lncRNA Kcnq1ot1 promotes bone formation by inhibiting miR-98-5p/Tbx5 axis in MC3T3-E1 cells

FURONG WANG, FUCAI ZHANG and FENG ZHENG

Department of Orthopedics, Qinghai Provincial People's Hospital, Chengdong, Xining, Qinghai 810007, P.R. China

Received June 18, 2021; Accepted July 30, 2021

DOI: 10.3892/etm.2022.11117

Abstract. Long non-coding (lnc)RNA KCNQ1 opposite strand/antisense transcript 1 (Kcnq1ot1) has been shown to regulate multiple biological processes. However, the functional role of Kcnq1ot1 in osteoporosis and the underlying mechanism are still unclear. The present study aimed to investigate the function of lncRNA Kcnq1ot1 in osteogenic differentiation. Alkaline phosphatase (ALP) activity was measured using an ALP assay kit. Western blotting was performed to assess the expression levels of osteogenic differentiation-associated proteins. Reverse transcription-quantitative PCR was performed to detect Kcnq1ot1, microRNA (miR)-98-5p and T-box transcription factor 5 (Tbx5) expression levels. The binding of Kcnq1ot1 with miR-98-5p and that of miR-98-5p with Tbx5 were predicted by starBase and TargetScan databases, respectively, and verified using dual luciferase reporter assays. The mineralization of MC3T3-E1 cells was observed using an Alizarin red S staining assay. The results revealed that expression of Kcnq1ot1 was increased and that of miR-98-5p was decreased during osteogenic differentiation. Additionally, Kcnq1ot1 was shown to target miR-98-5p and inhibit its expression. Inhibiting miR-98-5p reversed the inhibitory effect of Kcnq1ot1 knockdown on osteogenic differentiation and mineralization of MC3T3-E1 cells. Furthermore, Kcnq1ot1 regulated Tbx5 expression via miR-98-5p. Overexpressing miR-98-5p or downregulating Tbx5 expression reversed the promotive effect of Kcnq1ot1 overexpression on osteogenic differentiation and mineralization of MC3T3-E1 cells. In conclusion, these findings suggested that Kcnq1ot1 may promote bone formation by inhibiting miR-98-5p and upregulating Tbx5. Kcnq1ot1, miR-98-5p and Tbx5 may therefore serve as promising targets for the treatment of osteoporosis.

Correspondence to: Dr Feng Zheng, Department of Orthopedics, Qinghai Provincial People's Hospital, 2 Gonghe Road, Chengdong, Xining, Qinghai 810007, P.R. China
E-mail: hydgk2011@126.com

Key words: long non-coding RNA KCNQ1 opposite strand/antisense transcript 1, microRNA-98-5p, T-box transcription factor 5, osteogenesis, bone formation

Introduction

Osteoporosis is a 'silent disease' that results in fragile bones that are prone to fracture and lacks obvious symptoms at the beginning of the pathological process (1). The loss of bone, decrease in bone mass, destruction of bone microstructure and increase in bone brittleness can lead to systemic bone disease in patients with osteoporosis (2). Osteoporosis can be classified as primary or secondary according to its etiology. The etiology of primary osteoporosis is still not clear, but secondary osteoporosis is often caused by endocrine metabolic disease (such as hyperthyroidism and hyperparathyroidism) and can also be caused by certain drugs (such as glucocorticoids) that affect bone metabolism (3-5). In general, adequate calcium intake, a healthy diet and physical activity contribute to good bone health and decrease the risk of osteoporosis (6-8). However, age is associated with the onset of osteoporosis, which is why this disease is considerably more common in older individuals (9). In China, the prevalence of osteoporosis in the elderly (>60 years old) is 36%, amongst which, the incidence is ~23% in males and ~49% in females (10-11). Fractures are a serious consequence of osteoporosis. In 2010, the number of fractures caused by osteoporosis in China reached 2.23 million, of which 1.11 million were spinal fractures and 360,000 were pelvic fractures (12). According to various reports, osteoporosis has become one important factor affecting the quality of life of middle-aged and elderly individuals (13,14). Therefore, it is necessary to identify diagnostic markers and therapeutic targets for improved diagnosis and treatment of osteoporosis.

Long non-coding RNA (lncRNA) serves a role in cell proliferation, migration and differentiation, among numerous other processes (15,16). lncRNA KCNQ1 opposite strand/antisense transcript 1 (Kcnq1ot1) is an imprinted antisense lncRNA in the human KCNQ1 locus and is associated with bone formation (17). Additionally, Kcnq1ot1 has been shown to facilitate osteogenic differentiation of human bone marrow-derived mesenchymal stem cells via the microRNA (miRNA or miR)-320a/Smad5 axis (18). Furthermore, miR-98-5p has been reported to participate in myocardial differentiation of bone marrow mesenchymal stem cells via the regulation of T-box transcription factor 5 (Tbx5) (19). In addition, this miRNA may target high mobility group AT-hook 2 to inhibit the osteogenic differentiation of MC3T3-E1 osteoblasts, thereby obstructing bone regeneration (20). Thus, it was

hypothesized that Kcnq1ot1 may affect osteogenic differentiation by binding to miR-98-5p. The aim of the present study was to verify this hypothesis to clarify the role of miR-98-5p in osteogenic differentiation and identify novel therapeutic targets for the treatment of osteoporosis.

Materials and methods

Bioinformatics analysis. starBase database (starbase.sysu.edu.cn/index.php/) was used to predict the binding site of Kcnq1ot1 and miR-98-5p. miR-98-5p and Tbx5 target site was predicted using TargetScan (targetscan.org/vert_72/).

Cell culture. The MC3T3-E1 mouse pre-osteoblast cell line was sourced from the American Type Culture Collection (cat. no. CRL-2594) and cultured in α-Minimum Essential Medium (cat. no. A1049001) supplemented with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate and 10% FBS (all Gibco; Thermo Fisher Scientific, Inc.) in the absence of ascorbic acid. Cells were cultured at 37˚C in a humidified incubator with 5% CO_2 and the medium was replaced every 2-3 days.

Induction of osteogenic differentiation. Osteogenic differentiation of MC3T3-E1 cells was induced by incubation with osteogenesis-inducing medium at 80% confluence for 14 days at 37˚C, as previously described (18). The medium consisted of 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 5 mM L-glycerophosphate (Sigma-Aldrich; Merck KGaA), 100 nM dexamethasone (AmyJet Scientific, Inc.) and 50 mg/ml ascorbic acid (Shanghai Aladdin Biochemical Technology Co., Ltd.).

Cell transfection. Short hairpin (sh)RNA plasmid for Kcnq1ot1 (sh-Kcnq1ot1; 5'-GCAGAACCAUCGAUGGUGCGU-3'), shRNA targeting Tbx5 (sh-Tbx5; 5'-CGGCUGCUAGUGUCUAUGUUU-3'), shRNA-negative control (sh-NC; 5'-AGUGCUGCGCACGUGUCUCAU-3'), pcDNA3.1(+)/Kcnq1ot1 plasmid (pc-Kcnq1ot1; 5'-GGGGTACCCCAGGTGACAAGGTGCAGGCGC-3'), pcDNA3.1 (5'-AUCUCCGGGGUUUACGUAUAC-3'), miR-98-5p antagomir (antagomiR-98-5p; 5'-AACAAUACAACUUACUACCUCA-3'), antagomiR-NC (5'-UCACAACCUCCUAGAAAGAGUAGA-3'), miR-98-5p agomir (agomiR-98-5p; 5'-UGAGGUAGUAAGUUGUAUUGUU-3') and agomiR-NC (5'-UCGCUUGGUGCAGGUCGGG-3') were constructed by Shanghai GenePharma, Co., Ltd. A final concentration of 100 nM shRNA, pcDNA, antagomir or agomir of miR-98-5p and NC was transfected into MC3T3-E1 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. At transfection, cells were harvested, and reverse transcription-quantitative PCR (RT-qPCR) was performed to assess the transfection efficiency. On the 7th day of osteogenic differentiation, transfection as aforementioned was performed to maintain the altered gene expression.

Alkaline phosphatase (ALP) activity assay. ALP activity was measured to assess differentiation of MC3T3-E1 cells. Briefly, cells were seeded into 12-well plates at a density of $4x10^4$ cells/ml 37˚C for 7 days. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature and treated with 0.3 nitro-blue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich; Merck KGaA) at room temperature for 2 h. Cells were then washed with deionized water and observed under an inverted light microscope (Nikon Corporation; magnification, x200) at a wavelength of 405 nm.

Western blotting. Total protein was extracted from MC3T3-E1 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was determined using a BCA assay kit (Beyotime Institute of Biotechnology). A total of 30 μg protein/well was resolved using 10% SDS-PAGE and transferred to a PVDF membrane. Subsequently, 5% non-fat milk was used to block the membrane at 37˚C for 1 h, followed by incubation at room temperature for 1 h with primary antibodies as follows: Runt-related transcription factor 2 (RUNX2; 1:1,000; cat. no. 12556; Cell Signaling Technology, Inc.), collagen type I α 1 (COL1A1; 1:1,000; cat. no. ab34710; Abcam), osteopontin (1:1,000, cat. no. ab214050; Abcam), osteocalcin (1:1,000, cat. no. ab133612; Abcam), Tbx5 (cat. no. ab259980; Abcam) and GAPDH (1:2,500, cat. no. ab9485; Abcam). The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2,000, cat. no. #5127; Cell Signaling Technology, Inc.) at room temperature for 2 h. The bands were visualized by using an enhanced chemiluminescence (ECL) reagent kit (Shanghai Yeasen Biotechnology Co., Ltd.) and semi-quantified with Image J software (Version 1.49; National Institutes of Health).

RT-qPCR. Total RNA was extracted from MC3T3-E1 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed to synthesize cDNA from total RNA, using a PrimeScript RT Reagent kit, according to the manufacturer's protocol (Takara Bio, Inc.). A SYBR green PCR Master Mix kit (cat. no. SR1110; Beijing Solarbio Science & Technology Co., Ltd.) was used for cDNA amplification by qPCR in accordance with the manufacturer's instructions on an AFD9600 PCR system (Hangzhou AGS BioTech Co., Ltd.). The primer sequences for PCR were as follows: Kcnq1ot1 forward, 5'-ACTCACTCACTCACTCACT-3' and reverse, 5'-CTGGCTCCTTCTATCACATT-3'; miR-98-5p forward, 5'-ATCCAGTGCGTGTCGTG-3' and reverse, 5'-TGCTTGAGGTAGTAAGTTG-3'; Tbx5 forward, 5'-AAGTAAAGAATATCCCGTGGTC-3' and reverse, 5'-AGACTCGCTGCTGAAAGG-3'; GAPDH forward, 5'-GGGAAACTGTGGCGTGAT-3' and reverse, 5'-GAGTGGGTGTCGCTGTTGA-3' and U6 forward, 5'-CTCGCTTCGGCAGCACATATA-3' and reverse, 5'-ACGCTTCACGAATTTGAGTGTC-3'. The thermocycling conditions were as follows: 94˚C for 60 sec, followed by 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 60 sec. The $2^{-\Delta\Delta Cq}$ method (21) was used to calculate relative gene expression. GAPDH was used as an internal reference for Kcnq1ot1 and Tbx5, while U6 was used as the control gene for miR-98-5p.

Dual luciferase reporter assay. The wild-type (Kcnq1ot1-WT or Tbx5-WT) and mutant (Kcnq1ot1-MUT or Tbx5-MUT) 3'-untranslated regions were cloned into a pmirGLO vector

(Shanghai GenePharma Co., Ltd.). For luciferase reporter analysis, MC3T3-E1 cells were co-transfected with luciferase reporter vectors, agomiR-98-5p (5'-UGAGGUAGUAAG UUGUAUUGUU-3') and agomiR-NC (5'-UCGCUUGGU GCAGGUCGGG-3') using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h after transfection, the relative luciferase activities were measured by using a Dual-Luciferase Reporter Assay (Promega Corporation) and normalized to *Renilla* luciferase reporter activity according to the manufacturer's protocol.

Alizarin red S (ARS) staining assay. An ARS staining kit (GuideChem) was used to detect the formation of mineralized nodules in MC3T3-E1 osteoblasts. Following induction of osteogenesis and cell fixation with 95% ethanol for 20 min at room temperature, the cells were washed with PBS (Shanghai Aladdin Biochemical Technology Co., Ltd.) three times and stained with ARS staining solution for 30 min at room temperature. The cells were observed and images were obtained using a light microscope (magnification, x200).

Statistical analysis. All experiments were performed independently three times. GraphPad Prism version 6.0 (GraphPad Software, Inc.) was used to analyze the data. Data are presented as the mean ± SD. One-way ANOVA followed by Tukey's post hoc test was used for comparisons between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Kcnq1ot1 expression is increased and miR-98-5p expression is decreased during osteogenic differentiation. A time-dependent increase in the relative activity of ALP was observed during the incubation of MC3T3-E1 osteoblasts in osteogenesis-inducing medium, indicating the occurrence of osteogenic differentiation (Fig. 1A). The protein expression levels of osteogenic differentiation-associated proteins, RUNX2, COL1A1, osteopontin and osteocalcin, were also increased in a time-dependent manner following incubation of MC3T3-E1 cells in osteogenesis-inducing medium (Fig. 1B and C). The expression of Kcnq1ot1 was elevated, and that of miR-98-5p was decreased as the duration of osteogenesis induction increased (Fig. 1D). These results suggested that Kcnq1ot1 expression increased and miR-98-5p expression decreased during osteogenic differentiation.

Kcnq1ot1 targets and inhibits miR-98-5p. According to starBase, Kcnq1ot1 was predicted to bind to miR-98-5p (Fig. 2A). A dual luciferase reporter assay showed decreased luciferase activity in MC3T3-E1 cells co-transfected with agomiR-98-5p and Kcnq1ot1-WT compared with that in cells co-transfected with agomiR-98-5p and Kcnq1ot1-MUT, which verified the binding between Kcnq1ot1 and miR-98-5p (Fig. 2B). Furthermore, following successful knockdown of Kcnq1ot1 (Fig. 2C), the expression of miR-98-5p was significantly upregulated compared with the control group (Fig. 2D). These results suggest that Kcnq1ot1 may target and inhibit miR-98-5p in MC3T3-E1 cells.

Antagonizing miR-98-5p reverses the inhibitory effect of Kcnq1ot1 knockdown on osteogenic differentiation. To determine the role of the interaction between miR-98-5p and Kcnq1ot1 in osteogenic differentiation, MC3T3-E1 cells were transfected with sh-Kcnq1ot1 or co-transfected with sh-Kcnq1ot1 and antagomiR-98-5p. The knockdown effect of antagomiR-98-5p was detected by RT-qPCR (Fig. 3A). miR-98-5p expression was increased by sh-Kcnq1ot1; this was rescued following co-transfection of sh-Kcnq1ot1 and antagomiR-98-5p (Fig. 3B). Kcnq1ot1 knockdown also decreased ALP activity, whereas inhibition of miR-98-5p restored ALP activity (Fig. 3C). Kcnq1ot1 knockdown significantly inhibited the formation of mineralized nodules, which was rescued following co-transfection of sh-Kcnq1ot1 and antagomiR-98-5p in MC3T3-E1 cells (Fig. 3D). Kcnq1ot1 knockdown also significantly inhibited the expression of RUNX2, COL1A1, osteopontin and osteocalcin, whereas antagonizing miR-98-5p in the presence of Kcnq1ot1 knockdown significantly upregulated expression of these proteins (Fig. 3E). These results indicated that antagonizing miR-98-5p may reverse the inhibitory effect of Kcnq1ot1 knockdown on osteogenic differentiation.

Kcnq1ot1 regulates Tbx5 expression via miR-98-5p. TargetScan software was used to predict the binding sites between Tbx5 and miR-98-5p (Fig. 4A). The transfection efficiency of pc-Kcnq1ot1 and agomiR-98-5p was detected by RT-qPCR (Fig. 4B and C). A dual luciferase reporter assay confirmed the results of the TargetScan prediction as significantly decreased relative luciferase activity was observed in MC3T3-E1 cells co-transfected with Tbx5-WT and agomiR-98-5p. Furthermore, MC3T3-E1 cells transfected with Tbx5-WT, agomiR-98-5p and pc-Kcnq1ot1 exhibited increased luciferase activity compared with the Tbx5-WT + agomiR-98-5p group (Fig. 4D). The results of RT-qPCR and western blotting both showed that Tbx5 expression was significantly downregulated by agomiR-98-5p but upregulated following Kcnq1ot1 overexpression (Fig. 4E and F). These results indicated that Kcnq1ot1 may exert a regulatory effect on Tbx5 expression via modulating miR-98-5p.

miR-98-5p overexpression and Tbx5 knockdown reverse the promotive effect of Kcnq1ot1 overexpression on osteogenic differentiation and mineralization. To determine the role of the interaction between miR-98-5p and Kcnq1ot1, as well as that between Tbx5 and Kcnq1ot1, in osteogenic differentiation and mineralization, agomiR-98-5p or sh-Tbx5 were transfected into MC3T3-E1 cells overexpressing Kcnq1ot1. The transfection efficiency of sh-Tbx5 was detected by RT-qPCR (Fig. 5A). Tbx5 expression was decreased in both the pc-Kcnq1ot1 + agomiR-98-5p and pc-Kcnq1ot1 + sh-Tbx5 groups (Fig. 5B). Meanwhile, the relative increase in ALP activity following Kcnq1ot1 overexpression was decreased following miR-98-5p overexpression or Tbx5 knockdown (Fig. 5C). Kcnq1ot1 overexpression significantly increased mineralization of MC3T3-E1 cells, and this was decreased following transfection with agomiR-98-5p or sh-Tbx5 (Fig. 5D and E). These results suggested that overexpressing miR-98-5p or knocking down Tbx5 may reverse the promotive effect of Kcnq1ot1 overexpression on osteogenic differentiation and mineralization.

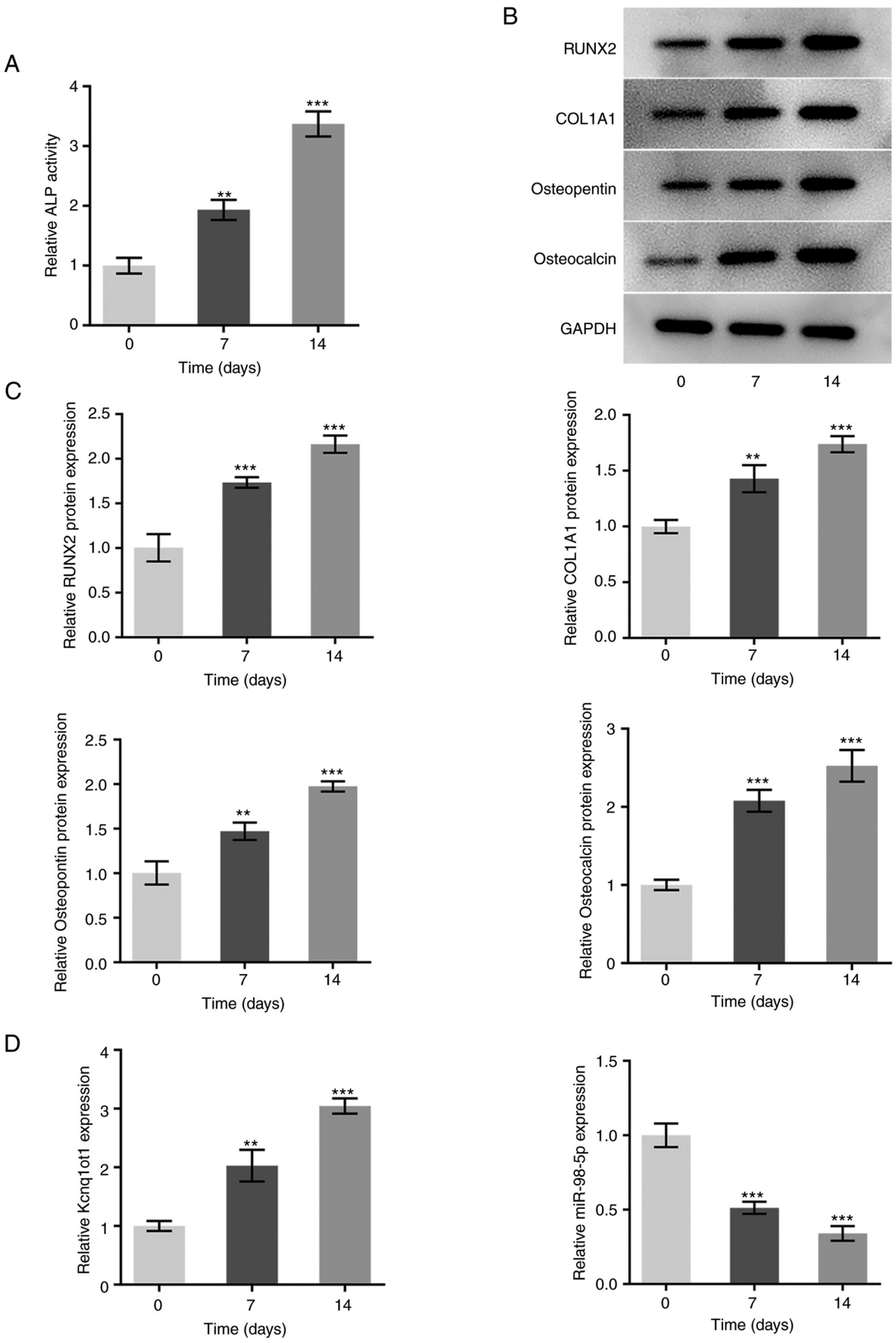

Figure 1. Increased Kcnq1ot1 expression and decreased miR-98-5p in osteogenic differentiation. (A) Relative ALP activity detected by ALP assay kit. (B) Western blotting was performed to determine (-C) protein expression of osteogenic differentiation-associated RUNX, COL1A1, osteopontin and osteocalcin detected by western blotting. (D) mRNA expression of Kcnq1ot1 and miR-98-5p detected by reverse transcription-quantitative PCR in MC3T3-E1 cells cultured in osteogenesis-inducing medium. Data are expressed as mean ± SD. **P<0.01, ***P<0.001 vs. 0. Kcnq1ot1, long non-coding RNA KCNQ1 opposite strand/antisense transcript 1; miR, microRNA; ALP, alkaline phosphatase; RUNX, Runt-related transcription factor 2; COL1A1, collagen type I α 1.

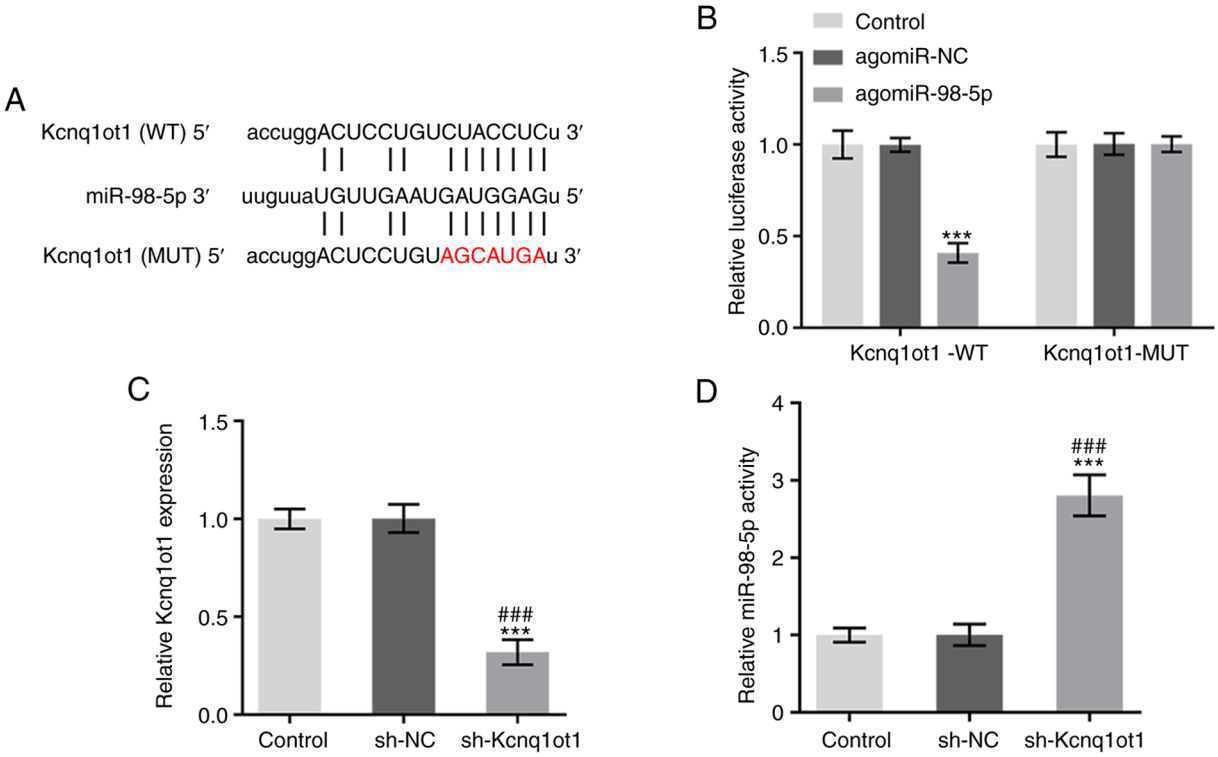

Figure 2. Kcnq1ot1 targets and inhibits miR-98-5p. (A) StarBase prediction of the shared binding sites between Kcnq1ot1 and miR-98-5p. (B) Relative luciferase activity in MC3T3-E1 cells transfected with control, Kcnq1ot1-WT + agomiR-NC or agomiR-98-5p, Kcnq1ot1-MUT + agomiR-NC or agomiR-98-5p, detected by dual-luciferase reporter assay. Relative mRNA expression of (C) Kcnq1ot1 and (D) miR-98-5p in MC3T3-E1 cells transfected with control, sh-NC or sh-Kcnq1ot1, detected by RT-qPCR. Data are expressed as mean ± SD. ***P<0.001 vs. control; $^{###}$P<0.001 vs. sh-NC. Kcnq1ot1, long non-coding RNA KCNQ1 opposite strand/antisense transcript 1; miR, microRNA; WT, wild-type; NC, negative control; MUT, mutant; sh, short hairpin.

miR-98-5p overexpression and Tbx5 knockdown reverse the promotive effect of Kcnq1ot1 overexpression on osteogenic differentiation-associated protein expression. The expression levels of osteogenic differentiation-associated proteins, RUNX2, COL1A1, osteopontin and osteocalcin, were detected following co-transfection of pc-Kcnq1ot1 with agomiR-98-5p or sh-Tbx5. The expression levels of differentiation-associated proteins were increased by Kcnq1ot1 knockdown and inhibited by co-transfection of pc-Kcnq1ot1 and agomiR-98-5p or pc-Kcnq1ot1 and sh-Tbx5 (Fig. 6). These findings suggested that miR-98-5p overexpression and Tbx5 knockdown may reverse the promotive effect of Kcnq1ot1 overexpression on osteogenic differentiation-associated protein expression levels.

Discussion

Osteoporosis is a metabolic bone disease that is characterized by a severe decrease in bone density and mass (22). Dysfunction of either osteoblasts or osteoclasts can affect bone formation and resorption, ultimately leading to metabolic bone disease (22,23). In osteoporosis, back pain or body aches decrease quality of life, whereas spinal deformities and fractures can be disabling, limiting mobility and the ability to function independently (24). In addition, osteoporosis increases the risk of lung infection and bedsores when patients are bedridden for long periods of time. These not only seriously threaten quality of life and survival rate of patients, but also pose a heavy economic burden to individuals, families and society (25).

The involvement of miRNAs, lncRNAs and circular RNAs in various types of disease, including osteoporosis, has been reported by an increasing number of studies (26-28). A recent study showed that adipogenesis and osteogenesis of tendon stem cells are inhibited by Kcnq1ot1 knockdown, which exerts its effect via indirect inhibition of the miR-138 target genes peroxisome proliferator activator receptor γ and RUNX2 (29). Kcnq1ot1 has also been shown to positively regulate osteogenic differentiation of bone marrow mesenchymal stem cells by sponging miR-214 (30). The proliferative capacity of human osteoblastic cell line is less than that of mouse MC3T3-E1 cells, so human cell lines were not suitable for the present study. In addition, induction of osteogenic differentiation in human cells is harder than in mouse cell lines (31). Human and mouse genes are highly homologous, thus MC3T3-E1 cell line was investigated in the present study rather than human osteoblastic cells (32). In the present study, increased expression of Kcnq1ot1 was observed in MC3T3-E1 cells. Kcnq1ot1 silencing significantly inhibited osteogenic differentiation and mineralization in MC3T3-E1 cells, which was consistent with a previous report (33). According to starBase, Kcnq1ot1 shares binding sites with miR-98-5p. A dual luciferase reporter assay was then performed, which verified the binding between Kcnq1ot1 and miR-98-5p. Furthermore, the expression of miR-98-5p was significantly upregulated in MC3T3-E1 cells following Kcnq1ot1 knockdown. miR-98-5p overexpression inhibits osteogenic differentiation and proliferation of MC3T3-E1 osteoblasts by targeting high mobility group AT-Hook2, thereby obstructing bone regeneration (20).

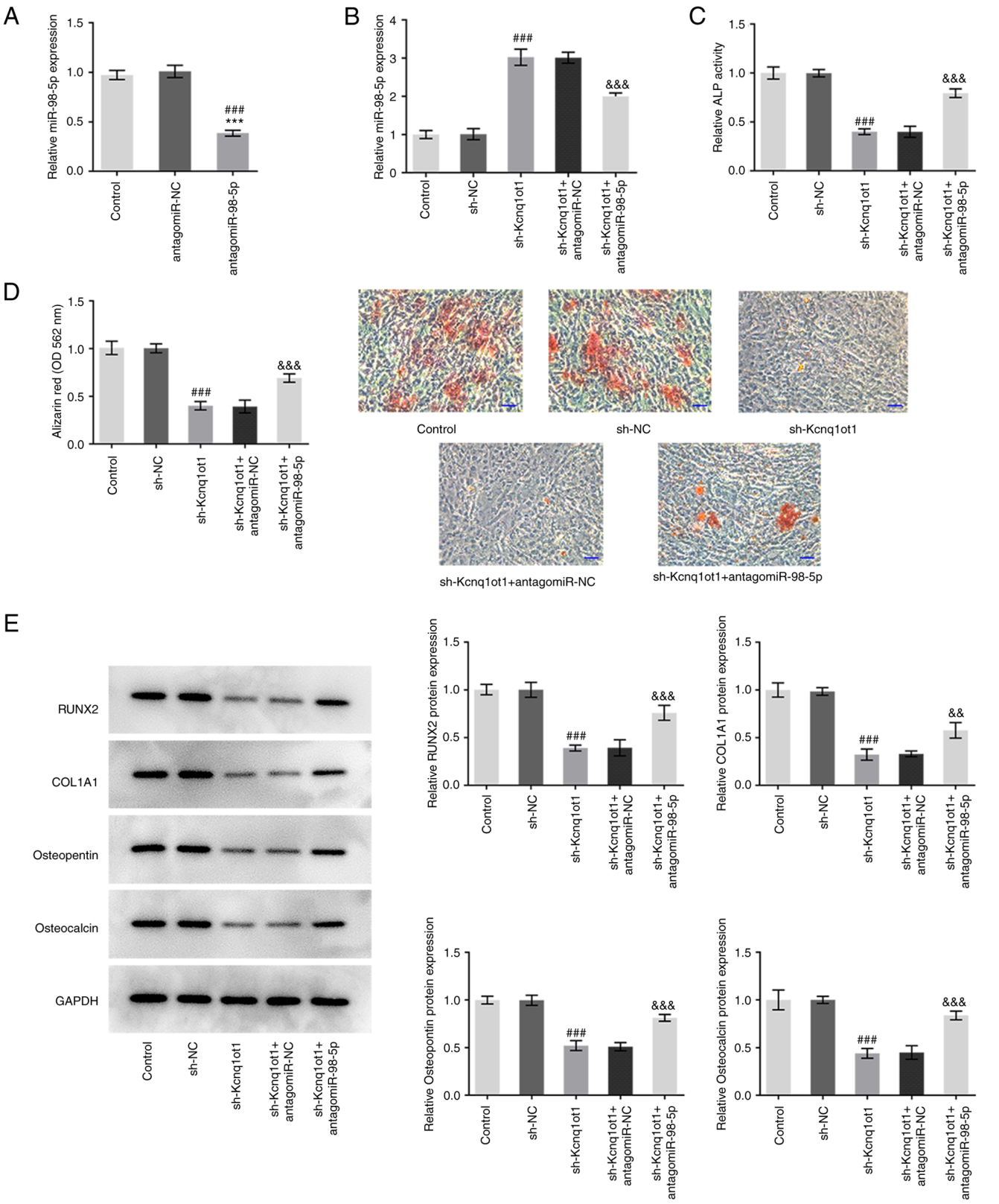

Figure 3. Antagonizing miR-98-5p reverses the inhibitory effect of Kcnq1ot1 knockdown on osteogenic differentiation. (A) Relative mRNA expression of miR-98-5p in MC3T3-E1 cells was detected by reverse transcription-quantitative PCR following transfection with (A) antagomiR-98-5p or (B) control, sh-NC, sh-Kcnq1ot1, sh-Kcnq1ot1 + antagomiR-NC or sh-Kcnq1ot1 + antagomiR-98-5p. (C) Relative ALP activity in MC3T3-E1 cells transfected with control, sh-NC, sh-Kcnq1ot1, sh-Kcnq1ot1 + antagomiR-NC or sh-Kcnq1ot1 + antagomiR-98-5p detected by ALP assay kit. (D) Formation of mineralized nodules in MC3T3-E1 cells transfected with control, sh-NC, sh-Kcnq1ot1, sh-Kcnq1ot1 + antagomiR-NC or sh-Kcnq1ot1 + antagomiR-98-5p, detected by alizarin red S staining assay. Scale bar, 50 μm. (E) Relative protein expression of osteogenic differentiation-associated RUNX, COL1A1, osteopontin and osteocalcin in MC3T3-E1 cells transfected with control, sh-NC, sh-Kcnq1ot1, sh-Kcnq1ot1 + antagomiR-NC or sh-Kcnq1ot1 + antagomiR-98-5p detected by western blotting. Data are expressed as mean ± SD. ***P<0.001 vs. control; $^{###}$P<0.001 vs. antagomiR-NC or sh-NC; $^{&&}$P<0.01, $^{&&&}$P<0.001 vs. sh-Kcnq1ot1 + antagomiR-NC. Kcnq1ot1, long non-coding RNA KCNQ1 opposite strand/antisense transcript 1; miR, microRNA; ALP, alkaline phosphatase; RUNX, Runt-related transcription factor 2; COL1A1, collagen type I α 1; sh, short hairpin; NC, negative control; OD, optical density.

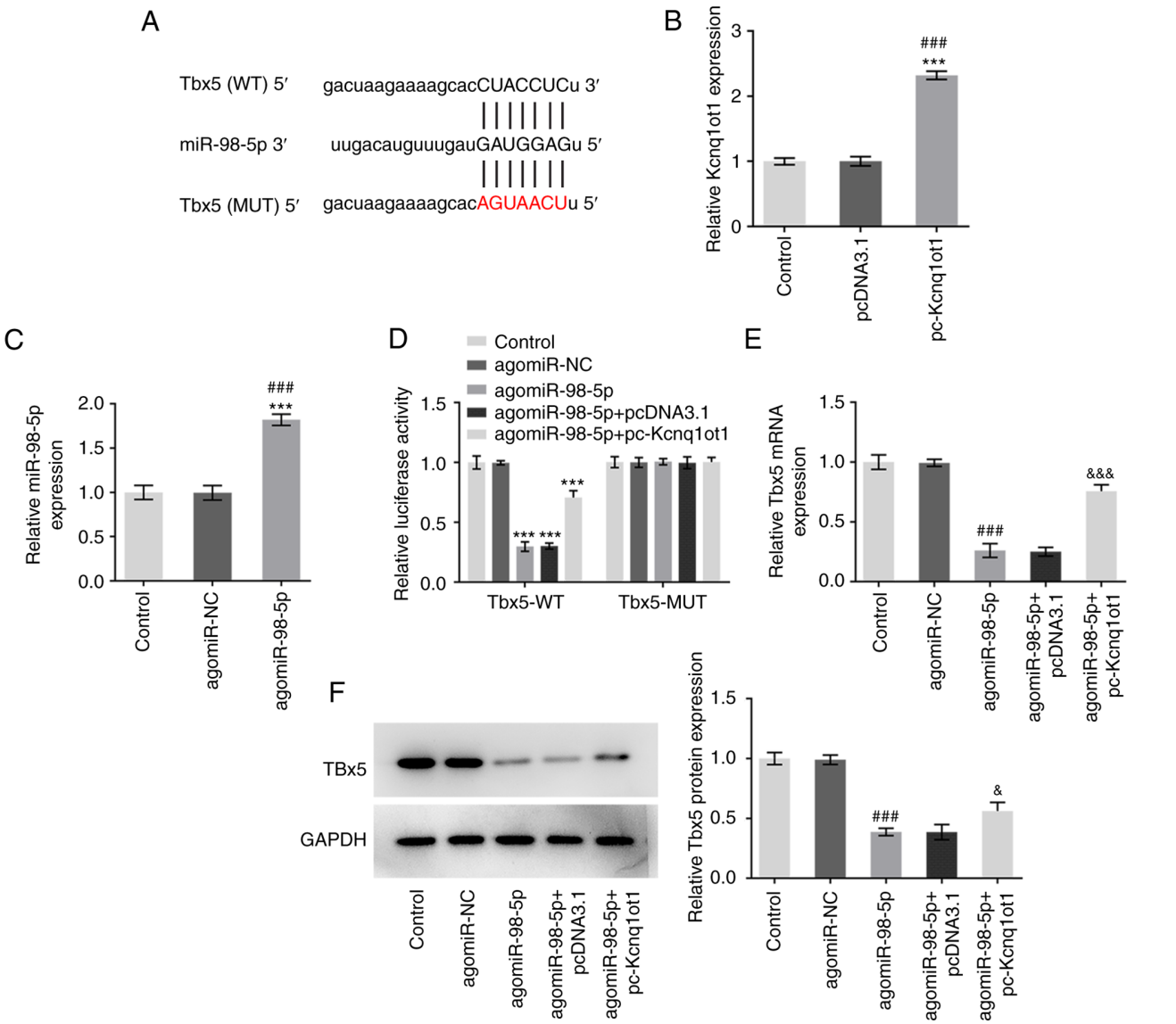

Figure 4. Kcnq1ot1 regulates Tbx5 expression via miR-98-5p. (A) TargetScan prediction of the shared binding sites between miR-98-5p and Tbx5. Expression of (B) Kcnq1ot1 and (C) miR-98-5p was measured by RT-qPCR following transfection with pc-Kcnq1ot1 and agomiR-98-5p. (D) Relative luciferase activity in MC3T3-E1 cells transfected with control, Tbx5-WT + agomiR-NC, Tbx5-WT + agomiR-98-5p, Tbx5-WT + agomiR-98-5p + pcDNA3.1, Tbx5-WT + agomiR-98-5p + pc-Kcnq1ot1, Tbx5-MUT + agomiR-NC, Tbx5-MUT + agomiR-98-5p, Tbx5-MUT + agomiR-98-5p + pcDNA3.1, Tbx5-MUT + agomiR-98-5p + pc-Kcnq1ot1 detected by dual-luciferase reporter assay. Relative expression of Tbx5 in MC3T3-E1 cells transfected with control, agomiR-NC, agomiR-98-5p, agomiR-98-5p + pcDNA3.1 or agomiR-98-5p + pc-Kcnq1ot1, detected by (E) RT-qPCR and (F) western blot analysis. Data are expressed as mean ± SD. ***P<0.001 vs. control; $^{###}$P<0.001 vs. pcDNA3.1 or agomiR-NC; $^{&}$P<0.05, $^{&&&}$P<0.001 vs. agomiR-98-5p + pcDNA3.1. Kcnq1ot1, long non-coding RNA KCNQ1 opposite strand/antisense transcript 1; miR, microRNA; sh, short hairpin; NC, negative control; Tbx5, T-box transcription factor 5; RT-q, reverse transcription-quantitative; WT, wild-type; MUT, mutant.

In agreement with the aforementioned studies, miR-98-5p silencing rescued the inhibitory effect of Kcnq1ot1 knockdown on osteogenic differentiation and mineralization, suggesting that Kcnq1ot1 silencing may serve a suppressive role in bone formation by upregulating miR-98-5p expression.

The present study investigated the interaction between Kcnq1ot1 and miR-98-5p and the mechanism of miR-98-5p in osteogenic differentiation. According to a previous study, miR-98-5p targets the transcription factor Tbx5 and obstructs the transition of rat mesenchymal stem cells into cardiomyocytes (19). Furthermore, TargetScan predicted binding of miR-98-5p to Tbx5. Tbx5 has been reported to promote the differentiation of 5-azacytidine-treated cardiac fibroblasts into cardiomyocytes (34). Another study showed that miR-10-5p impedes myocardial differentiation of bone marrow mesenchymal stem cells via inhibition of Tbx5 (35). Therefore, it was hypothesized that Kcnq1ot1 may target miR-98-5p and upregulate expression of Tbx5, thereby promoting differentiation. In the present study, the dual luciferase reporter assay confirmed the binding between miR-98-5p and Tbx5. The data revealed that knockdown of miR-98-5p significantly decreased Tbx5 expression, which was rescued by Kcnq1ot1 overexpression. This suggested that Kcnq1ot1 regulated Tbx5 expression via miR-98-5p. In addition, less osteogenic differentiation and mineralization, as well as decreased expression levels of RUNX2, COL1A1, osteopontin and osteocalcin in MC3T3-E1 cells, were observed following Kcnq1ot1 overexpression or transfection with agomiR-98-5p or sh-Tbx5. These results

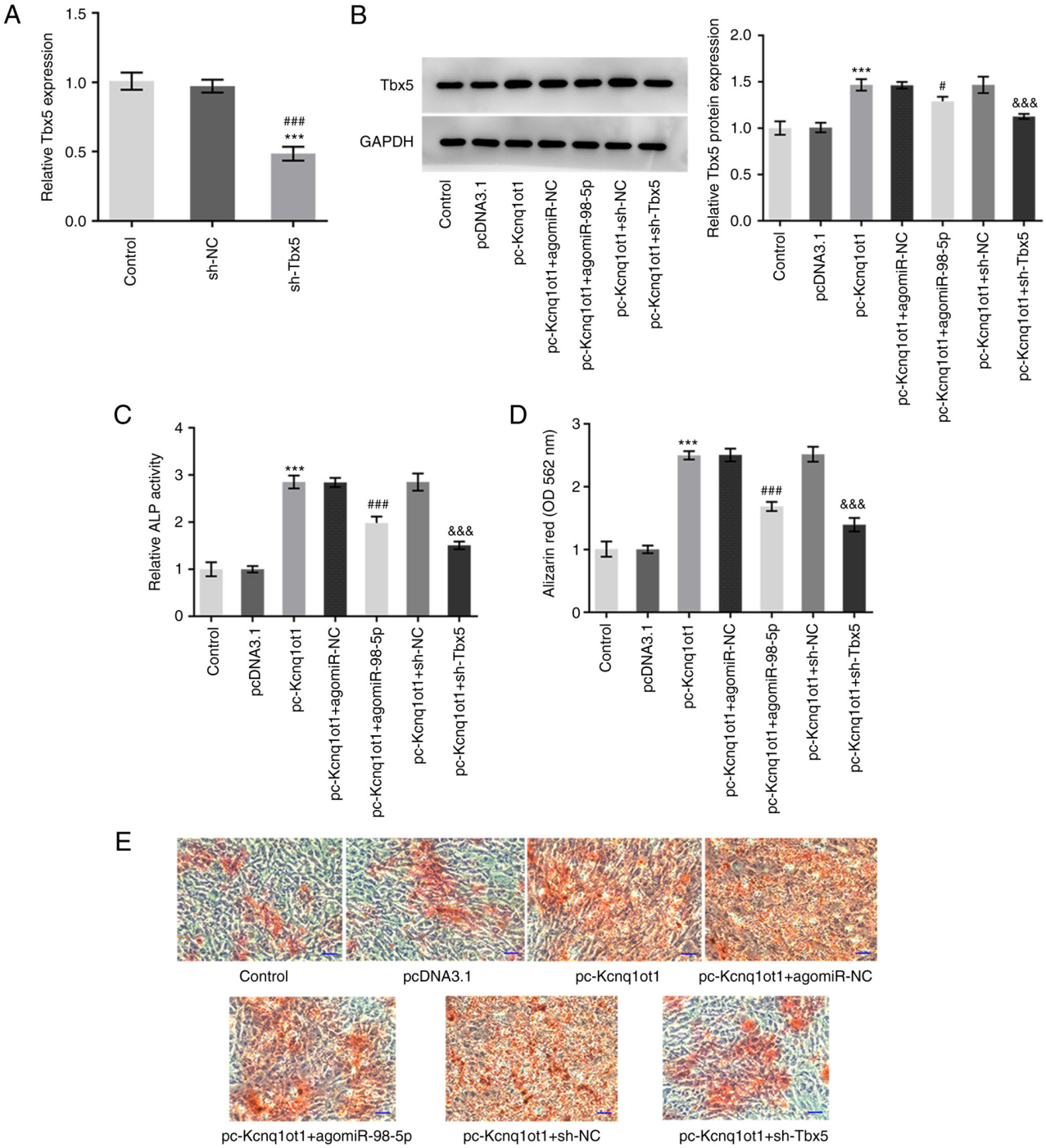

Figure 5. miR-98-5p overexpression and Tbx5 knockdown reverse the promotive effect of Kcnq1ot1 overexpression on osteogenic differentiation and mineralization. (A) Relative mRNA expression of Tbx5 in MC3T3-E1 cells was detected by reverse transcription-quantitative PCR following transfection with sh-Tbx5. (B) Relative protein expression of Tbx5 in MC3T3-E1 cells transfected with control, pcDNA3.1, pc-Kcnq1ot1, pc-Kcnq1ot1 + agomiR-NC, pc-Kcnq1ot1 + agomiR-98-5p, pc-Kcnq1ot1 + sh-NC or pc-Kcnq1ot1 + sh-Tbx5 detected by western blotting. (C) Relative ALP activity in MC3T3-E1 cells transfected with control, pcDNA3.1, pc-Kcnq1ot1, pc-Kcnq1ot1 + agomiR-NC, pc-Kcnq1ot1 + agomiR-98-5p, pc-Kcnq1ot1 + sh-NC or pc-Kcnq1ot1 + sh-Tbx5 detected by ALP assay kit. (D) Formation of mineralized nodules in MC3T3-E1 cells transfected with control, pcDNA3.1, pc-Kcnq1ot1, pc-Kcnq1ot1 + agomiR-NC, pc-Kcnq1ot1 + agomiR-98-5p, pc-Kcnq1ot1 + sh-NC or pc-Kcnq1ot1 + sh-Tbx5 (E) detected by alizarin red S staining assay. Scale bar, 50 μm. Data are expressed as mean ± SD. ***P<0.001 vs. pcDNA3.1; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. sh-NC or pc-Kcnq1ot1 + agomiR-NC; $^{\&\&\&}$P<0.001 vs. pc-Kcnq1ot1 + sh-NC. Kcnq1ot1, long non-coding RNA KCNQ1 opposite strand/antisense transcript 1; miR, microRNA; sh, short hairpin; NC, negative control; Tbx5, T-box transcription factor 5; ALP, alkaline phosphatase; OD, optical density.

suggested that miR-98-5p overexpression or Tbx5 knockdown may reverse the promotive effect of Kcnq1ot1 overexpression on the osteogenic differentiation of MC3T3-E1 cells. The present results also revealed that the increase in Tbx5 expression levels induced by Kcnq1ot1 overexpression was significantly suppressed by sh-Tbx5. However, the suppressive effect of agomiR-98-5p on the increased Tbx5 level was marginal. It was hypothesized that other unidentified specific pathways regulate the Kcnq1ot1/miR-98-5p/Tbx5 axis. This should be confirmed *in vivo* and in clinical practice. The

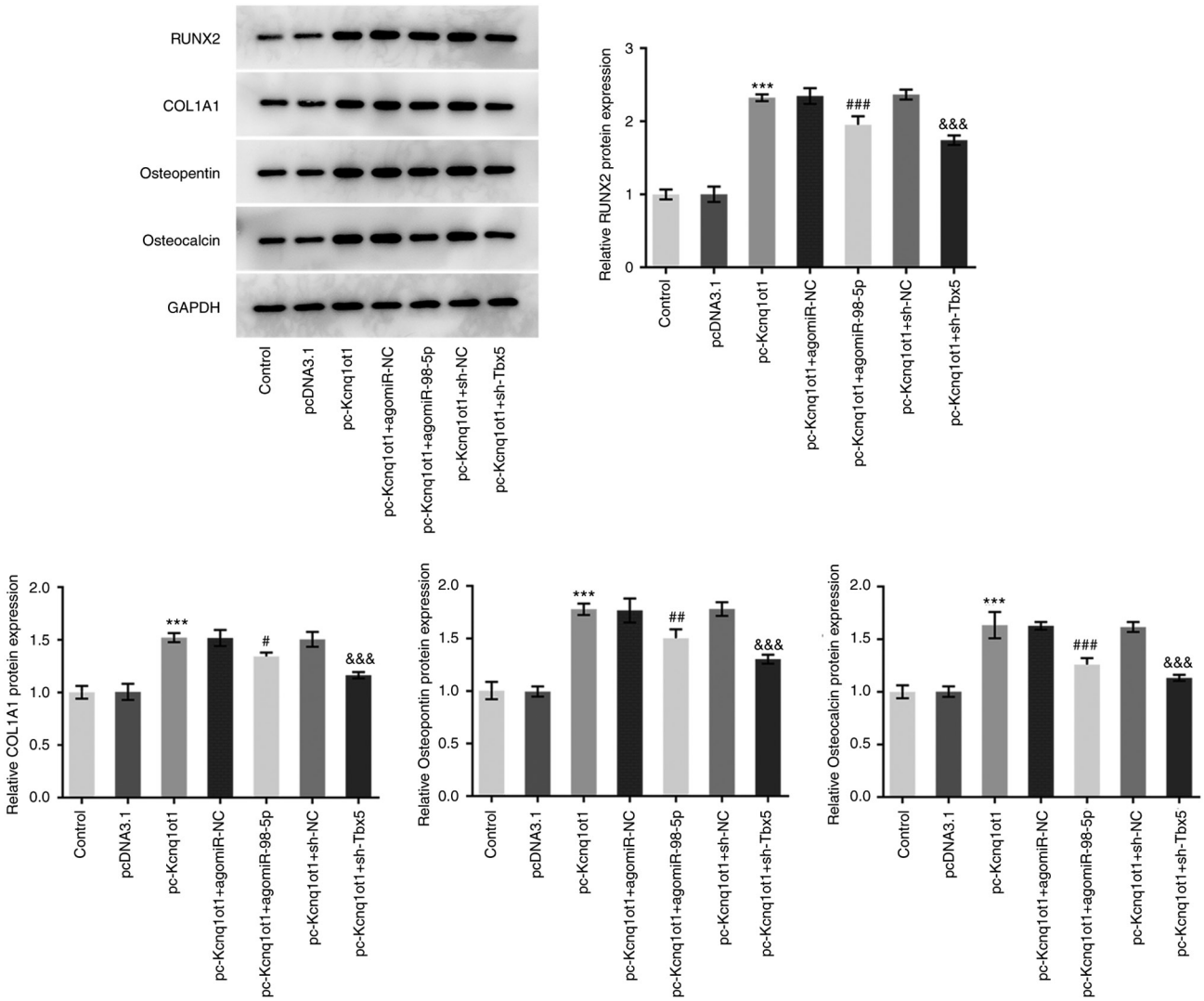

Figure 6. miR-98-5p overexpression and Tbx5 knockdown reverse the promotive effect of Kcnq1ot1 overexpression on osteogenic differentiation-associated protein expression. Relative protein expression of osteogenic differentiation-associated RUNX2, COL1A1, osteopontin and osteocalcin in MC3T3-E1 cells transfected with control, pcDNA3.1, pc-Kcnq1ot1, pc-Kcnq1ot1 + agomiR-NC, pc-Kcnq1ot1 + agomiR-98-5p, pc-Kcnq1ot1 + sh-NC or pc-Kcnq1ot1 + sh-Tbx5 detected by western blotting. Data are expressed as mean ± SD. ***P<0.001 vs. pcDNA3.1; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. pc-Kcnq1ot1 + agomiR-NC; $^{\&\&\&}$P<0.001 vs. pc-Kcnq1ot1 + sh-NC. Kcnq1ot1, long non-coding RNA KCNQ1 opposite strand/antisense transcript 1; miR, microRNA; sh, short hairpin; NC, negative control; Tbx5, T-box transcription factor 5; RUNX, Runt-related transcription factor 2; COL1A1, collagen type I α 1.

MC3T3-E1 cell line is a classical and common cell model used to study osteogenic differentiation. Thus, MC3T3-E1 was selected as a representative to investigate the role of Kcnq1ot1 in bone formation and the underlying mechanism (36,37). Human osteoblast cell lines should be used to verify the results. Utilization of multiple cell lines may better reveal the mechanism underlying the effect of Kcnq1ot1 on osteogenic differentiation. However, the present study focused on the target and mechanism by which osteogenic differentiation regulates osteoporosis in the present study. Another limitation of the study was that the expression of Kcnq1ot1 and miR-98-5p was not assessed at early timepoints (such as day 1-3). The biological effects of Kcnq1ot1/miR-98-5p in other pre-osteoblast cell lines and other potential mechanisms should be investigated in future.

In conclusion, the present study showed that Kcnq1ot1 serves a role in regulating osteogenic differentiation and mineralization of MC3T3-E1 by modulating expression of miR-98-5p/Tbx5. Kcnq1ot1 may be a potential effective therapeutic molecular biomarker for treatment of osteoporosis to improve patient quality of life.

Acknowledgements

Not applicable.

Funding

The present study was supported by Basic Research Project of Qinghai Provincial Science and Technology Department, China (grant no. 2020-ZJ-755).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FW and FZhe designed the experiments and wrote the manuscript. FW, FZha and FZhe performed the experiments and analyzed the data. FZhe revised the manuscript and supervised the experiments. FW and FZha confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Coughlan T and Dockery F: Osteoporosis and fracture risk in older people. Clin Med (Lond) 14: 187-191, 2014.
2. Lamichhane AP: Osteoporosis-an update. JNMA J Nepal Med Assoc 44: 60-66, 2005.
3. Compston J: Glucocorticoid-induced osteoporosis: An update. Endocrine 61: 7-16, 2018.
4. Lane NE: Glucocorticoid-induced osteoporosis: New insights into the pathophysiology and treatments. Curr Osteoporos Rep 17: 1-7, 2019.
5. Stein E and Shane E: Secondary osteoporosis. Endocrinol Metab Clin North Am 32: 115-134, 2003.
6. Gass M and Dawson-Hughes B: Preventing osteoporosis-related fractures: An overview. Am J Med 119 (Suppl 1): S3-S11, 2006.
7. Delaney MF: Strategies for the prevention and treatment of osteoporosis during early postmenopause. Am J Obstet Gynecol 194 (2 Suppl): S12-S23, 2006.
8. Carmona RJ and Adachi JD: Calcium and vitamin D for osteoporotic fracture prevention. Pol Arch Med Wewn 117: 441-442, 2007.
9. Aspray TJ and Hill TR: Osteoporosis and the ageing skeleton. Subcell Biochem 91: 453-476, 2019.
10. Wang Y, Tao Y, Hyman ME, Li J and Chen Y: Osteoporosis in china. Osteoporos Int 20: 1651-1662, 2009.
11. Chen P, Li Z and Hu Y: Prevalence of osteoporosis in China: A meta-analysis and systematic review. BMC Public Health 16: 1039, 2016.
12. Zeng Q, Li N, Wang Q, Feng J, Sun D, Zhang Q, Huang J, Wen Q, Hu R, Wang L, *et al*: The prevalence of osteoporosis in China, a nationwide, multicenter DXA survey. J Bone Miner Res 34: 1789-1797, 2019.
13. Shoback D, Rosen CJ, Black DM, Cheung AM, Murad MH and Eastell R: Pharmacological management of osteoporosis in postmenopausal women: An endocrine society guideline update. J Clin Endocrinol Metab 105: dgaa048, 2020.
14. Johnston CB and Dagar M: Osteoporosis in older adults. Med Clin North Am 104: 873-884, 2020.
15. Chen X and Yan GY: Novel human lncRNA-disease association inference based on lncRNA expression profiles. Bioinformatics 29: 2617-2624, 2013.
16. Khorkova O, Hsiao J and Wahlestedt C: Basic biology and therapeutic implications of lncRNA. Adv Drug Deliv Rev 87: 15-24, 2015.
17. Wang SZ, Jia J and Chen CH: LncRNA-KCNQ1OT1: A potential target in exosomes derived From ADSCs for the treatment of osteoporosis. Stem Cells Int 19: 7690006, 2021.
18. Wang JL, Wei X, Wang AG, Bai Y and Wu XJ: Kcnq1ot1 regulates osteogenic differentiation of hBMSC by miR-320a/Smad5 axis. Eur Rev Med Pharmacol Sci 24: 2843-2854, 2020.
19. Sun HH, Sun PF and Liu WY: MiR-98-5p regulates myocardial differentiation of mesenchymal stem cells by targeting Tbx5. Eur Rev Med Pharmacol Sci 22: 7841-7848, 2018.
20. Zheng F, Wang F and Xu Z: MicroRNA-98-5p prevents bone regeneration by targeting high mobility group AT-Hook 2. Exp Ther Med 18: 2660-2666, 2019.
21. Wang GL, Xia XL, Li XL, He FH and Li JL: Identification and expression analysis of the MSP130-related-2 gene from Hyriopsis cumingii. Genet Mol Res 14: 4903-4913, 2015.
22. Chen X, Wang Z, Duan N, Zhu G, Schwarz EM and Xie C: Osteoblast-osteoclast interactions. Connect Tissue Res 59: 99-107, 2018.
23. Mazess RB: Fracture risk: A role for compact bone. Calcif Tissue Int 47: 191-193, 1990.
24. Miller PD: Management of severe osteoporosis. Expert Opin Pharmacother 17: 473-488, 2016.
25. Srivastava M and Deal C: Osteoporosis in elderly: Prevention and treatment. Clin Geriatr Med 18: 529-555, 2002.
26. Yang Y, Yujiao W, Fang W, Linhui Y, Ziqi G, Zhichen W, Zirui W and Shengwang W: The roles of miRNA, lncRNA and circRNA in the development of osteoporosis. Biol Res 53: 40, 2020.
27. Jin D, Wu X, Yu H, Jiang L, Zhou P, Yao X, Meng J, Wang L, Zhang M and Zhang Y: Systematic analysis of lncRNAs, mRNAs, circRNAs and miRNAs in patients with postmenopausal osteoporosis. Am J Transl Res 10: 1498-1510, 2018.
28. Li Y, Li J, Chen L and Xu L: The roles of long non-coding RNA in osteoporosis. Curr Stem Cell Res Ther 15: 639-645, 2020.
29. Yu Y, Chen Y, Zhang X, Lu X, Hong J, Guo X and Zhou D: Knockdown of lncRNA Kcnq1ot1 suppresses the adipogenic and osteogenic differentiation of tendon stem cell via downregulating miR-138 target genes PPARgamma and RUNX2. Cell Cycle 17: 2374-2385, 2018.
30. Wang CG, Liao Z, Xiao H, Liu H, Hu YH, Liao QD and Zhong D: LncRNA Kcnq1ot1 promoted BMP2 expression to regulate osteogenic differentiation by sponging miRNA-214. Exp Mol Pathol 107: 77-84, 2019.
31. Czekanska EM, Stoddart MJ, Richards RG and Hayes JS: In search of an osteoblast cell model for in vitro research. Eur Cell Mater 24: 1-17, 2012.
32. Prasad A, Kumar SS, Dessimoz C, Bleuler S, Laule O, Hruz T, Gruissem W and Zimmermann P: Global regulatory architecture of human, mouse and rat tissue transcriptomes. BMC Genomics 14: 716, 2013.
33. Gao X, Ge J, Li W, Zhou W and Xu L: LncRNA Kcnq1ot1 promotes osteogenic differentiation to relieve osteolysis via wnt/beta-catenin activation. Cell Biosci 8: 19, 2018.
34. Jia Y, Chang Y, Guo Z and Li H: Transcription factor Tbx5 promotes cardiomyogenic differentiation of cardiac fibroblasts treated with 5-azacytidine. J Cell Biochem 120: 16503-16515, 2019.
35. Li M, Zhang YL, Huang H and Xiong Y: MicroRNA-10-5p regulates differentiation of bone marrow mesenchymal stem cells into cardiomyocytes by targeting Tbx5. Eur Rev Med Pharmacol Sci 23: 479-485, 2019.
36. Li W, Zhang S, Liu J, Liu Y and Liang Q: Vitamin K2 stimulates MC3T3-E1 osteoblast differentiation and mineralization through autophagy induction. Mol Med Rep 19: 3676-3684, 2019.
37. Lu X, Lu J, Zhang L and Xu Y: Effect of ANGPTL7 on proliferation and differentiation of MC3T3-E1 cells. Med Sci Monit 25: 9524-9530, 2019.