

Exogenous transforming growth factor- β 1 prevents the inflow of fluoride to ameloblasts through regulation of voltage-gated chloride channels 5 and 7

MEI JI, XUEJING DUAN, XIAOHUI HAN, JING SUN and DONGSHENG ZHANG

Department of Stomatology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, P.R. China

Received March 22, 2020; Accepted March 15, 2021

DOI: 10.3892/etm.2021.10047

Abstract. Dental fluorosis is a global issue. Although there are multiple causes of dental fluorosis, the precise mechanism remains controversial. Previous studies have demonstrated that extracellular fluoride may promote an accumulation of fluoride ions in ameloblasts, which may induce oxidative and endoplasmic reticulum stresses, leading to dental fluorosis. However, the exact process by which fluoride ions enter cells has not been determined. In the present study, intracellular fluoride concentration was determined using a newly developed specific fluorescent probe called probe 1. Under high extracellular fluoride concentrations, the fluorescence intensity of the ameloblasts increased, however, exogenous transforming growth factor- β 1 (TGF- β 1) was able to inhibit the increase. Furthermore, changes in the expression of the voltage-gated chloride channels 5 and 7 (CIC5 and CIC-7), which are responsible for the transport of fluoride were investigated. The results indicated that fluoride reduced the expression of endogenous TGF- β 1 and increased the expression of CIC-5 and CIC-7. Additionally, exogenous TGF- β 1 reduced the expression of CIC-5 and CIC-7. The results of the present study indicate that exogenous TGF- β 1 may prevent accumulation of fluoride in

ameloblasts through the regulation of CIC-5 and CIC-7 under high extracellular fluoride concentrations.

Introduction

The supplementation of drinking water with fluoride is an established method to prevent dental caries (1). According to the Dean index, the optimal concentration of fluoride in drinking water is 1 ppm (2). Nevertheless, in many areas of the world, the natural fluoride levels in drinking water exceed the recommended concentration (1). In some areas, the natural fluoride levels in drinking water are extremely high, such as in Colorado (11.2 mg/l), Oklahoma (12.0 mg/l), New Mexico (13.0 mg/l) and Idaho (15.9 mg/l) (3). With the development of dentistry, fluoride has also been added to dental materials, including toothpaste, varnish, foam and dental resin, to prevent the occurrence of caries. All these fluoride additions increase the morbidity of dental fluorosis (3), which is currently a worldwide problem. A small 'pea-sized' amount of toothpaste would contain 0.36-0.72 mg fluoride, which would increase the risk of dental fluorosis in children (4). In America, the prevalence of dental fluorosis in children aged 15-17 years increased from 22.6% in the 1986-1987 to 40.6% in the 1999-2004, which was provided by the National Health and Nutrition Examination Survey (5). Although there are multiple causes of dental fluorosis, the precise mechanism remains controversial (6,7). The regulation of intracellular and extracellular pH is an important mechanism that may lead to dental fluorosis. According to the acid hypothesis (8), fluoride ions are unable to enter the cell, whereas hydrogen fluoride (HF) can readily cross the cell membrane under acidic conditions (9). An increased fluoride concentration in the cytoplasm may induce oxidative stress by reducing the activity of antioxidant enzymes, thereby affecting a variety of structures and processes of normal cells due to reactive oxygen species accumulation (8,10-12). Furthermore, fluoride in the cytoplasm may induce endoplasmic reticulum (ER) stress, including the phosphorylation of the eukaryotic initiation factor 2 (eIF2), which may result in a decrease in the overall protein production, including secretion of the protease kallikrein 4 (KLK4) (8,13-15). However, studies on fluoride transport have primarily used fibroblasts and epithelial cells from the rat stomach and urinary bladder,

Correspondence to: Professor Dongsheng Zhang, Department of Stomatology, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan, Shandong 250021, P.R. China
E-mail: ds63zhang@sdu.edu.cn

Abbreviations: CIC-5, voltage-gated chloride channel 5; CIC-7, voltage-gated chloride channel 7; CICs, voltage-gated chloride channels; CFTR, cystic fibrosis transmembrane conductance regulator; CLCA, calcium-activated chloride channels; CLICs, chloride intracellular channels; ECM, extracellular matrix; HA, hydroxyapatite; HF, hydrogen fluoride; KLK4, kallikrein 4; LS8, the ameloblast-like cell line; RT-qPCR, reverse transcription-quantitative PCR; TGF- β 1, transforming growth factor- β 1

Key words: fluoride, chloride channels, transforming growth factor- β 1, ameloblasts, probe

hamster cheek pouch and renal tubules of several species, and very little research has been carried out in ameloblasts (9,16). Sharma *et al* (13) reported that a low pH environment in the maturation stage of ameloblasts facilitated the uptake of fluoride. This hypothesis was tested by measuring changes in stress-related genes and proteins. Fluorescent probes are a more intuitive method to observe the accumulation of fluoride in ameloblasts (17). However, limitations remain in the majority of the reported fluorescent fluoride probes in terms of determination and bioimaging (17-20). For example, pyrene-based dye lies within the ultraviolet-visible light range, increasing the difficulties in targeting fluoride in living animals (17,18). Although the probes with the lipophilic TBDPS moiety have high selectivity, their performance in apparent optical signal changes to the naked-eye remains poor (17,19,20). To improve on these methods, a highly selective ratiometric visual and red-emitting fluorescent dual-channel, probe 1, was developed. The probe contains the hydrophilic compound 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF) and the lipophilic tert-butyldiphenylsilyl (TBDPS); therefore, it can enter cells easily (21,22). Additionally, probe 1 possesses a latent internal charge transfer structure, which makes it detect signals to the naked-eye (21,22). Through cleavage of the Si-O bond, fluoride transforms probe 1 into a red-emitting fluorescent form. Therefore, probe 1 reflects the distribution of the fluoride ion may be used to determine fluoride concentration (17,21,22).

The epithelial transport process of fluoride relies on ion channels. To the best of our knowledge, no specialized fluoride channels have been identified. It is generally accepted that fluoride ions pass through the membrane via chloride channels (23). There are differences in the permeability and conductivity of fluoride between different chloride channels (23). Simchowicz (24) found that human neutrophils were selective for haloid ions, and the order of affinity for these ions was $\text{Cl} > \text{Br} > \text{F} > \text{I}^-$, which were reverse-transported in a 1:1 ratio to extracellular Cl^- . Studies on the epithelium of the respiratory tract have revealed that its chloride channels are highly selective for anions and that their ionic permeability to halogens is in the order of $\text{I} > \text{Br} \geq \text{Cl} > \text{F}^-$ (25,26). Additionally, Anderson *et al* (27) demonstrated that the order of selectivity of cystic fibrosis transcription factor for anions was $\text{Br} > \text{Cl} > \text{I} > \text{F}^-$. In mammals, several classes of chloride channels have been found, including voltage-gated chloride channels (ClCs), the cystic fibrosis transmembrane conductance regulator (CFTR), calcium-activated chloride channels (CLCAs) and chloride intracellular channels (CLICs) (28). There are nine ClCs, namely ClC-1 to ClC-7, ClC-Ka, and ClC-Kb. ClCs-3-7 function as the main Cl^-/H^+ -exchangers, and facilitate vesicular acidification by shunting currents of proton pumps and by increasing vesicular Cl^- concentration (29,30). ClCs control the electrical excitability of muscles or neurons, extra- and intracellular ion homeostasis and transepithelial transport (30). Hou *et al* (31) found that ClCs-1-7 were expressed in tooth germs. ClC-5 has been reported in the dental lamina, inner enamel epithelium, stratum intermedium, outer enamel epithelium, odontoblasts and ameloblasts (32). CLCN7 mRNA has been reported in ameloblasts at the maturation-stage (33). CFTR plays an important role in transportation of bicarbonate into the enamel space to buffer protons in ameloblasts and is

located on the apical plasma membrane during the maturation stage of ameloblasts (34). Lacruz *et al* (35) reported that the mRNA level of CFTR in rat ameloblasts was higher at the early-mid and mid-late maturation stages than at the secretory stage. Furthermore, previous studies have indicated that CFTR is a critical factor in the regulation of pH during the maturation of ameloblasts and is essential for enamel mineralization (34,36,37). Duan *et al* (38) demonstrated that CFTR inhibition and treatment with CFTR siRNA may increase intracellular pH. In our previous study, ClC-5 and ClC-7, which are Cl^-/H^+ -exchangers related to tooth development and pH regulation were the focus (30).

As a member of the transforming growth factor- β (TGF- β) superfamily, TGF- β 1 regulates cellular biological processes, such as proliferation, differentiation, apoptosis and extracellular matrix (ECM) protein production (39). Furthermore, it plays an important role in tooth development (32,40). Duan *et al* (32) indicated that ClC-5 regulates dentine development through the TGF- β 1 pathway. Previous studies have revealed that fluoride affects enamel protein content via TGF- β 1-mediated KLK4 inhibition (41,42). TGF- β 1 may also impair the expression and function of chloride ion channels in other epithelial cells, including alveolar, bronchial, vas deferens and nasal polyp epithelial cells (43-46). However, whether the expression and function of chloride ion channels in ameloblasts is regulated by TGF- β 1 remains to be elucidated.

In the present study the correlation between TGF- β 1 and the intracellular fluoride concentration at a high extracellular fluoride concentration was investigated. Furthermore, the effects of exogenous TGF- β 1 on ClC-5 and ClC-7 under a high extracellular fluoride concentration were studied.

Materials and methods

Cell culture. The ameloblast-like cell line (LS8) was a gift from Prof. Malcolm L. Snead (University of Southern California, USA), and was cultured in DMEM (Corning, Inc.) supplemented with 10% fetal bovine serum (FBS; Biological Industries) and antibiotics (100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin; Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37°C in a 5% CO_2 incubator and the medium was replaced with fresh medium every 2 days. The experiments were performed in triplicate.

Reverse transcription-quantitative PCR (RT-qPCR). The following groups of ameloblast-like LS8 cells were established: Control; high fluoride, treated with a high fluoride concentration (2.0 mM F^- ; Sigma-Aldrich; Merck KGaA); TGF- β 1, treated with 10 ng/ml TGF- β 1 (Peprotech Inc.); and FT, treated with 2.0 mM F^- + 10 ng/ml TGF- β 1. Cells were plated into six-well plates at a density of 1×10^6 cells/ml and treated with different conditions as aforementioned for 24 h. Total RNA of different groups were extracted using RNAiso (Takara Bio, Inc.) according to the manufacturer's instructions. cDNA was synthesized using the PrimeScriptTM RT reagent kit with gDNA Eraser using the temperature protocols of 37°C for 15 min and 85°C for 5 sec (Takara, Bio, Inc.). Mouse-specific primers for GAPDH (47), TGF- β 1, ClC-5 and ClC-7, were used in conjunction with SYBR Premix Ex TaqTM II (Takara Bio., Inc.) for RNA measurement. All qPCR reactions were conducted under

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Forward sequence (5'-3')	Reverse sequence 5'-3'
TGF- β 1	CTTCAATACGTCAGACATTTCGGG	GTAACGCCAGGAATTGTTGCTA
CIC-5	GAGGAGCCAATCCCTGGTGTA	TTGGTAATCTCTCGGTGCCTA
CIC-7	CGCCAGTCTCATTCTGCACT	GAGGATCGACTTCCGGGTC
GAPDH	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG

TGF- β 1, transforming growth factor- β 1; CIC-5, voltage-gated chloride channel 5; CIC-7, voltage-gated chloride channel 7.

the following conditions: Hot start for 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. The primer sequences used are presented in Table I. In the next step, the target mRNA expression levels were determined using the LightCycler 480 Real-Time PCR System (Roche Diagnostics). The relative expression of target genes was calculated using the $2^{-\Delta\Delta C_q}$ method (48). Each group was set with three repeated wells and their mean values were calculated.

Western blot analysis. Total protein was extracted from 60 mm dishes (Corning, Inc.) containing the ameloblast-like LS8 cells with the seeding density of 2×10^6 cells/ml and treated as described in the previous section. The protein concentration of each sample was determined with a bicinchoninic acid assay (Beijing Solar Bio Science & Technology Co., Ltd.). To separate TGF- β 1, CIC-5 and CIC-7, equal amounts of proteins (40 μ g) were loaded onto 10% SDS-PAGE gels and then the proteins were transferred onto PVDF membranes. Membranes were blocked in Tris-buffered saline Tween (hereafter called TBST) with 1% Tween and 5% non-fat dry milk for 1 h at room temperature and the membranes were labelled with antibodies against TGF- β 1, CIC-5 and CIC-7 at 4°C overnight. The probed membranes were washed with TBST three times and incubated with appropriate secondary antibodies for 1 h at room temperature. Protein bands were visualised using the enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Inc.) detection system Amersham Imager 600 (GE Healthcare; Cytiva). The primary antibodies used were all rabbit-derived and were anti-GAPDH at a ratio of 1:500 (cat. no. BA2913; Wuhan Boster Biological Technology Co., Ltd.), anti-TGF- β 1 at a ratio of 1:1,000 (cat. no. ab92486; Abcam), anti-CIC-5 at a ratio of 1:1,000 (cat. no. ab188503; Abcam), anti-CIC-7 at a ratio of 1:1,000 (cat. no. ab136016; Abcam). Secondary antibodies used were horseradish peroxidase-labelled-goat-derived anti-rabbit at a ratio of 1:5,000 (cat. no. BA1054; Wuhan Boster Biological Technology Co., Ltd.) and horseradish peroxidase-labelled rabbit-derived anti-goat at a ratio of 1:5,000 (cat. no. BA1060; Wuhan Boster Biological Technology Co., Ltd.).

Intracellular fluoride detection through confocal laser scanning microscopy. LS8 cells were seeded into 35 mm glass bottom dishes (cat. no. P35G-1.5-14-C; MatTek Corporation) at a density of 4×10^5 cells/dish. After adhesion, cells were treated for 24 h, as described for RT-qPCR, before 10 μ l the probe 1 was added to each dish and the dishes maintained at room temperature for 30 min (49). The emitted fluorescence

intensity was determined using a confocal laser scanning microscope (model no. LSM 780; Zeiss AG) at 543 nm. The mean fluorescence intensities were calculated by ImageJ 1.52v. Probe 1 was provided by Professor Baocun Zhu (Jinan University, Shandong, China).

Statistical analysis. All values are presented as the mean \pm the standard deviation (SD). Western blot and RT-qPCR results were analysed by one-way ANOVA. Multiple comparisons between groups were performed using the Tukey's test. $P < 0.05$ was considered statistically significant and $P < 0.01$ values was considered highly statistically significant.

Results

Fluoride treatment reduces TGF- β 1 expression in LS8 cells. Fig. 1A indicates the relative value (compared with the internal reference GAPDH) of TGF- β 1 mRNA expression. In the samples from the high fluoride group, endogenous TGF- β 1 mRNA expression levels were significantly lower than those of the control group. Fig. 1B indicates the relative value (compared with the internal reference GAPDH) of TGF- β 1 protein expression. Consistent with the RT-qPCR results, endogenous TGF- β 1 protein expression in the high fluoride group was significantly lower than that of the control group.

Fluoride increases the expression levels of CIC-5 and CIC-7 in LS8 cells. Fig. 2A indicates the relative values (compared with the internal reference GAPDH) of CIC-5 and CIC-7 mRNA expression levels. In the high fluoride group, CIC-5 and CIC-7 mRNA expression levels were significantly higher than those of the control group. Fig. 2B indicates the relative values (compared with the internal reference GAPDH) of CIC-5 and CIC-7 protein expression. CIC-5 protein expression in the high fluoride group was significantly higher than that in the control group. CIC-7 protein expression in the high fluoride group was higher than that in the control group.

Exogenous TGF- β 1 reduces the expression of CIC-5 and CIC-7 in LS8 cells. Fig. 3A illustrates the relative values (compared with the internal reference GAPDH) of CIC-5 and CIC-7 mRNA expression levels. In the high fluoride group the CIC-5 and CIC-7 mRNA expression levels were significantly higher than those of the control group. In the exogenous TGF- β 1 group, CIC-5 and CIC-7 mRNA expression levels were significantly lower than those of the control group. In the FT group, CIC-5 and CIC-7 mRNA expression levels were significantly lower

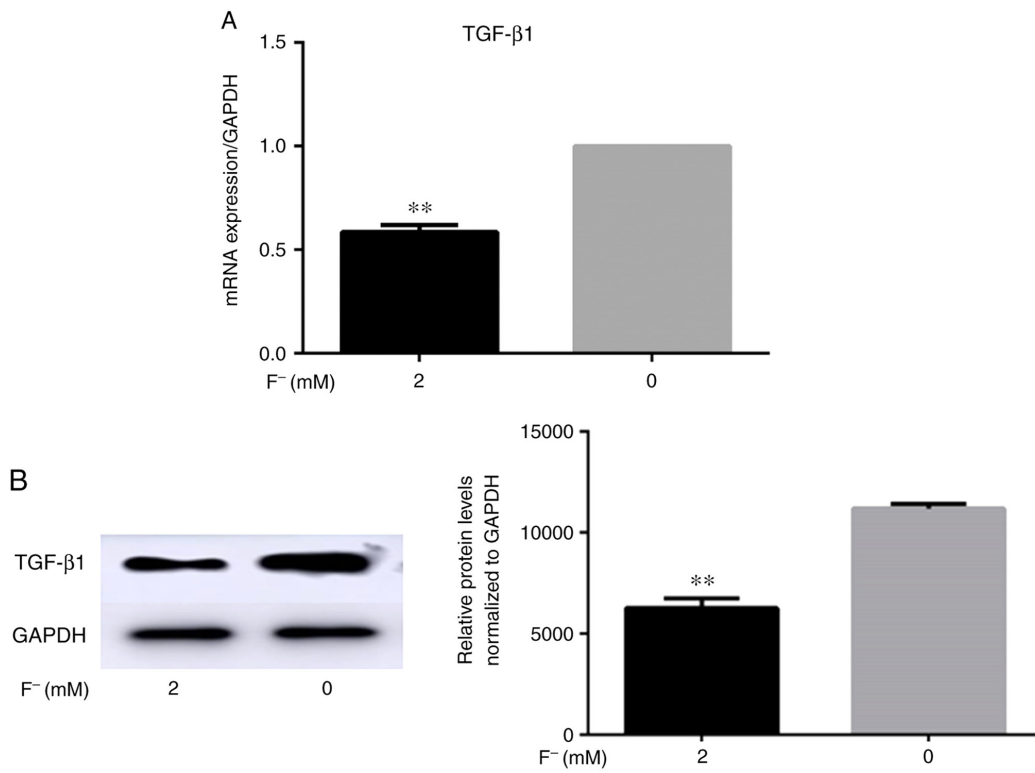


Figure 1. Effect of high fluoride on the TGF- β 1 expression in ameloblasts. (A) TGF- β 1 mRNA levels and (B) TGF- β 1 protein levels were determined in control and high fluoride treated cells. Data are presented as the mean \pm the standard deviation of three repeated experiments. ** $P < 0.01$ vs. control. TGF- β 1, transforming growth factor- β 1.

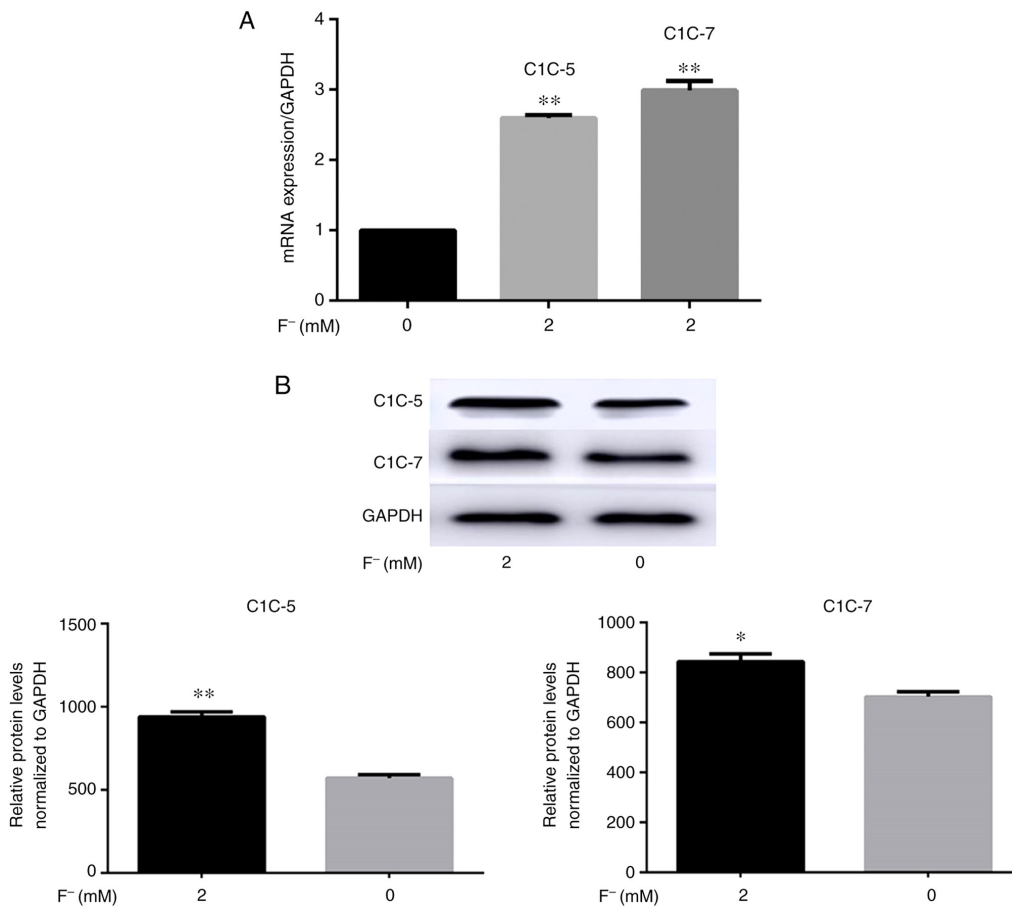


Figure 2. Effects of high fluoride on CIC-5 and CIC-7 expression levels in ameloblasts. Expression levels of CIC-5 and CIC-7 (A) mRNA and (B) protein were determined in control and high fluoride treated cells. Data are presented as the mean \pm the standard deviation of three repeated experiments. * $P < 0.05$, ** $P < 0.01$ vs. control. TGF- β 1, transforming growth factor- β 1; CIC-5, voltage-gated chloride channel 5; CIC-7, voltage-gated chloride channel 7.

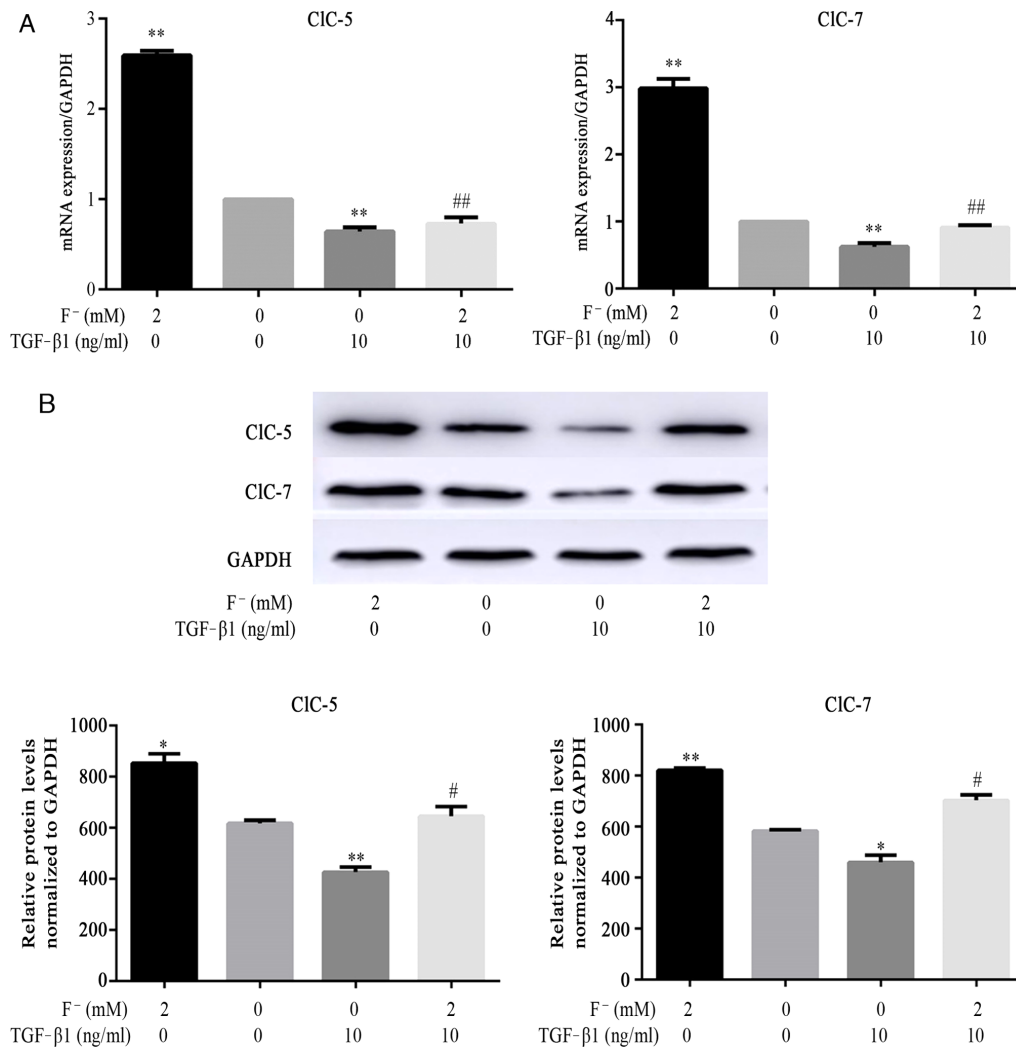


Figure 3. Effects of TGF-β1 on CIC-5 and CIC-7 expression levels in ameloblasts. (A) mRNA and (B) protein levels of CIC-5 and CIC-7 in ameloblasts following treatment with high or low levels of fluoride and exogenous TGF-β1 or a control. *P<0.05, **P<0.01 vs. 0 F⁻ + 0 TGF-β1; #P<0.05, ##P<0.01 vs. high fluoride. TGF-β1, transforming growth factor-β1; CIC-5, voltage-gated chloride channel 5; CIC-7, voltage-gated chloride channel 7.

than those of the high fluoride group. Fig. 3B illustrates the relative values (compared with the internal reference GAPDH) of CIC-5 and CIC-7 protein expression. In the high fluoride group, CIC-5 protein expression was higher than that of the control group. In the exogenous TGF-β1 group, CIC-5 protein expression was significantly lower than that of the control group. In the FT group, CIC-5 protein expression was lower than that of the high fluoride group. In those in the high fluoride group, CIC-7 protein expression levels were significantly higher than that of the control group. In the exogenous TGF-β1 group, CIC-7 protein expression levels were lower than those of the control group. In the FT group, CIC-7 protein expression levels were lower than those of the high fluoride group.

Exogenous TGF-β1 decreased the fluorescence intensity of LS8 cells. Fig. 4 demonstrates the fluorescence intensity of LS8 cells exposed to different treatments. The fluorescence intensity of the high fluoride group was significantly higher than that of the control group. The fluorescence intensity of the exogenous TGF-β1 group was significantly lower than that of the control group. In the FT group, the fluorescence intensity was significantly lower than that of the high fluoride group.

Discussion

The results of the present study indicated that a high extracellular fluoride concentration had two effects on ameloblasts, an increase in intracellular fluoride concentration and an increase in CIC-5 and CIC-7 expression. Using probe 1, the red fluorescence intensity of intracellular fluoride in the high fluoride group was indicated to be stronger than that in the control group. Fluoride cannot enter ameloblasts directly and must be converted to HF beforehand (8). Protons are released during the process of hydroxyapatite (HA) deposition. Depending on the phosphate precursor, the precipitation of HA releases 8-14 moles of hydrogen ions per mole of HA, which acidifies the enamel matrix (8,13,50). Additionally, a number of *in vitro* and *in vivo* studies have proved that fluoride can accelerate crystal formation and induce hypermineralized lines in secretory enamel (51,52). Crystal growth produces a large number of protons, which can acidify the microenvironment (53). The low pH of the microenvironment promotes the conversion of fluoride to HF. According to the Henderson-Hasselbalch equation, more than 25-fold the amount of HF is produced at pH 6.0, compared with that at pH 7.4 (8,13). Owing to the

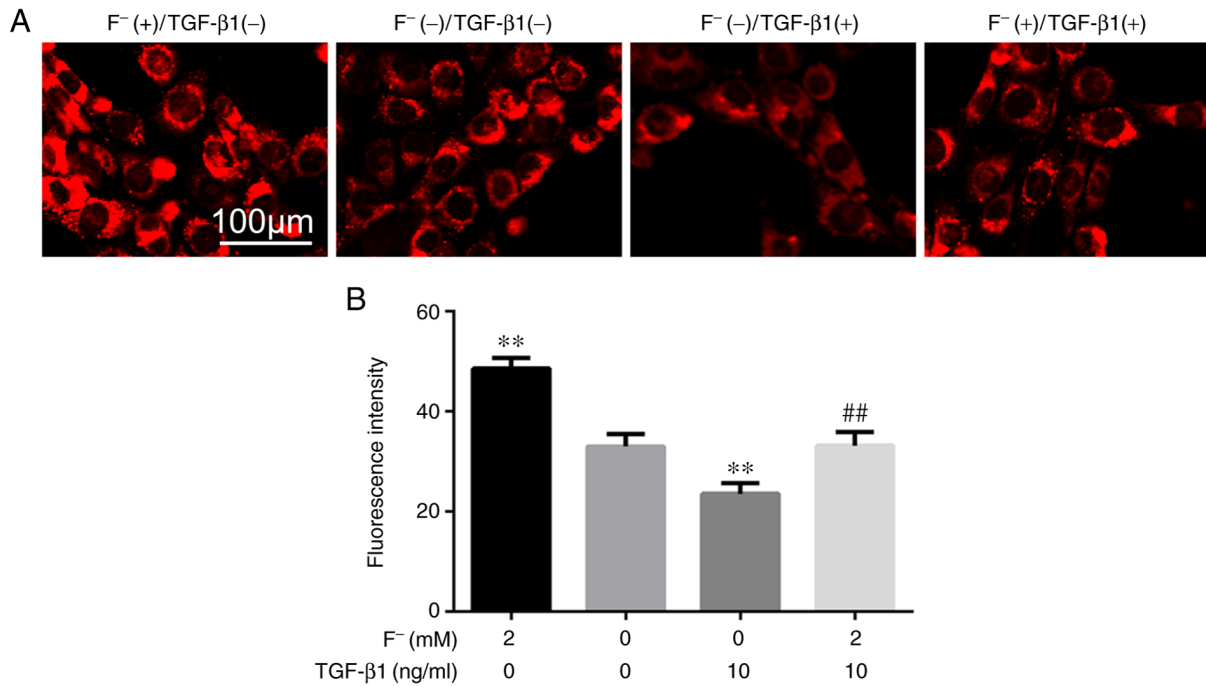


Figure 4. Effects of TGF- β 1 on fluorescence intensity in ameloblasts. (A) Fluorescence images and (B) intensities of probe 1 in ameloblasts following treatment with high or low levels of fluoride and exogenous TGF- β 1 or a control. TGF- β 1, transforming growth factor- β ; **P<0.01 vs. control; ##P<0.01 vs. the high fluoride group.

pH gradient, HF may diffuse easily into the cytoplasm from the enamel matrix and revert to fluoride ions in the neutral cytoplasm; consequently, it cannot easily diffuse out of the cell (8,9,13,16). Increased fluoride concentrations in the cytoplasm may induce oxidative stress and endoplasmic reticulum stress, both of which increase the occurrence of dental fluorosis (8,10-12,14,15).

In the present study, probe 1 was employed to determine the intracellular fluoride concentration. Probe 1 is a highly selective ratiometric visual and red-emitting fluorescent dual-channel probe, which can detect fluoride in aqueous solution and living systems (49). In comparison to conventional technology, a ratiometric probe could eliminate most or all ambiguities by self-calibration at two wavelength intensities, which may avoid interference due to instrumental stability, sample environments and probe distribution (17,21,49,54). Additionally, a ratiometric probe may display a large wavelength shift (158 nm) for practical use in ratiometric determination. The 158 nm shift caused by fluoride changes the solution containing probe 1 from yellow to blue (49). Thus probe 1 may serve as a visual probe for fluoride because it can be used to determine fluoride level changes by the naked-eye (49). Additionally, probe 1 could detect fluoride levels on the fluorescence spectrum, due to considerable fluorescence enhancement at 612 nm caused by the addition of fluoride (49). Furthermore, the probe 1 is a rapid analytical method for the detection of fluoride. Previous research suggested that the fluorescence intensity at 612 nm increased with reaction time and then leveled off at a reaction time >>25 min (49), which made probe 1 use easier. On the basis of this finding 30 min was chosen as the time point to assess the fluorescence intensity of fluoride using probe 1 in the present study.

The second effect of fluoride on ameloblasts an increase in CIC-5 and CIC-7 expression. As voltage-gated chloride

channels, CIC-5 and CIC-7 are responsible for exchanging Cl⁻ and H⁺ to maintain cell acidification. High expression levels of CIC-5 promote the influx of fluoride into the cytoplasm. CIC-7 localizes in vesicles of the endocytotic-lysosomal pathway in different cell types (30). It has been indicated that during the maturation stage of ameloblasts, the highest levels of CIC-7 are localized in ameloblast vesicles (33,55). Thus, the high expression of CIC-7 promotes the influx of fluoride into the endosome/lysosome. This may induce toxicity in ameloblasts, thereby leading to dental fluorosis (8,13). CFTR stimulates the transport activity of Slc26a members, which leads to bicarbonate efflux (37,56,57). Additionally, CFTR is permeable to bicarbonate (37,58,59). Although the results of previous studies have indicated that CFTR is more permeable to Cl⁻ than to bicarbonate, studies have revealed that CFTR may be responsible for more than 50% of the total bicarbonate efflux in pancreatic duct cells (37,53,58). The low pH caused by HA deposition may lead to an increase in expression of the electrolyte transporter responsible for the efflux of bicarbonate (60,61). The released bicarbonate may neutralize protons to produce a microenvironment that favors crystal nucleation. However, in the present study, no significant changes in CFTR were identified. This may be due to the use of different cell types or low expression of CFTR in LS8 cells. Further research is needed to better understand the mechanism underlying CFTR expression. Duan *et al* (38) found that excess fluoride inhibited endocytic activity of ameloblasts through the CFTR chloride channel or other chloride channels. CFTR siRNA and CFTR-specific channel inhibitor were employed to disturb the function of CFTR, which could examine the effects on the transport activity in presence of fluoride (38). Thus, future work will assess the effects of CICs inhibitors

or silencing with siRNAs on the expression and transport activity of ameloblasts.

TGF- β 1 is distributed in most cells and is activated through the release of latency-associated protein 1 and latent TGF- β -binding protein 1 (62-64). Active TGF- β 1 may activate and affect organic homeostasis by binding to the transforming growth factor- β receptor 1 (TGF- β R1) through the TGF- β 1-Smad signal pathway. Previous studies have shown that fluoride may repress the expression of TGF- β 1 (13,41,65,66) and the present study confirmed this result. In the present study, fluoride significantly reduced the expression levels of TGF- β 1 in ameloblasts at the gene and protein levels. TGF- β 1 may also impair the expression and function of chloride ion channels in other epithelial cells, including alveolar, bronchial, vas deferens and nasal polyp epithelial cells (43-46). To investigate the relationship between TGF- β 1 and chloride ion channels, exogenous TGF- β 1 was added to the culture medium. The present results suggested a largely negative correlation between them. Exogenous TGF- β 1 significantly reduced the expression levels of CIC-5 and CIC-7 in comparison with a control. When TGF- β 1 and high fluoride levels were added to the medium at the same time, the increase in the expression levels of CIC-5 and CIC-7 caused by fluoride was markedly reduced compared with high fluoride treatment alone. These results suggested that the addition of exogenous TGF- β 1 may compensate for the changes in CIC-5 and CIC-7 caused by the decrease of endogenous TGF- β 1 induced by fluoride. With the inhibition of CIC-5 and CIC-7 expression, the red fluorescence intensity of intracellular fluoride was weaker compared with that of the control group.

Both the decreased pH and the highly expressed CIC-5 and CIC-7 levels caused by the high fluoride concentration accelerated the accumulation of fluoride in ameloblasts. If the buffering capacity of ameloblasts is overwhelmed by an excessive amount of fluoride, hypomineralization occurs and may cause dental fluorosis. The present study may provide a new perspective for the prevention of dental fluorosis through TGF- β -mediated reduction of fluoride accumulation. The present study assessed the effect of exogenous TGF- β 1 on preventing the inflow of fluoride to ameloblasts through regulation of CIC-5 and CIC-7 for 24 h. A longer time course will be utilized in future studies. Additionally, further studies are required to investigate the precise mechanism by which the ions are transported. Studies will also be conducted to confirm this hypothesis *in vivo*.

In conclusion, with the use of probe 1, it was demonstrated that exogenous TGF- β 1 may prevent the accumulation of intracellular fluoride through the regulation of CIC-5 and CIC-7 in ameloblasts under high extracellular fluoride concentrations.

Acknowledgements

The authors would like to thank Professor Malcolm L. Snead (University of Southern California, USA) and Professor Baocun Zhu (Jinan University, Shandong, China) for kindly donating LS8 cells and probe 1 respectively.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DZ and XD contributed to the conception and design of the present study. MJ, XH and JS conducted all the experiments. MJ and XD drafted the manuscript and revised it critically for important intellectual content. MJ, XH and JS interpreted and analyzed the data. MJ and XH were responsible for assessing the authenticity of all the raw data. All authors read and approve the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Truman BI, Gooch BF, Sulemana I, Gift HC, Horowitz AM, Evans CA, Griffin SO, Carande-Kulis VG and Task Force on Community Preventive Services: Reviews of evidence on interventions to prevent dental caries, oral and pharyngeal cancers, and sports-related craniofacial injuries. *Am J Prev Med* 23 (Suppl 1): S21-S54, 2002.
- Dean T, Jay P, Arnold F and Elvove E: Domestic water and dental caries. II. A study of 2832 white children, aged 12-14 years, of 8 suburban communities, including *Lactobacillus acidophilus* studies of 1761 children. *Public Health Reports* 56: 761-792, 1941.
- Denbesten P and Li W: Chronic fluoride toxicity: Dental fluorosis. *Monogr Oral Sci* 22: 81-96, 2011.
- Institute of Medicine: Dietary reference intakes for calcium, phosphorus, magnesium, Vitamin D, and Fluoride. National Academies Press, Washington, DC, 1997.
- Beltrán-Aguilar ED, Barker LK, Canto MT, Dye BA, Gooch BF, Griffin SO, Hyman J, Jaramillo F, Kingman A, Nowjack-Raymer R, *et al*: Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis-United States, 1988-1994 and 1999-2002. *MMWR Surveill Summ* 54: 1-43, 2005.
- Bawden JW, Crenshaw MA, Wright JT and LeGeros RZ: Consideration of possible biologic mechanisms of fluorosis. *J Dent Res* 74: 1349-1352, 1995.
- Robinson C, Connell S, Kirkham J, Brookes SJ, Shore RC and Smith AM: The effect of fluoride on the developing tooth. *Caries Res* 38: 268-276, 2004.
- Sierant ML and Bartlett JD: Stress response pathways in ameloblasts: Implications for amelogenesis and dental fluorosis. *Cells* 1: 631-645, 2012.
- Kawase T and Suzuki A: Studies on the transmembrane migration of fluoride and its effects on proliferation of L-929 fibroblasts (L cells) *in vitro*. *Arch Oral Biol* 34: 103-107, 1989.
- Mittal M and Flora SJ: Effects of individual and combined exposure to sodium arsenite and sodium fluoride on tissue oxidative stress, arsenic and fluoride levels in male mice. *Chem Biol Interact* 162: 128-139, 2006.
- Jin XQ, Xu H, Shi HY, Zhang JM and Zhang HQ: Fluoride-induced oxidative stress of osteoblasts and protective effects of baicalin against fluoride toxicity. *Biol Trace Elem Res* 116: 81-89, 2007.

12. Varol E, Icli A, Aksoy F, Bas HA, Sutcu R, Ersoy IH, Varol S and Ozaydin M: Evaluation of total oxidant status and total antioxidant capacity in patients with endemic fluorosis. *Toxicol Ind Health* 29: 175-180, 2013.
13. Sharma R, Tsuchiya M, Skobe Z, Tannous BA and Bartlett JD: The acid test of fluoride: How pH modulates toxicity. *PLoS One* 5: e10895, 2010.
14. Sharma R, Tsuchiya M and Bartlett JD: Fluoride induces endoplasmic reticulum stress and inhibits protein synthesis and secretion. *Environ Health Perspect* 116: 1142-1146, 2008.
15. Kubota K, Lee DH, Tsuchiya M, Young CS, Everett ET, Martinez-Mier EA, Snead ML, Nguyen L, Urano F and Bartlett JD: Fluoride induces endoplasmic reticulum stress in ameloblasts responsible for dental enamel formation. *J Biol Chem* 280: 23194-23202, 2005.
16. He H, Ganapathy V, Isales CM and Whitford GM: pH-dependent fluoride transport in intestinal brush border membrane vesicles. *Biochim Biophys Acta* 1372: 244-254, 1998.
17. Jiao Y, Zhu B, Chen J and Duan X: Fluorescent sensing of fluoride in cellular system. *Theranostics* 5: 173-187, 2015.
18. Gai L, Chen H, Zhou B, Lu H, Lai G, Li Z and Shen Z: Ratiometric fluorescence chemodosimeters for fluoride anion based on pyrene excimer/monomer transformation. *Chem Commun (Camb)* 48: 10721-10723, 2012.
19. Kim SY, Park J, Koh M, Park SB and Hong J: Fluorescent probe for detection of fluoride in water and bioimaging in A549 human lung carcinoma cells. *Chem Commun (Camb)* 21: 4735-4737, 2009.
20. Ke B, Chen W, Ni N, Cheng Y, Dai C, Dinh H and Wang B: A fluorescent probe for rapid aqueous fluoride detection and cell imaging. *Chem Commun (Camb)* 49: 2494-2496, 2013.
21. Li Y, Zhang X, Zhu B, Yan J and Xu W: A highly selective colorimetric and 'off-on-off' fluorescent probe for fluoride ions. *Anal Sci* 26: 1077-1080, 2010.
22. Zhu B, Yuan F, Li R, Li Y, Wei Q, Ma Z, Du B and Zhang X: A highly selective colorimetric and ratiometric fluorescent chemodosimeter for imaging fluoride ions in living cells. *Chem Commun (Camb)* 47: 7098-7100, 2011.
23. Dawson DC, Smith SS and Mansoura MK: CFTR: Mechanism of anion conduction. *Physiol Rev* 79 (Suppl 1): S47-S75, 1999.
24. Simchowit L: Interactions of bromide, iodide, and fluoride with the pathways of chloride transport and diffusion in human neutrophils. *J Gen Physiol* 91: 835-860, 1988.
25. Ran S, Fuller CM, Arrate MP, Latorre R and Benos DJ: Functional reconstitution of a chloride channel protein from bovine trachea. *J Biol Chem* 267: 20630-20637, 1992.
26. Duszyk M, Liu D, Kamosinska B, French AS and Man SF: Characterization and regulation of a chloride channel from bovine tracheal epithelium. *J Physiol* 489: 81-93, 1995.
27. Anderson M, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE and Welsh MJ: Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 253: 202-205, 1991.
28. Jentsch TJ, Stein V, Weinreich F and Zdebek AA: Molecular structure and physiological function of chloride channels. *Physiol Rev* 82: 503-568, 2002.
29. Stauber T, Weinert S and Jentsch TJ: Cell biology and physiology of CLC chloride channels and transporters. *Compr Physiol* 2: 1701-1744, 2012.
30. Duan X: Ion channels, channelopathies, and tooth formation. *J Dent Res* 93: 117-125, 2014.
31. Hou J, Situ Z and Duan X: CIC chloride channels in tooth germ and odontoblast-like MDPC-23 cells. *Arch Oral Biol* 53: 874-878, 2008.
32. Duan X, Mao Y, Yang T, Wen X, Wang H, Hou J, Xue Y and Zhang R: CIC-5 regulates dentin development through TGF- β 1 pathway. *Arch Oral Biol* 54: 1118-1124, 2009.
33. Lacruz RS, Brookes SJ, Wen X, Jimenez JM, Vikman S, Hu P, White SN, Lyngstadaas SP, Okamoto CT, Smith CE and Paine ML: Adaptor protein complex 2-mediated, clathrin-dependent endocytosis, and related gene activities, are a prominent feature during maturation stage amelogenesis. *J Bone Miner Res* 28: 672-687, 2013.
34. Wright JT, Kiefer CL, Hall KI and Grubb BR: Abnormal enamel development in a cystic fibrosis transgenic mouse model. *J Dent Res* 75: 966-973, 1996.
35. Lacruz RS, Smith CE, Moffatt P, Chang EH, Bromage TG, Bringas P Jr, Nanci A, Baniwal SK, Zabner J, Welsh MJ, *et al*: Requirements for ion and solute transport, and pH regulation during enamel maturation. *J Cell Physiol* 227: 1776-1785, 2012.
36. Sui W, Boyd C and Wright JT: Altered pH regulation during enamel development in the cystic fibrosis mouse incisor. *J Dent Res* 82: 388-392, 2003.
37. Bronckers A, Kalogeraki L, Jorna HJ, Wilke M, Bervoets TJ, Lyaruu DM, Zandieh-Doulabi B, Denbesten P and de Jonge H: The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in maturation stage ameloblasts, odontoblasts and bone cells. *Bone* 46: 1188-1196, 2010.
38. Duan X, Mao Y, Wen X, Yang T and Xue Y: Excess fluoride interferes with chloride-channel-dependent endocytosis in ameloblasts. *J Dent Res* 90: 175-180, 2011.
39. Maeda H, Wada N, Tomokiyo A, Monnouchi S and Akamine A: Prospective potency of TGF- β 1 on maintenance and regeneration of periodontal tissue. *Int Rev Cell Mol Biol* 304: 283-367, 2013.
40. Zhao H, Oka K, Bringas P, Kaartinen V and Chai Y: TGF- β 1 type I receptor *Alk5* regulates tooth initiation and mandible patterning in a type II receptor-independent manner. *Dev Biol* 320: 19-29, 2008.
41. Suzuki M, Shin M, Simmer JP and Bartlett JD: Fluoride affects enamel protein content via TGF- β 1-mediated *KLK4* inhibition. *J Dent Res* 93: 1022-1027, 2014.
42. Den Besten PK: Mechanism and timing of fluoride effects on developing enamel. *J Public Health Dent* 59: 247-251, 1999.
43. Roux J, Carles M, Koh H, Goolaerts A, Ganter MT, Chesebro BB, Howard M, Houseman BT, Finkbeiner W, Shokat KM, *et al*: Transforming growth factor β 1 inhibits cystic fibrosis transmembrane conductance regulator-dependent cAMP-stimulated alveolar epithelial fluid transport via a phosphatidylinositol 3-kinase-dependent mechanism. *J Biol Chem* 285: 4278-4290, 2010.
44. Snodgrass SM, Cihil KM, Cornuet PK, Myerburg MM and Swiatecka-Urban A: Tgf- β 1 inhibits Cfrt biogenesis and prevents functional rescue of Δ F508-Cfrt in primary differentiated human bronchial epithelial cells. *PLoS One* 8: e63167, 2013.
45. Yi S, Pierucci-Alves F and Schultz BD: Transforming growth factor- β 1 impairs CFTR-mediated anion secretion across cultured porcine vas deferens epithelial monolayer via the p38 MAPK pathway. *Am J Physiol Cell Physiol* 305: C867-C876, 2013.
46. Pruliere-Escabasse V, Fanen P, Dazy AC, Lechapt-Zalcman E, Rideau D, Edelman A, Escudier E and Coste A: TGF- β 1 downregulates CFTR expression and function in nasal polyps of non-CF patients. *Am J Physiol Lung Cell Mol Physiol* 288: L77-L83, 2005.
47. Faibish D, Suzuki M and Bartlett JD: Appropriate real-time PCR reference genes for fluoride treatment studies performed in vitro or in vivo. *Arch Oral Biol* 62: 33-42, 2016.
48. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
49. Zhu B, Kan H, Liu J, Liu H, Wei Q and Du B: A highly selective ratiometric visual and red-emitting fluorescent dual-channel probe for imaging fluoride anions in living cells. *Biosens Bioelectron* 52: 298-303, 2014.
50. Smith CE, Chong DL, Bartlett JD and Margolis HC: Mineral acquisition rates in developing enamel on maxillary and mandibular incisors of rats and mice: Implications to extracellular acid loading as apatite crystals mature. *J Bone Miner Res* 20: 240-249, 2005.
51. Lyaruu DM, Medina JF, Sarvide S, Bervoets TJ, Everts V, Denbesten P, Smith CE and Bronckers AL: Barrier formation: Potential molecular mechanism of enamel fluorosis. *J Dent Res* 93: 96-102, 2014.
52. Bronckers AL, Lyaruu DM, Jansen ID, Medina JF, Kellokumpu S, Hoeben KA, Gawenis LR, Oude-Elferink RP and Everts V: Localization and function of the anion exchanger *Ae2* in developing teeth and orofacial bone in rodents. *J Exp Zool B Mol Dev Evol* 312B: 375-387, 2009.
53. Ji M, Xiao L, Xu L, Huang S and Zhang D: How pH is regulated during amelogenesis in dental fluorosis. *Exp Ther Med* 16: 3759-3765, 2018.
54. Fan J, Hu M, Zhan P and Peng X: Energy transfer cassettes based on organic fluorophores: Construction and applications in ratiometric sensing. *Chem Soc Rev* 42: 29-43, 2013.
55. Leisle L, Ludwig CF, Wagner FA, Jentsch TJ and Stauber T: CIC-7 is a slowly voltage-gated 2Cl(-)/1H(+)-exchanger and requires *Ostm1* for transport activity. *EMBO J* 30: 2140-2152, 2011.
56. Ko SB, Zeng W, Dorwart MR, Luo X, Kim KH, Millen L, Goto H, Naruse S, Soyombo A, Thomas PJ and Muallem S: Gating of CFTR by the STAS domain of SLC26 transporters. *Nat Cell Biol* 6: 343-350, 2004.

57. Mount DB and Romero MF: The SLC26 gene family of multi-functional anion exchangers. *Pflugers Arch* 447: 710-721, 2004.
58. Ishiguro H, Steward MC, Naruse S, Ko SB, Goto H, Case RM, Kondo T and Yamamoto A: CFTR functions as a bicarbonate channel in pancreatic duct cells. *J Gen Physiol* 133: 315-326, 2009.
59. Shcheynikov N, Kim KH, Kim KM, Dorwart MR, Ko SB, Goto H, Naruse S, Thomas PJ and Muallem S: Dynamic control of cystic fibrosis transmembrane conductance regulator Cl⁻/HCO₃⁻ selectivity by external Cl⁻. *J Biol Chem* 279: 21857-21865, 2004.
60. Paine ML, Snead ML, Wang HJ, Abuladze N, Pushkin A, Liu W, Kao LY, Wall SM, Kim YH and Kurtz I: Role of NBCe1 and AE2 in secretory ameloblasts. *J Dent Res* 87: 391-395, 2008.
61. Zheng L, Zhang Y, He P, Kim J, Schneider R, Bronckers AL, Lyaruu DM and DenBesten PK: NBCe1 in mouse and human ameloblasts may be indirectly regulated by fluoride. *J Dent Res* 90: 782-787, 2011.
62. Lawrence DA, Pircher R and Jullien P: Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. *Biochem Biophys Res Commun* 133: 1026-1034, 1985.
63. Munger JS, Harpel JG, Gleizes PE, Mazzieri R, Nunes I and Rifkin DB: Latent transforming growth factor-beta: Structural features and mechanisms of activation. *Kidney Int* 51: 1376-1382, 1997.
64. Oklu R and Hesketh R: The latent transforming growth factor beta binding protein (LTBP) family. *Biochem J* 352 Pt 3: 601-610, 2000.
65. Suzuki M, Everett ET, Whitford GM and Bartlett JD: 4-phenylbutyrate mitigates fluoride-induced cytotoxicity in ALC cells. *Front Physiol* 8: 302, 2017.
66. Zhang X, Zhang Y, Xi S, Cheng G and Guo X: The effect of different fluoride concentrations on the expression of transforming growth factor-beta1 in ameloblast of rat incisor. *Hua Xi Kou Qiang Yi Xue Za Zhi* 30: 434-438, 2012 (In Chinese).



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.