK4) is critical for proper dental enamel formation. Klk4 null mice, and humans with two defective KLK4 alleles have obvious enamel defects, with no other apparent phenotype. KLK4 mRNA or protein is reported to be present 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 mice were examined by X-gal histochemistry. After 5 h of incubation, X-gal staining was observed specifically in the nuclei of maturation-stage ameloblasts in molars and incisors from Klk4−/−lacZ mice and was detected weakly in the nuclei of salivary gland ducts and in 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.

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 humans 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-related peptidase 4 is a glycosylated, chymotrypsin-like serine protease that is expressed and secreted by murine maturation-stage ameloblasts (22–25). Kallikrein-related peptidase 4 degrades enamel proteins (26), which facilitates their reabsorption by maturation-stage ameloblasts (27). In the absence of KLK4, accumulated enamel proteins are retained in the matrix and the crystals do not fully mature (28). Klk4 null mice (28) and humans 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).

Kallikrein-related peptidase 4 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. Immunohistological analyses of 37 tissues from healthy adults found that KLK4 was not abundant in any adult tissue, but was highest in liver, colon or pituitary (31). Immunohistochemistry of tissue microarrays were positive for KLK4 in healthy adult kidney, liver, and prostate tissue, but negative for KLK4 in 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 with the levels in developing teeth. Human KLK4 expression was also surveyed by quantitative PCR, which detected the 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 that 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 can be used to assay Klk4 expression using β-galactosidase histochemistry (28). In this study, we used these Klk4 knockout/lacZ knockin mice to investigate the expression of Klk4 in developing teeth, adult prostate, liver, kidneys, submandibular salivary glands, prostate, ovaries, testis, vas deferens, and epididymis.

**Material and methods**

**Breeding and genotyping**

Klk4lacZ/lacZ mice were mated with Klk4lacZ/lacZ mice. Genotyping was by 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'-AACCTAAGGGACAGGCGAT and 5'-TGAGGTGGTACACAGGGTCA; 550-bp amplicon). To detect the knockin gene (Klk4lacZ), we used a PCR primer pair that annealed to the Klk4 upstream region and to the NLS-lacZ coding region (5'-TGCCCTCAAACCAGATAGGTTC and 5'-GACAGTATCGGCCTCAG GAA; 595-bp amplicon). The wild-type mice were strain C57BL/6.

**Tissue processing for histochemistry**

Mice (1 yr of age) were anaesthetized 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 PBS (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na 2HPO4, 1.4 mM Na 2H2PO4, 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

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**Fig. 1.** Kallikrein-related peptidase 4 (KLK4) expression in day 5–14 maxillary first molars. Sections from postnatal day 5, 6, 7, 8, and 14 wild-type (WT) mice are shown in the left column. Comparable sections from Klk4-null mice are shown in the right column. No lacZ histostaining was observed in the sections from wild-type mice, 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 that the red counterstaining of enamel proteins was diminished in day 8 and absent in enamel of day-14 wild-type mice, but persisted in the Klk4 null mice through day 14. Am, ameloblasts; D, dentin; E, enamel; Od, odontoblasts; P, pulp. Bars = 100 μm.
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) medium, and stored at −80°C. The blocks were cryosectioned at 8 μm at −20 to −22°C on a Leica cryostat. The 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 4–5 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 8–9 d for day-5 mice, 19–21 d for days 6, 7, and 8 mice, and 30 d for day-14 mice. The samples were washed in PBS at 4°C 4–5 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, Buffalo Grove, IL, USA. The slides were stored at −80°C until required for staining.

**X-gal staining**

The slides were removed from the −80°C freezer 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, three times, for 5 min each wash, with 0.1 M HEPES containing 2 mM MgCl₂ (pH 7.3). 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⁻¹ of X-gal substrate; pH 8.0) for 5 h or overnight at 45°C, washed several times in PBS, 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**

β-galactosidase histostaining of wild-type and *Klk4lacZ/lacZ* maxillary first molars was carried out on sections from days 5, 6, 7, 8, and 14 (Fig. 1), which covers the period

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**Fig. 2.** Kallikrein-related peptidase 4 (KLK4) expression in day-14 mandibular first and second molars and continuously erupting incisor. (A) Mandibular longitudinal section through the incisor and three molars of a day-14 *Klk4lacZ/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.
when secretory ameloblasts first transition into maturation-stage ameloblasts (39). No β-galactosidase histo-
staining was observed in any of the wild-type molar sections or in the maxillary first molars of day-5 Klk4lacZ/lacZ mice. 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 Klk4lacZ/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 β-galactosidase histostaining was observed throughout the ameloblast layer. No staining was observed in cells besides amelo-
bysts. Notably there was no staining in secretory-stage ameloblasts, odontoblasts, bone, or along the developing roots. At all time-points, the Klk4lacZ/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 Klk4lacZ/lacZ maxillary first molars for days 5 to 7. By day 8 the residual enamel proteins near the cusp tips were reduced and by day 14 had disappeared. The presence of enamel proteins in the maturation-stage enamel matrix past day 8 is caused by the absence of KLK4 expression. β-galactosidase histostaining of the continuously growing incisor of day-14 Klk4lacZ/lacZ mouse 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**

β-galactosidase histostaining of the maxillary first molars of day-14 Klk4lacZ/lacZ mice was carried out in parallel with the histostaining of selected non-dental tissues obtained from 1-yr-old wild-type and Klk4lacZ/lacZ mice (Fig 3). Positive β-galactosidase nuclear histostaining was observed in the striated ducts of the submandibular salivary gland (Fig. 3C,D) and in localized areas of prostate epithelia (Fig. 3F–H). Nuclear staining was stronger in the salivary gland than in the prostate and clearly above background levels. Localized endogenous β-galactosidase staining (background) was evident in prostate epithelia in wild-type mice when the incubation was allowed to run overnight (Fig. S1). The nuclei in adult liver (Fig. 4A–C), kidney (Fig. 4D–F), testis (Fig. 4G–I), ovary (Fig. 4J,K), and ovarian duct (Fig. 4J,L) were stain-negative. The wild-type and Klk4-null mice were also negative for these tissues in the overnight incubation (Fig. S2). Cytoplasmic β-galactosidase histo-
staining was detected in the epididymis (Fig. 5A) and vas

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**Fig. 3.** Kallikrein-related peptidase 4 (KLK4) expression in submandibular salivary gland and prostate. (A, B) Submandibular salivary gland from wild-type mouse shows no endogenous (background) staining for β-galactosidase after a 5-h incubation. (C, D) Intralobular (striated) duct cells show β-galactosidase positive nuclei in the Klk4lacZ/lacZ null mouse. (E) Day-14 maxillary molars were 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 μm; B, D, F, 50 μm; H, 50 μm.
deferens (Fig. 5B), but this clearly arose from endogenous galactosidase activity as the same level of staining was found in those tissues in wild-type controls.

Discussion

This is the first survey to compare KLK4 expression in developing teeth with that in healthy adult tissues. We confirmed the results of previous in situ hybridization studies which showed that KLK4 is expressed by transition-stage and maturation-stage ameloblasts (22–25). The expression of KLK4 by maturation-stage ameloblasts was far stronger than that in 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 adult mice, 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 or 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 expression of KLK4 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 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 six additional KLK4 transcripts out of the more than 4 million characterized from all other tissues combined. By comparison, the EST profile for PSA (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 transcripts from prostate and 44 from other tissues.

Even though the number of KLK4 mRNA transcripts in non-dental tissues is very low, PCR 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 detectable in prostate extracts (being present at a concentration 10^4 times lower than PSA) and in seminal plasma (present at a concentration 10^6 times lower than PSA and 10^4 times lower than KLK2) (41). Our findings of only trace expression of KLK4 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 level of 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 KLK4) 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 such as histatins, is easy to imagine. However, lacZ histostaining shows only a low level of expression of KLK4, 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 signalling cascades. Despite this irreversible mechanism of activation, signalling 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 signalling may not be a part of the normal physiological activity of KLK4, aberrantly expressed KLK4 is able to signal via aberrantly expressed PAR1 in colon tumorigenesis (47). The expression of PARs (e.g. PAR1 to 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 in any of the adult tissues examined. As the number of Klk4/ 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 that KLK4 functions as a tooth-specific protease.

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Conflicts of interest – No author declared any competing interests.
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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 Klk4lacZ/lacZ null mouse tissues.

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

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