

# Nicotinamide mononucleotide attenuates glucocorticoid-induced osteogenic inhibition by regulating the SIRT1/PGC-1 $\alpha$ signaling pathway

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Received August 19, 2019; Accepted March 3, 2020

DOI: 10.3892/mmr.2020.11116

**Abstract.** Long-term and high-dose glucocorticoid treatment is recognized as an important influencing factor for osteoporosis and osteonecrosis. Nicotinamide mononucleotide (NMN) is an intermediate of NAD<sup>+</sup> biosynthesis, and is widely used to replenish the levels of NAD<sup>+</sup>. However, the potential role of NMN in glucocorticoid-induced osteogenic inhibition remains to be demonstrated. In the present study, the protective effects of NMN on dexamethasone (Dex)-induced osteogenic inhibition, and its underlying mechanisms, were investigated. Bone mesenchymal stem cells were treated with Dex, which decreased the levels of the osteogenic markers alkaline phosphatase, Runt-related transcription factor 2 and osteocalcin. NMN treatment attenuated Dex-induced osteogenic inhibition and promoted the expression of sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 $\alpha$ . SIRT1 knockdown reversed the protective effects of NMN and reduced the expression levels of PGC-1 $\alpha$ . Collectively, the results of the present study reveal that NMN may be a potential therapeutic target for glucocorticoid-induced osteoporosis.

## Introduction

Osteoporosis is a chronic disease with a heavy global socioeconomic burden. It is defined as a skeletal disorder, characterized by decreased bone strength, which in turn predisposes affected individuals to fractures (1) and damage to the bone microarchitecture (2). Osteoporosis is diagnosed based on the presence of fragility fractures in the absence of other metabolic bone disorders, or a T-score of  $\leq 2.5$  in the lumbar

spine (anteroposterior), femoral neck and total hip, and/or 33% (one-third) radius, even in the absence of a prevalent fracture. Osteoporosis may also be diagnosed in patients with osteopenia and increased fracture risk, using FRAX<sup>®</sup> country-specific thresholds. Osteopenia is defined as T-score between -1.0 and -2.5, based on bone mineral density testing (3). There are two major categories of osteoporosis: Primary and secondary. Sex and age are the primary influencing factors for primary osteoporosis, whereas secondary osteoporosis is associated with long-term and high-dose glucocorticoid treatment (4). Glucocorticoids such as dexamethasone (Dex) and hydrocortisone are widely used to treat inflammation and immunological rejection, as well as autoimmune diseases (5). These diseases not only include rheumatoid arthritis and systemic lupus erythematosus, but also asthma, chronic obstructive pulmonary disease, Crohn's disease and ulcerative colitis (6-12). Dex-induced osteogenic inhibition has been considered as the most severe side-effect of this particular treatment type (13), and long-term administration of glucocorticoids can result in osteoporosis or osteonecrosis (14). However, the molecular mechanism underlying glucocorticoid-induced osteogenic inhibition is not clear.

Nicotinamide mononucleotide (NMN) is an important NAD<sup>+</sup> intermediate whose levels decrease with age. As such, NMN administration is an effective treatment for age-related diseases and bone metabolism (Table I), and can be used to alleviate age-related type 2 diabetes, ischemia-reperfusion injury and Alzheimer's disease (15). NMN has previously been reported to improve osteogenesis by regulating sirtuin 1 (SIRT1) (16). The present study aimed to investigate the role of NMN against the glucocorticoid-induced loss of bone cell viability via mesenchymal stromal cell (MSC) regulation. MSCs are non-hematopoietic pluripotent stem cells with regenerative capacity, and with age, a reduction in the number or function of MSCs severely limits tissue regeneration (17). However, the mechanism of action of NMN in glucocorticoid-induced loss of bone cell viability remains unclear.

SIRT1, also known as silent mating type information regulation 2 homolog, was discovered in humans in 1999 (18), and is an NAD-dependent class III protein deacetylase (19). As a deacetylase, SIRT1 is closely associated with various other proteins such as p53, Ku70, forkhead box protein O1,

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**Key words:** nicotinamide mononucleotide, osteoporosis, sirtuin 1, peroxisome proliferator-activated receptor gamma coactivator

NF- $\kappa$ B, peroxisome proliferator-activated receptor- $\gamma$  and p300, and is involved in the regulation of cell senescence and apoptotic death under stress conditions, thereby enhancing cellular activity, self-healing and survival capacity (20-23). Numerous studies have reported the involvement of SIRT1 in bone metabolism, and as a mediator of bone mass regulation (24-26). Conditional knockout of SIRT1 leads to low bone density and mass, and a significant increase in body weight, skeletal size, bone volume, osteoblast numbers, alkaline phosphatase (ALP)- and type I collagen-positive areas in SIRT1 transgenic mice (27).

In the present study, the effects of NMN on glucocorticoid-induced loss of bone cell viability, and its underlying mechanisms, underwent preliminary investigation. It was hypothesized that NMN plays a protective role in glucocorticoid-induced osteoporosis, and the present study provides a potential therapeutic method for glucocorticoid-induced osteoporosis.

## Materials and methods

**Cell culture and osteogenic induction.** Bone mesenchymal stem cells (BMSCs) at passage 6 were obtained from Cyagen Biosciences, Inc. The cells were cultured in C57BL/6 Mouse Mesenchymal Stem Cell growth medium (Cyagen Biosciences, Inc.) containing 10% FBS, 1% glutamine and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere (5% CO<sub>2</sub>). For osteogenic induction, BMSCs were seeded into 6-well plates at a density of 2x10<sup>5</sup> cells per well. The culture medium was substituted for C57BL/6 Mouse Mesenchymal Stem Cell osteogenic differentiation medium (Cyagen Biosciences, Inc.; 10% FBS, 1% penicillin-streptomycin, 0.2% ascorbate, 1% glutamine, 10<sup>-10</sup> M Dex and 10 mM  $\beta$ -glycerophosphate), and the cells were incubated until reaching 80% confluence. The negative control group was incubated in osteogenic differentiation medium supplemented with 10<sup>-10</sup> M Dex. For subsequent experiments, BMSCs were used between passages 7 and 10. The osteogenic induction medium was replaced every 3 days. After incubation for 7 days, subsequent experiments were performed.

**Nicotinamide mononucleotide (NMN) treatment.** BMSCs were treated with 1, 5 or 10 mM NMN (cat. No. 1094-61-7) for 7 days. The vehicle group was cultured with osteogenic differentiation medium for 7 days. The Dex groups were cultured with osteogenic differentiation medium containing 10<sup>-6</sup> M Dex for 7 days. The NMN groups were cultured with osteogenic differentiation medium containing 10<sup>-6</sup> M Dex and 1, 5 or 10 mM NMN for 7 days.

**RNAi and transfection.** SIRT1-small interfering (si)RNA and their negative control (NC) siRNA were purchased from Shanghai GenePharma Co., Ltd. BMSCs were transfected with 5  $\mu$ l SIRT1 siRNA (si-SIRT1: 5'-CCACCUGAGUUG GAUGAUA-3'; 20  $\mu$ M) or NC siRNA (5'-ACGUGACACGUU CGGAGAATT-3'; 20  $\mu$ M) were transfected into BMSCs using Lipofectamine<sup>®</sup> RNAi Max reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After 48 h, the effect of knockdown was confirmed by reverse transcription-quantitative PCR (RT-qPCR) and western blotting.

**RT-qPCR analysis.** Total RNA was isolated from cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using an absorbance measurement at a wavelength of 260 nm. The RNA was reverse-transcribed into cDNA using a SYBR<sup>®</sup> PrimeScript<sup>™</sup> RT-PCR kit (Takara Bio, Inc.) The specific primers for mouse ALP, Runt-related transcription factor 2 (Runx2), osteocalcin (OCN), SIRT1 and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 $\alpha$  are listed in Table II. qPCR was performed using SYBR<sup>®</sup> Premix Ex Taq (Takara Bio, Inc.) with the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions used for qPCR were as follows: Initial denaturation at 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and a final extension at 72°C for 20 sec, as previously described (28). Melting curve analysis was used to analyze the specificity of transcript amplification, and target gene expression was quantified using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method and normalized to the internal reference gene GAPDH (29).

**Protein extraction and western blotting.** For nuclear and cytoplasmic proteins, the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology) with 1% Triton X-100 (Beyotime Institute of Biotechnology) was added to cells in order to solubilize plasma membrane and keep the nuclear membrane intact. The supernatant was incubated at 4°C for 20 min, and then 500  $\mu$ l nuclear isolation buffer was added. Next, homogenates were centrifuged at 600 x g (10 min, 4°C) for separation into the supernatant cytosolic fraction and pellet nuclear fraction. Proteins were then lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) (30). Total protein was extracted from cells using RIPA lysis buffer containing 10% phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Protein concentration was assessed using a bicinchoninic acid kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Protein samples (30  $\mu$ g) were separated by 8-12% SDS-PAGE and electro-transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk for 2 h at room temperature, and then incubated with primary antibodies against ALP (cat. no. ab229126, 1:1,000), Runx2 (Abcam; cat. no. ab192256, 1:1,000), SIRT1 (Abcam; cat. no. ab110304, 1:1,000), proliferating cell nuclear antigen (PCNA; Abcam; cat. no. ab29, 1:1,000), proliferator-activated receptor gamma coactivator (PGC)-1 $\alpha$  (Abcam; cat. no. ab54481, 1:1,000) and GAPDH (Beyotime Institute of Biotechnology; cat. no. AF5009, 1:1,000) at 4°C overnight. The membranes were washed three times with TBS-Tween 20 (1% Tween) and incubated with a horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (H+L) (Beyotime Institute of Biotechnology; cat. no. A0208; 1:2,000) or HRP-labeled goat anti-mouse IgG (H+L) (Beyotime Institute of Biotechnology; cat. no. A0216; 1:2,000) for 1 h at room temperature. The bands were visualized using the Western Chemiluminescent HRP Substrate kit (EMD Millipore), and ImageJ software (version 1.8.0; National Institutes of Health) was used for densitometric quantification.

**Alizarin red and ALP staining.** Following osteogenic induction, BMSCs were washed three times with PBS and fixed with 4%

Table I. Study characteristics.

Study	Type	Primary findings	(Refs.)
Song <i>et al</i> , 2019	Research	NMN promotes osteogenesis via SIRT1	(16)
Zainabadi, 2019	Review	NMN improve osteogenesis	(45)
Liang <i>et al</i> , 2019	Research	NMN alleviates aluminum-induced bone loss by inhibiting the thioredoxin-interacting protein-NLRP3 inflammasome	(42)
Hassan <i>et al</i> , 2018	Research	Nicotinamide phosphoribosyltransferase expression in osteoblasts controls osteoclast recruitment in alveolar bone remodeling	(46)
Mills <i>et al</i> , 2016	Research	Long-term NMN administration significantly improves bone density	(47)
Baek <i>et al</i> , 2017	Research	Nicotinamide phosphoribosyltransferase inhibits receptor activator of nuclear factor- $\kappa$ B ligand-induced osteoclast differentiation <i>in vitro</i>	(48)
Abed <i>et al</i> , 2014	Research	Low SIRT1 levels in human osteoarthritis subchondral osteoblasts lead to abnormal sclerostin expression which decreases Wnt/ $\beta$ -catenin activity	(49)

NMN, nicotinamide mononucleotide; SIRT1, sirtuin 1; NLRP3, nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3.

Table II. Primers used for qPCR.

Gene	Primer sequence (5'→3')	
	Forward	Reverse
Runx2	GACGAGGCAAGAGTTTCACC	GGACCGTCCACTGTCACTTT
ALP	TCGGGACTGGTACTCGGATAAC	GTTCACTGCGGTTCCAGACATAG
OCN	CAAGCAGGGAGGCAATAAGG	CGTCACAAGCAGGGTTAAGCQ
SIRT1	CACATGCCAGAGTCCAAGTT	AAATCCAGA TCCTCCAGCAC
PGC-1 $\alpha$	AACCACACCCACAGGATCAGA	TCTTCGCTTTATTGCTCCATGA
GAPDH	AGGAGCGAGACCCCACTAACA	AGGGGGGCTAAGCAGTTGGT

Runx2, Runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin; SIRT1, sirtuin 1; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$ .

paraformaldehyde for 10 min at room temperature. Alizarin working solution (1%; 1 g Alizarin diluted in aqueous solution; Cyagen Biosciences, Inc.) was used to perform Alizarin staining for 3-5 min, and a 5-bromo, 4-chloro, 3-indolyphosphate/Nitro-Blue Tetrazolium ALP Color Development kit (Beijing Leagene Biotechnology, Co., Ltd.) was used to perform ALP staining as previously described (31). After washing three times, images of the stained cells were immediately captured. Alizarin red staining was used to determine the effectiveness of osteogenic differentiation by the number and size of red calcium nodules. ALP staining was used to determine the ALP of BMSCs according to color shade. Stained cells were observed using a BX51 light microscope (Olympus Corporation; magnification, x100).

**Cell viability assay.** Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. BMSCs were cultured and induced in 96-well plates at density of  $5 \times 10^3$  cells/well for 7 days. Subsequently,  $10 \mu\text{l}$  CCK-8 solution was added to each well and cultured for 1 h. The optical

density of each well at a wavelength of 450 nm was measured using a microplate reader and analyzed using GraphPad Prism software (version 8.0; GraphPad Software, Inc.).

**ALP activity assay.** After osteogenic induction, BMSCs were harvested and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation at  $18407 \times g$  at  $4^\circ\text{C}$  for 10 min, the lysate supernatants were collected and added to 96-well plates. ALP activity was detected with the ALP Assay kit (Beyotime Institute of Biotechnology) using the p-nitrophenylphosphate method, according to the manufacturer's instructions. The cells were incubated at  $37^\circ\text{C}$  for 30 min, and absorbance was measured with a microplate reader (Omega Bio-Tek, Inc.) at 405 nm. The ALP level was normalized to the total protein content, and ALP activity was demonstrated as a fold change over the corresponding control group.

**Immunofluorescence assay.** After osteogenic induction and treatment, BMSCs were washed with PBS and then fixed in 4% paraformaldehyde for 15 min at room temperature. The cells

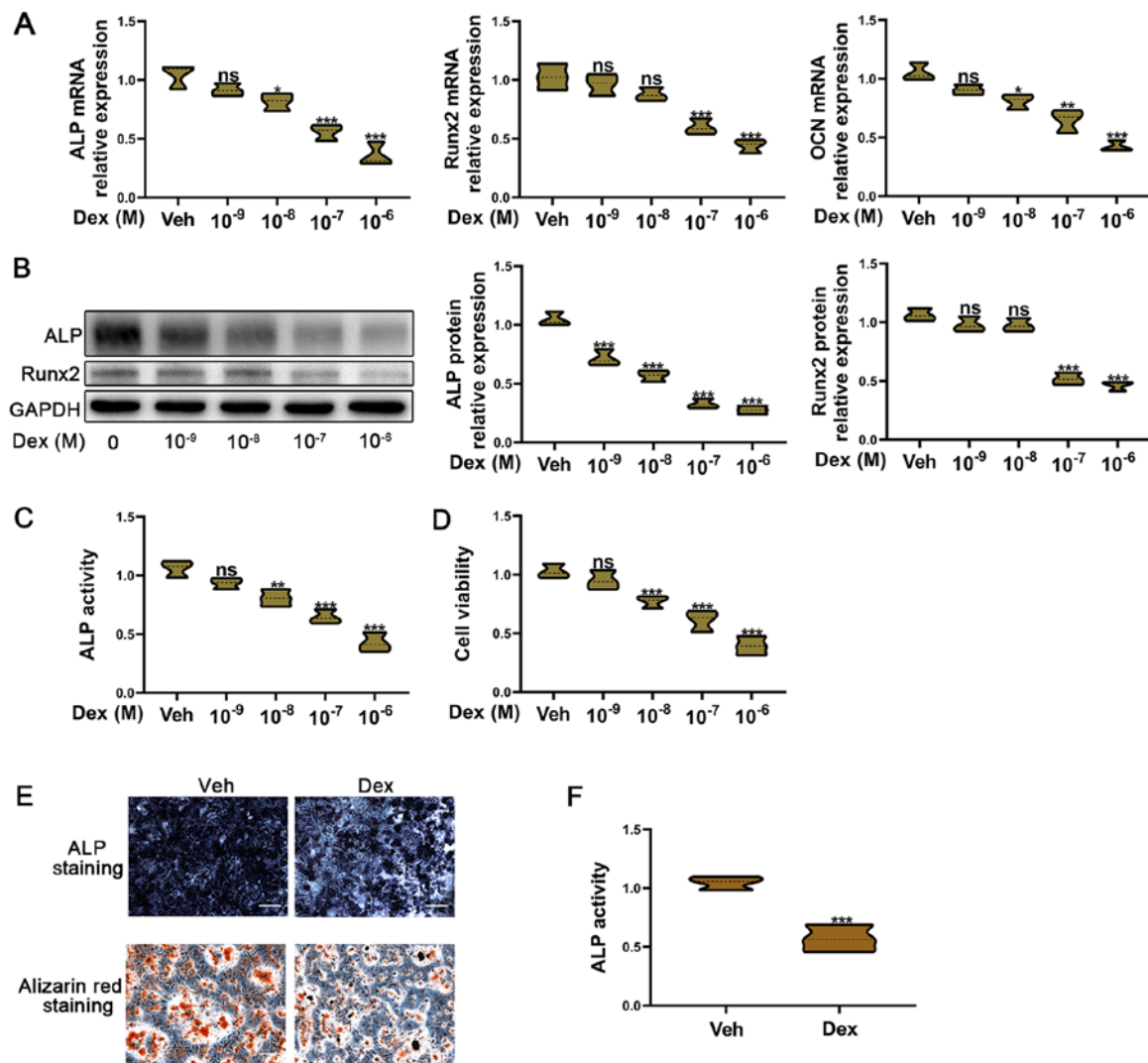


Figure 1. Dex-induced osteogenic inhibition in BMSCs. BMSCs were exposed to Dex (range, 10<sup>-9</sup>-10<sup>-6</sup> M) for 7 days. (A) Reverse transcription-quantitative PCR was used to detect Runx2, ALP and OCN mRNA expression in BMSCs. (B) Western blotting was used to determine the Runx2 and ALP protein expression levels in BMSCs. (C) Relative ALP activity. (D) Cell Counting Kit-8 was used to assess the viability of BMSCs. (E) Alizarin red and ALP staining assays were used to measure the osteogenic function of BMSCs treated with 10<sup>-6</sup> M Dex for 7 days. Scale bar, 20  $\mu$ m. (F) Relative ALP activity of BMSCs treated with 10<sup>-6</sup> M Dex for 7 days. All experiments were performed  $\geq 3$  times. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 and <sup>ns</sup>P $\geq$ 0.05 vs. Veh. Dex, dexamethasone; BMSCs, bone mesenchymal stem cells; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; OCN, osteocalcin; Veh, vehicle.

were permeabilized with 0.1% Triton-X 100 in PBS for 10 min. After blocking with 5% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, the cells were incubated with primary antibodies against SIRT1 (Abcam; cat. no. ab110304, 1:2,000) and PCNA (Abcam; cat. no. ab92552, 1:500) at 4°C overnight. The cells were then incubated with DAPI, Alexa Fluor® 488-AffiniPure goat anti-mouse IgG [H+L] (Jackson ImmunoResearch Europe, Ltd.; cat. no. 115-545-003, 1:200) and Cy3-AffiniPure goat anti-rabbit IgG [H+L] (Jackson ImmunoResearch Europe, Ltd.; cat. no. 111-165-003, 1:200) at room temperature for 1 h. Images of cells were captured using a fluorescence microscope (Olympus Corporation; magnification, x1,000).

**Statistical analysis.** All independent experiments were performed  $\geq 3$  times and the data are presented as the mean  $\pm$  SEM. Statistical differences were analyzed using a student's t-test or one-way ANOVA followed by Tukey's multiple

comparison post hoc test. Statistical analysis was carried out using Prism 8 (GraphPad Software, Inc.), and P<0.05 was considered to indicate a statistically significant difference.

## Results

**Dex inhibits the osteogenic function of BMSCs.** A number of studies have reported that glucocorticoid treatment can suppress the osteogenic function of osteoblasts, and lead to osteoporosis and osteonecrosis (13,32-34). To confirm this hypothesis, BMSCs were treated with Dex (dose range, 10<sup>-9</sup>-10<sup>-6</sup> M). The mRNA expression levels of the osteogenic markers ALP, Runx2 and OCN were decreased with increasing concentrations of Dex (Fig. 1A). Moreover, ALP and Runx2 protein expression was also reduced with an increasing concentration gradient of Dex (Fig. 1B). ALP activity can reflect the osteogenic function of osteoblasts, and a decrease in ALP activity was observed with an increasing concentration

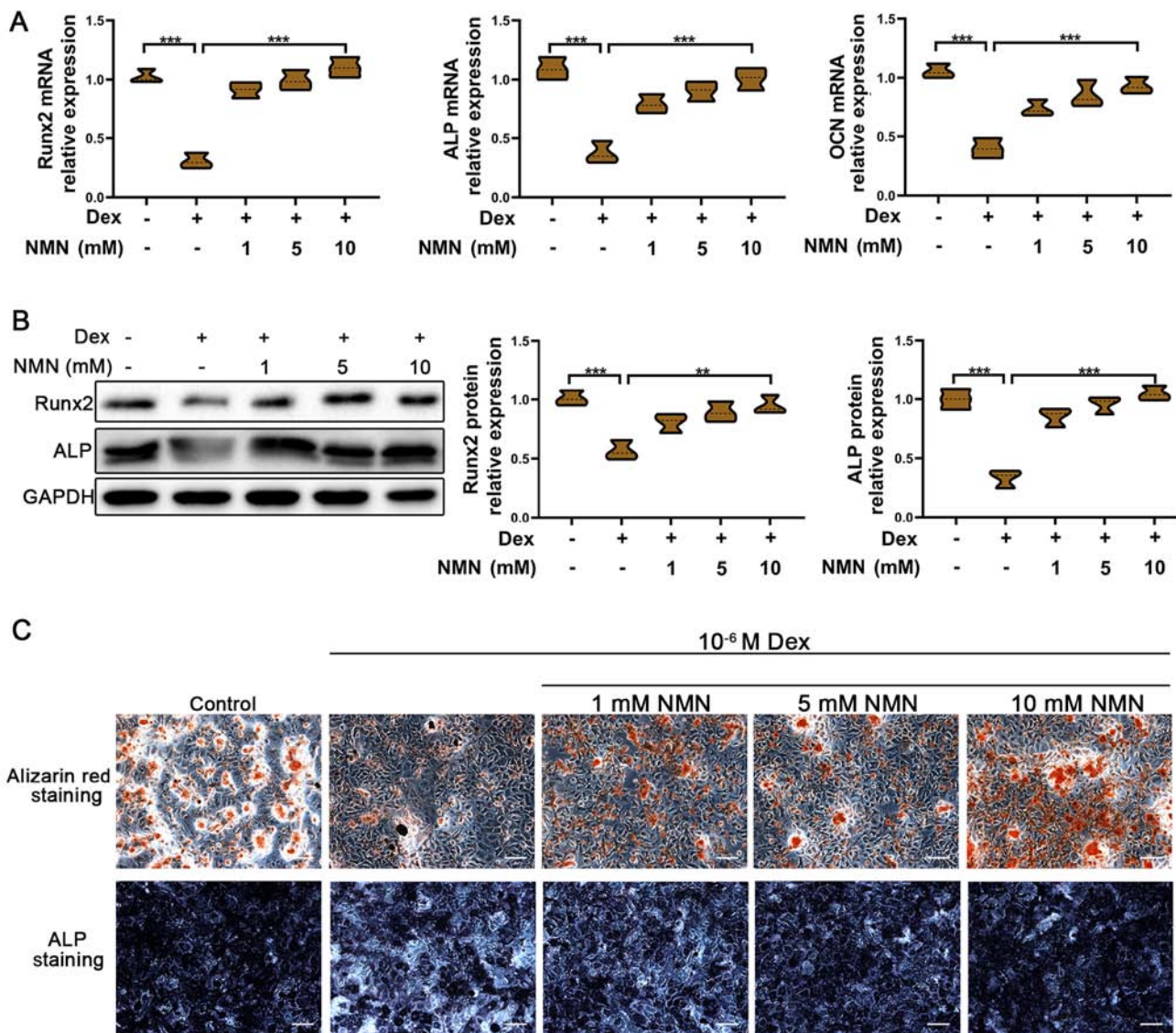


Figure 2. Role of NMN in Dex-induced osteogenic inhibition of BMSCs. BMSCs were exposed to  $10^{-5}$  M Dex and NMN (1, 5 and 10 mM). (A) Reverse transcription-quantitative PCR was used to detect Runx2, ALP and OCN mRNA expression in BMSCs. (B) Western blotting was used to determine the Runx2 and ALP protein expression levels in BMSCs. (C) Alizarin red and ALP staining assays were used to assess the osteogenic function of BMSCs treated with  $10^{-5}$  M Dex for 7 days. Scale bar=20  $\mu$ m. All experiments were performed  $\geq 3$  times.  $P < 0.05$  was considered to indicate a statistically significant difference. \*\*\* $P < 0.001$  and \*\* $P < 0.01$ , as indicated. NMN, nicotinamide mononucleotide; Dex, dexamethasone; BMSC, bone mesenchymal stem cells; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; OCN, osteocalcin.

gradient of Dex (Fig. 1C). Dex also decreased the viability of BMSCs (Fig. 1D). A decrease in ALP activity and cell viability resulted in a subsequent decrease in the osteogenic function of osteoblasts. According to these results,  $10^{-6}$  M Dex was used to treat BMSCs in subsequent experiments, confirming that the osteogenic ability of BMSCs was markedly inhibited by  $10^{-6}$  M Dex (Fig. 1E and F).

*NMN attenuates Dex-induced osteogenic inhibition of BMSCs.* A recent study demonstrated that NMN could improve osteogenesis and reduce the adipogenesis of BMSCs in aging bone marrow (16). However, the role of NMN in the glucocorticoid-induced osteogenic inhibition of BMSCs remains unknown. To investigate whether NMN could promote the osteogenic ability of glucocorticoid-treated BMSCs, BMSCs were cultured in medium containing Dex

and NMN. The mRNA expression levels of the osteogenic markers Runx2, ALP and OCN were reduced following Dex treatment; however, this was attenuated by NMN (Fig. 2A). The western blotting results also confirmed that NMN could enhance the osteogenic ability of BMSCs exposed to Dex, as ALP and Runx2 protein expression was increased compared with Dex-only cells at 1, 5 and 10 mM NMN (Fig. 2B). The mineralization ability and ALP activity of BMSCs were also enhanced by NMN treatment (Fig. 2C). These results suggest that NMN promotes the osteogenic ability of glucocorticoid-treated BMSCs.

*NMN promotes the osteogenic ability of glucocorticoid-repressed osteoblasts via the SIRT1/PGC-1 $\alpha$  signaling pathway.* SIRT1 is considered to be an important regulator of cellular metabolism. Numerous studies have suggested that



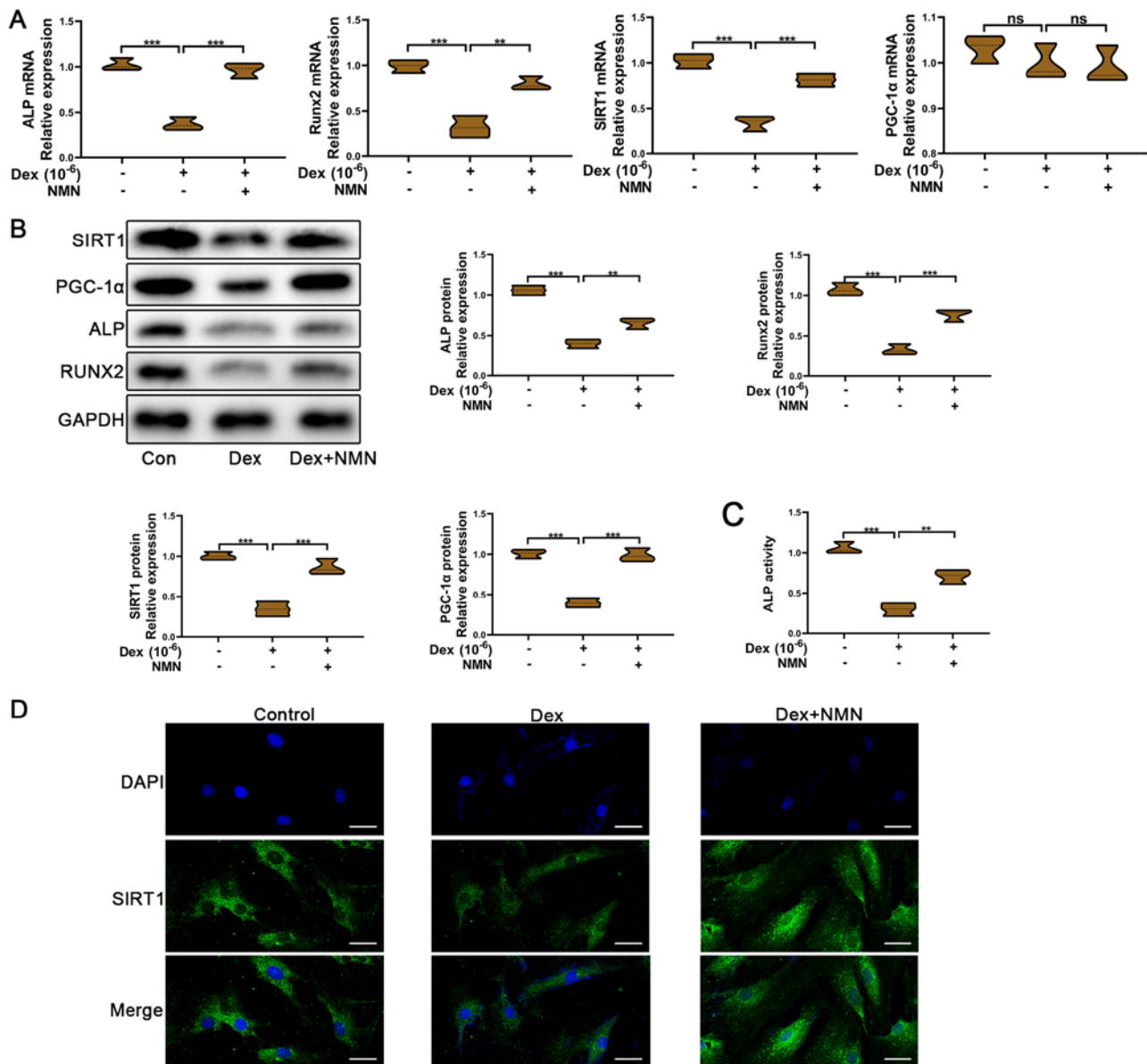


Figure 3. SIRT1/PGC-1 $\alpha$  signaling is involved in the protective effects of NMN on Dex-induced osteogenic inhibition. BMSCs were treated with control, Dex and Dex + NMN for 7 days. (A) Reverse transcription-quantitative PCR was used to detect the SIRT1, PGC-1 $\alpha$ , Runx2 and ALP mRNA expression levels in BMSCs. (B) Western blotting was used to determine the SIRT1, PGC-1 $\alpha$ , Runx2 and ALP protein expression levels in BMSCs. (C) Relative ALP activity. (D) Immunofluorescence was used to detect SIRT1 protein expression in BMSCs following 2 days of treatment. Scale bar, 5  $\mu$ m. All experiments were performed  $\geq 3$  times. \*\*\* $P < 0.001$  and \*\* $P < 0.01$ , as indicated. SIRT1, sirtuin 1; PGC, peroxisome proliferator-activated receptor gamma coactivator; NMN, nicotinamide mononucleotide; Dex, dexamethasone; BMSC, bone mesenchymal stem cells; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2.

NMN protects cells from stress stimuli, such as oxidative stress, aging and toxicity stress, by regulating SIRT1 (35). To determine whether NMN represses glucocorticoid-induced osteogenic inhibition by regulating SIRT1 expression, the mRNA and protein expression of SIRT1 were detected in BMSCs following Dex administration. SIRT1 mRNA expression was decreased after Dex treatment; however, NMN treatment restored these expression levels (Fig. 3A). PGC-1 $\alpha$ , which is downstream of SIRT1, is an important regulator that is involved in the development of various diseases (36). In the present study, the PGC-1 $\alpha$  mRNA expression level was unchanged by Dex treatment (Fig. 3A); the protein expression of PGC-1 $\alpha$  was decreased following treatment with Dex but increased by Dex + NMN (Fig. 3B). This trend was also

observed for SIRT1 protein expression, which was increased by Dex + NMN treatment compared with the Dex-only experiment. Immunofluorescence assays and western blotting were performed to further confirm these changes in SIRT1 protein expression (Fig. 3D). SIRT1 is expressed in the cytoplasm, but not in the nucleus, and was decreased in BMSCs treated with Dex, while NMN treatment promoted SIRT1 protein expression (Fig. S1A and B). These results show that NMN may promote the osteogenic ability of Dex-treated BMSCs by inducing SIRT1 mRNA and protein expression, as well as regulating the protein, but not the mRNA expression, of PGC-1 $\alpha$ .

*SIRT1 knockdown reverses the protective effect of NMN on glucocorticoid-induced osteogenic inhibition in BMSCs.* To

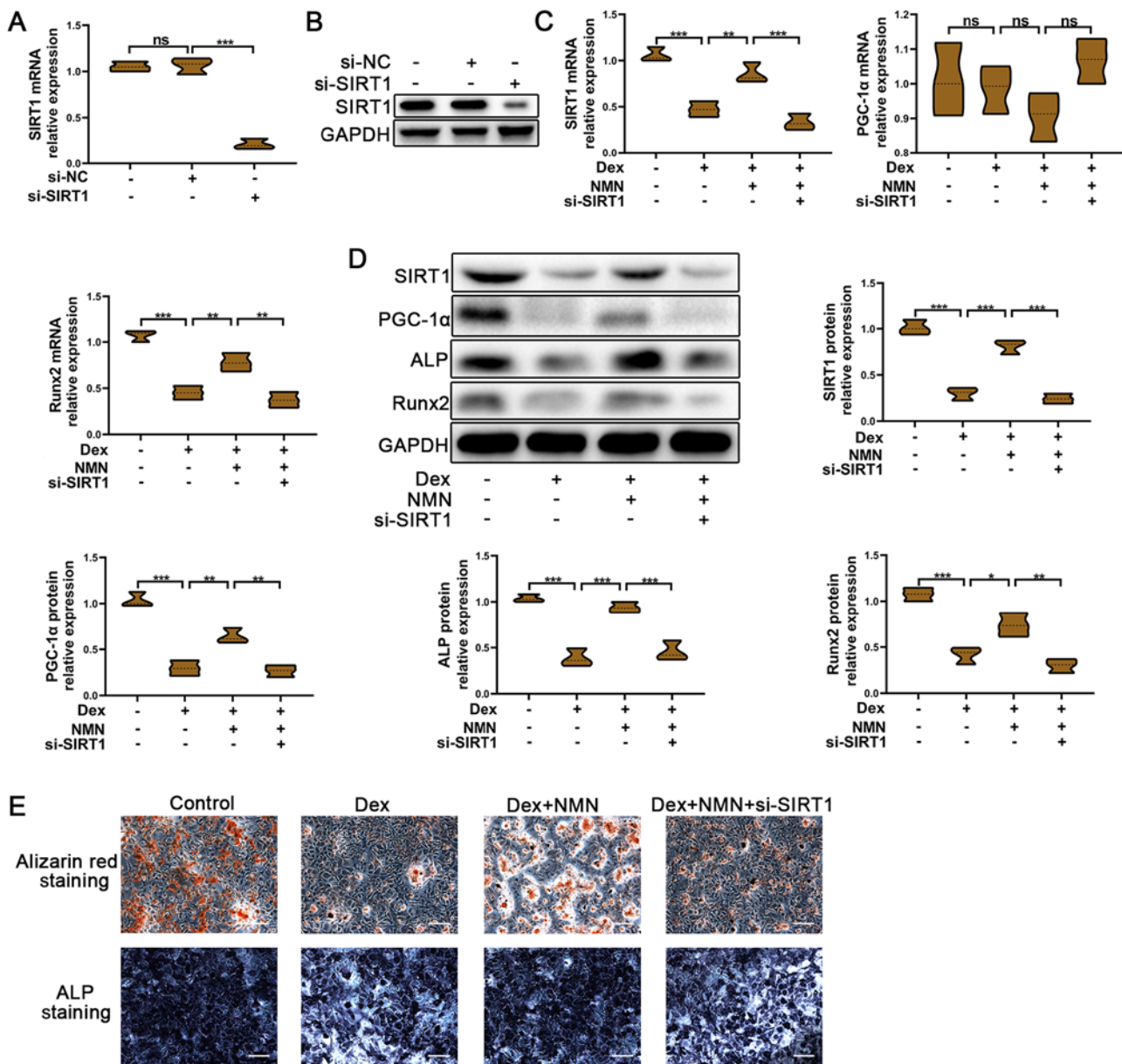


Figure 4. SIRT1 knockdown reduces the protective effects of NMN on Dex-induced osteogenic inhibition. Si-NC or si-SIRT1 were transfected into BMSCs before Dex and NMN treatment. SIRT1-knockdown was confirmed by (A) RT-qPCR and (B) western blotting. (C) RT-qPCR was used to detect the SIRT1, PGC-1 $\alpha$  and Runx2 mRNA expression levels in BMSCs. (D) Western blotting assays were used to determine the SIRT1, PGC-1 $\alpha$ , ALP and Runx2 protein expression levels in BMSCs. (E) Alizarin red and ALP staining assays were used to assess the osteogenic ability of BMSCs. Scale bar, 20  $\mu$ m. All experiments were performed  $\geq 3$  times. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$  and  $^{\#}P \geq 0.05$ , as indicated. Si, small interfering; NC, negative control; NMN, nicotinamide mononucleotide; Dex, dexamethasone; BMSC, bone mesenchymal stem cells; SIRT1, sirtuin 1; PGC, peroxisome proliferator-activated receptor gamma coactivator; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; RT-qPCR, reverse transcription-quantitative PCR.

further investigate whether NMN enhanced the osteogenic ability of Dex-treated BMSCs, siRNA was used to inhibit SIRT1 expression. Firstly, the silencing effects of si-SIRT1 were determined by RT-qPCR. SIRT1 mRNA expression was decreased in BMSCs transfected with si-SIRT1, compared with si-NC (Fig. 4A). Secondly, western blotting assays were used to elucidate the effects of si-SIRT1 on protein expression. Following knockdown, SIRT1 protein expression was decreased compared with cells treated with si-NC (Fig. 4B). Finally, SIRT1 mRNA expression was decreased in Dex + NMN + si-SIRT1 cells (Fig. 4C). The PGC-1 $\alpha$  mRNA expression level was not altered by Dex + NMN + si-SIRT1. Importantly, mRNA expression of Runx2 was also reduced by Dex + NMN + si-SIRT1.

Furthermore, the protein expression levels of SIRT1, PGC-1 $\alpha$  and Runx2 were promoted by Dex + NMN, and reduced in the Dex + NMN + si-SIRT1 treatment group (Fig. 2D). ALP activity and the mineralization ability of BMSCs were enhanced in the Dex + NMN, but inhibited in the Dex + NMN + si-SIRT1 group. These results further support that NMN alleviates the glucocorticoid-induced osteogenic inhibition of osteoblasts by regulating SIRT1/PGC-1 $\alpha$  signaling.

## Discussion

BMSCs possess the potential to differentiate into osteoblasts, a process that can be suppressed by glucocorticoid use (32,37,38).

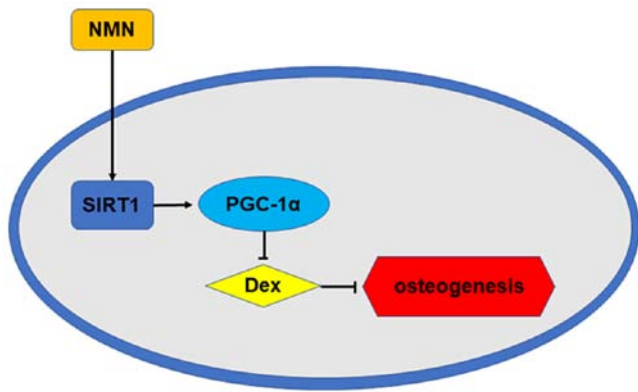


Figure 5. NMN alleviates Dex-induced osteogenesis by regulating the SIRT1/PGC-1 $\alpha$  signaling pathway. NMN, nicotinamide mononucleotide; Dex, dexamethasone; SIRT1, sirtuin 1; PGC, peroxisome proliferator-activated receptor gamma coactivator.

Inhibiting osteogenesis increases the risk of osteoporosis and osteonecrosis, resulting in bone fracture (39). Due to glucocorticoid-induced osteogenic inhibition of BMSCs, long-term and high-dose administration of glucocorticoids can lead to serious side effects, such as osteoporosis (40). In the present study, a potential therapeutic method for glucocorticoid-induced osteogenic inhibition was investigated.

The present study suggested NMN as a potential therapeutic target for Dex-induced inhibition of osteogenesis, and that SIRT1 was an important downstream target of NMN. The expression of BMSC osteogenic markers was decreased following exposure to Dex (range,  $10^{-9}$ - $10^{-6}$  M); these included ALP, Runx2 and OCN. ALP staining and alizarin red staining also confirmed above results. These results suggest that Dex, as a glucocorticoid, can inhibit the differentiation and osteogenesis of BMSCs.

Various studies have demonstrated that the administration of NMN significantly increases the intracellular levels of NAD<sup>+</sup>. Much evidence has also confirmed that intracellular NAD<sup>+</sup> is closely associated with bone diseases. Li *et al* (41) demonstrated that intracellular NAD<sup>+</sup> levels were enhanced during osteogenic differentiation. NAD<sup>+</sup> is also involved in the maintenance of osteoblast differentiation, and an increase in the intracellular levels of NAD<sup>+</sup> is a necessary event for the development of senile osteoporosis. Liang *et al* (42) reported that NMN attenuates aluminum-induced bone loss. However, the protective effects of NAD<sup>+</sup> depletion on glucocorticoid-induced osteogenic inhibition have yet to be elucidated. In the present study, NMN was found to alleviate Dex-induced osteogenic inhibition. NMN treatment was able to promote osteogenic marker expression in BMSCs pre-treated with Dex. Furthermore, the mineralization ability and ALP activity of Dex-treated BMSCs was enhanced by NMN. These results suggest that NMN protects against Dex-induced osteogenic impairment, although the exact mechanism requires further investigation.

A previous study found that in aged bone marrow, NMN improved osteogenesis and reduced adipogenesis by regulating MSCs via the SIRT1 pathway (17). NMN, a key NAD<sup>+</sup> intermediate, can stimulate BMSC differentiation and osteogenesis (17). SIRT1 is an NAD<sup>+</sup>-dependent

deacetylase, which regulates metabolism in a variety of cell types (21,22). Qu *et al* (43) revealed that SIRT1 was involved in osteogenic proliferation and differentiation by regulating miR-132-3p. Furthermore, Wang *et al* (44) demonstrated that SIRT1 promotes osteogenic differentiation and increases alveolar bone mass via B cell-specific Moloney murine leukemia virus integration site 1. In the present study, the expression of SIRT1 and its downstream target PGC-1 $\alpha$  was discovered to be decreased in osteoblasts exposed to Dex. Therefore, it was speculated that the SIRT1/PGC-1 $\alpha$  signaling pathway played an important role in the protective effects of NMN in Dex-treated BMSCs. In the present study, NMN treatment was found to increase the mRNA and protein expression levels of SIRT1. The protein expression of PGC-1 $\alpha$  was also enhanced by NMN treatment, whereas the mRNA levels remained unchanged. Thus, it was hypothesized that SIRT1 regulates the expression of PGC-1 $\alpha$  protein by deacetylation, while leaving mRNA expression unaltered. Meanwhile, the results of immunofluorescence also indicated that Dex treatment decreased protein expression of SIRT1 in the cytoplasm, while NMN promoted the SIRT1 expression in BMSCs treated with Dex.

To further confirm the role of SIRT1 in this process, si-SIRT1 was used to knock down SIRT1, which inhibited the protective effect of NMN in glucocorticoid-induced osteogenic inhibition. Knockdown of SIRT1 was found to reduce the expression of the osteogenic markers that were increased with NMN treatment. Alizarin red and ALP staining also confirmed the importance of SIRT1 in the protective effect of NMN in Dex-treated BMSCs. Importantly, SIRT1 knockdown was able to reduce the protein expression of PGC-1 $\alpha$  improved by NMN treatment in BMSCs exposed to Dex. Together, these results suggest that NMN attenuates Dex-induced osteogenic inhibition by regulating the SIRT1/PGC-1 $\alpha$  signaling pathway, and that SIRT1 regulates this process through improving the protein expression of PGC-1 $\alpha$  rather than PGC-1 $\alpha$  mRNA (Fig. 5).

Song *et al* (16) reported that NMN could promote osteogenesis in aged bone marrow. However, the effect of NMN in glucocorticoid-induced osteoporosis was unknown. Our work confirmed the effect of NMN in glucocorticoid-induced osteogenic inhibition. Together, these two studies suggest a therapeutic role of NMN in osteoporosis caused by age and glucocorticoids. However, as the present study was performed *in vitro*, future *in vivo* studies will be required to validate the findings.

In conclusion, the results of the present study show that Dex is capable of inhibiting the differentiation and mineralization of BMSCs. Moreover, NMN can alleviate Dex-induced osteogenic inhibition by regulating SIRT1/PGC-1 $\alpha$  expression. These findings provide a novel mechanism to improve the understanding of glucocorticoid-induced osteogenic inhibition, and indicate that NMN may be a potential therapeutic target.

#### Acknowledgements

The authors would like to thank Professor Yan Jiang (The Second Affiliated Hospital of Nanchang University) for



provided suggestions regarding experimental design and article writing.

### Funding

No funding was received.

### Availability of data and materials

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

### Authors' contributions

RH performed the experiments and collected the data. JT designed the study, analyzed the data and drafted the manuscript. 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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