European Journal of
Oral Sciences

Eur J Oral Sci 2011; 119 (Suppl. 1): 226–233
DOI: 10.1111/j.1600-0722.2011.00834.x
Printed in Singapore. All rights reserved

© 2011 Eur J Oral Sci
European Journal of
Oral Sciences

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

James P. Simmer[1], Amelia S. Richardson[1], Charles E. Smith[1,2], Yuanyuan Hu[1], Jan C-C. Hu[1]

[1]Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, MI, USA; [2]Facility for Electron Microscopy Research, Department of Anatomy & Cell Biology, and Faculty of Dentistry, McGill University, Montreal, QC, Canada

Simmer JP, Richardson AS, Smith CE, Hu Y, Hu JC-C. Expression of kallikrein-related peptidase 4 in dental and non-dental tissues.
Eur J Oral Sci 2011; 119 (Suppl. 1): 226–233. © 2011 Eur J Oral Sci

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*$^{lacZ/lacZ}$ 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*$^{lacZ/lacZ}$ 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.

James P. Simmer, Department of Biologic and Materials Sciences, University of Michigan Dental Research Laboratory, 1210 Eisenhower Place, Ann Arbor, MI 48108, USA

Telefax: +1–734–9759329
E-mail: jsimmer@umich.edu

Key words: enamel; kallikrein; proteases; amelogenesis imperfecta; submandibular salivary gland

Accepted for publication May 2011

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. Immunoassays of 38 healthy adult tissues detected KLK4 primarily in prostate, with no expression in liver, colon or pituitary (32). 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 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 *lac*Z 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

Klk4$^{lacZ/lacZ}$ mice were mated with *Klk4*$^{lacZ/lacZ}$ 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′-AACCTAAGGGACAGGGCAGT and 5′-TGAGGTGG-TACACAGGGTCA; 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′-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_2HPO_4, 1.4 mM $Na_2H_2PO_4$, 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

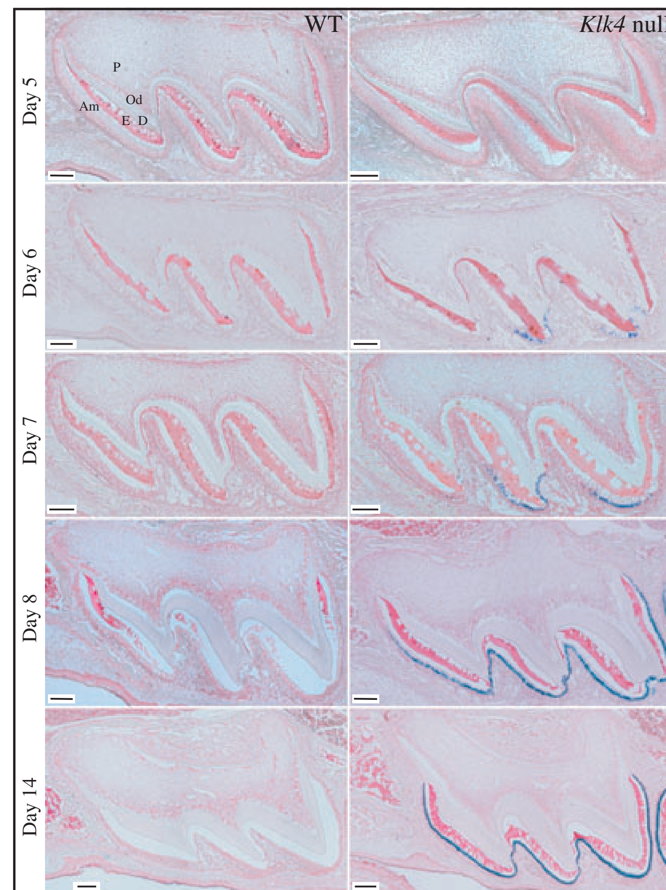

Fig. 1. Kallikrein-related peptidase 4 (KLK4) expression in day 5–14 maxillary first molars. Sections from postnatal day 5, 6, 7, 8, and 14 wild-type (WT) mice are shown in the left column. Comparable sections from *Klk4*-null mice are shown in the right column. No lacZ histostaining was observed in 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 $MgCl_2$, 0.5% glutaraldehyde, pH 7.3] and then washed, three times, for 5 min each wash, with 0.1 M HEPES containing 2 mM $MgCl_2$ (pH 7.3). 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, 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 $Klk4^{lacZ/lacZ}$ maxillary first molars was carried out on sections from days 5, 6, 7, 8, and 14 (Fig. 1), which covers the period

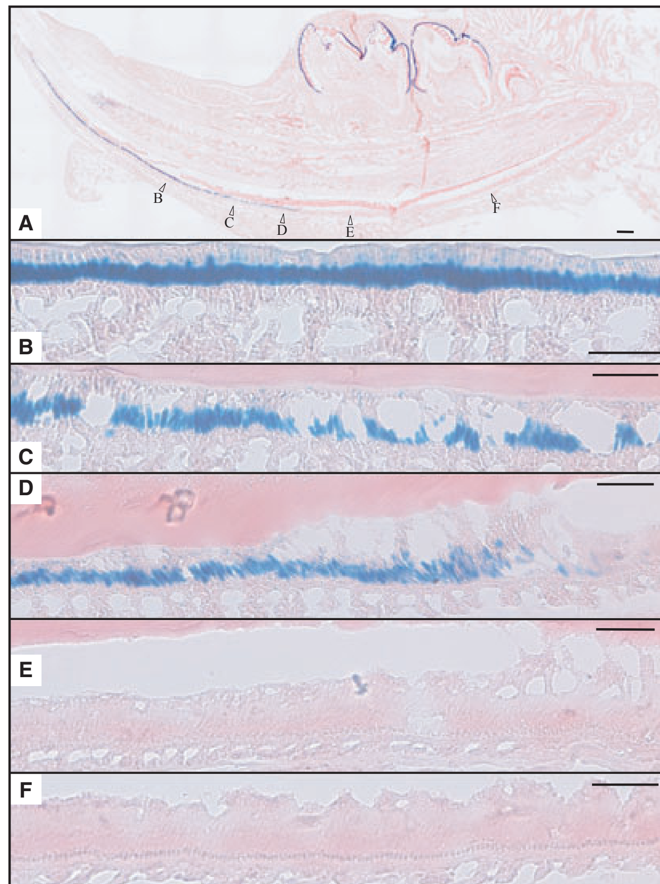

Fig. 2. Kallikrein-related peptidase 4 (KLK4) expression in day-14 mandibular first and second molars and continuously erupting incisor. (A) Mandibular longitudinal section through the incisor and three molars of a day-14 $Klk4^{lacZ/lacZ}$ null mouse. Arrowheads indicate positions of the higher-magnification 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.

when secretory ameloblasts first transition into maturation-stage ameloblasts (39). No β-galactosidase histostaining was observed in any of the wild-type molar sections or in the maxillary first molars of day-5 *Klk4*$^{lacZ/lacZ}$ 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 *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 β-galactosidase histostaining was observed throughout the ameloblast layer. No staining was observed in cells besides ameloblasts. Notably there was no 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 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 *Klk4*$^{lacZ/lacZ}$ 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 *Klk4*$^{lacZ/lacZ}$ mice 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 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. 4 J,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 histostaining was detected in the epididymis (Fig. 5A) and vas

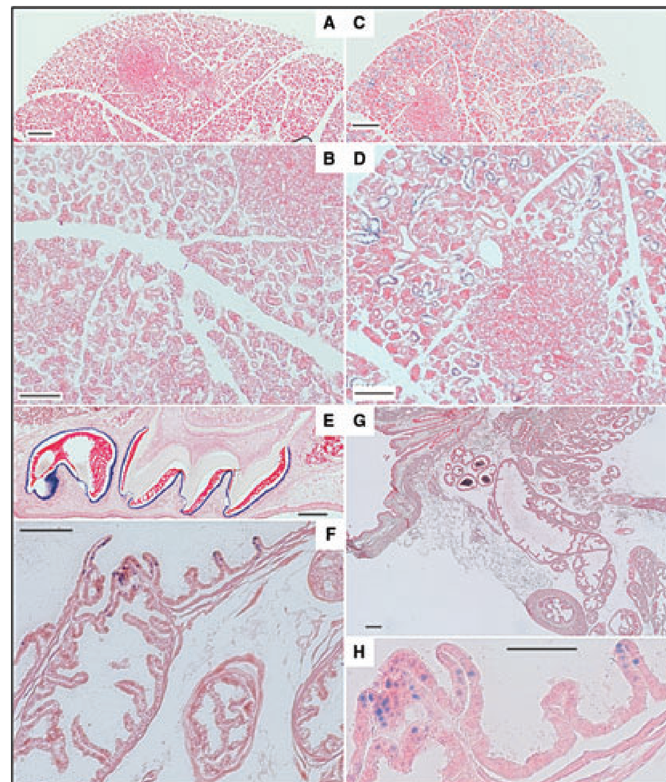

Fig. 3. Kallikrein-related peptidase 4 (KLK4) expression in submandibular salivary gland and prostate. (A, B) Submandibular salivary gland from wild-type mouse shows no endogenous (background) staining for β-galactosidase after a 5-h incubation. (C, D) Intralobular (striated) duct cells show β-galactosidase positive nuclei in the *Klk4*$^{lacZ/lacZ}$ 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.

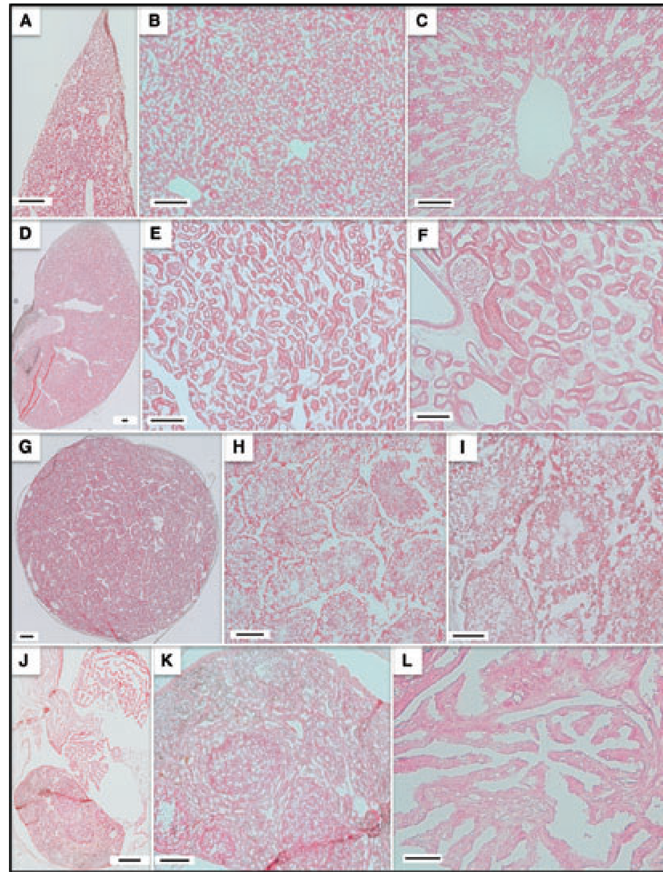

Fig. 4. Lack of kallikrein-related peptidase 4 (KLK4) expression (no nuclear staining) in liver, kidney, testis, ovary, and oviduct. Sections of these tissues from wild-type mice were stain-negative (data not shown). All sections shown are from $Klk4^{lacZ/lacZ}$ null mice: (A–C) liver; (D–F) kidney; (G–I) testis; (J, K) ovary; and (L) oviduct. Bars on the left = 200 μm. Bars in the middle = 100 μm. Bars on the right = 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

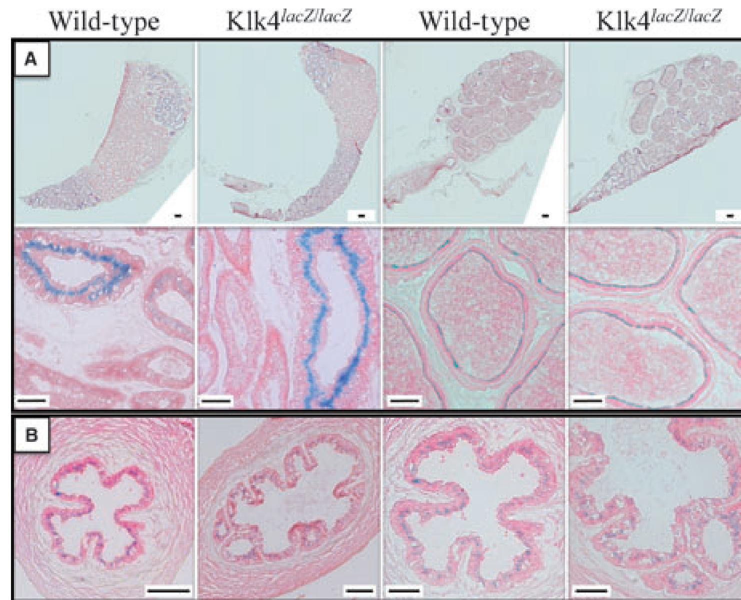

Fig. 5. Lack of kallikrein-related peptidase 4 (KLK4) expression (no nuclear staining) in epididymis and vas deferens. Positive staining for β-galactosidase activity was observed in the epididymis and vas deferens but in the cytoplasm rather than in the nuclei and at similar locations in $Klk4^{lacZ/lacZ}$ null and wild-type mice. (A) Strong endogenous staining for β-galactosidase activity 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 μm; second row = 50 μm. (B) Weak endogenous staining for β-galactosidase activity in the epithelium of the vas deferens. Bars: left pair = 100 μm; right pair = 50 μm.

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 PAR_1 and PAR_2 (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 PAR_1 in colon tumorigenesis (47). The expression of PARs (e.g. PAR_1 to PAR_4) 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.

Acknowledgements – This investigation was supported by USPHS Research Grant DE019775 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 29892, USA.

Conflicts of interest – No author declared any competing interests.

References

1. Fincham AG, Moradian-Oldak J, Simmer JP. The structural biology of the developing dental enamel matrix. *J Struct Biol* 1999; **126**: 270–299.
2. Smith CE, Pompura JR, Borenstein S, Fazel A, Nanci A. Degradation and loss of matrix proteins from developing enamel. *Anat Rec* 1989; **224**: 292–316.
3. Uchida T, Tanabe T, Fukae M, Shimizu M, Yamada M, Miake K, Kobayashi S. Immunochemical and immunohistochemical studies, using antisera against porcine 25 kDa amelogenin, 89 kDa enamelin and the 13-17 kDa nonamelogenins, on immature enamel of the pig and rat. *Histochemistry* 1991; **96**: 129–138.
4. Fukae M, Tanabe T, Uchida T, Yamakoshi Y, Shimizu M. Enamelins in the newly formed bovine enamel. *Calcif Tissue Int* 1993; **53**: 257–261.
5. Fincham AG, Belcourt AB, Termine JD, Butler WT, Cothran WC. Dental enamel matrix: sequences of two amelogenin polypeptides. *Biosci Rep* 1981; **1**: 771–778.
6. Fincham AG, Moradian-Oldak J. Amelogenin post-translational modifications: carboxy-terminal processing and the phosphorylation of bovine and porcine "TRAP" and "LRAP" amelogenins. *Biochem Biophys Res Commun* 1993; **197**: 248–255.
7. Yamakoshi Y, Tanabe T, Fukae M, Shimizu M. Porcine amelogenins. *Calcif Tissue Int* 1994; **54**: 69–75.
8. Fincham AG, Moradian-Oldak J. Comparative mass spectrometric analyses of enamel matrix proteins from five species suggest a common pathway of post-secretory proteolytic processing. *Connect Tissue Res* 1996; **35**: 151–156.
9. Yamakoshi Y, Hu JC-C, Ryu OH, Tanabe T, Oida S, Fukae M, Simmer JP. A comprehensive strategy for purifying pig enamel proteins. In: Kobayashi I, Ozawa H, eds. *Biomineralization: formation, diversity, evolution and application Proceedings of the 8th International Symposium on Biomineralization*, Niigata, Jpn, Sept 25–28, 2001. Hadano, Japan: Tokai University Press, 2003; 326–332.
10. Begue-Kirn C, Krebsbach PH, Bartlett JD, Butler WT. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *Eur J Oral Sci* 1998; **106**: 963–970.
11. Ryu OH, Fincham AG, Hu CC, Zhang C, Qian Q, Bartlett JD, Simmer JP. Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. *J Dent Res* 1999; **78**: 743–750.
12. Nagano T, Kakegawa A, Yamakoshi Y, Tsuchiya S, Hu JC, Gomi K, Arai T, Bartlett JD, Simmer JP. Mmp-20 and Klk4 cleavage site preferences for amelogenin sequences. *J Dent Res* 2009; **88**: 823–828.
13. Iwata T, Yamakoshi Y, Hu JC, Ishikawa I, Bartlett JD, Krebsbach PH, Simmer JP. Processing of ameloblastin by MMP-20. *J Dent Res* 2007; **86**: 153–157.
14. Chun YH, Yamakoshi Y, Yamakoshi F, Fukae M, Hu JC, Bartlett JD, Simmer JP. Cleavage Site Specificity of MMP-20 for Secretory-stage Ameloblastin. *J Dent Res* 2010; **89**: 785–790.
15. Caterina JJ, Skobe Z, Shi J, Ding Y, Simmer JP, Birkedal-Hansen H, Bartlett JD. Enamelysin (matrix metalloproteinase 20)-deficient mice display an amelogenesis imperfecta phenotype. *J Biol Chem* 2002; **277**: 49598–49604.
16. Kim JW, Simmer JP, Hart TC, Hart PS, Ramaswami MD, Bartlett JD, Hu JC. MMP-20 mutation in autosomal recessive pigmented hypomaturation amelogenesis imperfecta. *J Med Genet* 2005; **42**: 271–275.
17. Papagerakis P, Lin HK, Lee KY, Hu Y, Simmer JP, Bartlett JD, Hu JC. Premature stop codon in MMP20 causing amelogenesis imperfecta. *J Dent Res* 2008; **87**: 56–59.
18. Ozdemir D, Hart PS, Ryu OH, Choi SJ, Ozdemir-Karatas M, Firatli E, Piesco N, Hart TC. MMP20 active-site mutation in hypomaturation amelogenesis imperfecta. *J Dent Res* 2005; **84**: 1031–1035.
19. Lee SK, Seymen F, Kang HY, Lee KE, Gencay K, Tuna B, Kim JW. MMP20 hemopexin domain mutation in amelogenesis imperfecta. *J Dent Res* 2010; **89**: 46–50.
20. Smith CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med* 1998; **9**: 128–161.
21. Simmer JP, Papagerakis P, Smith CE, Fisher DC, Rountrey AN, Zheng L, Hu JC. Regulation of dental enamel shape and hardness. *J Dent Res* 2010; **89**: 1024–1038.
22. Hu JC, Ryu OH, Chen JJ, Uchida T, Wakida K, Murakami C, Jiang H, Qian Q, Zhang C, Ottmers V, Bartlett JD, Simmer JP. Localization of EMSP1 expression during tooth formation and cloning of mouse cDNA. *J Dent Res* 2000; **79**: 70–76.
23. Hu JC, Zhang C, Sun X, Yang Y, Cao X, Ryu O, Simmer JP. Characterization of the mouse and human PRSS17 genes, their relationship to other serine proteases, and the expression of PRSS17 in developing mouse incisors. *Gene* 2000; **251**: 1–8.
24. Hu JC, Sun X, Zhang C, Liu S, Bartlett JD, Simmer JP. Enamelysin and kallikrein-4 mRNA expression in developing mouse molars. *Eur J Oral Sci* 2002; **110**: 307–315.
25. Simmer JP, Sun X, Yamada Y, Zhang CH, Bartlett JD, Hu JC-C. Enamelysin and kallikrein-4 expression in the mouse incisor. In: Kobayashi I, Ozawa H, eds. *Biomineralization: formation, diversity, evolution and application Proceedings of the 8th International Symposium on Biomineralization, Niigata, Jpn, Sept 25-28, 2001*. Hadano, Japan: Tokai University Press, 2004; 348–352.
26. Ryu O, Hu JC, Yamakoshi Y, Villemain JL, Cao X, Zhang C, Bartlett JD, Simmer JP. Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts. *Eur J Oral Sci* 2002; **110**: 358–365.
27. Smith CE, Richardson AS, Hu Y, Bartlett JD, Hu JC-C, Simmer JP. Effects of loss of function of kallikrein 4 on mineralization of enamel: comparison to mice lacking matrix metalloproteinase 20. *J Biol Chem* 2011; **286**(20): 18149–18160.
28. Simmer JP, Hu Y, Lertlam R, Yamakoshi Y, Hu JC. Hypomaturation enamel defects in Klk4 knockout/LacZ knockin mice. *J Biol Chem* 2009; **284**: 19110–19121.
29. Hart PS, Hart TC, Michalec MD, Ryu OH, Simmons D, Hong S, Wright JT. Mutation in kallikrein 4 causes autosomal recessive hypomaturation amelogenesis imperfecta. *J Med Genet* 2004; **41**: 545–549.
30. Lu Y, Papagerakis P, Yamakoshi Y, Hu JC, Bartlett JD, Simmer JP. Functions of KLK4 and MMP-20 in dental enamel formation. *Biol Chem* 2008; **389**: 695–700.
31. Meredith RW, Gatesy J, Cheng J, Springer MS. Pseudogenization of the tooth gene enamelysin (MMP20) in the common ancestor of extant baleen whales. *Proc Biol Sci* 2011; **278**(1708): 993–1002.
32. Obiezu CV, Shan SJ, Soosaipillai A, Luo LY, Grass L, Sotiropoulou G, Petraki CD, Papanastasiou PA, Levesque MA, Diamandis EP. Human kallikrein 4: quantitative study in tissues and evidence for Its secretion into biological fluids. *Clin Chem* 2005; **51**: 1432–1442.
33. Shaw JL, Diamandis EP. Distribution of 15 human kallikreins in tissues and biological fluids. *Clin Chem* 2007; **53**: 1423–1432.
34. Seiz L, Kotzsch M, Grebenchtchikov NI, Geurts-Moespot AJ, Fuessel S, Goettig P, Gkazepis A, Wirth MP, Schmitt M, Lossnitzer A, Sweep FC, Magdolen V. Polyclonal antibodies against kallikrein-related peptidase 4 (KLK4): immunohistochemical assessment of KLK4 expression in healthy tissues and prostate cancer. *Biol chem* 2010; **391**: 391–401.
35. Day CH, Fanger GR, Retter MW, Hylander BL, Penetrante RB, Houghton RL, Zhang X, Mcneill PD, Filho AM, Nolasco M, Badaro R, Cheever MA, Reed SG, Dillon DC, Watanabe Y. Characterization of KLK4 expression and detection of KLK4-specific antibody in prostate cancer patient sera. *Oncogene* 2002; **21**: 7114–7120.
36. Nelson PS, Gan L, Ferguson C, Moss P, Gelinas R, Hood L, Wang K. Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. *Proc Natl Acad Sci USA* 1999; **96**: 3114–3119.
37. Takayama TK, Mcmullen BA, Nelson PS, Matsumura M, Fujikawa K. Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type

plasminogen activator and degradation of prostatic acid phosphatase. *Biochemistry* 2001; **40**: 15341–15348.
38. Williams SA, Xu Y, De Marzo AM, Isaacs JT, Denmeade SR. Prostate-specific antigen (PSA) is activated by KLK2 in prostate cancer ex vivo models and in prostate-targeted PSA/KLK2 double transgenic mice. *Prostate* 2010; **70**: 788–796.
39. Hu JC, Sun X, Zhang C, Simmer JP. A comparison of enamelin and amelogenin expression in developing mouse molars. *Eur J Oral Sci* 2001; **109**: 125–132.
40. Dong Y, Kaushal A, Bui L, Chu S, Fuller PJ, Nicklin J, Samaratunga H, Clements JA. Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. *Clin Cancer Res* 2001; **7**: 2363–2371.
41. Obiezu CV, Soosaipillai A, Jung K, Stephan C, Scorilas A, Howarth DH, Diamandis EP. Detection of human kallikrein 4 in healthy and cancerous prostatic tissues by immunofluorometry and immunohistochemistry. *Clin Chem* 2002; **48**: 1232–1240.
42. Kita M, Okumura Y, Ohdachi SD, Oba Y, Yoshikuni M, Nakamura Y, Kido H, Uemura D. Purification and characterisation of blarinasin, a new tissue kallikrein-like protease from the short-tailed shrew Blarina brevicauda: comparative studies with blarina toxin. *Biol Chem* 2005; **386**: 177–182.
43. Aminetzach YT, Srouji JR, Kong CY, Hoekstra HE. Convergent evolution of novel protein function in shrew and lizard venom. *Curr Biol* 2009; **19**: 1925–1931.
44. Ramsay AJ, Dong Y, Hunt ML, Linn M, Samaratunga H, Clements JA, Hooper JD. Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression. *J Biol Chem* 2008; **283**: 12293–12304.
45. Mize GJ, Wang W, Takayama TK. Prostate-specific kallikreins-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through protease-activated receptors-1 and -2. *Mol Cancer Res* 2008; **6**: 1043–1051.
46. Ossovskaya VS, Bunnett NW. Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* 2004; **84**: 579–621.
47. Gratio V, Beaufort N, Seiz L, Maier J, Virca GD, Debela M, Grebenchtchikov N, Magdolen V, Darmoul D. Kallikrein-related peptidase 4: a new activator of the aberrantly expressed protease-activated receptor 1 in colon cancer cells. *Am J Pathol* 2010; **176**: 1452–1461.

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.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.