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Inhibitory Effect of Sirtuin6 (SIRT6) on Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABD Fei Xiao
CEF Yun Zhou
CDE Yongfu Liu
FG Mian Xie
AEF Guancheng Guo

Department of Emergency Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, P.R. China

Corresponding Author: Guancheng Guo, e-mail: guanchengguoo@163.com
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Background: The imbalance between bone resorption and formation is the basic mechanism underlying osteoporosis in the elderly. Osteogenesis is the differentiation of human mesenchymal stem cells (hMSCs) into osteoblasts. Sirtuin6 (SIRT6) regulates various biological functions, including differentiation. Transient receptor potential cation channel subfamily V member 1 (TRPV1) is a non-selective cation channel that can be activated by physical and chemical stimulation. However, experimental data supporting the role of SIRT6 in osteogenic differentiation (OD) of hMSCs are lacking.

Material/Methods: Differentiation of hMSCs was induced. The expressions of SIRT6, TRPV1, and CGRP were detected by Q-PCR, Western blot, and ELISA, respectively. SIRT6 was overexpressed in hMSCs by transfection. ALP activity and Alizarin Red staining were utilized to detect the effect of SIRT6 on hMSC OD. Then, capsaicin and capsazepine, the TRPV1 agonist and antagonist, respectively, were administrated to assess the role of TRPV1.

Results: SIRT6 expression was downregulated during hMSC differentiation. SIRT6 overexpression was accompanied by reduced expression of specific genes and alkaline phosphatase (ALP) activity in osteoblasts. Furthermore, TRPV1 channel was also reduced by SIRT6 overexpression via ubiquitinating TRPV1. Capsaicin was utilized in SIRT6-overexpressed cells. Capsaicin therapy counteracted the effect of SIRT6 overexpression on OD, and markedly decreased OD.

Conclusion: The SIRT6-TRPV1-CGRP signal axis is the key to regulating OD in hMSCs, which could be a potential therapeutic target for osteoporosis and bone loss-related diseases.

MeSH Keywords: Osteosarcoma • Sirtuin 1 • Sirtuins

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Background

Osteoporosis is associated with deteriorating bone structure and low bone mass, leading to increased bone vulnerability and fracture susceptibility [1]. This disease can be divided into postmenopausal (primary type 1), senile (primary type 2), and steroid- or glucocorticoid-induced (secondary type). Osteoporosis is caused by an imbalance between bone resorption and formation, mediated by osteoblasts in the bone marrow microenvironment [2,3]. Osteogenesis is the differentiation of mesenchymal stem cells (MSCs) into osteoblasts [4,5]. Generation of different phenotypes in human MSCs (hMSCs) is regulated by various signaling pathways and growth factors related to precursors in fat, marrow, and specific other tissues [6].

Previous studies have focused on the increase of bone resorption induced by osteoclast, but few studies have assessed bone formation induced by osteoblast cells. MSCs are the source of osteoblasts in bone tissue, and their number and function directly affect the number and function of osteoblasts [7]. Studies found that the proliferation of MSCs in osteoporosis was reduced, and the ability of ALP and calcified nodule induced *in vitro* was decreased [8]. Another osteoporosis study showed that estrogen deficiency decreased the capacity of osteogenic differentiation of MSCs through repressing proliferation and inducing apoptosis [9]. Recent studies have shown that osteoporosis is caused by abnormal differentiation of MSCs, which can lead to decreased numbers of bone marrow cells and increased numbers of bone marrow adipocytes [9]. MSCs also exert a crucial role in bone remodeling, especially in maintaining the balance between bone formation and resorption. However, the specific biological regulatory mechanism of abnormal differentiation of BMSCs is not fully documented.

Sirtuins (SIRTs) are essential for human physiology and disease pathogenesis [10]. SIRT6 is a member of the SIRTs and mainly exists in the nucleus [11]. Increasingly evidence indicates that SIRTs play an important role in various differentiation processes by promoting or inhibiting many signaling pathways [12,13]. SIRT6 is expressed in both bone marrow stroma cells and bone-related cells in mouse and human models. SIRT6-KO mice exhibit a significant decrease in body weight and remarkable dwarfism, while their skeleton is deficient in cartilage and mineralized bone tissue, suggesting that SIRT6 is an important regulator of bone metabolism [14]. SIRT6 expression was upregulated in cyclic strain in differentiation of vascular smooth muscle cells (VSMCs) [15]. However, the mechanism of SIRT6 on bone marrow MSCs during OD has not been fully investigated.

TRPV1 is mainly expressed in the nerve endings of pain receptors detecting external stimuli [16]. TRPV1 channel is an inflammation-mediated molecular sensor of allergic reactions [17]. Reportedly, TRPV4 was reported to be involved in early OD of hMSCs induced by flow shear stress (FSS). Early OD was activated by FSS, as confirmed by osterix (Osx) of early OD markers and staining of alkaline phosphatase (ALP) [18]. A previous study also demonstrated that TRPV1 is expressed in bone tissues, and the antagonists to TRPV1 downregulated the expression of osteoblast and osteoclast regulators in tail-suspended mice [19]. TRPV1 activation induced production of calcitonin gene-related peptide (CGRP), which is a neuropeptide widely distributed in the peripheral and central nervous systems [20]. It has been previously reported that the local treatment of capsaicin, a TRPV1 agonist, triggered the release of CGRP from sensory nerve endings [21], and that capsazepine, a TRPV1 antagonist, reversed this effect [22]. Previous studies also indicated that the local release of CGRP from sensory nerve endings can modulate vasodilation [23], inflammatory responses [24], and osteogenesis [25]. These findings suggest that nerve fibers, via the release of CGRP, can regulate bone remodeling [26]. However, the role of TRPV1-CGRP signaling in OD of hMSCs remains unclear.

In the present study, the expressions of SIRT6 and TRPV1 in hMSCs during OD were assessed, and the effect of hMSCs on osteogenic differentiation was studied. We found that the expression of SIRT6 in hMSCs during OD was decreased, while TRPV1 was increased. In hMSCs during OD, TRPV1 was ubiquitinated in the presence of SIRT6. These results further elucidate the pathogenesis of osteoporosis and provide a new therapeutic target for clinical treatment.

Material and Methods

Cell culture and differentiation

Telomerase-immortalized hMSCs (obtained from primary hMSCs [27]) were incubated in DMEM medium (Thermo Fisher, cat. no. 41965-120). Induction of differentiation was carried out in accordance with previously described methods [28,29]. Osteoblasts were identified via ALP activity following incubation [30]. Matrix mineralization was assessed using Alizarin Red (AR) staining at day 14 after incubation. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Transfection

The overexpression vector, pcDNA3-SIRT6, was prepared, and cell transfection was carried out using vector and Lipofectamine 2000 (Invitrogen, cat. no. 11668-030).

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Western blotting (WB)

Radio-immunoprecipitation buffer was used to prepare hMSC lysates. Proteins were separated via 10% SDS-PAGE, and transferred into polyvinylidene fluoride membranes. Next, 5% BSA was diluted with PBST buffer and used to block non-binding sites on the membranes for 1 h. Proteins were detected via primary antibody probes at 4°C overnight, and then incubated with secondary antibodies. Primary antibodies included: Anti-SIRT6 antibody (Abcam, ab88494, 1: 1000), Anti-TRPV1 antibody (Thermo Fisher, PA1-748, 1: 1000), Anti-Ubiquitin antibody (Abcam, ab7780, 1: 3000), and Anti-GAPDH antibody (Abcam, ab9485, 1: 5000). Protein bands were stained and a C-DiGit blot scanner was used to measure corresponding gray values. The WB analysis for each protein were repeated at least 3 times independently.

Extraction of RNA and quantitation PCR (qRT-PCR)

Total RNA was extracted according to standard procedures, and the internal reference was GAPDH. A SYBR-Green kit (Thermo Fisher, cat. no. 4385612) was used for qRT-PCR in a 20-µl system. Quantitative analysis was based on the $2^{-\Delta\Delta CT}$ method, normalized to GAPDH. The primer sequences were as follows:
OCN F: 5'-CCG GGA GCA GTG TGA GCT TA-3',
R: 5'-AGG CGG TCT TCA AGC CAT ACT-3';
OPN F: 5'-TAC GAC CAT GAG ATT GGC AGT GA-3',
R: 5'-TAT AGG ATC TGG GTG CAG GCT GTA A-3';
BSP F: 5'-GTC CAG GGA GGC AGT GAC-3',
R: 5'-GAG AGT GTG GAA AGT GTG GAG-3';
RUNX2 F: 5'-CAT TTG CAC TGG GTC ACA CGT A-3,
R: 5'-GAA TCT GGC CAT GTT TGT GCT C-3';
SIRT6 F: 5'-ACG CGG ATA AGG GCA AGT-3',
R: 5'-CTC CCA CAC CTT GCG TTC-3';
GAPDH F: 5'-TGC TGT CCC TGT ATG CCT CT-3',
R: 5'-TGC TGT CCC TGT ATG CCT CT-3'.
The qRT-PCR analysis for each gene were repeated at least 3 times independently.

ELISA

Released CGRP levels were detected using an ELISA kit (LifeSpan BioSciences, cat. no. LS-F12515-1), and absorbance at 543 nm was detected via a microplate reader. The ELISA for CGRP examination was repeated at least 3 times independently.

Immunoprecipitation

Cells were rinsed in PBS and lysed with RIPA buffer containing protease inhibitor. Lysates were centrifuged at 4°C for 15 min at 12 000 g, and supernatants were collected. Protein concentration was quantified and normalized using Bradford assay. Samples were immunoprecipitated with anti-TRPV1 antibodies (2 µl/ml) and protein G-Sepharose beads (1:1 ratio) overnight at 4°C. Samples were washed 5 times, stored at –20°C, and separated by SDS-PAGE and Western blot analysis.

Statistical analysis

All *in vitro* experiments were repeated at least 3 times independently. All values are expressed as mean ±SD. One-way ANOVA and 2-tailed *t* test were used for comparative analyses. Statistical significance was set at $P<0.05$.

Results

Reduction of protein and mRNA expression levels of SIRT6 in OD of hMSCs

The OD of hMSCs occurred during incubation in osteoblast-inducing medium. Genes involved in OD, such as *OPN, OCN, RUNX2, ALP,* and *BSP*, were upregulated (Figure 1A). The increase in ALP activity, compared with the non-induced group, was confirmed to be a typical osteoblast phenotype (Figure 1B, 1C).

Moreover, Q-PCR data showed that SIRT6 expression was downregulated on day 14 following induction, and that OD of hMSCs was induced (Figure 2A). In addition, Western blot analysis data further confirmed that SIRT6 protein levels were decreased following induction of OD (Figure 2B).

SIRT6 overexpression inhibits OD of hMSCs

The hMSCs were transfected with the pcDNA3-SIRT6 or pcDNA3-empty (NC), resulting in upregulation of SIRT6 expression levels. The effect of SIRT6 on OD of hMSCs was evaluated. SIRT6 expression in each group was detected via Q-PCR and Western blot analysis (Figure 3A, 3B). Significant upregulation of ALP, BSP, OCN, OPN, and RUNX2 expression was noted in cells induced via NC vector transfection, while their expression in the overexpression group was decreased (Figure 3C). ALP activity was reduced by pcDNA3-SIRT6 transfection (Figure 3D). SIRT6 overexpression decreased matrix mineralization (Figure 3E). These data suggest that SIRT6 inhibits OD of hMSCs.

Downregulation of TRPV1 expression by SIRT6 overexpression

Capsaicin, which is a TRPV1 agonist, activates sensory nerve endings to release CGRP [31]. Our results indicated that while TRPV1 channel protein expression was remarkably increased during OD, SIRT6 overexpression downregulated TRPV1 levels (Figure 4A). We also found that SIRT6 overexpression upregulated TRPV1 ubiquitination (Figure 4B), possibly leading

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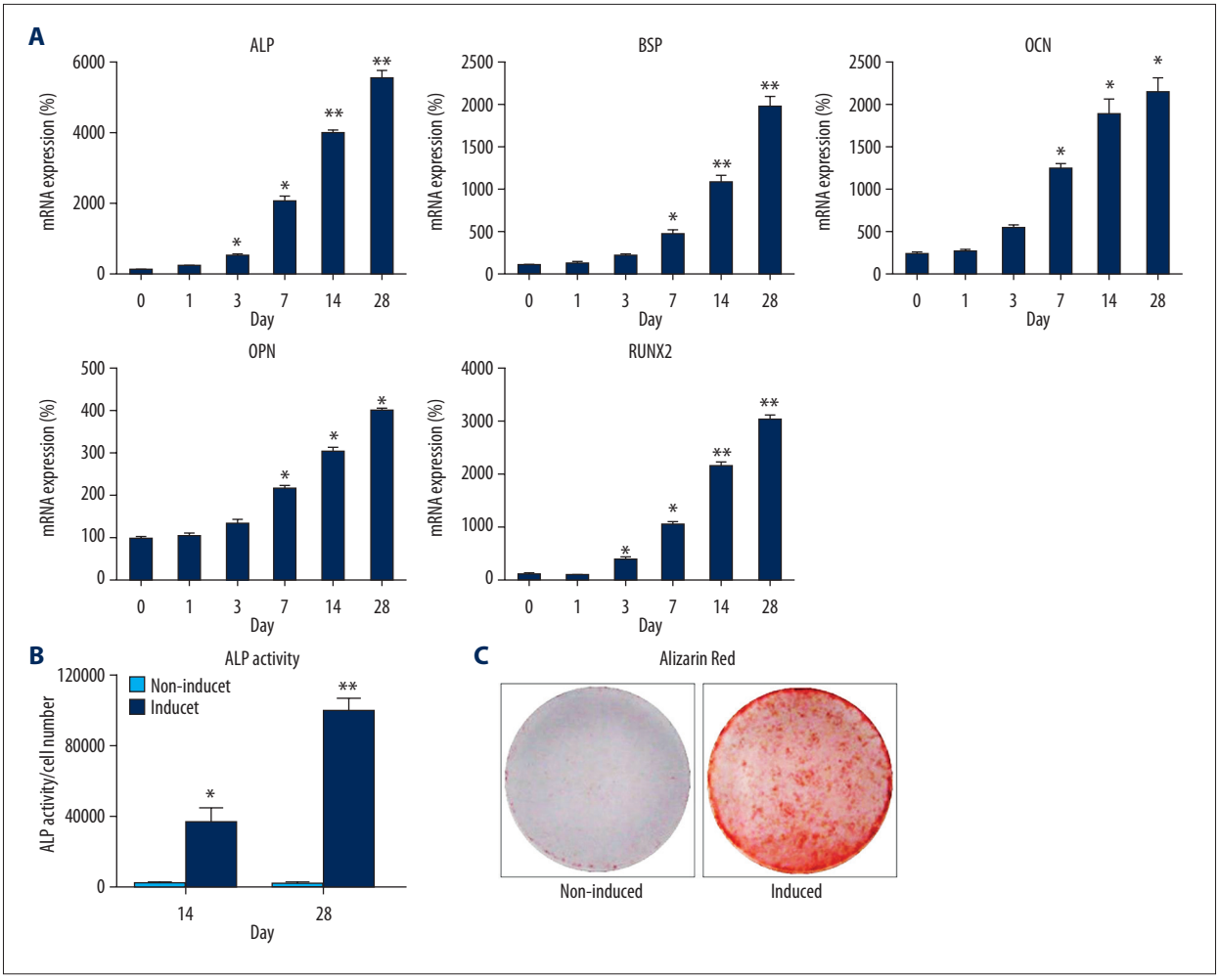

Figure 1. OD of hMSCs. Occurrence of OD in hMSCs. (**A**) qPCR of *OPN*, *OCN*, *BSP*, *RUNX2*, and *ALP* normalized to *GAPDH* at different time points following induction. (**B**) Activity of ALP in OD. (**C**) Results of AR staining on day 14 following induction. N=3. The 2-tailed *t* test was used for comparative analyses of the data in (**A**), and one-way ANOVA was used for comparative analyses of the data in (**B**). * P<0.05, ** P<0.01 *vs.* non-induced groups.

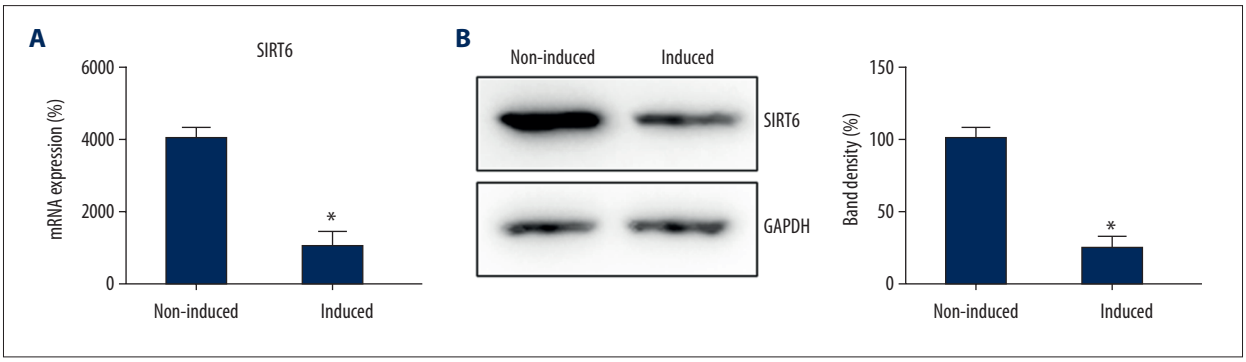

Figure 2. Expression of SIRT6 during OD of hMSC. (**A**) qPCR and (**B**) Western blot analysis were used to detect SIRT6 expression in OD. N=3. The 2-tailed *t* test was used for comparative analyses of the data in (**A**). * P<0.05 *vs.* indicated groups.

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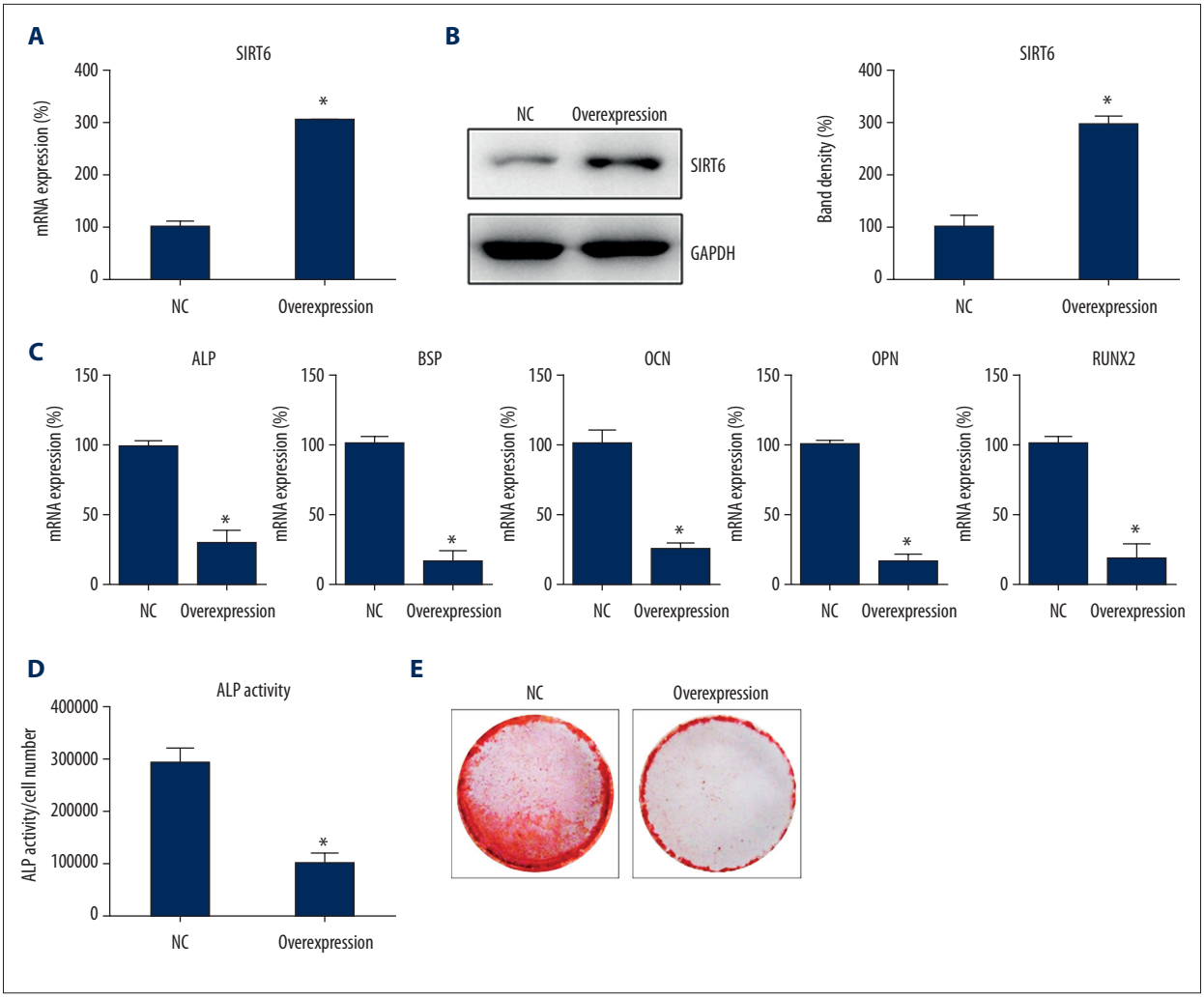

Figure 3. Inhibition of SIRT6 overexpression in OD of hMSCs. The hMSCs were transfected with SIRT6 expression vector and induced via OD. The results of (**A**) qPCR and (**B**) Western blot analysis of SIRT6 levels on day 14 are shown. (**C**) On the 14th day after induction, the expressions of *OPN*, *OCN*, *BSP*, *RUNX2*, and *ALP* in each group were detected by qPCR. (**D**) ALP activity in each group on day 14 after induction. (**E**) Results of AR staining on day 14 following induction. N=3. A 2-tailed *t* test was used for comparative analyses of the data in (**A, C, D**) * P<0.05 *vs.* NC groups.

to TRPV1 degradation. We also examined release of CGRP via ELISA, showing that SIRT6 upregulation also blocked CGRP release (Figure 4C). These findings indicate that SIRT6 overexpression inhibits activity of the TRPV1 channel.

Cells were treated with 0.1 μM capsaicin (a TRPV1 channel agonist) to study the effect of the TRPV1 channel on OD of hMSCs. CGRP levels in the capsaicin group were increased, while TRPV1 and SIRT6 levels remained unchanged (Figure 5A, 5B). The levels of mRNAs related to osteoblast differentiation, such as *OPN*, *OCN*, *BSP*, *RUNX2*, and *ALP*, were detected. The expression levels of mRNAs in the capsaicin group were increased (Figure 5C). In addition, increased ALP activity and matrix mineralization were observed during the treatment of OD of hMSCs with capsaicin (Figure 5D, 5E).

Induced OD upregulated the expression TRPV1, while SIRT6 downregulated TRPV1. Activation of TRPV1 counteracted the inhibitory effect on OD by SIRT6 overexpression. Therefore, we hypothesized that TRPV1 deactivation suppresses OD. To assess the effect of TRPV1 deactivation on OD, hMSCs were treated with 5 μM capsazepine, a TRPV1 antagonist, to block TRPV1, showing that the CGRP level was significantly reduced by blocking TRPV1 (Figure 6A). The expression of 5 OD markers (ALP, BSP, OCN, OPN, and RUNX2 mRNA) and ALP activity were obviously decreased (Figure 6B), indicating that TRPV1 positively modulated the OD process.

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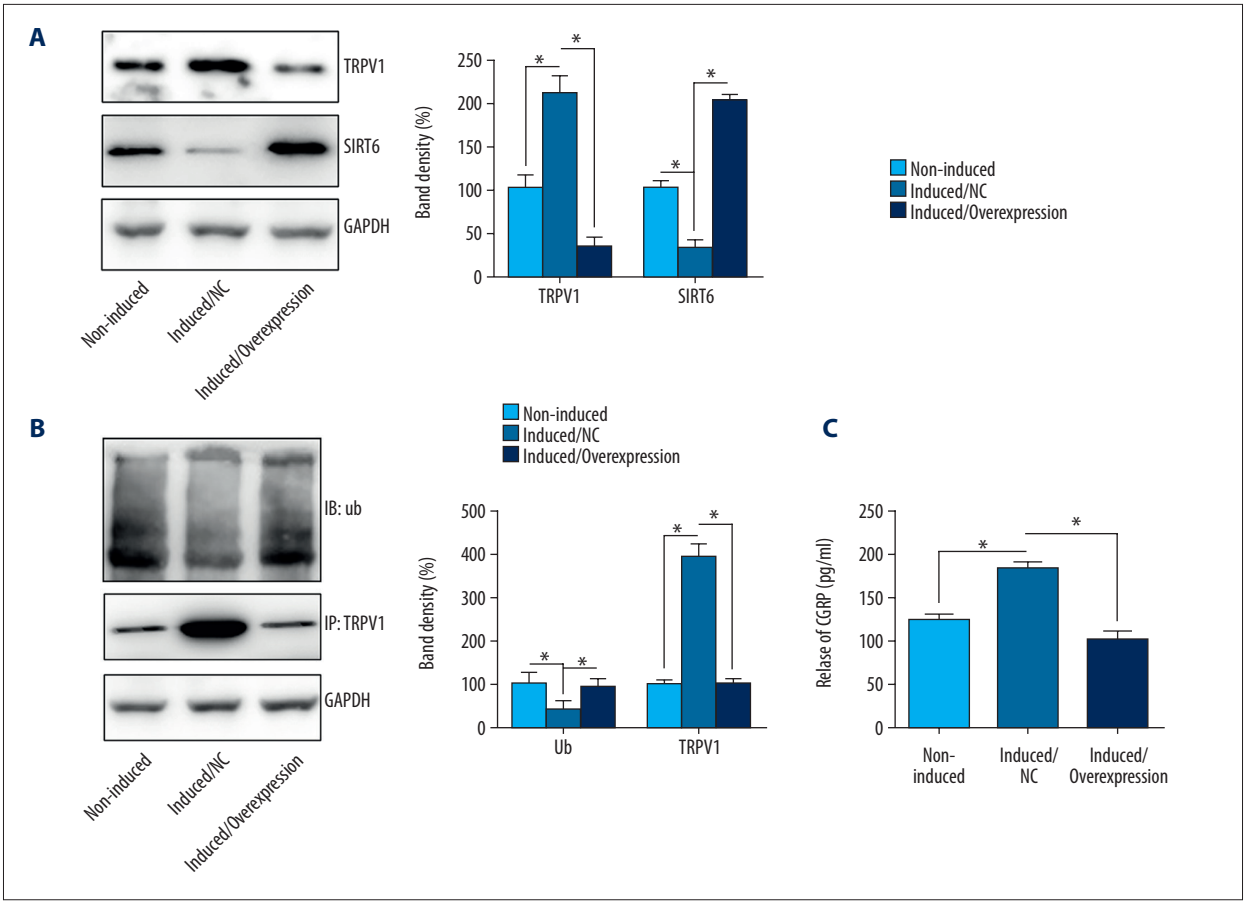

Figure 4. Downregulation of TRPV1 by SIRT6 overexpression. The hMSCs were transfected with SIRT6-expressing vector, and OD was induced. (**A**) Western blot analysis results showing TRPV1 expression during SIRT6 overexpression. (**B**) Western blot analysis results revealed the ubiquitination level of TRPV1 under SIRT6 overexpression. (**C**) ELISA was utilized to detect released CGRP. N=3. One-way ANOVA was used for comparative analyses of the data in (**C**). * P<0.05 *vs.* NC groups.

Discussion

Our results show that SIRT6 is a novel OD regulator. SIRT6 overexpression increases TRPV1 ubiquitination by targeting the TRPV1 protein. In addition, when SIRT6 was overexpressed via transfection, ALP activity and genes inhibiting OD were also upregulated. Activation of capsaicin reversed the effect of SIRT6 on TRPV1, suggesting that inactivation of the TRPV1 channel, via ubiquitination of SIRT6, partially inhibits osteoblast differentiation of hMSCs (Figure 7). These results also suggest a potential treatment for osteoporosis, and provide sufficient reasons to explore the interaction between SIRT6 and TRPV1 during the process of OD.

Previous studies have shown that the absence of SIRT6 (a chromatin-related deacetylase) in mice is a severe premature aging phenotype, including osteopenia [14]. In particular, the lack of SIRT6 in mice leads to low-turnover osteopenia caused by reduced bone formation and resorption. In terms of mechanism, SIRT6 interacts with Runx2 and Osx, which are transcriptional regulators of osteoblast formation for deacetylation of histone H3 at lysine 9 (H3K9) of their promoter. Therefore, the excessive increase of Runx2 and Osx in SIRT6 (–/–) osteoblasts inhibited osteoblast formation. In addition, the absence of SIRT6 induced hyperacetylation of H3K9 in the promoter of Dickkopf-related protein 1 (Dkk1), which is an effective negative regulator of osteoblast formation. Osteoprotegerin (OPG) is an inhibitor of osteoclast formation [32]. Knockdown of SIRT6 also promotes cell aging of hMSCs [33]. Further studies have shown that by reducing osteoclasts, SIRT6 ablation in hematopoietic cells, including osteoclast precursors, led to an increase of bone volume. Overexpression of SIRT6 led to an increase of osteoclast formation, and osteoclast precursor cells with SIRT6 absence could not effectively differentiate [34]. SIRT6 regulates bone morphogenetic protein (BMP) signal and P300/CBP-associated factor (PCAF) to regulate osteogenic differentiation *in vitro*, which was not related to deacetylase activity [35]. In summary, SIRT6 play an unclear regulatory role in bone formation and loss. In our study, it was found that SIRT6 expression was downregulated in the

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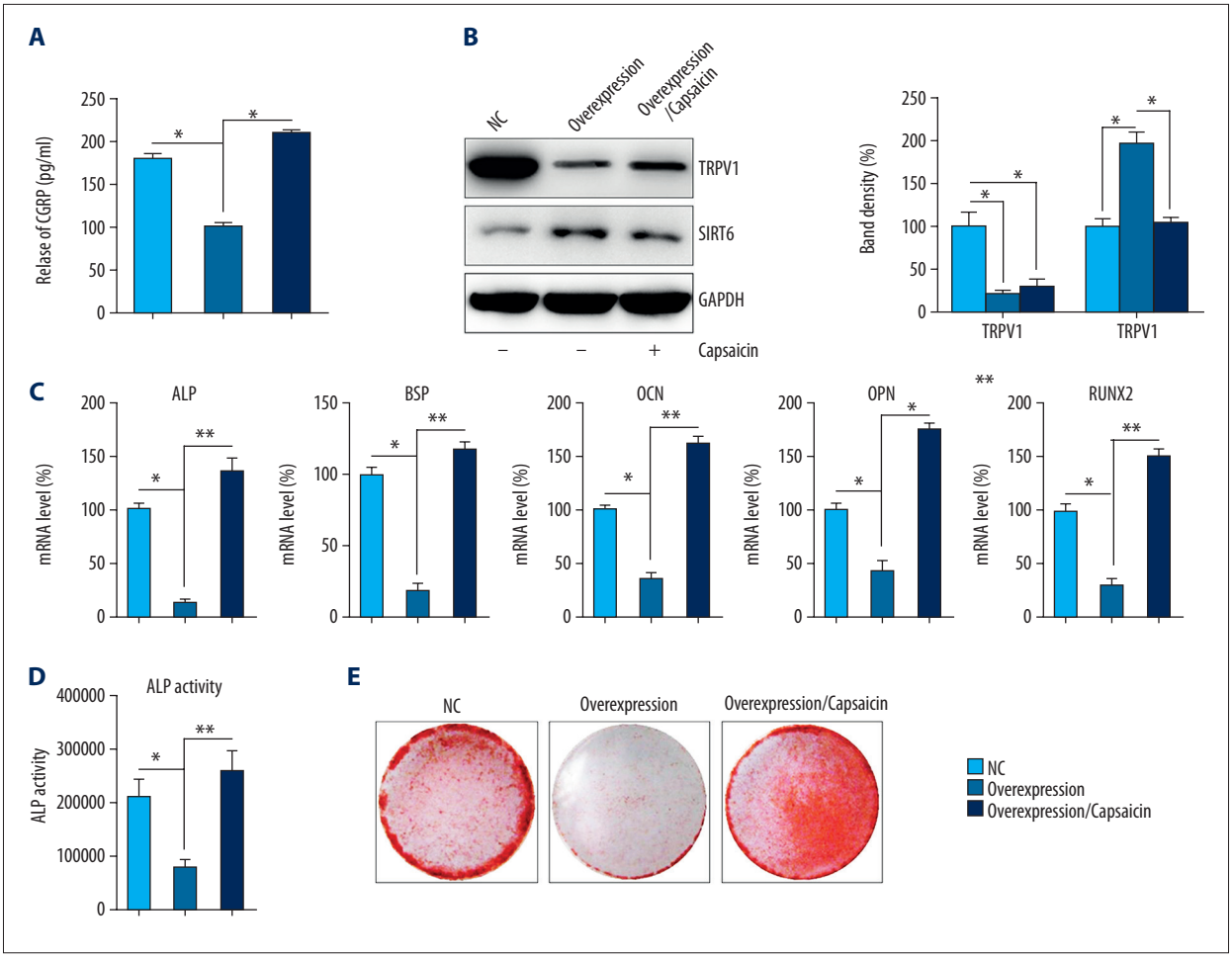

Figure 5. Evaluation of the effect of capsaicin on SIRT6-mediated OD. The hMSCs were transfected with SIRT6 overexpression vector, and treated with 10 μM capsaicin for 12 h. (**A**) ELISA was utilized to detect released CGRP. (**B**) Western blot analysis was performed to detect SIRT6 and TRPV1 protein levels. (**C**) Levels of the osteoblastic markers *OPN*, *OCN*, *BSP*, *RUNX2*, and *ALP* were determined via qPCR for each group on day 14 after induction. (**D**) The activity of ALP was detected in each group in OD. (**E**) AR staining was performed to detect matrix mineralization of hMSCs between different treatments on day 14 following induction. N=3. One-way ANOVA was used for comparative analyses of the data in (**A, C, D**). * P<0.05 *vs.* indicated groups.

OD process of HMSCs, while overexpression of SIRT6 inhibited the expression of OD markers and ALP activity, which indicated that SIRT6 inhibits the OD of HMSCs by reducing the expression of osteoblast markers.

TRPV1 is a TRP protein and cationic permeation channel. TRPV1 is a non-selective cationic channel with high permeability to Ca^{2+} and Na^{+} [36–38]. CGRP expression was upregulated by the CRTC1/CREB signaling pathway, which is regulated by TRPV1, which induces neurogenic inflammation [39]. Capsaicin, the main ingredient of capsicum, is a natural and specific TRPV1 channel agonist. Capsaicin can induce pain and heat reactions, which manifest as a 'hot taste' [39]. It can upregulate TRPV1 channel activity, which induces spontaneous inflammation and nerve injury [40,41]. Capsazepine is TRPV1 ion channel antagonist. Previous studies have shown that capsazepine inhibits osteoclast formation and resorption in a dose-dependent manner in co-culture of osteoblasts and osteoclasts produced by RANKL, while capsaicin, a TRPV1 agonist, promotes osteoclast formation. In the culture of calvarial osteoblasts (COBs), capsazepine also induces apoptosis of mature osteoclasts, inhibiting ALP activity and bone nodule formation. Capsazepine also inhibits bone resorption and formation *in vivo* [42]. In a periodontitis model, knockout of TRPV1 gene in mice resulted in severe bone loss. Chemical ablation of TRPV1-expressing sensory neurons was performed to reproduce the phenotype of TRPV1 knockout mice, which suggests a functional relationship between TRPV1 signal transduction and periodontal bone loss [43]. In another study, wild-type mice had completely remodeled fracture callus, while TRPV1

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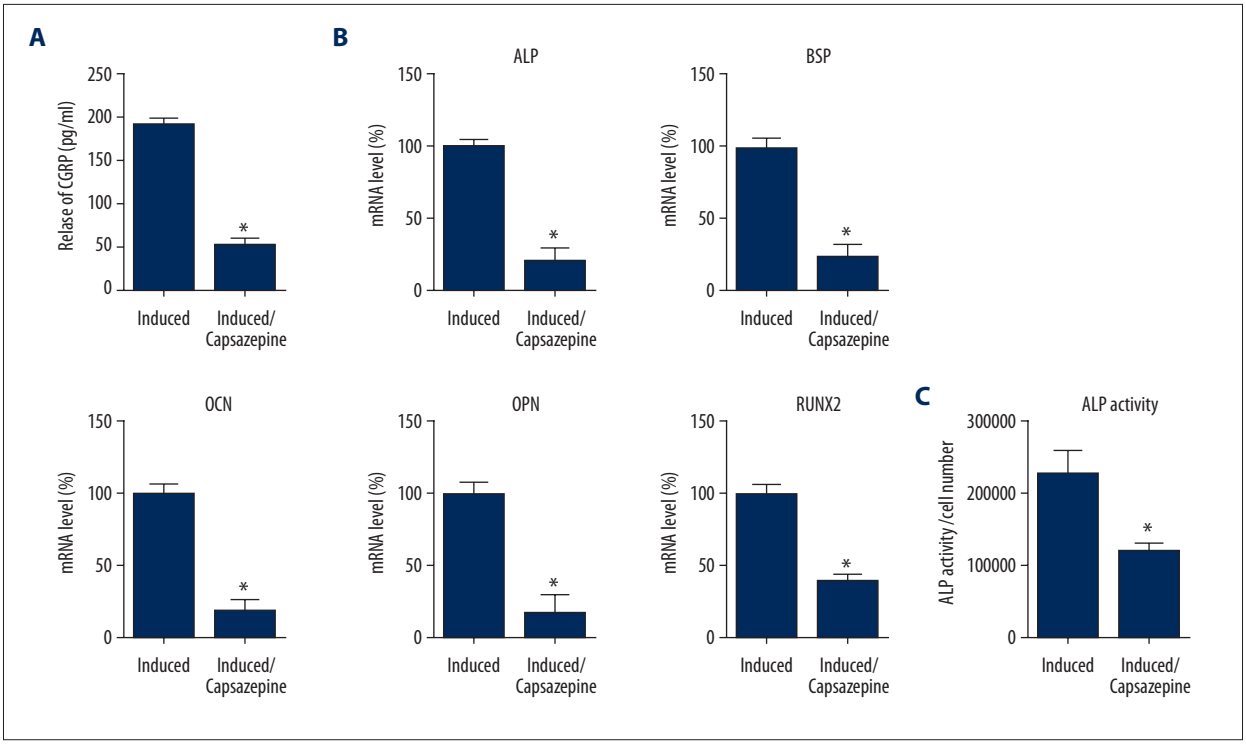

Figure 6. Evaluation of the effect of capsazepine on OD of hMSCs. The hMSCs were transfected with SIRT6 overexpression vector, and treated with 5 μM capsazepine for 12 h. (**A**) ELISA was utilized to detect released CGRP. (**B**) Levels of the osteoblastic markers *OPN*, *OCN*, *BSP*, *RUNX2*, and *ALP* were determined via qPCR for each group on day 14 after induction. (**C**) The activity of ALP was detected in each group in OD. N=3. Two-tailed *t* test were used for comparative analyses of the data in (**A, B, C**). * P<0.05 *vs.* indicated groups.

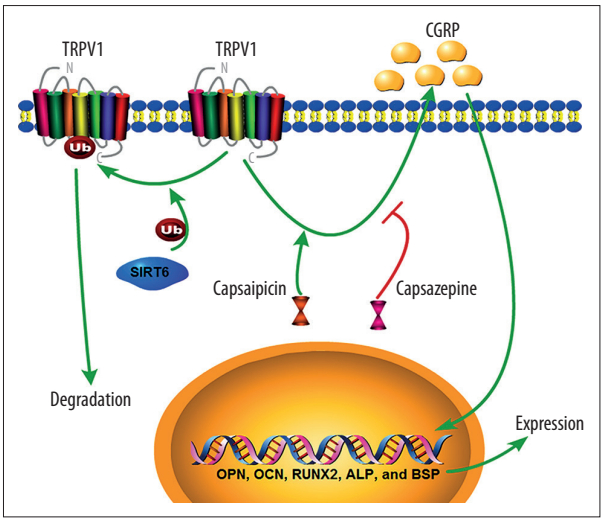

Figure 7. Schematic diagram of the mechanism of SIRT6-mediated TRPV1-CGRP pathway in hMSCs. In induced hMSCs, SIRT6 increased TRPV1 ubiquitination, and led to degradation. CGRP was released after TRPV1 activation and subsequently promoted the OD process. Administration of capsaicin or capsazepine activated or blocked the TRPV1 channel, and therefore mediated OD progression.

knockout mice still had a significant fracture gap and unabsorbed soft callus at 4 weeks after fracture. TRPV1 knockout decreased osteoclasts in fracture callus, resulting in impaired osteoclast formation and resorption *in vitro*. Loss of TRPV1 decreases the frequency of calcium oscillation and peak concentration of the osteoclast precursor cytoplasm, then decreases the expression and nuclear translocation of NFATc1, and downregulates DC-stamp, cathepsin K, and ATP6V [44]. Previous studies showed that CGRP has remarkable effects on bone metabolism. Several functional receptors are produced by osteoclasts for CGRP [45]. CGRP-immunoreactive nerve fibers are widely found in bone tissue such as bone marrow [46]. CGRP-deficient animal showed osteopenia and accelerated bone loss with aging [47]. The administration of CGRP showed anti-osteoclastogenic effects both *in vitro* [25] and *in vivo* [48]. In the present study, we showed a regulatory role for TRPV1 and CGRP in OD by highlighting the advantageous effect of CGRP in ALP activity and osteoblasts markers expression. Induced OD of hMSCs upregulated TRPV1 expression and activity, which was accompanied by SIRT6 downregulation. These data show that the expression and release of CGRP can be induced in OD. SIRT6 overexpression inhibits TRPV1 expression by ubiquitinating it and thereby repressing CGRP release (Figure 7). To verify that TRPV1 was the cause of SIRT6-mediated OD, cells were treated with

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capsaicin or capsazepine. After capsaicin treatment, osteoblastic marker expression was upregulated and ALP activity was increased, suggesting that capsaicin promotes the OD process. After administration of capsazepine, we found an obvious reduction in osteoblastic marker expression. This is consistent with previous studies, suggesting that TRPV1 and CGRP signaling regulate bone metabolism. These results suggest that activation of the TRPV1 channel and CGRP induces OD of hMSCs, and that SIRT6 inhibits OD by repressing TRPV1 expression.

Conclusions

This study shows that SIRT6 may be a negative regulator of OD. Low expression of SIRT6 and high expression of TRPV1 in hMSCs may be closely related to specific osteogenesis-related genes, as well as activity of ALP and matrix mineralization. The findings also suggested that TRPV1 and CGRP show potential as biomarkers of clinical osteoporosis and bone loss. The SIRT6-TRPV1-CGRP signal axis may be a potential therapeutic target for diseases related to osteoporosis and bone loss.

Conflict of interests

None.

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