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Curculigoside attenuates osteoporosis through regulating DNMT1 mediated osteoblast activity

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Abstract

This work aims to study the function of curculigoside in osteoporosis and explore whether DNMT1 is closely involved in osteoblast activity. After OB-6 osteoblasts were treated with hydrogen peroxide (H_2O_2), a curculigoside treatment group was set up and a series of biological tests including MTT, flow cytometry, western blotting, ROS fluorescence intensity, mitochondrial membrane potential, and ELISA experiments were performed to verify the effect of curculigoside on the activity of osteoblasts. Then, alkaline phosphatase (ALP) activity, alizarin red staining, PCR, and western blotting assays were performed to detect the effects of curculigoside on osteoblast function. By constructing DNMT1 knockdown and overexpression OB-6 cell lines, the effect of DNMT1 on osteoblast function was verified. In addition, the expression level of Nrf2 in each group was detected to speculate the mechanism of DNMT1 in osteoporosis. The cell activity and level of bcl-2 and SOD were significantly increased; the cell apoptosis, ROS fluorescence intensity, mitochondrial membrane potential, MDA and level of caspase-3, Bax, and CAT was reduced in curculigoside treatment group compared with H_2O_2-induced OB-6 osteoblasts. Meanwhile, the ALP activity, number and area of bone mineralized nodules, and gene and protein expression of OSX and OPG were significantly elevated in curculigoside group. Moreover, DNMT1 knockdown had a similar promotion effect on osteoblast function as curculigoside, and DNMT1 overexpression could reverse the promotion effect of curculigoside on osteoblast function. Further mechanistic studies speculated that DNMT1 might play a role in osteoporosis by affecting Nrf2 methylation. Curculigoside enhances osteoblast activity through DNMT1 controls of Nrf2 methylation.

Keywords Curculigoside · Osteoporosis · Osteoblast activity · DNMT1 · Nrf2 · Methylation

Introduction

The skeleton is the hard organ that makes up the vertebrate endoskeleton and has functions such as movement, support, and protection of the body. The thickness of human bones is genetically determined and influenced by lifestyle. The degeneration of bones affects the function of the whole body (Cymet et al. 2000). Old age, trauma, braking, and viscera dysfunction and other factors result in bone thinning atrophy, known as osteoporosis (Gosset et al. 2021). The danger of fractures caused by osteoporosis is enormous and is one of the main causes of disability and death in elderly patients (Alexiou et al. 2018; Lai et al. 2022). The study of the occurrence, development, and pathological characteristics of osteoporosis is important for finding more effective treatments for osteoporosis.

Osteoporosis is a common clinical category of orthopedic diseases, and calcium deficiency is only the surface cause of osteoporosis; the most important cause is the change of

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bone mechanism in the body (Fang et al. 2022). The human skeleton undergoes a dynamic process of change at all times, as old bone is continuously absorbed and degraded by osteoclasts, while new bone is remodeled by osteoblasts derived from bone marrow mesenchymal stem cells (McDonald et al. 2021; Fischer and Haffner-Luntzer 2022). The pathological basis for the development of osteoporosis is the disruption of the balance of bone reconstruction due to the imbalance of osteoclast and osteoblast activities (Chen et al. 2022). Since the functional decline of osteoblasts is an important cause of the imbalance of bone metabolism and bone loss, improving the bone-forming capacity of osteoblasts has direct implications for the prevention and treatment of osteoporosis.

Curculigo orchioides is a perennial herb in the Lithospermum family, and its medicinal part is the rhizome (Valls et al. 2006). It is rich in chemical components, such as phenols, phenolic glycosides, and lignans (Zuo et al. 2010). Among them, curculigoside is the main active component of *curculigo orchioides* (Zuo et al. 2010). Curculigoside is a benzoate derivative with a molecular formula of $C_{22}H_{26}O_{11}$ and a molecular weight of 466.44. Research shown that curculigoside can improve learning and memory and has anti-inflammatory, antioxidant, immune enhancement, protection against ischemia/reperfusion injury, and other pharmacological effects (Han et al. 2020; Liu et al. 2021). Moreover, Zhao et al. 2015 found that curculigoside can increase bone density and serum SOD and CAT levels in ovariectomized rats with osteoporosis (Zhao et al. 2015). Thus, we conducted a cell experiment to study the function of curculigoside in osteoporosis and explore the potential mechanism through OB-6 osteoblasts.

Materials and methods

Cell transfection and groups OB-6 osteoblastic cells were obtained from the BFB (Shanghai, China) and cultured as described previously (Guo et al. 2017). Then, OB-6 osteoblastic cells were incubated with or without 300 μm H_2O_2 (Sigma-Aldrich, St Louis, MO) for 12 h or 10 μM curculigoside (curculigoside A, MCE, Sigma-Aldrich) for another 60 h (Zhang et al. 2019; Lian et al. 2021). For further mechanistic studies, OB-6 osteoblastic cells were transfected with DNMT1 siRNA (OBiO, Shanghai, China) or DNMT1 mimics through Lipofectamine™ 3000 transfection reagent (Invitrogen, Grand Island, NY). At 48 h after DNMT1 mimics transfection, the cells were treated with 10 μM curculigoside for another 60 h.

Cell viability assay The MTT assay was used to study the cell viability. The MTT absorbance optical density (OD) at 570 nm was measured.

Cell apoptosis assay After centrifugated and resuspended, cell suspension of different group was pipetted and incubated with Annexin V-PE/7-AAD. Then, the cell apoptosis was analyzed using a flow cytometry (Agilent Technologies, Santa Clara, CA).

Western blot Cellular proteins were determined by bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA), resolved with SDS-PAGE electrophoresis (Beyotime Biotechnology Co., Ltd., Shanghai, China), and transferred to PVDF (Millipore, USA). Then, proteins were sealed with 5% skim milk and immunodetected with primary antibodies against caspase-3 (ab184787, 1:2000, Abcam, Cambridge, UK), Bax (ab32503, 1:10,000, Abcam), bcl-2 (ab182858, 1:2000, Abcam), osterix (OSX, ab209484, 1:1000, Abcam), osteoprotegerin (OPG, ab73400, 1:1000, Abcam), and GAPDH (60,004–1-Ig, 1:50,000, PTG). After incubated with peroxidase conjugate secondary antibody containing horseradish (ZB-2305, 1:10,000, ZSGB, China), protein bands were analyzed through Image Lab Software (Bio-Rad, Hercules, CA).

Cell ROS and mitochondrial membrane potential detection The cells in each group were rinsed with PBS twice. After that, samples were processed and flow cytometry was performed according to the instructions of ROS detection kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) and mitochondrial membrane potential detection kit (Elabscience Biotechnology Co.,Ltd, Wuhan, China), respectively.

Enzyme-linked immunosorbent assay The activity of superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT) was detected through enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China).

Alkaline phosphatase activity assay Cells were lysed with 100 μL of western and IP cell lysate combined with 1% of 0.01 mol/L PMSF; the cell lysate was collected. The alkaline phosphatase (ALP) activity was expressed as p-nitrophenol (μmol/L) released per milligram of protein, and the ALP activity of the cells in the blank control group was expressed as 100%, and the ratio of ALP activity of the other treated groups to the ALP activity of the blank control group × 100%. One hundred percent indicates the relative ALP activity of each group of cells after treatment.

Alizarin red staining The formation of bone mineralization nodules in osteoblasts was observed by alizarin red staining. Cells were fixed for 10 min with precooled 10%

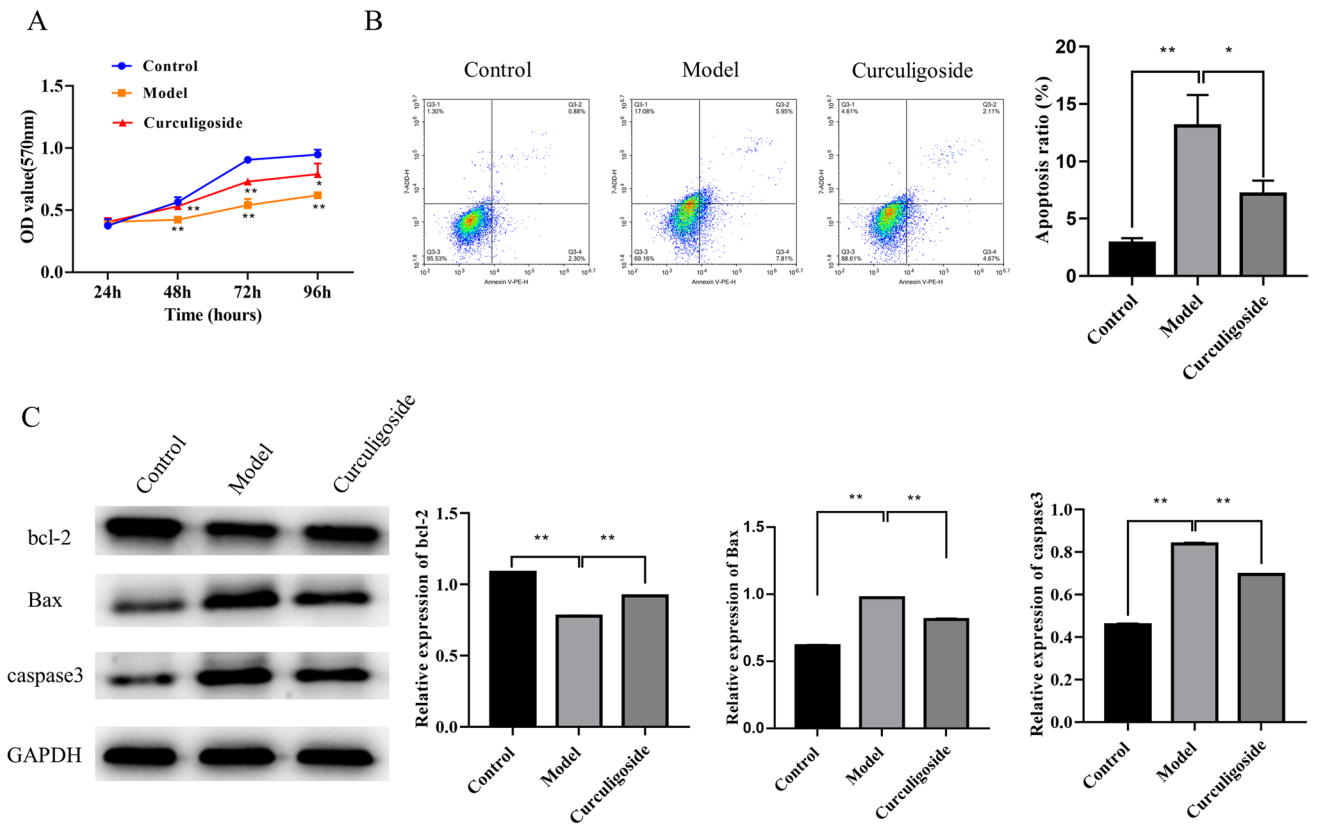

Figure 1. Curculigoside promotes proliferation and inhibits apoptosis of OB-6 osteoblasts. (*A*) MTT assay. (*B*) Flow cytometry assay. (*C*) Western blot assay. *$p < 0.05$, **$p < 0.01$. Control group: OB-6 osteoblastic cells were incubated without 300 μm H_2O_2. Model group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2. Curculigoside group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2 and 10 μM curculigoside.

neutral formaldehyde solution before staining. After 3 times of rinsing with PBS, the cells were incubated with 0.1% Tris–HCl alizarin red dye. Finally, the cells were rinsed with PBS until the floating color was removed, then dried and photographed.

QRT-PCR Total RNA was extracted through TRIzon Reagent (Thermo Fisher Scientific, Waltham, MA) and reversed transcription into cDNA. Then, SYBR Green PCR Master Mix (Thermo Fisher Scientific) was used to perform real-time PCR. The relative expression of DNMT1, OSX, and OPG was calculated through $2^{-\Delta\Delta CT}$ method with GAPDH as normalizing control.

Immunofluorescence Cells were fixed with 4% paraformaldehyde and treated with 0.1% TritonX-100. After being blocked with bovine serum albumin, cells were incubated with anti-Nrf2 antibody (16,396–1-AP, PTG) at 4°C overnight. After incubated with Alexa Fluor 488 coupled (Invitrogen), cells were observed under a fluorescence microscope.

Statistical methods All experiments were repeated at least three times. GraphPad software was applied and p-value < 0.05 was considered statistically significant. All data were expressed as mean ± SD and analyzed by t-test between two groups and LSD test following ANOVA between multiple groups.

Results

Effect of curculigoside on osteoblast activity The proliferation and apoptosis in OB-6 osteoblasts was analyzed using MTT (Fig. 1*A*), flow cytometry (Fig. 1*B*), and western blotting (Fig. 1*C*), and the results showed that the cell proliferation and bcl-2 protein expression were significantly increased and the cell apoptosis and expression of caspase-3 and Bax protein was markedly reduced in the curculigoside treatment group compared with the model group ($p < 0.05$, respectively). The findings suggested that curculigoside

could promote proliferation and inhibit apoptosis of OB-6 osteoblasts co-treated with H_2O_2.

Effect of curculigoside on oxidative stress in OB-6 osteoblasts Oxidative stress is a causative factor in osteoporosis (Kimball et al. 2021). Figure 2 *A–C* reveal that compared with normal control group, the SOD level was observably decreased and the ROS fluorescence intensity, mitochondrial membrane potential, and level of MDA and CAT was markedly increased in model group ($p < 0.05$, respectively). However, curculigoside treatment could reversed those oxidative stress indexes in OB-6 osteoblasts. All these results suggested that curculigoside could ameliorate the oxidative damage induced by H_2O_2 in OB-6 osteoblasts.

Effect of curculigoside on the function of osteoblast ALP activity (Fig. 3*A*), alizarin red staining (Fig. 3*B*), western blotting (Fig. 3*C*), and PCR (Fig. 3*D*) assays were performed to detect the effects of curculigoside on osteoblast function. Compared with the control group, the concentration of ALP, number and area of bone mineralized nodules, and protein and mRNA expression of OSX and OPG were markedly decreased in model group ($p < 0.05$, respectively). Moreover, the concentration of ALP, area of bone mineralized nodules, and protein and mRNA expression of OSX and OPG were significantly elevated in curculigoside group compared with that in model group ($p < 0.05$, respectively). All those findings indicated that curculigoside could improve the osteoblast function of OB-6 osteoblasts.

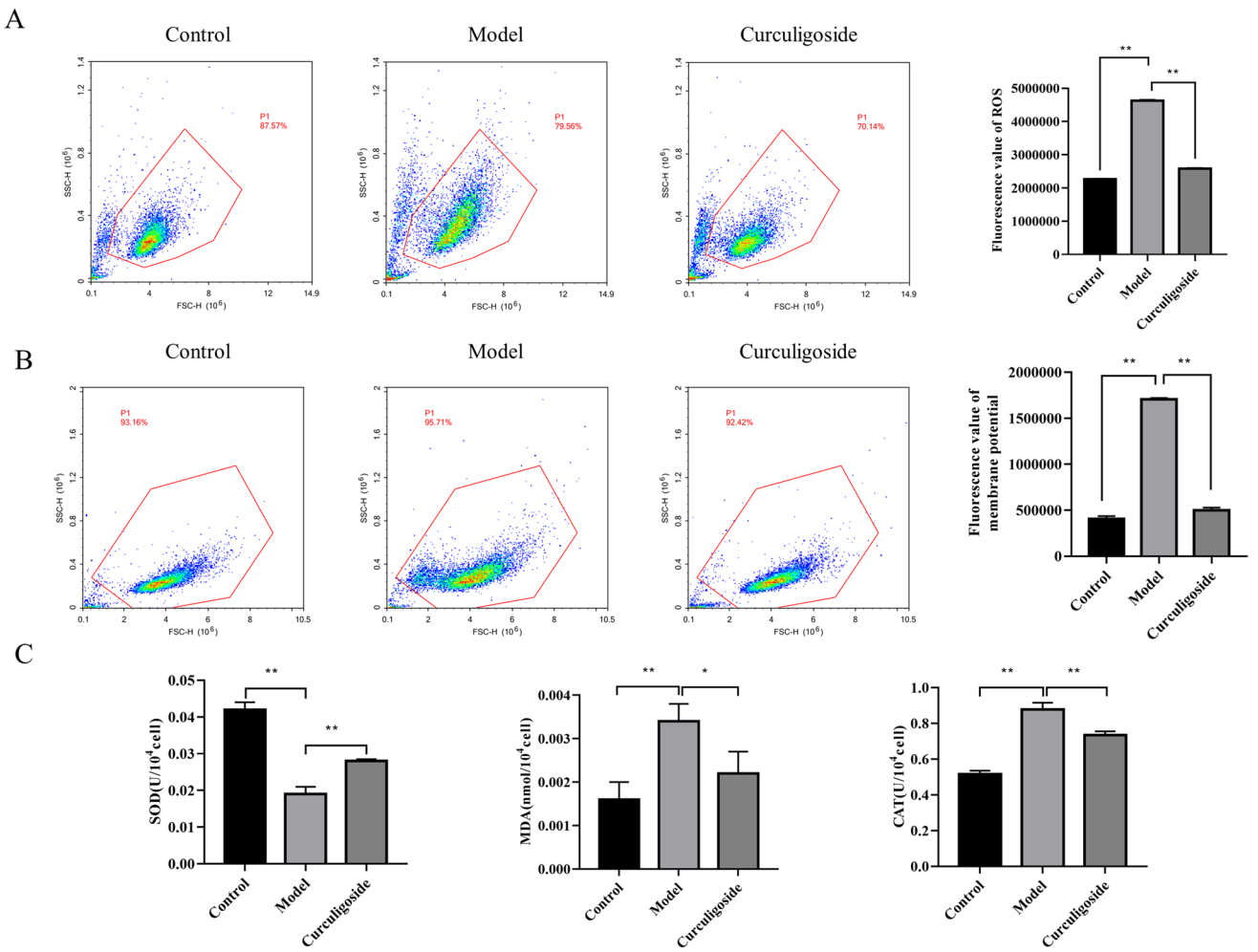

Figure 2. Curculigoside ameliorates the oxidative damage induced by H_2O_2 in OB-6 osteoblasts. (*A*) Flow cytometry assay was used to detect ROS fluorescence intensity. (*B*) Flow cytometry assay was used to detect mitochondrial membrane potential. (*C*) ELISA assay. $^*p < 0.05$, $^{**}p < 0.01$. Control group: OB-6 osteoblastic cells were incubated without 300 μm H_2O_2. Model group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2. Curculigoside group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2 and 10 μM curculigoside.

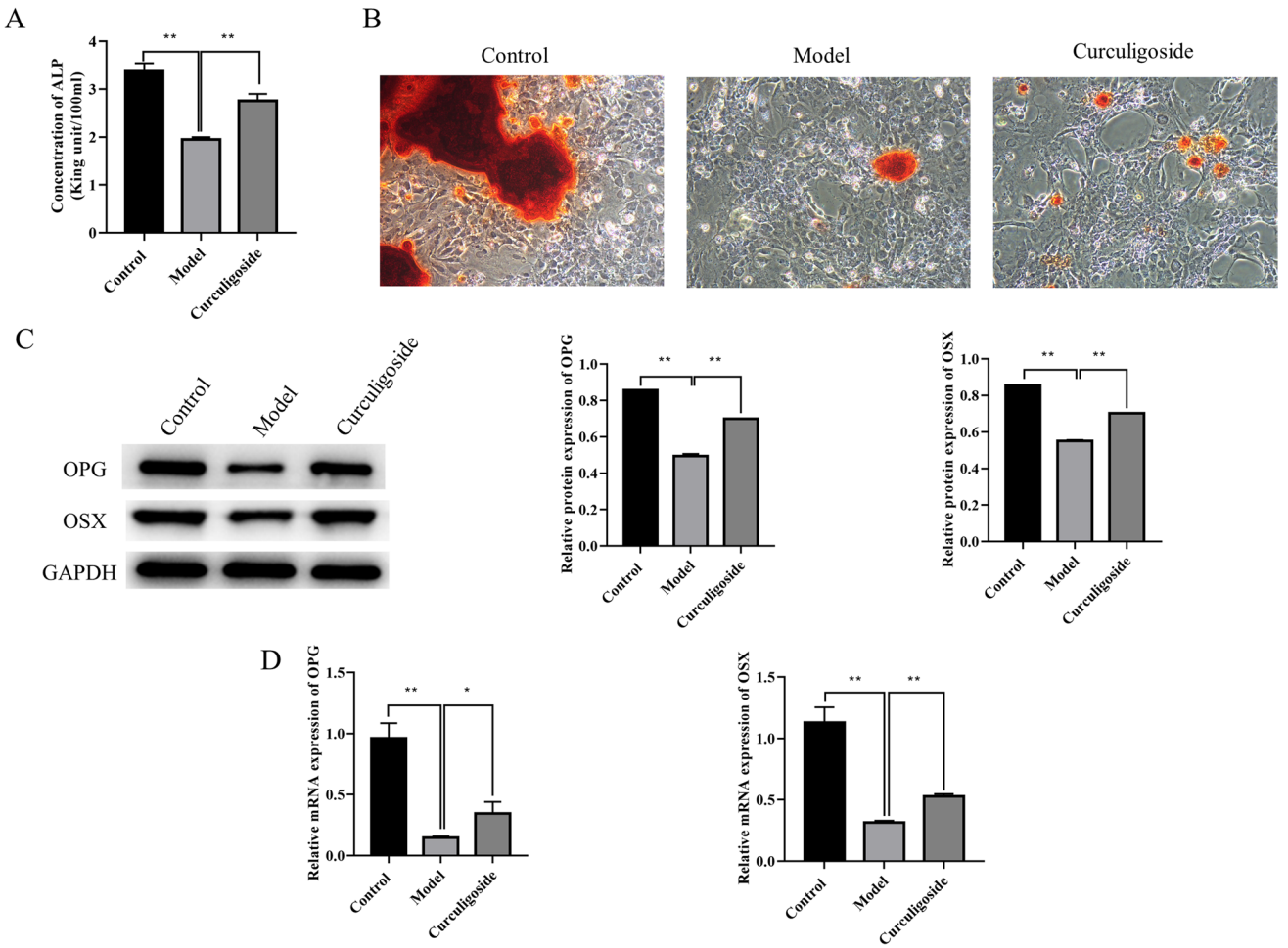

Figure 3. Curculigoside improves the osteoblast function of OB-6 osteoblasts. (*A*) ALP activity assay. (*B*) Alizarin red assay (200×). (*C*) Western blot assay. (*D*) PCR assay. *$p < 0.05$, **$p < 0.01$. Control group: OB-6 osteoblastic cells were incubated without 300 μm H_2O_2. Model group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2. Curculigoside group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2 and 10 μM curculigoside.

Effect of silencing DNMT1 on the function of osteoblasts The level of DNMT1 in control, model, and curculigoside groups was detected and the results showed that the level of DNMT1 mRNA increased significantly in the model group than in the control group ($p < 0.05$, Fig. 4*A*). Therefore, DNMT1 knockdown OB-6 cell lines were established and the effect of DNMT1 on osteoblast function was verified. DNMT1 siRNA was found to cause a dramatical decrease of DNMT1 in OB-6 osteoblasts than that in sh-NC group ($p < 0.05$, Fig. 4*A*), which means that the mold is successful. Moreover, compared with the model group, the number and area of bone mineralized nodules and the expression of OSX and OPG were markedly increased, while the cell apoptosis and ROS fluorescence intensity were markedly reduced in DNMT1-KO group ($p < 0.05$, respectively, Fig. 4*B–E*). The results indicated that silencing DNMT1 could improve the osteoblast function of OB-6 osteoblasts.

Effect of DNMT1 overexpression on the function of osteoblasts DNMT1 overexpression OB-6 cell lines were further established and DNMT1 mimics was found to cause a significantly increased of DNMT1 in OB-6 osteoblasts than that in NC group ($p < 0.05$, Fig. 5*A*). Meanwhile, compared with the curculigoside group, the gene expression of OSX and OPG was markedly decreased, and the cell apoptosis and ROS fluorescence intensity were markedly increased in curculigoside + DNMT1 mimics group ($p < 0.05$, respectively, Fig. 5*A–C*). The results suggested that DNMT1 overexpression could disrupt the enhancing effect of curculigoside on osteoblast function.

Curculigoside may enhance osteoblast activity through DNMT1 controls of Nrf2 methylation To further speculate the mechanism of curculigoside in osteoporosis, the

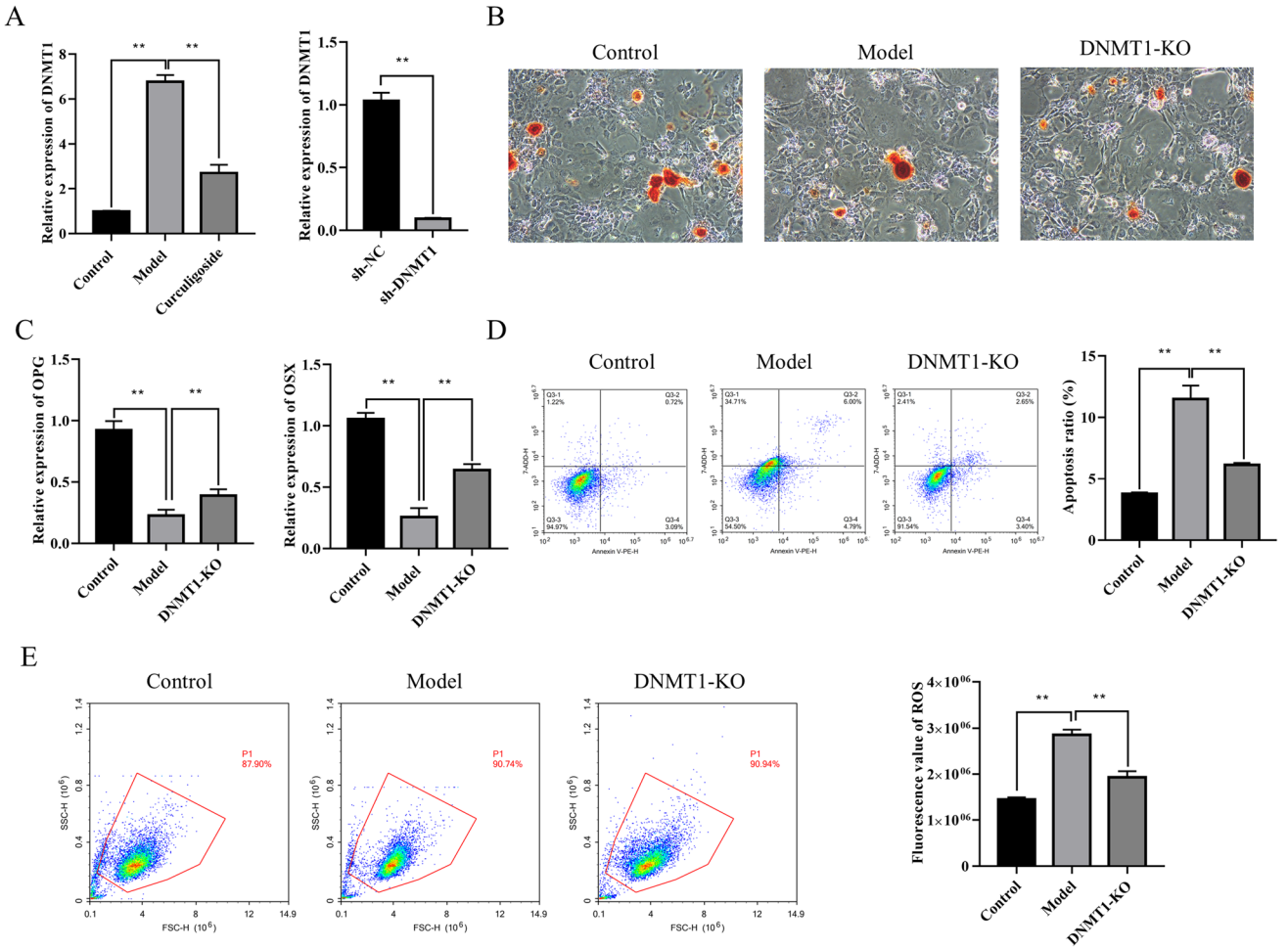

Figure 4. Silencing DNMT1 improves the osteoblast function of OB-6 osteoblasts. (*A*) PCR assay was used to detect DNMT1 expression. (*B*) Alizarin red assay (200×). (*C*) PCR assay was used to detect OPG and OSX expression. (*D*) Flow cytometry assay was used to detect apoptosis. (*E*) Flow cytometry assay was used to detect ROS fluorescence intensity. $^{**}p < 0.01$. Control group: OB-6 osteoblastic cells were incubated without 300 μm H_2O_2. Model group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2. DNMT1-KO group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2 and transfected with DNMT1 siRNA. Sh-NC group: OB-6 osteoblastic cells were transfected with DNMT1 siRNA NC. Sh-DNMT1 group: OB-6 osteoblastic cells were transfected with DNMT1 siRNA.

expression of Nrf2 in control, model, and curculigoside groups was studied. Compared with the control group, the gene (Fig. 6*A*), protein (Fig. 6*B*), and fluorescence intensity (Fig. 6*C*) of Nrf2 were markedly reduced in the model group ($p < 0.05$, respectively). Moreover, the gene, protein, and fluorescence intensity of Nrf2 were markedly increased in the curculigoside group ($p < 0.05$, respectively). Taken together, curculigoside may enhance osteoblast activity through DNMT1 controls of Nrf2 methylation.

Discussion

Osteoporosis is a multifactorial systemic bone metabolic disease characterized by a decrease in bone mass per unit volume and destruction of bone tissue microarchitecture (Alexiou et al. 2018; Lai et al. 2022). Almost all bones are at risk of fracture due to osteoporosis. Therefore, it is of great social value and significance to study the pathogenesis and pathological characteristics of osteoporosis in order to find more effective treatment methods.

The decreased osteogenic capacity and increased osteoclastic capacity of bone lead to a rate of bone resorption that exceeds the rate of bone formation (Chen et al. 2017). The disturbances and imbalances in bone metabolism is the fundamental causes of osteoporosis in middle-aged and elderly people (Chen et al. 2017). Therefore, promoting new bone formation to replace lost bone tissue is the key to the treatment of osteoporosis. In recent years, oxidative stress as a risk factor of osteoporosis has been paid more and more attention. Oxidative stress can promote the occurrence and development of osteoporosis (Agidigbi and Kim

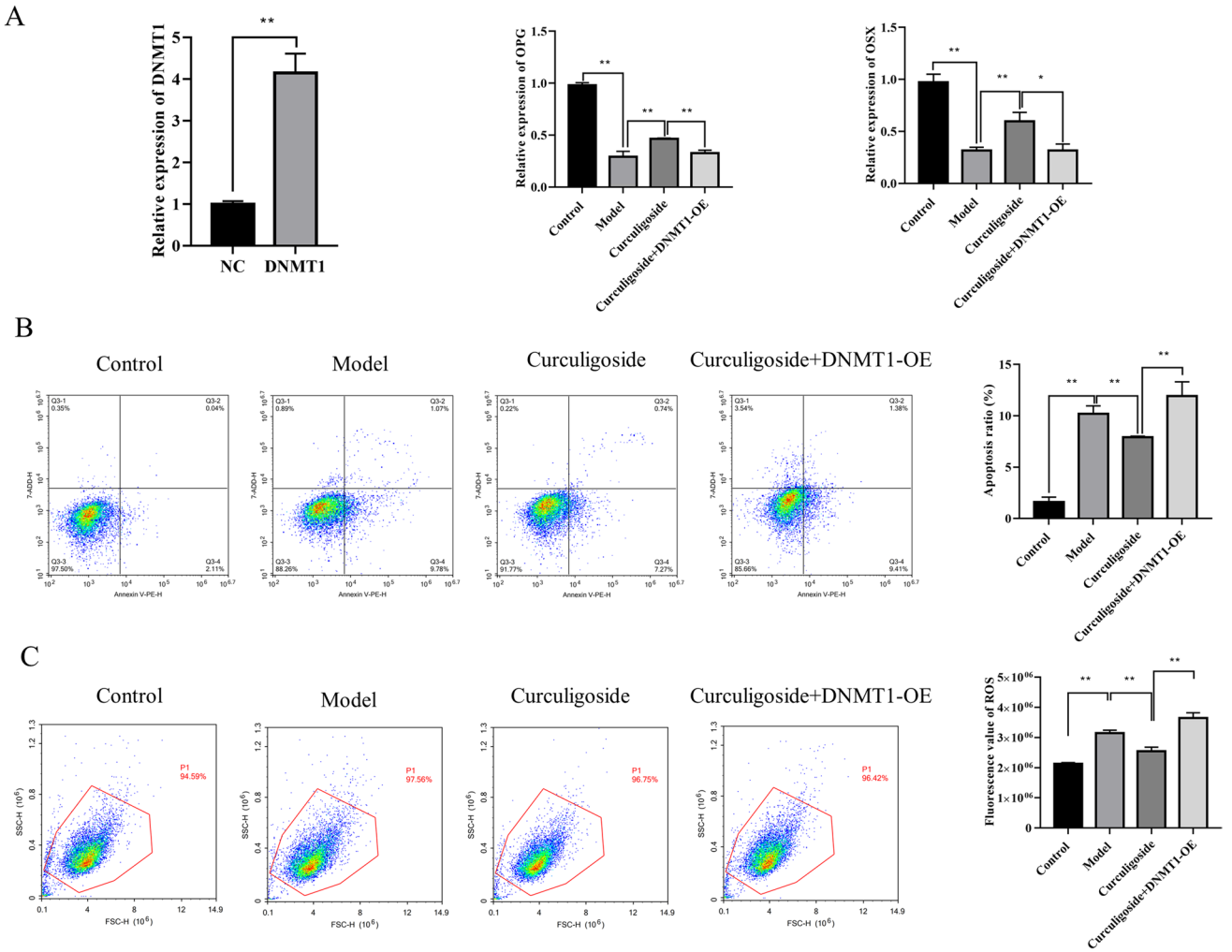

Figure 5. DNMT1 overexpression disrupts the enhancing effect of curculigoside on osteoblast function. (*A*) PCR assay was used to detect OPG and OSX expression. (*B*) Flow cytometry assay was used to detect apoptosis. (*C*) Flow cytometry assay was used to detect ROS fluorescence intensity. $*p<0.05$, $**p<0.01$. Control group: OB-6 osteoblastic cells were incubated without 300 μm H_2O_2. Model group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2. Curculigoside group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2 and 10 μM curculigoside. Curculigoside + DNMT1-OE group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2 and 10 μM curculigoside after DNMT1 mimics transfection. NC group: OB-6 osteoblastic cells were transfected with DNMT1 mimics NC. DNMT1 group: OB-6 osteoblastic cells were transfected with DNMT1 mimics.

2019; Torres et al. 2021). The overproduction of ROS that is not balanced by adequate levels of antioxidants can lead to oxidative stress, which in turn causes oxidative damage to cells (Daenen et al. 2019). At the same time, as the body ages, estrogen levels decline and ROS production increases, and excessive ROS inhibit bone formation by osteoblasts, leading to bone loss and osteoporosis (Lephart and Naftolin 2021). It has been found that curcuma zedoary saponins can reduce oxidative stress and osteoclast formation (Wang et al. 2010). In view of this, we investigated the role of curcumin in osteoporosis and demonstrated that curcumin alleviates osteoporosis by regulating osteoblast activity.

The DNMT1 gene uses information about DNA methylation patterns in the parent chain to methylate the daughter strand in freshly copied hemimethylated DNA (Svedružić ŽM 2011). DNMT1 can also regulate gene expression independent of its catalytic activity and is involved in multiple processes (Mohan 2022). The present study identified upregulated expression of DNMT1 in H_2O_2-induced OB-6 osteoblasts, suggesting that DNMT1 may be a functional gene in osteoporosis. Based on the transfected with DNMT1 siRNA or DNMT1 mimics, the results found that silencing DNMT1 improves the osteoblast function of OB-6 osteoblasts and DNMT1 overexpression disrupts the enhancing effect of curculigoside on osteoblast function. In addition, Nrf2 is a

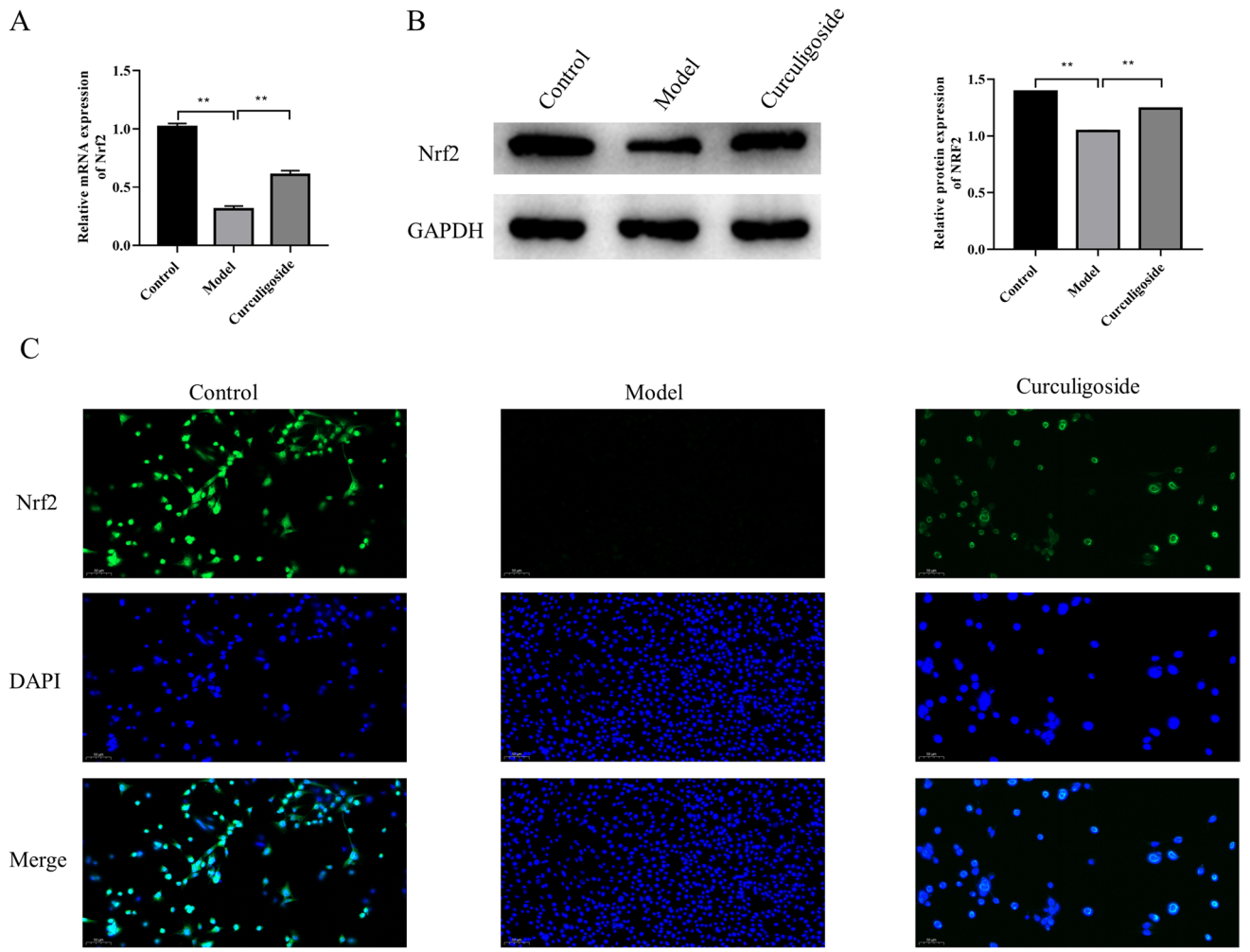

Figure 6. Curculigoside may enhance osteoblast activity through DNMT1 controls of Nrf2 methylation. (*A*) PCR assay. (*B*) Western blot assay. (*C*) Immunofluorescence assay. $^{**}p < 0.01$. Control group: OB-6 osteoblastic cells were incubated without 300 μm H_2O_2. Model group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2. Curculigoside group: OB-6 osteoblastic cells were incubated with 300 μm H_2O_2 and 10 μM curculigoside.

major regulator of oxidative stress and plays a key role in the pathogenesis of osteoporosis (Chen et al. 2021). As a key anti-osteoporotic factor, the hypermethylation of Nrf2 promoter caused by abnormal increase of DNMT may be the pathogenesis of osteoporosis (Chen et al. 2021). Consistent with previous study, we found that curculigoside may enhance osteoblast activity through the control of Nrf2 methylation by DNMT1.

Conclusions

In conclusion, we found that curculigoside has a protective effect against osteoporosis. In-depth studies revealed that curculigoside attenuates osteoporosis through regulating DNMT1-mediated osteoblast activity. These events, together with the fact that curculigoside modulates Nrf2 level and there is an interaction between DNMT1 and Nrf2, further speculated that curculigoside may enhance osteoblast activity through DNMT1 controls of Nrf2 methylation. These data are important for understanding the progression of osteoporosis and help explain the mechanisms by which curculigoside plays a protective role in osteoporosis. At the same time, it also provides an experimental basis for the use of curculigoside in the prevention and treatment of osteoporosis.

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Data Availability All data generated or analysed during this study are included in this published article.

Declarations

Competing interests The authors declare no competing interests.

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