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ABSTRACT

Dental fluorosis is caused by chronic high-level fluoride (F⁻) exposure during enamel development, and fluorosed enamel has a higher than normal protein content. Matrix metalloproteinase 20 cleaves enamel matrix proteins during the secretory stage, and KLK4 further cleaves these proteins during the maturation stage so that the proteins can be reabsorbed from the hardening enamel. We show that transforming growth factor β 1 (TGF- β 1) can induce *Klk4* expression, and we examine the effect of F⁻ on TGF- β 1 and KLK4 expression. We found that *in vivo* F⁻ inhibits *Klk4* but not *Mmp20* transcript levels. LacZ-C57BL/6-*Klk4*^{+LacZ} mice have LacZ inserted in frame at the *Klk4* translation initiation site so that the endogenous *Klk4* promoter drives LacZ expression in the same temporal/spatial way as it does for *Klk4*. KLK4 protein levels in rat enamel and β -galactosidase staining in LacZ-C57BL/6-*Klk4*^{+LacZ} mouse enamel were both significantly reduced by F⁻ treatment. Since TGF- β 1 induces *KLK4* expression, we tested and found that F⁻ significantly reduced *Tgf- β 1* transcript levels in rat enamel organ. These data suggest that F⁻-mediated down-regulation of TGF- β 1 expression contributes to reduced KLK4 protein levels in fluorosed enamel and provides an explanation for why fluorosed enamel has a higher than normal protein content.

KEY WORDS: amelogenin, matrix metalloproteinases, proteases/proteinases, signal transduction, cell stress, gene expression.

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Fluoride Affects Enamel Protein Content via TGF- β 1-mediated KLK4 Inhibition

INTRODUCTION

Community water fluoridation is recommended by the U.S. Public Health Service because appropriate levels (0.7 ppm) of fluoride (F⁻) prevent dental caries (Centers for Disease Control and Prevention, 2001). However, exposure to excessive amounts of F⁻ (>1.5 ppm) during enamel development causes dental fluorosis (Dean and Elvove, 1936). The mineral content of fluorosed enamel is reduced, and the enamel retains greater than normal protein levels. Fluorosed teeth may appear mottled, discolored, and porous and may be more susceptible to decay (DenBesten, 1999).

Enamel development occurs in stages, as defined by the morphology of the ameloblasts responsible for enamel formation. Secretory-stage ameloblasts secrete matrix metalloproteinase 20 (MMP20) and enamel proteins that combine to form a mineralization front that promotes appositional growth until the enamel layer reaches full thickness (Simmer *et al.*, 2012; Bartlett, 2013). Maturation-stage ameloblasts secrete KLK4, reabsorb protein degradation products, and promote mass mineral deposition as the enamel hardens into its final form (Hu *et al.*, 2007). Ameloblasts are exquisitely sensitive to environmental stress. High fever during enamel development (Tung *et al.*, 2006) and even the process of being born (Zanolli *et al.*, 2011) can result in enamel defects.

Previously, we proposed an “acid hypothesis” to explain why fluorosis occurs mainly during the maturation stage. The hypothesis proposes that the mass mineral precipitation of this stage creates an acid environment in which greater amounts of F⁻ become highly toxic hydrofluoric acid. Hydrofluoric acid easily passes through the ameloblast membrane down a steep concentration gradient from the acid environment into the neutral cytosol, and this elicits an ameloblast stress response (Sharma *et al.*, 2010). The acid hypothesis predicts that maturation-stage ameloblast cell functions are more severely affected by F⁻ exposure than are secretory-stage cell functions. For these reasons, we chose to examine the effect of F⁻ on kallikrein 4 (KLK4) expression.

KLK4 is a glycosylated, chymotrypsin-like serine protease that is expressed and secreted by maturation-stage ameloblasts (Simmer *et al.*, 1998; Hu *et al.*, 2000). KLK4 degrades enamel matrix proteins (Ryu *et al.*, 2002) before their export from the hardening enamel (Smith *et al.*, 2011). Ablation of *Klk4* in mice results in abnormally soft enamel with a higher than normal protein content (Simmer *et al.*, 2009), and fluorosed enamel displays the same characteristics. Previously, we showed that murine *Klk4* transcript levels were reduced after F⁻ treatment (Sharma *et al.*, 2010), and here we propose a mechanism by which this occurs and demonstrate that protein levels are similarly affected.

MATERIALS & METHODS

Animals

All animals were treated humanely as approved by the Forsyth Institutional Animal Care and Use Committee. The Forsyth Institute is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, which follows the National Research Council's *Guide for the Care and Use of Laboratory Animals*. The *Klk4^{+/lacZ}* mice are knock-in mice with *LacZ* inserted in frame into the *Klk4* translation initiation site such that the endogenous upstream *Klk4* promoter drives *LacZ* expression (Simmer *et al.*, 2009). Six-week-old Sprague-Dawley rats and *Klk4^{+/lacZ}* mice were provided *ad libitum* water containing F⁻ (0, 50, or 100 ppm) as NaF. After 6 wk of F⁻ treatment, animals were euthanized, and tissues were obtained for quantitative real-time polymerase chain reaction (qPCR), Western blots, and histochemistry.

Cell Culture

Mouse ameloblast-lineage cells (ALCs; Nakata *et al.*, 2003) were cultured at pH 7.4 in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. The human prostate carcinoma cell line (LNCaP) was purchased from ATCC (Manassas, VA, USA) because it expresses KLK4 (Xi *et al.*, 2004). These cells were cultured at pH 7.4 in modified (ATCC) RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. LNCaP cells (3.0×10^5 cells) were seeded into 6-well plates and after 48 hr were stimulated with either human recombinant transforming growth factor β 1 (TGF- β 1; Sigma, St. Louis, MO, USA) or vehicle (4 mM HCL + 1 mg/mL BSA) at the indicated concentrations. After 24 hr, total RNA was extracted. ALCs (3.0×10^5) were seeded into 6-well plates and after 24 hr were treated with F⁻ (0, 1, 3 or 5mM) as sodium F⁻ (Fisher Scientific, Pittsburg, PA, USA) for 24 hr, followed by total RNA extraction.

qPCR Analysis

Total RNA was extracted from LNCaP cells, ALCs, and secretory- and maturation-stage rat mandibular incisor enamel organs (EOs) *via* Direct-zol RNA MiniPrep (Zymo Research Corp., Irvine, CA, USA). Total RNA (1 μ g) was reverse transcribed into cDNA with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Minneapolis, MN, USA). The cDNA was subjected to qPCR amplification on a LightCycler 480 Real-Time PCR System (Roche Diagnostics). The relative expression of target genes was determined by the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001). For cultured cells, cDNA from 3 different samples for each treatment group was assayed 3 times in duplicate. For rat EOs, cDNA from 4 rat incisors for each treatment group was assayed in duplicate. The internal reference control gene was *Eef1a1* because of its consistent expression with variations in F⁻ treatment. For human recombinant TGF- β 1 experiments, *Rn18S* was the control gene. Primers (Invitrogen) and their sequences were as follows:

Rat EO: Klk4, forward: 5'-GCTGCTGTATGACCCCGT G-3', reverse: 5'-CCCCCAGAGTCCCCATTG-3'; *Mmp20*, forward: 5'-CCCACCTACAAGCCATCC-3', reverse: 5'-CTGGTGATGGTGTCTGGGCC-3'; *Tgf- β 1*, forward: 5'-AGGACCTGGGTTGGAAGTGGAT-3', reverse: 5'-AAGCGCCCGGGTTGTGTT-3'; *Eef1a1*, forward: 5'-TGATGCCCCAGGACACAGAGACT-3', reverse: 5'-GATACCAGCTTCAAATTCCTCAACAC-3'.
Murine ALCs: Tgf- β 1, forward: 5'-AGGACCTGGGTTGG AAGTGGAT-3', reverse: 5'-AAGCGCCCGGGTTGT GTT-3'; *Eef1a1*, forward: 5'-GTCGCAGGGGCTTGT CAGTT-3'.
Human LNCaP cells: KLK4, forward: 5'-CGAACGGCA GAATGCCTACC-3', reverse: 5'-CAAGGCCCTGCA AGTACCCG-3'; *18S*, forward: 5'-GTAACCCGTTGA ACCCCATT-3', reverse: 5'-CCATCCAATCGGTAGT AGCG-3'.

Western Blot Analysis

Bulk secretory and maturation-stage enamel from extracted mandibular incisors was dissolved by incubation in 2 mL of 0.17 M HCl/0.98% formic acid for 2 hr at 4°C. Undissolved material was removed by centrifugation at 5,000 g for 10 min at 4°C. The supernatants were neutralized by dialyzing against 50 mM Tris-HCl buffer (pH 7.4). For amelogenin, samples were aliquoted and lyophilized. For KLK4, samples were fractionated with 20% ammonium sulfate, and precipitates were removed by centrifugation. Supernatants were dialyzed against 0.1 M acetic acid and lyophilized. KLK4 protein (10 μ g) and amelogenin protein (1 μ g) were used for blotting procedures with polyclonal antibodies as described previously (Yamakoshi *et al.*, 2011).

Histochemistry and X-gal Staining

Incisors from *Klk4^{+/LacZ}* mice were fixed in 5% formalin overnight at 4°C, followed by decalcification with 10% EDTA at 4°C for 2 wk. Samples were then embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and frozen at -80°C. Serial sagittal sections were stained with X-gal as described previously (Simmer *et al.*, 2011). The mean intensity of X-gal staining was quantified by use of ImageJ software (Schneider *et al.*, 2012).

Statistical Analysis

The qPCR results and X-gal staining intensity were analyzed by 1-way analysis of variance with Fisher's protected least significant difference *post hoc* test. Values of $p < .05$ were considered statistically significant.

RESULTS

F⁻ Decreases *Klk4* but Not *Mmp20* Expression in Rat EO

F⁻ treatment significantly decreased *Klk4* transcript levels (Fig. 1A, $p < .01$) during the maturation stage of enamel development.

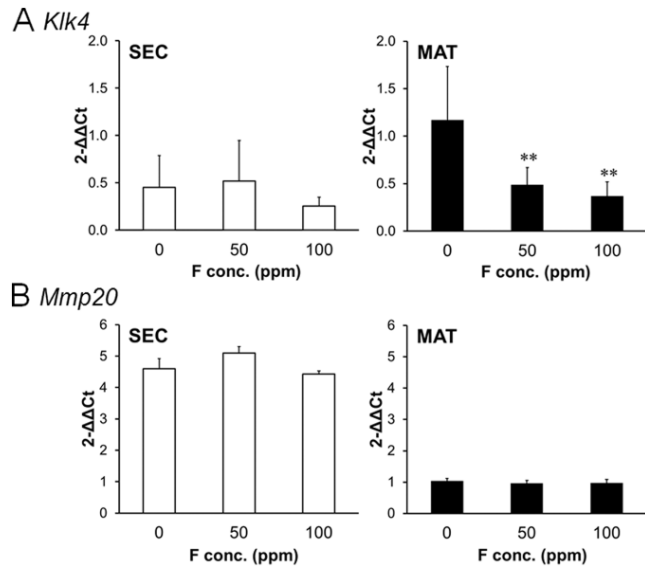


Figure 1. Fluoride decreased *Klk4* gene expression in maturation-stage rat enamel organ. Rats were supplied *ad libitum* fluoride (0, 50, or 100 ppm) as NaF in drinking water for 6 wk. Quantitative real-time polymerase chain reaction was performed on secretory-stage (SEC; left panels) and maturation-stage (MAT; right panels) enamel organs. (A) *Klk4* expression and (B) *Mmp20* expression were quantified by quantitative real-time polymerase chain reaction by use of the $\Delta\Delta C_T$ method. *Eef1a1* served as the reference gene control. cDNA from 4 rats in each treatment group were assayed in duplicate. All results were normalized to the 0 ppm maturation-stage result of *Klk4* or *Mmp20* respectively. Data are expressed as mean \pm SD. * $p < .05$, ** $p < .01$, vs. 0 ppm.

Conversely, *Mmp20* is expressed during the secretory stage, and F^- did not significantly affect *Mmp20* expression during this stage (Fig. 1B). The low levels of expression observed for *Klk4* during the secretory stage and for *Mmp20* during the maturation stage were likely due to a small overlap in the separation of these stages during the EO dissection.

Decreased Quantities of KLK4 Protein Were Present in F^- -Treated Rat Incisor Enamel

To determine if F^- treatment also reduced rat enamel KLK4 protein levels, we performed immunoblots on total protein extracted from rat mandibular incisors. Compared with the F^- controls (0 ppm), KLK4 protein levels were attenuated in the F^- treatment groups (50 and 100 ppm; Fig. 2; upper panel). In contrast, amelogenin, which is expressed during the secretory stage of development, showed no obvious decrease in protein levels among F^- treatment groups (Fig. 2; lower panel). These data support the acid hypothesis, postulating that the acidic environment of the maturation stage makes ameloblasts more susceptible to the toxic effects of F^- exposure.

LacZ Expression in *Klk4^{+LacZ}* Mice Treated with F^-

In *Klk4^{+LacZ}* knock-in mice, one allele expresses *Klk4* and the other expresses LacZ via the endogenous *Klk4* upstream

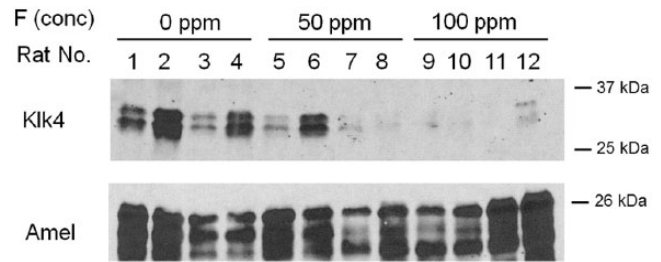


Figure 2. Fluoride attenuates KLK4 protein levels in rat incisor enamel. Rats were supplied *ad libitum* fluoride (0, 50, or 100 ppm) in drinking water for 6 wk. Protein was extracted from mandibular incisor enamel and assessed by Western blot procedures by use of antibodies specific for KLK4 (upper panel) or amelogenin (lower panel). KLK4 protein levels were assessed in incisors from 4 rats per treatment group (fluoride treatment groups: 0 ppm, lanes 1-4; 50 ppm, lanes 5-8; 100 ppm, lanes 9-12).

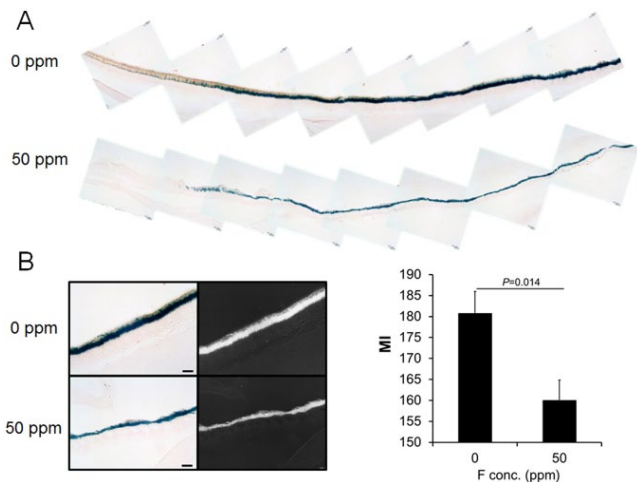


Figure 3. *Klk4^{+LacZ}* knock-in mice had reduced LacZ expression after fluoride treatment. These knock-in mice have 1 *Klk4* allele with *LacZ* inserted in frame at the *Klk4* translation initiation site. Therefore, the upstream promoter elements were identical for *Klk4* and *LacZ*. The *Klk4^{+LacZ}* mice were treated with fluoride (0 or 50 ppm) *ad libitum* in drinking water for 6 wk. (A) β -galactosidase histostaining of maxillary incisors from *Klk4^{+LacZ}* mice treated with 0-ppm (upper panel) or 50-ppm (lower panel) fluoride. (B) High magnification of β -galactosidase staining (left panel) in maturation-stage ameloblasts after the indicated fluoride treatment. Bar represents 30 μ m. Mean intensity (MI) as quantified by ImageJ analysis of β -galactosidase staining in maturation-stage ameloblasts (right panel). Sections from 3 mice in each group were analyzed in duplicate, and representative images are shown. Data are expressed as mean \pm SE.

promoter (Simmer *et al.*, 2009). The knock-in LacZ has a nuclear localization signal to help distinguish its expression from endogenous lysosomal β -galactosidase. F^- -treated *Klk4^{+LacZ}* mouse maxillary incisors were stained with X-gal to quantify the level of LacZ expression. Staining was observed in ameloblast nuclei from the transition through maturation stages of development. Representative histostained cryosections of *Klk4^{+LacZ}* are shown for control and F^- -treated incisors (Fig. 3A), along with higher magnification images of nuclei staining (Fig. 3B, left panels). Mean intensity of staining was quantified

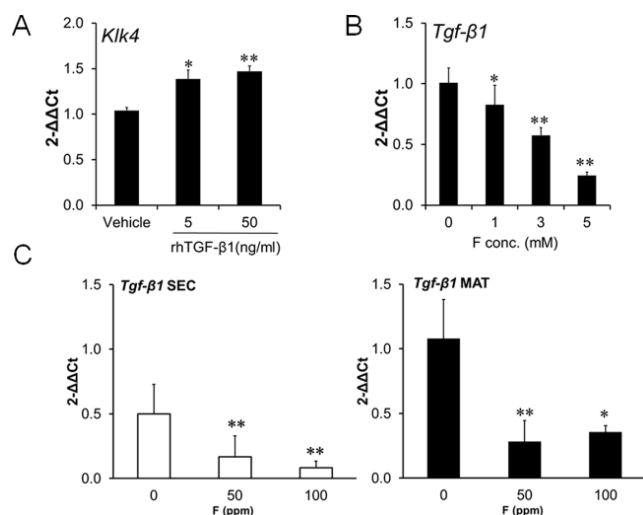


Figure 4. hrTGF-β1 treatment increased *KLK4* expression in human LNCaP cells, and fluoride decreased *Tgf-β1* expression in mouse ALC cells and in rat enamel organ. Gene expression was assessed by quantitative real-time polymerase chain reaction. (A) *KLK4* expression in LNCaP cells treated for 24 hr with either vehicle or hrTGF-β1 (5 and 50 ng/ml). (B) Treatment of ALC cells with 0, 1, 3, or 5 mM fluoride as NaF for 24 hr significantly reduced ALC *Tgf-β1* expression. (C) *Tgf-β1* expression was significantly reduced in secretory-stage (SEC) and maturation-stage (MAT) rat enamel organ after treatment with fluoride (0, 50, or 100 ppm) for 6 wk. *Rn18s* was the internal reference control gene for hrTGF-β1 treatments, and *Eef1α1* was the control gene for fluoride treatment. For the cell culture experiments, cDNA from 3 samples in each experimental treatment group were assayed 3 times in duplicate and data expressed as mean ± SE (A and B). For the rat enamel organ experiments, cDNA from 4 rats in each treatment group were assayed in duplicate and data expressed as mean ± SD (C). **p* < .05, ***p* < .01, vs. control.

by use of ImageJ (Fig. 3B, graph). Mean intensity was significantly decreased with F⁻ treatment (0 ppm, 181 ± 5.2; 50 ppm, 160 ± 4.8).

TGF-β1 Stimulates *KLK4* Expression and Both Genes Are Downregulated by F⁻ Exposure

Previously, we reported that TGF-β1 is upregulated in maturation-stage EO. To determine if TGF-β1 signaling upregulates *KLK4* expression, we treated LNCaP cells with human recombinant TGF-β1 (0, 5, or 50 ng/mL) for 24 hr. LNCaP cells were derived from a human prostatic adenocarcinoma, and they express *KLK4*. Human recombinant TGF-β1 significantly increased LNCaP *KLK4* transcript levels (Fig. 4A) indicating that *KLK4* is regulated by TGF-β1 signaling. Next, we asked if F⁻ suppresses *Tgf-β1* expression in ALCs or rat EO. ALCs were treated with the indicated F⁻ concentrations for 24 hr, and subsequent qPCR results demonstrated that *Tgf-β1* expression was significantly decreased (Fig. 4B). Consistent with previous data, *Tgf-β1* expression significantly increased during the rat maturation stage compared with the secretory stage (Fig. 4C). F⁻ treatment for 6 wk significantly attenuated *Tgf-β1* expression in both secretory- and maturation-stage rat EO (Fig. 4C). These data

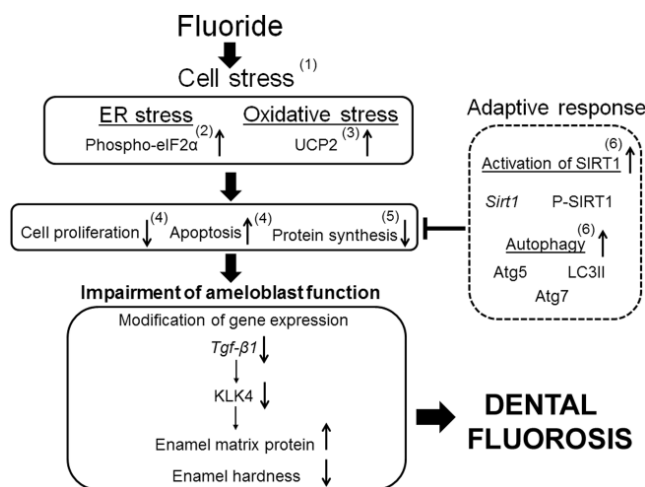


Figure 5. Presented is a schematic outlining our prior and current research results that characterize the mechanism of how fluoride causes dental fluorosis. Thick arrows indicate the progression of events leading to the impairment of ameloblast function. The sideways “T” indicates how activation of specific genes alleviate the toxic effects of fluoride-induced cell stress. Thin arrows indicate the direction of gene expression. (1) *Cells*. 1:631-645, 2012. (2) *Environ Health Perspect*. 116:1142-1146, 2008. (3) *Connect Tiss Res*. 55(Suppl 1):25-28, 2014. (4) *J Biol Chem*. 280:23194-23202, 2005. (5) *PLoS One*. 5:e10895, 2010; *Methods Enzymol*. 491:111-125, 2011. (6) *Biochim Biophys Acta*. 842:245-255, 2014.

suggest that the F⁻-mediated downregulation of TGF-β1 likely contributes to repressed *KLK4* expression.

DISCUSSION

Here we demonstrate that F⁻ downregulates *KLK4* gene expression during the maturation stage but does not downregulate amelogenin or *Mmp20* expression during the secretory stage of enamel development. We show that the F⁻-mediated *KLK4* downregulation is reflected by an overall decrease in *KLK4* protein within rat incisor enamel and that endogenous *KLK4* promoter-driven LacZ expression is significantly reduced by F⁻ exposure in mouse incisors. In contrast, during the secretory stage, amelogenin protein levels remained steady regardless of F⁻ treatment. Furthermore, we show *in vitro* that TGF-β1 induces *KLK4* expression and that the presence of F⁻ also significantly reduces *Tgf-β1* expression. We confirm that *Tgf-β1* is upregulated as rodent ameloblasts enter the maturation stage, and we demonstrate that F⁻ significantly reduces *Tgf-β1* expression during enamel development. Taken together, our data support the acid hypothesis stipulating that ameloblasts are more sensitive to the toxic effects of F⁻ during the maturation stage when mass mineral precipitation occurs accompanied by mass proton release. Our data also suggest that *Tgf-β1* is an important regulator of *KLK4* expression. Figure 5 presents a summary of our current and prior data demonstrating that F⁻ causes cell stress, that SIRT1 and autophagy alleviate F⁻-mediated cell stress, and that unresolved cell stress impairs ameloblast function.

Supporting evidence that TGF- β 1 signaling promotes *Klk4* expression was published using mice with amelogenin promoter-driven *Cre* recombinase and floxed *Tgf- β* receptor II. These mice had enamel with decreased mineral content and increased attrition, as well as thin enamel crystallites. Importantly, *Klk4* mRNA levels were significantly reduced in these animals. It was concluded that TGF- β signaling through the expression of *Klk4* plays an important role in ameloblast function and in enamel maturation (Cho *et al.*, 2013). The elevated expression of *Tgf- β* 1 in mouse maturation-stage ameloblasts is accompanied by phosphorylation of Smad 2/3 (Tsuchiya *et al.*, 2009). TGF- β regulates Smad2/3-mediated downstream signaling, and *Smad3*-null mice had hypomineralized enamel (Yokozeki *et al.*, 2003). Since ablation of *Klk4* also results in hypomineralized enamel (Simmer *et al.*, 2009), these studies provide compelling evidence suggesting that TGF- β signaling in maturation-stage EO plays an important role in regulation of KLK4 expression during enamel development.

In contrast, Gao *et al.* (2009) showed that for ALCs, TGF- β 1 did not induce KLK4 expression but did induce MMP20 expression. In their study, MMP20 and KLK4 transcript levels were assessed by performing reverse transcription PCR with scanning of the resulting bands for quantification of gene expression. In an attempt to confirm their results, we did assess MMP20 and KLK4 expression in ALCs by use of quantitative real-time PCR, but we did not reach the cycle thresholds for MMP20 and KLK4 transcripts by our 35-cycle cutoff point. This is why we used the human prostate carcinoma cell line LNCaP, which expresses KLK4 (Xi *et al.*, 2004), for our TGF- β 1/KLK4 expression-level studies.

Previously, we demonstrated that F⁻ causes endoplasmic reticulum stress in ameloblasts responsible for enamel formation (Kubota *et al.*, 2005); that F⁻-induced endoplasmic reticulum stress inhibits protein synthesis and secretion (Sharma *et al.*, 2008); that, in the presence of F⁻, culture medium at a moderately low pH increases the level of the stress response (Sharma *et al.*, 2010); and, recently, that Sirt1 and autophagy protect cells from F⁻-induced cell stress (Suzuki and Bartlett, 2014). Additionally, we have focused on determining if MMP20 or KLK4 plays a role in dental fluorosis. We showed that when wild-type and *Mmp20* heterozygous mice were treated for 6 wk with F⁻, mice with only 1 functional *Mmp20* allele had enamel hardness values similar to wild-type enamel (Sharma *et al.*, 2011). Strikingly, we performed exacting enzyme kinetics studies by use of Michaelis-Menten plots while recombinant enzymes were incubated with quenched fluorescent peptides to demonstrate that F⁻ concentrations of up to 10mM had no effect on MMP20 or KLK4 enzymatic activity (Tye *et al.*, 2011). Although KLK4 activity is not affected by F⁻ treatment, its expression levels at both the mRNA level and the protein level are affected, which is likely why fluorosed enamel has a higher than normal protein content and is softer than normal. The significantly reduced KLK4 expression level precludes the ability to adequately cleave enamel matrix proteins so that they can be properly exported from the hardening enamel.

Since the acid hypothesis predicts that fluorosis caused by hydrofluoric acid will occur predominantly during the acidic

maturation stage of enamel development, an unexpected result was that F⁻ treatment reduced *Tgf- β* 1 mRNA levels during the neutral secretory stage as well as during the acidic maturation stage. Expression levels of *Mmp20* and amelogenin were not reduced by F⁻ during the secretory stage, so it was surprising that this occurred for *Tgf- β* 1. Perhaps, since TGF- β 1 can induce growth arrest or cell death (Siegel and Massagué, 2003), its expression is more easily reduced so that cells can better adapt to environmental stresses, such as F⁻ exposure. However, when this occurs during the maturation stage of enamel development, a negative consequence may be that not enough KLK4 is expressed.

In conclusion, we demonstrate that F⁻ decreases *Klk4* mRNA and protein levels in maturation-stage ameloblasts and in enamel, respectively. We also provide insight into a potential mechanism by which F⁻ attenuates KLK4 expression through diminished TGF- β 1 signaling. These results suggest that down-regulation of KLK4 by F⁻ is one of the critical factors causing dental fluorosis.

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