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Original Article

E26 transformation-specific 1 is implicated in the inhibition of osteogenic differentiation induced by chronic high glucose by directly regulating Runx2 expression

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Abstract

Chronic high glucose (HG) plays a crucial role in the pathogenesis of diabetes-induced osteoporosis by inhibiting the differentiation and proliferation of osteoblasts. This study aims to examine the role of E26 transformation-specific 1 (ETS1) in the inhibition of osteoblast differentiation and proliferation caused by chronic HG, as well as the underlying mechanism. Chronic HG treatment downregulated ETS1 expression and inhibited differentiation and proliferation of MC3T3-E1 cells. Downregulation of ETS1 expression inhibited the differentiation and proliferation of MC3T3-E1 cells under normal glucose conditions, and ETS1 overexpression attenuated the damage to cells exposed to chronic HG. In addition, ETS1 overexpression reversed the decrease in runt-related transcription factor 2 (Runx2) expression in MC3T3-E1 cells treated with chronic HG. Using chromatin immunoprecipitation (ChIP) and luciferase reporter assays, we confirmed that ETS1 directly bound to and increased the activity of the *Runx2* promoter. In summary, our study suggested that ETS1 was involved in the inhibitory effect of chronic HG on osteogenic differentiation and proliferation and may be a potential therapeutic target for diabetes-induced osteoporosis.

Keywords: ETS1, Runx2, chronic high glucose, osteoblast, differentiation, proliferation

Introduction

Osteoporosis is a systemic, metabolic bone disease characterized by bone loss, mineral density reduction and fractures, always resulting in health problems and poor quality of life[1–2]. Similarly, diabetes mellitus (DM) is a metabolic disease inflicting millions of individuals worldwide[3–4]. Chronic high glucose (HG) can inhibit osteogenic differentiation and induce apoptosis of osteoblasts, thus disrupting bone matrix deposition and bone formation[5–9]. Therefore, a better understanding of the mechanisms underlying chronic HG-inhibited osteogenic differentiation and proliferation may have clinical significance in the

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prevention and treatment of diabetes-induced osteoporosis[10].

Transcription factor E26 transformation-specific 1 (ETS1), a member of the ETS family, is involved in various cellular behaviors, including proliferation, differentiation, metastasis, apoptosis, and angiogenesis[11–14]. Recent studies revealed that ETS1 plays a critical role in osteogenic differentiation and bone formation[12,15]. ETS1 expression is significantly increased in BMP-2-stimulated MC3T3-E1 cells during proliferation[16]. Furthermore, ETS1 has received attention due to its role in the progression of diabetic complications, as ETS1 is involved in diabetes-induced vascular disorders[17–18]. Additionally, the expression level of ETS1 is reported to be regulated by chronic HG in various cells, such as mesangial cells, pancreatic β-cells, and cardiomyocytes[19–21]. However, it is unknown whether ETS1 expression is regulated by chronic HG in osteoblasts.

In this study, we measured the expression of ETS1 in MC3T3-E1 cells by Western blotting. Our results showed that the expression of ETS1 was obviously downregulated in osteoblasts. Alkaline phosphatase activity and alizarin red staining indicated that downregulation of ETS1 expression in osteoblasts could inhibit osteogenic differentiation and proliferation. Moreover, we found that overexpression of ETS1 in osteoblasts could reverse the suppressive effect of chronic HG on osteogenic differentiation and proliferation. These results suggest that ETS1 may be a potential therapeutic target for diabetic osteoporosis.

Materials and methods

Cell culture and treatment

MC3T3-E1 cells were gifted by the Institute of Oral Diseases, Nanjing Medical University, and routinely cultured in ascorbic acid (AA)-free modified α-MEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Thermo Fisher Scientific, USA). A final concentration of 25 mmol/L glucose was used to generate the HG condition. To produce this medium, 19.5 mmol/L D-glucose powder (Biofroxx, China) was added to α-MEM medium, which already contains 5.5 mmol/L D-glucose. For osteogenic differentiation, MC3T3-E1 cells were cultured in OriCell MC3T3-E1 cell osteogenic differentiation medium containing 50 μg/mL ascorbic acid, 4 mmol/L β-glycerolphosphate, and 100 nmol/L dexamethasone (Cyagen Biosciences, China) for 14 days with or without HG treatment. Fresh medium was applied every three days.

Overexpression and silencing of ETS1 in MC3T3-E1 cells

Recombinant adenoviruses carrying the *Ets1* gene or shRNA targeting *Ets1* were constructed by Vigene Biosciences (China) and were transduced into MC3T3-E1 cells using ADV-HR (transduction promoting agents, Vigene Biosciences) according to the manufacturer's instructions. Briefly, MC3T3-E1 cells were seeded in 6-well plates 24 hours prior to the transfection experiments. Then, ETS1 or shRNA was diluted in FBS free α-MEM. The vectors and ADV-HR were added to tubes containing FBS free α-MEM. The solution was mixed, added to the cells, and incubated for 12 hours. Then, the medium was removed and replaced with fresh complete culture medium.

CCK-8 assay

After treatment with HG for 7 days, the viability of MC3T3-E1 cells was measured by using CCK-8 assay. The cells were treated as indicated in the text and then incubated with CCK8 (10 μL/well, Roche Diagnosis, Germany) for an additional 2 hours. The absorbance at 450 nm was then measured using a microplate absorbance reader.

Western blotting analysis

At the end of the indicated treatments, RIPA lysis buffer (Beyotime, China) was used to extract the total proteins from MC3T3 E1 cells. A total of 20 μg of protein was subjected to 10% SDS-PAGE and transferred to PVDF membranes. Having been blocked with 5% nonfat milk, the membranes were incubated with primary antibodies against ETS1, Runx2, ALP, and GAPDH (all from ABclonal Technology, China) at 4 °C overnight. The membranes were then washed three times with TBST, and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) were added and incubated. Enhanced ECL reagent (Pierce, USA) was used to detect the protein bands of interest.

Reverse transcriptase PCR

To determine the mRNA level of *Ets1*, TRIzol reagent (Invitrogen, USA) was used to extract the total RNA from MC3T3-E1 cells at the end of the indicated treatment. Superscript Ⅱ reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA according to the manufacturer's instructions. The cDNAs were amplified at 94 °C for 2 minutes, followed by 28 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute. The PCR

products were analyzed by electrophoresis on 2% agarose gels. The primer sequences were obtained from Genepharma (China) and listed as follows: *Ets1* sense, 5′-CTCTCCAGACAGACACCTTGC-3′; *Ets1* antisense, 5′-AGCACGGTCACGCACATA-3′; *Gapdh* sense, 5′-CATCACTGCCACCCAGAAGACTG-3′; *Gapdh* antisense, 5′-ATGCCAGTGAGCTTCCCGTTCAG-3′.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was assessed on day 14 after osteogenic induction using an ALP activity kit (Beyotime) according to the manufacturer's protocol. Briefly, the cells were lysed with cell lysis buffer, and the cell lysates were incubated with para-nitrophenyl phosphate for 30 minutes at 37 °C. Subsequently, the reaction was stopped by adding 0.1 mol/L NaOH, and the absorbance at 405 nm was detected using a spectrophotometer.

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay kit (Beyotime) was used to validate the binding relationship between ETS1 and Runx2. Briefly, MC3T3-E1 cells were crosslinked with 1% formaldehyde for 10 minutes, and then, the reaction was quenched with glycine (0.125 mol/L) for 5 minutes. Having been rinsed with ice-cold 1× PBS, the cells were resuspended in lysis buffer followed by ultrasonic treatment four times at setting 3 (model 550 sonic dismembrane; Fisher) for 10 seconds. The A/G agarose magnetic beads were conjugated to 4 μg anti-ETS1 (ABclonal Technology) or anti-IgG (Beyotime), and then incubated overnight at 4 °C with the cell lysate. After the reversal of cross-links at 65 °C for 4 hours, the DNA was recovered by phenol/chloroform extraction and the DNA enriched in the elution was analyzed. The enrichment of the *Runx2* promoter was determined by PCR. The corresponding primer sequences used were as follows: forward: 5′-AGGGAGAGGACAACAGAAGAGAA-3′; reverse: 5′-TCAAAGTAAAGTGGGACTGCCTAC-3′.

Luciferase reporter assay

The promoter sequence of *Runx2* was inserted into the pGL3-Basic luciferase reporter vector (Promega, USA). When MC3T3-E1 cells reached 80%–90% confluence, the constructed luciferase reporter vector, Renilla luciferase reference vector (pRLh-null-Renilla), and ETS1 expression plasmid or control plasmid (vector) were cotransfected in each well using Lipofectamine 2000. After 24 hours of transfection, the cells were harvested using passive lysis buffer (Promega), and the luciferase activity of the cell lysates was evaluated using the Dual-Luciferase Reporter Kit (Promega) according to the manufacturer's instructions. Luciferase activity was normalized to values for Renilla luciferase.

Alizarin red staining

At the end of the indicated time points, alizarin red staining was performed using a specific kit (Cyagen Biosciences) according to the manufacturer's instructions. Briefly, MC3T3-E1 cells were fixed with 4% neutral formaldehyde solution for 30 minutes at room temperature. The cells were then stained with Alizarin red dye for 5 minutes at room temperature. The dye was then removed, and the cells were rinsed with PBS. The cells were observed under a fluorescence microscope.

Statistical analysis

Values were shown as the mean±standard deviation (SD) and were analyzed by SPSS software version 15.0 (SPSS, USA). Student's *t*-test and one-way analysis of variance (ANOVA) were used to analyze the differences between two or multiple groups. $P<0.05$ was considered statistically significant.

Results

Chronic HG downregulated ETS1 expression and inhibited the differentiation and proliferation of MC3T3-E1 cells

As shown in ***Fig. 1A***, HG treatment significantly reduced the expression level of ETS1. HG treatment also significantly reduced the viability of MC3T3-E1 cells (***Fig. 1B***). As shown in ***Fig. 1C***, the activity of ALP was obviously decreased after HG treatment. In addition, decreased mineralization was observed in the HG group compared with the control group (***Fig. 1D***). The above data suggested that HG downregulated ETS1 expression and inhibited the differentiation and proliferation of MC3T3-E1 cells.

Downregulation of ETS1 expression inhibited the differentiation and proliferation of MC3T3-E1 cells

To determine the role of ETS1 in the differentiation and proliferation of MC3T3-E1 cells, endogenous ETS1 expression was inhibited by *Ets1* specific shRNA. A marked downregulation of ETS1 expression was observed in MC3T3-E1 cells transduced with *Ets1* shRNA (***Fig. 2A***). The viability

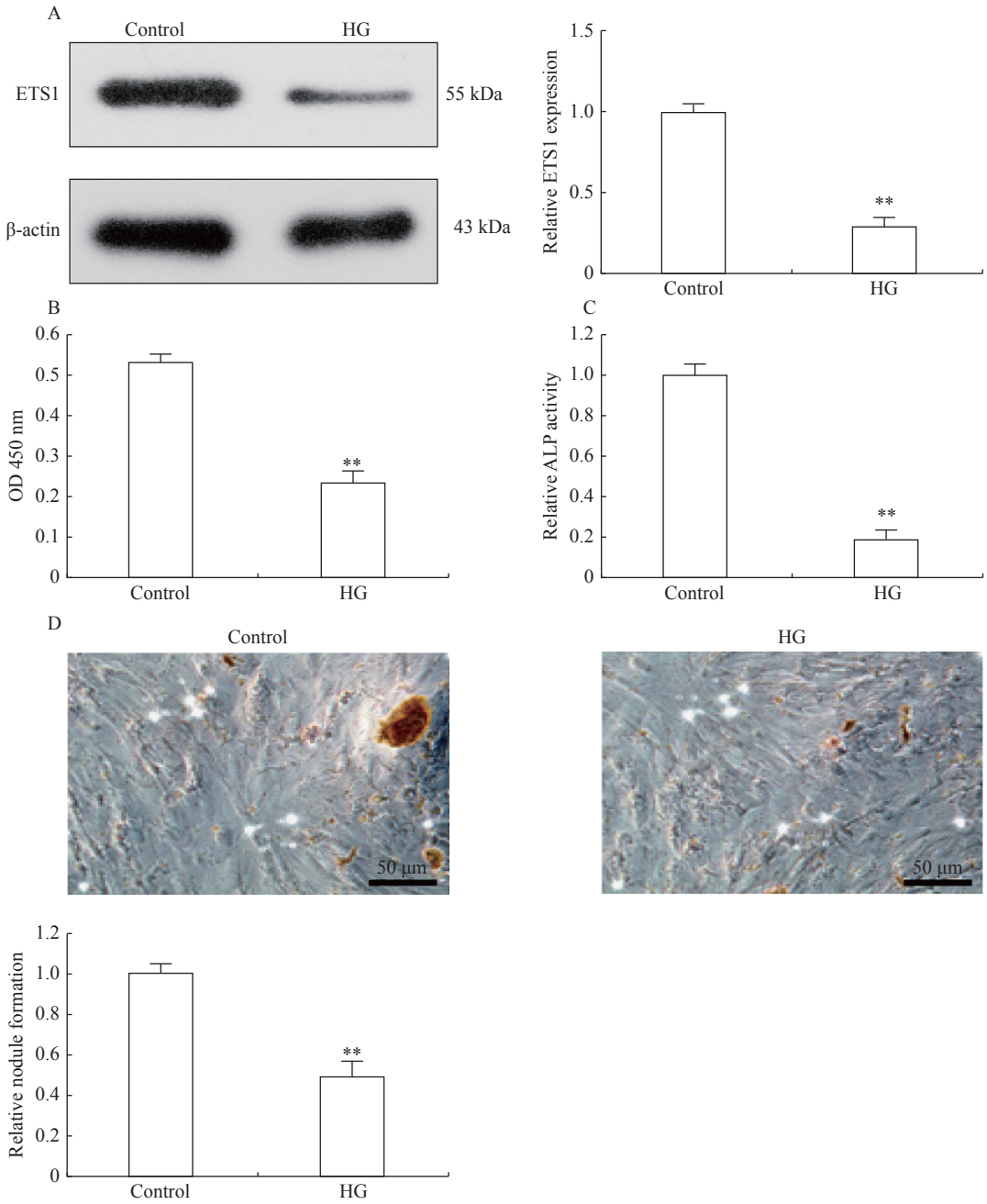

Fig. 1 **Effects of HG on ETS1 expression, proliferation and differentiation in MC3T3-E1 cells.** A and B: MC3T3-E1 cells were incubated under normal (Control) or HG conditions, the protein level of ETS1 was measured by Western blotting (A), and cell viability was measured by CCK-8 assay (B). C and D: MC3T3-E1 cells were cultured in osteogenic differentiation medium with or without HG for 14 days, the activity of ALP was determined with an ALP assay kit (C), and the level of calcification was examined using alizarin red staining (D). Scale bar: 50 μm. Data are presented as mean±SD and were analyzed using unpaired two-tailed Student's *t*-test. $^{**}P<0.01$ *vs*. Control, $n=3$. HG: High glucose; ALP: alkaline phosphatase.

of MC3T3-E1 cells was significantly decreased after transduction with *Ets1* shRNA (***Fig. 2B***). *Ets1* shRNA also obviously inhibited the activity of ALP (***Fig. 2C***). The alizarin red staining results showed that *Ets1* shRNA significantly suppressed the mineralization of MC3T3-E1 cells (***Fig. 2D***). The above data suggested that downregulation of ETS1 expression inhibited the differentiation and proliferation of MC3T3-E1 cells.

Overexpression of ETS1 attenuated HG-induced inhibition of differentiation and proliferation

To investigate the effects of ETS1 on the HG-induced inhibition of differentiation and proliferation, ETS1 expression was upregulated by transducing MC3T3-E1 cells with the ETS1-overexpressed vector. As shown in ***Fig. 3A***, the ETS1-overexpressing vector markedly upregulated ETS1 expression under HG

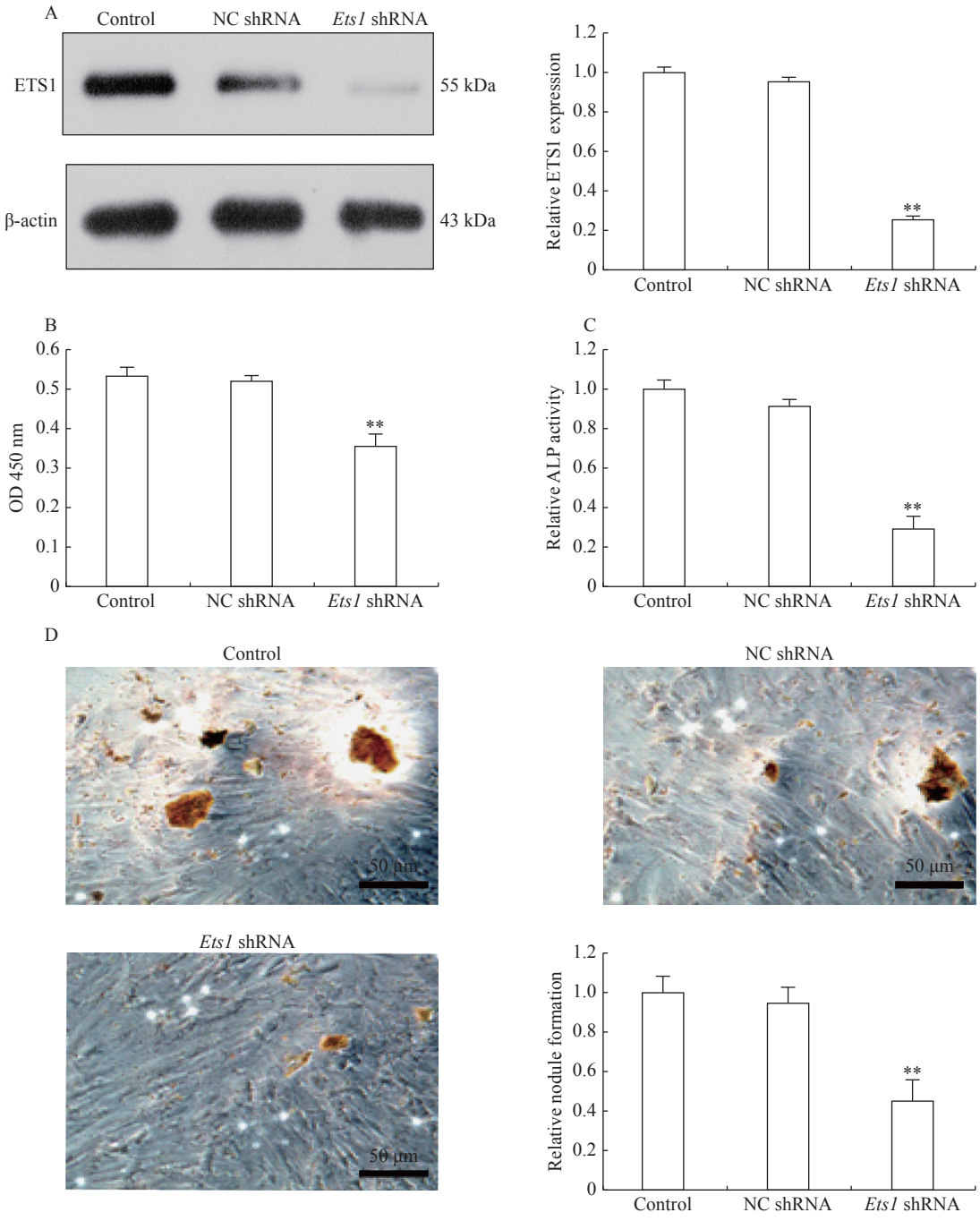

Fig. 2 **Effects of *Ets1* shRNA on the proliferation and differentiation of MC3T3-E1 cells.** MC3T3-E1 cells were transduced with recombinant adenovirus carrying negative control (NC) shRNA, *Ets1* shRNA or without transduction (Control). A and B: The protein level of ETS1 was measured by Western blotting (A), and cell viability was measured by CCK-8 assay (B). C and D: After 14 days of osteogenic differentiation, the activity of ALP was determined with an ALP assay kit (C), and the level of calcification was examined using alizarin red staining (D). Scale bar, 50 μm. Data are presented as mean±SD and were analyzed using unpaired two-tailed Student's *t*-test. $^{**}P<0.01$ *vs.* NC shRNA, $n=3$. ALP: alkaline phosphatase.

conditions. ETS1 overexpression also significantly increased the viability of MC3T3-E1 cells under HG conditions (***Fig. 3B***). In addition, in HG-treated MC3T3-E1 cells, the expression and activity (***Fig. 3A*** and ***C***) of ALP, as well as mineralization (***Fig. 3D***), were obviously increased by ETS1 overexpression. The above data suggested that ETS1 overexpression attenuated HG-induced inhibition of differentiation and proliferation of MC3T3-E1 cells.

ETS1 mediated the HG-induced inhibition of differentiation by directly regulating Runx2 expression

Previous studies demonstrated that Runx2 plays key

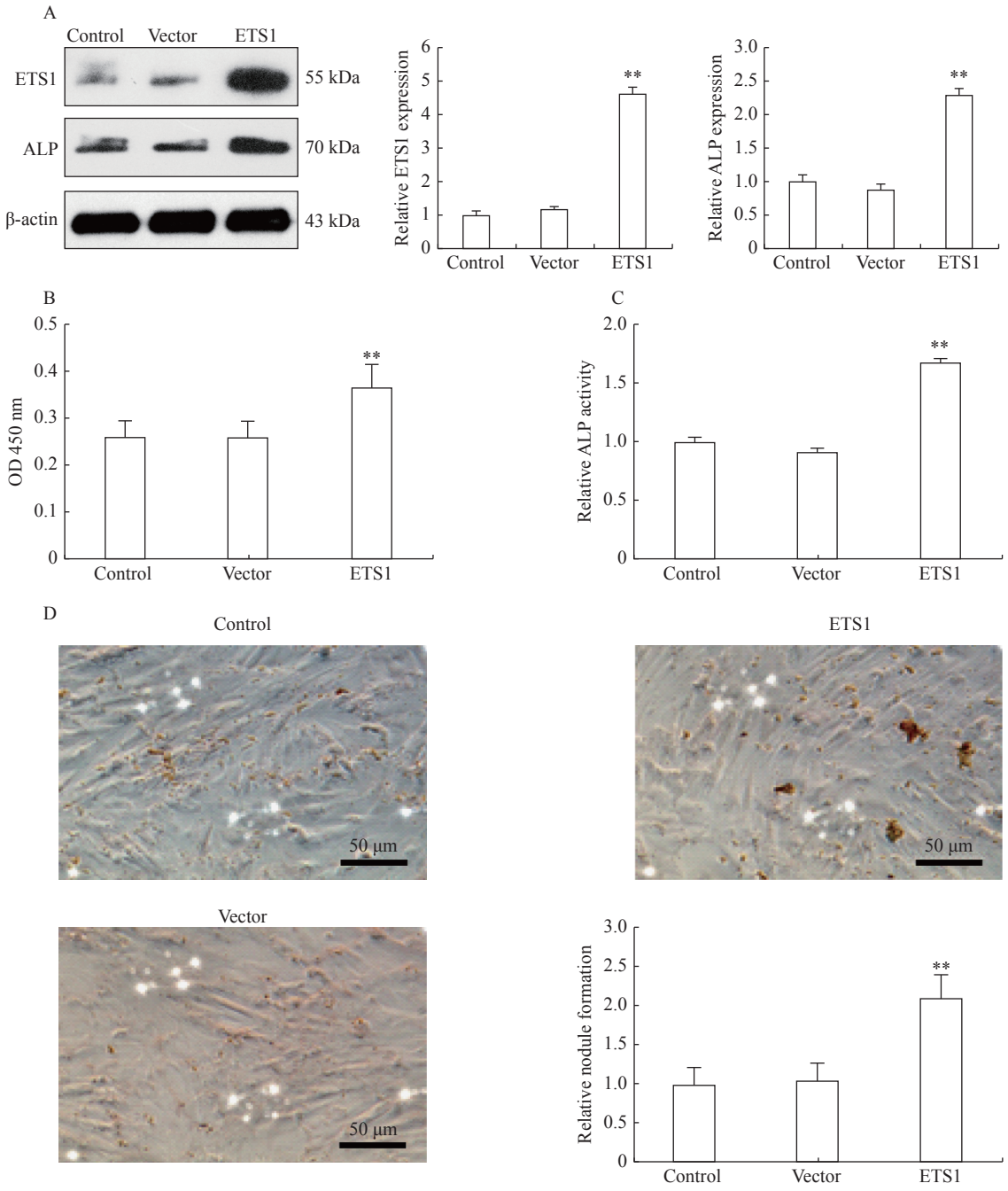

Fig. 3 **Effects of ETS1 overexpression on the proliferation and differentiation of MC3T3-E1 cells under HG conditions.** MC3T3-E1 cells were transduced with recombinant adenovirus carrying empty vector (Vector) or ETS1-overexpressed vector (ETS1), or without transduction (Control), the cells were then cultured under HG conditions. A and B: The protein levels of ETS1 and ALP were determined by Western blotting (A), and cell viability was determined by CCK-8 assay (B). C and D: The transduced cells were cultured in osteogenic differentiation medium with HG. After 14 days of osteogenic differentiation, the activity of ALP was determined with an ALP assay kit (C), and the level of calcification was examined using alizarin red staining (D). Scale bar, 50 μm. Data are presented as mean±SD and were analyzed using unpaired two-tailed Student's *t*-test. $^{**}P<0.01$ *vs.* vector, $n=3$. Vector: empty vector; HG: high glucose; ALP: alkaline phosphatase.

roles in the proliferation, differentiation, and function of osteoblasts, and that Runx2 expression is regulated by ETS1[22–24]. Therefore, we investigated whether Runx2 is involved in the ETS1-mediated inhibition of osteogenic differentiation and proliferation under HG conditions. We first examined the effect of ETS1 on Runx2 expression in HG-treated MC3T3-E1 cells. The results showed that ETS1 overexpression increased the protein and mRNA levels of *Runx2* in MC3T3-E1 cells under HG conditions (***Fig. 4A*** and ***B***), suggesting that ETS1 regulates Runx2 expression at the transcriptional level. We then investigated

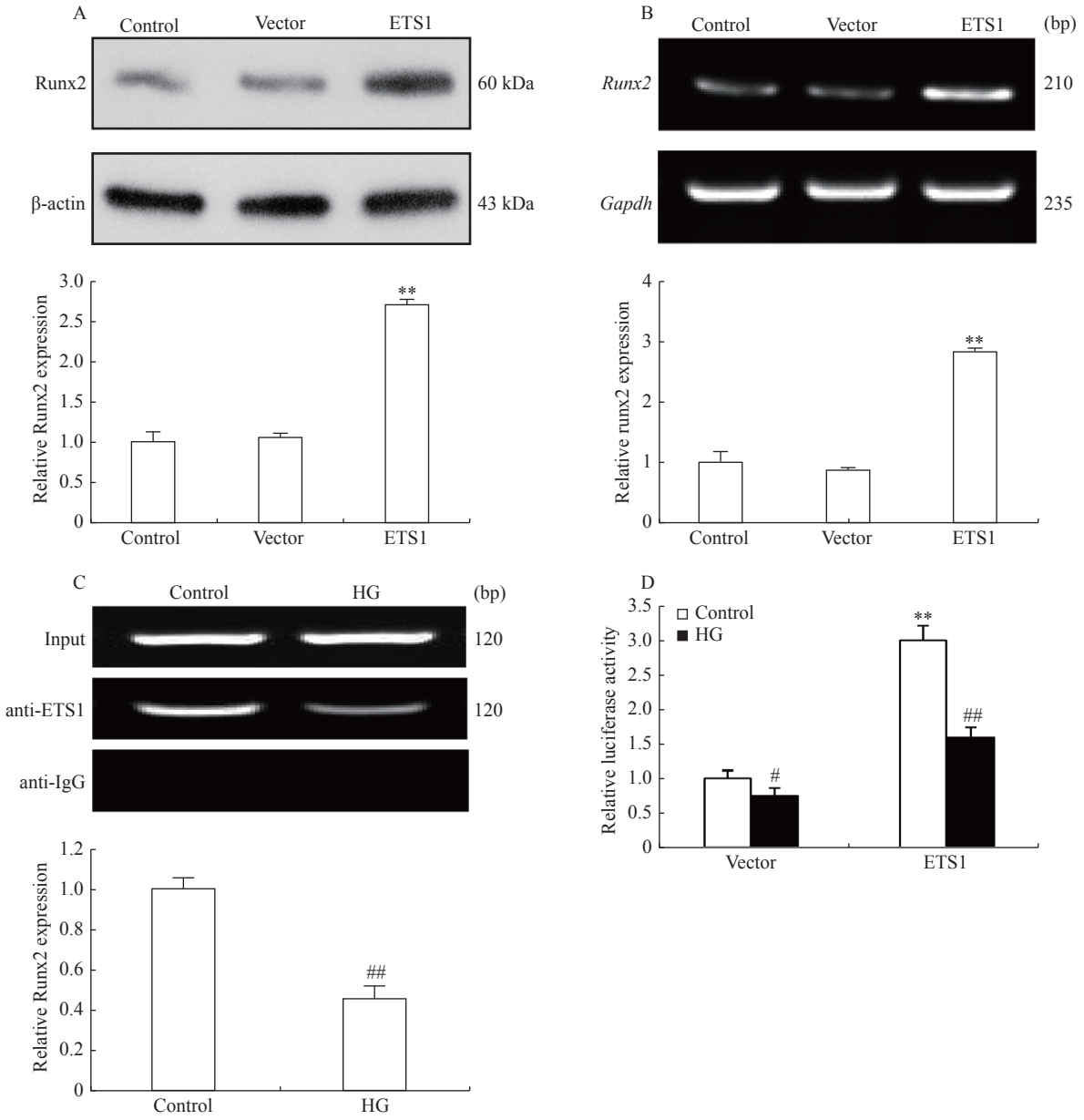

Fig. 4 **Effects of ETS1 overexpression on the expression and promoter activity of *Runx2*.** MC3T3-E1 cells were transduced with recombinant adenovirus containing empty vector (Vector) or ETS1-overexpressed vector (ETS1), or without transduction (Control), and then cultured under HG conditions. A and B: The protein expression level of Runx2 was examined by Western blotting (A), and the mRNA expression level of *Runx2* was examined by reverse transcriptase PCR and *Gapdh* was used as an internal reference (B). C: ETS1 enrichment in the *Runx2* promoter region in MC3T3-E1 cells under normal (Control) and HG conditions was analyzed by chromatin immunoprecipitation assay. D: MC3T3-E1 cells cotransfected with *Runx2*-Luc and plasmid expressing ETS1 or empty vector were cultured under normal (Control) or HG conditions for 24 hours, and then, the luciferase activity was examined using a luciferase reporter assay. Data are presented as mean±SD and were analyzed using unpaired two-tailed Student's *t*-test. $^{**}P<0.01$ *vs.* Vector; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* Control; $n=3$. Vector: empty vector; HG: high glucose.

whether ETS1 acts as a transcriptional activator of the *Runx2* gene. Data from ChIP assays showed that HG reduced the binding of ETS1 to the promoter of *Runx2* (***Fig. 4C***). Moreover, the luciferase reporter assay showed that ETS1 overexpression increased the promoter activity of *Runx2* in MC3T3-E1 cells, which was inhibited by HG treatment (***Fig. 4D***). These results suggest that ETS1 directly regulates Runx2 expression in HG-treated MC3T3-E1 cells.

Discussion

Our findings demonstrated that chronic HG downregulated ETS1 expression and subsequent Runx2 expression, thus inhibiting the differentiation and proliferation of osteoblasts. To the best of our knowledge, our study is the first to report the role of ETS1 in the development of diabetic osteogenesis, as well as the potential mechanisms.

A previous study reported that ETS1 expression was upregulated in HG-treated rat mesangial cells[19]. Wang *et al* reported that HG treatment induced cardiomyocyte apoptosis by upregulating ETS1 expression[20]. The above findings suggested that the effect of HG on the expression level of ETS1 may depend on the cell type. In osteoblasts, we found that chronic HG exposure led to a decrease in ETS1 expression. Upregulation of ETS1 expression could promote osteogenic differentiation[12,15], and downregulation of ETS1 expression in osteoblasts could lead to the inhibition of osteogenic differentiation and proliferation (***Fig. 2***). Thus, we proposed that the decrease in osteogenic differentiation and proliferation induced by chronic HG was due to reduced ETS1 expression. Previous studies showed that TGF-β upregulated ETS1 expression in hepatocytes by promoting binding of Smad2/3 to the promoter region of *ETS1*[22]. It has been demonstrated that platelet-derived growth factor (PDGF) maintains the high expression of ETS1 in human arterial vascular smooth muscle cells by continuously activating the mTOR signaling pathway[23]. In addition, it has been reported that HG induces the upregulation of ETS1 expression by increasing the levels of acetylation of histone H3 and H4 in the *ETS1* gene promoter in pancreatic β-cells[21]. In osteoblasts, it is unclear whether HG regulates the expression of ETS1 through the above mechanism, and this issue will be investigated in our further work.

Runx2, a key transcription factor, acts as a modulator that can regulate the proliferation, differentiation, and function of osteoblasts[24–26]. Previous studies demonstrated that Runx2 could alleviate the HG-induced inhibition of osteogenic differentiation in different types of cells, such as rat bone mesenchymal stem cells (rBMSCs), periodontal ligament stem cells (PDLSCs), and human dental pulp stem cells (hDPSCs)[27–29]. In osteoblasts, a correlation between ETS1 expression and Runx2 expression was observed, which has been implicated in osteogenic differentiation[15]. Moreover, basal Runx2 gene transcription is regulated by ETS1 during the progression of osteogenesis[30]. In the present study, ETS1 overexpression upregulated the mRNA and protein levels of Runx2 in HG-treated MC3T3-E1 cells. Using ChIP and luciferase reporter assays, we found that the binding of ETS1 to the *Runx2* promoter was impeded under HG conditions. As shown here and in previous studies, we established a connection between chronic HG and a reduction in ETS1 and Runx2 expression. ETS1 was responsible for the decrease in Runx2 expression in osteoblasts under HG conditions.

In conclusion, our findings indicated that chronic HG repressed ETS1 expression, leading to a reduction in Runx2 expression by inhibiting its promoter activity, and finally suppressing the differentiation and proliferation of osteoblasts.

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