cAMP regulates 11β-hydroxysteroid dehydrogenase-2 and Sp1 expression in MLO-Y4/MC3T3-E1 cells

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Abstract. 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) is one of the key enzymes in glucocorticoid metabolism, which can inactivate local corticosterone and regulate the level of active glucocorticoid in tissues. The expression of 11β-HSD2 and its regulatory pathway serve an important role in the apoptosis of steroid induced osteonecrosis of the femoral head (SANFH). The present study aimed to identify the regulatory effects of cAMP on the expression of Sp1 transcription factor (Sp1) and 11β-HSD2 in osteocytes at the cellular level. Murine long bone osteocyte Y4 (MLO-Y4) clone cells and mouse embryo osteoblast-like (MC3T3-E1) cells were cultured *in vitro* with adenylate cyclase activator or inhibitor (forskolin and SQ22536, respectively) to investigate the effects of alterations to intracellular cAMP levels. mRNA and protein expression levels of Sp1 and 11β-HSD2 were detected by reverse transcription-quantitative PCR and western blotting, respectively. Compared with the negative control group, the mRNA and protein expression levels of Sp1 were significantly increased in the activation group, whereas Sp1 expression levels were significantly decreased in the inhibition group. Similarly, compared with the negative control group, the mRNA and protein expression levels of 11β-HSD2 were significantly increased in the activator group, but significantly decreased in the inhibitor group. The aforementioned results indicated that intracellular cAMP levels significantly regulated the expression of Sp1 and 11β-HSD2 in mouse osteocytes and osteoblasts. Therefore, the present study suggested a potential therapeutic strategy for the prevention of osteonecrosis of the femoral head.

Introduction

Steroid-induced avascular necrosis of the femoral head (SANFH) is a serious orthopedic disease associated with the clinical application of steroid hormones, characterized by progressive osteonecrosis leading to structural alterations to the femoral head, which can ultimately result in femoral head collapse (1). According to the duration and dosage of the steroid hormone, femoral head necrosis occurs in 5-40% of patients a few years after the onset of the disease (2-4). Although the mechanism underlying ischemic necrosis of the femoral head is not completely understood (1), the mechanism underlying osteocyte apoptosis has received increased attention (5-8). Glucocorticoid-induced osteocyte apoptosis is a cumulative and irreparable deficiency defect that destroys the mechanical sensory function of the osteocyte-lacunar-tubule system, thereby initiating a succession of events that inevitably lead to joint collapse (9). A possible mechanism resulting in apoptosis is that the long-term use of glucocorticoids can lead to excessive oxidative stress, which may activate apoptosis-associated signaling pathways when the antioxidant capacity of the cells is exceeded (6). Another study demonstrated that glucocorticoids are able to induce apoptosis via Dickkopf-1 (DKK-1)-mediated blocking of the Wnt/β-catenin signaling pathway, a process that reduces osteoblast proliferation, activates apoptosis and induces osteoclast differentiation and maturation, subsequently disrupting bone homeostasis and leading to a series of bone diseases, including osteoporosis and osteonecrosis (10). DKK-1 regulates myeloma bone disease by disrupting osteoblast differentiation and function. A study conducted by Lu *et al* (11) described a DKK1 vaccine that displayed therapeutic effects against the established disease via induction of active immunity. However, apoptosis is mediated by the strict control of multiple genes, which involves the activation, expression and regulation of a series of genes. In a mouse model of steroid-induced osteonecrosis of the femoral head, Zhang *et al* (12) demonstrated that aggravation of femoral head cell apoptosis may be associated with down-regulated 11β-hydroxysteroid dehydrogenase (11β-HSD)-2 expression, providing insights into the mechanism underlying cell apoptosis during SANFH.

The regulation of glucocorticoid levels in humans and other animals depends predominantly on 11β-HSD, an enzyme that catalyzes the mutual transformation of active and inert glucocorticoids. 11β-HSD has two isozymes, 11β-HSD1 and 11β-HSD2, which are involved in regulation of the aforementioned reaction (13). Compared with 11β-HSD1, 11β-HSD2 has a higher affinity for its steroid substrate and relies on NAD^+ to convert active glucocorticoids into their

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inactive metabolites (14,15), thereby preventing the body from accumulating large amounts of active hormones. 11β-HSD2 is widely distributed in tissues such as the kidney, placenta, heart and colon (16); therefore, it has been hypothesized that low expression levels of 11β-HSD2 are unfavorable to the body.

Several overlapping binding sites for Sp1 transcription factor (Sp1) have been identified in multiple CpG islands in the promoter region of 11β-HSD2 (17). Sp1 is a member of the zinc-finger transcription factor family that binds to a consistent promoter site, including a central CpG dinucleotide (18,19). In particular, Sp1 specifically binds to the promoter region of 11β-HSD2 and recruits the transcriptional co-activator p300, which affects histone acetylation modification of the 11β-HSD2 promoter region, by increasing H3K9Ac and decreasing H3K9me2, to promote the expression of 11β-HSD2 (20). Wang *et al* (21) reported that cortisol-induced aromatase is expressed in human placental syncytiotrophoblast cells via the cAMP/Sp1 signaling pathway. A previous study demonstrated that the tissues of the femoral head also expressed 11β-HSD, and in a model of steroid-induced osteonecrosis of the femoral head, the expression of 11β-HSD1 was increased, whereas the expression of 11β-HSD2 was decreased, which lead to further increases in the local GC concentration of the femoral head tissue (22). Therefore, the present study aimed to investigate the effects of altering cAMP levels on the expression of Sp1 and 11β-HSD2 in osteocytes and osteoblasts.

Materials and methods

Cell culture and grouping. MLO-Y4 cells were purchased from Procell Life Science & Technology Co., Ltd and MC3T3-E1 cells were purchased from iCell. MLO-Y4 cells can be used as representative models of primary osteocytes (23) and MC3T3-E1 cells are a reliable substitute *in vitro* model of human osteoblasts (24). Cells were cultured in MEM-α medium (Hyclone; GE Healthcare Life Sciences) containing 10% FBS (Zhejiang Tianhang Biotechnology Co., Ltd.) and 1% penicillin/streptomycin at 37°C with 5% CO_2. At 80% confluence, cells were subcultured. Trypsin-EDTA (0.25% trypsin and 0.02% EDTA; Genom Biotech Pvt., Ltd.) was added to the cells for digestion purposes. Subsequently, the cell layer was infiltrated and observed under a light microscope (magnification, x20) until the cells became round and dispersed. The cell suspension was centrifuged at 300 x g for 5 min at room temperature, the supernatant was discarded and the cells were suspended in fresh MEM-α medium. Cells were subsequently cultured at 37°C with 5% CO_2. Cells in the exponential phase of growth were seeded into 6-well plates with 2x10⁵ cells per well and cultured at 37°C for 24 h. Subsequently, cells were divided into three groups: i) The NC group (negative control group), which was incubated with 100 μmol PBS; ii) the activator group, which was incubated with 100 μmol forskolin (Selleck Chemicals); and iii) the inhibitor group, which was incubated with 100 μmol SQ22536 (Selleck Chemicals). Following incubation at 37°C for 24 h, cells were used for subsequent experiments.

Assessment of cellular viability. Cells in the exponential phase of growth were digested and collected. A cell suspension

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')	PCR product size, bp
GAPDH	F: TGAAGGGTGGAGCCAAAAG	227
	R: AGTCTTCTGGGTGGCAGTGAT	
Sp1	F: AACCTCAGTGCATTGGGTACTTC	164
	R: CTATTCTCTCCTTCTCCACCTGC	
11βHSD2	F: TGCTTCAAGACAGATGCAGTGAC	175
	R: CAACTGGGCTAAGGTCAGGC	

Sp1, Sp1 transcription factor; 11βHSD2, 11β hydroxysteroid dehydrogenase 2; F, forward; R, reverse.

(1x10⁵ cells/ml) was prepared and 100 μl/well cell suspension was added to a plate. Cells were cultured at 37°C for 24 h to allow the cells to adhere. Subsequently, 10 μl medium containing forskolin or SQ22536 (0, 5, 10, 50, 100 and 200 μmol) was added to each well. For the negative control group, 10 μl medium containing solvent was added to each well. The blank group consisted of wells containing 10 μl cell-free medium only. Following incubation at 37°C for 24 h, 10 μl CCK-8 solution (Shanghai Rebiosci Biotech Co., Ltd.) was added to each well at 37°C for 2 h. The absorbance of each well was measured at a wavelength of 450 nm using an ELISA reader (Diatek Healthcare Pvt. Ltd.).

Quantitative determination of cAMP levels in each group by ELISA. Cells were centrifuged at 2,000 x g at 4°C for 10 min to remove insoluble cell debris. Subsequently, the supernatant was separated and analyzed using the mouse cAMP ELISA kit [cat. no. ELK8116; Elk (Wuhan) Biotechnology Co. Ltd.], according to the manufacturer's protocol.

RNA extraction and RT-qPCR. Cells were washed with pre-cooled PBS. Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with genomic (g)DNA Eraser (Clontech Laboratories, Inc.) according to the manufacturers protocol. To remove gDNA, the reaction was performed on ice. Subsequently, qPCR was performed on a StepOne™ Real-Time PCR machine (Thermo Fisher Scientific, Inc.) using the SYBR® Premix Ex Taq™ kit (Clontech Laboratories, Inc.) with three replicate wells per sample. The following thermocycling conditions were used for qPCR: Incubation at 95°C for 1 min; followed by 40 cycles of 95°C for 15 sec, 58°C for 20 sec and 72°C for 45 sec. The primers used for qPCR were synthesized by Wuhan GeneCreate Biological Engineering Co., Ltd. and are presented in Table I. Following qPCR, agarose gel (2.0%) electrophoresis of the PCR products was performed to observe band intensity. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene GAPDH (25). RT-qPCR was performed in triplicate.
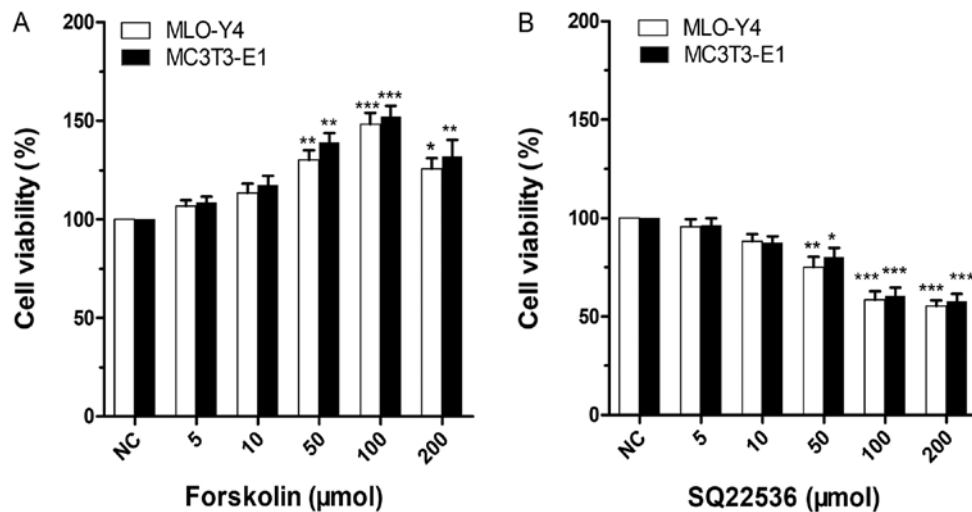
Figure 1. Effects of forskolin and SQ22536 on MLO-Y4 and MC3T3-E1 cell viability. The NC group was treated with 0 μmol forskolin or SQ22536. (A) Effect of forskolin on MLO-Y4 and MC3T3-E1 cell viability. Compared with the NC group, the 100 μmol forskolin group exhibited the most significant increase in MLO-Y4 cell viability (P<0.001), followed by the 50 μmol forskolin group (P<0.01). Moreover, 200 μmol forskolin significantly increased MLO-Y4 cell viability compared with the NC group (P<0.05). In MC3T3-E1 cells, compared with the NC group, the 50 and 200 μmol forskolin groups exhibited significantly enhanced cell viability (P<0.01). The 100 μmol forskolin group also significantly increased MC3T3-E1 cell viability compared with the NC group (P<0.001). (B) Effect of SQ22536 on MLO-Y4 and MC3T3-E1 cell viability. In MLO-Y4 and MC3T3-E1 cells, the 100 and 200 μmol SQ22536 groups displayed significantly decreased cell viability compared with the NC group (P<0.001). Moreover, compared with the NC group, MLO-Y4 cell viability was significantly decreased in the 50 μmol SQ22536 group (P<0.01). Similarly, MC3T3-E1 cell viability was significantly decreased in the 50 μmol SQ22536 group (P<0.05). *P<0.05, **P<0.01, ***P<0.001 vs. NC (0 μmol). NC, negative control.

Protein extraction and western blotting. Total protein was extracted from cells using RIPA protein lysate (Wuhan Aspen Biotechnology Co., Ltd.) and quantified using a bicinchoninic acid protein concentration assay kit (Wuhan Aspen Biotechnology Co., Ltd.). Subsequently, the protein expression levels of 11β-HSD2 and Sp1 were detected following a standard western blotting protocol. Proteins (40 μg) were added to an appropriate amount of protein loading buffer (5X) and incubated at 95-100°C in a boiling water bath for 5 min. Subsequently, proteins were separated via 8% SDS-PAGE and transferred onto methanol pre-activated PVDF membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk at room temperature for 1 h. Subsequently, the membranes were incubated overnight with the following primary antibodies: Anti-Sp1 (cat. no. ab227383; 1:5000; Abcam), anti-11β-HSD2 (cat. no. ab80317; 1:1,000; Abcam) and anti-GAPDH (cat. no. ab37168; 1:10,000; Abcam). After washing three times with TBS containing the 0.05% of Tween-20 (TBST), the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (cat. no. AS1107; 1:10,000; Wuhan Aspen Biotechnology Co., Ltd.) for 30 min. Protein bands were visualized using the ECL system (Thermo Fisher Scientific, Inc.).The optical density of the target bands was assessed using the AlphaEaseFC™ 4.0 software processing system (ProteinSimple) with GAPDH as the loading control. Western blotting was performed in triplicate.

Statistical analysis. All experiments were performed at least three times. Data are presented as the mean ± SEM. One-way ANOVA followed by Tukey's post hoc test was used to analyze the cell viability data. One-way ANOVA followed by the Student-Newman-Keuls post hoc test was used to analyze
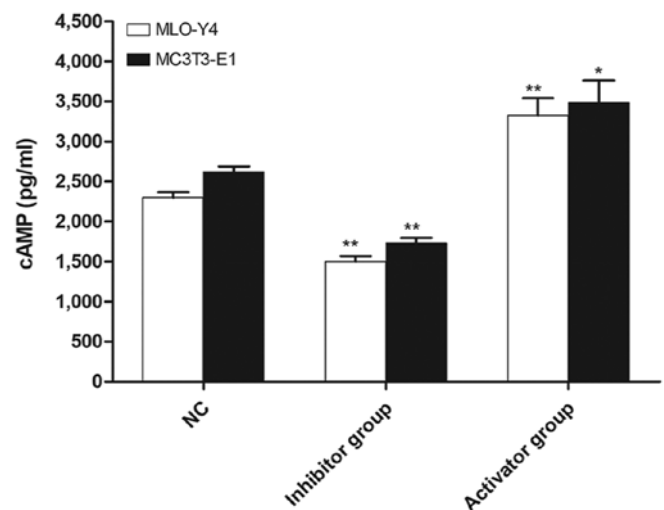

Figure 2. Detection of cAMP content in MLO-Y4 and MC3T3-E1 cells. Compared with the NC groups, the intracellular cAMP content was significantly decreased in the inhibitor groups (P<0.01). Compared with the NC group, the intracellular cAMP content in the activator group was significantly increased in MLO-Y4 (P<0.01) and MC3T3-E1 cells (P<0.05). *P<0.05, **P<0.01 vs. NC. NC, negative control.

the ELISA, RT-qPCR and western blotting data. Statistical analyses were performed using GraphPad Prism software (version 5; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of forskolin and SQ22536 on MLO-Y4/MC3T3-E1 cell activity. The effects of forskolin and SQ22536 on MLO-Y4 and MC3T3-E1 cell viability were assessed using the Cell
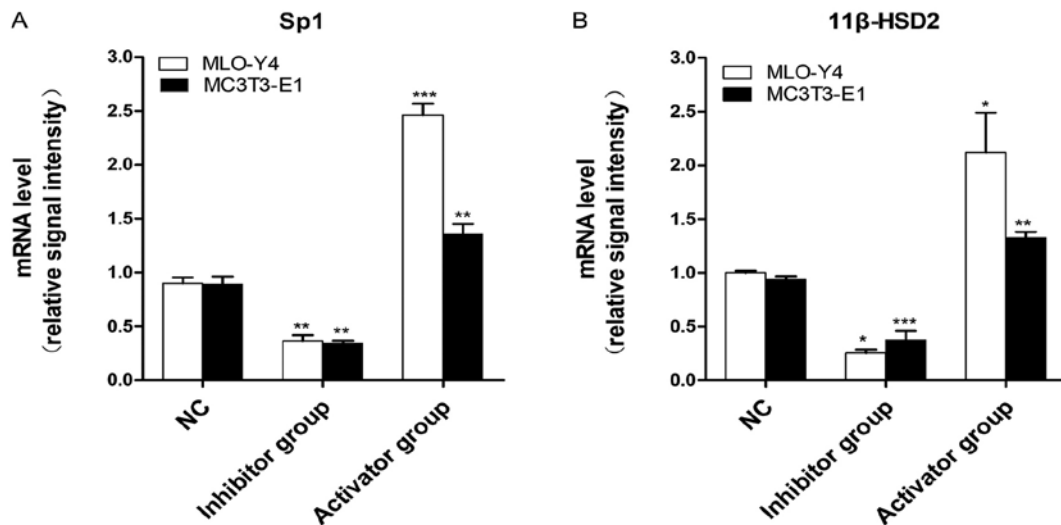
Figure 3. Sp1 and 11β-HSD2 expression levels in MLO-Y4 and MC3T3-E1 cells. (A) Sp1 mRNA expression levels in MLO-Y4 and MC3T3-E1 cells. Compared with the NC group, the expression of Sp1 was significantly reduced in the inhibitor group (P<0.01). By contrast, the expression of Sp1 was significantly increased in the activator group compared with the NC group in MLO-Y4 (P<0.001) and MC3T3-E1 cells (P<0.01). (B) 11β-HSD2 mRNA expression levels in MLO-Y4/MC3T3-E1 cells. In MLO-Y4 cells, compared with the NC group, the expression of 11β-HSD2 in the inhibitor and activator groups was decreased and increased, respectively (P<0.05). Compared with the NC group, the expression of 11β-HSD2 in MC3T3-E1 cells was significantly decreased in the inhibitor group (P<0.001), and significantly increased in the activator group (P<0.01). *P<0.05, **P<0.01, ***P<0.001 vs. NC. Sp1, Sp1 transcription factor; 11β-HSD2, 11β-hydroxysteroid dehydrogenase-2; NC, negative control.

Counting Kit-8 assay (Fig. 1). Compared with the 0 μmol forskolin group, cell viability was increased in the 10 μmol forskolin group following incubation for 24 h (P>0.05). Cell viability in the 50, 100 and 200 μmol forskolin groups was significantly increased compared with the 0 μmol forskolin group (P<0.05). However, the 100 μmol forskolin group displayed the most significant increase in cell viability compared with the 0 μmol forskolin group; therefore, 100 μmol forskolin was selected for subsequent experiments (Fig. 1A). Furthermore, compared with the 0 μmol SQ22536 group, cell viability was significantly decreased in the 100 and 200 μmol SQ22536 groups (P<0.001), but there was no significant difference between the two groups (P>0.05); therefore, 100 μmol SQ22536 was selected for subsequent experiments (Fig. 1B).

Detection of cAMP expression in MLO-Y4 and MC3T3-E1 cells by ELISA. Subsequently, whether forskolin and SQ22536 had a significant effect on the expression of cAMP in MLO-Y4 and MC3T3-E1 cells was investigated. Compared with the NC group, the content of cAMP in the activator group was significantly increased (P<0.05), whereas the content of cAMP was significantly decreased in the inhibitor group (P<0.01). The results suggested that the adenylate cyclase activator and inhibitor significantly upregulated and downregulated the content of cAMP, respectively, in mouse osteoblast-like and bone-like cells (Fig. 2).

Sp1 and 11β-HSD2 expression levels in MLO-Y4 and MC3T3-E1 cells. The effect of upregulation and down-regulation of cAMP content on the expression levels of Sp1 and 11β-HSD2 were detected by RT-qPCR (Fig. 3). The expression levels of Sp1 and 11β-HSD2 were significantly increased in the activator group compared with the NC group (P<0.05). By contrast, the expression levels of Sp1 and 11β-HSD2 in the

inhibitor group were significantly decreased compared with the NC group (P<0.05).

Protein expression levels of Sp1 and 11β-HSD2. Western blotting was performed to detect whether alterations to cAMP content had an effect on the expression levels of Sp1 and 11β-HSD-2 (Fig. 4). The expression levels of Sp1 and 11β-HSD2 in the activator group were significantly increased compared with the NC group (P<0.05). Moreover, the expression levels of Sp1 and 11β-HSD2 were significantly decreased in the inhibitor group compared with the NC group (P<0.05).

Discussion

The results of the present study supported the hypothesis that, compared with the negative control group, the relative expression of Sp1 and 11β-HSD2 in the activator group was significantly increased. In contrast, the relative expression of Sp1 and 11β-HSD2 in the inhibitor group was significantly decreased compared with the negative control group. Specifically, the data indicated that upregulating or downregulating intracellular cAMP levels in MLO-Y4 and MC3T3-E1 cells increased or decreased the expression levels of Sp1 and 11β-HSD2, respectively. Therefore, the results suggested that in osteoblasts and osteocytes, cAMP mediated the expression of the transcription stimulator Sp1 and the glucocorticoid-regulated key enzyme 11β-HSD2, which is closely related to SANFH-associated apoptosis.

Increasing evidence has suggested that activation of the cAMP signaling pathway serves an important role in hormone metabolism. Cabrera-Sharp *et al* (26) reported that cortisol-cortisone metabolism in boar testis and caput epididymis could be regulated by the cAMP-protein kinase A (PKA) signaling pathway via their selective effects on the reductase activity of 11β-HSD. Prenatal ethanol exposure
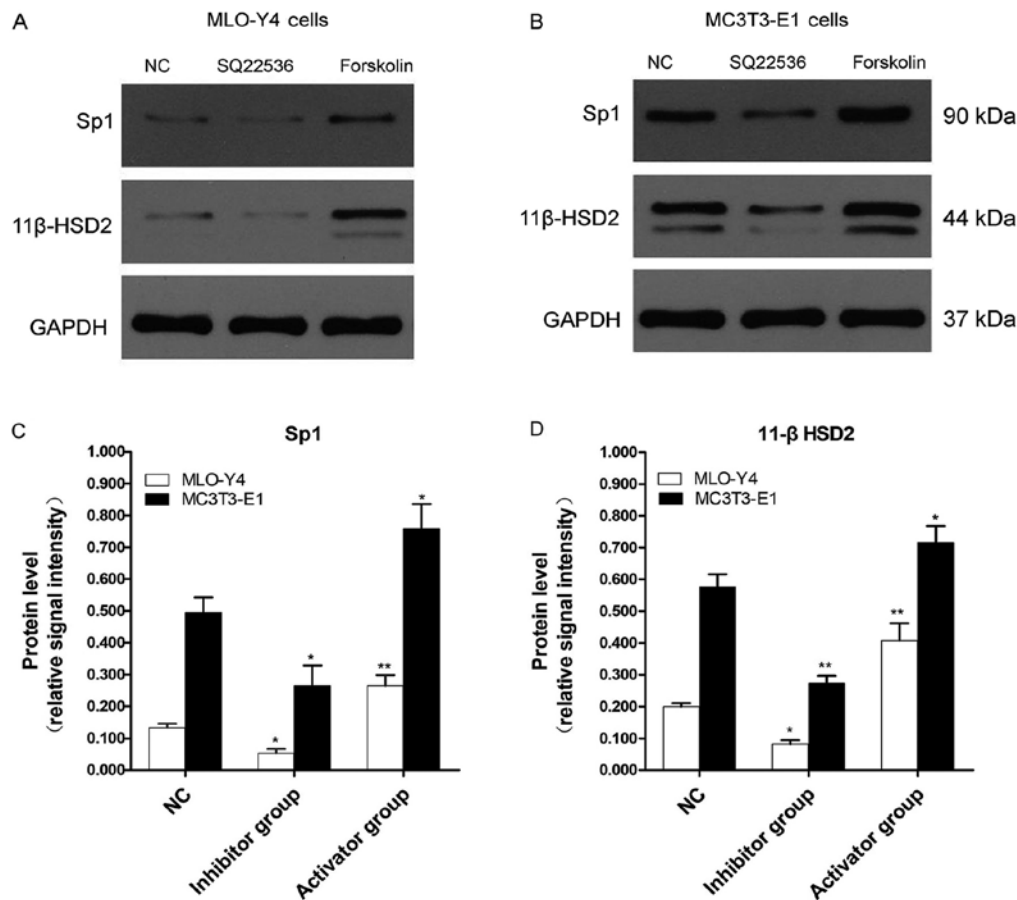
Figure 4. Western blotting was performed to measure the relative protein expression of Sp1 and 11β-HSD2 in MLO-Y4 and MC3T3-E1 cells. Protein expression levels were determined by western blotting in (A) MLO-Y4 and (B) MC3T3-E1 cells, and (C) Sp1 and (D) 11β-HSD2 expression levels were quantified. In MLO-Y4 cells, compared with the NC group, Sp1 protein expression was significantly decreased (P<0.05) and increased (P<0.01) in the inhibitor and activator groups, respectively. In MC3T3 cells, Sp1 protein expression in the inhibitor and activator groups was significantly decreased (P<0.05) and increased (P<0.05) compared with the NC group, respectively. Similarly, in MLO-Y4 cells, compared with the NC group, 11β-HSD2 expression was significantly decreased (P<0.05) and increased (P<0.01) in the inhibitor and activator groups, respectively. Furthermore, in MC3T3-E1 cells, 11β-HSD2 expression was significantly decreased (P<0.01) and increased (P<0.05) in the inhibitor and activator groups compared with the NC group, respectively. *P<0.05, **P<0.01 vs. NC. Sp1, Sp1 transcription factor; 11β-HSD2, 11β-hydroxysteroid dehydrogenase-2; NC, negative control.

reduced placental 11β-HSD2 expression, which was controlled via the mechanism underlying ethanol-induced histone modification alterations to the 11β-HSD2 promoter via the cAMP/PKA signaling pathway (27). However, biological analysis of the promoter sequence of the 11β-HSD2 gene indicated that the 11β-HSD2 promoter did not contain a cAMP-response element; therefore, the effect of cAMP on the expression of 11β-HSD2 was potentially indirectly mediated via other transcription factors (28). Sp1 is a sequence-specific DNA-binding protein that regulates the transcription of cellular and viral genes containing GC-rich promoters (29). A previous study suggested that the expression of 11β-HSD2 is highly tissue-specific *in vivo* (16). 11β-HSD2 is highly expressed in target organs containing mineralocorticoid receptors that are involved in mediating steroid resistance, and the tissue-specific profile of 11β-HSD2 expression has been attributed to differential methylation of the CpG island-rich 11β-HSD2 promoter (16,30). Sp1 domain B is a high affinity DNA-binding structure that includes a PKA-compliant phosphorylation site at Thr366 (31). The cAMP/PKA signaling pathway regulates the transactivation of Sp1 and DNA-binding activity, and PKA stimulates transcription of the Sp1-dependent promoter

in intact cells and activates the DNA-binding activity of Sp1 *in vitro* (32). In a study investigating the mechanism by which the placental glucocorticoid barrier is established, it was reported that human chorionic gonadotropin altered histone modification and increased the expression of Sp1 via activation of the cAMP/PKA signaling pathway, which activated 11β-HSD2 transcription (33). A further study demonstrated that 11β-HSD2 expression was influenced by increased Sp1 expression following activation of the cAMP signaling pathway, rather than the differential methylation of the 11β-HSD2 promoter (20). The interaction between Sp1 and PKA is similar to the complex formed between the PKA catalytic subunit and NF-κB (34). Another previous study reported that Sp1 is the substrate of DNA-dependent protein kinase *in vitro*, and is phosphorylated at multiple sites in HeLa nuclear extract; however, the study did not determine the degree of DNA binding, or Sp1-dependent transcription or specificity (35).

A previous study confirmed the expression of 11β-HSD2 in necrotic tissue of the femoral head, which was increased in a model of steroid-induced femoral head necrosis, whereas the expression of 11β-HSD2 was decreased (22). Furthermore,

it was reported that when the transfected recombinant adeno-virus Adb-11β-HSD2 entered into MLO-Y4 and MC3T3-E1 cells, the expression of 11β-HSD2 was upregulated, and the number of apoptotic cells and the relative expression of apoptotic proteins decreased, reflecting a decrease in apoptosis (12). However, the regulatory mechanism underlying 11β-HSD2 has not been previously reported.

In the present study, forskolin and SQ22536 were used as a cAMP activator and inhibitor, respectively. ELISA was performed to identify alterations to intracellular cAMP content, and RT-qPCR and western blotting were performed to measure the relative expression levels of Sp1 and 11β-HSD2. Compared with the negative control group, the expression levels of Sp1 and 11β-HSD2 were significantly increased in the activator group, and the opposite results were observed in the inhibitor group. The results preliminarily suggested that the expression of Sp1 and 11β-HSD2 was regulated by cAMP in osteocytes and osteoblasts. However, the mechanism underlying Sp1-mediated regulation of 11β-HSD2 was not identified. Sp1 regulates gene expression via two main mechanisms. One mechanism involves recruitment of basic transcriptional device proteins and direct interaction with certain proteins in transcriptional complexes, which activates gene transcription by recruiting or promoting the assembly of the basic transcription device (36). The other mechanism involves alterations to chromosome modification. Sp1 can recruit histone acetylase and deacetylase to regulate the acetylation state of the histone at the gene promoter, thereby activating or inhibiting gene expression (37). However, Sp1-mediated regulation of 11β-HSD2 expression requires further investigation.

Apoptosis is an important mechanism associated with steroid-induced femoral head necrosis; furthermore, osteocyte and osteoblast apoptosis result in reduced bone formation, as well as inadequate bone remodeling and repair (38). The aim of the present study was to identify a potential mechanism underlying 11β-HSD2 involvement in steroid-induced bone necrosis at the cellular level. However, combinations of Sp1 and 11β-HSD2 promoter regions, post-modification alterations and interactions *in vivo* have not yet been studied; therefore, future studies are required.

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Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZP made substantial contributions to the conception and design of the current study and gave final approval of the version to be published. DL analyzed and interpreted data, and drafted the manuscript. DL, YW, ZH and FC performed the experiments. ZP supervised the project. All authors read and approved the final 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.

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