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Original Article

Long non-coding RNA LncHIFCAR promotes osteoarthritis development via positively regulating HIF-1α and activating the PI3K/AKT/mTOR pathway

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Abstract: Osteoarthritis (OA) is a progressive disease that characterized by synovial inflammation and loss of cartilage in the joint. This study aimed to elucidate the potential role of long non-coding RNA LncHIFCAR in the development of osteoarthritis (OA). Expression of LncHIFCAR and HIF-1α in OA and normal cartilage tissues were determined. ATDC5 chondrocyte cells were cultured under hypoxia condition to establish a cell model of OA. Additionally, LncHIFCAR was suppressed in ATDC5 cells and the effects of LncHIFCAR suppression on hypoxia-induced cell injury were investigated by assessing cell proliferation, apoptosis, inflammatory response, and matrix synthesis. Furthermore, interactions between LncHIFCAR and HIF-1α as well as HIF-1α target genes (VEGF and BNIP3) were explored. Additionally, whether LncHIFCAR affected the expression of PI3K/AKT/mTOR pathway-related proteins was detected. LncHIFCAR and HIF-1α were up-regulated in OA tissues. Hypoxia induced ATDC5 cell injury and increased LncHIFCAR expression. Suppression of LncHIFCAR significantly improved hypoxia-induced cell injury by promoting cell proliferation, inhibiting apoptosis, decreasing the secretion of TNF-α and IL-6, and suppressing the synthesis of MMPs. In addition, LncHIFCAR positively regulated HIF-1α and HIF-1α target genes (VEGF and BNIP3). LncHIFCAR promoted hypoxia-induced inflammatory response and matrix synthesis by upregulation of VEGF, and induced hypoxia-induced apoptosis via upregulation of BNIP3. Furthermore, LncHIFCAR significantly inhibited hypoxia-induced activation of PI3K/AKT/mTOR pathway. Our results indicate that LncHIFCAR is up-regulated in OA tissues and suppression of LncHIFCAR may improve hypoxia-induced cell injury via positively regulating HIF-1α and HIF-1α target genes (VEGF and BNIP3). The PI3K/AKT/mTOR pathway may thus be a possible mechanism to mediate LncHIFCAR function in OA development.

Keywords: Osteoarthritis, LncHIFCAR, HIF-1α, VEGF, BNIP3, PI3K/AKT/mTOR pathway

Introduction

Osteoarthritis (OA) is a progressive disease that characterized by synovial inflammation and loss of cartilage in the joint [1, 2]. It always results in joint pain, stiffness, and even disability [3, 4]. It is estimated that approximately 10% of males and 18% of females are affected among populations over 60 years old [5]. The global incidence of OA rises quickly and limited effective therapy is available for this disease [6, 7]. Therefore, elucidation of key mechanism underlying OA has great significance for the development the effective therapeutic strategies for OA.

Long noncoding RNAs (lncRNAs) represent a subgroup of non-coding RNAs more than 200 nucleotides in length and are emerging as key regulators in a variety of human diseases [8, 9]. Aberrant expression of lncRNAs has been reported to play a crucial role in OA development [10, 11]. For instance, lncRNA UFC1 can increase chondrocyte proliferation in OA [12] and CIR can promote chondrocyte extracellular matrix degradation in this disease [13]. Recently, LncHIFCAR (long noncoding HIF-1α co-activating RNA), a specific, hypoxia-inducible lncRNA has been shown to play an oncogenic role in driving oral cancer progression [14]. Nevertheless, the function of LncHIFCAR in the pathogenesis of OA has not been well investigated to our knowledge.

The present intended to investigate the effects of LncHIFCAR on hypoxia-induced ATDC5 cell injury by assessing cell proliferation, apoptosis, inflammatory response, and matrix synthesis. Additionally, the interactions between LncHIFCAR and HIF-1α as well as HIF-1α target genes (VEGF and BNIP3) were explored. Additionally, whether LncHIFCAR affected the expression of PI3K/AKT/mTOR pathway-related proteins was detected to further elucidate the possible mechanism of LncHIFCAR on regulating hypoxia-induced ATDC5 cell injury. Our findings will help to identify novel therapeutic targets for OA.

Materials and methods

Articular cartilage tissues

Twelve OA patients (3 men and 9 women) who underwent total knee arthroplasty were enrolled in this study, and OA cartilage tissues were then harvested from the knee joints of these patients. Normal articular cartilage tissues isolated from the knee joints of 10 trauma patients (4 men and 6 women) were used as controls. All tissue were graded in accordance with a modified Mankin scale [15], for which < 2 points was < 3 points was considered normal and ≥5 points represented OA. This study was approved by the Ethics Committee of our hospital, and all patients included in this study provided their informed consent.

Cell culture

The ATDC5 chondrocyte cell line was propagated in Dulbecco's modified Eagle's medium (DMEM) : Ham's F-12 mix (1:1) (Gibco, Invitrogen, Carlsbad, USA) containing 5% fetal bovine serum (FBS) (Gibco), 1% antibiotic-antimycotic (AB) (Gibco), 10 μg/ml human transferring, and 30 mM sodium selenite (Sigma-Aldrich, St Louis, MO, USA) for 12 h, 24 h and 48 h, respectively. For physiological hypoxia (5% O_2) or normoxia treatment, cells were maintained in Anoxomat chambers (Mart Microbiology, Lichtenvoorde, Netherlands) containing 5% or 21% O_2 at 37°C for 0, 12, 24 and 48 h.

Cell transfection

To overexpress LncHIFCAR, VEGF and BNIP3, full-length cDNAs of LncHIFCAR, VEGF and BNIP3 were amplified and then cloned into pcDNA3.1 (Invitrogen). For knockdown of LncHIFCAR, shRNA oligonucleotides targeting LncHIFCAR (sh-HIFCAR) were synthesized and purified by Genepharma (Suzhou, Jiangsu, China). For cell transfection, cells were cultured in a 6-well cell culture plate for 24 h and then transfected with pc-HIFCAR, sh-HIFCAR, pc-VEGF, pc-BNIP3, and their corresponding controls using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocols.

CCK-8 assay

Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, cells were seeded in 96-well plate with a density of 5000 cells/well. After different treatments, CCK-8 solution was added to each well and incubation continued for 1 h in a 37°C humidified atmosphere with 5% CO_2. The absorbance was then detected at 470 nm with a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Cell apoptosis was analyzed by flow cytometry. After different treatments, cells were fixed in 70% ethanol, followed by double staining with propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated Annexin V in the presence of 50 μg/ml RNase A (Sigma-Aldrich). These cells were then incubated for 1 h at 37°C in the dark. Flow cytometry analysis for detecting apoptotic cells was conducted using a FACS can (Beckman Coulter, Fullerton, CA, USA) and the obtained data were then analyzed by a FlowJo software (Tree Star, San Carlos, Calif.).

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α and IL-6 in the culture medium of different treated groups were measured using the corresponding ELISA kits (R&D Systems, Minneapoils, MN) following the instructions provided by the supplier.

Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted from cartilage tissues (OA and normal) and different treated cells using Trizol (Gibco, Invitrogen, Carlsbad, USA). Reverse-transcription for cDNA synthesis was the performed by means of M-MLV reverse transcriptase (Promega, Madison, WI, USA).

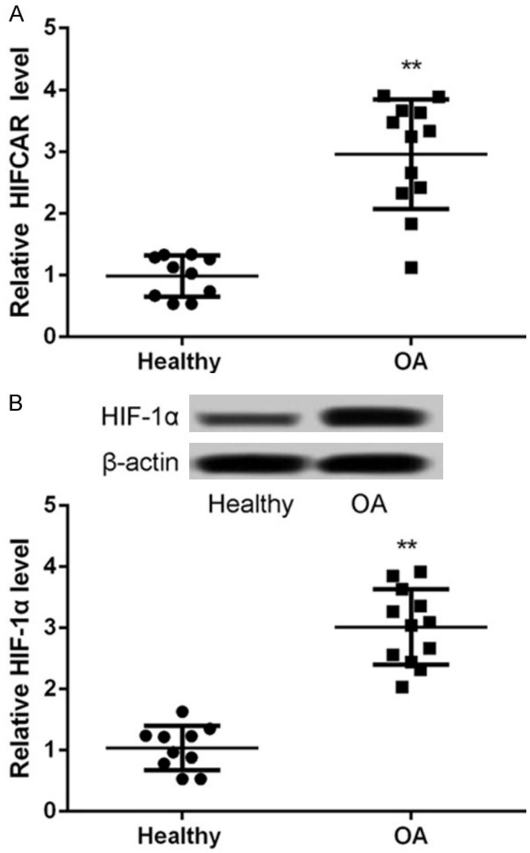

Figure 1. Expression of LncHIFCAR (A) and HIF-1α (B) in cartilage tissues isolated from the knee joints of osteoarthritis (OA) patients and normal trauma patients. The collected data are presented as the mean ± SD. ** means P < 0.01 compared with healthy controls.

qPCR reaction was then carried out following the manufacturer's protocols of two-step Stemaim-It miR qRT-PCR Quantitation Kit (SYBR Green) (Novland, shanghai, China). Data analysis was conducted with the MxPro qPCR system software (Stratagene). Levels of gene expression were calculated by relative quantification ($2^{-\Delta\Delta CT}$) method using GAPDH as the internal control.

Western blot

Protein was extracted from different treated cells in using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) mixed with protease mimics (Roche, Guangzhou, China) and the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was applied to detect the concentration of extracted proteins. Using a Bio-Rad BisTris Gel system, Western blot system was performed. Primary antibodies to HIF-1α, Bcl-2, BAX, Cytochrome C, TNF-α, IL-6, MMP1, MMP3, MMP13, VEGF, BNIP3, p-PI3K, PI3K, p-AKT, AKT, p-mTOR, mTOR, β-actin (Santa Cruz, CA, USA) were prepared in 5% blocking buffer at a dilution of 1:1,000 and then used to incubate with the polyvinylidene difluoride (PVDF) membranes (Millpore, Billerica, MA, USA) at 4°C overnight, followed by incubation with horseradish peroxidase-marked secondary antibody (Santa Cruz, CA, USA) for 1 h at 37°C. After rinsing, the membranes were transferred into the Bio-Rad ChemiDoc™ XRS system, and then visualized by incubation with Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA). The intensity of protein signals was then obtained and quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. The collected data are presented as the mean ± SD. Statistical analyses were performed using one-way ANOVA by means of SPSS 19.0 statistical software. A *P*-value of < 0.05 indicated a statistically significant result.

Results

LncHIFCAR and HIF-1α were up-regulated in OA tissues

To investigate whether LncHIFCAR played a role in OA, we detected expression of LncHIFCAR and HIF-1α in OA and normal cartilage tissues. The results showed that the expression levels of LncHIFCAR and HIF-1α in OA cartilage tissues were all significantly higher than those in normal cartilage tissues (P < 0.01, **Figure 1**).

Hypoxia induced ATDC5 cell injury and increased the expression levels of LncHIFCAR and HIF-1α

To establish the cell model of OA, the ATDC5 chondrocyte cells were cultured in hypoxia condition. The results showed that hypoxia treatment significantly inhibited the proliferation (**Figure 2A**), induced cell apoptosis (**Figure 2B**), increased the secretion of TNF-α and IL-6 (**Figure 2C**), and promoted the synthesis of MMPs (MMP1, MMP3 and MMP13) (**Figure 2D**) in a time-dependent manner within a certain experimental period (48 h) (all P < 0.05), indicating that hypoxia induced ATDC5 cell injury. Moreover, we found that hypoxia treatment sig-

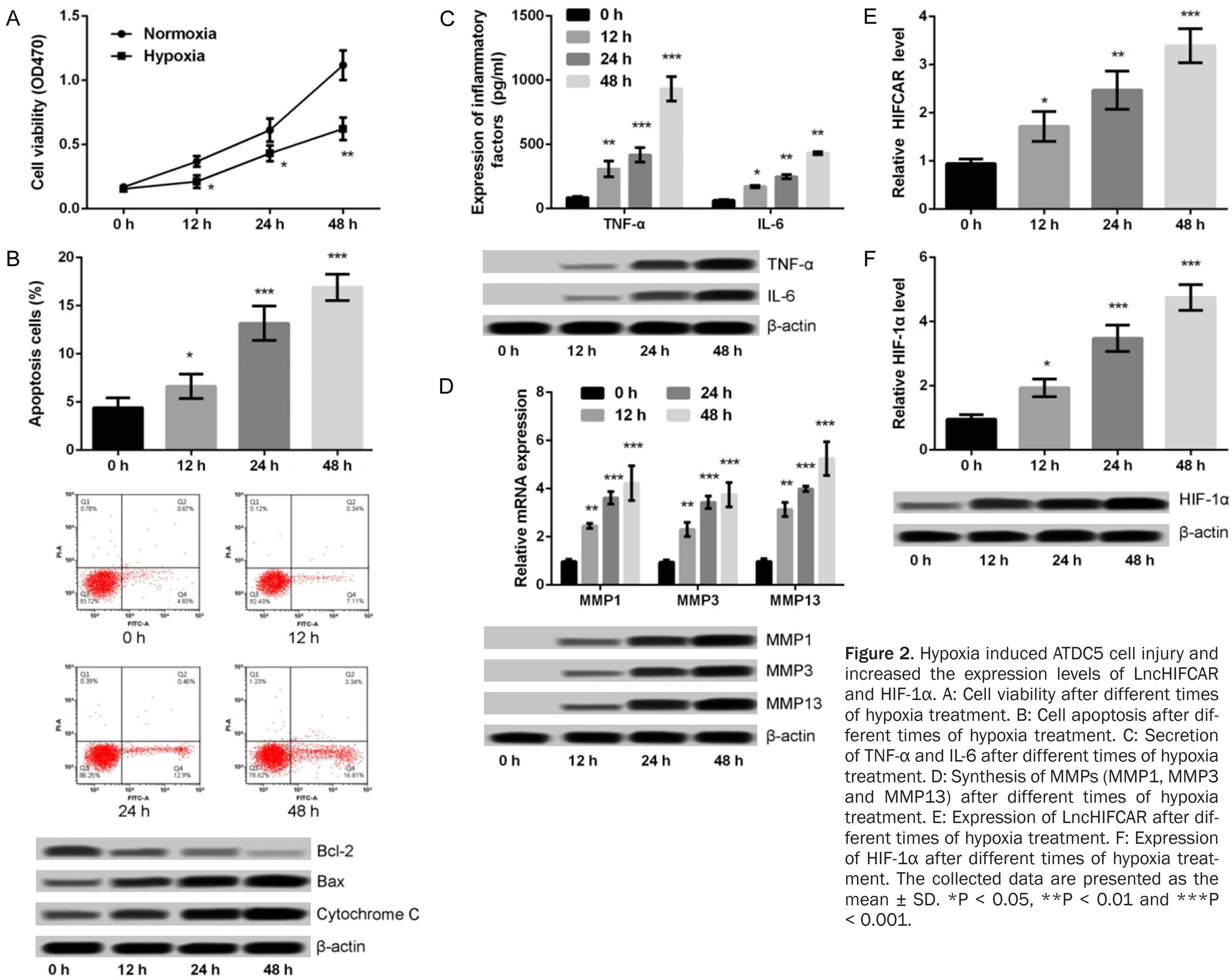

Figure 2. Hypoxia induced ATDC5 cell injury and increased the expression levels of LncHIFCAR and HIF-1α. A: Cell viability after different times of hypoxia treatment. B: Cell apoptosis after different times of hypoxia treatment. C: Secretion of TNF-α and IL-6 after different times of hypoxia treatment. D: Synthesis of MMPs (MMP1, MMP3 and MMP13) after different times of hypoxia treatment. E: Expression of LncHIFCAR after different times of hypoxia treatment. F: Expression of HIF-1α after different times of hypoxia treatment. The collected data are presented as the mean ± SD. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

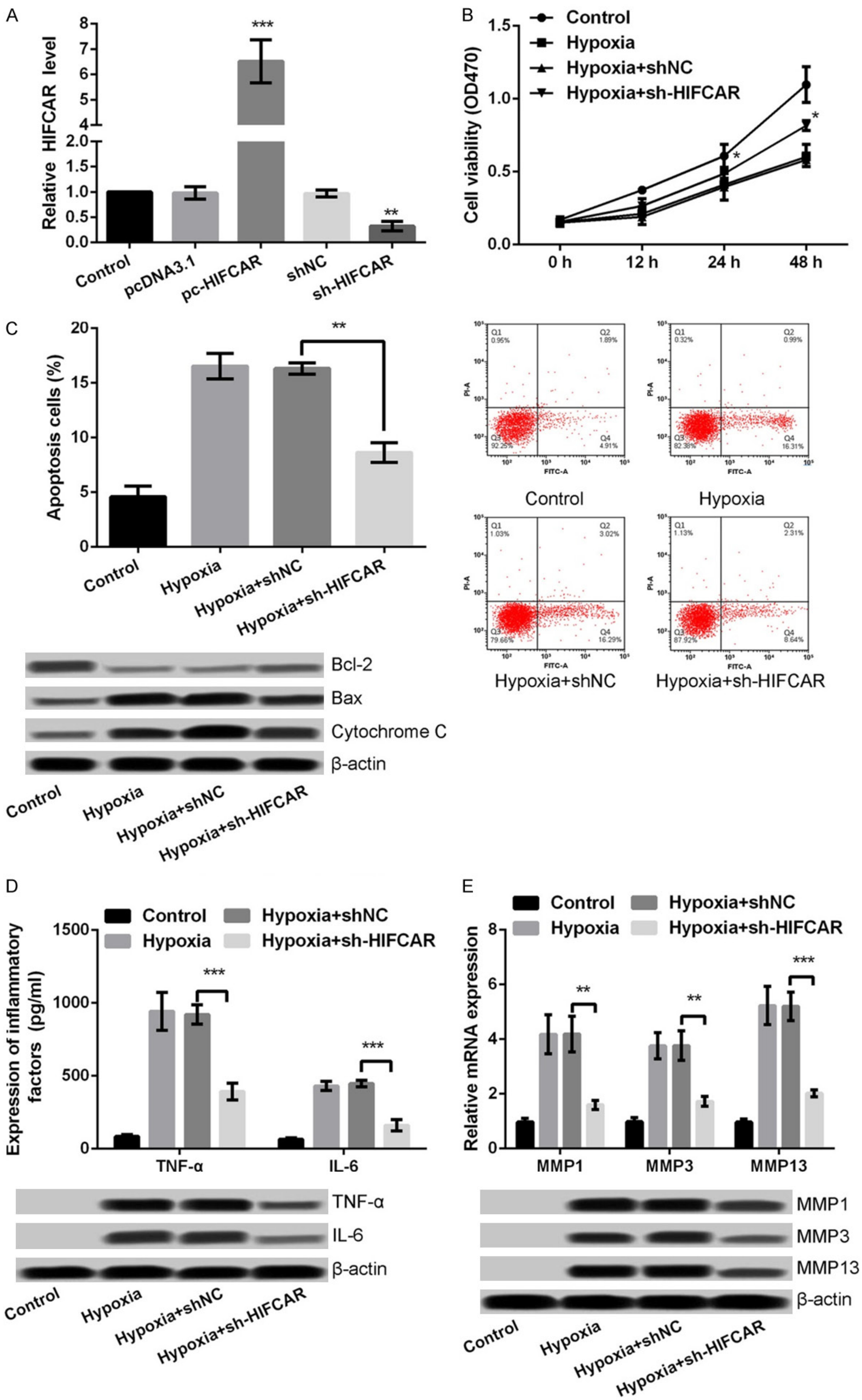
A
Relative HIFCAR level
Control
pcDNA3.1
pc-HIFCAR
shNC
sh-HIFCAR
B
Control
Hypoxia
Hypoxia+shNC
Hypoxia+sh-HIFCAR
Cell viability (OD470)
0 h
12 h
24 h
48 h
C
Apoptosis cells (%)
Control
Hypoxia
Hypoxia+shNC
Hypoxia+sh-HIFCAR
Bcl-2
Bax
Cytochrome C
β-actin
D
Expression of inflammatory factors (pg/ml)
TNF-α
IL-6
E
Relative mRNA expression
MMP1
MMP3
MMP13

Figure 3. Effects of aberrant expression of LncHIFCAR on hypoxia-induced cell injury. A: Expression of LncHIFCAR after different transfections. B: Cell viability of different groups. C: Cell apoptosis of different groups. D: Secretion of TNF-α and IL-6 of different groups. E: Synthesis of MMPs (MMP1, MMP3 and MMP13) of different groups. The collected data are presented as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

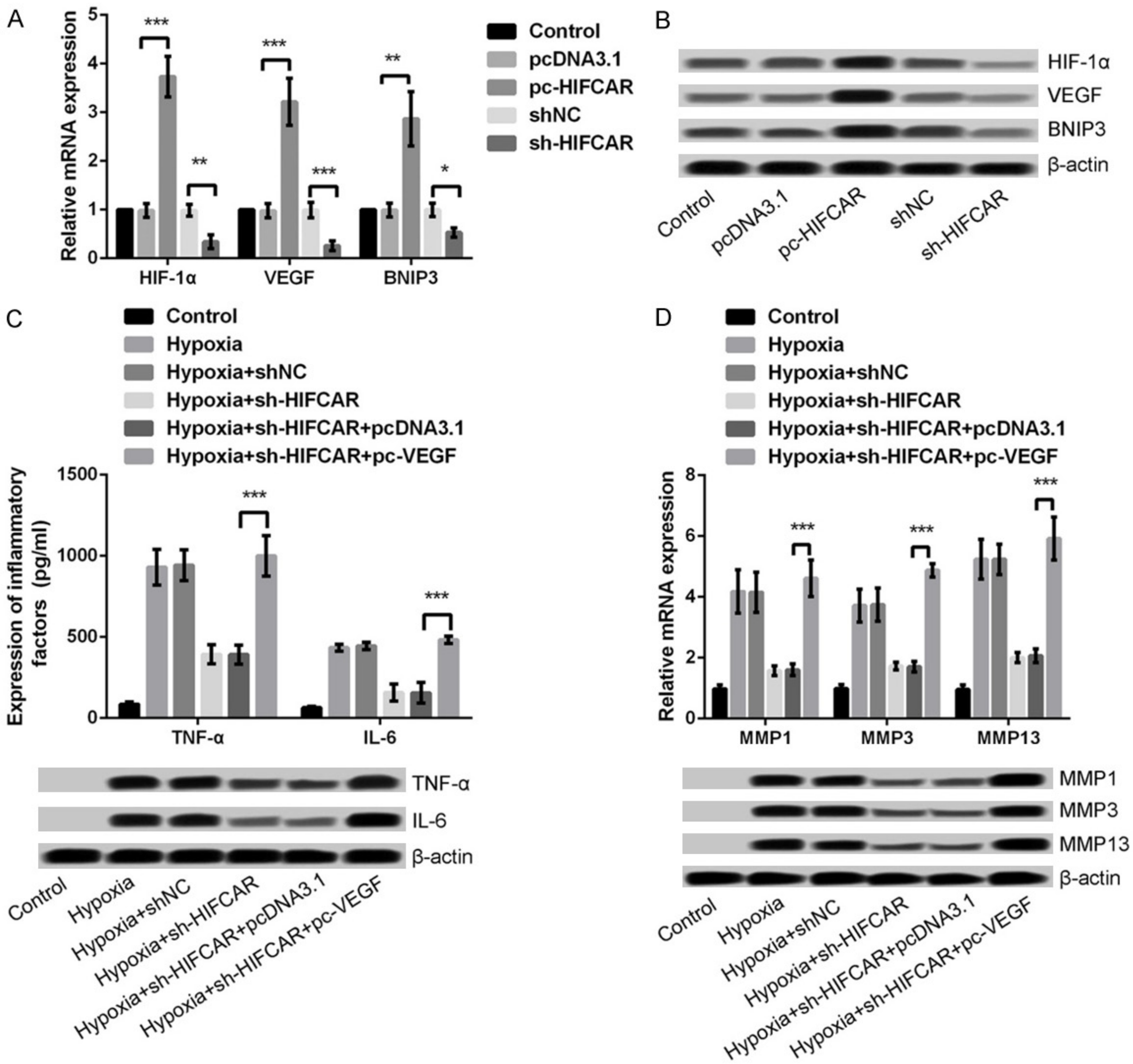

Figure 4. LncHIFCAR promoted hypoxia-induced inflammatory response and matrix synthesis possible by upregulation of VEGF. A, B: Expression levels of HIF-1α and HIF-1α target genes (VEGF and BNIP3) after overexpression and suppression of LncHIFCAR. C: Secretion of TNF-α and IL-6 of different groups. D: Synthesis of MMPs (MMP1, MMP3 and MMP13) of different groups. The collected data are presented as the mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001.

nificantly induced the expression of LncHIFCAR (**Figure 2E**) and HIF-1α (**Figure 2D**) in a time-dependent manner.

Effects of aberrant expression of LncHIFCAR on hypoxia-induced cell injury

To explore the role of LncHIFCAR in OA, we overexpressed and suppressed the expression of LncHIFCAR in ATDC5 chondrocyte cells. The results showed that the LncHIFCAR expression in pc-HIFCAR group was markedly higher than that in pcDNA3.1 group, whereas the LncHIFCAR expression in sh-HIFCAR group was markedly lower than that in sh-NC group (P < 0.01, **Figure 3A**). We then explored the effects of LncHIFCAR suppression on hypoxia-induced cell injury. The results showed that suppression of LncHIFCAR significantly improved hypoxia-induced cell injury by promoting cell prolifer-

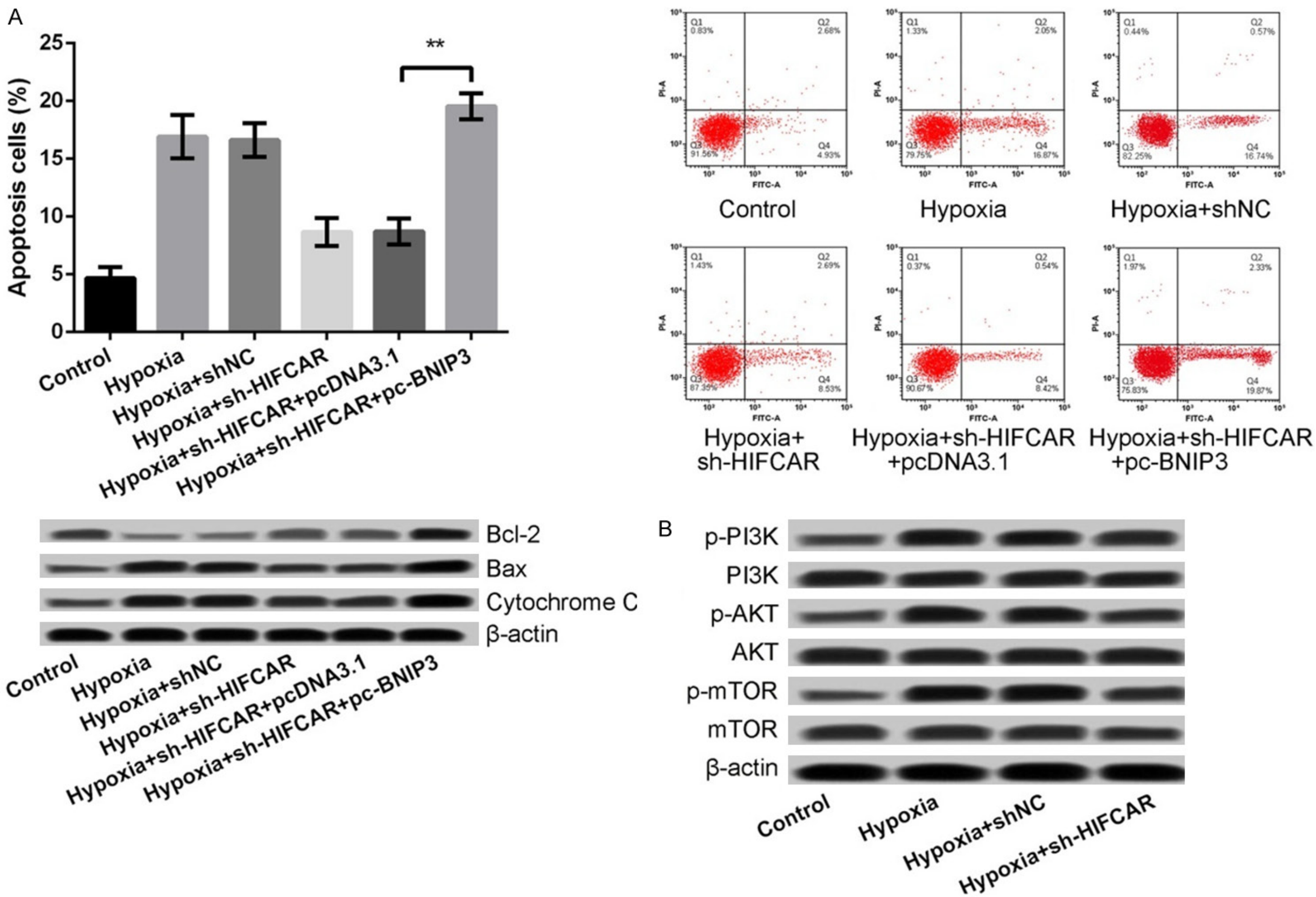

Figure 5. LncHIFCAR induced hypoxia-induced apoptosis possible by upregulation of BNIP3 and PI3K/Akt/mTOR signal pathway. A: Apoptotic cells and the cell apoptosis-related protein expression of LncHIFCAR in regulating BNIP were respectively presented in A. B: LncHIFCAR regulated the progression of OA by suppressing the activation of PI3K/Akt/mTOR signal pathway. The collected data are presented as the mean ± SD. $^{**}P < 0.01$.

ation (**Figure 3B**), inhibiting cell apoptosis (**Figure 3C**), decreasing the secretion of TNF-α and IL-6 (**Figure 3D**) and suppressing the synthesis of MMPs (MMP1, MMP3 and MMP13) (**Figure 3E**) (all $P < 0.05$).

LncHIFCAR positively regulated HIF-1α and HIF-1α target genes

We also detected the regulatory relationship between LncHIFCAR and HIF-1α as well as HIF-1α target genes (VEGF and BNIP3). The results showed that the expression of HIF-1α, VEGF and BNIP in pc-HIFCAR group were all significantly higher than those in pcDNA3.1 group, whereas the expression of these molecules in the sh-HIFCAR group was markedly lower than those in sh-NC group ($P < 0.05$, **Figure 4**), indicating that LncHIFCAR positively regulated HIF-1α and HIF-1α target genes.

LncHIFCAR promotes hypoxia-induced inflammatory response and matrix synthesis possible by upregulation of VEGF

To explore the downstream mechanism of LncHIFCAR inflammatory response and matrix synthesis, we investigated the regulatory relationship between LncHIFCAR and VEGF. The results showed that under hypoxia condition, overexpression of VEGF could significantly reverse the effects of LncHIFCAR suppression on the secretion of TNF-α and IL-6 (**Figure 4C**) and the synthesis of MMPs (MMP1, MMP3 and MMP13) (**Figure 4D**) (all $P < 0.05$), indicating that LncHIFCAR might promoted hypoxia-induced inflammatory response and matrix synthesis by upregulation of VEGF.

LncHIFCAR induces hypoxia-induced apoptosis by upregulation of BNIP3

To explore the downstream mechanism of LncHIFCAR on cell apoptosis, we investigated the regulatory relationship between LncHIFCAR and BNIP3. The results showed that overexpression of BNIP3 could significantly reverse the effects of LncHIFCAR suppression on hypoxia-induced cell apoptosis (**Figure 5A**) (all $P < 0.05$), indicating that LncHIFCAR might induced hypoxia-induced apoptosis through upregulation of BNIP3.

LncHIFCAR inhibits hypoxia-induced activation of PI3K/AKT/mTOR pathway

To further explore the mechanism of LncHIFCAR in OA, we detected that whether LncHIFCAR affected the expression of PI3K/AKT/mTOR pathway-related proteins. Expression levels of p-PI3K, p-AKT and p-mTOR were markedly increased under hypoxia conditions (**Figure 5B**), indicating that hypoxia could activate PI3K/AKT/mTOR pathway. However, suppression of LncHIFCAR markedly decreased the hypoxia-induced expression levels of p-PI3K, p-AKT and p-mTOR (**Figure 5B**), implying that suppression of LncHIFCAR inhibited hypoxia-induced activation of PI3K/AKT/mTOR pathway.

Discussion

In the current study, we investigated the effects of LncHIFCAR in OA development. The results show that hypoxia increased LncHIFCAR expression and suppression of LncHIFCAR significantly improved hypoxia-induced cell injury by promoting cell proliferation, inhibiting apoptosis, decreasing the secretion of TNF-α and IL-6, and suppressing the synthesis of MMPs. In addition, LncHIFCAR positively regulated HIF-1α and HIF-1α target genes (VEGF and BNIP3). The effects of LncHIFCAR suppression on hypoxia-induced inflammatory response and matrix synthesis could be reversed by upregulation of VEGF, and the effects on hypoxia-induced apoptosis could be reversed by upregulation of BNIP3. Furthermore, LncHIFCAR significantly inhibited hypoxia-induced activation of the PI3K/AKT/mTOR pathway. These preliminary data elucidate the possible mechanism of LncHIFCAR in OA development.

In line with a previous finding that LncHIFCAR could act as a HIF-1a co-activator to play an oncogenic role in oral cancer [14], we also found that LncHIFCAR positively regulated HIF-1α and HIF-1α target genes (VEGF and BNIP3). VEGF, an angiogenic factor, has been found to be involved in the osteoarthritic degradation of cartilage in OA [16]. Lingaraj et al. demonstrated that VEGF was expressed during the growth of osteoarthritic articular cartilage [17] and Yuan et al. reported that enhanced levels of VEGF could promote the pathogenesis of OA [18]. Based on our results, we speculate that LncHIFCAR may regulate hypoxia-induced inflammatory response and matrix synthesis by upregulation of VEGF. Furthermore, BNIP3 is a pro-apoptotic mitochondrial protein classified in the Bcl-2 family [19]. A previous study reported that hypoxia induced autophagic cell death in apoptosis-competent cells through regulating the pro-apoptotic gene BNIP3 [20]. Ma et al.

revealed that BNIP3 induced apoptosis and autophagy in esophageal squamous cell carcinoma cells under hypoxia conditions [21]. Also, Baicalein is shown to induce the apoptosis of human osteosarcoma MG-63 cells via ROS-induced BNIP3 expression [22]. Give the key role of BNIP3 in regulating apoptosis, we speculated that LncHIFCAR may induce hypoxia-induced apoptosis by upregulation of BNIP3.

Furthermore, we found that LncHIFCAR inhibited hypoxia-induced activation of PI3K/AKT/mTOR pathway. The PI3K/Akt/mTOR pathway has been shown to be implicated in diverse cell processes, such as cell proliferation and metabolism [23-25]. Inhibition of the PI3K/AKT/mTOR signaling pathway inhibits inflammatory response and activation of the autophagy of articular chondrocytes in rats with OA [26]. He et al. confirmed that miR-20 suppression can increase proliferation and autophagy in articular chondrocytes through regulating PI3K/AKT/mTOR pathway [27]. Based on our study, we speculate that the crucial roles of LncHIFCAR in OA development may be associated with the PI3K/AKT/mTOR pathway. Notably, inhibition of the PI3K/Akt/mTOR pathway is considered as a promising strategy for OA therapy [28]. LncHIFCAR is found to inhibit hypoxia-induced activation of PI3K/AKT/mTOR pathway in our study, implying that LncHIFCAR might serve as a therapeutic target for the treatment of OA.

Taken together, our results reveal that LncHIFCAR is up-regulated in OA tissues and suppression of LncHIFCAR may improve hypoxia-induced cell injury via positively regulating HIF-1α and HIF-1α target genes (VEGF and BNIP3). LncHIFCAR may promote the inflammatory response and matrix synthesis possible by regulating VEGF and may induced hypoxia-induced apoptosis possible by upregulation of BNIP3. The PI3K/AKT/mTOR pathway may be a possible regulatory mechanism to mediate the role of LncHIFCAR in OA development. Our findings provide a theoretical basis for designing an effective strategy for OA therapy.

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Disclosure of conflict of interest

None.

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