Cyclooxygenase-2 negatively regulates osteogenic differentiation in murine bone marrow mesenchymal stem cells via the FOXO3a/p27^{kip1} pathway

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Aims

Cyclooxygenase-2 (COX-2) is an enzyme that synthesizes prostaglandins from arachidonic acid. Previous reports have indicated that COX-2 is constitutively expressed in osteogenic cells instead of being expressed only after pathogenic induction, and that it facilitates osteoblast proliferation via PTEN/Akt/p27^{kip1} signalling. However, the role of COX-2 in osteogenic differentiation of murine bone marrow mesenchymal stromal cells (BMSCs) remains controversial. In this study, we investigated the function of COX-2 in the osteogenic differentiation of BMSCs.

Methods

COX-2 inhibitor, COX-2 overexpression vector, and p27^{kip1} small interfering RNA (siRNA) were used to evaluate the role of COX-2 in osteogenic differentiation and related signalling pathways in BMSCs.

Results

We found that the messenger RNA (mRNA) and protein levels of COX-2 decreased gradually during osteogenic differentiation. Inhibition of COX-2 activity promoted FOXO3a and p27^{kip1} expression and simultaneously enhanced osteogenesis, as indicated by increased osteogenic gene expression and mineralization in BMSCs. Furthermore, when p27^{kip1} was silenced, the suppressive effects of COX-2 on osteogenesis were reversed. It demonstrated that the negative regulatory effect of COX-2 on osteogenesis was mediated by p27^{kip1}. In addition, our results showed that overexpression of COX-2 reduced the mRNA and protein levels of FOXO3a and p27^{kip1}, and thus attenuated osteogenic gene expression. These results indicate that COX-2 negatively regulates osteogenic differentiation by reducing the expression of osteogenic genes via the FOXO3a/p27^{kip1} signalling pathway.

Conclusion

Together with the findings from previous and current studies, these results indicate that COX-2 has a different role in proliferation versus differentiation during osteogenesis via FOXO3a/p27^{kip1} signalling in osteoblasts or BMSCs.

Bone & Joint Research

Article focus

- To investigate the function of cyclooxygenase-2 (COX-2) in the osteogenic differentiation of murine bone marrow mesenchymal stromal cells (BMSCs).

Key messages

- COX-2 acts as a negative regulator of osteogenesis in BMSCs by activating the FOXO3a/p27^{kip1} pathway, and p27^{kip1} is an important regulator of osteogenic gene expression.

Strengths and limitations

- We suggest that constitutive COX-2-mediated FOXO3a/p27^{kip1} signalling may play an important role in promoting osteogenic cell proliferation and decreasing osteogenic differentiation. Together with the findings from previous studies and this study, these results indicate that COX-2 acts as a switch in regulating BMSCs from the proliferation stage to the differentiation stage.
- Although cyclooxygenase-2 negatively regulates osteogenic differentiation in murine BMSCs via the FOXO3a/p27^{kip1} pathway, how FOXO3a/p27^{kip1} promotes the expression of osteogenic genes is still unclear. It is worth further investigating the regulation between FOXO3a/p27^{kip1} and osteogenic genes.

Introduction

Cyclooxygenases (COXs), including COX-1, COX-2, and COX-3, are enzymes that synthesize prostaglandins from arachidonic acid. COX-2 is recognized as an inducible enzyme when cells or tissues are stimulated by inflammation, growth factors, sudden injury, or tumorigenesis. It has been recognized that the anti-inflammatory effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on relieving inflammation and pain is mainly due to their suppression of COX-2 generation, which is dramatically increased during inflammation.[1,2] Although non-selective NSAIDs can relieve pain and inflammation in patients with bone disorders or osteoarthritis, some studies have shown that NSAIDs reduce bone repair and normal remodelling.[3-8] In our previous studies, indometacin and ketorolac were found to inhibit bone repair in the early stage.[5,9] In primary cultured rat osteoblasts, NSAIDs inhibited osteoblast viability and proliferation, but stimulated intracellular alkaline phosphatase (ALP) activity and type I collagen formation at an earlier stage of differentiation.[8] Therefore, although inhibition of COX-2 activity by NSAIDs can effectively reduce inflammation and pain during bone injury, this treatment can also disrupt various processes of osteogenic cells, such as viability, proliferation, and differentiation, during bone repair.

Although COX-2 is considered an inducible enzyme, several studies have indicated that COX-2 is constitutively expressed in several organs, such as nerves, kidneys, and gonads, and has physiological functions.[10-13] In our previous study, COX-2 was found to be constitutively expressed in osteoblasts adjacent to the trabeculae, periosteum, and endosteum in mouse femora.[14] We also demonstrated that constitutive COX-2 expression promoted mouse and human osteogenic cell proliferation. However, the effect of constitutive COX-2 expression on osteogenic differentiation of osteogenic cells remains unclear.

Bone marrow mesenchymal stromal cells (BMSCs) can differentiate into osteogenic, chondrogenic, and adipogenic cells. Osteogenic differentiation in particular is critical for bone tissue regeneration and repair.[15] Bioengineering approaches for bone healing often use autologous bone marrow aspirate concentrate (BMAC) or BMSCs with biocompatible scaffolds. While BMAC is promising, its efficacy varies due to differences in BMSC concentration, robustness, and immune cell composition. Understanding interactions between macrophages, lymphocytes, and BMSCs could reveal new strategies to enhance bone repair.[16] Therefore, in this study, investigating COX-2-induced inflammation and the role of COX-2 in BMSCs is crucial. Although our previous study showed that constitutively expressed COX-2 in osteoblasts facilitates proliferation via PTEN/Akt/p27^{kip1} signalling,[14] the role of these molecules in the osteogenic differentiation of BMSCs remains unclear.

p27^{kip1} is a cyclin-dependent kinase inhibitor (CDKI) that classically binds and inhibits multiple cyclin-dependent kinases and arrests cell cycle progression. Our previous study demonstrated that constitutively expressed COX-2 promoted human osteoblastic proliferation via the activation of p-PTEN and p-Akt, and further inhibited FOXO3a and p27^{kip1} expression.[14] In contrast, proliferation was suppressed by anti-inflammatory drugs via the PI3K/Akt/FOXO3a/p27^{kip1} pathway.[17] Our findings indicated that FOXO3a and p27^{kip1} play important roles in the regulation of proliferation. However, it is not clear whether COX-2 regulates osteogenic differentiation. Therefore, we focused on whether osteogenic differentiation is regulated by COX-2 in this study. A previous study indicated that p27^{kip1} is a critical regulator of osteogenesis in BMSCs and bone formation.[18] Another report showed that increased p27^{kip1} expression promotes bortezomib-induced osteogenic differentiation of mouse BMSCs via cell cycle exit with a significant reduction in cell proliferation.[15] Accordingly, p27^{kip1} may be an important factor that regulates both the proliferation and differentiation of osteoblasts and BMSCs. In this study, we investigated whether COX-2 regulates osteogenic differentiation through p27^{kip1}-related signalling in murine BMSCs, and further investigated whether these effects occur through the FOXO3a/p27^{kip1} signalling pathway.

Methods

Cell culture

Murine BMSCs (D1 cells, CRL-12424; American Type Culture Collection (ATCC), USA) were used in this study. The cells were cultured in basal medium (low-glucose Dulbecco's Modified Eagle Medium; Gibco and Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS), 50 μg/ml L-ascorbic acid (Sigma-Aldrich, USA), 100 mg/ml nonessential amino acid solution (Thermo Fisher Scientific), and 50 U/ml penicillin/streptomycin solution (Thermo Fisher Scientific). The cultured cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.[19-22] For all experiments, the cells were cultivated under these conditions for four subcultures, and the medium was changed every two days. The doubling time of D1 cells was 20 to 24 hours under the experimental conditions.

For osteoinduction, the D1 cells cultured in basal medium were changed to osteoinduction medium (OIM; basal medium supplemented with 10 mM β-glycerophosphate

Table I. Real-time polymerase chain reaction sequences.

Gene name	Gene bank number	Primer sequences (5'-3')	Product size, bp	Tm, °C	GC content, %*	Efficiencies, %
		Forward: AGC TGC AAG AGA CAC CCTTTG	178	59.5	52.4	100.33
BMP-2	BC100344.1	Reverse: AGC ATG CCT TAG GGA TTT TGG A		62.8	45.5	
		Forward: CCC AGC CAC CTT TAC CTA CA	150	59.0	55.0	98.15
Runx-2	NM_001146038.3	Reverse: TAT GGA GTG CTG CTG GTC TG		59.4	55.0	
		Forward: AAC CCA GAC ACA AGC ATT CC	121	58.3	50.0	97.15
ALP	NM_007431.3	Reverse: GTC AGT CAG GTT GTT CCG ATT CAA		61.2	45.0	
		Forward: GAG GGC AAT AAG GTA GTG AAC A	110	56.8	55.0	100.29
OC	BC160309.1	Reverse: AAG CCA TAC TGG TCT GAT AGC TCG		61.1	57.1	
		Forward: AGA AGG AAA TGG CTG CAG AA	174	57.5	45.0	103.76
COX-2	NM_011198.5	Reverse: GCT CGG CTT CCA GTA TTG AG		57.5	55.0	
		Forward: CAG AAT CAT AAG CCC CTG GA	170	57.5	50.0	98.96
$p27^{kip1}$	NM_009875.4	Reverse: GGT CCT CAG AGT TTG CCT GA		57.3	55.0	
		Forward: GGT CCT CAG AGT TTG CCT GA	88	57.3	55.0	93.73
β-actin	NM_007393.8	Reverse: ACC AGA GGC ATA CAG GGA CA		57.4	55.0	

*Percentage of nitrogenous bases in a DNA or RNA molecule that are either guanine (G) or cytosine (C).
ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein 2; COX-2, cyclooxygenase-2; OC, osteocalcin; Runx-2, Runt-related transcription factor 2; Tm, melting temperature.

disodium salt hydrate and 100 nM dexamethasone in basal medium (both Sigma-Aldrich)) to induce osteogenic differentiation.[23,24]

COX-2 inhibitor treatment

The COX-2 inhibitor NS398 (10 mM) was dissolved in DMSO as a stock solution. The concentrations of NS398 used to treat D1 cells ranged from 10 to 50 μM. The final concentration of DMSO in each treatment group was ≤ 0.1% to reduce the effect on the cells. For the osteogenic differentiation experiments, when D1 cells reached subconfluence, the cells were treated with NS398 in OIM for one to seven days to investigate gene expression, protein levels, and mineralization. The cells were harvested to determine osteogenic gene expression and protein levels on days 1, 3, 5, or 7. Mineralization (calcium deposition) was evaluated by using Alizarin Red S staining.

$p27^{kip1}$ siRNA transfection of cells treated with the COX-2 inhibitor NS398

D1 cells were incubated in antibiotic-free culture medium for 24 hours before small interfering RNA (siRNA) transfection. $p27^{kip1}$ siRNA (Santa Cruz Biotechnology, USA) or the RNA interference (RNAi) universal negative control (Invitrogen, USA) was transfected into D1 cells by using Lipofectamine RNAiMAX reagent (Invitrogen) in serum-free Opti-MEM (Life Technologies, USA). After transfection, the cells were treated with NS398 in OIM for one to five days, and messenger RNA (mRNA) expression of osteogenic genes and osteogenic differentiation was assessed.

COX-2 overexpression in D1 cells

COX-2 overexpression vectors were constructed by GENEWIZ (USA). In brief, murine COX-2 complementary DNA (cDNA) (17 to 1,951 bp) was amplified by polymerase chain reaction (PCR) and subcloned and inserted into the pUC57-Amp vector. After the COX-2-pUC57-Amp vector was amplified in *Escherichia coli*, the COX-2 fragment was cut, and the 1.9 kb *Hind*III/*Xba*I COX-2 fragment was inserted into the pcDNA3.1(+) vector and subcloned into *E. coli* JM108. Then, the COX-2 pcDNA3.1(+) vector was purified and stored at -20°C for further experiments. For the overexpression experiment, D1 cells were incubated in antibiotic-free culture medium for 24 hours before COX-2 pcDNA3.1(+) vector transfection. The pcDNA3.1(+) vector was used as a negative control. The COX-2 pcDNA3.1(+) vector or pcDNA3.1(+) vector was transfected into D1 cells by using Lipofectamine 2000 reagent (Invitrogen) in serum-free Opti-MEM (Life Technologies). After transfection for 18 hours, the culture medium was replaced with OIM for one to three days to examine the osteogenic gene expression and protein levels.

Reverse-transcription polymerase chain reaction and real-time PCR

Total RNA from D1 cells was extracted using TRIzol Reagent (Cat. 15596026; Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis kit (Applied Biosystems, Thermo Fisher Scientific, USA) under the following conditions: ten minutes at 25°C, 120 minutes at 37°C, and five minutes at 72°C. The specific PCR products were detected by quantitative real-time PCR performed in a CFX Connect Real-Time PCR System (Bio-Rad Laboratories, USA) by measuring the

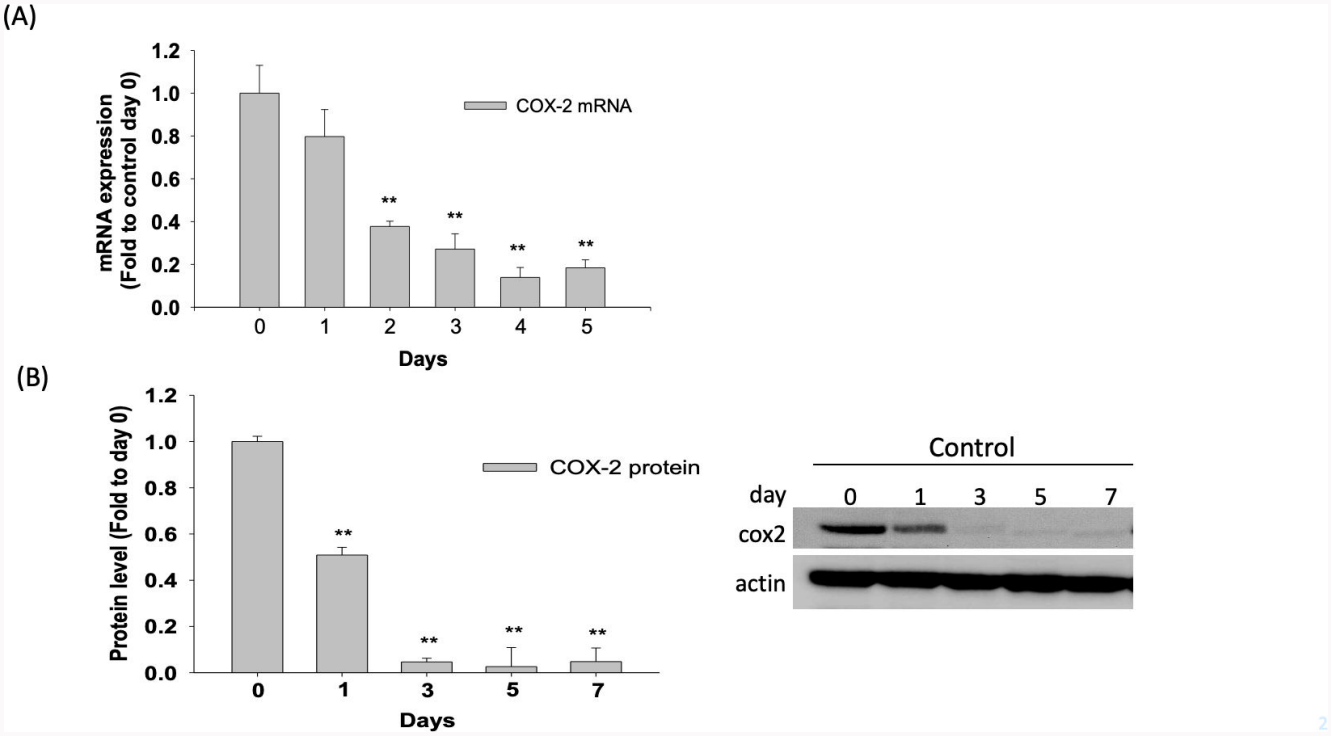

Fig. 1

Gene expression and protein levels of cyclooxygenase-2 (COX-2) during osteogenesis. a) COX-2 gene expression decreased from 100% on day 0 (subconfluence) to approximately 40% on day 2 (confluence) and decreased gradually until day 5. b) The protein level of COX-2 also decreased from day 0 to day 3 and was maintained until day 7 (**p < 0.01; n = 4; one-way analysis of variance and Scheffe's method). mRNA, messenger RNA.

fluorescence of SYBR Green, a double-stranded DNA-binding dye (Bio-Rad Laboratories). Reactions were performed in a 25 μl mixture containing cDNA, primers specific for each gene, and the iQSYBR Green Supermix. The mRNA levels of bone morphogenetic protein 2 (BMP-2), Runt-related transcription factor 2 (Runx-2), ALP, osteocalcin (OC), COX-2, $p27^{kip1}$, and β-actin were quantified using the specific quantitative polymerase chain reaction (qPCR) primer pairs that were used in our previous study (Table I).[25] The primer efficiencies for each gene were evaluated, and the calibration curves for each quantified target are presented in Supplementary Figure a. The primer efficiencies ranged from 93.73% to 103.76%, indicating their suitability for qPCR analysis. The following cycling conditions were used: incubation at 94°C for one minute, followed by 35 cycles of denaturation at 94°C for 30 seconds and annealing and extension at 61°C for 30 seconds. The relative mRNA expression level was calculated from the quantification cycle (Cq) value of each PCR product in CFX Connect Real-Time PCR System software, and normalized to that of β-actin for each sample by using the comparative Cq method. The five reference genes (β-actin, 18 S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Tbp, and Rplp0) were evaluated using RefFinder (Xie, China)[26] to identify the most stable reference gene. Based on the results, β-actin was selected as the reference gene (Supplementary Figure b). The mean gene expression in control group was assigned a value of 1, and the gene expression level of each experimental group was calculated relative to the control. All PCR amplifications were performed in triplicate, and the experiments were repeated at least three times.

Protein extraction and western blotting

For analysis of the protein expression of cell cycle regulators, protein was extracted from D1 cells at specific times. The cell protein was extracted using PhosphoSafe extraction reagent (Merck, Germany). The protein concentration was determined using a protein assay dye reagent (Bio-Rad Laboratories). Furthermore, 50 mg of total protein was separated by 10% sodium dodecyl sulphate polyacrylamide (SDS) gel electrophoresis at 90 volts for two hours, and transferred to a polyvinylidene fluoride (PVDF) membrane at 90 voltage for 1.5 hours. COX-2 (1;1,000), $p27^{kip1}$ (1;1,000), FOXO3a (1;1,000), and β-actin (1;5,000; all Cell Signalling Technology, USA) were used as primary antibodies. These antibodies were detected using horseradish peroxidase-conjugated anti-mouse (1:5,000; Invitrogen, #62-6520) and anti-rabbit antibodies (1;5000; Jackson ImmunoResearch, USA, #111-035-003). The secondary antibodies were detected through enhanced chemiluminescence (RPN2235; GE HealthCare) and an AutoChemi image and analysis system (Vilber, Fusion FX, France).

Alizarin Red S staining

Mineralization of extracellular matrix (ECM) after osteogenic induction was determined by Alizarin Red S staining. The cells were fixed with 10% formalin-saline at room temperature for ten minutes. After one wash with double-distilled water (ddH_2O), 200 μl of Alizarin Red S (Santa Cruz Biotechnology) solution (1% in ddH_2O, pH 4.2) was added to each well of a 24-well plate. The staining solution was removed ten minutes later, and each well was washed with H_2O. The fixed and stained plates were then air-dried at room temperature.

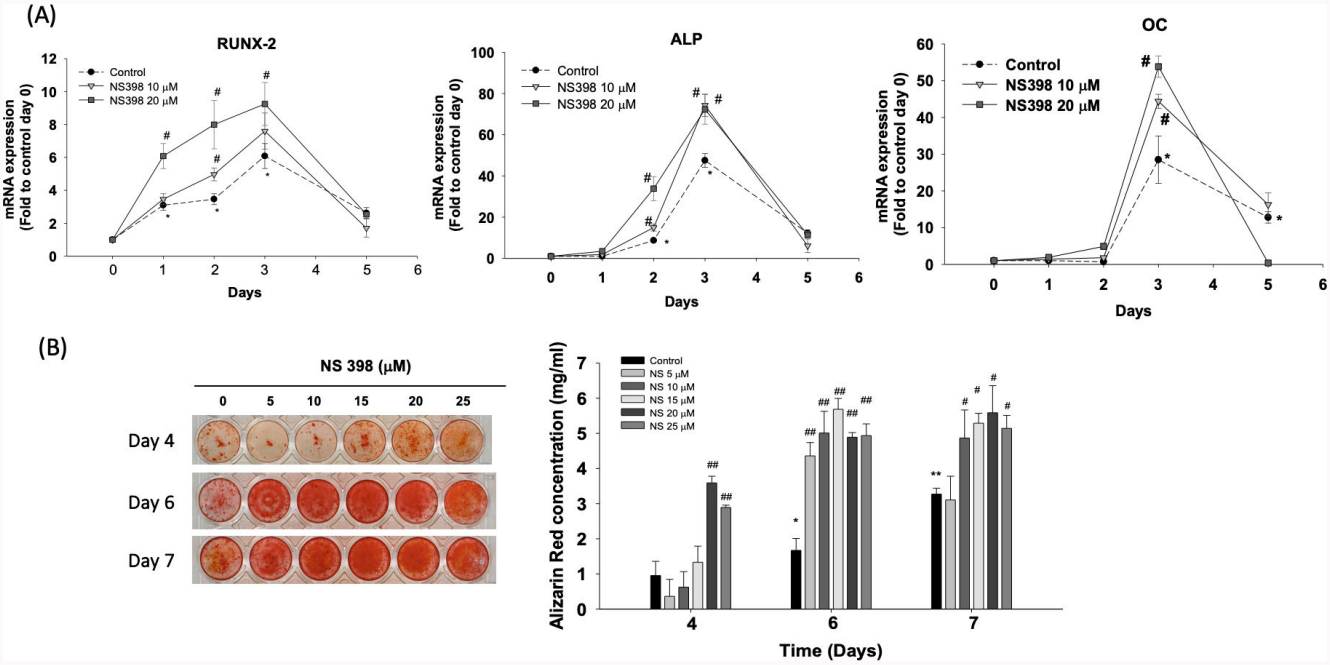

Fig. 2

Cyclooxygenase-2 (COX-2) inhibitor NS398 promotes osteogenic gene expression and mineralization. a) The gene expression levels of Runx-2, osteocalcin (OC), and alkaline phosphatase (ALP) increased in osteoinduction medium (OIM) from day 1 to day 3 and then decreased on day 5 (**p < 0.01 compared to the day 0 control group). After 10 μM and 20 μM NS398 treatment, the gene expression levels of Runx-2, OC, and ALP were significantly greater on days 1 to 3 than those in the control group on the same day (#p < 0.05, ##p < 0.01 compared to the control group on the same day). b) Mineralization was increased by 5 to 25 μM NS398 on days 4, 6, and 7 (*p < 0.05, **p < 0.01 compared to the day 0 control group; #p < 0.05, ##p < 0.01 compared to the control group on the same days). All p-values calculated using two-way analysis of variance and Scheffe's method.

The amount of mineralization was determined by dissolving the cell-bound Alizarin Red S in 10% acetic acid and then quantified spectrophotometrically at 415 nm.

Statistical analysis

For each study group, the data are shown as the mean and standard error. Each experiment was performed at least three times. Before performing analysis of variance (ANOVA), we evaluated the normality of the data using the Shapiro-Wilk test in SPSS v.20 (IBM, USA). The results confirmed that all samples across treatments were normally distributed. Significant differences between groups were identified using one-way or two-way ANOVA, and multiple comparisons were performed using Scheffe's method. The thresholds for significance and high significance were $p < 0.05$ and $p < 0.01$, respectively.

Results

Gene expression and protein level of COX-2 during osteogenesis in D1 cells

D1 cells were seeded in basal medium, and the medium was changed to OIM to induce osteogenic differentiation when the D1 cells reached 80% confluence. The cells were harvested to evaluate gene expression and protein levels. COX-2 gene expression decreased from 100% on day 0 (subconfluence) to approximately 20% on day 2 (confluence), and was maintained until day 5 (Figure 1a, Supplementary Figure c). Similarly, the protein level of COX-2 decreased from day 0 to day 3 and was maintained until day 7 (Figure 1b). These results showed that the gene expression and protein level of COX-2 decreased gradually in a time-dependent manner during osteogenic differentiation.

COX-2 inhibitor NS398 promotes osteogenic gene expression and mineralization

Before investigating osteogenic differentiation, we assessed the cell viability of the COX-2 inhibitor NS398 using an MTS assay. NS398 concentrations between 0 and 50 μM showed no significant effect on D1 cell viability (Supplementary Figure d), although a slight decrease was observed at 50 μM. Therefore, we selected a concentration range of 0 to 20 μM for further experiments. We evaluated the expression of the osteogenic genes Runx-2, ALP, and OC in D1 cells treated with the COX-2 inhibitor NS398 in OIM by real-time qPCR during osteogenesis. NS398 increased osteogenic gene expression. The gene expression levels of Runx-2, OC, and ALP were increased in OIM from day 1 to day 3 and then decreased on day 5 ($p = 0.012$ compared to the day 0 control group, two-way ANOVA and Scheffe's method). Following treatment with 10 μM and 20 μM NS398, Runx-2 gene expression levels in OIM increased significantly compared to controls on same days. With 10 μM NS398 treatment, Runx-2 expression increased from day 2 to day 3 (1.43- and 1.24-fold vs controls, respectively; $p = 0.016$, two-way ANOVA and Scheffe's method). With 20 μM NS398 treatment, Runx-2 expression increased from day 1 to day 3 (1.96- to 2.30-fold vs controls, respectively; $p = o.o15$, two-way ANOVA and Scheffe's method) (Figure 2a). Similarly, ALP gene expression levels in OIM increased from day 2 to day 3, with a 1.73- and 1.52-fold increase for 10 μM NS398 ($p = o.021$) and a 3.90- and 1.56-fold increase for 20 μM NS398 ($p = 0.017$, compared to controls on the same days, both

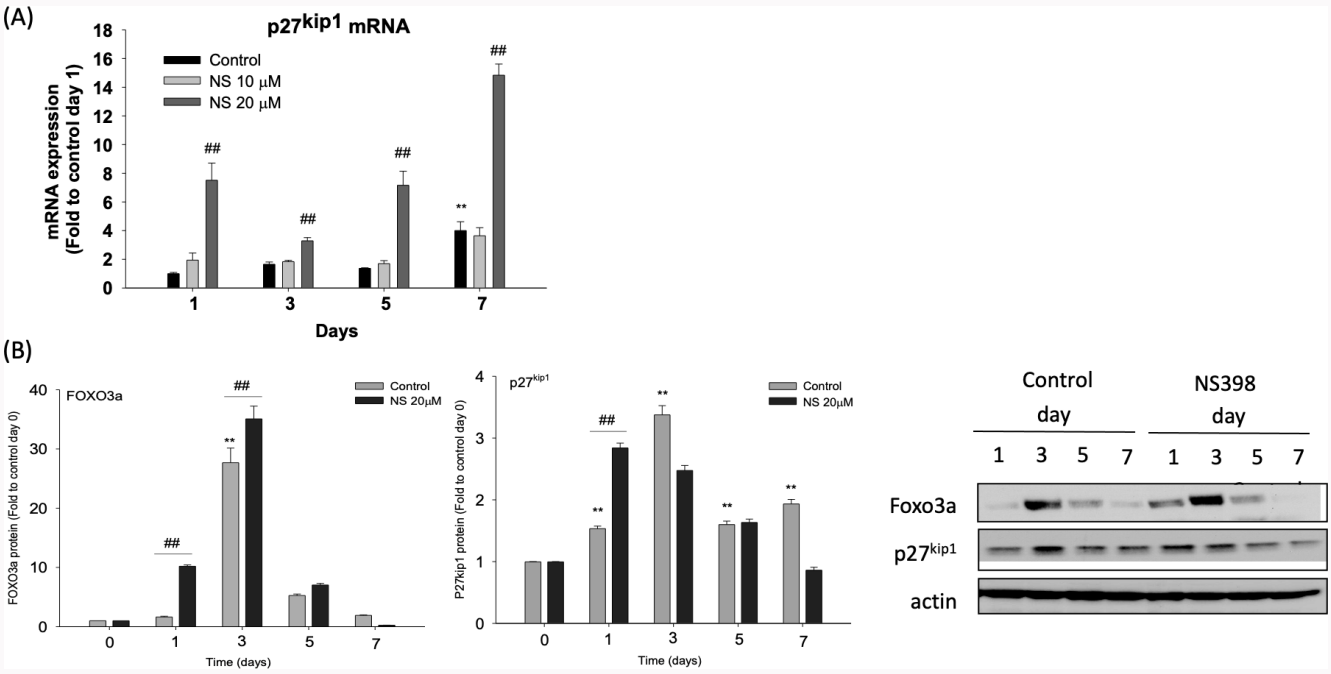

Fig. 3

Cyclooxygenase-2 (COX-2) inhibitor promoted $p27^{kip1}$ and FOXO3a messenger RNA (mRNA) and protein expression. a) $p27^{kip1}$ gene expression was increased during osteogenic differentiation from day 1 to day 7 (**p < 0.01 compared to the day 1 control group). After treatment with 20 μM NS398, the gene expression of $p27^{kip1}$ increased from day 1 to day 7 (##p < 0.01 compared to the control group on the same day). b) The protein levels of FOXO3a and $p27^{kip1}$ increased from day 1 to day 3, and then decreased from day 5 to day 7 during osteogenesis (**p < 0.01 compared to the day 0 control group). Compared with those in the control group, the FOXO3a and $p27^{kip1}$ protein levels in the NS398 treatment group were also increased on day 1 or day 3 (##p < 0.01, compared with the control group on the same day). All p-values calculated using two-way analysis of variance and Scheffe's method.

two-way ANOVA and Scheffe's method). OC gene expression levels also increased on day 3 with both 10 μM and 20 μM NS398 treatments, reaching 1.55- and 1.89-fold compared to controls, respectively (p = 0.014, compared to controls on the same days, two-way ANOVA and Scheffe's method) (Figure 2a). The gene expression of BMP-2 was not significantly different between treatments (Supplementary Figure e).

Alizarin Red S staining demonstrated that NS398 significantly increased mineralization from day 4 to day 7 (p = 0.006, two-way ANOVA and Scheffe's method). In the control group, mineralization progressively increased from day 4 to day 7 (p = 0.023 at day 6 and p = 0.001 at day 7 compared to the control group at day 4, two-way ANOVA and Scheffe's method). At day 4, both 20 and 25 μM NS398 significantly enhanced mineralization (p = 0.001 compared to the control group at day 4, two-way ANOVA and Scheffe's method). Additionally, NS398 concentrations from 10 to 25 μM significantly increased mineralization at days 6 and 7 (p = 0.004 to p = 0.001 compared to the control group on the same days, two-way ANOVA and Scheffe's method), with no significant differences observed among the different NS398 concentrations. This showed that high concentrations of NS398 lead to earlier mineralization in D1 cells, resulting plateau of mineralization in day 7, with no further differences among treatments of NS398. These data demonstrated that inhibition of COX-2 promoted osteogenic differentiation.

COX-2 negatively regulates the gene expression and protein levels of $p27^{kip1}$ and FOXO3a

$p27^{kip1}$ gene expression was increased during osteogenic differentiation at day 7 (p = 0.001 compared with the day 1 control group, two-way ANOVA and Scheffe's method). After treatment with 20 μM NS398, the gene expression of $p27^{kip1}$ increased from day 1 to day 7 (p = 0.001, compared with that in the control group on the same day, two-way ANOVA and Scheffe's method) (Figure 3). During osteogenic differentiation, the FOXO3a protein levels in the NS398 treatment group were greater than those in the control group at days 1 and 3 (p = 0.001 and p = 0.009, compared with the control group on the same day, two-way ANOVA and Scheffe's method), and $p27^{kip1}$ protein levels in the NS398 treatment group were greater than those in the control group on day 1 but not on day 3 (p = 0.001, compared with the control group on the same day, two-way ANOVA and Scheffe's method) (Figure 3b), and the protein levels decreased at day 5 and day 7. Thus, the FOXO3a and $p27^{kip1}$ proteins were dynamically expressed during osteogenesis. The COX-2 inhibitor at 20 μM increased FOXO3a protein levels on days 1 and 3. It also elevated $p27^{kip1}$ gene expression from day 1 to day 7, and increased $p27^{kip1}$ protein levels on day 1.

COX-2 negatively regulates osteogenic differentiation

To investigate whether COX-2 negatively regulates osteogenic differentiation via $p27^{kip1}$, we used $p27^{kip1}$ siRNA to examine gene expression and mineralization. The gene expression of $p27^{kip1}$ was significantly reduced after D1 cells were transfected with 10 or 20 nM $p27^{kip1}$ siRNA from day 1 to day 5 (p

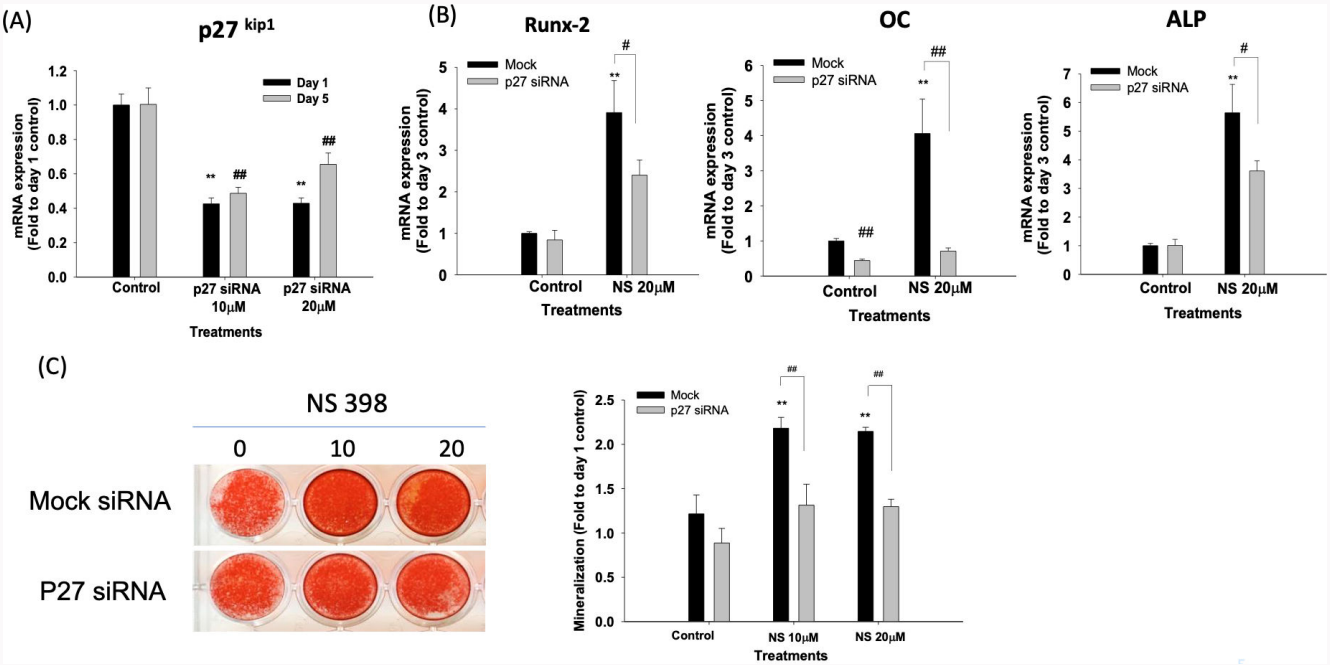

Fig. 4

p27^{kip1} silencing reduces cyclooxygenase-2 (COX-2) inhibition-promoted osteogenesis. a) The gene expression of p27^{kip1} was significantly reduced after D1 cells were transfected with 10 or 20 nM p27^{kip1} small interfering RNA (siRNA) from day 1 to day 5 (**p < 0.01, compared to the mock control group day 1; ##p < 0.01, compared to the mock control group day 5). b) The gene expression levels of Runx-2, osteocalcin (OC), and alkaline phosphatase (ALP) were increased after 20 μM NS398 treatment, but the expression levels of these genes were decreased after combined p27^{kip1} siRNA and NS398 treatment (**p < 0.01, NS398 group compared to the mock siRNA group; ##p < 0.01, the mock control with NS398 group compared with the siRNA p27^{kip1} with NS398 group). c) Quantification of the increase in mineralization after treatment with 10 and 20 NS398 and mock siRNA (**p < 0.01, compared to the 0 μM NS control group). However, after treatment with p27^{kip1} siRNA, NS398-induced mineralization was attenuated (##p < 0.01, compared to the mock siRNA group at the same concentration in the NS398 group). All p-values calculated using two-way analysis of variance and Scheffe's method. mRNA, messenger RNA.

= 0.001, compared to the mock control group days 1 and 5, two-way ANOVA and Scheffe's method; Figure 4). Osteogenic gene expression was also attenuated by p27^{kip1} siRNA treatment on day 3. The gene expression levels of Runx-2, OC, and ALP increased after NS398 treatment (p = 0.001, compared to the mock siRNA group, two-way ANOVA and Scheffe's method; Figure 4b), but decreased after combined p27^{kip1} siRNA and NS398 treatment (p = 0.001, compared to the mock siRNA with NS398 group, two-way ANOVA and Scheffe's method; Figure 4b). BMP-2 didn't show a significant difference (Supplementary Figure e). After silencing p27^{kip1} or addition of the mock control, D1 cells were treated with 10 and 20 μM NS398 to examine mineralization. The amount of mineralization increased after treatment with NS398 (10 to 20 μM) and mock siRNA (p = 0.001, compared to the 0 μM NS control group, two-way ANOVA and Scheffe's method; Figure 4c). The data are similar to those from the cells treated with NS398 alone. This finding indicated that the mock siRNA did not affect mineralization. However, after treatment with p27^{kip1} siRNA, the NS398-induced increase in mineralization was attenuated (p = 0.001, compared to the mock siRNA group, two-way ANOVA and Scheffe's method; Figure 4c). These data showed that the COX-2 inhibitor-induced osteogenesis was reversed by p27^{kip1}.

Overexpression of COX-2 reduces osteogenic differentiation

D1 cells were successfully transfected with COX-2 overexpression vectors. COX-2 gene expression continued to increase over time from day 1 to day 3 (p = 0.001, two-way ANOVA and Scheffe's method; Figure 5). COX-2 gene expression was significantly increased by 200-fold at three days. We further explored whether COX-2 gene overexpression reduced the expression of osteogenic genes. The results showed that the gene expression levels of Runx-2, BMP-2, OC, and ALP were significantly decreased after overexpression of the COX-2 gene on days 2 and 3 (50% to 90% reduction, p = 0.001, compared with those in the vehicle control group, two-way ANOVA and Scheffe's method; Figures 5b to 5e). Therefore, COX-2 overexpression reduced osteogenic gene expression.

Protein levels of FOXO3a and p27^{kip1} are reduced after COX-2 overexpression

In both the control vehicle group and the COX-2 overexpression group, the protein levels of COX-2 decreased in a time-dependent manner (p = 0.001 compared with the day 0 vehicle control group; p = 0.001 compared with the day 0 COX-2 overexpression group, two-way ANOVA and Scheffe's method) (Figures 6a and 6b, Supplementary Figure g). However, the protein level of COX-2 was significantly increased after transfection with the COX-2 overexpression vector in day 0 to day 1 (p = 0.001 compared with the control group at the same day, two-way ANOVA and Scheffe's method; Figures 6a and 6b). We investigated whether FOXO3a and p27^{kip1} are affected by COX-2 overexpression. According to the western blotting results (Figure 6a), the FOXO3a protein levels gradually increased over time in the control group (p = 0.001

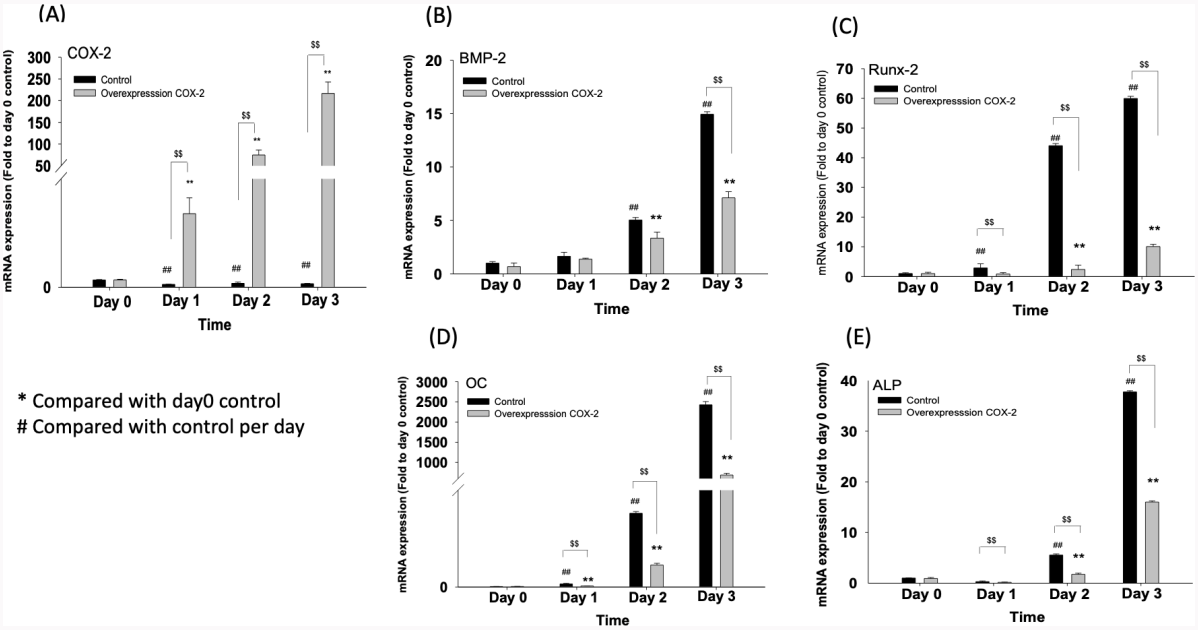

Fig. 5

Overexpression of cyclooxygenase-2 (COX-2) reduced osteogenic gene expression. a) In the control group, COX-2 gene expression was reduced from day 1 to day 3 during osteogenic differentiation (##p < 0.01 compared with the day 0 vehicle control group). COX-2 gene expression was significantly increased by 200-fold three days after COX-2 overexpression (**p < 0.01, compared with the day 0 COX-2 overexpression group). b) to e) The gene expression levels of Runx-2, bone morphogenetic protein 2 (BMP-2), osteocalcin (OC), and alkaline phosphatase (ALP) were significantly increased during osteogenic differentiation, but the expression of these genes decreased after overexpression of the COX-2 gene on days 2 and 3 ($$p < 0.01, compared with the vehicle control group). All p-values calculated using two-way analysis of variance and Scheffe's method.

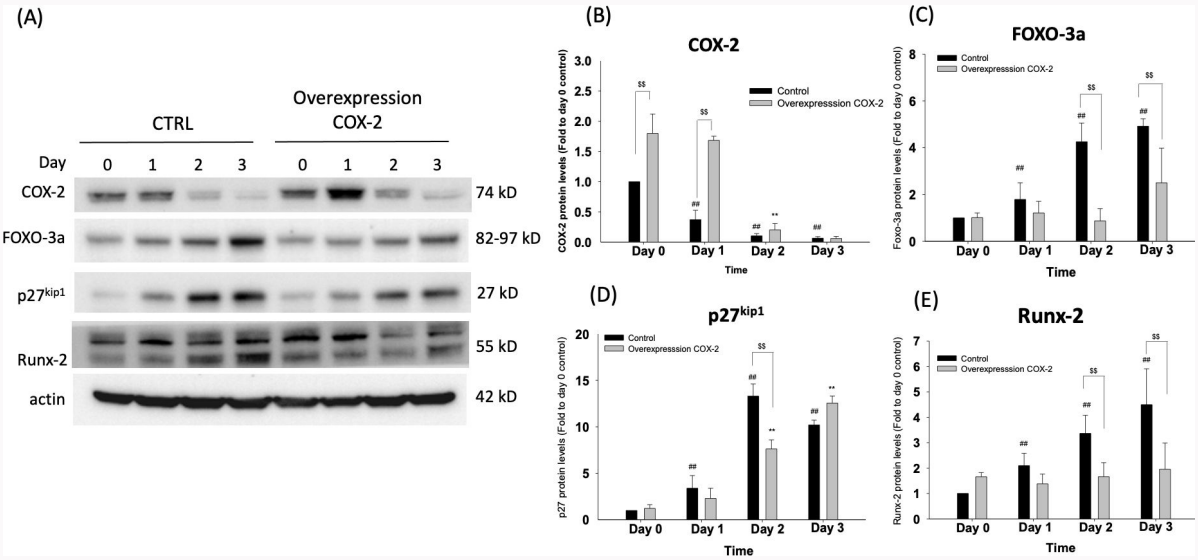

Fig. 6

Cyclooxygenase-2 (COX-2) overexpression reduced FOXO3a and p27^{kip1} protein levels. a) The western blotting showed the protein levels of COX-2, FOXO3a, p27^{kip1}, and Runx-2. The protein levels of actin were included as the internal control. b) The protein levels of COX-2 decreased in a time-dependent manner in the vehicle control group and in the COX-2 overexpression group (##p < 0.01 compared with the day 0 vehicle control group; **p < 0.01 compared with the day 0 COX-2 overexpression group). COX-2 protein levels were significantly increased after transfection with the COX-2 overexpression vector in day 0 and day 1 ($$p < 0.01 compared with the control group on the same day). c) Western blotting revealed that the FOXO3a protein level increased with time in the vehicle control group, but decreased with time after COX-2 was overexpressed that compared with the control group on the same day (##p < 0.01 compared with the day 0 vehicle control group; $$p < 0.01 compared with the vehicle control group on the same day). d) The protein level of p27^{kip1} increased in the control group in a time-dependent manner, but decreased with time after COX-2 overexpression (##p < 0.01 compared with the day 0 vehicle control group; $$p < 0.01 compared with the vehicle control group on the same day). e) Runx-2 protein levels were also reduced after COX-2 was overexpressed compared with the control group on the same day (##p < 0.01 compared with the day 0 vehicle control group; $$p < 0.01 compared with the vehicle control group on the same day). All p-values calculated using two-way analysis of variance and Scheffe's method.

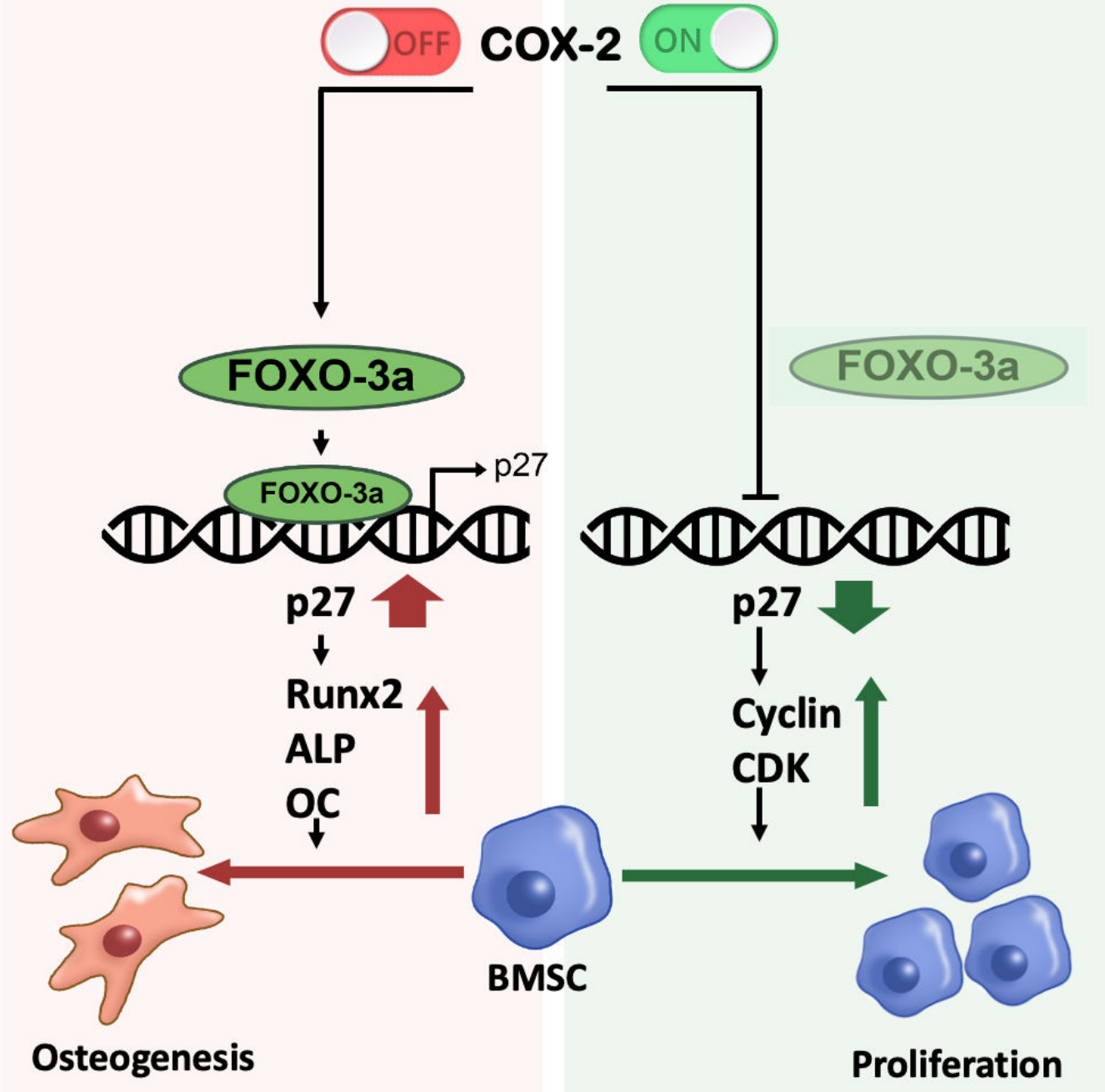

Fig. 7
Diagram illustrating the molecular mechanism by which cyclooxygenase-2 (COX-2) negatively regulates osteogenic differentiation via the FOXO3a/$p27^{kip1}$ pathway. ALP, alkaline phosphatase; BMSC, bone marrow mesenchymal stromal cell; CDK, cyclin-dependent kinase; OC, osteocalcin; Runx2, Runt-related transcription factor 2.

compared with control group in day 0, two-way ANOVA and Scheffe's method) (Figure 6c). The level of FOXO3a protein did not significantly change after COX-2 overexpression, but was reduced compared to the control group in the same day ($p = 0.001$ compared with the control group at the same day, two-way ANOVA and Scheffe's method; Figure 6c). Moreover, $p27^{kip1}$ protein levels similarly increased in the control group in a time-dependent manner ($p = 0.001$ compared with control group at day 0, two-way ANOVA and Scheffe's method). The $p27^{kip1}$ protein also increased after COX-2 overexpression at day 2 and day 3 ($p = 0.001$ compared with the day 0 COX-2 overexpression group, two-way ANOVA and Scheffe's method), but was reduced compared with the control group in the same day ($p = 0.001$ compared with the control group at the same day, two-way ANOVA and Scheffe's method; Figure 6d). These data showed that COX-2 overexpression reduced FOXO3a and $p27^{kip1}$ protein levels. Moreover, the Runx-2 protein levels in COX-2 overexpression group were reduced compared with control group ($p = 0.001$ compared with the control group in the same day, two-way ANOVA and Scheffe's method; Figure 6e).

Discussion

Our previous study demonstrated that constitutively expressed COX-2 promoted human osteoblastic proliferation via PTEN/Akt/p27^{kip1} signalling.[14] In this study, we found that the mRNA and protein levels of COX-2 gradually decreased during osteogenic progression in BMSCs (D1 cells). We further demonstrated that overexpression of COX-2 decreased osteogenic differentiation, while treatment with COX-2 inhibitors upregulated osteogenic genes such as Runx-2, ALP, and OC, and promoted mineralization in D1 cells. More importantly, this study demonstrated that COX-2 negatively regulates osteogenic differentiation via the FOXO3a/p27^{kip1} signalling pathway (Figure 7). Therefore, we inferred that COX-2 is an important regulator of osteogenic cells because it promotes proliferation and decreases osteogenic differentiation. Together with the findings from previous and current studies, these findings imply that COX-2 plays a crucial role in regulating stromal cells from differentiating down an osteogenic lineage via the FOXO3a/p27^{kip1} signalling pathway.

Mesenchymal stem cells (MSCs) are adult stem cells that can differentiate into specialized mesodermal cells such as adipocytes, chondrocytes, and osteogenic cells.[27] Although the differentiation of MSCs is tightly controlled by specific transcription factors and their related signalling pathways, there are many important connections between cell fate decisions and the cell cycle machinery in MSCs.[28,29] Cell differentiation is usually associated with cell cycle exit, and mitotic arrest in the G0/G1 phase plays an essential role in cell cycle exit and initiation of differentiation.[15,28] Among the complicated regulatory pathways, the FOXO3a/p27^{kip1} pathway is important for regulating cell proliferation. p27^{kip1} is not only a CDKI that arrests cell cycle progression and inhibits cell proliferation,[30] but is also involved in cell differentiation and apoptosis.[31] In addition, FOXO3a is a key transcription factor that promotes p27^{kip1} gene expression and downstream signalling and eventually inhibits proliferation.[14,32] Previously, we found that COX-2 is a positive regulator of human osteoblast proliferation through PI3K/Akt/FOXO3a/p27^{kip1} signalling.[17] By contrast, in this study, we found that COX-2 also regulates FOXO3a/p27^{kip1} signalling during osteogenic differentiation in BMSCs, although COX-2 is a negative regulator. This role of COX-2 was demonstrated by gene overexpression or COX-2 inhibition. COX-2 downregulated the mRNA and protein expression of FOXO3a and p27^{kip1}, as well as the expression of osteogenic genes. This effect of COX-2 was largely abolished when p27^{kip1} was silenced, indicating that the suppressive effect of COX-2 occurs through FOXO3a/p27^{kip1} signalling. More importantly, we found that COX-2 expression decreased gradually with the time of osteogenic differentiation in BMSCs. It is possible that certain mechanisms during osteogenesis may decrease the transcription and/or translation of COX-2, or increase COX-2 protein degradation. This may be the reason that during overexpression of COX-2, mRNA levels were significantly increased from day 0 to day 3, but protein levels only increased from day 0 to day 1 and declined on days 2 and 3. This finding indicated that changes in COX-2 expression control the process of osteogenesis. Together with previous findings on the role of COX-2 in osteoblast proliferation,[14] these results suggest that COX-2 is a regulator to switch the proliferation to differentiation in osteogenic cells through FOXO3a/p27^{kip1} signalling.

Our study showed that osteogenic gene expression increased from day 1 to day 3, then decreased by day 5 in D1 cell cultures, which was different from that in human BMSCs. The D1 cell line we used to study the osteogenic differentiation is a unique group of murine BMSCs, which have the ability to undergo rapid mineralization in osteogenic induction medium, as reported in previous studies.[23,24,33] Accordingly, the expressions of osteogenic genes are also dynamically changed during osteogenesis. In this study, we observed an increase in osteogenic gene expression on day 3, followed by a decrease by day 5 during mineralization. The decrease may be due to differentiation being terminated, and is accompanied by decreased osteogenic gene expression and mineralization. Our study also showed that FOXO3a/p27^{kip1} signalling is important for COX-2-regulated osteogenic differentiation. Moreover, FOXO3a overexpression increases p27^{kip1} promoter activity and protein levels in osteogenic cells,[14,34] and the SIRT1-FOXO3a complex increases Runx-2 promoter activity and further upregulates Runx-2 gene expression in human embryonic stem cell-derived mesenchymal progenitors.[35] Additionally, the SIRT1-FOXO3a complex promotes osteogenesis and reduces osteogenic cell senescence in MSCs.[36] Our study suggested that blocking COX-2 increases osteogenic differentiation by increasing FOXO3a gene expression, leading to increased p27^{kip1} promoter activity. Accordingly, we suggest that when activated by COX-2 inhibition, FOXO3a not only induces cell cycle arrest through p27^{kip1}, but also promotes osteogenesis by upregulating the Runx-2 gene.

Our study showed that inhibition of COX-2 promoted osteogenic differentiation by increasing p27^{kip1} expression in murine BMSCs. However, a previous study showed that the body size of *p27*$^{-/-}$ mice is greater than that of wild-type mice.[18] Deletion of *p27*^{kip1} promoted not only cell proliferation, but also osteogenic differentiation in osteogenic cells derived from the bone marrow of *p27*$^{-/-}$ mice.[18,37–41] These findings are consistent with our results regarding the impact of p27^{kip1} on cell proliferation; however, there are significant differences in the effects of p27^{kip1} on osteogenic differentiation. Based on our study design, murine BMSCs were induced to undergo osteogenic differentiation when transitioning from subconfluence to confluence. This phenomenon allowed us to clearly differentiate between the proliferation and differentiation stages. Therefore, we concluded that the increase in mouse body size and ossification in *p27*$^{-/-}$ mice may be attributed to cell proliferation rather than the inherent ossification ability of the cells. A study demonstrated a similar phenomenon, wherein elevated p27^{kip1} expression promoted bortezomib-induced osteogenic differentiation of mouse BMSCs by inhibiting cell proliferation.[15] This finding suggested that p27^{kip1} is a key factor in COX-2-regulated cellular fate, directing the transition from proliferation towards osteogenic differentiation.

COX-2 has been found to play an essential role in both endochondral and intramembranous bone formation during skeletal repair in *COX-2*$^{-/-}$ mice.[41] COX-2 inhibitors also suppress bone repair in the early stages.[6,25] In addition to its role in osteogenic cells, COX-2 plays a key regulatory role at the critical point of chondrocyte differentiation; for example, COX-2 facilitates chondrocyte terminal differentiation in

epiphyseal growth plates during bone growth.[42,43] By contrast, in articular chondrocytes, COX-2 plays an important role in maintaining chondrocyte function by upregulating PTHrP, and may also be involved in switching from normal to degenerative phenotypes.[44] The diverse roles of COX-2 in skeletal system cells may depend on different mediators and different cell biological statuses. Although COX-2 negatively regulates osteogenic differentiation in D1 cells via the FOXO3a/p27^{kip1} pathway, the mechanism by which FOXO3a/p27^{kip1} promotes osteogenic gene expression remains unclear. Some studies suggest that microRNAs (miRNAs) or long noncoding RNAs (lncRNAs) may influence osteogenic differentiation,[45–48] and they may regulate osteogenesis through the PTEN/PI3K/AKT pathway.[48] Therefore, further investigation is needed to determine whether specific miRNAs or lncRNAs are involved in COX-2-mediated regulation of osteogenesis.

In conclusion, our results demonstrated that COX-2 acts as a negative regulator of osteogenesis in BMSCs by activating the FOXO3a/p27^{kip1} pathway, and that p27^{kip1} is an important regulator of osteogenic gene expression. Together with our previous findings regarding cell proliferation,[8,9,14,17,25] and osteogenic differentiation in this study, we suggest that constitutive COX-2-mediated FOXO3a/p27^{kip1} signalling may play an important role in promoting osteogenic cell proliferation and decreasing osteogenic differentiation. These results indicate that COX-2 acts as a switch in regulating osteogenic cells from the proliferation stage to the differentiation stage (Figure 7). This finding may provide insight into the role of COX-2 in the proliferation and osteogenic differentiation of osteogenic cells.

Supplementary material

Supplementary material includes primer efficiency data for each gene, analysis of the most stable reference gene, cell viability following NS398 treatment, bone morphogenetic protein 2 (BMP-2) gene expression levels, and the original images of the western blot results.

References

1. **Soliman S**, **Ahmed RM**, **Ahmed MM**, **Attia A**, **Soliman AR**. Non-steroidal anti-inflammatory drugs: what is the actual risk of chronic kidney disease? A systematic review and meta-analysis. *Rom J Intern Med*. 2024.
2. **Takefuji Y**. Drug-induced inflammation: a review of literature. *Chem Biol Interact*. 2024;404:111282.
3. **Hadjicharalambous C**, **Alpantaki K**, **Chatzinikolaidou M**. Effects of NSAIDs on pre-osteoblast viability and osteogenic differentiation. *Exp Ther Med*. 2021;22(1):740.
4. **Yoon DS**, **Yoo JH**, **Kim YH**, **Paik S**, **Han CD**, **Lee JW**. The effects of COX-2 inhibitor during osteogenic differentiation of bone marrow-derived human mesenchymal stem cells. *Stem Cells Dev*. 2010;19(10):1523–1533.
5. **Chang JK**, **Li CJ**, **Liao HJ**, **Wang CK**, **Wang GJ**, **Ho ML**. Anti-inflammatory drugs suppress proliferation and induce apoptosis through altering expressions of cell cycle regulators and pro-apoptotic factors in cultured human osteoblasts. *Toxicology*. 2009;258(2–3):148–156.
6. **Chang JK**, **Wu SC**, **Wang GJ**, **Cho MH**, **Ho ML**. Effects of non-steroidal anti-inflammatory drugs on cell proliferation and death in cultured epiphyseal-articular chondrocytes of fetal rats. *Toxicology*. 2006;228(2–3):111–123.
7. **Chang JK**, **Wang GJ**, **Tsai ST**, **Ho ML**. Nonsteroidal anti-inflammatory drug effects on osteoblastic cell cycle, cytotoxicity, and cell death. *Connect Tissue Res*. 2005;46(4–5):200–210.
8. **Ho ML**, **Chang JK**, **Chuang LY**, **Hsu HK**, **Wang GJ**. Effects of nonsteroidal anti-inflammatory drugs and prostaglandins on osteoblastic functions. *Biochem Pharmacol*. 1999;58(6):983–990.
9. **Ho ML**, **Chang JK**, **Wang GJ**. Antiinflammatory drug effects on bone repair and remodeling in rabbits. *Clin Orthop Relat Res*. 1995;313:270–278.
10. **Martin F**, **Fletcher D**, **Chauvin M**, **Bouhassira D**. Constitutive cyclooxygenase-2 is involved in central nociceptive processes in humans. *Anesthesiology*. 2007;106(5):1013–1018.
11. **O'Neill GP**, **Ford-Hutchinson AW**. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. *FEBS Lett*. 1993;330(2):156–160.
12. **Harris RC**, **McKanna JA**, **Akai Y**, **Jacobson HR**, **Dubois RN**, **Breyer MD**. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J Clin Invest*. 1994;94(6):2504–2510.
13. **Yasojima K**, **Schwab C**, **McGeer EG**, **McGeer PL**. Distribution of cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins in human brain and peripheral organs. *Brain Res*. 1999;830(2):226–236.
14. **Li CJ**, **Chang JK**, **Wang GJ**, **Ho ML**. Constitutively expressed COX-2 in osteoblasts positively regulates Akt signal transduction via suppression of PTEN activity. *Bone*. 2011;48(2):286–297.
15. **Zhang D**, **Fan R**, **Lei L**, **et al**. Cell cycle exit during bortezomib-induced osteogenic differentiation of mesenchymal stem cells was mediated by Xbp1s-upregulated p21^{Cip1} and p27^{Kip1} *J Cell Mol Med*. 2020;24(16):9428–9438.
16. **Murayama M**, **Chow SK**, **Lee ML**, **et al**. The interactions of macrophages, lymphocytes, and mesenchymal stem cells during bone regeneration. *Bone Joint Res*. 2024;13(9):462–473.
17. **Li CJ**, **Chang JK**, **Chou CH**, **Wang GJ**, **Ho ML**. The PI3K/Akt/FOXO3a/p27Kip1 signaling contributes to anti-inflammatory drug-suppressed proliferation of human osteoblasts. *Biochem Pharmacol*. 2010;79(6):926–937.
18. **Wu J**, **Wang R**, **Kan X**, **et al**. A Sonic Hedgehog-Gli-Bmi1 signaling pathway plays A critical role in p27 deficiency induced bone anabolism. *Int J Biol Sci*. 2022;18(3):956–969.
19. **Diduch DR**, **Coe MR**, **Joyner C**, **Owen ME**, **Balian G**. Two cell lines from bone marrow that differ in terms of collagen synthesis, osteogenic characteristics, and matrix mineralization. *J Bone Joint Surg Am*. 1993;75-A(1):92–105.
20. **Cui Q**, **Wang GJ**, **Su CC**, **Balian G**. The Otto Aufranc Award. Lovastatin prevents steroid induced adipogenesis and osteonecrosis. *Clin Orthop Relat Res*. 1997;344:8–19.
21. **Chen CH**, **Ho ML**, **Chang JK**, **Hung SH**, **Wang GJ**. Green tea catechin enhances osteogenesis in a bone marrow mesenchymal stem cell line. *Osteoporos Int*. 2005;16(12):2039–2045.
22. **Chuang SC**, **Chen CH**, **Chou YS**, **Ho ML**, **Chang JK**. G protein-coupled estrogen receptor mediates cell proliferation through the cAMP/PKA/CREB pathway in murine bone marrow mesenchymal stem cells. *Int J Mol Sci*. 2020;21(18):6490.
23. **Chuang S-C**, **Chen C-H**, **Fu Y-C**, **et al**. Estrogen receptor mediates simvastatin-stimulated osteogenic effects in bone marrow mesenchymal stem cells. *Biochem Pharmacol*. 2015;98(3):453–464.
24. **Li X**, **Cui Q**, **Kao C**, **Wang GJ**, **Balian G**. Lovastatin inhibits adipogenic and stimulates osteogenic differentiation by suppressing PPARgamma2 and increasing Cbfa1/Runx2 expression in bone marrow mesenchymal cell cultures. *Bone*. 2003;33(4):652–659.
25. **Chang J-K**, **Li C-J**, **Wu S-C**, **et al**. Effects of anti-inflammatory drugs on proliferation, cytotoxicity and osteogenesis in bone marrow mesenchymal stem cells. *Biochem Pharmacol*. 2007;74(9):1371–1382.
26. **Xie F**, **Xiao P**, **Chen D**, **Xu L**, **Zhang B**. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol Biol*. 2012;80(1):75–84.
27. **Krause DS**, **Theise ND**, **Collector MI**, **et al**. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*. 2001;105(3):369–377.
28. **Ruijtenberg S**, **van den Heuvel S**. Coordinating cell proliferation and differentiation: antagonism between cell cycle regulators and cell type-specific gene expression. *Cell Cycle*. 2016;15(2):196–212.
29. **Dalton S**. Linking the cell cycle to cell fate decisions. *Trends Cell Biol*. 2015;25(10):592–600.
30. **Abbastabar M**, **Kheyrollah M**, **Azizian K**, **et al**. Multiple functions of p27 in cell cycle, apoptosis, epigenetic modification and transcriptional

regulation for the control of cell growth: a double-edged sword protein. *DNA Repair (Amst)*. 2018;69:63–72.
31. **Sun W**, **Wu J**, **Huang L**, **et al**. PTHrP nuclear localization and carboxyl terminus sequences modulate dental and mandibular development in part via the action of p27. *Endocrinology*. 2016;157(4):1372–1384.
32. **Liu Y**, **Ao X**, **Ding W**, **et al**. Critical role of FOXO3a in carcinogenesis. *Mol Cancer*. 2018;17(1):104.
33. **Honda Y**, **Ding X**, **Mussano F**, **Wiberg A**, **Ho C-M**, **Nishimura I**. Guiding the osteogenic fate of mouse and human mesenchymal stem cells through feedback system control. *Sci Rep*. 2013;3:3420.
34. **Lees SJ**, **Childs TE**, **Booth FW**. Age-dependent FOXO regulation of p27Kip1 expression via a conserved binding motif in rat muscle precursor cells. *Am J Physiol Cell Physiol*. 2008;295(5):C1238–46.
35. **Tseng P-C**, **Hou S-M**, **Chen R-J**, **et al**. Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis. *J Bone Miner Res*. 2011;26(10):2552–2563.
36. **Sun W**, **Qiao W**, **Zhou B**, **et al**. Overexpression of Sirt1 in mesenchymal stem cells protects against bone loss in mice by FOXO3a deacetylation and oxidative stress inhibition. *Metabolism*. 2018;88:61–71.
37. **Chien WM**, **Garrison K**, **Caufield E**, **Orthel J**, **Dill J**, **Fero ML**. Differential gene expression of p27Kip1 and Rb knockout pituitary tumors associated with altered growth and angiogenesis. *Cell Cycle*. 2007;6(6):750–757.
38. **Drissi H**, **Hushka D**, **Aslam F**, **et al**. The cell cycle regulator p27kip1 contributes to growth and differentiation of osteoblasts. *Cancer Res*. 1999;59(15):3705–3711.
39. **Fero ML**, **Rivkin M**, **Tasch M**, **et al**. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell*. 1996;85(5):733–744.
40. **Zhu M**, **Zhang J**, **Dong Z**, **et al**. The p27 pathway modulates the regulation of skeletal growth and osteoblastic bone formation by parathyroid hormone-related peptide. *J Bone Miner Res*. 2015;30(11):1969–1979.
41. **Zhang X**, **Schwarz EM**, **Young DA**, **Puzas JE**, **Rosier RN**, **O'Keefe RJ**. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest*. 2002;109(11):1405–1415.
42. **Gu J**, **Lu Y**, **Li F**, **et al**. Identification and characterization of the novel Col10a1 regulatory mechanism during chondrocyte hypertrophic differentiation. *Cell Death Dis*. 2014;5(10):e1469.
43. **Welting TJM**, **Caron MMJ**, **Emans PJ**, **et al**. Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *Eur Cell Mater*. 2011;22(420–36):420–436.
44. **Chang LH**, **Chen CH**, **Wu SC**, **Chang JK**, **Ho ML**. Cyclooxygenase-2 regulates PTHrP transcription in human articular chondrocytes and is involved in the pathophysiology of osteoarthritis in rats. *J Orthop Translat*. 2021;30:16–30.
45. **Dai Z**, **Chen Y**, **He E**, **et al**. Interleukin-19 promotes bone resorption by suppressing osteoprotegerin expression in BMSCs in a lipopolysaccharide-induced bone loss mouse model. *Bone Joint Res*. 2023;12(11):691–701.
46. **Hu X**, **Yang L**, **Du Y**, **Meng X**, **Shi Y**, **Zeng J**. Astragalus polysaccharide promotes osteogenic differentiation of human bone marrow derived mesenchymal stem cells by facilitating ANKFY1 expression through miR-760 inhibition. *Bone Joint Res*. 2023;12(8):476–485.
47. **Li Z**. LncRNA PCBP1-AS1 induces osteoporosis by sponging miR-126-5p/PAK2 axis. *Bone Joint Res*. 2023;12(6):375–386.
48. **Yao C**, **Sun J**, **Luo W**, **et al**. Down-expression of miR-494-3p in senescent osteocyte-derived exosomes inhibits osteogenesis and accelerates age-related bone loss via PTEN/PI3K/AKT pathway. *Bone Joint Res*. 2024;13(2):52–65.

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

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