MiR-181a Promotes Hypoxia-Induced Apoptosis and Mitochondrial Damage Through Regulating Higd-1a

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Abstract

Aberrant microRNAs are strongly associated with cardiac diseases. However, the regulation mechanism of MiR-181a in hypoxia-induced cardiomyocytes apoptosis and mitochondrial fragmentation have not been clarified. In the present study, we investigated the function of miR-181a in cardiomyocytes under hypoxic conditions. Cell viability, apoptosis, mitochondrial fragmentation, ROS level, activity of caspase 3 and 9, mitochondrial membrane potential, as well as primary antibodies Bcl-2, Bax, Drp1, MFN2, and Higd-1a levels in treated cells were tested. The results showed that overexpression of miR-181a led to an increase in apoptosis, ROS production, and mitochondrial membrane potential loss. Mechanistically, miR-181a promotes mitochondrial fission through targeting Higd-1a, and the effects of miR-181a could be rescued by Higd-1a. Collectively, our results are beneficial to understand the function of miR-181a in hypoxia-induced apoptosis and mitochondrial damage, which might become a novel direction for related diseases.

Keywords

MiR-181a, Hypoxia, Higd-1a, Apoptosis, Mitochondrial fragmentation

Introduction

Mitochondria are important subcellular organelles regulating oxidative phosphorylation and apoptosis. The balance between fusion and fission is essential for maintaining the morphology and function of mitochondria [1]. Unbalanced fission and fusion contribute to the occurrence of neurodegenerative and cardiac disease-related disorders [2,3]. For instance, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) protected against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydrodropyridine-induced neurotoxicity [4]. Mitochondrial fusion and fission are essential for its function. The dynamin-related protein 1 (DRP1) can assemble into multimeric ring-like structures and wrap around the constriction sites of dividing mitochondria to regulate mitochondrial fission [5]. Mitochondrial outer membrane dynamin-like guanosine triphosphatases (GTPases) mitosis MFN-1 and MFN-2 contribute to mitochondrial docking and fusion [6]. Mitochondrial inner membrane protein optic atrophy 1 (OPA1) is encoded by eight mRNA splice forms, which are produced by differential splicing. Evidence suggests that cleavage of OPA1 is required for inner mitochondrial membrane fusion [7].

Hypoxia is closely associated with cardiac diseases and the tumor microenvironment [8]. Studies have revealed that hypoxia induces mitochondrial fragmentation [9]. The HIG1 hypoxia inducible domain (HIGD) gene family is conserved throughout evolution. HIGD genes expressions were induced by hypoxia. Expression of Higd-1a is a survival factor for pancreatic beta cells and is regulated by hypoxia-inducible factor-1 (HIF1) under hypoxic conditions [10]. In addition, Higd-1b regulates cell viability and apoptosis in cardiomyocytes through interacting with OPA1 [11]. MicroRNAs (miRNAs), a class of 17-23 nucleotide non-coding RNAs, have been documented to be involved in diverse biological and pathological processes by binding to 3'-UTR (3'-untranslated region) of the target mRNAs, thus inducing

the translational inhibition and degradation of mRNAs [12].

Previous studies have established a link between aberrant miRNAs expression and hypoxia. Under hypoxic conditions, HIF-1α (Hypoxia-Inducible Factor-1α) enhances macrophage necroptosis through upregulating miR-210 and downregulating miR-383 [13]. It was reported that miRNAs regulate apoptosis of cardiac cells by regulating mitochondrial fission and fusion and reactive oxygen species (ROS) production [14]. MiR-181 was also regarded as involved in regulation of the aldosterone-mineralocorticoid receptor (Aldo-MR) pathway during myocardial infarction [15]. In addition, miR-181a regulated the reproduction and migration potentials of cardiac progenitor cells through targeting LYRM1 in hypoxia [16]. However, the role and mechanism of miR-181a in mitochondrial fusion and fission have not been explored.

The exploration of miR-181a in mitochondrial dynamics is crucial as it might reveal novel therapeutic targets for diseases related to mitochondrial dysfunction, such as heart failure and neurodegenerative disorders. In this study, the biological function of miR-181a in hypoxia-induced mitochondrial fragmentation was characterized, and the underlying mechanisms were investigated. Furthermore, the functional relationship between miR-181a and Higd-1a was assessed. Our experiments, results, and analysis may provide valuable information on the roles of miR-181a in hypoxia-induced mitochondrial fusion and fission in cardiac cells.

Materials and methods

Cell culture and transfection

AC16 human cardiomyocytes were purchased from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37˚C in a humidified incubator with 5% CO2. For hypoxia experiments, cells were cultured in a hypoxia chamber with 1% oxygen (5% CO2 and 94% N2). All transfection experiments were carried out with Lipo 3000 transfection reagent (Thermo Fisher, USA) according to the manufacturer's instructions. The miR-181a inhibitor, mimic, and negative control oligonucleotides were purchased from Sangon Biotech Shanghai (China, Shanghai). For Higd-1a overexpression, the coding sequence of Higd-1a was inserted in pcDNA6-Flag vector to generate pcDNA6-Higd1a-Flag.

Flow analysis

Apoptotic cells were assessed by flow cytometric analysis using an Annexin V-FITC Apoptosis Detection kit (Solaibao Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's protocol. Early and late apoptotic cells (Annexin V+/PI− and Annexin V+/PI+) were evaluated using a flow cytometer (FACSCanto II; BD Bioscience), and the flow cytometry data was analyzed using FlowJo v10 (FlowJo, LLC). For mitochondrial membrane potential analysis, cells were incubated with JC-1 solution at 37˚C for 15 min and washed twice with JC-1 dye buffer. After JC-1 staining, the fluorescence intensity of JC-1 was analyzed using a fluorescence microscope. The JC-1 monomer (Red): JC-1 aggregate (Green) ratio was calculated to determine the loss of mitochondrial membrane potential.

Intracellular ROS levels were measured using a Reactive Oxygen Species Assay Kit (Solaibao Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions. After treatment, cells were incubated with 5 μM DCFH-DA at 37˚C for 30 min and then analyzed by flow cytometry. Caspase 3 and 9 activity was measured with Fluorescent Assay kits (cat. nos.C1168S and C1157; Beyotime Institute of Biotechnology).

Cell viability assay

Cell viability was measured by using an MTT Cell Proliferation and Cytotoxicity Assay Kit (Solaibao Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions. After treatment, AC16 cells were seeded on a 96-well plate. Subsequently, MTT solution was added, and the reaction system was incubated for 2 h at 37˚C. The relative viability of cells was obtained at 490 nm absorbance. For crystal violet staining assay, cells were fixed with 4% formaldehyde at room temperature for 15 min, followed by staining with a crystal violet solution (crystal violet 0.2%, ethanol 2%) at room temperature for 10 min, and then the colonies were photographed using a dissection microscope.

Luciferase reporter assay

The target gene of miR-181a was confirmed by 3'-untranslated region (UTR) luciferase, wherein the wild-type (WT, 5'-acUCCCUGUUUGCUGCAGAAUGUc-3') and mutant (MU, 5'-acUCCCUGUUUGCUGCAC-UUACAc-3') 3'-UTR of Higd-1a were cloned into the downstream of a luciferase reporter gene in the pair GLO

vector (Promega, USA). Cells were co-transfected with luciferase constructs and miR-181a mimic. After 48 h, a dual-luciferase reporter assay system (Promega, USA) was used to study the luciferase activity.

Western blot analysis

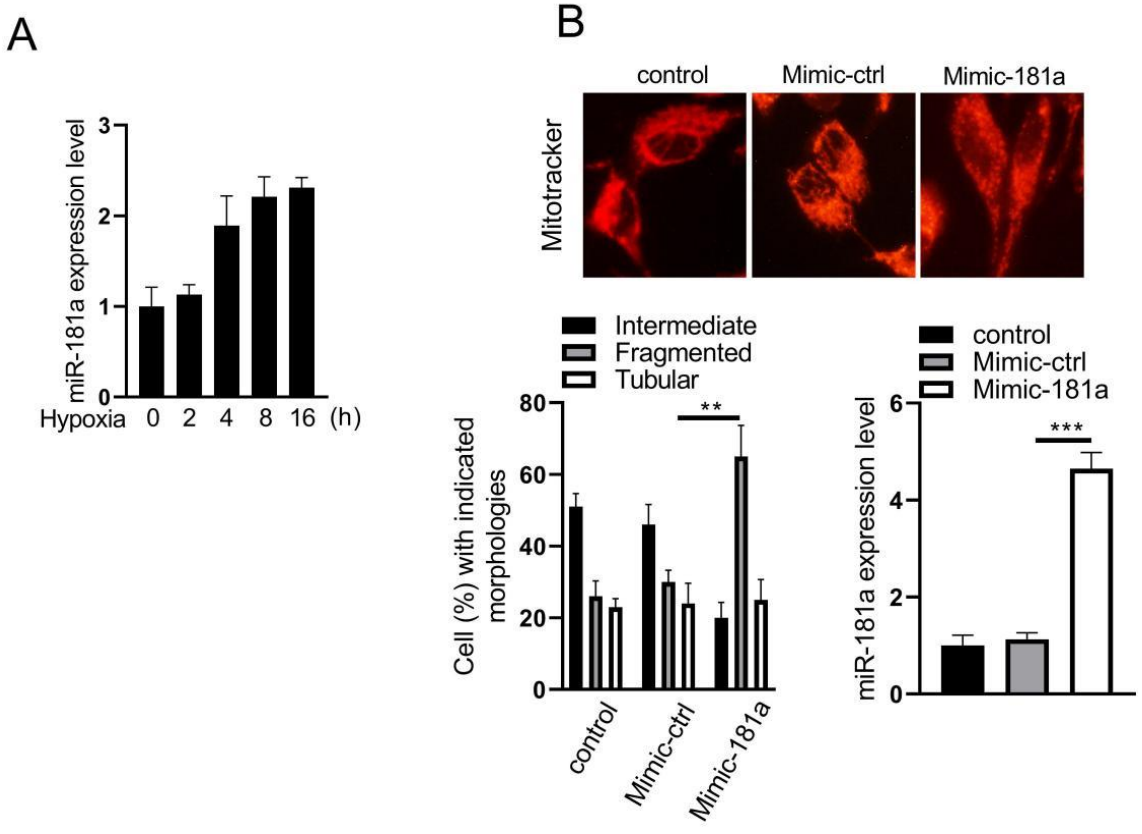
Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer (Solaibao Biotechnology Co., Ltd., Beijing, China) with protease inhibitor and phosphatase inhibitor cocktail (Solaibao Biotechnology Co., Ltd., Beijing, China) for 20 min on ice and then centrifuged for 10 min at 10,000×g and 4˚C. Protein concentration was measured using bicinchoninic acid (BCA) protein assay kits (Solaibao Biotechnology Co., Ltd., Beijing, China). Equal amounts of the protein samples were separated via 10% SDS-PAGE gel and then transferred onto polyvinylidene difluoride (PVDF) membranes for 1 h at 100 V on ice. Following blocking with 5% nonfat milk in Tris-buffered saline and Tween 20 (TBST) for 1 h at room temperature, the membranes were incubated overnight at 4°C with the indicated primary antibody. After washing 3 times with TBST, the membranes were incubated with the respective secondary antibody at room temperature for 1 h. The membranes were incubated with ECL reagent (Millipore) to visualize proteins. Primary antibodies: Higd-1a (1:2,000; cat. no. ab117456; Abcam), Bcl-2 (1:2,000; cat. no. sc-7382; Santa Cruz Biotechnology, Inc.), Bax (1:2,000; cat. no.5023S; Cell Signaling Technology, Inc.), beta Actin (1:5,000; cat. no. ab8226; Abcam), MFN2 (1:2,500; cat. no. ab205236; Abcam), Drp1 (1:2,000; cat. no. ab184247; Abcam).

Immunofluorescence

AC16 cells were plated on glass coverslips at 37˚C for 48 h, and stained with 100 NM Mito Tracker red CMXRos at 37˚C for 30 min, then fixed with 4% formaldehyde at 4˚C for 30 min. To determine the morphology of the mitochondria, the cells were classified into three types under a fluorescence microscope: Tubular, intermediate, and fragmented. “Tubular” referred to the cells that exhibited the most interconnected mitochondria, while “fragmented” referred to cells that primarily contained spherical mitochondrial segments.

Statistical analysis

Quantification of immuno-blots was performed with ImageJ 1.51p. Altered levels of protein were presented as normalized fold change compared to the control value. Statistical analysis was performed using ANOVA followed by Tukey’s post hoc test. P-values below 0.05 were considered significantly different. As shown Figure 1.



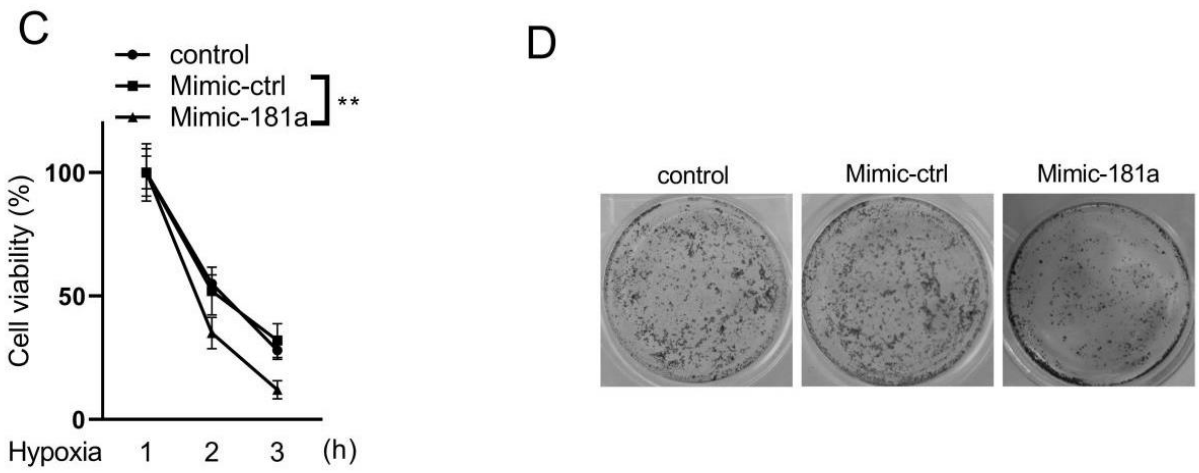


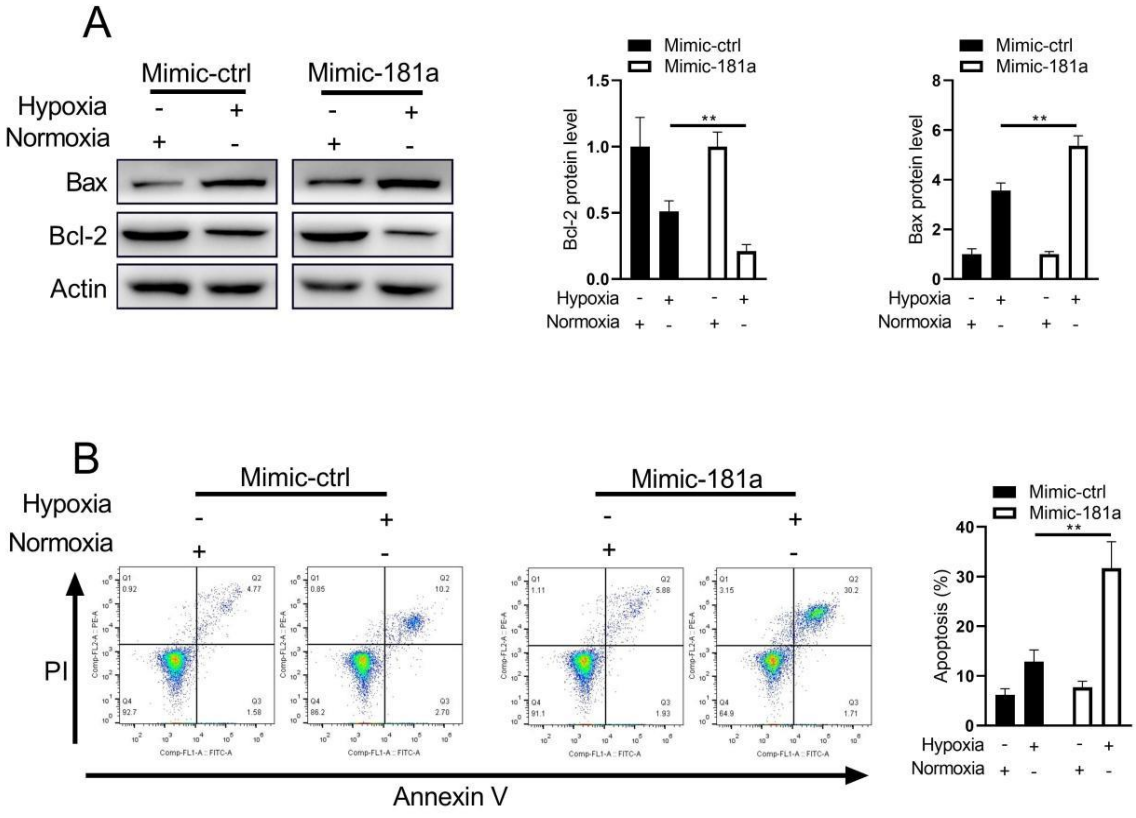
Figure 1. Overexpression of miR-181a induces mitochondrial fragmentation.

Note: (A) miR-181a expression level in AC16 cells. (B) Microscopy graphs of cells after stained with MitoTracker. Scale bar, 2 μm. (C) Cell viability. (D) The visualized cells using crystal violet. The data are presented as the mean ± SD. **P<0.01, ***P<0.001.

Overexpression of miR-181a promotes hypoxia-induced apoptosis

Next, the effects of miR-181a on hypoxia-induced apoptosis were assessed. Under hypoxic conditions, an increase in level of pro-apoptotic protein Bax and a concomitant decrease in level of Bcl-2, anti-apoptotic proteins, was observed. However, overexpression of miR-181a led to a further increase in Bax and a decrease in Bcl-2 (Figure 2A).

The results of Annexin V/PI staining showed that overexpression of miR-181a promoted hypoxia-induced apoptosis (Figure 2B), and miR-181a-overexpression cells exhibited higher caspase 3 and 9 activity compared with control cells, indicating that miR-181a promoted apoptosis via the caspase-dependent pathway (Figure 2C). In addition, miR-181a overexpression further enhanced hypoxia-induced ROS generation (Figure 2D), suggesting that miR-181a regulated hypoxia-induced apoptosis via the mitochondrial pathway.



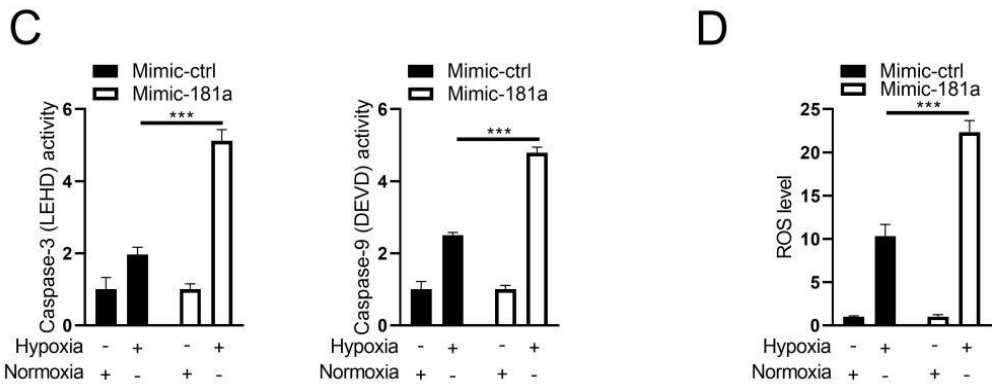


Figure 2. Overexpression of miR-181a promotes hypoxia-induced cell death.

(Note: (A) Detection of Bax and Bcl-2 protein expression levels. (B) Annexin V/PI staining. (C) Caspase 3 and 9 activities. (D) ROS level. The data are presented as the mean ± SD. **P<0.01, ***P<0.001).

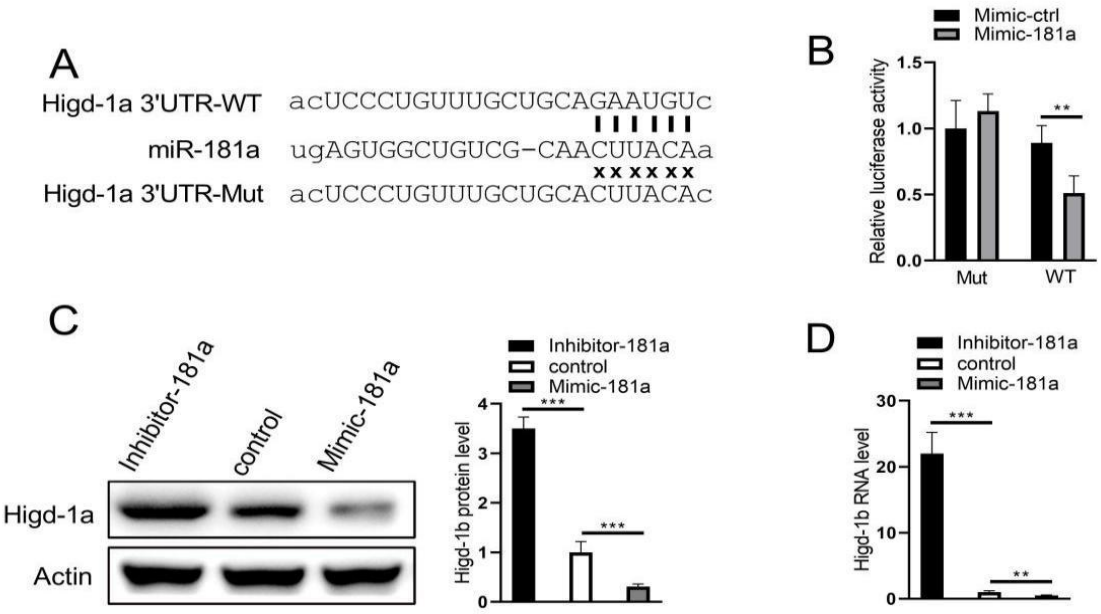
Higd-1a is a direct downstream target of miR-181a

To predict the targets of miR-181a, we performed bioinformatics analysis using ENCORI database (https://starbase.sysu.edu.cn/index.php).

The results showed that potential miR-181a binding sites were found within 6002 genes. Among the candidate target genes, we focus on Higd-1a, which has been shown to be involved in regulation of mitochondrial fusion and fission [17].

Therefore, we selected Higd-1a and studied the relationship between it and miR-181a. The predictive binding site for miR-181a in the 3'-UTR of Higd-1a mRNA were imported from ENCORI database (Figure 3A). The direct reaction between miR-181a and Higd-1a by dual-luciferase reporter assay. The function of luciferase was inhibited in cells transfected with miR-181a mimic fused to the 3'-UTR of Higd-1a, comparing to the control groups (Figure 3B). It was observed that the protein and RNA levels of Higd-1a were reduced in the miR-181a mimic group, while it was enhanced in the miR-181a inhibitor group (Figure 3C and D). To verify whether miR-181a regulated hypoxia-induced apoptosis through Higd-1a, the rescue assay was performed. As expected, overexpression of miR-181a results in an increase in Bax and a decrease in Bcl-2 under hypoxic conditions, while Higd-1a overexpression could restore these proteins expression (Figure 3E). Similar results were also obtained when testing the apoptosis through flow cytometry analysis (Figure 3F).

Therefore, these results together showed that miR-181a regulated hypoxia-induced apoptosis through targeting Higd-1a.



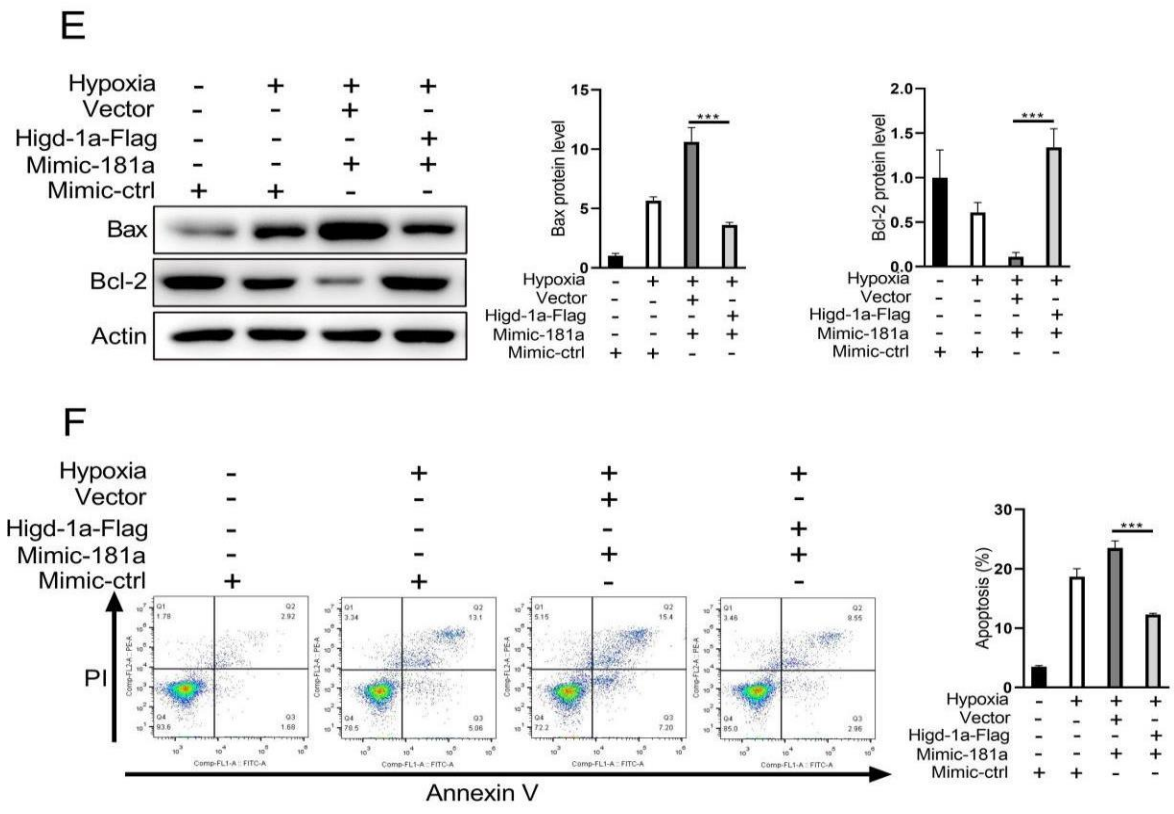


Figure 3. Higd-1a is a direct target of miR-181a.

Note: (A) The 3'-untranslated region (UTR) of Higd-1a harbor potential miR-181a binding sites. (B) Luciferase activity. (C) The protein level of Higd-1a. (D) The RNA level of Higd-1a. (E) The protein levels of Bax and Bcl-2. (F) Cell apoptosis. The data are presented as the mean ± SD. **P<0.01, ***P<0.001.

MiR-181a influenced hypoxia-induced mitochondrial damage through targeting Higd-1a

The results demonstrated that miR-181a was involved in hypoxia-induced apoptosis and mitochondrial fragmentation; the role of miR-181a in fusion- and fission-associated protein expression levels was therefore investigated. As shown in Figure 4A, the protein expression level of the mitochondrial fusion protein MFN2 was observably lessened in AC16 cells under hypoxic conditions, and this reduction was further exacerbated when cells were transfected with miR-181a mimic to achieve miR-181a overexpression. In contrast, the expression trend of the mitochondrial fission protein Drp1 was completely opposite to that of MFN2: hypoxia alone significantly upregulated Drp1 expression, and miR-181a overexpression further amplified this upregulation, driving a shift toward excessive mitochondrial fission. However, restoration of Higd-1a expression in miR-181a-overexpression cells through transfection with pcDNA-Higd1a-Flag vector effectively reversed these effects, increasing MFN2 expression and decreasing Drp1 levels to restore the balance of mitochondrial dynamics. These results indicated that miR-181a promoted mitochondrial fission through Higd-1a (Figure 4A).

It was also found that miR-181a overexpression significantly elevated hypoxia-induced ROS generation, exacerbating oxidative stress in the mitochondria, while Higd-1a overexpression effectively inhibited this excessive ROS production, mitigating oxidative damage (Figure 4B). Under hypoxic conditions, the number of JC-1 monomer-positive cells increased, indicating a marked loss of mitochondrial membrane potential, a key early marker of apoptosis and mitochondrial dysfunction. AC16 cells transfected with miR-181a mimic showed further aggravated mitochondrial membrane potential loss, which was effectively restored by Higd-1a overexpression (Figure 4C).

Furthermore, transfection with miR-181a mimic resulted in a higher proportion of fragmented mitochondria in AC16 cells under hypoxia, while overexpression of Higd-1a significantly inhibited this effect, preserving the normal tubular mitochondrial morphology. Based on these findings, it was concluded that miR-181a exacerbated hypoxia-induced mitochondrial damage through downregulating Higd-1a, disrupting

mitochondrial dynamics, increasing oxidative stress, and impairing mitochondrial function.

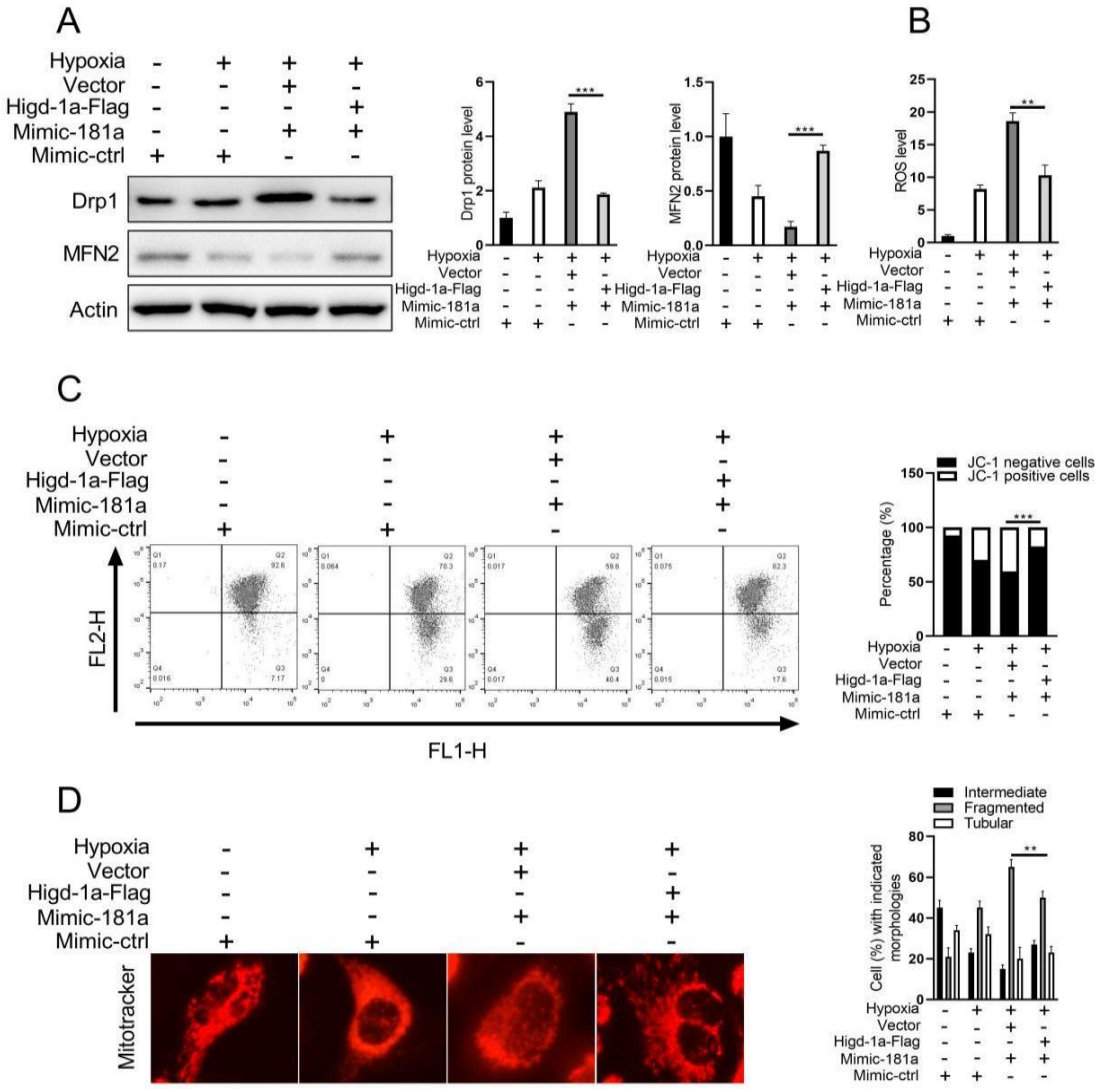


Figure 4. Mir-181a enhances hypoxia-induced mitochondrial damage through Higd-1a.

Note: (A) The protein levels of Drp1 and MFN2. (B) The ROS levels. (C) JC-1 staining. (D) Stained cells with Mito Tracker. The data are presented as the mean ± SD. **P<0.01, ***P<0.001.

Discussion

Mitochondrial fusion is required for cardiomyocyte differentiation and heart development, although it occurs rarely. Hypoxia can induce mitochondrial dysfunctions and apoptosis in cardiomyocytes. In-depth understanding of molecular mechanisms of mitochondrial fusion and fission is important for the novel methods development of diseases caused by hypoxia in cardiomyocytes. This study was performed to investigate the roles of miR-181a in apoptosis and mitochondrial fragmentation of AC16 in hypoxia, with a conclusion that overexpression of miR-181a could enhance hypoxia-induced apoptosis and mitochondrial fission via downregulating Higd-1a expression.

It was shown that miR-181a expression in AC16 cells had a slight upregulation during hypoxia [18]. Overexpression miR-181a resulted in a decrease in cell viability and mitochondrial fragmentation, suggesting that miR-181a increases hypoxia-induced cell damage, which is consistent with the results of previous study, indicating that miR-181a may have adverse effects on the survival of cardiomyocytes [19].

The expression ratio of Bcl-2 and Bax is involved in hypoxia-induced apoptosis. Downregulation of Bax in caspase 3 activity following hypoxia-ischemia compared with wild-type mice [20,21]. The results of the present study demonstrated that miR-181a promoted hypoxia-induced apoptosis by changing expression levels of Bcl-2 and Bax: miR-181a-overexpression cells exhibited significantly increased protein expression levels of Bax

and decreased expression levels of Bcl-2. Moreover, miR-181a-overexpression cells exhibited more caspase 3 and 9 activity. Therefore, miR-181a regulated mitochondrial apoptosis involving caspase proteins. Different molecular mechanisms for miR-181a with apoptosis have been identified in different cell models. For example, miR-181a is upregulated in hydrogen peroxide-treated granulosa cells and promotes apoptosis through regulating Foxo1 acetylation via SIRT1. In laryngeal cancer cells, miR-181a regulates apoptosis by directly targeting NPM1. According to bioinformatics analyses using ENCORI database, Higd-1a was suggested to be a potential target of miR-181a. The present study also provided evidence that miR-181a negatively regulated the expression of Higd-1a. Further rescue experiments revealed the antagonistic roles of miR-181a and Higd-1a in the regulation of hypoxia-induced apoptosis. It was reported that Higd-1a co-localized with mitochondria and served an anti-apoptotic function in response to hypoxia [22]. The Knockdown of Higd-1a gene has been found to alter mitochondria membrane potential or cellular ATP. Furthermore, Higd-1a was required for optimal fusion of mitochondria in Drp1-silenced cells.

Mitochondrial fission and fusion can affect apoptotic cell death. Drp1 is a mitochondrial fission protein, which can be recruited to mitochondria, and divides the outer and inner membranes of the mitochondrion [23]. Mitochondrial fusion protein MFN2 is required for outer membrane fusion [24]. It was shown that upregulation of miR-181a increased Drp1 expression and decreased MFN2, while Higd-1a overexpression restored these proteins expression. The excessive ROS accumulation can lead to even more mitochondrial damage and mitochondrial membrane potential loss. It was observed that miR-181a overexpression increased ROS generation and decreased mitochondrial membrane potential. However, Higd-1a attenuated these effects of miR-181a under hypoxia conditions, indicating that miR-181a can exacerbate hypoxia-induced mitochondrial damage by downregulating Higd-1a.

Conclusion

Summarily, the present study demonstrated that overexpression of miR-181a promotes hypoxia-induced cell death and mitochondrial fragmentation. Additionally, miR-181a overexpression promotes mitochondrial fission through suppressing Higd-1a expression. Overall, the results of the present study may help to understand the molecular mechanisms by which hypoxia-induced mitochondrial damage.

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Conflicts of Interest

The authors declare no conflict of interest.

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