

Title: The MAP kinase phosphatase MKP-1 regulates p-ERK1/2 signaling pathway with fluoride treatment

Running title: Fluoride upregulates MKP-1 via inhibiting p-ERK1/2 signaling pathway

Lin Zhao^{1#}, Jiali Su^{2#}, Sijia Liu¹, Yang Li¹, Tao Xi¹, Malcolm L. Snead³, Kristina Xiao Liang^{4*}, Ruizhe Huang^{5*}

¹ Department of Oral Pathology, College of Stomatology, Ningxia Medical University, China.

² Yinchuan Stomatology Hospital, Yinchuan, China.

³ University of Southern California, Herman Ostrow School of Dentistry of USC, Center for Craniofacial Molecular Biology, Los Angeles, USA.

⁴ Center of Excellence for Clinical Research in Neurological Diseases, Haukeland University Hospital, Norway; Department of Clinical Medicine, University of Bergen, Bergen, Norway.

⁵ Clinical Research Center of Shaanxi Province for Dental and Maxillofacial Diseases. Department of Preventive Dentistry, Stomatology Hospital, Xi'an Jiaotong University, Xi'an, China.

*These first authors contributed equally to this work*

*** *These corresponding authors contributed equally***

Corresponding author:

Ruizhe Huang

Department of Preventive Dentistry,
Stomatology Hospital, Xi'an Jiaotong University,
Xiwu Road no. 98, 710004 Xi'an, Shaanxi, China
E-mail: hrzh8897@sina.com

Co-corresponding author:

Kristina Xiao Liang

Department of Clinical Medicine,
University of Bergen,
PO Box 7804,
5020 Bergen, Norway;
Tel. +47 55 97 50 96
E-mail: xiao.liang@uib.no

Abstract

Dental fluorosis is characterized by hypomineralization of tooth enamel caused by ingestion of excessive fluoride during enamel formation. Excess fluoride could have effects on the ERK signaling, which is essential for the ameloblasts differentiation and tooth development. MAP kinase phosphatase-1 (MKP-1) plays a critical role in regulating ERK related kinases. However, the role of MKP-1 in ameloblast and the mechanisms of MKP-1/ERK signaling in the pathogenesis of dental fluorosis are incompletely understood. Here, we adopted an *in vitro* fluorosis cell model using murine ameloblasts-like LS8 cells by employing sodium fluoride (NaF) as inducer. Using this system, we demonstrated that fluoride exposure led to an inhibition of p-MEK and p-ERK1/2 with a subsequent increase in MKP-1 expression in a dose-dependent manner. We further identified, under high dose fluoride, MKP-1 acted as a negative regulator of the fluoride-induced p-ERK1/2 signaling, leading to downregulation of CREB, c-myc, and Elk-1. Furthermore, we evaluated the effects of the ERK or MAPK kinases inhibitor PD98059 and ERK signaling inducer curcumin on fluoride-induced MKP-1 activation and found the treatment abolished the response of fluoride-induced MKP-1 activation and the regulation of the downstream transcription factors. Our results identify a novel MKP-1/ERK signaling mechanism that regulates dental fluorosis and provide a framework for studying the molecular mechanisms of intervention and fluorosis remodeling under normal and pathological conditions. MKP-1 inhibitors may prove to be a benefit therapeutic strategy for dental fluorosis treatment.

Key words: MKP-1, ERK1/2, NaF, LS8, Dental Fluorosis.

Highlights:

1. Fluoride exposure led to a inhibition of phosphorylation of MEK and ERK1/2 with a subsequent increase in MKP-1 expression in a dose-dependent manner.
2. MKP-1 acted as a negative regulator of the fluoride-induced p-ERK1/2 signaling.
3. MKP-1 negatively mediates the downstream regulation of transcription factors CREB, c-myc and Elk-1.
4. PD98059 and curcumin treatment attenuates the gene expression changes of transcript factors in response to high dose fluoride exposure.
5. MKP-1 inhibitors may prove to be a benefit therapeutic strategy for dental fluorosis treatment.

Abbreviations:

MKP-1: MAP kinase phosphatase-1

NaF: sodium fluoride

ERK-MAPK: extracellular signal-regulated kinase/mitogen-activated protein kinase

RTK: receptor tyrosine kinase

MKPs: MAPK phosphatases

MAPK: Mitogen-activated protein kinase

DUSPs: dual-specificity phosphatases

siRNA: silencing RNA

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS: Tris-buffered saline

qRT-PCR: quantitative real-time polymerase chain reaction

p-MEK: phosphorylated form of MEK

p-ERK1/2: phosphorylated forms of ERK1/2

p-MKP-1: phosphorylation of MKP-1

CREB: cAMP-response element-binding protein

c-myc: c- myelocytomatosis oncogene cellular homolog

SRE: serum effect factors

1. Introduction

Fluoride plays a dual role in tooth development. Fluoride at low concentrations can strengthen enamel and prevent tooth decay. When permanent teeth are under development, high exposure to fluoride leads to dental fluorosis, as referred to a condition characterized by staining and pitting of the teeth that affects millions of people worldwide. Although much research has been conducted, the mechanisms underlying its onset and progression remains unknown.

The signaling networks responsible for properly building the dentition have been heavily investigated and the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK-MAPK) pathway. This molecular cascade is initiated by binding of a growth factor to a receptor tyrosine kinase (RTK), leading to the increased phosphorylation of successive kinases, activated effector kinases and the transcription of target genes [1]. Previous research has reported a probable link between fluoride exposure and ERK-MAPK pathway [2]. Investigation of the effects of fluoride on enamel-forming cells isolated from rats (primary enamel cells) revealed ERK pathway as an important regulator during tooth development [3]. Moreover, phosphorylated ERK1/2 are highly expressed in ameloblasts and odontoblasts in mandibular molars and incisors [3]. The finding from a mouse-derived enamel cell line known as LS8 provided an *in vitro* model for understanding the molecular basis of dental fluorosis due to its relative ease of handling in the lab compared with primary enamel cells. Through our previous study of fluoride-treated LS8 cell, we identified a fluoride-induced downregulation of p-ERK1/2 which is further involved in apoptosis [1].

MAPK phosphatases (MKPs) is a negative regulator for Mitogen-activated protein kinase (MAPK) activity via dual-specificity phosphatases (DUSPs). Lately, MAPK phosphatase 1 (MKP-1) has emerged as the main counter-regulator of MAPK signaling [2]. MKP-1 locates in the nuclear region and controls gene expression by inactivating the subcellular group of MAPKs [3]. MKP-1 is the original member of a family of dual-specificity phosphatases that can remove phosphates from tyrosine and threonine in ERK and related kinases [4]. MKP-1 activity can manifest positively or negatively the signaling outcomes through a particular pathway, which varies in different cell types either as a function of the relative activities of the various MAPKs and/or abundance of the MAPK substrate [5]. However, the effect of MKP-1 in ameloblast and how MKP-1 regulates ERK signaling together with their downstream regulation of transcription factors in dental fluorosis are unclear.

In the present study, we apply an established *in vitro* fluorosis system by using murine ameloblasts-like LS8 cells and employed NaF as an inducer for dental fluorosis. We show that fluoride exposure inactivates both MEK and ERK1/2 pathways with a subsequent active in MKP-1, which negatively mediates the downstream regulation of transcription factors cAMP-response element-binding protein (CREB), c-mycelocytomatosis oncogene cellular homolog (c-myc) and Elk-1. Moreover, blocking or enhancing either the ERK pathway attenuates the changes of MKP-1 in response to

high dose fluoride exposure. Together, these data provide evidence MKP-1/ERK mediated pathways contribute to dental fluorosis pathogenesis and, importantly, indicating that MKP-1 inhibitors may prove to be benefit therapeutic strategy for dental fluorosis treatment.

Materials and Methods

Cell culture and treatments

The mouse ameloblast-like cell line (LS8) was cultured in DMEM supplemented with 10% FBS and 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, CA, USA). The incubator atmosphere was humidified and adjusted at 5% CO₂ and 95% air at 37°C. When reached 70–80% confluence, the cells were incubated with serum-free medium containing the indicated concentrations (0~2 mM) of NaF. After the treatment, the cells were incubated for 24 hours or 48 hours at 37°C.

Chemicals and antibodies and antibodies

Rabbit Anti-phospho-p44/42 ERK (#9101), rabbit Anti-phospho-MEK (#9154), Anti-ERK (#9102), and GAPDH (#97166) were purchased from Cell Signaling (Beverly, MA, USA). MKP-1 (#373841) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All antibodies were used at a dilution ratio of 1:500~1:1000 for western blot analysis. Inhibitor PD98059 was purchased from Cell Signaling (Boston, MA, USA). Activator curcumin was purchased from Sigma Aldrich Fluka (St. Louis., MO, USA). The inhibitor and activator were dissolved separately in dime thylsulfoxide (DMSO, Sigma, MO, USA) immediately before use. Anti-rabbit and anti-mouse secondary antibody were purchased from Zhongshan Biological Manufacture (Zhongshan Co., Ltd, Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was supplied by Thermo Scientific Company (Logan, Utah, USA). Fetal bovine serum (FBS) was supplied by Gibco (GIBCO, Invitrogen, CA, USA), Lipofectamine 2000 Transfection Reagent was purchased from Invitrogen (Carlsbad, CA, USA). Trizol Reagent was purchased from Invitrogen (Carlsbad, CA, USA). MKP-1-mus-773 siRNA that specifically target mouse MKP-1 and control siRNA, or FAM-labeled negative control siRNA were purchased from Gene Pharma (Gene Pharma Co., Ltd, Shanghai, China). All procedures were conducted with approval from the Ethics Committee at Xi'an Jiaotong University, Xi'an, China.

Transient transfection siRNA

At 40% confluence, LS8 cells were transfected with silencing RNA (siRNA) against MKP-1, using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA) according to the manufacturer's instruction. Briefly, MKP-1-siRNA was diluted in serum-free culture medium with the transfection reagent, mixed by vortexing and incubated for 20 minutes at room temperature to allow the formation of the transfection complex. Then the MKP-1-siRNA was added to the cells for 24 hours. The

effectiveness of gene silencing was monitored by measuring the MKP-1 levels in relation to GAPDH, as analyzed by qRT-PCR for mRNA expression level. Cells transfected with MKP-1-siRNA, were further exposed to NaF for 24 hours. The mRNA expression level of c-myc, CREB, Elk-1 was determined by using qRT-PCR.

Protein extraction

Cells were washed with chilled PBS and lysed in ice-cold RIPA buffer as previously described [4], consisting of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 10 mM EGTA, 2 mM sodium pyrophosphate, 4 mM paranitrophenyl phosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin and 2 µg/ml pepstatin. Cell lysate was collected using a cells scraper (Corning, Acton, MA) and the homogenates sonicated on ice for 30 min. The lysate was collected by centrifugation at 12,000×g for 15 min at 4°C. The protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL) by extrapolation to dye binding for a standard series of known protein concentration using spectrophotometry.

Western blotting

A volume of supernatant corresponding to an equal mass of protein for each experimental condition was mixed with loading buffer (5-sodium dodecyl sulfate, 5% v/v) and denatured by heating the samples at 95°C for 5 min. Lysate proteins were resolved to size by electrophoresis using 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.22 µm polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a semi-dry blotting system (Bio-Rad, Hercules, CA, USA). Non-specific absorption by the membranes was blocked by incubation with 5g% (w/v) skin milk in Tris-buffered saline (TBS, 500 mM NaCl, 20 mM Tris-HCl pH 7.5) with 0.05% (v/v) Tween-20 for 2 hours. Samples were incubated overnight with one of the following primary antibodies at 4°C: Anti-phospho-ERK and total ERK (1:1000), Anti-phospho-MEK (1:1000), Anti-MKP-1 (1:500), GAPDH (1:1000), each diluted in TBS with 5% (v/v) bovine serum containing 0.1% Tween-20 for 24 hours at room temperature with gentle shaking. The membranes were washed using 0.1% Tween-20 TBS three times for 10 min each and incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse secondary antibody (1:10000), as appropriate for each primary antibody, for 1 hour at room temperature. After washing in TBS-0.1% Tween-20 three times, an enhanced chemiluminescence kit (Millipore, MA, USA) was used to detect immunoreactive protein bands. Blots were immuno-detected with an anti-GAPDH antibody (1:1000) to confirm equal mass of protein loaded among samples. The intensity for each immunoreactive protein band was quantified using a Quantity One densitometer (BioRad, Hercules, USA).

RNA Extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of the cells was extracted using Trizol reagent (Carlsbad, CA, USA) according to the manufacturer's instruction. The quality and quantity of the isolated RNA was examined using a NanoDrop 2000/2000C spectrophotometer with measuring absorbance at 260/280nm. First-strand c-DNA synthesis was performed on 2 µg of total RNA using reverse transcription with Real Master Mix (Thermo Fisher Scientific, Logan, Utah, USA). QRT-PCR was performed in a 10 µL reaction mixture system using an Applied Bio systems 7500 Real-Time PCR System (Thermo Fisher, Waltham, MA, USA) with an initial denaturation of 5 min at 95°C, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Primers used in the amplification were designed and synthesized by Gene Pharma (Gene Pharma Inc., Shanghai, China). The sequences of the PCR primers were as follow: MKP-1 F:5' CCCCTGAGTACTAGTGTGCCTGAC 3', MKP-1 R:5' AGCTGAAGTTC GGGGAGATGATAC 3'; C-myc F:5' GCT GCA TGA GGA GAC ACC 3', c-myc R:5' GTG CGG AGG TTT GCT GTG 3'; CREB F:5' ACA GAT TGC CAC ATT AGC 3', CREB R:5' GGA CTT GTG GAG ACT GGA 3'; Elk-1 F:5' ATATCATCCGCAAGGTG AGC3', Elk-1 R:5' ATGGCCGAGGTTACAGACAC3'; GAPDH F:5' GCTGA GTATGTCGTGGAGT3, 'GAPDH R:5' GTTCACACCCATCACAAAC3; were used as the internal control. A melting curve analysis was performed on each amplicon to ensure amplification of a single PCR product. The relative expression levels were calculated using the comparative threshold cycle ($\Delta\Delta CT$) method.

Statistical analysis

Statistical analyses were performed using SPSS software, Version 18.0 (SPSS Inc; Chicago, IL). All data were expressed as mean \pm standard deviation (SD) with each experiment performed in triplicates. Differences among groups were tested by one-way ANOVA or two-way ANOVA, and the T test was used for two individual comparisons. For all analyses, two-tailed *p* values of less than 0.05 were considered significant.

More details on the descriptions of chemicals and antibodies, transfection siRNA, protein extraction, western blotting, RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) are listed in supplementary material.

Results

Fluoride exposure inhibits phosphorylation of MEK and ERK1/2 with a subsequent increase in MKP-1 expression via a dose-dependent manner.

Previously, our group has established the *in vitro* dental fluorosis model by treating NaF in murine ameloblasts-like LS8 cells [1]. Briefly, LS8 cells were incubated with NaF at a serial concentration of 0 mmol/L, 1 mmol/L and 2 mmol/L for 48 hours. We observed that NaF treatment induced a significant decrease on cell number with a dosage-dependent manner (Figure 1A). However, the cell morphology was not changed after the NaF treatment (Figure 1A).

By using this model, we examined whether fluoride treatment in our cell system leads to MEK and ERK activation. LS8 cells were treated with NaF for 48 hours with various concentrations (0 mmol/L, 1 mmol/L, 2 mmol/L), and total cell lysates were subjected to western blot analysis using an antibody against the phosphorylated form of MEK (p-MEK) and antibodies against phosphorylated forms of ERK1/2 (p-ERK1/2). As shown in Figure 1, NaF treatment led to a significant downregulated expression level of p-MEK (Figure 1A, a&b) and p-ERK1/2 (Figure 1B, a&b) in NaF-treated cells compared to un-treated group in a dose-dependent manner. MAPKs are deactivated by members of the MAPK phosphatases such as MKP-1, which is vital in ERK and related kinases activation and plays a critical role in regulation of MAPK signaling in various peripheral tissues. Therefore, we investigated the MKP-1 expression in the cells in the present of NaF. In contrast, we showed that the phosphorylation of MKP-1 (p-MKP-1, Figure 1C, a&b) was strongly activated in fluoride-treated cells than those in control via a dose-dependent manner, suggesting the negative feedback regulation of the MAPK/ ERK pathway. These results indicate that fluoride exposure inhibits phosphorylation of MEK and ERK1/2 with a subsequent increase in MKP-1 expression via a dose-dependent manner.

High dose fluoride exposure activates *MKP-1* gene transcription and induces downregulation of downstream transcription factors.

MKP-1 is an important kinase as the downstream regulators of both p38 and ERK1/2 [4]. Activation of MKP-1 is regulated through phosphorylation of several transcription factors, including CREB, c-myc and Elk-1. The severity of dental fluorosis is dependent upon fluoride dose and the timing and duration of fluoride exposure. Therefore, we used the LS8 cells with fluoride treatment under a high dose (2 mmol/L) to mimic the *in vivo* dental fluorosis and investigated whether this condition activates *MKP-1* gene expression and their downstream transcription factors c-myc, CREB and Elk-1. QRT-PCR measurement demonstrated a significantly increased mRNA expression of *MKP-1* (Figure 2A), confirming the positive feedback control mechanism induced by fluoride treatment as demonstrated above in Figure 1C. Quantification of mRNA expression of the downstream molecule CREB displayed a downregulation of *CREB* gene level in fluoride treated LS8 cells versus control cells, with similar change in both concentration (Figure 2B). Furthermore, a significantly decreased level of *c-myc* (Figure 2C) and *Elk-1* (Figure 2D) transcription expression was identified in both NaF-treated groups compared to un-treated cells with a dosage-dependent pattern. These data suggest a negative feedback between MKP-1 signaling and downstream regulation of transcription factors *CREB*, *c-myc* and *Elk-1*.

PD98059 and curcumin treatment attenuates the changes of MKP-1 protein expression in response to high dose fluoride exposure.

PD98059 is a potent and selective inhibitor of MAPK kinases via binding to the inactive form of MAPK and prevents activation by upstream activators, such as c-Raf [10]. Curcumin, one of the main bioactive components extracted from a traditional Chinese

medicinal herb, induces ERK/MARK activation with up-regulating phospholortated ERK1/2 signaling [11]. To further confirm the linkage between MARK and ERK signaling in dental fluorosis, the LS8 cells were treated with 50 μ M PD98059 for 1 hour and then incubated with 2mM NaF for 48 hours. Western blot assessment was then applied to reveal the protein expression changes of p-ERK1/2 and MKP-1 for the cells under different combinations of the treatment. Our results showed, compared to the control and NaF group, a decreased expression of p-ERK1/2 in 9PD98059, 9PD98059 and NaF treated groups, suggesting the treatment of PD98059 were able to inhibit p-ERK1/2. In addition, we demonstrated that PD98059 and NaF treated groups showed significantly lower levels of p-ERK1/2 than that with 9PD98059 alone (Figure 3A). In contrast, the protein expression level of MKP-1 increased gradually from the cells treated with 9PD98059 alone, 9PD98059 and NaF or NaF alone (Figure 3B), where 9PD98059 and NaF exposed cells demonstrated a significantly increased level of MKP-1 than that exposed in NaF alone (Figure 3B). To validate this observation, we used 1 μ M curcumin to treat the LS8 cells for 1 hours before NaF treatment and we found that, curcumin treated cells exhibited significantly higher p-ERK1/2 expression when normalized with total expression of ERK1/2 than untreated cells (Figure 3C), suggesting treatment of curcumin actives the ERK1/2 signaling in LS8 cells. When the cells treated with curcumin alone, MKP-1 expression elevated in the all three treated groups (Figure 3D) with a gradual decrease from the cells treated with curcumin alone, curcumin and NaF or NaF alone. In addition, curcumin and NaF treated groups showed significantly higher level of MKP-1 than that with NaF alone (Figure 3D). These finding further confirm the effect of the MKP-1 on negative regulation phosphorylation ERK signaling pathway in the cells with fluoride exposure, as described above (Figure 1).

PD98059 and curcumin treatment attenuates the gene expression changes of transcript factors in response to high dose fluoride exposure.

Our findings in Figure 2 suggested a negative feedback between MKP-1 signaling and downstream regulation of transcription factors *CREB*, *c-myc* and *Elk-1*. To further validate this observation, qRT-PCR measurement was applied to measure the of mRNA expression of *c-myc*, *CREB*, *Elk-1* in LS8 cells pre-incubated with PD98059 (50 μ M) or curcumin (1 μ M) for 1 hour, followed by incubation with 2.0 mM NaF for 24 hours. Our data showed that mRNA expression of *c-myc* and *Elk-1* decreased in the cells treated with PD98059, PD98059 and NaF than NaF group and control group, whereas the mRNA expression of *CREB* dropped in the group in the present of PD98059, PD98059 and NaF, and NaF than control group (Figure 4A). In contrast, curcumin treated cells showed a significantly highest transcriptional expression level for *c-myc*, *CREB* and *Elk-1* among the cells treated with control, curcumin and NaF, and NaF (Figure 4B). To further confirm whether lack of MKP-1 protein expression in response to fluoride treatment is regulated through a transcriptional mechanism via modulating downstream transcription factors c-myc, CREB and Elk-1, we then transfected the LS8 cells with siRNA targeting MKP-1 to know-out the MKP-1 expression before the qRT-

PCR was performed. MOCK siRNAs with the sequences that do not target any gene product was used for negative control. After transfection of GFP siRNA-MKP-1 for 6 hours, we detected a bright green fluorescent signal, indicating the cells expressed MKP-1 after transfection (Figure 4C). We demonstrated that mRNA expression of *c-myc*, *CREB* and *Elk-1* increased in LS8 cells treated with siRNA-MKP-1 than all the other groups (Figure 4C). Similarly, a significantly upregulated mRNA expression of *c-myc*, *CREB* and *Elk-1* was identified in LS8 cells transfected with siRNA-MKP-1 compared to that in MOCK control group ($P < 0.05$, Figure 4C). More importantly, curcumin and NaF treated cells showed a significant increase of *c-myc*, *CREB* and *Elk-1* mRNA expression than NaF treat cells ($P < 0.05$, Figure 4E). These evidences suggest that MKP-1 can facilitate gene transcription through modulating downstream transcription factors *c-myc*, *CREB* and *Elk-1* in fluoride treated cells. Therefore, the previous observations in the qRT-PCR have been confirmed on this point.

5. Discussion

Our previous evidence showed the activation of ERK1/2 and other MAP Kinase in dental pulp cells [12] and excess fluoride have effects on the ERK signaling [1]. MKP-1, as an inhibitor of MAPKs, plays an essential role in regulating ERK related kinases. However, it remains unclear on how MKP-1 is regulated in dental fluorosis. Here, we present compelling evidence that treatment with fluoride in vitro at the millimolar concentrations markedly activated MKP-1 expression and decreased MEK and ERK1/2 phosphorylation level in a dose-dependent manner in mouse ameloblasts-like LS8 cells [1], indicating a negative feedback between MKP-1 and MEK/ERK signaling. We further use high dose fluoride to induce the dental fluorosis and demonstrated a negative regulatory role of MKP-1 in the fluoride-induced p-ERK1/2 signaling. We also found that MKP-1 downregulated ERK/MAPK-mediated CREB, *c-myc*, and *Elk-1* transcriptions. Our study clearly demonstrates the effect of MKP-1 on dental fluorosis and its therapeutic potential for the treatment of dental fluorosis.

In our previous work, we reported a positive expression of p-ERK, p-JNK, p-p38 and fluoride-induced apoptosis led to the decreased phosphorylation and deactivation of ERK and JNK signaling cascade in a concentration- and time-dependent manner in ameloblasts cells, indicating a linkage of MAPK signaling on dental fluorosis [1]. In present study, we confirm the MEK and ERK1/2 phosphorylation in ameloblasts and their downregulation induced by fluoride exposure.

MAPK is a serine/threonine protein kinase that is widely present in eukaryotic cells. Previous studies found four different MAPKs, including ERK, *c*-JNK, ERK5 and p38 MAPK (p38) [13]. ERK cascade reaction can be activated by various stimuli, such as RTK and G protein-coupled receptors. After activation, it can regulate the proliferation, differentiation and apoptosis. Ras/Raf/MEK/ERK cascade reaction is an important signaling pathway in MAPKs. Various stimuli can activate the corresponding cell surface receptors that, in turn, activates the signal transduction pathway and produce an appropriate biological response. The Ras/Raf/MEK/ERK cascade reaction is the key

factor in integrating the signal transduction pathway. Previous observation of individuals with orofacial and craniofacial disorders identified an association of Ras/Raf/MEK/ERK pathway and dental malformations [14]. MKP-1 has the capacity to bind and dephosphorylate ERK, and subsequently p38 and JNK kinases. MKP-1 exerts dual effects under different physiological conditions. Previous studies have suggested that MKP-1 at relatively low level has a weak binding affinity for ERK kinases and exerts little effect on their function, whereas MKP-1 is activated and shows a higher binding affinity for p38 and JNK kinases than ERK kinases under other physiological conditions [5, 6]. When MKP-1 levels are high, it also inhibits ERK kinases, although to a lesser extent than MARK/p38 and JNK, and thus provides a negative feedback loop on many cellular processes. Consistent with these findings, here, we report a gradual upregulation of MKP-1 and inhibition of MEK and ERK expression from low to high fluoride exposure. Furthermore, we show that the relative high phosphorylation level of MKP-1 is associated with the negative feedback regulation of the MAPK/ERK pathway, which may contribute to the pathogenesis in dental fluorosis.

ERK-induced Elk-1 phosphorylation leads to enhanced DNA-binding and TCF-mediated transcriptional activation. Several early genes, such as c-jun, c-fos, and c-myc, are regulated upstream by Ras/ERK and p38. They were found to be involved in controlling the cell growth and metabolism [15]. Elk-1, after phosphorylation by ERK, binds to the SRE cis-acting element in the promoter region of c-fos and induces its transcription. Transcription factor Elk-1 is a part of the ternary complex which can be combined with serum effect factors (SRE) to regulate gene activity in order to reply to the serum and growth factors [16]. The activation of ERK1/2 is transferred from the cytoplasmic to nuclear and activates its downstream substrate Elk-1, and the phosphorylation of Elk-1 will promote the function of cell differentiation, proliferation and apoptosis [17-19]. In addition, CREB and c-myc are also recognized as putative active specific transcriptional factor substrates which mediate indirectly by extracellular signal-regulated kinase such as ERK [7]. Activated phosphorylation of CREB is reported in human molar odontoblasts and cementoblasts in vivo [20]. Study in osteoblast cells reports that ERK/CREB signaling can inhibit cellular oxidative stress [21]. However, some studies reported that MEK/ERK/CREB signaling pathway may not be the solely mediator in the signal transduction pathways in dental pulp cells [22]. The myelocytomatosis oncogene cellular homolog c-myc family consists of transcription factors which modulates genes responsible for cellular homeostasis [8]. *C-myc* gene transcription and protein stabilization and accumulation [9] are sustained by the hyper-activated RAS/MEK/ERK pathway. In this study, we demonstrate that MKP-1 reduction results in the deactivation of ERK-induced Elk-1 phosphorylation in cytoplasm, which consequently leads to downregulate the downstream substrates CREB and c-myc.

In summary, we determine that upregulation of MKP-1 induced by fluoride treatment via negatively regulating cellular p-MEK1/2 levels following feedback-regulated p-ERK1/2 signaling, which consequently facilitates gene transcription through

modulating specific transcription factor substrates c-myc, CREB and Elk-1 in fluoride treated cells. These findings provide novel insights into the role of MKP-1 in the pathogenesis of dental fluorosis and its potential as a new target for dental fluorosis therapy.

Figure Legends:

Figure 1. The study design and the effect of NaF on the expression of MKP-1, p-ERK1/2, p-MEK proteins in LS8 cells. (A). The study design demonstrates that LS8 cells are treated with NaF under at a serial final concentration to model the dental fluorosis. Western blotting analysis is used to measure p-MEK, p-ERK1/2 and MKP-1 expression. QRT-PCR is performed to demonstrate the mRNA expression for transcription factors CREB, c-myc and Elk-1 after the treatment. Inhibitor PD98059 and activator Curcumin for ERK1/2 signaling were added for further validation. (B). Bright-field images for the cells treated with NaF at different concentration (0 mmol/L, 1 mmol/L and 2 mmol/L) for 48 hours. Magnification is 100X. (C) - (E): Western blot analysis of protein expression of p-MEK (C), p-ERK1/2 (D) and MKP-1 (E). Each value (mean \pm SD) is expressed as the ratio of the phosphorylated ERK1/2 protein level to the corresponding total ERK1/2 protein level, p-MEK and MKP-1 to GAPDH protein level. Statistical significance of: *, $p < 0.05$. when $p < 0.05$ was determined by calculation using values obtained from untreated control group (NaF 0 mM).

Figure 2. mRNA expression of MKP-1, c-myc, CREB, Elk-1 changes in LS8 treated with NaF. (A): QRT-PCR measurement of mRNA expression of MKP-. (B) - (C): QRT-PCR analysis of c-myc, CREB, Elk-1 mRNA level. Cells were incubated in 1mmol/L and 2 mmol/L NaF after 48 hours before applied to qRT-PCR. The data presented as the Each value (mean \pm SD). Statistical significance of: *, $p < 0.05$, **, $p < 0.01$, when $p < 0.05$ and $p < 0.01$ are determined by calculation using values obtained from untreated control group (NaF 0 mM).

Figure 3. Protein expression after PD98059 and curcumin exposure on NaF-treated LS8 cells. (A) - (B). Western blot analysis for p-ERK1/2 (A) and MKP-1 (B) expression in LS8 cells treated with PD98059 (50 μ M) alone, PD98059 + NaF or NaF alone. (C) Western blot analysis for MKP-1 expressions in LS8 cells treated with curcumin (1 μ M) alone, curcumin (1 μ M) + NaF (2 mM) or NaF (2 mM) alone. (C) Western blot analysis for the p-ERK1/2 and ERK1/2 expressions in LS8 cells with and without curcumin (1 μ M) treatment. The cells were treated either with PD98059 (A, B) or curcumin for 1 hour and then incubated with 2 mM NaF for 48 hours. The value (mean \pm SD) in A and C presents the ratio of p-ERK1/2 to total ERK1/2 expression and value in B and D is expressed as the ratio of MKP-1 to GAPDH expression and the value. Statistical significance of: *, $p < 0.05$, experimental value versus untreated control group if not stated.

Figure 4. mRNA-expression of *c-myc*, *CREB*, *Elk-1* after PD98059, curcumin and siRNA MKP-1 exposure on NaF treated LS8 cells. (A) - (B): QRT-PCR measurement

of *c-myc*, *CREB*, *Elk-1* mRNA expression in LS8 cells pre-incubated with PD98059 (A) and curcumin (B) for 1 hour, followed by incubation with 2.0 mM NaF for 48 hours. (C) Phase-contrast and fluorescent microscopy display the GFP signal in the LS8 cells transfected with siRNA-MKP-1 for 6 hours. Magnification is 100X. (D) - (E): QRT-PCR measurement of mRNA expression of *c-myc*, *CREB*, *Elk-1* in siRNA-MKP-1 transfected - cells (D) and in NaF-post treated cells (E). The data presented as mean value (mean \pm SD). Statistical significance of: *, $p < 0.05$, experimental value versus untreated control group if not stated.

Submission declaration and verification

This work described has not been published previously

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Disclosure statement

The authors report no conflicts of interest.

Author's contributions:

L.Z, J.S, S.L, Y.L, T. X and M. L.S contribute to the experimental investigation; L.Z and K.L contribute to the writing original draft; all the authors contribute to review and edit; R.H contribute the resources, supervision and funding acquisition.

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