BMC Musculoskeletal Disorders

RESEARCH

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Loss of BACH1 improves osteogenic differentiation in glucocorticoid-induced hBMSCs through restoring autophagy

ShuYing Xiao[1], GuoJuan Li[1], MeiHua Tan[1], Wen Liu[1] and WenJin Li[2*]

Abstract

Background Glucocorticoid-induced osteoporosis (GIOP) is the most common type of secondary osteoporosis. Recently, autophagy has been found to be related with the development of various diseases, including osteoporosis and osteoblast differentiation regulations. BTB and CNC homology 1 (BACH1) was a previously confirmed regulator for osteoblast differentiation, but whether it's could involve in glucocorticoid-induced human bone mesenchymal stem cells (hBMSCs) differentiation and autophagy regulation remain not been elucidated.

Methods hBMSCs were identified by flow cytometry method, and its differentiation ability were measured by ARS staining, oil O red, and Alcian blue staining assays. Gene and proteins were quantified via qRT-PCR and western blot assays, respectively. Autophagy activity was determined using immunofluorescence. ChIP and dual luciferase assay validated the molecular interactions.

Results The data revealed that isolated hBMSCs exhibited positive of CD29/CD44 and negative CD45/CD34. Moreover, BACH1 was abated gradually during osteoblast differentiation of hBMSCs, while dexamethasone (Dex) treatment led to BACH1 upregulation. Loss of BACH1 improved osteoblast differentiation and activated autophagy activity in Dex-challenged hBMSCs. Autophagy-related proteins (ATG3, ATG4, ATG5, ATG7, ATG12) were repressed after Dex treatment, while ATG3, ATG7 and BECN1 could be elevated by BACH1 knockdown, especially ATG7. Moreover, BACH1 could interact ATG7 promoter region to inhibit its transcription. Co-inhibition of ATG7 greatly overturned the protective roles of BACH1 loss on osteoblast differentiation and autophagy in Dex-induced hBMSCs.

Conclusion Taken together, our results demonstrated that silencing of BACH1 mitigated Dex-triggered osteogenic differentiation inhibition by transcriptionally activating ATG7-mediated autophagy, suggesting that BACH1 may be a therapeutic target for GIOP treatment.

Highlight

- BACH1 was gradually reduced during osteogenic differentiation and induced by Dex.
- Loss of BACH1 impaired Dex-induced osteogenic differentiation and autophagy inhibition.
- BACH1 transcriptionally inhibited ATG7 expression.

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• The helpful effect of BACH1 silence on osteogenic differentiation and autophagy in Dex-induced BMSCs were reversed by ATG7 inhibition.

Keywords Glucocorticoid-induced osteoporosis, Osteoblast differentiation, BACH1, Autophagy, ATG7

Introduction

Osteoporosis (OP) is a kind of systematic disease characterized by a decline in bone mass and density,, leading to an elevated fracture risk, significant morbidity, mortality, and a substantial public health burden [1]. This systemic disease is further categorized into primary and secondary OP. Primary OP, particularly postmenopausal OP, arises from numerous endogenous factors, primarily hormonal imbalances such as deficiencies in estrogen or vitamin D, coupled with secondary hyperparathyroidism [2]. Turning to secondary OP, glucocorticoid-induced osteoporosis (GIOP) emerges as the predominant subtype [3]. Research has underscored that individuals on oral glucocorticoids face nearly tripled risks of vertebral fractures and double the risk of hip fractures [4]. Contemporary studies have illuminated various mechanisms through which glucocorticoids instigate osteoporosis, implicating the dysregulation of bone formation [5], bone resorption and bone metabolisms [6]. However, the intricate pathophysiology underlying the development of GIOP by glucocorticoids remains to be fully elucidated.

Autophagy, a cellular regulatory process involving the lysosomal degradation of cytoplasmic components [7], is vital for maintaining cellular homeostasis and safeguarding against stress induced by infections, nutritional imbalances, and metabolic disorders. Its significance extends to the pathology of numerous diseases, including osteoporosis [8]. Recently, accumulating evidence underscored the pivotal function of autophagy in governing the progression of GIOP [9]. For instance, Huang et al's work demonstrated that geniposide administration could alleviate OP by activating osteoblastic autophagy, thereby preventing from glucocorticoid-induced apoptosis, endoplasmic reticulum stress and bone loss [10]. Similarly, the activation of AMPK/mTOR/p70S6K signaling remarkably promotes osteoblast autophagy to prevent cells from dexamethasone (Dex)-induced apoptosis [11]. Of utmost importance, the activation of autophagy in bone mesenchymal stem cells (BMSCs) could greatly counteract Dex-triggered osteogenic differentiation inhibition, leading to the improvement of GIOP [12]. Uncovering the intricate molecular controls governing autophagy in GIOP is imperative and may pave the way for identifying therapeutic targets for clinical management of this condition.

BTB and CNC homology 1 (BACH1), a member of the Cap 'n' Collar and basic region leucine zipper family (CNC-bZip) of transcription factors, is ubiquitously expressed in mammalian cells and functions primarily as a transcriptional repressor [13]. It has been documented that BACH1 regulates a broad spectrum of cellular and molecular activities, encompassing cell cycle, metabolism, oxygen species production, heme homeostasis, and immune responses, etc [14]. A wealth of literature positions have shown that BACH1was a key transcription factor upstream of HO-1 [15, 16], with HO-1 and its associated Nrf2 signaling cascade being recognized as key facilitators of autophagy induction [17]. Intriguingly, depletion of BACH1 in osteoarthritis mice was accompanied by autophagy-related LC3B elevation [18], implying the potential involvement of BACH1 in autophagy regulation. Moreover, silencing BACH1 play an antioxidant role, shielding steoblast from oxidative stress damage [19], while concurrently inhibiting osteoclastogenesis and bone loss [20, 21]. However, the exact roles and mechanism of BACH1 in GIOP, and specifically whether its modulatory actions interface with autophagic pathways, warrant further investigation.

In the present study, hBMSCs was isolated and subjected to Dex treatment to construct a GIOP cellular model in vitro. Further experiments demonstrated that BACH1 was suppressed during osteogenic differentiation of hBMSCs, and is further induced after Dex administration. Moreover, ablation of BACH1 greatly impeded Dex-triggered inhibition of osteogenic differentiation and autophagy. Delving into the underlying mechanisms, autophagy-related gene-7 (ATG7) was proved to be a transcriptionally target of BACH1 in hBMSCs. Overall, our data uncovered that BACH1 restrained osteogenesis in GIOP by transcriptionally suppressing ATG7-activated autophagy, pinpointing a promising new molecular target for therapeutic intervention in GIOP management.

Materials and methods

Isolation and characterization of human BMSC

Bone marrow samples were kindly furnished by three hospitalized patients (comprising 1 male and 2 females, aged between 30 and 48 years) diagnosed with femoral head necrosis based on MRI assessments at The Affiliated Nanhua Hospital of Hengyang Medical College, University of South China. Inclusion criteria mandated the absence of femoral head height loss, whereas patients presenting with a history of trauma, cardiovascular diseases, or evidence of tumor infiltration were deliberately excluded from this investigation. All the experimental procedures have been approved by the ethics committee of The Affiliated Nanhua Hospital, Hengyang Medical School, University of South China, which was conducted in accordance with the principles of the Declaration of

Helsinki, and all participants in the study gave a written informed consent.

For hBMSCs isolation, approximately 15 mL of bone marrow samples were obtained from healthy donors under aseptic conditions. Then, BMSCs was isolated by Percoll solution (Sigma-Aldrich, St. Louis, MO, USA) and growth in complete DMEM medium (Gibco, Carlsbad, USA) in a humidified incubator with 5% CO_2 for two days. The culturing medium was removed and supplied with a fresh medium every 48 h. When the cell density reached 80 ~ 90%, BMSCs were digested with 0.25% trypsin and cultured by passage.

For the characterization of BMSCs, the BMSCs at passage 3 were digested and collected for flow cytometry analysis. After washing and resuspending by PBS solution, BMSCs (1×10^5 cells) were stained with the antibodies of CD29 (Cat #14-0299-82), CD44 (Cat#12-0441-82), CD45 (Cat#12-0451-82) or CD34 (Cat#11-0349-42) for 30 min in a dark condition, respectively. After incubation, the stained BMSCs were analyzed by flow cytometry using a Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL, USA). All antibodies were acquired from Thermo Fisher Scientific (Waltham, MA, USA).

Differentiation potential of hBMSCs Validation•

Osteogenic Differentiation: hBMSCs were incubated in in osteogenesis induction medium (complete DMEM supplemented with 100 ng/mL recombinant human BMP-2 (R&D systems, Minneapolis, MN, USA)) for different times (0, 3, 7, 14 days), with media refreshment every 2–3 days. Briefly, after osteogenic induction, hBMSCs were harvested and washed with PBS, followed by an immobilization with 10% neutral formalin for 20 min. Then, the cells were incubated with Alizarin red staining solution (Sigma-Aldrich) for 10 min. Finally, the positive cells were observed and imaged by an inverted microscope (IXplore standard, Olympus, Japan).

Adipogenic Differentiation: hBMSCs were cultured in adipogenic Induction Medium (AIM, Cyagen Biosciences), changing the medium every 3 days over a 21-day period. Cells were then fixed with 4% PFA, stained with Oil Red O (Solarbio) for 15 min, and examined under an inverted microscope to observe lipid droplet formation.

Chondrogenic Differentiation: hBMSCs were seeded in Mesenchymal Stem Cell Chondrogenic Differentiation Medium (Cyagen Biosciences) for 21 days, with medium renewal every 3 days. Following incubation, cells were stained with Alcian Blue 8GX (Sigma-Aldrich) for 1 h, rinsed, and inspected under an optical microscope (Olympus) to confirm chondrogenesis.

Table 1 The primer sequences used in qRT-PCR

Gene name	Primer sequences
ALP forward	CCTGGAGAAGGGTGGTTTCC
ALP reverse	TTTGGCTCTCGACCAGTGTC
RUNX2 forward	CGCCTCACAAACAACCACAG
RUNX2 reverse	TCACTGTGCTGAAGAGGCTG
OCN forward	GGCAGCGAGGTAGTGAAGAG
OCN reverse	CTAGACCGGGCCGTAGAAG
BACH1 forward	TGCACAAGGCTGTCGATCAT
BACH1 reverse	GTGTTTGTCTCCAACACGGC
ATG3 forward	GATTAGTGGCAAAATGGCTGTT
ATG3 reverse	GAGCTACTGCTACACATGGGG
ATG4 forward	GACTGGGAAGATGGACGCAG
ATG4 reverse	ATCATCTGTCCACACCGCAG
ATG5 forward	GAAGCTGTTTCGTCCTGTGG
ATG5 reverse	TCTGTTGGCTGTGGGATGAT
ATG7 forward	TGCCTCACCAGGTTCTTGAT
ATG7 reverse	CGCTCATGTCCCAGATCTCA
ATG12 forward	CTGGAGGGGAAGGACTTACG
ATG12 reverse	ACTTCTTGGTCTGGGGAAGG
BECN1 forward	GAGAACCTCAGCCGAAGACT
BECN1 reverse	CCTCTAGTGCCAGCTCCTTT
GAPDH forward	CCAGGTGGTCTCCTCTGA
GAPDH reverse	GCTGTAGCCAAATCGTTGT

Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted using TRIzol™ regent (Invitrogen, Carlsbad, CA, USA). The RNA was reverse-transcribed using Superscript IV reverse transcriptase (Thermo Fisher Scientific), according to the manufacturer's instructions. qRT-PCR was performed by a One Step SYBR® Ex Taq qRT-PCR Kit (Takara, Dalian, China), using an Applied Biosystems 7000 (Life Technologies, Gaithersburg, MD, USA). The primers were customized ordered from Genwitz (Genwitz, Suzhou, China). The primer sequences were provided in Table 1.

Western blot

After indicated treatment, hBMSCs were collected and lysed in cell lysis buffer (Thermo Fisher Scientific) containing protease/ phosphatase inhibitors. Post-centrifugation, cell supernatants were collected, and the protein contents were determined using a BCA protein assay kit (Thermo Fisher Scientific). Protein samples were separated using 10% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (PVDF, Thermo Fisher Scientific). Membranes were blocked with TBS-T (5% milk in TBS containing 0.1% Tween 20) for 60 min at room temperature. Afterwards, the membranes were incubated with primary antibodies overnight at 4 °C. The primary antibodies including BACH1 (Cat #ab128486, 1:1000, Abcam, Cambridge, UK), β-actin (Cat#ab8227, 1:2000, Abcam), LC3II/I (Cat#ABC929, 1:1000, Sigma-Aldrich) and ATG7 (Cat# ab52472, 1:1000, Abcam). After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG (ab288151, 1:5000, Abcam) for 1 h at room temperature. The band signals

were enhanced using a chemiluminescence system (Bio-Rad, California, USA). The band of immune blotting was imaged by iBright Analysis Software (Thermo Fisher Scientific) and quantified by Image J software.

Immunofluorescence staining

Following varied treatments, hBMSCs underwent a series of procedures: triple rinsing with PBS, fixation in 4% paraformaldehyde for 10 min, and subsequent incubation in blocking buffer (3% BSA in PBS) for 1 h at room temperature. Next, cells were incubated with indicated primary anti-LC3B (Cat#ab48394, Abcam) for 2 h at 4 °C. After washing by PBS, cells were incubated with secondary goat Anti-Rabbit IgG H&L, Alexa Fluor® 488) (Abcam, Cat#ab150077) for 1 h. Fluorescence images from 5 fields/well were scanned using an automated microscope IN Cell Analyzer 6000 (GE Healthcare, Bucks, UK).

shRNA for gene knockdown

The short hairpin RNAs (shRNAs) against for BACH1 (shBACH1) and ATG7 (shATG7), as well as their negative control (shNC) were all customized ordered from Genscript (Nanjing, China). Then, the above shRNAs were inserted into pLVX-Puro plasmids (Takara, Tokyo, Japan) to construct recombinant plasmids. Subsequently, the lentiviral plasmids were co-transfected alongside packaging vectors (psPAX2) into 293T cells to facilitate amplification. After a 48-hour incubation period, the cell supernatant, enriched with recombinant lentivirus, was harvested. Afterwards, the recombinant lentivirus plasmids were further transfected into cardiomyocytes by using Lipofectamine 2000 (Thermo Fisher Scientific).

Dual luciferase assay

The transcriptional relationship between ATG7 and BACH1 were validated by dual luciferase assay. The ATG7 promoter region was cloned into pGL3 luciferase vector (Promega, Madison, WI, USA) to synthesized a recombinant luciferase pGL3 plasmids. The empty pGL3 or recombinant pGL3-ATG7 were co-transfected with lentivirus packing sh-BACH1 or shNC into hBMSCs, respectively. Post-of 48 h transfection, cells were harvest, and the luciferase activities from hBMSCs were tested by a Dual-Luciferase® Reporter Assay System (Promega) according to the instructions of manufacturer.

Chromatin immunoprecipitation (ChIP) assay

hBMSCs were harvested and a chromatin immunoprecipitation assay was performed using ChIP Assay Kit (Millipore, Billerica, MA, USA) according to the manufacturer's protocol. hBMSCs were treated with formaldehyde to cross-link protein and DNA, and the chromatin was then fragmented by ultrasound. The cross-linked chromatin was immunoprecipitated with the BACH1 antibody (Cat# ab264132, Abcam) or IgG isotype control (Cat # ab172730, Abcam). The immunoprecipitated DNA was extracted and determined by qRT-PCR assay.

Data analysis

All data were shown as Mean ± SD value and analyzed by GraphPad Prism 6.0 software. Data comparison between two group was analyzed by Student t test, and data comparison among multiple groups were analyzed by One-way followed by Tukey's post hoc test. *P* values of the plotted data ≤ 0.05 were considered statistically significant. All statistical analyses were generated based on at least three independent experiments.

Results

BACH1 was gradually suppressed during osteogenic differentiation of hBMSCs

Human BMSCs were isolated from healthy donors, and and cultivated under in vitro conditions for propagation and expansion. hBMSCs were authenticated through flow cytometry by using antibodies against the markers. The results indicated the hBMSCs were positive for CD29 and CD44, while being negative for CD45 and CD34 (Fig. 1A). Furthermore, hBMSCs have also been demonstrated to possess the capacity for osteogenic, adipogenic, and chondrogenic differentiation (Fig. 1B). Next, hBMSCs were induced osteogenic differentiation for 0, 3, 7, 14 days. As shown in Fig. 1C, ARS staining suggested a significant mineralized nodule positive in hBMSCs after 7- and 14-days osteogenic induction. qRT-PCR test also displayed the notable up-regulations of ALP, RUNX2 and OCN expressions in a time dependent manner after osteogenic induction (Fig. 1D). Interestingly, we found that the expression of BACH1 was gradually down-regulated during the process of osteoblast differentiation (Fig. 1E-F).

Loss of BACH1 lightened Dex induced-suppression of hBMSCs osteoblast differentiation

Here, hBMSCs were stimulated with Dex to mimic GIOP cellular model in vitro. As indicated by qRT-PCR assay, a great increase of BACH1 mRNA level was observed upon Dex exposure (Fig. 2A). To validate the role of BACH1 in Dex-induced osteoblast suppression, BACH1 was knocked down by transfection with lentivirus-packed shBACH1. As shown Fig. 2B-C, the expression of BACH1 was knocked down significantly by shRNA-BACH1 transfection in hBMSCs. Next, ARS staining demonstrated that Dex treatment led to the decreased mineralized nodule deposition, while the inhibitory role was greatly reversed in the presence of shBACH1 (Fig. 2D). Similarly, the osteogenic differentiation-related gene markers including ALP, Runx2 and OCN were markedly

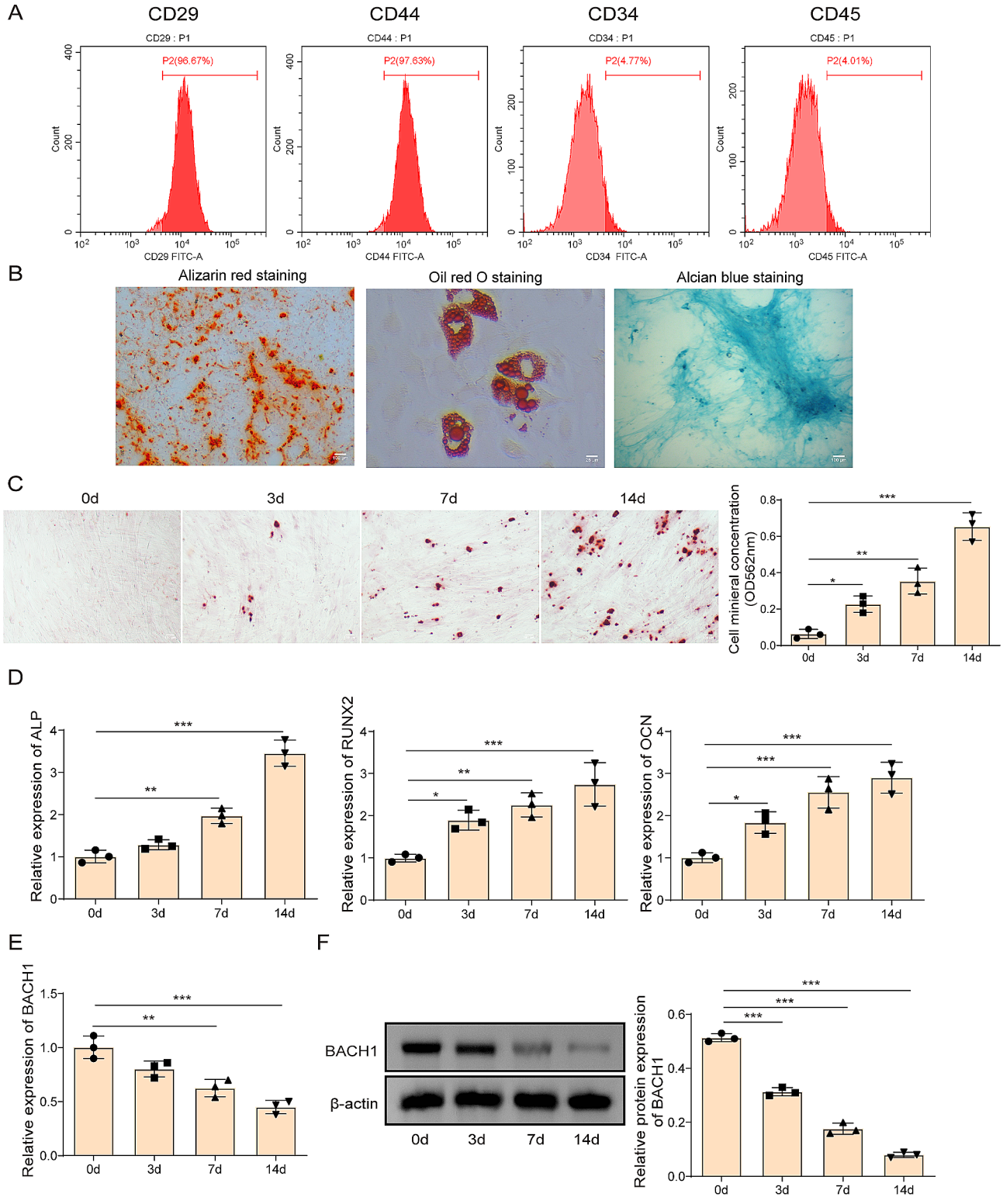

Fig. 1 BACH1 was gradually suppressed during osteogenic differentiation of hBMSCs. (**A**) hBMSCs were isolated and the cell surface markers including CD29, CD44, CD45 and CD34 were characterized by flow cytometry. (**B**) Representative micrographs showing osteogenic, adipogenic, and chondrogenic differentiation of hBMSCs at passage 3 by alizarin red staining assay, Oil red O staining, and Alcian blue staining assay, respectively. hBMSCs were cultivated in osteogenic induction medium for 0, 3, 7 days and 10 days, respectively. (**C**) Osteoblast differentiation of hBMSCs were measured by Alizarin Red staining. (**D**) The mRNA expressions of ALP, Runx2 and OCN were measured by qRT-PCR. (**E**) qRT-PCR was performed for measurement of BACH1 mRNA expression. (**F**) Protein expression of BACH1 was measured by western blot. Statistical data are presented as Mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

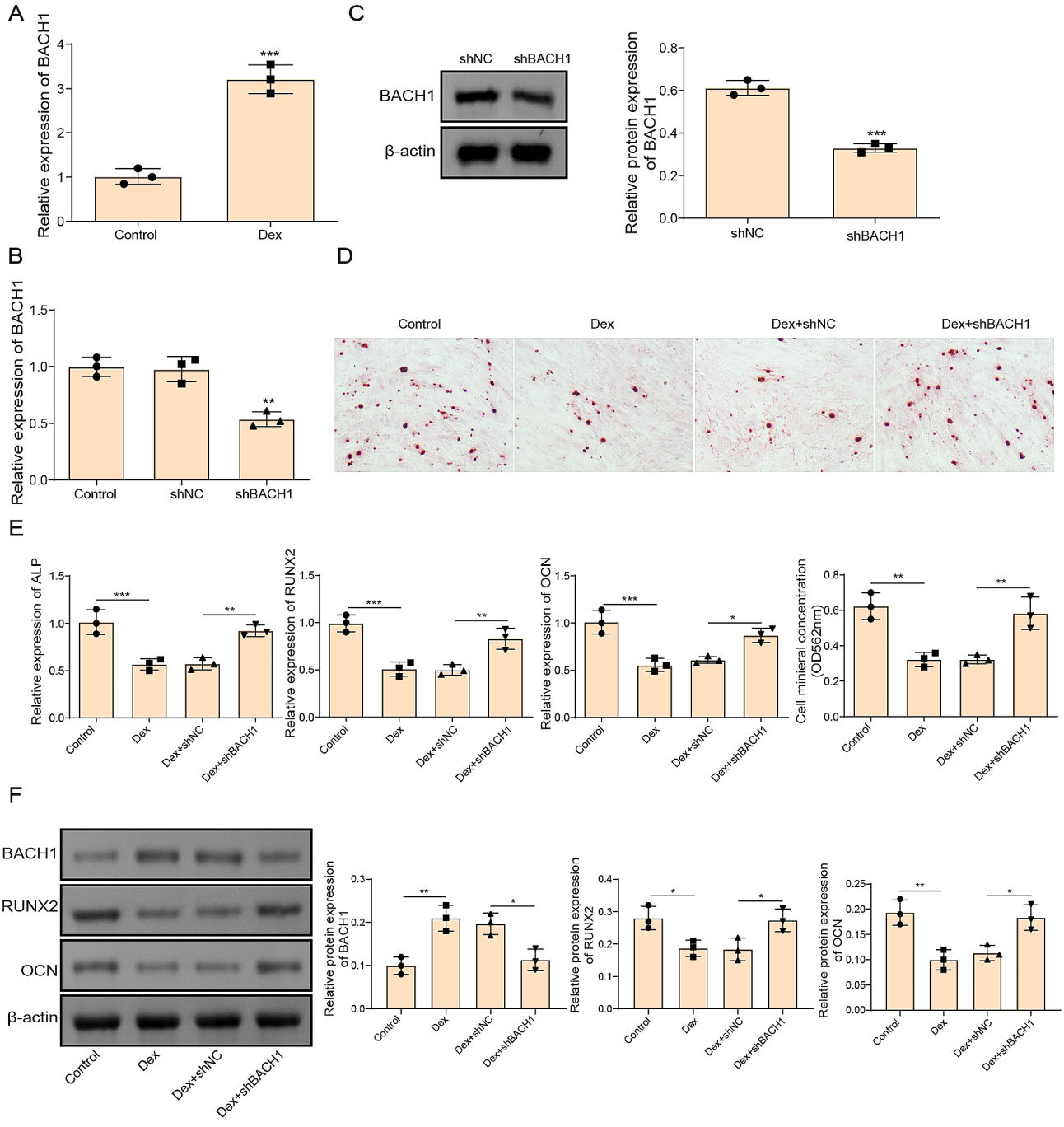

Fig. 2 Loss of BACH1 lightened Dex induced-suppression of hBMSCs osteoblast differentiation. (**A, B**) hBMSCs were stimulated by 10 μM Dex for 48 h, and then the mRNA level of BACH1 was revealed by qRT-PCR. (**B**) hBMSCs were transfected with lentivirus-packed shNC or shBACH1, and the transfection efficiency was evidenced by qRT-PCR. (**C**) The protein level of BACH1 in hBMSCs with shNC or shBACH1 transfection were determined by western blot. hBMSCs with/without BACH1 knockdown were exposed to Dex stimulation. After osteogenic induction, the following experiments were arranged. (**D**) Alizarin Red staining assessed the osteogenic differentiation of hBMSCs. (**E**) qRT-PCR measured the mRNA levels of ALP, Runx2 and OCN. (**F**) Western blot was conducted for detecting the protein levels of BACH1, Runx2 and OCN. Statistical data are presented as Mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

repressed by Dex, and BACH1 knockdown further strikingly reversed these alterations (Fig. 2E-F). These results suggested that BACH1 depletion relieved Dex induced inhibitory role on osteoblast differentiation.

BACH1 enhanced Dex induced suppression of osteogenic differentiation via repressing autophagy

In this part, we objected to explore whether the regulatory roles of BACH1 on osteogenic differentiation was related the autophagy. Firstly, the level of autophagy

marker LC3B was measured by immunofluorescence. As demonstrated in Fig. 3A, the level of LC3B in hBMSCs was suppressed by Dex treatment, however, it could be reversed by BACH1 knocked down. Western blot assay depicted that the protein expression of LC3II/I was suppressed by Dex treatment, and this change was abolished obviously by BACH1 loss (Fig. 3B). Next, we evaluated the expression changes of several autophagy associated proteins (ATG3, ATG4, ATG5, ATG7, ATG12 and BECN1) in hBMSCs. The results indicated that Dex treatment led to the decreases of ATG3, ATG4, ATG5, ATG7, ATG12 and BECN1 mRNAs, while these alterations were remarkably restored after BCAH1 reduction, especially of ATG7 (Fig. 3C-H). These data implied that the inhibition of BACH1 on Dex-induced osteogenic differentiation is potentially linked to the reduction of autophagy-related genes.

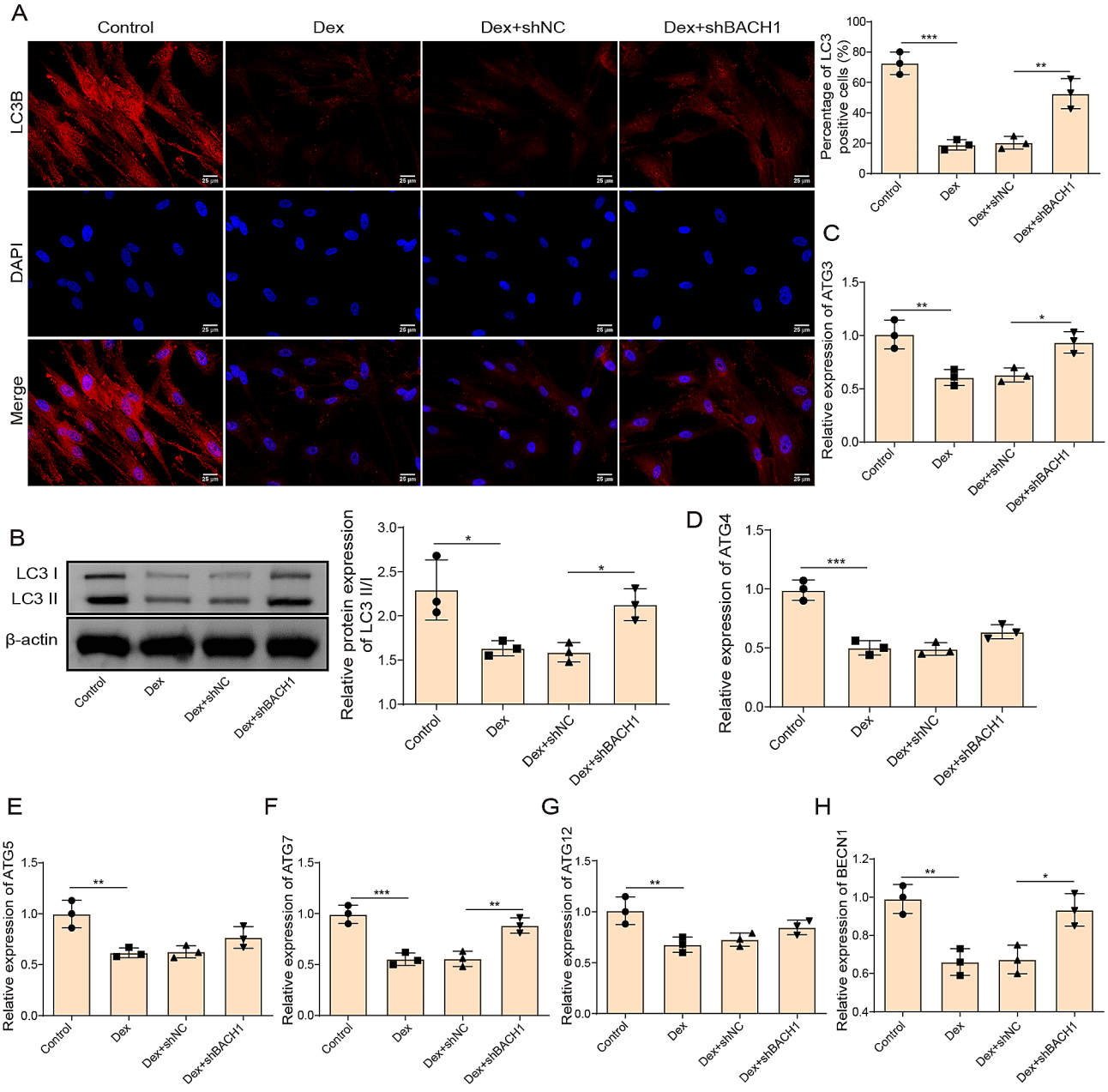

Fig. 3 BACH1 enhanced Dex induced suppression of osteogenic differentiation via repressing autophagy. hBMSCs with/without BACH1 knockdown were exposed to Dex stimulation. After osteogenic induction, the following experiments were arranged. (**A**) Immunofluorescence staining was conducted to detect LC3B level. (**B**) Protein level of LC3B was detected by western blot. (**C-H**) The mRNA expression of ATG3, ATG4, ATG5, ATG7, ATG12 and BECN1 were measured by qRT-PCR. Statistical data are presented as Mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

BACH1 inhibits the transcription of ATG7 to suppress its gene expression

Through the prediction of online Jaspar database, the transcriptional motif of BACH1 and its putative binding sites (P1, P2) on ATG7 promoter region were displayed in Fig. 4A-B. Validated by ChIP analysis, there were greatly enrichment of P1 and P2 fragments precipitated by specific BACH1 antibody (Fig. 4C). Moreover, dual luciferase assay also proved that the repression of BACH1 resulted in markedly enhanced luciferase activity of recombinant pGL3-ATG7 transfected cells compared to shNC group (Fig. 4D). Compared to shNC group, the mRNA and protein of ATG7 expression was pronouncedly elevated by depletion of BACH1 (Fig. 4E-F). Collectively, these results suggested that BACH1 inhibited the transcription of ATG7.

Inhibition of ATG7 reversed the BACH1 silence-mediated protection of hBMSCs osteogenic differentiation

Here, we endeavored to ascertain whether suppressing ATG7 influenced the effects of BACH1 knockdown on the osteogenic differentiation in hBMSCs. Firstly, shRNA targeting ATG7 was transfected into hBMSCs and remarkably downregulated ATG7 expression (Fig. 5A). As indicated by immunofluorescence assay, the up-regulation of LC3B regulated by BACH1 knockdown was reversed by ATG7 co-inhibition (Fig. 5B). Additionally, the elevated level of LC3II/I caused by BACH1 repression was also overturned by shATG7 co-transfection (Fig. 5C).

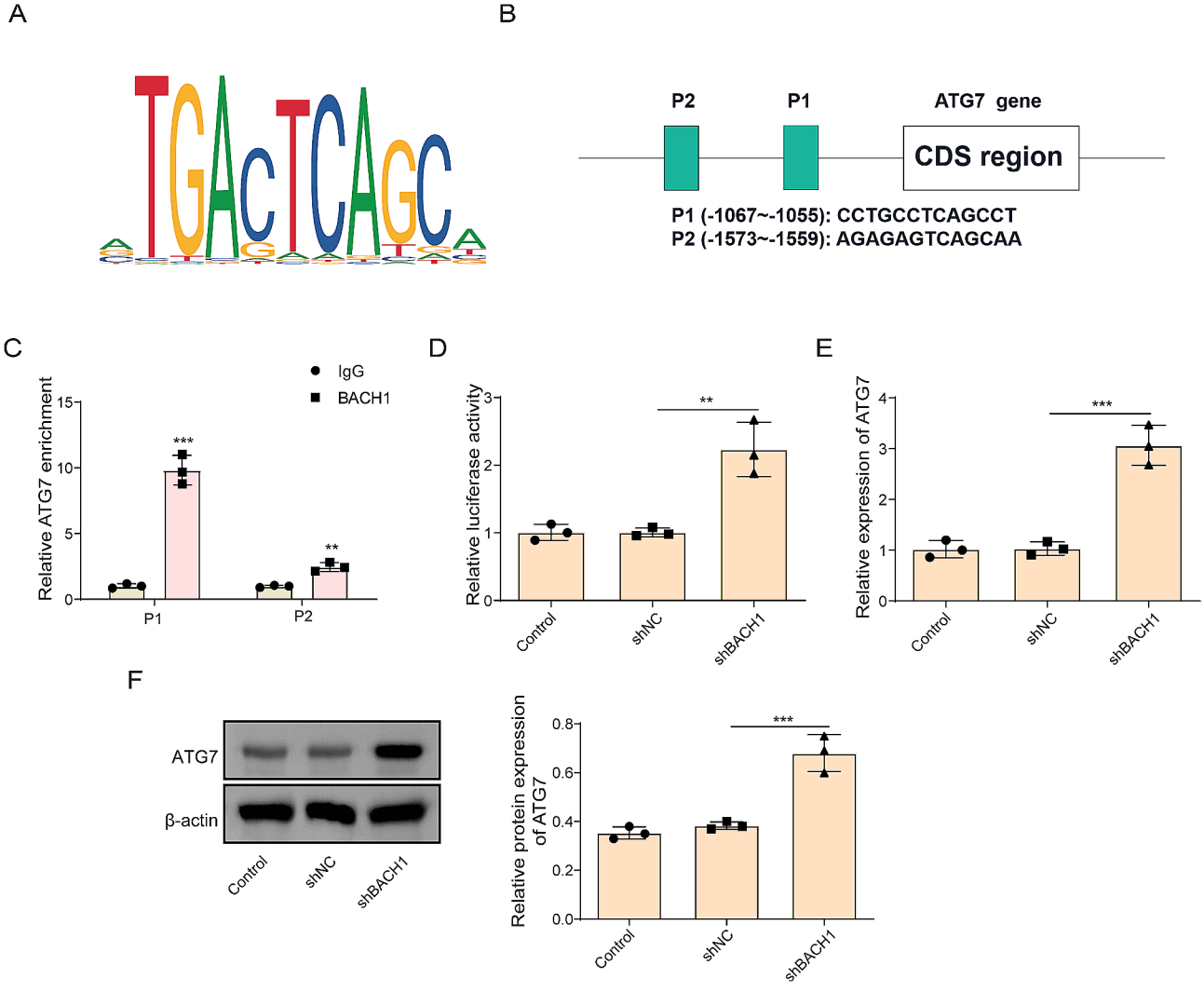

Fig. 4 BACH1 inhibits the transcription of ATG7 to suppress its gene expression. (**A**) Schematic diagram of BACH1 transcriptional motif. (**B**) Schematic diagram of transcriptional recognition sites of BACH1 on ATG7 promoter, which was predicted by Jaspar database. (**C**) ChIP was used to validate the binding relationship between ATG7 and BACH1. (**D**) Luciferase assay vefified the specific binding of BACH1 to ATG7 promoter region. hBMSCs were transfected with lentivirus-packed shNC or shBACH1, respectively. (**E-F**) ATG7 mRNA and protein expressions were detected by qRT-PCR and western blot assays. Statistical data are presented as Mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

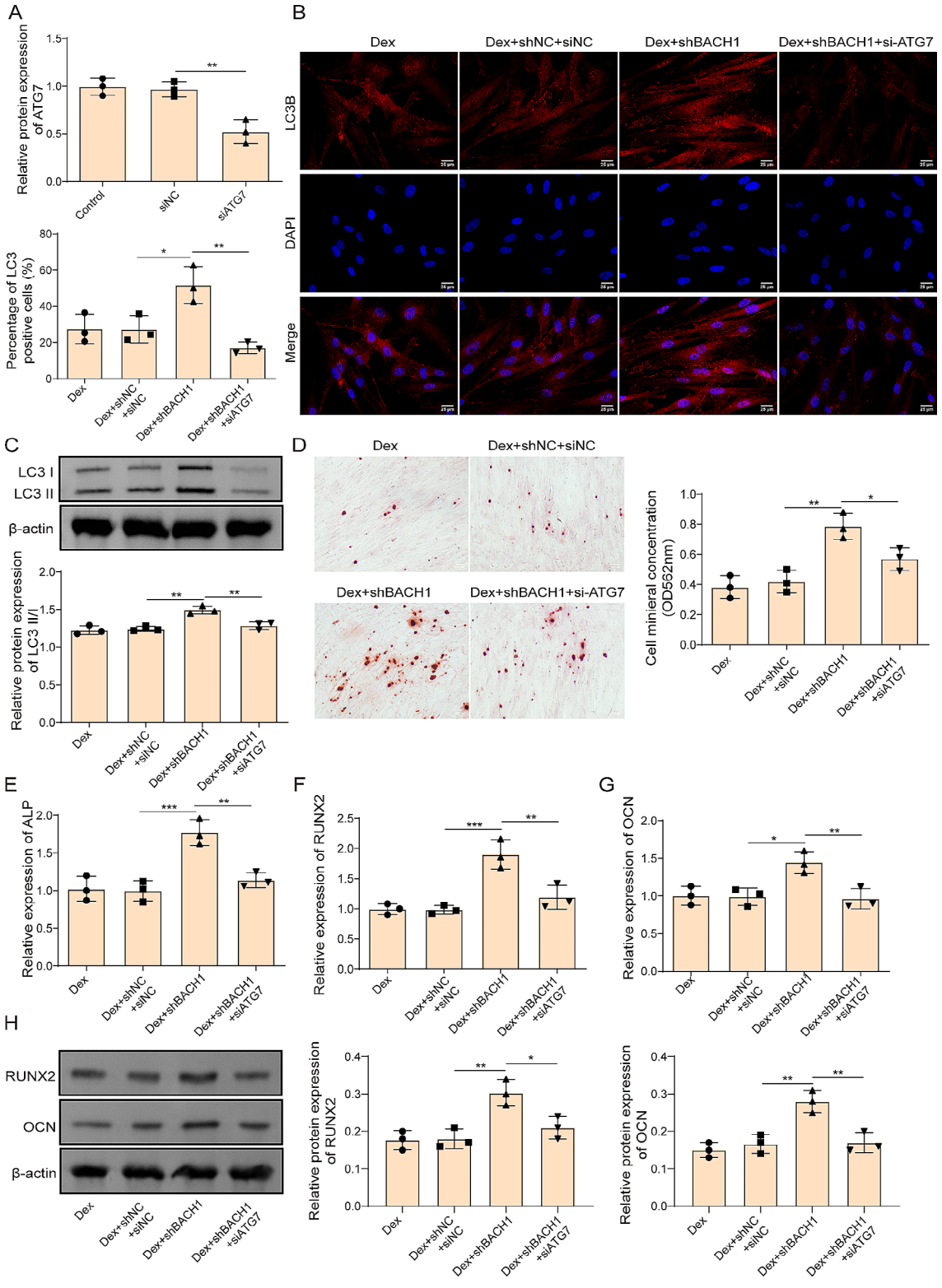

Fig. 5 Inhibition of ATG7 reversed the BACH1 silence-mediated protection of hBMSCs osteogenic differentiation. (**A**) hBMSCs were transfected with shNC or shATG7, respectively, and the transfection efficiency was determined by qRT-PCR. hBMSCs were treated with shBACH1 or co-transfected with shBACH1 plus shATG7, and followed by exposed to Dex condition. (**B**) Immunofluorescence staining was used to determine LC3B. (**C**) Western blot was used to determine LC3II/I level. (**D**) Alizarin Red staining was used to determine osteogenic differentiation of hBMSCs. (**E-G**) qRT-PCR was used to measure the mRNA levels of ALP, Runx2 and OCN. (**H**) Western blot was conducted for detecting the protein levels of Runx2 and OCN. Statistical data are presented as Mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Next, ARS staining discovered that loss of ATG7 dramatically diminished the promoting role of BACH1 depletion on mineral deposition (Fig. 5D). Similar phenomena were observed in osteogenic differentiation markers, which presented that the increases of ALP, OCN and RUNX2 in Dex-induced hBMSCs mediated by BACH1 knockdown were all restrained in the presence of shATG7 (Fig. 5E-H). These findings suggested that the protective roles of BACH1 loss on Dex-induced osteogenic differentiation inhibition was partly depended on ATG7-mediated autophagy activation.

Discussion

GIOP stands as a critical subtype of osteoporosis, posing significant challenges to public health due to its association with heightened fracture risks [22]. Existing references has shown that glucocorticoid was a main pathogenic reason for GIOP by inhibiting bone formation, which usually exhibited as the impaired proliferation, mineralization and osteogenic differentiation of osteoblasts [23, 24]. Given their multipotent differentiation potential, BMSCs are regarded as a vital osteoblast progenitor [25]. In the current study, hBMSCs was stimulated by Dex to mimic GIOP cellular model, and the acquired findings elucidated that BACH1 repressed ATG7 transcription, thereby suppressing osteoblast differentiation by inactivating autophagy. These findings provided a deep understanding of the molecular mechanisms of Dex-induced osteoblast suppression, and the unveiling a potential therapeutic target for GIOP treatment.

BACH1 has been identified to modulate a broad range of cellular activities, such as the production of ROS, cell cycle, heme homeostasis, hematopoiesis, and immunity [13]. In GIOP cellular models, we had found that BACH1 was gradually decreased during osteogenic induction in a time dependent manner, and its expression could be induced by Dex. Giving the consideration that downregulation of BACH1 could protect osteoblast by preventing hydrogen peroxide-induced oxidative damage [19], and inhibition of BACH1 could augment Nrf2-regulated antioxidant enzymes to diminish intracellular ROS signaling, thus improving osteoclast genesis [20]. We speculated that BACH1 might participate in the inhibition of osteogenic differentiation induced by glucocorticoids. In the present work, our data demonstrated for the first time that knockdown of BACH1 could reverse Dex induced the suppression of osteogenic differentiation. Therefore, it is possible to consider BACH1 as a therapeutic target for developing drugs to treat GIOP.

Recent studies demonstrate that autophagy plays essential role in regulating osteoblast differentiation and bone remodeling [26]. Previously, BACH1 deficiency could reduce the osteoarthritis phenotype by elevating autophagy-related LC3 expression in chondrocytes, thus protecting cartilage from degradation and damage [18]. Inhibition of BACH1 could rescue OGD-induced autophagy dysregulation in nucleus pulposus cells by upregulating HO-1 expression, thereby ameliorating intervertebral disc disorder [27]. Additionally, BACH1 could interact with NFE2L2-regulated enhancer antioxidant response elements (AREs), thus interfering with NFE2L2-mediated gene transcription, including a series of autophagy related genes [28–30]. Despite these insights, the direct involvement of BACH1 in regulating autophagy during osteoblast differentiation from hBMSCs remained unexplored. Our current study uncovered that Dex, a glucocorticoid, suppressed autophagy in hBMSCs, contributing to inhibited osteoblast differentiation. Importantly, we provided the first evidence that BACH1 knockdown in hBMSCs rescued Dex-induced autophagy suppression, as indicated by altered expressions of autophagy markers such as LC3B and ATG protein family. The present findings revealed a novel mechanism by which BACH1 was involved in BMSCs differentiation during GIOP. However, the precise mechanisms by which BACH1 modulation of autophagy impacts the osteogenic differentiation pathway BMSCs warrant further investigation.

In the current study, we attempted to explore the mechanism of BACH1 mediated regulation of autophagy in hBMSCs. As a transcriptional regulatory factor, BACH1 was reported to interact with a variety of chromatin modification related proteins such as HDAC1, PRC2, DNMTs and MAFs family proteins to inhibit gene transcription [9, 31, 32]. Through a series of experiments, we found that BACH1 silencing could promote the expression of autophagy related ATG3, ATG7 and BECN1. Among them, ATG7 is an essential effector enzyme for autophagy, and plays important roles in the regulation of immunity, cell death, cell cycle and apoptosis, etc. [33]. As an E1-like ligase, ATG7 conjugates ATG5 to ATG12 to facilitate the autophagosome biogenesis [7]. Therefore, it suggested that ATG7 plays a central role in the initiation of autophagy. Moreover, Li et al. reported that ATG7 cKO mice showed a reduced bone mass at both developmental and adult age, and deletion of ATG7 in osteoblast also led to endoplasmic reticulum stress, thus impeding osteoblast mineralization and promoted apoptosis [34]. Here, we further uncovered that ATG7 was a negative transcriptional target of BACH1 in hBMSCs. Loss of function of ATG7 greatly reversed the protective roles of BACH1 knockdown in Dex-induced the impairment of autophagy and osteogenesis. Therefore, we considered that BACH1 mediated suppression of ATG7 expression was a main reason for the inhibition of autophagy in Dex induce osteoblast inhibition.

Collectively, our data presents novel evidence, for the first time, implicating that Dex-induced BACH1

up-regulation was an important mechanism suppressing osteoblast differentiation inhibition, thereby highlighting BACH1 might be a potential therapeutic target for GIOP treatment. Through mechanistic exploration, we identified that BACH1 could transcriptionally suppressed ATG7 expression, leading to autophagy inhibition in Dex-challenged hBMSCs, aggravating GIOP development. This finding provided a theoretical reference for elucidating the pathogenesis of GIOP. However, our study is not without its limitations. For instance, further elucidation of our findings via animal experiments remains a desideratum. Additionally, the construction of BACH1 overexpression vector to definitively ascertain the impact of BACH1 upregulation on the progression of GIOP is another aspect yet to be addressed. Rest assured, we are committed to tackling these issues in our future endeavors.

Abbreviations

OP	Osteoporosis
GIOP	Glucocorticoid-induced osteoporosis
hBMSCs	Human bone marrow mesenchymal stem cells
BACH1	BTB and CNC homology 1
ATGs	Autophagy related genes
BECN1	Beclin-1
Dex	Dexamethasone
ARS	Alizarin red staining
ALP	Alkaline phosphatase, Runx2:Runt-related transcription factor 2
OCN	Osteocalcin
qRT-PCR	Quantitative real-time-PCR
ChIP	Chromatin immunoprecipitation
AREs	Antioxidant response elements

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12891-024-07761-y.

Supplementary Material 1

Acknowledgements
We would like to give our sincere gratitude to the reviewers for their constructive comments.

Author contributions
ShuYing Xiao: Conceptualization, Methodology, Data curation, Formal analysis, Writing- Reviewing and EditingGuoJuan Li: Data curation, Writing-Original draft preparationMeiHua Tan: SoftwareWen Liu: ValidationWenJin Li: Supervision, Writing- Reviewing and Editing.

Funding
None.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study of human BMSCs isolation has been approved by the ethics committee of The Affiliated Nanhua Hospital, Hengyang Medical School, University of South China, which was conducted in accordance with the principles of the Declaration of Helsinki, and all participants in the study gave a written informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 23 April 2024 / Accepted: 6 August 2024
Published online: 24 August 2024

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