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Original Article

Effects of fluoride on the proliferation and activation of osteoblasts by regulating methylation of the DNA repair genes MGMT and MLH1

Long Chen [a], Na Yin [a], Yi Ding [b], Mei-Lin Zhang [c], Min Li [b], Jin-Jie Zhong [d, **], Shu-Mei Feng [b, *]

[a] Functional Center, School of Basic Medical Sciences, Xinjiang Medical University, Urumqi, Xinjiang, 830011, China
[b] Department of Histology and Embryology, School of Basic Medical Sciences, Xinjiang Medical University, Urumqi, Xinjiang, 830011, China
[c] Cilinical Laboratoray, Urumqi Blood Center, Urumqi, Xinjiang, 830000, China
[d] Department of Basic Medicine Sciences, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310058, China

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ABSTRACT

Introduction: Fluoride can induce the proliferation and activation of osteoblasts, resulting in skeletal fluorosis progression; however, the specific mechanism is unclear.
Methods: Cell proliferation was examined using the MTT assay. Flow cytometry was performed to detect the cell cycle distribution. Alkaline phosphatase (ALP) was calculated to evaluate bone formation and turnover. Gene methylation was examined using the MSP assay. mRNA and protein expression levels were assessed using qRT-PCR and Western blot assays.
Results: Low-concentration NaF treatment promoted the cell cycle progression of osteoblasts to S-phase, thus accelerating cell proliferation and activation in a concentration-dependent manner. In addition, the methylation of the MGMT and MLH1 genes was increased, and their mRNA expression was reduced. Furthermore, the DNA methyltransferase inhibitor 5-AZA-dC suppressed cell viability, cell number in S-phase, ALP activity and osteogenesis-related protein levels in osteoblasts treated with low doses of NaF. Meanwhile, 5-AZA-dC suppressed the increase in MGMT and MLH1 gene methylation in osteoblasts treated with low doses of NaF, leading to enhanced expression of MGMT and MLH1 mRNA.
Conclusion: NaF treatment led to methylation of the DNA repair genes MGMT and MLH1 in osteoblasts, resulting in cell proliferation and activation and causing the development of skeletal fluorosis.

1. Introduction

Bone lesions of skeletal fluorosis are complicated and varied, due mainly to the destruction of bone formation and bone resorption balance and the acceleration of bone turnover [1]. The enhancement of osteogenic activity is an early and dominant link that becomes an important feature of the progression of skeletal fluorosis [2]. Previous studies have shown that the pathogenesis of skeletal fluorosis lesions is characterized mainly by aberrant proliferation and activation of osteoblasts, and the proliferation of osteoblasts is finely regulated by the cell cycle [3]. Daily intake of a small dose of fluoride can promote the normal growth and development of teeth and bones, as well as the normal activity of the enzyme system [4]. If fluoride is taken in large amounts over a long period of time, fluoride can cause abnormal proliferation and activation of osteoblasts and osteoclasts, leading to skeletal

Abbreviations: ALP, Alkaline phosphatase; MSP, Methylation specific PCR; NaF, Sodium fluoride; 5-AZA-dC, 5-aza-2-deoxycytidine; MGMT, O6-Methylguanine-DNA methyltransferase; BMP, Bone morphogenetic protein; BGP, bone gla protein; BLBC, Basal-like breast cancer.

* Corresponding author. Department of Histology and Embryology, School of Basic Medical Sciences, Xinjiang Medical University, No. 567, Shangde North Road, Xuelianshan Campus, Shuimogou Street, Shuimogou District, Urumqi, Xinjiang, 830011, China.

** Corresponding author. Department of Basic Medicine Sciences, Zhejiang University School of Medicine, No. 866, Yuhangtang Road, Xihu District, Hangzhou, Zhejiang, 310058, China.

E-mail addresses: 616504535@qq.com (J.-J. Zhong), 87391167@qq.com (S.-M. Feng).

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fluorosis, which can lead to pain and damage to joints and bones and even permanent disability [3,5].

In a previous study of this subject, the osteoblasts of primary cultured mice were found to always proliferate when the fluoride dose was 5–20 mg/L, and the proliferation of osteoblasts was the highest when the fluoride dose was 10 mg/L (preliminary research on this subject). The expression levels of genes related to the function of osteoblasts, such as Runx-2 and Osteocalcin, and genes related to proliferation, such as MCM3, were remarkably increased (preliminary research on this subject). In osteoblasts treated with NaF, excessive methylation of p16 has been reported to be induced, causing increased cell proliferation, prolonged S-phase of the cell cycle, and skeletal fluorosis progression, while the methylation inhibitor 5-aza-2-deoxycytidine (5-AZA-dC) reverses the hypermethylation of p16 induced by NaF [6].

O6-Methylguanine-DNA methyltransferase (MGMT) is a ubiquitous DNA repair protein that can correct the mismatch of O6 alkyl guanine and directly reverse DNA damage, which plays a key role in the early repair process of DNA damage [7]. In addition to its repair function, MGMT also protects DNA and protects chromatin from chemical carcinogens and cytotoxic attacks, maintaining the original appearance of the DNA [8]. The abnormal methylation of the mismatch repair gene MLH1 can lead to the transcriptional inactivation of mRNA and the loss of protein expression, which will result in defects in the mismatch repair function of the body, thus causing instability of the whole genome and eventually leading to the occurrence of tumours [9]. As demonstrated in previous reports, MGMT and MLH1 methylation increased in the blood of fluorosis patients [10]. However, studies of MGMT and MLH1 in osteoblast cells treated with fluoride have not been reported.

In this study, we hypothesized that in osteoblasts treated with fluoride, increased methylation of the MGMT and MLH1 genes was induced, leading to a decrease in their expression, thus causing abnormal proliferation and activation of osteoblasts and resulting in skeletal fluorosis progression.

2. Materials and methods

2.1. Cell culture and treatment

The human osteoblast cell lines MG63 and Saos2 were obtained from the Shanghai Institute of Cells, Chinese Academy of Sciences. The cells were cultured in DMEM (Gibco, US) supplemented with 10% FBS (Life Technologies, US) under environmental conditions of 37 °C and 5% CO_2. When the cells reached approximately 80% density, routine cell passage was carried out.

The dosage of NaF was set as 0, 2.5, 5, 10, 20 and 40 mg/L, and the osteoblasts were exposed to NaF at 24 h, 48 h and 72 h to evaluate the optimal concentration and time of NaF treatment on the proliferation and activation of MG-63 and SAOS-2 cells.

NaF (10 mg/L) together with 5-AZA-dC (Sigma, USA) at 5, 10 and 20 μmol/L were used to treat the human osteoblast cell lines MG63 and Saos2 for 72 h.

2.2. Detection of cell proliferation by MTT assay

Osteoblast cells were inoculated into a 96-well plate at a density of 5×10^4 cells/well. After treatment, cells were incubated with 10 μL of MTT reagent (Sigma, USA) for 4 h at 37 °C. After discarding the medium, 100 μL of DMSO (Sigma, USA) was added to each well, and the optical density (OD) value was evaluated by measuring the absorbance at 490 nm wavelength.

2.3. Determination of the cell cycle by flow cytometry

After treatment with NaF for 72 h, 1×10^6 osteoblast cells were collected and discarded after rinsing. Then, 1 mL of 70% precooled ethanol was added to the precipitate, which was oscillated and fixed at 4 °C overnight. Then, 0.5 mL of RNase solution was added to the cells and incubated at 37 °C for 30 min. Then, 1.5 mL of propidium iodide (PI) dyeing solution (Invitrogen, USA) was added and mixed well. After incubation in the dark for 30 min at 4 °C, the cell cycle distribution of osteoblasts with NaF treatment was detected by a FACScan flow cytometer (Beckman Coulter, USA). The proportion of each phase of the cell cycle was automatically fitted, and the average was calculated. The proliferation index (PID) was used to indicate the effect of NaF on cell division and proliferation of osteoblast cells. $PID = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$.

2.4. Alkaline phosphatase (ALP) staining

After treatment, the medium was removed, and the cells were harvested and fixed using 70% ethanol for 20 min. Subsequently, the cells were incubated with the ALP colour development solution BCIP/NBT (Sigma, USA). After incubation, the stain was extracted by cetylpyridinium chloride and quantified in a microplate reader at 540 nm.

2.5. MGMT and MLH1 gene methylation detection by methylation-specific (MSP) PCR

After treatment with sodium bisulfite, primers corresponding to the methylated or unmethylated predicted sequences of genomic DNA were synthesized by Beijing Institute of Genomics (China) (Table 1). The MSP reaction system with a total volume of 20 μL consisted of 2 μL of template, 200 nmol/L each primer, 1 × PCR buffer, 4 mmol/L $MgCl_2$, 200 mmol/L dNTPs and 1 U of Hot Star Taq DNA polymerase (Takara, China). The number of PCR cycles was adjusted to 40 cycles so that the amplification was within the linear range. The PCR conditions were 'hot started' at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and a final extension cycle of 72 °C for 10 min. The products of MSP were analyzed by agarose gel electrophoresis containing ethidium bromide (EB). The methylation status was determined using MSP, and the intensities of methylated (M) and unmethylated (U) bands were individually quantified with the aid of the Gel Pro Analyzer ver. 4. The relative amount of methylation in the sample was slightly modified and calculated using the following formula: methylation ratio = M/(M + U), where M and U indicated their band intensities measured by gel scanning.

2.6. Quantitative reverse transcript-PCR

Total RNA from the human osteoblast cell lines MG63 and Saos2 was extracted using TRIzol reagent (Sigma, USA). MGMT and MLH1 were reverse transcribed by using a Takara reverse transcription kit (Takara, China). The relative expression levels were determined by quantitative reverse transcript polymerase chain reaction (qRT-PCR) using SYBR Mix (Takara, China). β-Actin served as the internal

Table 1
Primer sequences for MSP.

Gene		Sequence (5'~3')	Tm	GC (%)
MGMT	forward	TGGTAAATTAAGGTATAGAGTTTTAGG	55.26	55.56
	reverse	AAAACCTAAAAAAAACAAAAAAAC	54.68	50.00
MLH1	forward	TTTTTTTAGGAGTGAAGGAGGTTA	57.71	54.17
	reverse	CCCAAAAAAAACAAAATAAAAATC	57.39	45.83

reference, and data were analyzed with the $2^{-\Delta\Delta Ct}$ method. Gene-specific primers for qRT-PCR analyses were listed as follows:

MGMT-F, 5′-AGTAGGAGGAGCGATGAGGAG-3′,
and MGMT-R, 5′-AGAAGCCACTCTTTCACAGTCT-3’;
MLH1-F, 5′-AGTGGCTGGACAGAGGAACA-3′,
and MLH1-R, 5′-GATCAGGCAGGTTAGCAAGC-3’;
β-actin-F, 5′-GGAGATTACTGCCCTGGCTCCTA-3′,
and β-actin-R, 5′-GACTCATCGTACTCCTGCTTGCTG-3’.

2.7. Western blot

The osteoblast cell lines were dissolved by using cold RIPA reagent (Beyotime, China), and protein concentrations were examined using a BCA kit (Bio-Rad Laboratories, USA), which was separated by SDS-PAGE and transferred onto PVDF membranes (Invitrogen, USA). Then, membranes were incubated overnight with antibodies against rabbit antiRUNX2 antibody (1:1000 dilution, Abcam, UK), rabbit antiALP antibody (1:500 dilution, Abcam) and rabbit antiOCN antibody (1:1000 dilution). After washing with PBS-T, membranes were then incubated with the corresponding secondary antibody. The membranes were visualized and imaged by a GEL imaging system (Bio-Rad, CA, USA). The density of the protein bands was analyzed using ImageJ software.

2.8. Statistical analysis

The SPSS 20.0 software package was used for statistical data analysis in this study. All the tests conducted were repeated at least three times with similar results. The measurement data are expressed as the mean ± standard deviation (SDs). Student's t-test was used for pairwise comparisons, and one-way ANOVA was applied for multigroup comparisons. $P < 0.05$ indicated that the difference was statistically significant.

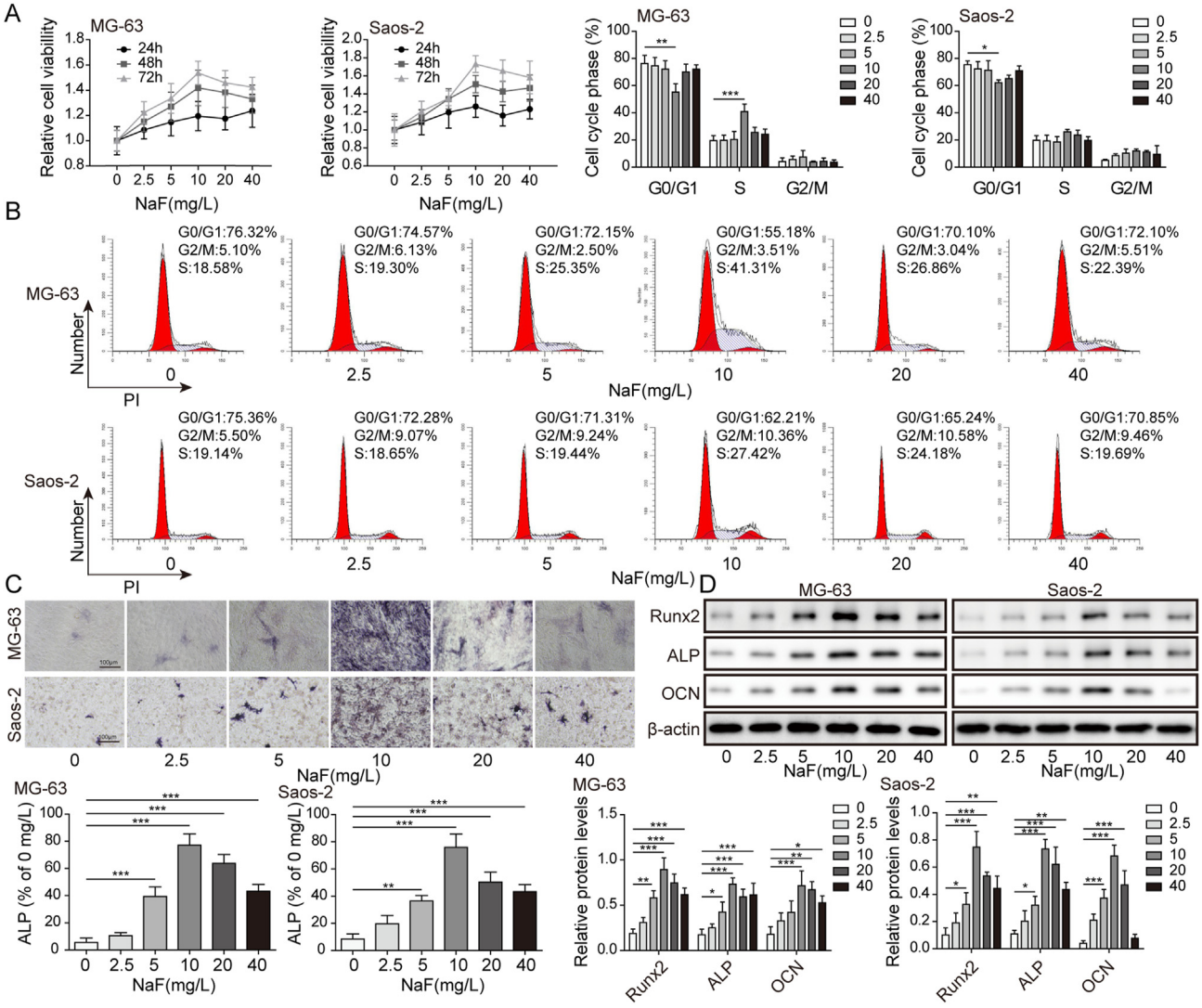

Fig. 1. Effects of different concentrations of NaF on the proliferation and activation of osteoblasts. Human osteoblasts were treated with NaF at concentrations of 0 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 20 mg/L and 40 mg/L for 24 h, 48 h and 72 h, respectively. (A) Cell viability was evaluated using MTT assay. (B) Cell cycle was measured by flow cytometry. (C) ALP activity of human osteoblasts. (D) RUNX2, ALP and OCN levels were detected by Western blot. The measurement data are expressed as the mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. All the above assays were executed three times.

3. Results

3.1. Dose-effect relationship between osteoblast proliferation, activation and NaF treatment

As shown in Fig. 1A, with increasing NaF dosage and time, the effect of NaF on osteoblast proliferation was different. Osteoblasts exposed to NaF at 10 mg/L for 72 h exhibited more cell proliferation compared with other treatment conditions. Therefore, osteoblast cells exposed to NaF for 72 h were selected for subsequent experiments. Low concentrations of NaF increased the number of osteoblasts in S phase and reduced the number of osteoblasts in G_0/G_1 phase in a concentration-dependent manner, and this effect was decreased when the NaF concentration exceeded 10 mg/L (Fig. 1B). ALP activity increased in a concentration-dependent manner when osteoblasts were treated with NaF at low concentrations, and this effect was reduced when the concentration of NaF reached 10 mg/L (Fig. 1C). The Western blot results showed that a low concentration of NaF elevated osteogenesis-related protein (RUNX2, ALP and OCN) levels in a concentration-dependent manner, and when the concentration of NaF reached 10 mg/L, this effect was lowered (Fig. 1D). All of the above data revealed that a low concentration of NaF promoted the cell cycle progression of osteoblasts to S-phase, thereby accelerating cell proliferation and activation in a concentration-dependent manner.

3.2. Induction of MGMT and MLH1 gene methylation in osteoblasts treated with low doses of NaF

As shown in Fig. 2A and C, treatment with a low concentration of NaF elevated MGMT and MLH1 methylation in osteoblasts, and the methylation ratio reached the highest when the NaF concentration was 10 mg/L. When the NaF concentration exceeded 10 mg/L, the methylation ratio gradually decreased. In addition, MGMT and MLH1 expression was reduced in osteoblasts treated with low concentrations of NaF. When the concentration of NaF reached 10 mg/L, the effect was the best, and when the concentration of NaF exceeded 10 mg/L, the effect was inhibited (Fig. 2B and D). Collectively, these results suggested that in osteoblasts treated with low doses of NaF, increased methylation of the MGMT and MLH1 genes was induced, leading to a decrease in their expression.

3.3. 5-AZA-dC inhibited proliferation and activation in osteoblasts treated with low doses of NaF

The methylation inhibitor 5-AZA-dC suppressed the viability of osteoblasts treated with low doses of NaF (10 mg/L) in a dose-dependent manner (Fig. 3A). Moreover, the results of flow cytometry proved that 5-AZA-dC restrained the increase in cell number in the S phase after low-dose NaF (10 mg/L) treatment, and the inhibitory effect was dose-dependent (Fig. 3B). In addition, ALP activity was inhibited in a dose-dependent manner in osteoblasts treated with 10 mg/L NaF together with 5-AZA-dC (Fig. 3C). Western blot assays indicated that 5-AZA-dC suppressed the expression levels of RUNX2, ALP, and OCN in osteoblasts treated with low doses of NaF (10 mg/L) in a dose-dependent manner (Fig. 3D). The above results suggested that the methylation inhibitor 5-AZA-dC restrained the proliferation and activation of osteoblasts with low-dose NaF treatment.

3.4. 5-AZA-dC reduced the methylation of the MGMT and MLH1 genes in osteoblasts treated with a low dose of NaF

As shown in Fig. 4A and C, 5-AZA-dC suppressed the methylation of MGMT and MLH1 in NaF-treated osteoblasts in a dose-dependent manner. Furthermore, 5-AZA-dC elevated MGMT and MLH1 expression in a dose-dependent manner (Fig. 4B and D). All the above data demonstrated that the methylation inhibitor 5-AZA-dC could inhibit the increase in MGMT and MLH1 gene methylation in osteoblasts treated with low-dose NaF, leading to enhanced expression of MGMT and MLH1 mRNA, and the effect was dose-dependent.

4. Discussion

The initial phase of skeletal fluorosis is asymptomatic, and imaging studies show a slight increase in bone mass [11]. The progression of the clinical stage of skeletal fluorosis is closely related to

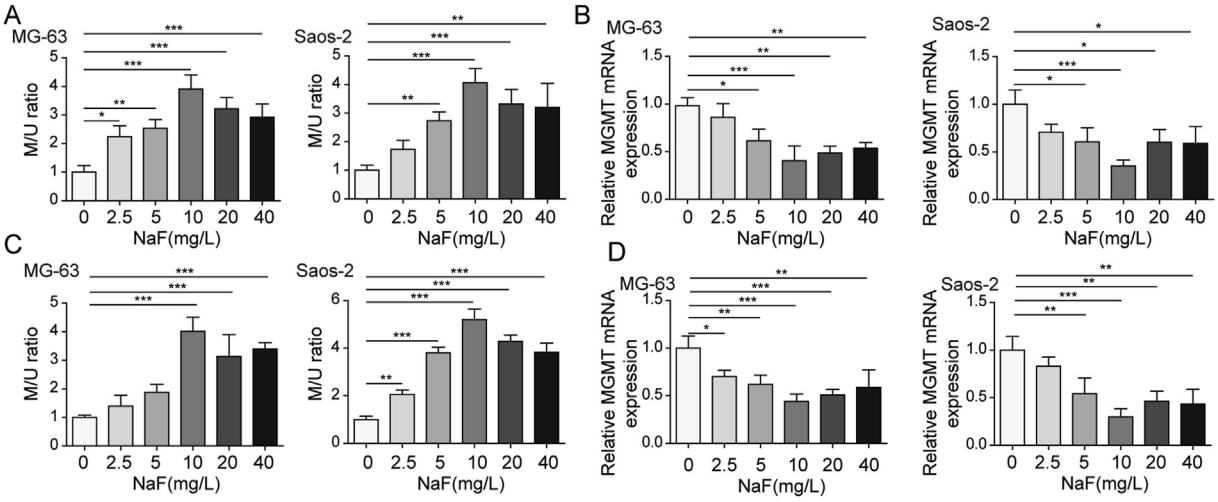

Fig. 2. Low doses of NaF induced increased methylation of the MGMT and MLH1 genes in osteoblasts. Human osteoblasts were treated with NaF at concentrations of 0 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 20 mg/L and 40 mg/L for 72 h. (A) The methylation level of the MGMT gene in MG63 and Saos2 cell lines exposed to NaF at different concentrations was detected by the MSP method. (B) The expression level of MGMT mRNA with different concentrations of NaF treatment was determined by qPCR assay. (C) The methylation level of the MLH1 gene in osteoblasts after exposure to different concentrations of NaF was assessed by the MSP method. (D) The mRNA expression level of MLH1 in MG63 and Saos2 cell lines exposed to NaF was measured by qPCR assay. The measurement data are presented as the mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. All the above assays were executed three independent times.

the measured fluoride load level in tissues [11]. Epidemiological studies have found that the onset of skeletal fluorosis takes a longer time to observe symptoms and may occur in people who consume between 36 and 54 mg of fluoride daily for 10 years or more [12]. Previous studies have shown that fluoride can promote the proliferation of osteoblasts, enhance bone mass and increase the activity of osteoblasts by upregulating alkaline phosphatase (ALP), bone morphogenetic protein (BMP) and bone gla protein (BGP) levels [12]. However, the specific mechanism of action of fluoride on osteoblasts is still not well known. This study proved that after treatment with a low dose of NaF in osteoblasts, MGMT and MLH1 methylation was promoted, resulting in a decrease in their expression, thereby enhancing osteoblastic proliferation and activation and leading to the development of skeletal fluorosis.

Evidence has proven that NaF promotes cell proliferation and suppresses apoptosis of osteoblasts [13]. After the treatment of osteoblasts with NaF, p16 showed hypermethylation and decreased expression levels, causing elevated cell proliferation, prolonged S-phase of the cell cycle, and eventual development of skeletal fluorosis [6]. DNA methylation is a main mechanism of epigenetic gene regulation, which is completed by DNA methyltransferase, and 5-AZA-dC has been proven to be a major methyltransferase-specific inhibitor [14,15]. In this work, we proved that NaF promoted the cell cycle progression of osteoblasts to S-phase, thereby accelerating cell proliferation and activation in a concentration-dependent manner, while the methylation inhibitor 5-AZA-dC restrained these effects.

In the laryngeal cancer Hep-2 cell line, the MGMT gene showed DNA methylation and histone H3–K9 hypermethylation, while 5-AZA-dC reversed H3–K9 methylation of the MGMT gene and upregulated the decreased gene expression of MGMT [16]. The methylation of the MLH1 gene suggested that it might be inactivated by epigenetic mechanisms [17]. The immunoreactive levels of MLH1 and MGMT in patients with basal-like breast cancer (BLBC) were lower than the immunoreactive levels of MLH1 and MGMT in nonBLBC patients, and the changes in MLH1 and MGMT had important implications for tumour progression and survival of patients with BLBC [18]. MGMT and MLH1 methylation in blood

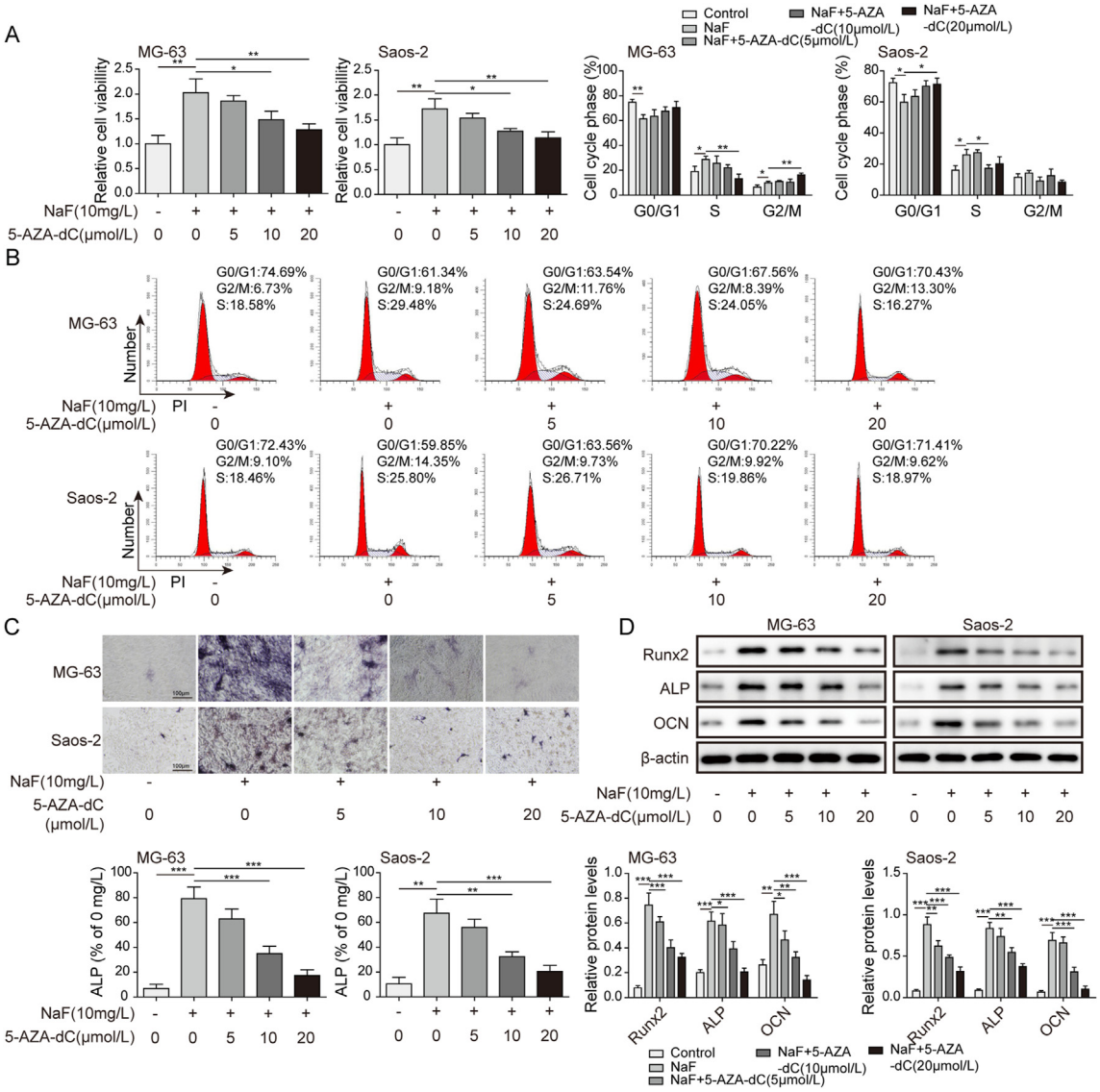

Fig. 3. Methylation inhibitor 5-AZA-dC suppressed proliferation and activation in osteoblasts treated with low-dose NaF. NaF (10 mg/L) together with 5-AZA-dC at 5, 10 and 20 μmol/L was used to treat the human osteoblast cell lines MG63 and Saos2 for 72 h. (A) The MTT assay was used to detect cell viability. (B) The cell cycle was determined using flow cytometry. (C) ALP activity of human osteoblasts. (D) RUNX2, ALP and OCN levels were examined using the Western blot assay. The measurement data are expressed as the mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. All the above assays were executed three times.

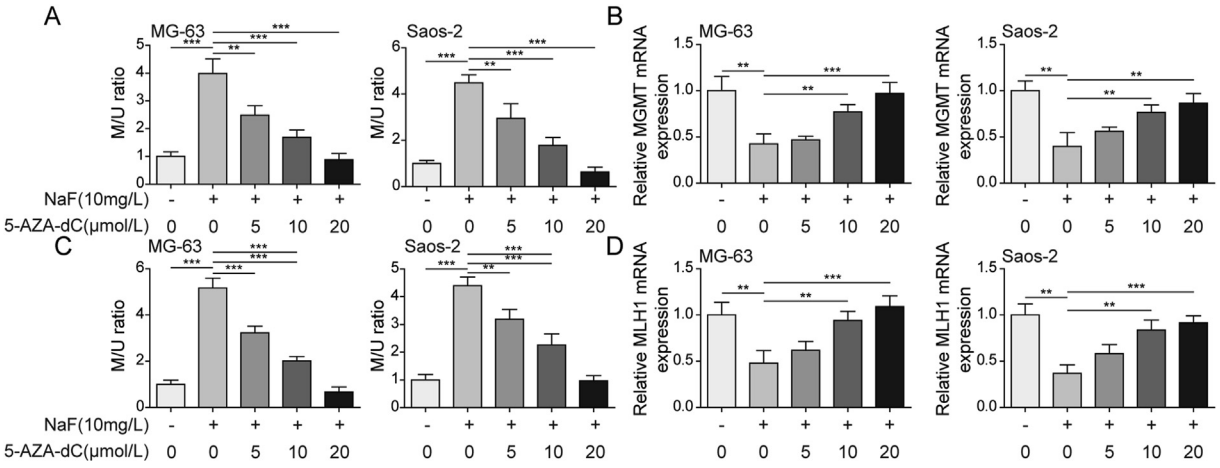

Fig. 4. 5-AZA-dC inhibited the increased methylation of the MGMT and MLH1 genes in osteoblasts treated with a low dose of NaF. NaF (10 mg/L) together with 5-AZA-dC at 5, 10 and 20 μmol/L was used to treat the human osteoblast cell lines MG63 and Saos2 for 72 h. (A) The methylation level of the MGMT gene in human osteoblasts treated with 5-AZA-dC and 10 mg/L NaF was detected by the MSP method. (B) The expression level of MGMT mRNA after 5-AZA-dC and NaF (10 mg/L) treatment was assessed using qPCR assay. (C) The MSP method was used to detect the effects of 5-AZA-dC on the methylation level of the MLH1 gene in osteoblasts treated with a low dose of NaF. (D) The mRNA expression level of MLH1 in MG63 and Saos2 cell lines treated with 5-AZA-dC and 10 mg/L NaF was measured by qPCR assay. The measurement data are presented as the mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. All the above assays were executed three independent times.

was elevated in patients with fluorosis, and the degree of methylation was positively correlated with the severity of fluorosis [3]. As demonstrated by Wu C. X. et al., the methylation degree of MGMT and MLH1 genes was changed in fluorosis disease, leading to changes in the expression of these genes, which played a role in the liver injury caused by fluoride [10]. In our study, we demonstrated that a low dose of NaF induced increased methylation of the MGMT and MLH1 genes, leading to a decrease in their expression, while 5-AZA-dC could inhibit these effects.

Overall, these findings illustrated that NaF acted on osteoblasts and led to methylation of the DNA repair genes MGMT and MLH1, causing proliferation and activation of osteoblasts, thus resulting in the development of skeletal fluorosis, which would provide a new perspective for understanding the mechanism of the action of fluoride on osteoblasts in the pathogenesis of skeletal fluorosis.

We reported for the first time that fluoride treatment promoted MGMT and MLH1 methylation in osteoblasts, leading to a decrease in their expression, resulting in abnormal proliferation and activation of osteoblasts and causing skeletal fluorosis progression, which would provide new insight for understanding the mechanism of action of fluoride in skeletal fluorosis.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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