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Long noncoding RNA ILF3-AS1 regulates myocardial infarction *via* the miR-212-3p/SIRT1 axis and PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: Myocardial infarction (MI) is a serious cardiac disease due to its high incidence and mortality worldwide. Long noncoding RNAs (lncRNAs) have been found to play an essential role in the pathological progress of various cardiovascular diseases. ILF3-AS1 is a newly identified lncRNA, and many studies have demonstrated that ILF3-AS1 affects the development of various malignancies. However, the biological function of ILF3-AS1 and its underlying mechanism in MI are still unknown. In the present study, the function of ILF3-AS1 and the possible mechanisms against hypoxia-induced apoptosis in H9c2 cells were investigated.

MATERIALS AND METHODS: H9c2 cells were exposed to hypoxia (1% O2) to mimic the in vitro model of MI. The levels of lncRNA ILF3-AS1 and microRNA miR-212-3p were measured by real-time PCR (RT-PCR). Transfection was performed to upregulate the levels of ILF3-AS1 and miR-212-3p. Western blot assays were carried out to measure protein expression. The relationship between ILF3-AS1 and miR-212-3p was verified by Dual-Luciferase reporter assay.

RESULTS: We found that ILF3-AS1 was downregulated by hypoxia. Overexpression of ILF3-AS1 resulted in the relief of hypoxia-induced damage to H9c2 cells by rescuing cell viability, migration, and invasion and suppressing apoptosis, while downregulation of ILF3-AS1 had the opposite effects. Moreover, ILF3-AS1 could negatively regulate miR-212-3p expression, and upregulation of ILF3-AS1 could alleviate hypoxic injury via downregulation of miR-212-3p. Moreover, miR-212-3p negatively regulated SIRT1 expression. Further investigations revealed that ILF3-AS1 activated PI3K/Akt signaling and that application of the PI3K inhibitor LY294002 could abrogate the protective effects of ILF3-AS1 against hypoxia.

CONCLUSIONS: In summary, we concluded that ILF3-AS1 provides protection against hypoxia-induced injury via the PI3K/Akt pathway, which may provide clues for the treatment of patients with MI.

Key Words:
Long noncoding RNA, ILF3-AS1, Hypoxia, MiR-212-3p, Apoptosis.

Introduction

Myocardial infarction (MI) is a leading cause of death worldwide and is characterized by insufficient cardiac blood supply, which causes damage to cardiac muscle and ultimately leads to heart failure[1]. MI induces the excessive death of cardiomyocytes via programmed cell death, leading to a reduction in the functional cell number within a short period[2]. In recent years, although the treatment of MI has been significantly improved, the deterioration of cardiac function still cannot be fully prevented[3]. Therefore, it is necessary to develop novel efficient treatment options for MI.

Long noncoding RNAs (lncRNAs) contain more than 200 nucleotides and most do not encode proteins[4]. LncRNAs play important roles in the physical and pathological processes of various human diseases, including cardiovascular system diseases. So, overexpression of lncRNA Gm2691 could protect cardiomyocytes from damage caused by MI via activation of the PI3K/Akt signaling pathway[6]. In contrast, lncRNA PCFL aggravates MI through the miR-378/GRB2 pathway[7]. LncRNA ILF3-AS1 (ILF3-AS1) is a newly found lncRNA that is located at chromosome 17p13.1 and has been demonstrated to exert various functions in different cellular processes[8]. Some studies have indicated that ILF3-AS1 functions as an oncogene in various cancers. In fact, upregulation of ILF3-AS1 promotes the proliferation, migration, and invasion of melanoma cells[8]. In addition, ILF3-AS1 also contributes to the progression of osteosarcoma via regulation of

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SOX5[9]. However, whether ILF3-AS1 has any effects on MI has never been investigated.

As hypoxia is the leading and underlying cause of MI, we applied hypoxia treatment to mimic the MI process in rat H9c2 cardiomyocytes. In the present study, we found that hypoxia induced myocardial injury in H9c2 cells and led to the downregulation of ILF3-AS1. Overexpression of ILF3-AS1 attenuated the hypoxia-induced injury of H9c2 cells. Moreover, ILF3-AS1 was shown to affect the expression of miR-212-3p, which negatively regulated SIRT1 levels. Furthermore, suppression of the PI3K/Akt signaling pathway abrogated the protective effects of ILF3-AS1 on hypoxia-induced H9c2 cell injury. We may provide a novel strategy for the treatment of MI injury.

Materials and Methods

Cell Culture and Treatment

The rat myocardial H9c2 cell line and human embryonic kidney HEK-293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humid atmosphere with 5% CO_2. Cells were exposed to hypoxic conditions (93% N_2, 2% O_2, and 5% CO_2) for 24 h, and cells incubated under normoxic conditions were used as control. The PI3K/Akt inhibitor LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA). After transfection, the cells were incubated with 10 μM LY294002 for 12 h. All other routine chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Transfection

Short-hairpin RNA (shRNA) targeting ILF3-AS1 (shILF3-AS1) and the corresponding negative control shCtrl were ligated into the U6/GFP/Neo plasmid (GenePharma, Suzhou, China). Full-length ILF3-AS1 and SIRT1 were inserted into the pcDNA3.1 and pEX plasmids (GenePharma, Suzhou, China), respectively, and the resultant plasmids were named pc-ILF3-AS1 and pEX-SIRT1. The empty vectors were used as negative controls. Cells were transfected with vectors by Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's guidelines. Real-time quantitative PCR (RT-PCR) analysis was performed to measure transfection efficacy.

Cell Viability Assay

After transfection, H9c2 cells were seeded into 96-well plates at a density of 5×10^3 cells/well. Then, the cells were cultured under hypoxic conditions as described above for 24 h, followed by incubation in normoxic conditions at 37°C for 24 h. After reoxygenation, 10 μL of CCK-8 solution (Solarbio Technologies, Beijing, China) was added to each well, followed by 4 h of incubation at 37°C. The absorbance at 450 nm was examined by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell Apoptosis Assay

Cell apoptosis was assayed by using an annexin V-FITC apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, cells were seeded into six-well plates at a density of 1×10^5 cells/well. After treatment, when cells reached approximately 80% confluence, cells were harvested and resuspended in staining buffer containing 10 μl annexin V-FITC. After incubation for 20 min at room temperature avoiding light, the cells were analyzed by a FACScan flow cytometer (BD Biosciences).

Cell Migration and Invasion Assays

The migration of H9c2 cells was measured by a chamber migration assay. In brief, after treatment, H9c2 cells were resuspended in 200 μl serum-free media and then seeded at a density of 1×10^5 cells/well in the upper chamber of a 24-well plate. Then, the lower compartment was filled with 500 μl of complete medium. After culturing for 48 h at 37°C, the cells migrated to the lower chamber were fixed with ethanol, stained with crystal violet, and counted under a microscope at 200× magnification (Olympus, Tokyo, Japan). The invasion assay was the same as the migration assay except that the insert was precoated with Matrigel (BD Biosciences). Each experiment was performed in triplicate.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from H9c2 cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's guide. The PrimeScript RT reagent Kit (TaKaRa, Dalian, China) was used to reverse transcribe RNA into complementary DNA. PCR amplification was subsequently performed to measure the expression of ILF3-AS1 and

SIRT1 using the SYBR PrimeScript PLUS RT-RNA PCR Kit (TaKaRa), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The expression of miR-212-3p was determined using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), and U6 was used as a control. The following primers were used: ILF3-AS1: 5′-TTTTCCCTGACACCCCTCTC-3′ (sense) and 5′-TAGCTTCCATACCCAGCACC-3′ (anti-sense); SIRT1: 5'-TCAGCGTCACTCCCAAG-3' (sense) and 5'-GCAATCCTGCTCCCTCC-3' (anti-sense); GAPDH : 5'-GCACCACCAACTGCTTAGCA-3' and 5'-GTCTTCTGGGTGGCAGTGATG-3'; miR-212-3p: 5'- GGTAACAGTCTCCAGTCA-3' (sense) and 5'- GCAATTGCACTGGATACG-3' (anti-sense); U6 5'-GCTTCGGCAGCACATATACTAAAAT-3' (sense) and 5'-CGCTTCACGAATTTGCGTGTCAT-3' (anti-sense). Fold changes of gene expression were then calculated by the relative quantification ($2^{-\Delta\Delta Ct}$) method.

Western Blot Assay

Total proteins were isolated by radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Beijing, China). The bicinchoninic acid assay (BCA) protein assay kit (Beyotime Biotechnology, Beijing, China) was used for protein quantification. Equal amounts of protein (20 μg) were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the PVDF membranes were incubated with primary antibodies overnight at 4°C and then probed with secondary antibodies at room temperature for 1 h. The protein bands were visualized by enhanced chemiluminescence (ECL) detection system (Millipore, Billerica, MA, USA). The intensity of the protein bands was measured using NIH ImageJ software (Bethesda, MD, USA). The primary antibodies against Bcl-2, Bax, GAPDH, caspase-3, and caspase-9 were all purchased from Cellular Signaling Technology (Danvers, MA, USA). The antibodies against phospho-PI3K, phospho-Akt, PI3K, Akt, phospho-GSK3ß, GSK3ß, and SIRT1 were purchased from Abcam (Cambridge, MA, USA).

Luciferase Reporter Assay

Using a bioinformatics program (www.targetscan.org), the putative binding site of ILF3-AS1 was predicted. The miR-212-3p target binding sequence in ILF3-AS1 and its mutant binding sites and the SIRT1 3'-untranslated region (3'-UTR) containing the predicted binding site were amplified by PCR and then subcloned into the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, Madison, WI, USA) to create the Luciferase reporter vectors. The ILF3-AS1 and SIRT1 3'-UTR carrying the mutated sequence in the complementary sites of miR-212-3p were generated by the Directed Mutagenesis System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's guidelines and inserted into the pmirGLO reporter vector. Subsequently, H9c2 cells were cotransfected with the Luciferase reporter vectors and miR-212-3p mimics or mimic NC, followed by detection of their Luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Data are presented as the mean ± standard deviation (SD) from three independently repeated experiments. Statistical analysis was performed using GraphPad Prism 7.0 statistical software (Tristar, Chicago, IL, USA), and the statistical differences between groups were analyzed by one-way analysis of variance (ANOVA). Tukey's post hoc test was used to examine pairwise differences. p-value<0.05 indicated a statistically significant result.

Results

Hypoxia Induces Myocardial Injury in H9c2 Cells

To establish a hypoxia injury model, we exposed rat cardiac H9c2 cells to hypoxia for 24 h. Cell viability and apoptosis were assessed. As indicated in Figure 1A and B, cell viability was significantly decreased, while cell apoptosis was increased after hypoxia treatment when compared to the control. Then, the migration and invasion of cells were measured, and the results showed that the migration and invasion of H9c2 cells were both significantly decreased after hypoxia treatment (Figure 1C). Furthermore, compared with the control, hypoxia treatment led to the downregulation of the anti-apoptotic protein Bcl-2 and the upregulation of the proapoptotic protein Bax. Moreover, cleavage of caspase-3 and caspase-9 was also observed after hypoxia treatment (Figure 1D). Therefore, it was confirmed that exposure to hypoxia results in H9c2 cell damage.

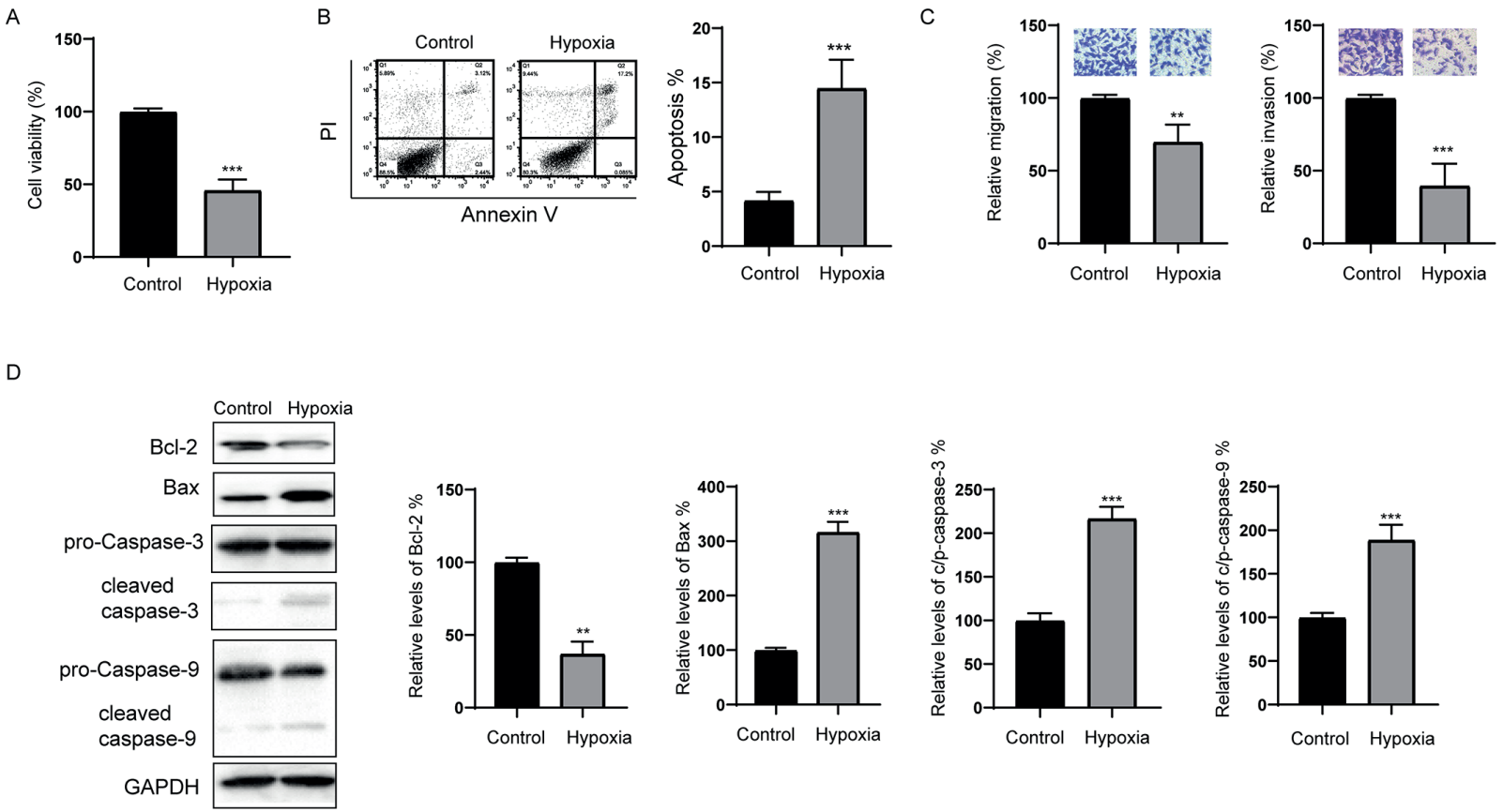

Figure 1. Hypoxia reduced the viability and induced apoptosis of H9c2 cells. **A**, Viability of H9c2 cells was valued by the CCK-8 assay. **B**, Apoptotic cells were assayed by the flow cytometry. **C**, Migration and invasion of cells were assayed (100 ×). **D**, Western blot was performed to measure the levels of apoptosis-related proteins. GAPDH was used as a control. Data are presented as mean ± SD from three independent experiments performed in duplicate. $^{**}p<0.01$, $^{***}p<0.001$. CCK-8, Cell Counting Kit-8.

Hypoxia Decreases the Expression of ILF3-AS1, Which is Involved in the Hypoxic Injury of H9c2 Cells

The expression of ILF3-AS1 was assayed after hypoxia treatment. As indicated in Figure 2A, hypoxia treatment resulted in a significant downregulation of ILF3-AS1 in comparison with the control ($p<0.001$). To explore whether ILF3-AS1 is involved in the hypoxic injury of H9c2 cells, we successfully used a pcDNA3.1 plasmid containing ILF3-AS1 or siRNA against ILF3-AS1 to overexpress or knock down ILF3-AS1 in H9c2 cells, and compared cells transfected with these plasmids with the corresponding control transfectants (Figure 2B, C). As shown in Figure 2D, when ILF3-AS1 was overexpressed or suppressed in H9c2 cells, cell viability was significantly increased or reduced after hypoxia treatment when compared with the control group. We also found that downregulation of ILF3-AS1 resulted in further decreases in the migration and invasion of H9c2 cells under hypoxic conditions, while overexpression of ILF3-AS1 reversed these effects of hypoxia (all $p<0.05$, Figure 2E, F). Furthermore, hypoxia-induced apoptosis of H9c2 cells was decreased after overexpression of ILF3-AS1, but it was markedly increased after silencing ILF3-AS1 ($p<0.05$, Figure 2G). Similarly, changes in the expression of apoptosis-related proteins under hypoxic conditions were also observed after dysregulation of ILF3-AS1 (Figure 2H). Taken together, these data indicated that overexpression of ILF3-AS1 could protect H9c2 cells from hypoxia-induced injury and silencing of ILF3-AS1 aggravated hypoxia injury.

ILF3-AS1 Negatively Regulates MiR-212-3p Expression in H9c2 Cells

Several studies[10,11] have indicated that lncRNAs act as miRNA sponges in various diseases, including hypoxia-induced injury of different tissues. Using a bioinformatics tool, we found that miR-212-3p was a predicted target of ILF3-AS1 (Figure 3A, left). To investigate the association between ILF3-AS1 and miR-212-3p, a Luciferase reporter assay was conducted. As a result, we found that the Luciferase activity of wild-type ILF3-AS1 but not mutant ILF3-AS1 could be specifically inhibited by miR-212-3p mimics and not by miR-NC (Figure 3A, right). It was also found that hypoxia treatment resulted in the upregulation of miR-212-3p (Figure 3B). Next, we investigated whether ILF3-AS1 regulated cell viability and apoptosis via inhibition of miR-212-3p in H9c2 cells. The miR-212-3p mimic and miR-212-3p inhibitor were transduced into H9c2 cells to upregulate or downregulate miR-212-3p, respectively (Figure 3C). Then, we assessed the effects of miR-212-3p expression on cell viability under hypoxic conditions. The data showed that cell viability was significantly decreased by the miR-212-3p mimic and significantly increased by the miR-212-3p inhibitor compared with the control ($p<0.05$, Figure 3D). Furthermore, we also investigated the effects of miR-212-3p expression on cell apoptosis under hypoxia treatment. As shown in Figure 3E, miR-212-3p mimics could promote apoptosis in H9c2 cells, while miR-212-3p inhibitor decreased apoptosis. Western blot analysis also indicated the effects of miR-212-3p on apoptosis in H9c2 cells under hypoxia treatment (Figure 3F). Furthermore, migration and invasion assays also revealed that miR-212-3p mimics aggravated hypoxia-induced injury, while miR-212-3p inhibitor alleviated the injury induced by hypoxia in H9c2 cells (Figure 3G, H). Taken together, these data suggested that ILF3-AS1 regulated hypoxia-induced injury possibly via the regulation of miR-212-3p.

SIRT1 is a Target of MiR-212-3p

We investigated the possible target of miR-212-3p. Using the bioinformatics tool TargetScan, we identified SIRT1 as a potential target of miR-212-3p (Figure 4A, left). Further Luciferase reporter assays confirmed the association between miR-212-3p and SIRT1. As shown in Figure 4A, the Luciferase activity of SIRT1-wt was significantly inhibited in miR-212-3p-transfected cells, while the Luciferase activity of SIRT1-mut did not exhibit any changes. Furthermore, the mRNA levels of SIRT1 were significantly decreased or increased in the miR-212-3p mimic or miR-212-3p inhibitor group, respectively (Figure 4B). These observations suggest a negative regulatory relationship between miR-212-3p and SIRT1. Then, we further dysregulated the expression of SIRT1 to investigate whether ILF3-AS1 affects hypoxic injury in H9c2 cells via regulation of miR-212-3p-mediated targeting of SIRT1. As showed in Figure 4C, qRT-PCR analysis showed that the mRNA levels of SIRT1 were successfully overexpressed or downregulated in H9c2 cells via transfection of pEX-SIRT1 or si-SIRT1, respectively (all $p<0.05$). Western blots also revealed that the change in protein levels was in line with the qRT-PCR results (Figure 4D). Then, we analyzed the effects of SIRT1 on hypoxia-induced injury. The results disclosed that cell viability was significantly in-

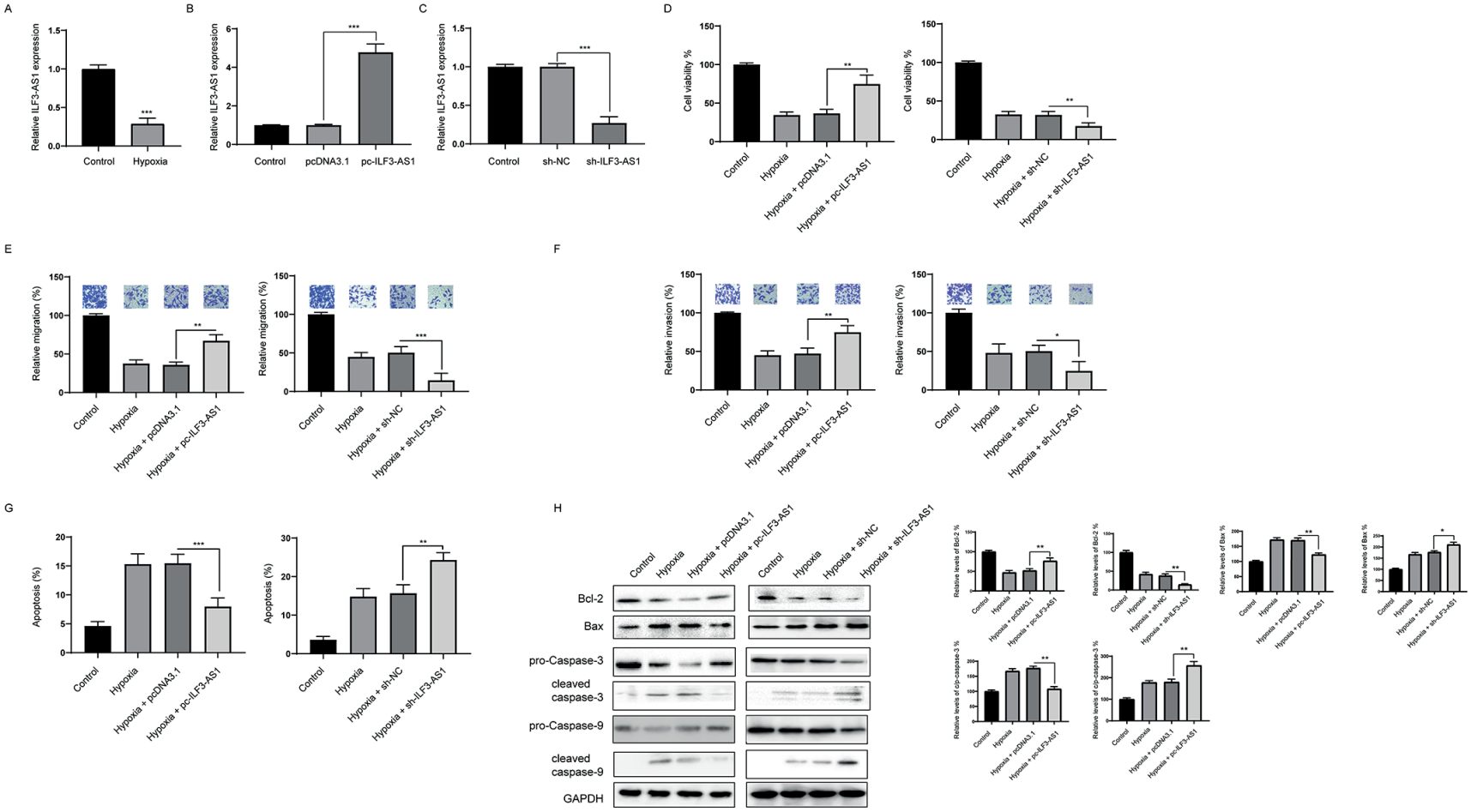

Figure 2. Hypoxia induced downregulation of ILF3-AS1 and overexpression of ILF3-AS1 alleviated hypoxia-induced injury in H9c2 cells, while suppression of ILF3-AS1 aggravated the injury. **A**, After hypoxia treatment, the levels of ILF3-AS1 were measured by RT-PCR. **B**, Expression of ILF3-AS1 in H9c2 cells were assayed after transfection with pc-ILF3-AS1 and vector control. **C**, Expression of ILF3-AS1 in H9c2 cells were assayed after transfection with sh-ILF3-AS1 and negative control. **D**, Cell viability was assayed after overexpression or downregulation of ILF3-AS1. **E**, Cell migration was assayed after overexpression or downregulation of ILF3-AS1 (100 ×). **F**, Cell invasion was assayed after overexpression or downregulation of ILF3-AS1 (100 ×). **G**, Cellular apoptosis was measured after overexpression or downregulation of ILF3-AS1. **H**, After treatment, Western blot was performed to measure the levels of apoptosis-related proteins. GAPDH was used as a control. Data are presented as mean ± SD from three independent experiments performed in duplicate. $^{**}p<0.01$, $^{***}p<0.001$. RT-PCR, Real-Time PCR.

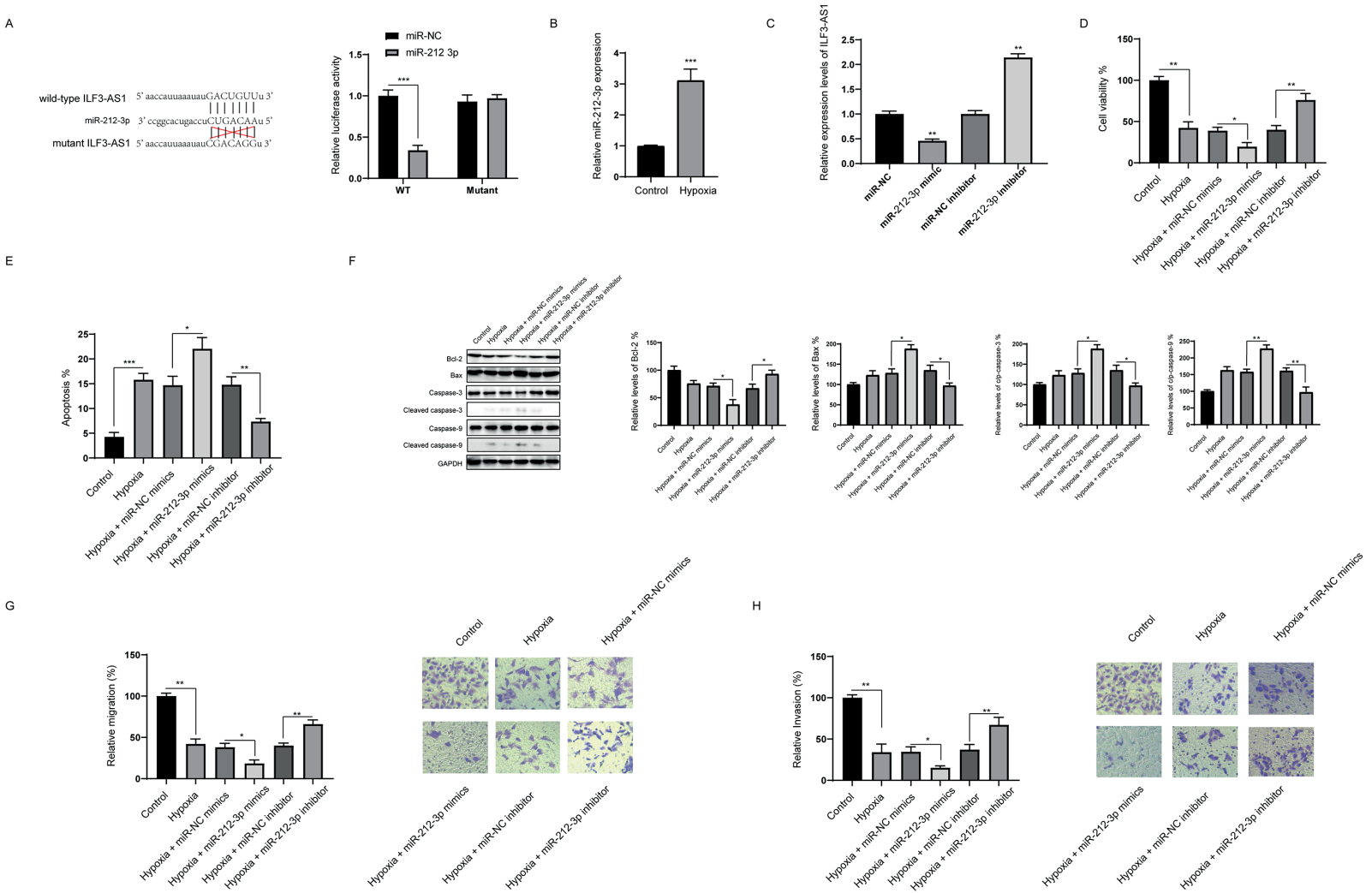

Figure 3. ILF3-AS1 negatively regulated the expression of miR-212-3p which also regulated the hypoxia-induced injury in H9c2 cells. **A**, Schematic picture of the predicted interaction sites between ILF3-AS1 and miR-212-3p. **B**, Luciferase activity analysis in HEK-293 cells co-transfected with reporter plasmid inserted with wild-type or mutated ILF3-AS1 sequences and miR-212-3p mimics or miR-NC. **C**, Levels of ILF3-AS1 were measured by RT-PCR in H9c2 cells after transfected with miR-212-3p mimics or inhibitors. **D**, H9c2 cells were treated as indicated, then cell viability was assayed. **E**, H9c2 cells were treated as indicated, then cell apoptosis was assayed. **F**, H9c2 cells were treated as indicated, cellular lysates were analyzed by Western blot for the expression of apoptosis related proteins. **G**, Cells were treated as indicated, then cellular migration was assayed (100 ×). **H**, Cells were treated as indicated, then cellular invasion was assayed (100 ×). Data are presented as mean ± SD from three independent experiments performed in duplicate. *p<0.05, **p<0.01, ***p<0.001.

creased or decreased after overexpression or downregulation of SIRT1 under hypoxic conditions (Figure 4E). We also assayed apoptosis after SIRT1 dysregulation and found that overexpression of SIRT1 could protect H9c2 cells from hypoxia-induced apoptosis, while downregulation of SIRT1 promoted hypoxia-induced apoptosis (Figure 4F). Western blot results showed that the change in apoptosis-related proteins was in accordance with the change in the apoptosis rate (Figure 4G). Then, the migration and invasion of H9c2 cells were investigated as well. It was found that overexpression of SIRT1 promoted cell migration and invasion, while SIRT1 silencing had the opposite effect (Figure 4H, I). Taken together, these data suggested that the effects of ILF3-AS1 on hypoxia injury were mediated via regulation of miR-212-3p-targeted SIRT1.

Inhibition of PI3K/Akt Signaling Abrogates the Protective Effects of ILF3-AS1 on H9c2 Injury Induced by Hypoxia

Brand et al[12] have observed that the PI3K/Akt signaling pathway and its downstream targets participate in the progression of MI and that activation of the PI3K/Akt pathway has protective effects on cardiomyocytes. Western blot analysis indicated that hypoxia caused a marked reduction in the phosphorylation of PI3K and Akt, while overexpression of ILF3-AS1 led to the upregulation of phosphorylation of PI3K and Akt (Figure 5A). To test whether the PI3K/Akt signaling pathway is involved in the cardioprotective function of ILF3-AS1, a specific PI3K/Akt inhibitor, LY294002, was applied. As shown in Figure 5A, treatment with LY294002 successfully restored the phosphorylation of PI3K and Akt. In addition, application of LY294002 decreased the viability of H9c2 cells even after overexpression of ILF3-AS1 under hypoxia treatment (Figure 5B). Moreover, application of LY294002 also restored the apoptosis levels after overexpression of ILF3-AS1 under hypoxia treatment (Figure 5C). The change in apoptosis-related proteins was in line with the apoptosis assay results (Figure 5D). Migration and invasion assays also revealed that LY294002 abrogated the protective effects of ILF3-AS1 on hypoxia-induced injury induced by hypoxia (Figure 5E, F). These findings indicate that overexpression of ILF3-AS1 prevents H9c2 cells from undergoing hypoxia-induced myocardial injury via regulation of the PI3K/Akt pathway.

Discussion

MI can lead to irreversible damage to the myocardium, which is a leading cause of morbidity and mortality worldwide[13]. However, the mechanisms of MI injury are largely elusive. In the present study, we found that overexpression of ILF3-AS1 protected H9c2 cells from hypoxic injury. ILF3 negatively regulated miR-212-3p expression, and inhibition of miR-212-3p also exerted similar protective effects against injury induced by hypoxia. Moreover, SIRT1 was identified as a target of miR-212-3p, and miR-212-3p negatively regulated the expression of SIRT1. Furthermore, it was also determined that overexpression of ILF3-AS1 activated the PI3K/Akt pathway in hypoxia-treated H9c2 cells, and inhibition of the PI3K/Akt pathway could abrogate the protective effects of ILF3-AS1. Therefore, the PI3K/Akt signaling pathway plays an essential role in the myoprotective effects of ILF3-AS1. These findings detected the key functions of ILF3-AS1 in regulating MI injury.

LncRNAs function as competitive endogenous RNA (ceRNA) to negatively regulate the expression of miRNA[14]. In line with a study[9] in which ILF3-AS1 was shown to act as a ceRNA of miR-212, we also found that ILF3-AS1 negatively regulated the expression of miR-212-3p in H9c2 cells. In our research, it was found that miR-212-3p regulates MI injury by affecting apoptosis. Of note, Liu et al[15] demonstrated that miR-212-3p had similar effects on MI injury via regulation of autophagy-dependent apoptosis. Moreover, miR-212-3p has also been shown to have essential roles in regulating cardiac hypertrophy[16]. It will be interesting to further investigate the role of miR-212-3p in cardiac physiology.

MiRNAs are known to regulate various biological processes by targeting the 3'-UTR of mRNA and thereby negatively regulating the miRNA target genes[17]. In line with previous findings showing that SIRT1 is a target of miR-212, we also confirmed the target relationship between SIRT1 and miR-212-3p[18,19]. SIRT1 is an NAD+-dependent histone deacetylase that plays an essential role in various cellular functions, such as metabolism, apoptosis, stress, and aging[20]. We found that overexpression of SIRT1 could protect H9c2 cells from hypoxia-induced injury. This finding is in line with previous studies[21,22] that also found that activation of SIRT1 can attenuate MI injury. However, we did not

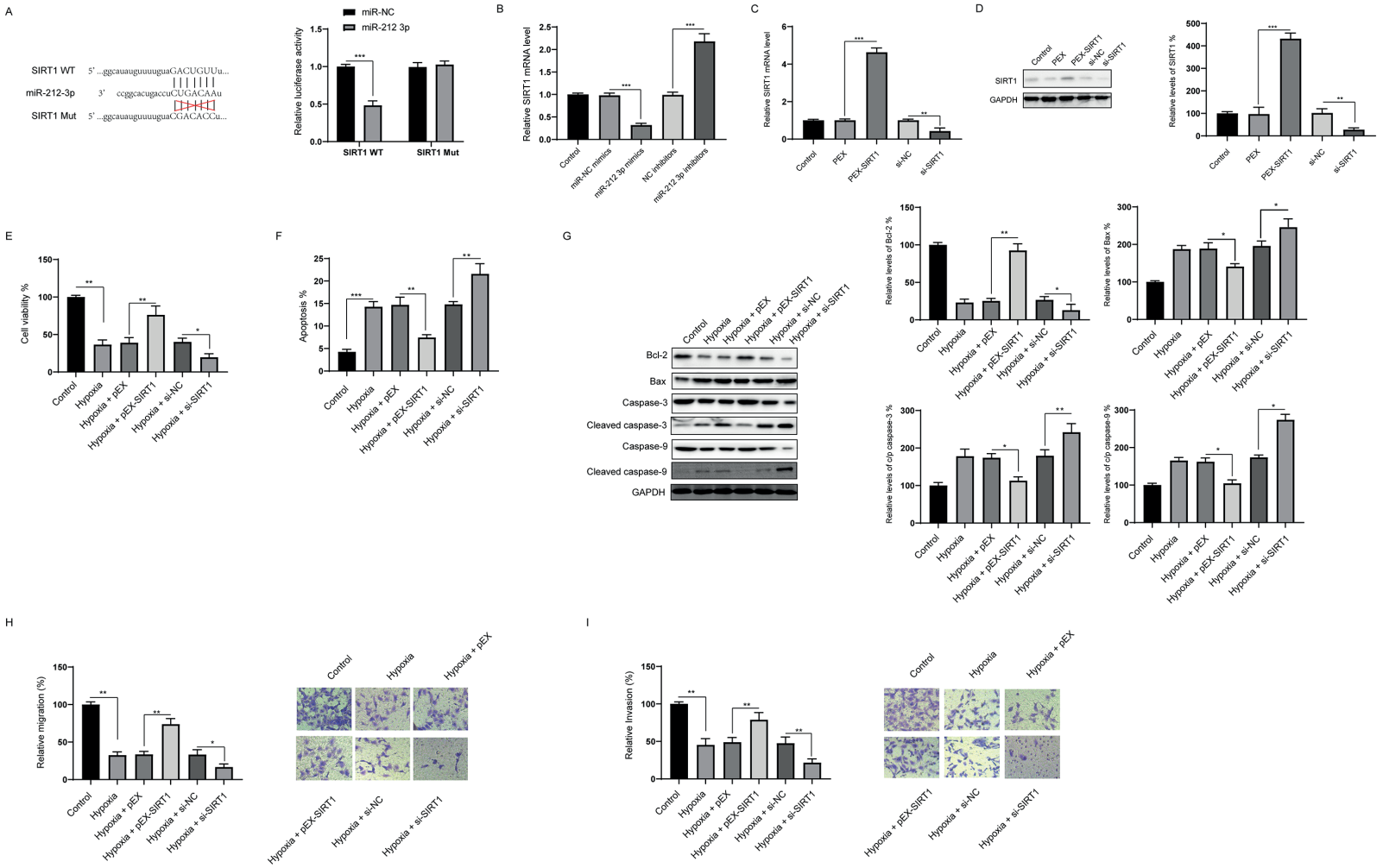

Figure 4. SIRT1 was a target of miR-212-3p. **A**, Schematic picture of the predicted interaction sites between SIRT1 and miR-212-3p (left) and the luciferase activity assay showed the target relationship between miR-212-3p and SIRT1 (right). **B**, After transfection of miR-212-3p mimics or inhibitors respectively in H9c2 cells, then the SIRT1 mRNA levels were assayed by RT-PCR. **C**, After transfection of SIRT1 overexpression vector or siRNA against SIRT1, then the SIRT1 mRNA levels were assayed by RT-PCR. **D**, After transfection of SIRT1 overexpression vector or siRNA against SIRT1, then the SIRT1 protein levels were assayed by Western blots. **E**, After treated as indicated, cell viability of H9c2 cells was assayed by CCK-8 assay. **F**, After treated as indicated, cellular apoptosis of H9c2 cells was assayed. **G**, After treated as indicated, apoptosis-related proteins were measured by Western blot. **H**, **I**, After treated as indicated, migration and invasion were measured respectively (100 ×). Data are presented as mean ± SD from three independent experiments performed in duplicate. $^{**}p<0.01$, $^{***}p<0.001$.

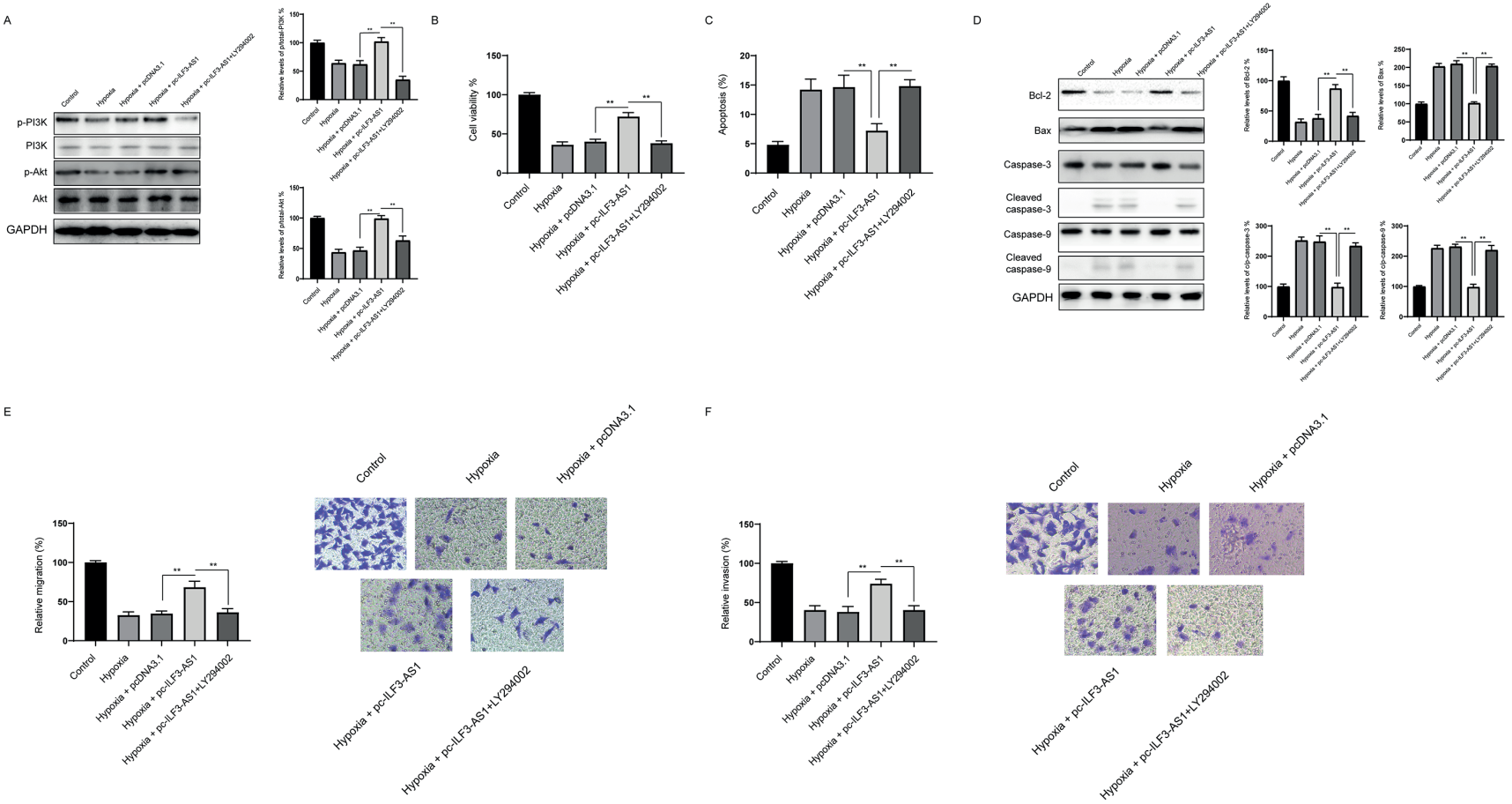

Figure 5. Akt/PI3K signaling pathway was involved in the protection effects of ILF3-AS1 against hypoxia. **A**, H9c2 cells were treated as indicated, then total cellular lysates were subjected to Western blot analysis. **B**, Cells were treated as indicated, then cell viability was assayed by the CCK-8 assay. **C**, Cells were treated as indicated, then cellular apoptosis was measured. **D**, Cells were treated as indicated, then total cellular lysates were subjected to Western blot analysis. **E**, **F**, Cells were treated as indicated, then migration and invasion were assayed, respectively (100×). Data are presented as mean ± SD from three independent experiments performed in duplicate. *p<0.05, **p<0.01, ***p<0.001.

investigate the mechanisms by which SIRT1 protects H9c2 from hypoxia-induced injury. Hsu et al[23] indicated that SIRT1 activation can lead to the activation of FOXO1 and thereby upregulate antioxidants and downregulate proapoptotic molecules, which ultimately results in the decrease of oxidative stress. Therefore, SIRT1 might be applied as a key indicator guiding the clinical diagnosis and treatment of heart disease.

PI3K/Akt signaling pathway plays an essential role in the regulation of myocardial survival[24-26]. Activation of the PI3K/Akt pathway could exert protective effects against ischemia/reperfusion injury[24]. The PI3K/Akt pathway also protects cardiomyocytes against hypoxia-induced apoptosis via translocation of pAkt to mitochondria[27]. Many studies have found that various lncRNAs affect the survival of cardiomyocytes via the PI3K/Akt signaling pathway. Shi et al[28] revealed that lncRNA FAF protected cardiomyocytes from hypoxia-induced apoptosis via activation of the PI3K/Akt pathway. LncRNA HOTAIR also increases the viability of cardiomyocytes via activation of PI3K/Akt and thereby improves diabetic cardiomyopathy[29]. In the present work, we found that hypoxia inhibited the phosphorylation of PI3K and Akt. Moreover, inhibition of PI3K by a specific inhibitor abrogated the cardioprotective function of ILF3-AS1 in hypoxia-induced H9c2 cells. To our knowledge, this is the first report showing that ILF3-AS1 affects the PI3K/Akt signaling pathway, and it will be interesting to test whether ILF3-AS1 also regulates PI3K/Akt signaling under other conditions.

Conclusions

We indicate that ILF3-AS1 may protect H9c2 cells from hypoxia-induced injury by regulating miR-212-3p/SIRT1, as well as the PI3K/Akt signaling pathway. Therefore, ILF3-AS1 may serve as a potential therapeutic target in myocardial ischemia. However, a drawback of our research is the lack of clinical and *in vivo* investigations of ILF3-AS1, and further investigations are still needed to confirm our findings.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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