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Silence of lncRNA BCAR4 alleviates the deterioration of osteoporosis

D.-J. LI[1], G.-Q. LIU[2], X.-J. XU[3]

[1]Trauma Department of Orthopedics, Dongying People's Hospital, Dongying, China
[2]Department of Spine Surgery, Dongying People's Hospital, Dongying, China
[3]Department of Emergency, Dongying People's Hospital, Dongying, China

Abstract. – OBJECTIVE: To illustrate the biological function of long non-coding RNA (lncRNA) BCAR4 in triggering osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs), thus mediating the progression of osteoporosis.

MATERIALS AND METHODS: Relative levels of BCAR4 in BMSCs undergoing indicated time points of osteogenic differentiation were examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). After intervening BCAR4 levels in osteogenically differentiated BMSCs, the relative levels of serum osteocalcin (OCN) and osteopontin (OPN) were detected, as well as ALP activity and mineralization capacity. Female Sprague Dawley (SD) rats were classified into sham group, BMSCs group (administration with BMSCs), RNAi group (administration with BMSCs transfected with si-BCAR4), and control group, with 10 rats per group. Osteoporosis model was generated in the latter three groups by resection of bilateral ovaries. Positive expressions of procollagen type I N propeptide (PINP) and β-C-terminal telopeptide (β-CTx), and β-CTx in rats were determined by enzyme-linked immunosorbent assay (ELISA). Bone density in rat femurs and bone biomechanics were examined using the dual energy X-ray bone densitometer and the three-point bending test, respectively.

RESULTS: BCAR4 was downregulated on the 3rd and 7th day of osteogenic differentiation in BMSCs. Overexpression of BCAR4 downregulated OCN and OPN. In the meantime, BCAR4 was able to weaken alkaline phosphatase (ALP) activity and mineralization capacity in BMSCs. The promotive effects of silenced BCAR4 on osteogenic potentials in BMSCs were abolished by overexpression of GLI2. In rats of RNAi group, positive expression of PINP and bone biomechanics were remarkably higher than BMSCs group, whereas they were lower than the sham group. Positive expression of β-CTx was declined in RNAi group compared with that of BMSCs group, and it was still higher than that of sham group.

CONCLUSIONS: BCAR4 is involved in the osteogenic differentiation of BMSCs. The knockdown of BCAR4 can alleviate the progression of osteoporosis.

Key Words:
Osteoporosis, BCAR4, GLI2, Osteogenic differentiation.

Introduction

Osteoporosis is a multi-gene metabolic bone disease in which decreased bone mass and abnormal microstructure of bones increases bone fragility and fracture risk[1]. The incidence of osteoporosis in China has annually increased due to the aging population, bringing huge burdens on home care and influencing the quality of life of the elderly[2]. Therefore, it is necessary to clarify the pathogenesis of osteoporosis and to develop preventive strategies.

Osteogenic differentiation is a complicated process, involving multiple transcription factors and pathways[3,4]. Co-regulation and interaction in regulatory factors dynamically maintain the balance of bone metabolism[5,6]. Osteoblasts are mainly differentiated from bone marrow mesenchymal stem cells (BMSCs) regulated by various transcription factors and cytokines[7]. BMSCs exert self-renewal capacity, and multi-potentials of differentiation to osteoblasts or steatoblasts[8]. Very recently, BMSCs have been extensively applied as seed cells in the fields of biomaterials and tissue engineering[9,10].

Long non-coding RNAs (lncRNAs), non-coding RNAs exceeding 200 nucleotides that cannot encode proteins[11], are previously considered as non-functional transcription noises[12]. LncRNAs are reported to be involved in the regulation of osteogenic and adipogenic differentiation of BMSCs[13]. By promoting the assembly of transcription coactivator with PDZ-binding motif and Runx2/PPARG, lncRNA Bmncr drives osteogenic differentiation[14]. LncRNA ADINR stimulates adipogenesis by binding PAl and recruiting MLL3/4[15].

Corresponding Author: Guangquan Liu, MM; e-mail: lgquan3319@163.com

LncRNA BCAR4 is closely linked to antiestrogen resistance in breast cancer[16]. Later, biological functions of BCAR4 in osteosarcoma[17] and non-small-cell lung carcinoma[18] have been identified. This study aims to detect the role of BCAR4 in osteogenic differentiation of BMSCs and the potential mechanism.

Materials and Methods

Cell Culture

BMSCs (Kanbei Biotechnology, Shanghai, China) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 1% penicillin, and 1% streptomycin were added in the medium. BMSCs were passaged until they were grown to 90% confluence.

Induction of Osteogenic Differentiation

Osteogenic differentiation in BMSCs was induced until they reached 70% confluence. BMSCs were cultivated in the abovementioned DMEM containing 10 nM dexamethasone, 10 mM β-glycerophosphate, and 0.2 mM ascorbic acid for indicated time points.

Cell Transfection

The third-generation BMSCs were implanted in 6-well plates (5×10^4/mL). At 60% confluence, BMSCs were cultivated in antibiotics-free DMEM and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 2 days, followed by 14-day induction of osteogenic differentiation. BCAR4 siRNA (5'-GCU GCG AGG GUA GAC AUC UCU GUU U-3') and si-NC (5'-UAA GGC UAU GAA GAG AUA C-3') were synthetized by GenePharma Co. Ltd. (Shanghai, China).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Cellular RNAs were isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and their concentrations were determined by NanoDrop 2000 (Invitrogen, Carlsbad, CA, USA). RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using the PrimeScript™RT Master Mix, which was subjected to amplification by the TBGreen®Premix Ex Taq™II (TaKaRa, Tokyo, Japan). QRT-PCR was conducted on the CFX-96 RT-qPCR system (Bio-Rad Technologies, Inc., Hercules, CA, USA) with the following conditions: 95°C pre-denaturation for 30 s and 40 cycles at 95°C for 5 s and 60°C for 30 s. β-actin was the internal reference. Primer sequences were as follows: BCAR4: 5›-ACA GCA GCT TGT TGC TCA TCT-3› (forward) and 5›-TTG CCT TGG GGA CAG TTC AC-3› (reverse); β-actin: 5›-TCT GGC TGA GGC TGG TTG AC-3› (forward) and 5›-CTC CTT AAT GTC ACG CAC GAT-3› (reverse); OCN: 5›-GAC AAG TCC CAC ACA GCA ACT-3› (forward) and 5›-GGA CAT GAA GGC TTT GTC AGA-3› (reverse); OPN: 5›-CAA ATA CCC AGA TGC TGT GGC-3› (forward) and 5›-TCC TGG CTG TCC ACA TGG AC-3› (reverse).

Western Blot

The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) on ice for 30 min. Cell lysate was centrifuged at 4°C, 1000 rpm for 10 min. Extracted protein samples were quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. The membranes reacted with primary and secondary antibodies for indicated time. Band exposure and analyses of grey values were finally conducted.

ALP Activity Determination

BMSCs in the 24-well plate were incubated with 125 μL of Triton X-100 (0.05%) per well and subjected to repeated freeze-thaw for three times. The cells were collected, and centrifuged at 4°C, 15,000 rpm for 15 min. ALP activity determination kit (Wako, Kanagawa, Japan) was used. Optical density at 405 nm was measured using the Synergy™ 2 microplate reader (BioTek, Biotek Winooski, VT, USA) and the corresponding protein concentration was detected for calculating the relative activity of ALP.

ALP and ARS Staining

Osteogenically differentiated BMSCs on the 7th day were collected for ALP staining. They were fixed in 10% formaldehyde for 1 min, washed in phosphate-buffered saline (PBS) for three times, and dyed with BCIP in the dark for 15 min.

Osteogenically differentiated BMSCs on the 14th day were collected for ARS staining. They were fixed in 4% paraformaldehyde for 30 min, washed in deionized water for three times, and dyed in ARS-Tris-

HCL solution (pH 4.1-4.3) in the dark for 45 min. Images were captured under an inverted microscope.

Animal Procedures

Forty female Sprague Dawley (SD) rats with 8-week-old weighing 220-250 g were provided by Experimental Animal Center of the Academy of Military Medical Sciences. This investigation was approved by the Animal Ethics Committee of Dongying People's Hospital Animal Center. They were classified into sham group, BMSCs group, RNAi group, and control group, with 10 rats per group. Osteoporosis model was established in the latter three groups. Briefly, the rats were intraperitoneally administrated with 5% chloral hydrate (0.6 mL/100 g) for anesthesia. After cutting a lumbar posterior median incision, the muscles were bluntly separated for resecting bilateral ovaries. Incision was sutured layer by layer. The rats in the control group were similarly operated for removing a little fatty tissue surrounding the ovary. The rats in the sham group were negative controls without specific treatment. For BMSCs treatment in rats of BMSCs group and RNAi group, BMSCs were cultivated in serum-free L-DMEM on the 5th day of osteoporosis model establishment. On the other day, the cells were incubated with 30 μl of Ad-si-BCAR4 per dish (100 mm), and L-DMEM containing 10% FBS was replaced 8 hours later. On the 7th day of osteogenesis model establishment, 2×10^6 treated BMSCs suspended in 1 ml of serum-free L-DMEM was administrated in rat tail of RNAi group. The rats in the BMSCs group were administrated with normally cultivated BMSCs. Five weeks later, all rats were sacrificed for collecting 5 ml of intraperitoneal blood, which were centrifuged at 3000 rpm for 10 min. The upper layer serum was stored at -20°C. Meanwhile, rat femurs were collected after peeling off muscles and soft tissues, and stored in the formalin at room temperature.

Enzyme-Linked Immunosorbent Assay (ELISA)

Positive expressions of the osteogenic index (PINP) and bone resorption index (β-CTx) in rats were detected using the commercial ELISA kits (KMinsw Biotechnology, Shanghai, China).

Determination of Bone Density and Biomechanics

Bone density (g/cm^2) in rat femurs was determined using the XR-46 dual energy X-ray bone densitometer. Bone biomechanics (N) in rats was examined by the Instron material test system.

Statistical Analysis

Data were expressed as mean±SD (standard deviation) and processed by Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA). The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). A significant difference was set at $p < 0.05$.

Results

Expression Change of BCAR4 During Osteogenic Differentiation of BMSCs

On the 3rd and 7th day of osteogenesis induction in BMSCs, BCAR4 was markedly downregulated compared with controls, indicating the involvement of BCAR4 in osteogenic differentiation of BMSCs (Figure 1A). Subsequently, we constructed si-BCAR4 and pcDNA-BCAR4, and their transfection performance was examined in BMSCs (Figure 1B, 1C).

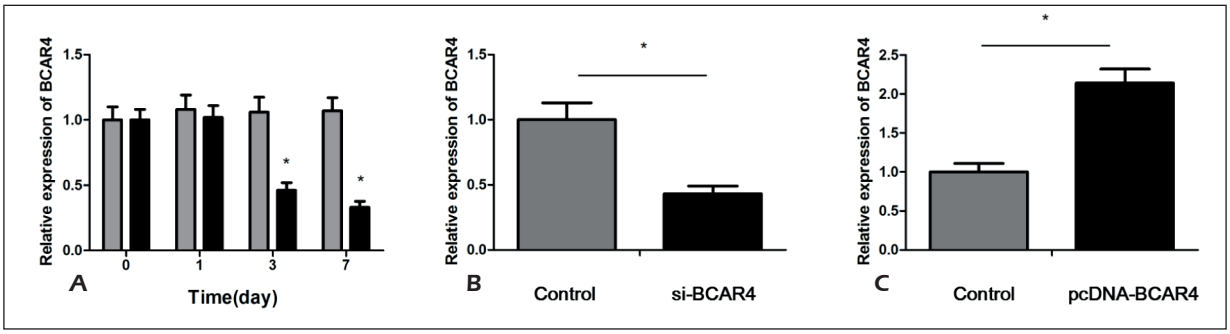

Figure 1. Expression change of BCAR4 during osteogenic differentiation of BMSCs. **A**, BCAR4 was downregulated on the 3rd and 7th day of osteogenesis induction in BMSCs. **B**, Transfection of si-BCAR4 significantly downregulated BCAR4 in BMSCs. **C**, Transfection of pcDNA-BCAR4 significantly upregulated BCAR4 in BMSCs.

Overexpression of BCAR4 Inhibited BMSCs Osteogenesis

In osteogenically differentiated BMSCs overexpressing BCAR4, osteogenesis indexes OCN and OPN were remarkably downregulated at both mRNA and protein levels (Figure 2A-2C). In addition, the overexpression of BCAR4 attenuated ALP activity and mineralization capacity in BMSCs undergoing osteogenic differentiation (Figure 2D, 2E). It is indicated that BCAR4 could intervene BMSCs osteogenesis.

Knockdown of BCAR4 Stimulated BMSCs Osteogenesis

To further validate the biological functions of BCAR4 in osteogenesis, its level was intervened in BMSCs by transfection of si-BCAR4. The knockdown of BCAR4, conversely, upregulated OCN and OPN in BMSCs (Figure 3A-3C). Meanwhile, ALP activity and mineralization capacity in BMSCs was stimulated by silence of BCAR4 (Figure 3D, 3E).

GLI2 Abolished the Role of BCAR4 In Regulating BMSCs Osteogenesis

It is previously reported that the binding between BCAR4 and GLI2 promoter activates the GLI2 signaling in osteosarcoma cells[19] and breast cancer cells[20]. Here, the involvement of GLI2 in BMSCs osteogenesis was assessed. It is found that upregulated OCN and OPN in BMSCs with BCAR4 knockdown were reversed by co-transfection of pcDNA-GLI2 (Figure 4A). As expected, increased ALP activity and mineralization capacity in BMSCs with BCAR4 knockdown was markedly reduced by overexpressed GLI2 (Figure 4B, 4C). The above results demonstrated that BCAR4 exerted its functions in influencing BMSCs osteogenesis *via* regulating GLI2.

Knockdown of BCAR4 Alleviated Osteoporosis

We established osteoporosis model in rats to clarify the *in vivo* functions of BCAR4. Twenty-eight days after administration of treated BMSCs, relative indicators reflecting osteoporosis conditions were determined. Compared with rats in control and BMSCs group, PINP level was higher in RNAi group and it was still lower than that in sham group. β-CTx level showed the opposite trend (Table I, II). Bone density and biomechanics in rats of each group were recorded. Bone density and biomechanical index were enhanced by the treatment of BMSCs transfected with si-BCAR4 (Table III, IV). Collectively, the knockdown of BCAR4 could alleviate the progression of osteoporosis.

Discussion

The growth of bones involves huge changes in bone structure and size, which is known as bone modeling. Maintaining the normal shape of mature bones has been accompanied by the process of bone reconstruction[21]. Bone reconstruction is a complicated process occurring in the basic multicellular unit, during which osteoblasts and osteoclasts are the most functional cells[22]. Four steps are involved in bone reconstruction, including (i) bone surface activation, (ii) osteoclast adhesion, and bone resorption, (iii) transformation from bone absorption to bone formation or from catabolism to anabolism, and (iv) osteoblast activation and osteogenesis.

Recent findings have shown the involvement of lncRNAs in stem cell differentiation and body development. They are able to regulate life activities and pathological processes by interacting with RNAs, enhancers and protein complexes modified chromatin[23]. Finally, Wang et al[24] demonstrated that lncRNA is of significance in BMSCs osteogenesis as a vital regulator. Zhu et al[25] pointed out that lncRNA ANCR catalyzes H3K27me3 by recruiting EZH2 to the promoter of Runx2, thereafter suppressing BMSCs osteogenesis. Feng et al[26] induces *in vitro* osteogenic differentiation in mouse BMSCs. Overexpression of BDNF-AS by lentivirus transfection remarkably downregulates OPN and Rtmx2, indicating that BDNF-AS inhibits bone formation by a negative feedback to the osteogenesis signaling. Consistently, our findings uncovered that BCAR4 was gradually downregulated during the process of osteogenic differentiation of BMSCs. In addition, BCAR4 could inhibit osteogenesis marker levels, ALP activity and mineralization capacity in BMSCs. It is suggested that BCAR4 suppressed osteogenesis in BMSCs.

GLI2 (glioma associated oncogene homolog) is the critical gene in the Sonic hedgehog signaling. It is capable of regulating cell apoptosis *via* targeting the activation and transcription of downstream genes[27]. Cai et al[28] has shown that the knockdown of GLI2 reverses the regulatory effects of BCAR4 on growth and migration of prostate cancer cells[28].

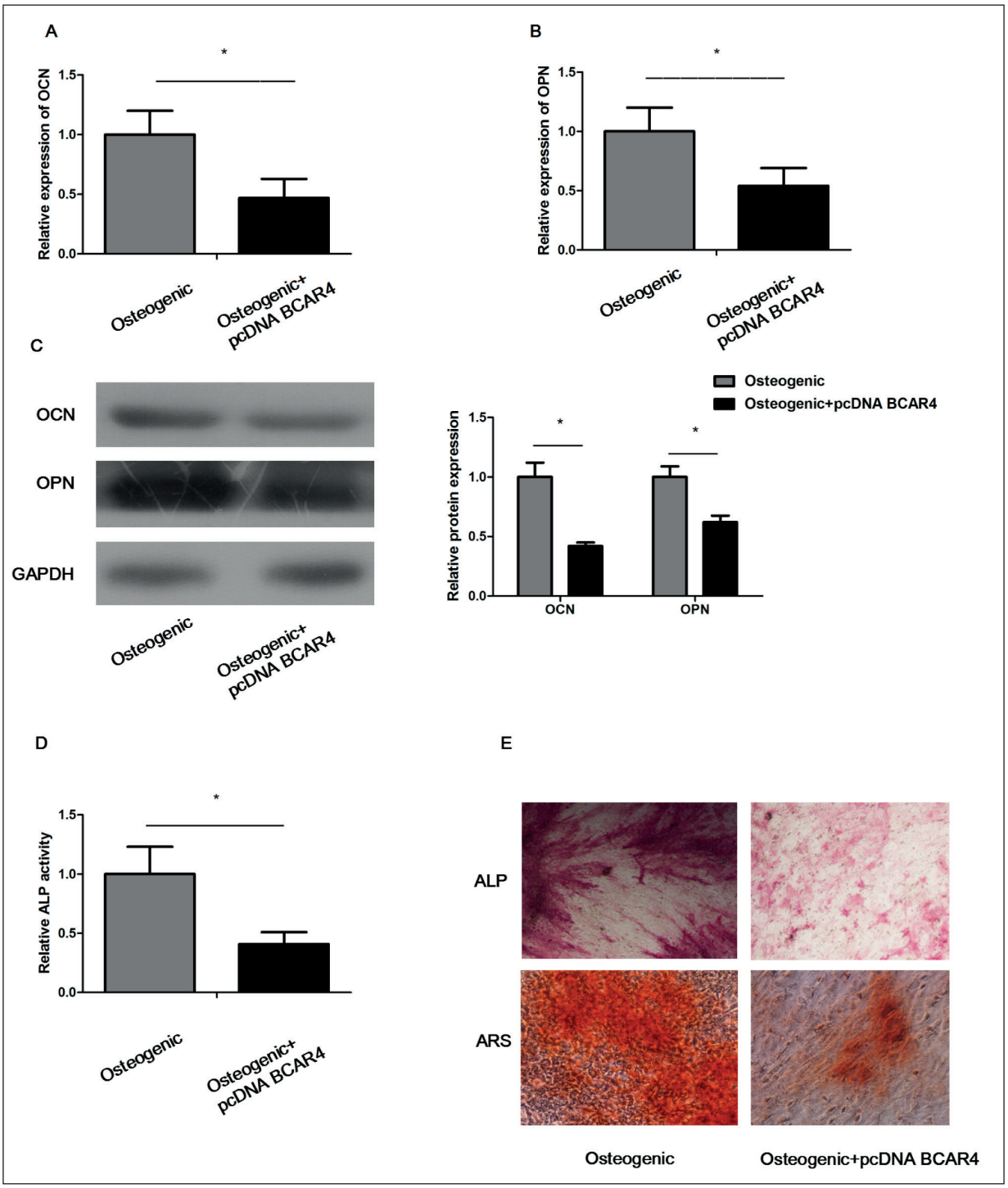

Figure 2. Overexpression of BCAR4 inhibited BMSCs osteogenesis. **A**, Overexpression of BCAR4 downregulated mRNA level of OCN in osteogenically differentiated BMSCs. **B**, Overexpression of BCAR4 downregulated mRNA level of OPN in osteogenically differentiated BMSCs. **C**, Overexpression of BCAR4 downregulated protein levels of OCN and OPN in osteogenically differentiated BMSCs. **D**, Overexpression of BCAR4 decreased ALP activity in osteogenically differentiated BMSCs. **E**, Overexpression of BCAR4 decreased ALP level and mineralization ability in osteogenically differentiated BMSCs, (magnification: 400×).

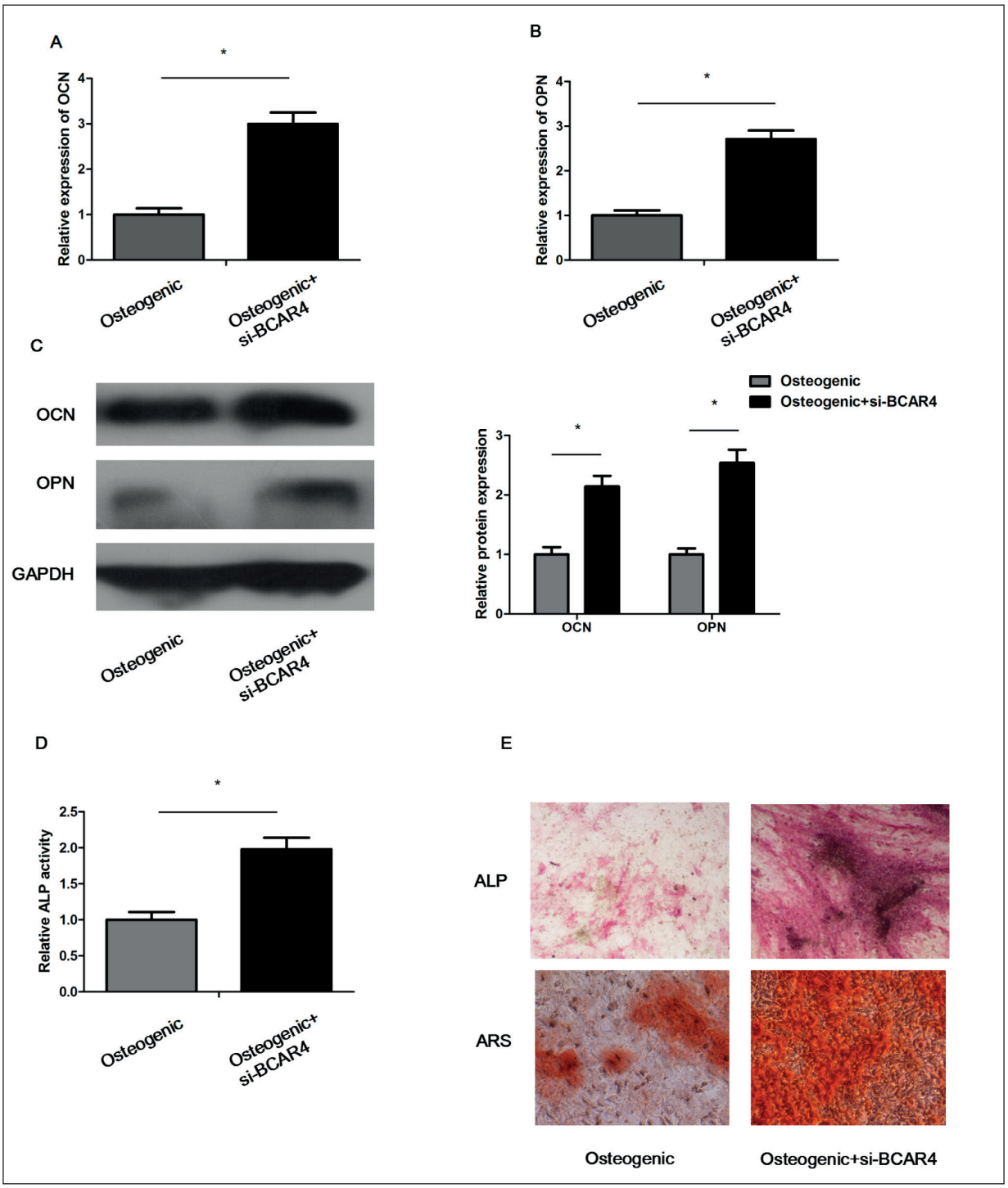

Figure 3. Knockdown of BCAR4 stimulated BMSCs osteogenesis. **A**, Knockdown of BCAR4 upregulated mRNA level of OCN in osteogenically differentiated BMSCs. **B**, Knockdown of BCAR4 upregulated mRNA level of OPN in osteogenically differentiated BMSCs. **C**, Knockdown of BCAR4 upregulated protein levels of OCN and OPN in osteogenically differentiated BMSCs. **D**, Knockdown of BCAR4 increased ALP activity in osteogenically differentiated BMSCs. E, Knockdown of BCAR4 increased ALP level and mineralization ability in osteogenically differentiated BMSCs, (magnification: 400×).

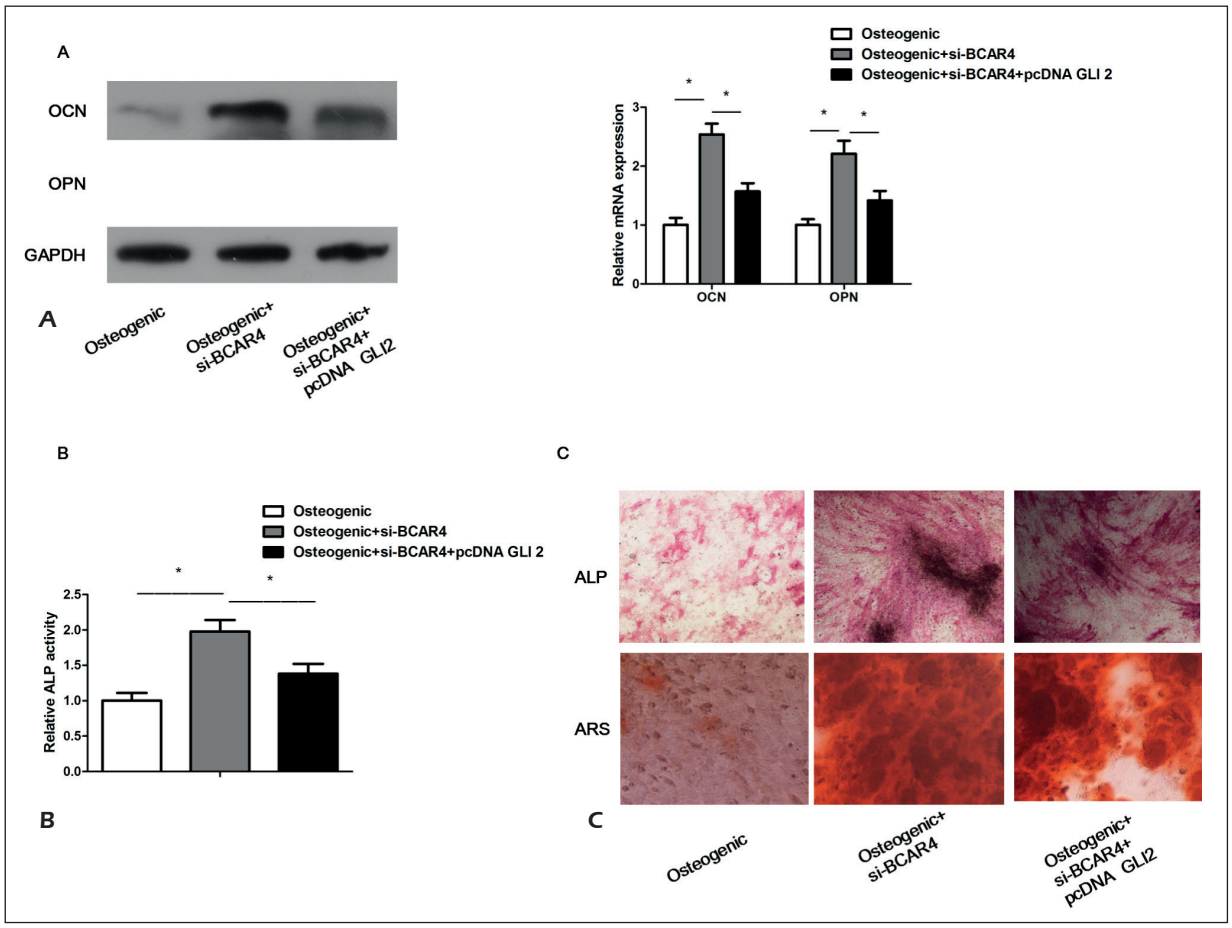

Figure 4. Knockdown of BCAR4 alleviated osteoporosis. **A**, Co-transfection of si-BCAR4 and pcDNA-GLI2 reduced OCN and OPN levels in osteogenically differentiated BMSCs than those transfected with si-BCAR4. **B**, Co-transfection of si-BCAR4 and pcDNA-GLI2 reduced ALP activity in osteogenically differentiated BMSCs than those transfected with si-BCAR4. **C**, Co-transfection of si-BCAR4 and pcDNA-GLI2 reduced ALP level and mineralization ability in osteogenically differentiated BMSCs than those transfected with si-BCAR4, (magnification: 400×).

Table I. Relative level of PINP in osteoporosis rats.

Groups	n	x±s (ng/mL)	F	p
RNAi	10	13.17±3.86*#&	6.83	<0.001
BMSCs	10	10.89±2.51*#		
Sham	10	15.27±3.99*		
Control	10	8.57±1.75		

*p<0.05 *vs.* Control group; #p<0.05 *vs.* Sham group; &p<0.05 *vs.* BMSCs group.

Table II. Relative level of β-CTx in osteoporosis rats.

Groups	n	x±s (ng/mL)	F	p
RNAi	10	12.56±2.87*#&	5.91	<0.001
BMSCs	10	17.88±3.01*#		
Sham	10	10.12±1.99*		
Control	10	23.46±3.13		

*p<0.05 *vs.* Control group; #p<0.05 *vs.* Sham group; &p<0.05 *vs.* BMSCs group.

Table III. Bone density in osteoporosis rats.

Groups	n	x±s (g/cm²)	F	p
RNAi	10	0.23±0.017*#&	7.41	<0.001
BMSCs	10	0.19±0.16*#		
Sham	10	0.263±0.22*		
Control	10	0.168±0.14		

*p<0.05 *vs.* Control group; #p<0.05 *vs.* Sham group; &p<0.05 *vs.* BMSCs group.

Table IV. Bone biomechanics in osteoporosis rats.

Groups	n	x±s (g/cm²)	F	p
RNAi	10	57.45±2.87*#&	11.68	<0.001
BMSCs	10	51.76±2.14*#		
Sham	10	66.32±4.23*		
Control	10	42.44±1.54		

*p<0.05 *vs.* Control group; #p<0.05 *vs.* Sham group; &p<0.05 *vs.* BMSCs group.

In this paper, the knockdown of BCAR4 and overexpression of GLI2 were simultaneously conducted in BMSCs. As we found, overexpression of GLI2 reversed the promotive effects of silenced BCAR4 on OCN and OPN expressions. Meanwhile, the enhanced ALP activity, ALP content, and calcification were abolished as well. It is indicated that BCAR4 induced osteogenesis in BMSCs through the GLI2 signaling.

PINP is a procollagen peptide that is decomposed from both ends of type I procollagen molecules. Serum level of PINP reflects the ability of osteoblasts to synthesize bone collagen, which can be used to sensitively and specifically examine osteoblast viability and bone formation. β-CTX exists in mature collagen. When the activity of osteoclasts is enhanced, a large amount of type I collagen is degraded. Later, formed C-terminal peptide is degraded into β-CTX, and thus releases into the blood. It is considered that β-CT is an important indicator for monitoring bone resorption and bone turnover, with a high specificity[29-32]. Furthermore, we established osteoporosis model in rats. *In vivo* results indicated that BCAR4 participated in osteogenic differentiation of BMSCs. The knockdown of BCAR4 alleviated the process of osteoporosis. It is concluded that BCAR4 may become an effective drug target for clinical treatment of osteoporosis.

Conclusions

Altogether these data demonstrated that BCAR4 is involved in the osteogenic differentiation of BMSCs. The knockdown of BCAR4 can alleviate the progression of osteoporosis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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