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Research Paper

Novel_circ_003686 regulates the osteogenic differentiation of MSCs in patients with myeloma bone disease through miR-142-5p/IGF1 axis

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HIGHLIGHTS

- Circ_003686 was significantly under-expressed in MBD-BMSC. miR-142-5p was over-expressed in BMD-BMSC.
- Overexpression of circ_003686 could significantly promoted osteogenic differentiation in BMD-BMSC.
- Circ_003686 could bind miR-142-5p to promote osteogenic differentiation.
- miR-142-5p could bind to IGF1 to promote osteogenic differentiation.
- Circ_003686/miR-142-5p/IGF1 axis meaningfully controlled the relative expression ratios of PI3K/AKT pathway.

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ABSTRACT

Objectives: Circ_003686 is a novel_circRNA with abnormally low expression found in the samples of multiple myeloma bone disease (MBD) patients. The current research intended to investigate the effects of novel_circ_003686 in osteogenesis-induced differentiation of bone marrow mesenchymal stem cells (BMSCs) in MBD.
Methods: BMSCs were extracted from MBD patients and normal participants, the pcDNA3.1 encoding the circ_003686 (ov-circ_003686), miR-142-5p-mimic/inhibitor and siRNA oligonucleotides targeting insulin like growth factor 1 (IGF1, si-IGF1) were applied to intervene circ_003686, miR-142-5p and IGF1 levels, respectively.
Results: Results showed that ov-circ_003686 could mediate the osteogenesis-induced differentiation of MBD-BMSC, and luciferase assay and RIP experiments confirmed that circ_003686 could bind to miR-142-5p. MiR-142-5p-inhibitor helped osteogenesis-induced differentiation, while miR-142-5p-mimic inhibited osteogenesis-induced differentiation and reversed the promoting effect of ov-circ_003686, suggesting that circ_003686/miR-142-5p axis participated in osteogenesis-induced differentiation of MBD-BMSC. In addition, miR-142-5p binds to the target gene IGF1 and negatively adjust its expression. Si-IGF1 significantly inhibited the osteogenesis-induced differentiation and reversed the promotion effects of miR-142-5p-inhibitor and ov-circ_003686. Moreover, circ_003686/miR-142-5p/IGF1 axis meaningfully regulates protein expressions in the PI3K/AKT pathway.
Conclusion: In conclusion, this research confirmed that circ_003686 regulated the osteogenesis-induced differentiation of MBD-BMSC by sponging miR-142-5p and mediating IGF1, and the PI3K/AKT pathway may also be involved.

1. Introduction

Multiple myeloma (MM) is a hematologic malignancy characterized by malignant proliferation of plasma cell, which is more common clinically and has an increasing incidence [1]. The most communal complication of MM is multiple myeloma bone disease (MBD), and the severity of bone damage is related to the quality of life and prognosis level of MM patients [2]. About 90 % of MM patients are complicated by

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osteopathy, and 80 % of them present with pathological fractures and bone pain during the course of the disease [3]. The pathogenesis of MBD is mainly the imbalance of osteoclasts and osteoblasts, that is, the number and activity of osteoclasts increase, while osteoblasts decrease [4]. Bone marrow mesenchymal stem cells (BMSCs) are a class of stem cells with the potential for self-renewal and multidirectional differentiation, including differentiation into osteoblasts [5]. Many studies have found that BMSCs from MM patients have abnormal immunophenotype and secretion of cytokines, and decreased osteogenic differentiation potential [6]. Therefore, it is critical to explore the possible mechanisms affecting BMSCs osteogenic differentiation in the disease progression of MM.

Circular RNA (circRNA) is a special class of covalently closed endogenous non-coding RNAs, which are tissue-specific and widely present in human cells [7]. CircRNA is expressed differently in tumor and other disease-related tissues and cells, suggesting that circRNA has a regulatory effect on tumor-related diseases [8]. Similarly, researches have confirmed that some circRNAs are differentially expressed in MM and may regulate some potential signaling pathways, which can be used as potential biomarkers [9,10]. In our previous study, a novel circRNA novel_circ_003686 (chr5: 65780179-65788484) was found to be abnormally low expressed in BMSCs from MBD patients (Shown in supplementary materials). Its role in BMSCs osteogenic differentiation and its potential regulatory mechanism need to be further studied. It is generally believed that circRNA can play the role of tumor regulation in various ways, but the current research is relatively clear about its function of microRNA (miRNA) sponge [11]. CircRNA competitively binds to target miRNA through miRNA sponge, resulting in degradation or loss of regulatory function of miRNA, thus changing the expression levels of downstream target genes of miRNA [12]. The CircRNA-miRNA-mRNA regulatory network show a key role in MBD and can be used as a research idea to explore the role of novel_circ_003686 in MBD.

Bioinformatics predicted that novel_circ_003686 had a potential targeting interaction with miR-142-5p, which was negatively associated with insulin-like growth factor-1 (IGF1), and there was also a potential binding site (Supplementary Fig. 2). Therefore, BMSCs obtained from MBD patients and normal participants were used in this study to discover the effects of novel_circ_003686/miR-142-5p/IGF1 in controlling osteogenesis-induced differentiation of BMSCs. Validating the value of novel_circ_003686 as a potential biomarker of MBD and its role in MBD treatment can offer novel biomarkers and intervention targets for MBD patients.

2. Material and methods

2.1. Clinical sample

Bone marrow from 5 patients (3 males, 2 females) diagnosed with Stage IIIA-IIIB MM (38–60 years) and 5 normal participants admitted to the General Hospital of Western Theater Command between January 2020 and January 2021 were used to extract BMSCs, which were permitted by the Ethics Committee of this hospital. The inclusion and exclusion criteria were the same as in our previous study [13], and all patients consented and signed informed consent.

2.2. Isolation and identification of BMSCs

Ficoll (Sigma, USA) was used to isolate BMSCs from MBD patients (MBD-BMSC) or normal participants (normal-BMSC). The bone marrow fluid was collected, and the same amount of Ficoll was added, centrifuged at room temperature for 20 min at 450 × *g*, the upper layer of separation fluid was removed to obtain monocytes. The isolated cells were transferred to DMEM (Hyclone, USA) with 10 % FBS (Gibco, USA) and cultured in a cell incubator. BMSCs morphology was observed, and the expressions of cell surface antigen (CD44, CD90, CD105, CD34 and CD45) were detected by flow cytometry. The CD34-PC5.5 (cat. no. 119311), CD44-APC (cat. no. 397505), CD45-FITC (cat. no. 982316), CD90-APC (cat. no. 328113) and CD105-PE (cat. no. 323205) antibodies were all purchased from BioLegend (California, USA), and the detailed detection method was referred to the previous published article [14]. In addition, BMSCs were induced to differentiate to identify their osteogenic ability.

2.3. Osteogenic differentiation

About 1×10^5 BMSCs were seeded in 6-well plates and cultured to 70 % cell density under conventional conditions. BMSCs were cultured with 2 mL of fresh DMEM containing FBS (10 %), dexamethasone (1×10^{-8} mol/L), vitamin C (50 μg/mL) and sodium β-glycerophosphate (10 mmol/mL) (osteogenic induction medium), replaced every-three days, and continuously cultured for 3 weeks to induce osteoblast differentiation of BMSCs [13,15].

2.4. Alizarin red S staining

The calcium deposition on BMSCs was observed to analyze osteogenic differentiation ability. After washing with PBS (Solarbio, China), BMSCs were fixed with 4 % paraformaldehyde (Solarbio, China) for 20 min, PBS washed off the fixative solution, and 1 % alizarin red S (Sigma-Aldrich, USA) stained for 5 min. The staining images were observed by an inverted microscope and photographed at ×100 magnification (Olympus, Japan).

2.5. Real-time quantitative polymerase chain reaction (RT-PCR)

The total RNA of BMSCs was extracted by an TRIzol reagent (Thermo Fisher, USA), and PrimeScript RT reagent kit (Takara, Dalian, China) was used to prepare cDNA. On this basis, gene expressions were analyzed with SYBR Premix Ex Taq II (Takara, China) in a ultimate volume of 20 μL using the normal procedure of the Real-Time PCR system (Applied Biosystems 7500; USA): 3 min at 94 °C; 40 cycles of 5 s at 94 °C and 30 s at 60 °C; then hold at 72 °C for 30 s. And miRNA expressions were analyzed with Bulge-Loop miRNA RT-qPCR Primer and Starter kit (RiboBio, China) with 10 min at 94 °C; 40 cycles of 2 s at 94℃, 20 s at 60℃ and 10 s at 70℃. Then, $2^{-\Delta\Delta Cq}$ method was applied to calculate the relative expression, and the endogenous normalized control for IGF1, alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC) was ACTIN, for miR-142-5p was U6, and for circ_003686 was GAPDH. The primer sequences were shown as Table 1.

2.6. Cell transfection

The pcDNA3.1 encoding the circ_003686 was used to overexpress circ_003686 (ov-circ_003686) and constructed by Ribobio (Guangzhou, China). The hsa-miR-142-5p-mimic (miR10000434-1-5), hsa-miR-142-5p-inhibitor (miR20000434-1-5), NC-mimic (miR1N0000001-1-5) and NC-inhibitor (miR2N0000001-1-5) were obtained from Ribobio. And three siRNA oligonucleotides targeting IGF1 were also synthesized by Ribobio. The sequences of siRNA were: si-IGF1-1: GGAUAAAGAUAUCAAUUUAAA; si-IGF1-2: GAAGAAUUGUGAAAGUUUAUG; si-IGF1-3: GGUUCUGUGGAAUAAGAUACU. About 1×10^5 DMSCs were cultured for a total of 24 h after inoculation in 6-well plates. Next, the ov-NC, ov-circ_003686, NC-mimic/inhibitor, miR-142-5p-mimic/inhibitor, si-NC or si-IGF1 were used to transfect DMSCs with Lipofectamine 2000 (Invitrogen, USA) in medium without penicillin–streptomycin and FBS for 6 h as required. Then, the transfection medium was altered to complete medium and the culture was continued for 42 h. Subsequent researches were performed after a total of 48 h of treatment.

2.7. Dual-luciferase reporter assay

The luciferase reporter plasmid was created by constructing the wild-

Table 1
The primer sequences used in the study.

	Forward primer	Reverse primer
IGF1	5′-ACATTGCTCTCAACATCTCCCA-3′	5′-TGGTGTGCATCTTCACCTTCA-3′
ALP	5′-GACCTCCTCGGAAGACACTCTG-3′	5′-CGCCTGGTAGTTGTTGTGAGC-3′
OPN	5′-GCCGACCAAGGAAAACTCACT-3′	5′-GGCACAGGTGATGCCTAGGA-3′
OC	5′-CCAGGCGCTACCTGTATCAATG-3′	5′-ATGTGGTCAGCCAACTCGTCA-3′
ACTIN	5′-TGGCACCCAGCACAATGAA-3′	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′
miR-142-5p	5′-CATAAAGTAGAAAGCACTAC-3′	5′-CTCAACTGGTGTCGTGGA-3′
U6	5′-ACAGAGAAGATTAGCATGGCC-3′	5′-GACCAATTCTCGATTTGTGCG-3′
circ_003686	5′-GCTACAGGAGAAGTATTGGGAC-3′	5′-TCAGGAAGAGCACCAAGTTCA-3′
GAPDH	5′-ATGGTTGCCACTGGGGATCT-3′	5′-TGCCAAAGCCTAGGGGAAGA-3′

Note: IGF1, insulin-like growth factors-1; ALP, alkaline phosphatase; OPN, osteopontin; OC, osteocalcin.

type (wt) and mutant-type (mut) sequences of circ_003686 and IGF-1 into the luciferase vector psi-CHECK2 (Hanbio Biotechnology, China). About 4 × 10^4 BMSCs were seeded in 24-well plates and cultured at 37 ℃ to about 70 % confluence, Lipofectamine 2000 was used to co-transfect luciferase reporter plasmids, NC-mimic or miR-142-5p-mimic. Luciferase activity was measured 24 h after transfection (Promega Corporation, USA).

2.8. RNA immunoprecipitation (RIP)

RIP experiment was completed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). Cell lysates from DMSCs were co-incubated with Ago2 antibodies or lgG. RNA was extracted from the pull-down complexes, and then RT-PCR was performed to detect circ_003686 and miR-142-5p expressions.

2.9. Western blot analysis (WB)

Radio immunoprecipitation assay lysis buffer (Beyotime, China) was applied to extract total cell proteins and quantified by BCA protein assay kit (Beyotime, China). The quantified proteins were separated by 10 % SDS-PAGE and transferred onto a PVDF membrane (EMD Millipore, USA). Protein immune responses were performed with antibodies. The enhanced chemiluminescence detection kit (Bio-Rad, USA) was applied to develop the color reaction of the protein bands, and the protein gray level was statistically analyzed by the all-purpose imager (ChemiDoc

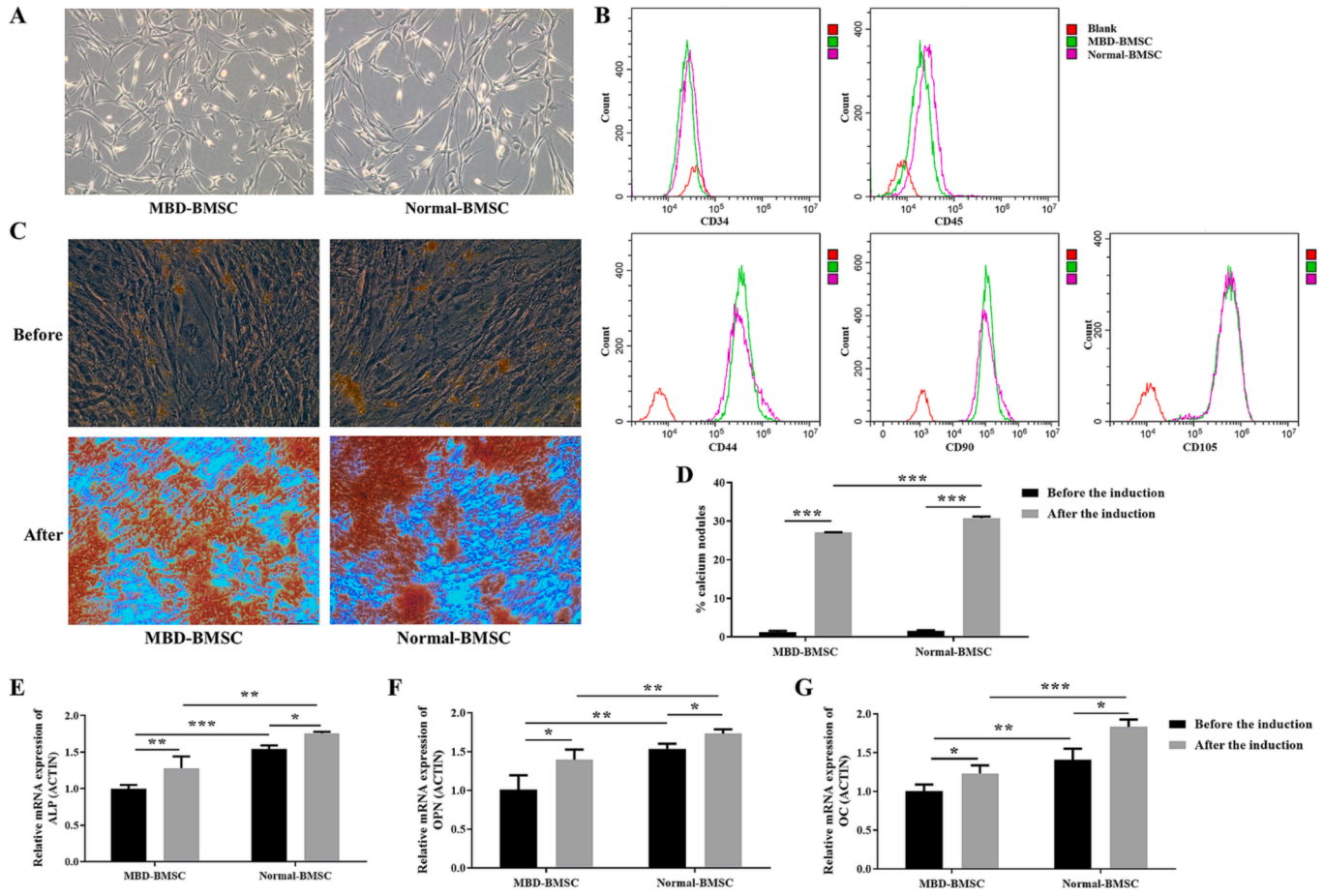

Fig. 1. Identification and osteogenic differentiation of BMSCs. (A) Cell morphology of MBD-BMDC and Normal-BMSC. Magnification, ×100. (B) Detection of surface antigens CD34, CD45, CD44, CD90 and CD105 in MBD-BMDC and Normal-BMSC. (C) Alizarin red staining of MBD-BMDC and Normal-BMSC before and after osteogenic differentiation. Magnification, ×100. The mRNA expressions of alkaline phosphatase (ALP) (D), osteopontin (OPN) (E) and osteocalcin (OC) (F) were detected by RT-PCR, and ACTIN was applied as the normalization standard. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MP, Bio-Rad). Primary antibodies IGF1 (DF6096), PI3K (AF6241), phospho-PI3K (p-PI3K, AF3242), phospho-AKT (AF0016), AKT (AF6261), phospho-mTOR (AF3308), mTOR (AF6308) and HRP labeled goat anti-rabbit secondary antibody (S0001) used in this experiment were purchased from Affinity (China).

2.10. Statistical analysis

All experiments in this research were independently replicated at least three times, with data expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and least-significant difference (LSD) post hoc used for statistical differences among multiple groups, and differences between two groups were analyzed by unpaired *t*-test, both using SPSS software version 20.0. When $P < 0.05$, the difference was statistically significant.

3. Results

3.1. Culture and identification of BMSCs

MBD-BMSC and normal-BMSC were derived from the bone marrow of MBD and normal participants, respectively. The cell morphology was shown in Fig. 1 A, most of which were long spindle shaped, and there was no obvious difference between MBD-BMSC and normal-BMSC. Flow cytometry analysis found that CD34 and CD45 expression in MBD-BMSC and normal-BMSC were negative, and CD44, CD90 and CD105 expression were positive (Fig. 1B), indicating that BMSCs were successfully extracted. Further induction of BMSCs osteogenic differentiation, and alizarin red staining showed that both MBD-BMSC and normal-BMSC had obvious calcium deposition after osteogenic induction (Fig. 1C). The results of RT-PCR experiments also confirmed that the gene levels of ALP, OPN and OC were significantly improved after osteogenesis-induced differentiation in both MBD-BMSC and normal-BMSC groups (Fig. 1D–F, $P < 0.05$), all the outcomes confirmed that both MBD-BMSC and normal-BMSC could normal osteogenic differentiation.

3.2. Circ_003686 mediated osteogenic differentiation of BMSCs

RT-PCR analysis gene levels of circ_003686 and miR-142-5p in MBD-BMSC and normal-BMSC and showed that circ_003686 was meaningfully higher in the normal-BMSC than in the MBD-BMSC, while miR-142-5p was meaningfully lower than MBD-DMSC (Fig. 2A, $P < 0.001$). In addition, circ_003686 expression was meaningfully increased in both MBD-BMSC and normal-BMSC groups after osteogenic induction compared with before induction (Fig. 2B, $P < 0.001$). To explain the role of circ_003686 in MBD-BMSC osteogenic differentiation, ov-circ_003686 was used to transfected MBD-BMSC, and RT-PCR showed that ov-circ_003686 significantly increased the expression of circ_003686 in BMSCs, confirming high transfection efficiency (Fig. 2C, $P < 0.001$). Alizarin red staining presented that the capability to differentiate into osteoblasts in the ov-circ_003686 group was meaningfully enhanced compared with the ov-NC group (Fig. 2D), and the levels of ALP, OPN and OC were also meaningfully increased after ov-circ_003686 transfection (Fig. 2E, $P < 0.01$). All outcomes confirmed that circ_003686 promoted the differentiation of BMSCs into osteoblasts.

3.3. Circ_003686 binds to miR-142-5p

The potential regulatory ceRNA network predicted the potential regulatory relationship between novel_circ_003686 and miR-142-5p, and further bioinformatics predicted the potential binding site between circ_003686 and miR-142-5p (Fig. 3A). RT-PCR detection of miR-142-5p-mimic transfection efficiency suggested that miR-142-5p-mimic meaningfully increased miR-142-5p levels, and the transfection

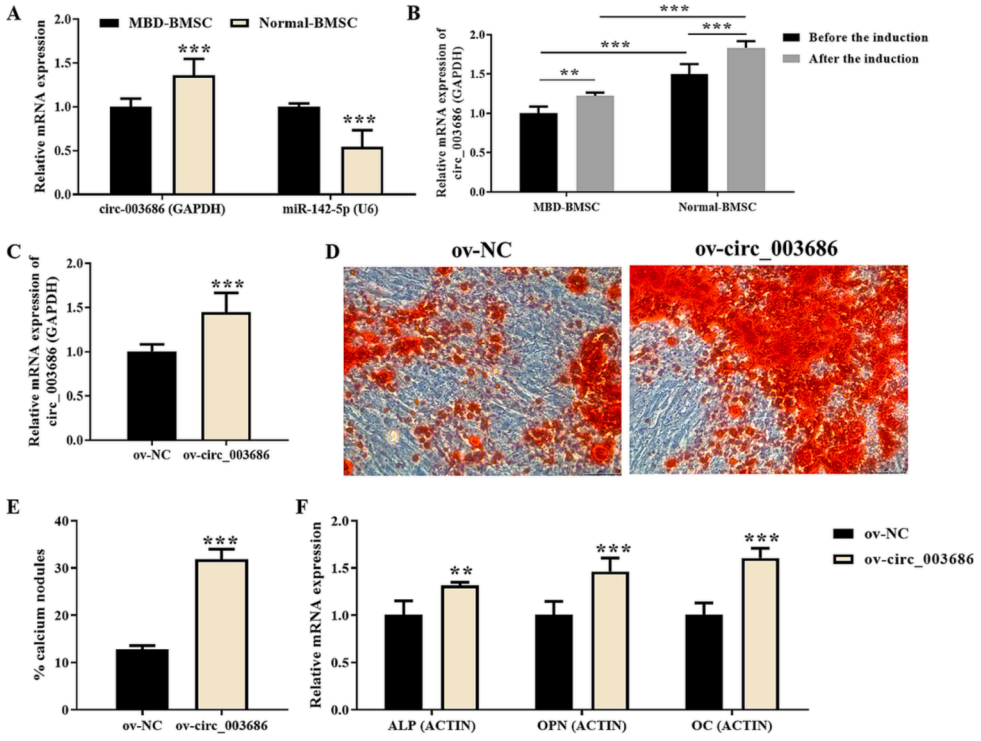

Fig. 2. Effects of circ_003686 on osteogenic differentiation of BMSCs. (A) RT-PCR was used to analyze circ_003686 and miR-142-5p level in MBD-BMSC and normal-BMSC, and GAPDH or U6 was the normalization standard. (B) Circ_003686 expression before and after osteogenic induction in MBD-BMSC and normal-BMSC was analyzed by RT-PCR. (C) Circ_003686 was overexpressed by the overexpression plasmid in MBD-BMSC, and the mRNA expression was detected by RT-PCR. (D) Alizarin red staining was used to analyze the effect of ov-circ_003686 on osteogenic differentiation. Magnification, ×100. (E) The mRNA levels of alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC) were detected by RT-PCR, and ACTIN was used as the normalization standard. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

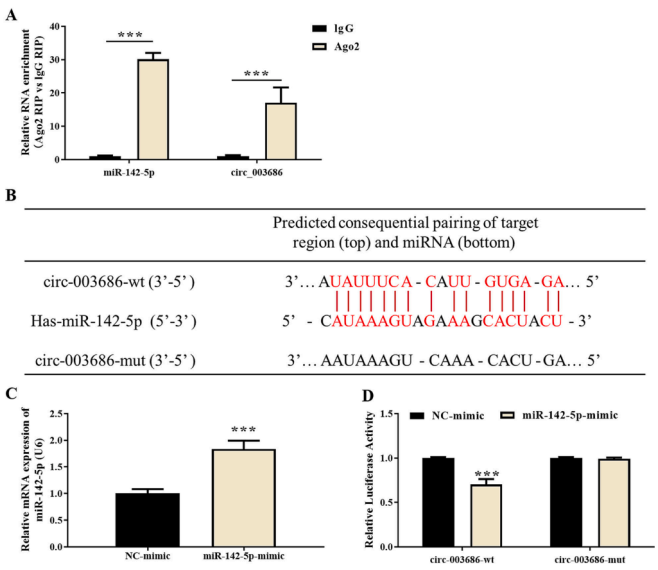

Fig. 3. Regulatory association between circ_003686 and miR-142-5p. (A) The binding site between circ_003686 and miR-142-5p. (B) After transfection with miR-142-5p-mimic, miR-142-5p level was detected by RT-PCR. (C) The binding of miR-142-5p to circ_003686 was verified by dual-luciferase reporter assay. (D) The enrichment levels of circ_003686 and miR-142-5p pulled down from Ago2 protein were analyzed by RIP. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

efficiency was high (Fig. 3B, $P < 0.001$). Luciferase experiments showned that miR-142-5p-mimic reduced the relative luciferase activity of circ_003686- wt, but has no effect on circ_003686-mut (Fig. 3C, $P < 0.001$), validating the binding relationship between circ_003686 and miR-142-5p. In addition, RIP assay was conducted for Ago2 protein, and RT-PCR analysis showed that Ago2 pellet enriched the expressions of endogenous circ_003686 and miR-142-5p pulled down from Ago2-expressed cells compared with the input lgG control (Fig. 3D, $P < 0.001$), confirming that circ_003686 could bind to miR-142-5p.

3.4. Circ_003686/miR-142-5p axis was involved in the osteogenic differentiation of BMSCs

In order to clarify the effects of circ_003686/miR-142-5p axis in osteogenesis-induced differentiation of BMSCs, ov-circ_003686, miR-142-5p-mimic/inhibitor were transfected into MBD-BMSC. Alizarin red staining showed that ov-circ_003686 meaningfully increased osteogenesis-induced differentiation ability of BMSCs, miR-142-5p-inhibitor also significantly improved osteogenic differentiation ability, while miR-142-5p-mimic significantly decreased osteogenic ability. And compared with ov-circ_003686 + NC-mimic group, co-transfection of ov-circ_003686 and miR-142-5p-mimic significantly reduced osteogenic ability (Fig. 4A). RT-PCR results also showed that transfection with ov-circ_003686 or miR-142-5p-inhibitor meaningfully rose the gene levels of ALP, OPN and OC, while the expression levels were significantly decreased after transfection with miR-142-5p-mimic. Meanwhile miR-142-5p-mimic also significantly reduced ov-circ_003686-induced ALP, OPN and OC gene expression (Fig. 4B–D, $P < 0.001$). These results indorsed that circ_003686/miR-142-5p axis was involved in osteogenesis-induced differentiation of BMSCs.

3.5. MiR-142-5p can bind to IGF1

Online TargetScan (https://www.targetscan.org/vert_72/) prediction indicated that miR-142-3p had a potential binding site with IGF1 mRNA (Fig. 5A). And co-transfection of miR-142-3p-mimic and IGF1-wt meaningfully reduced the activity of luciferase (Fig. 5B, $P < 0.001$), while transfection with IGF-mut did not change the activity of luciferase (Fig. 5B, $P > 0.05$), confirming that miR-142-3p could bind to IGF1 through the predicted binding site. In addition, after transfection with miR-142-3p-mimic, the IGF1 gene level was significantly decreased (Fig. 5C, $P < 0.001$), indicating that miR-142-3p targeted the binding site of IGF1 and negatively regulated its expression. To further demonstrate the role of IGF1, three siRNA oligonucleotides targeting IGF1 (si-IGF1) were used to interfere with IGF1 expression, and verify the transfection efficiency with WB and RT-PCR. The results presented that the three si-IGF1 could significantly decrease the protein and gene expression of IGF1, showing a good gene interference effect on IGF1 (Fig. 5D–F, $P < 0.001$).

3.6. Circ_003686/miR-142-5p/IGF1 axis regulates osteogenic differentiation of BMSCs

Consistent with previous results, alizarin red staining showed that transfection with miR-142-5p-inhibitor or ov-circ_003686 significantly increased osteogenic ability, and si-IGF1 significantly reduced the osteogenic differentiation ability. Similarly, si-IGF1 also significantly reduced the increase of osteogenic ability caused by miR-142-5p-inhibitor or ov-circ_003686 (Fig. 6A). In addition, RT-PCR results also showed that si-IGF1 significantly reduced the gene levels of ALP, OPN and OC compared with the si-NC group, and si-IGF1 + miR-142-5p-inhibitor or si-IGF1 + ov-circ_003686 significantly decreased the increase of ALP, OPN and OC gene expression caused by miR-142-5p-inhibitor or ov-circ_003686 (Fig. 6B–D, $P < 0.001$), confirming that circ_003686/miR-142-5p/IGF1 axis plays a vital role in osteogenesis-induced differentiation of BMSCs.

To further clarify the possible mechanism, the PI3K/AKT signaling pathway predicted by KEGG (Supplementary Fig. 3) was selected for analysis. And the results presented that miR-142-5p-inhibitor and ov-circ_003686 significantly increased the relative expression ratios of p-AKT/AKT, p-mTOR/mTOR and p-PI3K/PI3K compared with the NC group, while si-IGF1 meaningfully decreased the proteins expression of PI3K/AKT pathway. In addition, si-IGF1 + miR-142-5p-inhibitor or si-IGF1 + ov-circ_003686 also significantly reversed the increased of PI3K/AKT protein expressions caused by miR-142-5p-inhibitor or ov-circ_003686 (Fig. 6E–H, $P < 0.001$), confirming that circ_003686/miR-142-5p/IGF1 axis may regulate the ability of BMSCs to differentiate into osteoblasts by regulating PI3K/AKT signaling pathway.

4. Discussion

MBD is the main complications of MM [16]. The etiology of bone injury in MBD is osteoclast activation and osteoblast inhibition caused by tumor cell infiltration and bone marrow microenvironment changes [17]. BMSCs are important cellular components of bone marrow microenvironment and have the capability to differentiate into adipocytes, osteoblasts and chondrocytes [18]. As the main source of osteoblasts, promoting the osteogenesis-induced differentiation of BMSCs may be one of the feasible methods for the treatment of MBD [19]. In this study, BMSCs were isolated from MBD patients and normal participants, and both MBD-BMSC and normal-BMSC could successfully induced osteogenic differentiation. Circ_003686 as a novel circRNA, this research confirmed that it was significantly lower expressed in MBD-BMSC, and its expression was significantly increased after osteogenic differentiation of both MBD-BMSC and normal-BMSC. In addition, it was found that overexpression of circ_003686 could significantly encourage BMSCs differentiation into osteoblasts, and increase the expression of osteogenic differentiation genes ALP, OPN and OC, suggesting that circ_003686 plays a vital role in MBD.

CircRNA is a kind of single-stranded RNA molecule with closed ring structure, which is closely related to cell proliferation, differentiation and other life activities [20]. Previous studies have revealed a close relationship between CircRNA and MM [21]. CircRNA circ_0000190 can inhibit the development of MM by controlling miR-767-5p/MAPK4

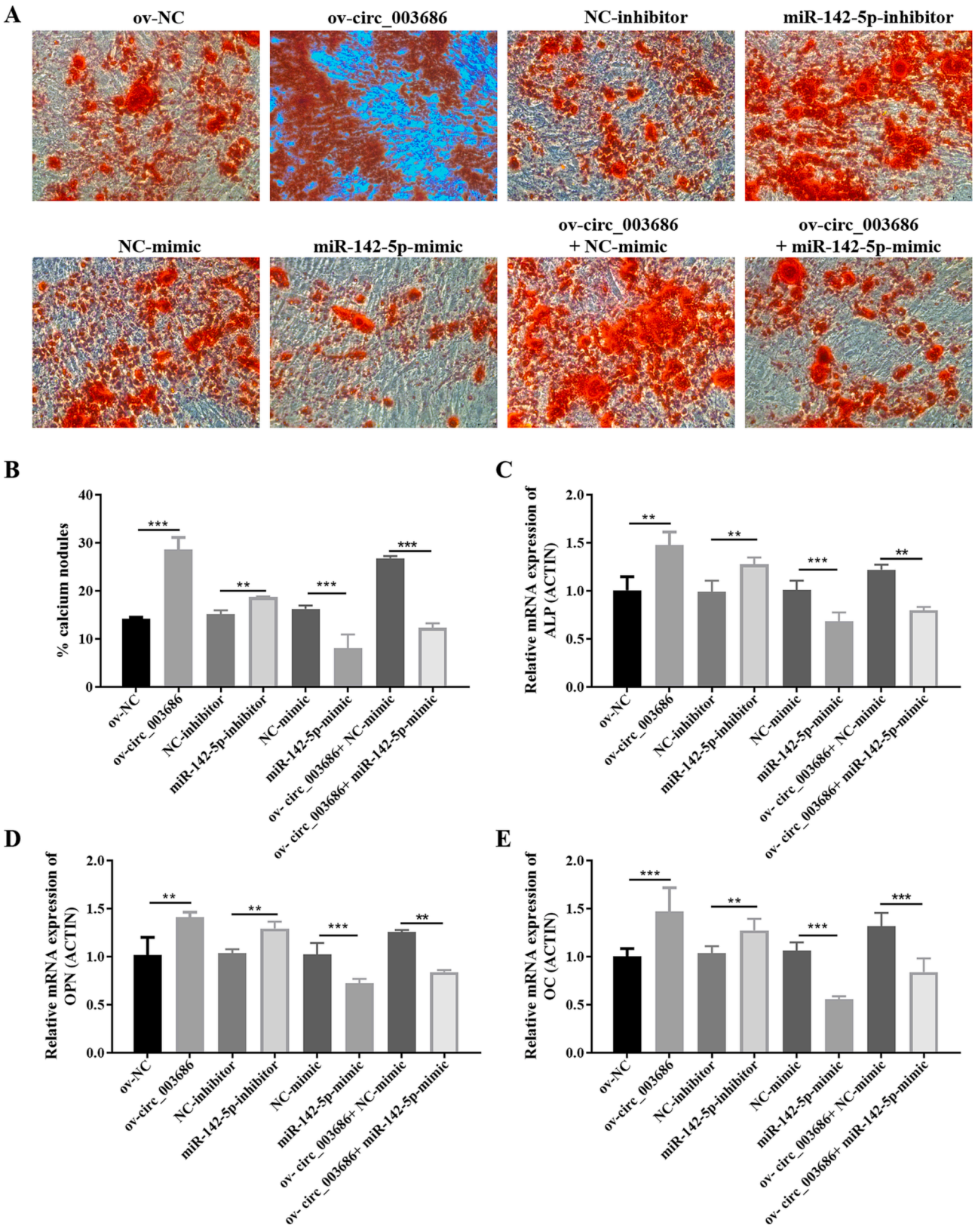

Fig. 4. Circ_003686/miR-142-5p axis was involved in the osteogenic differentiation of BMSCs. (A) Alizarin red staining was used to analyze osteogenesis-induced differentiation ability. The mRNA expressions of alkaline phosphatase (ALP) (B), osteopontin (OPN) (C) and osteocalcin (OC) (D) were detected by RT-PCR, and ACTIN as the normalization standard. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

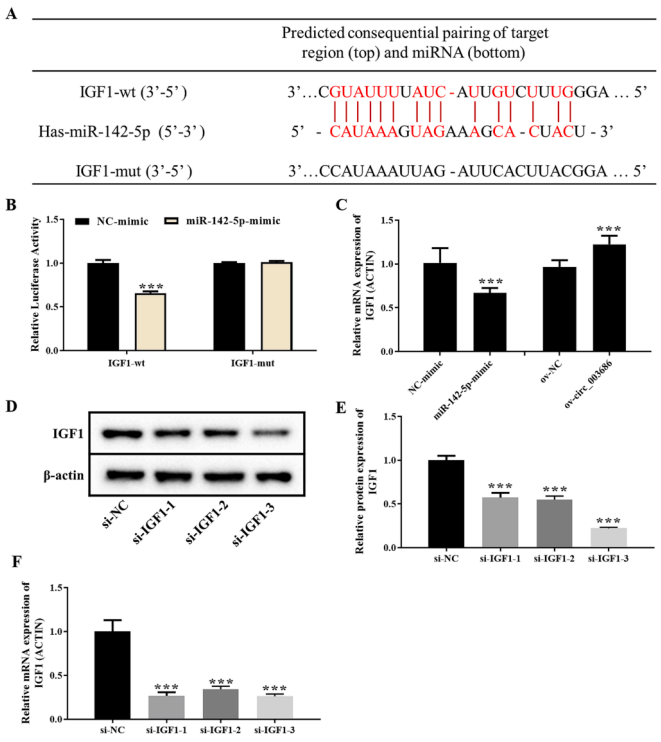

Fig. 5. Regulatory relationship between miR-142-3p and IGF1. (A) Online TargetScan (https://www.targetscan.org/vert_72/) suggests that miR-142-3p may bind to 3′ untranslated region (3′UTR) of IGF1 mRNA. (B) The binding of miR-142-3p to IGF1 was verified by dual-luciferase reporter assay. (C) IGF1 mRNA expression after miR-142-3p-mimic treatment was examined by RT-PCR analysis. (D-E) The IGF1 was silencing by si-IGF1-1, si-IGF1-2 or si-IGF1-3 in MBD-BMSC, and the protein expression was observed by WB. (F) IGF1 mRNA expression after IGF1 was silenced was examined by RT-PCR. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

pathway [22], and circBUB1B_544aa can aggravate MM by causing chromosomal instability [23]. Different CircRNA play different roles in MM, but their role in MBD is relatively less studied. It is generally considered that CircRNA functions through sponge adsorption of miRNA [24]. In the early stage of this research, novel_circ_003686 with abnormally low expression in MBD-BMSCs was screened out by RNA-seq sequencing analysis (Supplementary Fig. 1 and Supplementary Table1), and it was also prophesied that circ_003686 had a potential regulatory relationship with mir-142-5p (supplementary materials S1). This research experimentally confirmed that circ_003686 was significantly under-expressed in MBD-BMSCs and significantly overexpressed after osteogenic induction. Overexpression of circ_003686 can promote osteogenic differentiation of BMSCs. Similarly, we confirms that circ_003686 has a potential binding effect with miR-142-5p. Researched have confirmed that miR-142-5p exist an imperative role in control profibrogenic macrophage program [25], cervical cancer progression [26] and retinoblastoma [27]. In addition, miR-142-5p is also closely connected with bone metabolism and can promote the differentiation of osteoclast of bone marrow-derived macrophages [28]. And miR-142-5p could also promote bone repair by preserving osteoblast activity [29]. The present study found that miR-142-5p-inhibitor promoted osteogenic differentiation of BMSCs, while miR-142-5p-mimic inhibited osteogenic differentiation and reversed the promoting effect of circ_003686. These results suggested that circ_003686 may promote the osteogenic differentiation of MBD-BMSC through sponge adsorption of miR-142-5p. In addition, studies have shown that IGF1 is a downstream gene of miR-142-5p, and the miR-142-5p/IGF1 axis plays an vital role in diabetic retinopathy [30], periodontitis [31] and non-small cell lung cancer [32]. Similarly, this research established the potential communication between miR-142-5p and IGF1, and miR-142-5p could bind to IGF1 and negatively regulate its expression in MBD-BMSC.

IGF1 is a polypeptide substances with growth-promoting effect, belonging to the IGF family, and its secreting cells are widely distributed in many tissues of human body [33]. IGF has strong growth-promoting effect and regulates normal growth and differentiation in vivo [34]. Dysregulation of the IGF system has been demonstrated in the occurrence and progression of various types of cancers [35], and targeting the IGF axis can be used as a possible target for cancer treatment [36]. IGF1 can participate in the homing and invasion of MM cells, continuous proliferation, resistance to cell death and angiogenesis [37]. In addition, IGF1 has been proved to be an important protein in bone metabolism and plays a promoting effect in the process of osteogenic differentiation [38,39]. Our research found that si-IGF1 meaningfully repressed the osteogenic differentiation of MBD-BMSC, suggesting that IGF1 plays an imperative role in MBD. And si-IGF1 reversed the promoting effects of ov-circ_003686 and miR-142-5p-inhibitor on osteogenic differentiation, which was confirmed that circ_003686/miR-142-5p/IGF1 axis plays an imperative role in regulating osteogenic differentiation of MBD-BMSC. In addition, this study also confirmed that circ_003686/miR-142-5p/IGF1 axis significantly regulated the protein expressions of PI3K/AKT pathway, a differential expression signaling pathway predicted by KEGG. Numerous researches have confirmed the role of PI3K/AKT pathway in osteogenesis-induced differentiation [40]. It has also been shown that IGF-1 can reverse the inhibitory effect of dexamethasone on the osteogenesis-induced differentiation of BMP9-induced embryonic fibroblasts of mouse through the PI3K/AKT pathway [41]. Thus, the PI3K/AKT pathway may be a downstream pathway of circ_003686/miR-142-5p/IGF1 axis controlling the osteogenesis-induced differentiation of MBD-BMSC.

5. Conclusion

In conclusion, circ_003686 was significantly under-expressed in MBD-BMSC, while miR-142-5p was over-expressed in BMD-BMSC. Ov-circ_003686 significantly promoted osteogenic differentiation and improved the gene expression of ALP, OPN and OC in BMD-BMSC. In addition, circ_003686 was confirmed to bind miR-142-5p. MiR-142-5p-inhibitor significantly encouraged osteogenic differentiation, and miR-142-5p-mimic inhibited osteogenic differentiation and reversed the promoting effect of ov-circ_003686. Further studies discovered that IGF1 was a downstream gene of miR-142-5p, and miR-142-5p could bind to IGF1 and negatively regulate its expression. Si-IGF1 could significantly inhibit osteogenic differentiation and reverse the promoting effects of miR-142-5p-inhibitor and ov-circ_003686. In addition, this study also showed that circ_003686/miR-142-5p/IGF1 axis meaningfully controlled the relative expression ratios of PI3K/AKT pathway. Our study discovered that circ_003686/miR-142-5p/IGF1 axis plays an important role in regulating osteogenesis-induced differentiation of BMSCs.

Author's contributions

Q.L, M.L. and C.D designed the experiments and analyzed data. C.J. Z.N. performed the clinical samples. Q.L. C.D. performed isolation and identification of BMSCs. Q.L. M.L. performed osteogenic differentiation and Alizarin red S staining of BMSCs. C.D. Y.H. performed RT-PCR, cell transfection and dual-luciferase reporter assay. Q.L. W.X. performed RNA immunoprecipitation and WB analysis. and W.X. performed the public dataset analysis. and F.FY. supervised the whole study and wrote the paper. The author(s) read approved the final manuscript.

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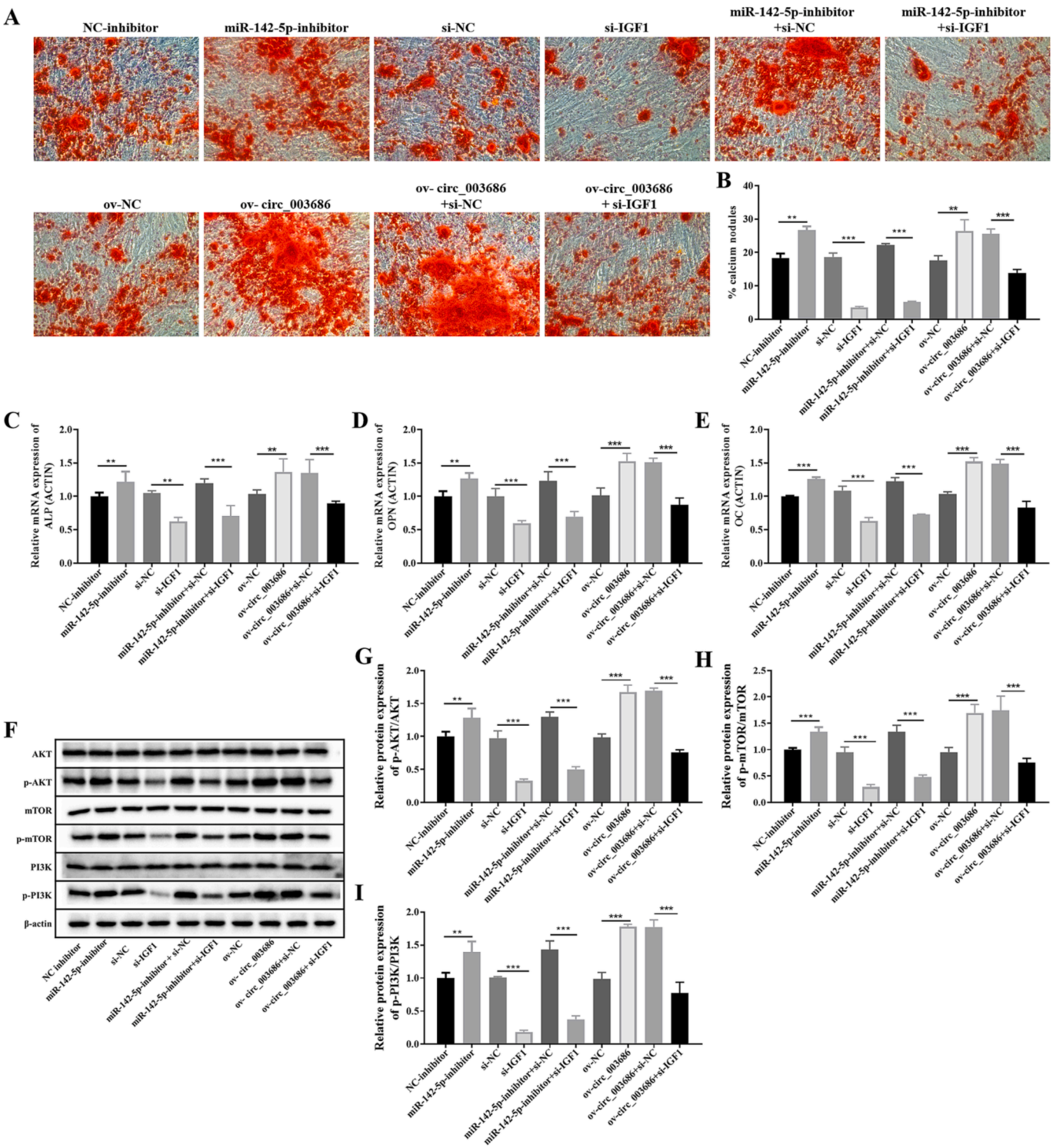

Fig. 6. Circ_003686/miR-142-5p/IGF1 axis regulates osteogenic differentiation of BMSCs. (A) Alizarin red staining was used to analyze osteogenesis-induced differentiation ability. The mRNA expressions of alkaline phosphatase (ALP) (B), osteopontin (OPN) (C) and osteocalcin (OC) (D) were detected by RT-PCR, and ACTIN was applied as the normalization standard. (E) The protein expressions of AKT-PI3K signaling pathway were detected by WB, and the densitometry of p-AKT/AKT (F), p-mTOR/mTOR (G) and p-PI3K/PI3K (H) was analyzed. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Ethics approval and consent to participate

Prior patient consent and approval were obtained from the Institutional Medical Research Ethics Committee of the General Hospital of Western Theater Command (Chengdu, China).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbo.2023.100509.

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