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Ischemia-reperfusion injury in human AC16 cardiomyocytes is modulated by AXIN1 depending on c-Myc regulation

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Objective: A major consequence of acute myocardial infarction is myocardial ischemia-reperfusion (I/R) injury. Collecting proof demonstrates that AXIN1 assume a basic part in different disease; however, the role of AXIN1 in I/R injury remains to a great extent obscure.

Methods: The I/R injury model on AC16 cells was constructed. siRNA transfection was used to knockdown AXIN1. The qRT-PCR assays and western blot assays were used to detect the expression level of AXIN1 and other key proteins. CCK-8 assays and cell apoptosis assays were used to detect cell proliferation and cell apoptosis.

Results: AXIN1 was significantly overexpressed in an in vitro model of I/R injury. Knockdown of AXIN1 significantly restored the cell proliferation inhibition caused by IR injury, while inhibiting apoptosis and inflammation. Further mechanistic studies revealed that the transcription factor c-Myc could regulate the expression of AXIN1. The effects of I/R injury on AC16 cells after overexpression of c-Myc were reversed by knockdown of AXIN1. Meanwhile, AXIN1 could regulate the SIRT1/p53/Nrf 2 pathway.

Conclusion: Our results show an important role for AXIN1 and provide new targets for avoiding and treating I/R injury.

Keywords: apoptosis, AXIN1, c-Myc, cell proliferation, myocardial I/R injury

Introduction

Myocardial ischemia-reperfusion (I/R) injury is on the rise in China and around the world. China's death rate has been rapidly increasing since 2005[1–3]. Acute reperfusion therapy is currently the most common and successful treatment for acute heart attack[4,5]. Rapidly restoring blood flow after a heart attack, on the other hand, may set off a chain reaction of unfavorable events such as cardiocyte apoptosis, inflammatory response, and oxidative stress, exacerbating rather than easing myocardial infarction[6]. Myocardial I/R injury is the medical term for this situation[7]. In the treatment of patients with severe myocardial dead tissue, myocardial I/R damage has recently become a serious concern[8]. Pretreatment or treatment with antiapoptotic, anti-mitigating, or antioxidative techniques may be essential for reducing the occurrence of myocardial I/R injury, which affects the clinical outcome of reperfusion therapy.

HIGHLIGHTS

- AXIN1 was significantly overexpressed in an in vitro model of ischemia-reperfusion (I/R) injury.
- AXIN1 regulated the SIRT1/p53/Nrf2 pathway in I/R injury.
- AXIN1 is regulated by the transcription factor c-Myc in I/R injury.

The growth silencer AXIN1 is a delegate of characteristically confused framework proteins[9]. Apart from its primary role in Wnt signaling[10], it also acts as a negative regulator of -catenin in the Wnt flagging pathway[11,12]. AXIN1 has been distinguished as a part of a few unique pathways, including p53, c-Myc, TGF-β, and JNK[13,14]. c-Myc directs a few cell capacities including cell development, expansion, apoptosis, separation, digestion, and neoplastic change[15,16]. The quality is broadly communicated during cell development and fast expansion[17,18]. Besides, it stays vague whether c-Myc can go about as a downstream go-between of AXIN1 guideline in myocardial I/R injury. The aim of our study was to investigate the role of AXIN1 and its specific molecular mechanisms in an *in vitro* I/R injury model, providing potential drug targets for the clinical treatment of myocardial I/R injury.

Methods

Cell culture and stimulation

Human AC16 ventricular cardiomyocytes (Tongpai Biotechnology) were grown in DMEM/F12 media supplemented with 5% FBS

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(Gibco), 100 U/ml penicillin (Sigma–Aldrich), and 100 g/ml streptomycin (Sigma–Aldrich) at 37°C in a humidified incubator with 95% air and 5% CO_2. The cells were typically cultivated in 6-well plates with a density of 6 104 cells per well (Corning). Sigma–Aldrich provided recombinant IL-1 with a purity of better than 98% (HPLC). For 24 h, cells were treated with 5 g/ml IL-1.

Establishment of an in vitro model of I/R injury

An in vitro model of I/R injury was laid out by treating AC16 cells with a state of oxygen glucose hardship followed by reperfusion (OGD/R). Momentarily, AC16 cells filled in 24-well cell culture dishes were kept up with in a sans glucose medium in an anoxic climate (95% N_2 and 5% CO_2) for 4 h. Then, the cells were filled in a total medium at 37°C in a 5% CO_2 and 95% air hatchery for 24 h. AC16 cells filled in normoxic conditions were utilized as controls. Also, an in vitro model of hypoxia injury was produced by refined AC16 cells with the sans glucose medium in an anoxic air (95% N_2 and 5% CO_2) for 4 h.

Cell transfection

GenePharma Co. generated the c-Myc-OE overexpression plasmid, human c-Myc sequence, and human AXIN1 sequence (Shanghai, China). The pEX-2 plasmid was used to insert full-length wide-type AXIN1 genes (GenePharma). As a negative control, an empty pEX-2 plasmid was transfected. AXIN1-specific siRNA was cloned into the U6/Neo plasmid (GenePharma). Under nonserum conditions, cell transfections were carried out with the Lipofectamine 3000 reagent (Invitrogen).

For transient transfection, a mixture of siRNA (80 nM) or/and miRNA mimic (30 nM) or/and miRNA inhibitor (50 nM) or/and plasmid (100 ng), Opti-MEM medium, and Lipofectamine 2000 (all from Thermo Fisher Scientific) was prepared as per the accompanying guidance and then dispensed into each well of 24-well cell culture dishes containing AC16 cells (5×105 per well).

CCK-8 assay

The transfected AC16 cells (5×10^3) in a 96-well plate and kept up with in a total medium. After one more 24 h of agonizing at new culture medium, cell appropriateness was studied by using CCK-8 (Dojindo Molecular Technologies). Immediately, the lifestyle medium was taken out, and the cells were washed two times with phosphate pad saline (PBS). 10 μl CCK-8 course of action was added, and the plates were incubated for 1 h at 37°C. The absorbance was assessed at 450 nm using a Microplate Reader (Bio-Rad).

Apoptosis assay

After transfection and IL-1 administration, the AC16 cells (5×10^5) in 6-well plates were examined for apoptosis using the Annexin V-FITC/PI apoptosis detection kit (Invitrogen). Within the sight of 50 g/ml RNase A, 1×10^5 cells from each example were washed two times with PBS and stained with Annexin V-FITC and PI as indicated by the producer's convention. (Sigma–Aldrich). The FACS can discriminate between apoptotic and nonapoptotic cells (Beckman Coulter). FlowJo software was used to quantify the data (Tree Star Inc.).

qRT-PCR

The RNA Pure Rapid Extraction Kit was used to extract total RNA from AC16 cells (Bioteke Corporation). The SuperScriptTM IV First-Strand Synthesis System was used to transcribe the cDNA (Invitrogen). The mRNA and miRNA expressions were detected using SYBRTM Green PCR Master Mix (Applied Biosystems).

Western blot

Full scale protein in AC16 cells after significant transfection and treatment were eliminated using a RIPA lysis pad (Beyotime). The goodness of proteins was estimated by the BCA Protein Assay Kit (Pierce). The western smudge structure was spread out using a Bio-Rad Bis-Tris Gel system (Bio-Rad Laboratories). Proteins in identical obsession were electrophoresed in PAGE-SDS and moved onto PDVF films (Millipore). Ensuing to blocking in the 5% impeding support (Beyotime), the movies were agonized with fundamental antibodies, which were prepared in the 5% ruining support at a debilitating of 1:1000. Subsequent to incubating with fundamental antibodies at 4°C present moment, the PDVF films were agonized with an assistant immunizer for 1 h at room temperature. Indications of the gatherings were discovered using the Bio-Rad ChemiDocTM XRS system (Bio-Rad). The force of the gatherings was estimated using Image Lab Software (Bio-Rad). The power of the groups was evaluated utilizing Image Lab Software (Bio-Rad).

Immunofluorescence confocal microscopy

Treated AC16 cells were gathered and cultivated onto glass coverslips uniquely ready for immunofluorescence. Subsequent to washing two times with cold PBS for 5 min, cells were fixed with 4% paraformaldehyde (PFA) for 25 min, and permeabilized with PBS containing 0.2% Triton X-100 for 25 min. Then, the cells were obstructed with 3% BSA in PBS for 1 h, and brooded with against caspase3 immunizer (1:50) at 4°C short-term. In the wake of washing threefold with PBS for 5 min, the cells were stained with FITC-formed enemy of bunny IgG immunizer (1:200) at 37° C for 1 h. In the wake of counterstaining with DAPI, (Invitrogen) for 20 min, the pictures were caught with an Olympus FV1000 confocal magnifying lens.

Dual-luciferase reporter assay

AC16 cells were co-transfected using Opti-MEM medium and Lipofectamine2000 in quintuplicate with the appropriate luciferase reporter (100 ng) and 30 nM of either a control mimic or an AXIN1 mimic. Cell lysates were used to evaluate the luciferase activity after 48 h using a dual-luciferase reporter system with a luminometer (Promega).

Chromatin immunoprecipitation (CHIP) assays

For CHIP experiments, 5×10^6 AC16 cells transfected with si-NC or si-c-Myc were lysed (0°C; 30 min) in 100–200 μl of RIPA buffer. In the meantime, a mixture of an antibody against AXIN1 (Abcam) or isotype IgG antibody (ab172730, Abcam) and protein A/G magnetic beads (MedChemExpress) was prepared and incubated for 4 h at 4°C. Following the cross-linking unfastening with the use of 0.2 μM NaCl at 65°C for 6 h, the pulled down fragment was subjected to a qPCR assay.

Statistical analysis

Except if part of the information is from Western smear examination, any remaining information is introduced as the mean ± SD. Measurements were examined by the one-way examination of change (ANOVA) in SPSS 19.0 factual programming (SPSS Inc.). A *P*-worth of lower than 0.05 was considered as a critical outcome.

Results

AXIN1 is upregulated in I/R-stimulated AC16 cells

To explain the meaning of AXIN1 in myocardial I/R injury, we first established the *in vitro* models of I/R injury. As exhibited by qRT-PCR and WB, AXIN1 was especially overexpressed in the myocardial I/R injury AC16 cells (Fig 1A and B).

Silencing AXIN1 lightens I/R-prompted injury in AC16 cells

To explore the role of AXIN1 in I/R injury, we silenced the expression of AXIN1 in AC16 cells with siRNA-AXIN1(si-AXIN1). In I/R-stimulated AC16 cells, transient transfection of si-AXIN1, but not si-NC controls, significantly reduced AXIN1 expression (Figs 2A and B). The cell viability of AXIN1 knock-down AC16 cells in the I/R injury model was measured by the CCK-8 assay. Cells were incubated for 0, 6, 12, and 24 h, respectively. As shown in Figures 2C, D and F, compared with the I/R models, knockdown of the expression of AXIN1 could enhance cell proliferation and repress cell apoptosis. The restraint of AXIN1 knockdown on cell apoptosis was additionally affirmed by the modification of Bax and Bcl-2 levels (Fig. 2E). AXIN1 knockdown reduced the production of IL-1, IL-6, and TNF in I/R-stimulated AC16 cells, implying that AXIN1 silencing inhibited cell inflammation (Fig. 2G).

AXIN1 is regulated by the transcription factor c-Myc

To understand how AXIN1 influenced I/R-induced injury in AC16 cells, we considered that c-Myc may be directly bound to AXIN1. We first overexpressed and silenced c-Myc in AC16 cells, respectively. The detection of c-Myc silencing and overexpression efficiency using Western blotting assays is shown in Figure 3A. Then, we constructed the AXIN1 wild-type or mutant luciferase plasmids and analyzed them by a double luciferase assay. Transfection of c-Myc specifically decreased and luciferase journalist quality merged on the wild-type target grouping, and this impact was protected by changes in the corresponding restricting site (Fig. 3B). CHIP experiments showed that transfection of c-Myc led to a sharp rise in the level of AXIN1 improvement in controls that had c-Myc (Fig. 3C). And then, we explored the expression of c-Myc in I/R-stimulated AC16 cells at different times. As shown in Figures 3D and E, c-Myc was overexpressed in I/R-stimulated AC16 cells, with a time-dependent relationship. We then found out if c-Myc addressed a utilitarian go between of AXIN1 in directing I/R injury. To address this, we co-transfected si-c-Myc and c-Myc-OE into AC16 cells before the I/R injury. AXIN1 articulation in AC16 cells was altogether decreased, and overexpression of c-Myc improved the outflow of AXIN1 (Fig. 3F).

Silencing of AXIN1 alleviates I/R injury in AC16 cells by upregulating c-Myc

In I/R-stimulated AC16 cells, transfected si-AXIN1 markedly reverses the effects of c-Myc-OE cell viability enhancement, inflammation inhibition, apoptosis defect, and proliferation promotion (Figs. 4A–D). Moreover, overexpression of c-Myc cancels out the power of reduced AXIN1 on TNF-α, IL-6, and IL-1β production levels in I/R-stimulated AC16 cells (Fig. 4E). In addition, we examined related pathway proteins and found the same effect. As shown in Figure 4F, Western blotting assays revealed that in I/R-treated AC16 cells, knockdown of AXIN1 offset the influence of c-Myc-OE, which reduced the expression of SIRT1 and Nrf 2, but enhanced p53.

Discussion

The system of myocardial I/R injury is a complex pathophysiological process that could prompt unfortunate patient results. Recently, it has become evident that c-Myc directs a few cell capacities, including cell development, expansion, apoptosis, separation, digestion, and neoplastic change[15,16]. Simultaneously, a significant appearance of myocardial I/R injury is the decline of myocardial cell suitability, the increment of apoptosis, and the presence of specific safe reactions. Knowing how c-Myc works at a molecular level will be crucial for developing molecularly focused therapies. In this article, our data showed that AXIN1 was overexpressed in I/R-stimulated AC16 cells. According to previous research, AXIN1 has a crucial role in cardiomyocyte apoptosis produced by I/R activation[19]. According to our findings, AXIN1 controlled I/R-induced cardiomyocyte damage by altering cell proliferation, inflammation, and apoptosis. At least in part, we offered a new molecular explanation for the critical control of AXIN1 in myocardial I/R

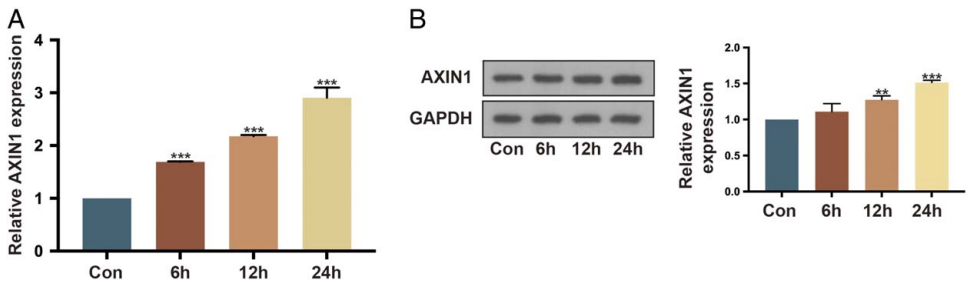

Figure 1. AXIN1 is overexpressed in I/R-stimulated AC16 cells. (A). The expression of AXIN1 in AC16 cells was measured by qRT-PCR, $N = 3$. (B) The expression of AXIN1 was monitored by Western Blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. I/R, ischemia-reperfusion.

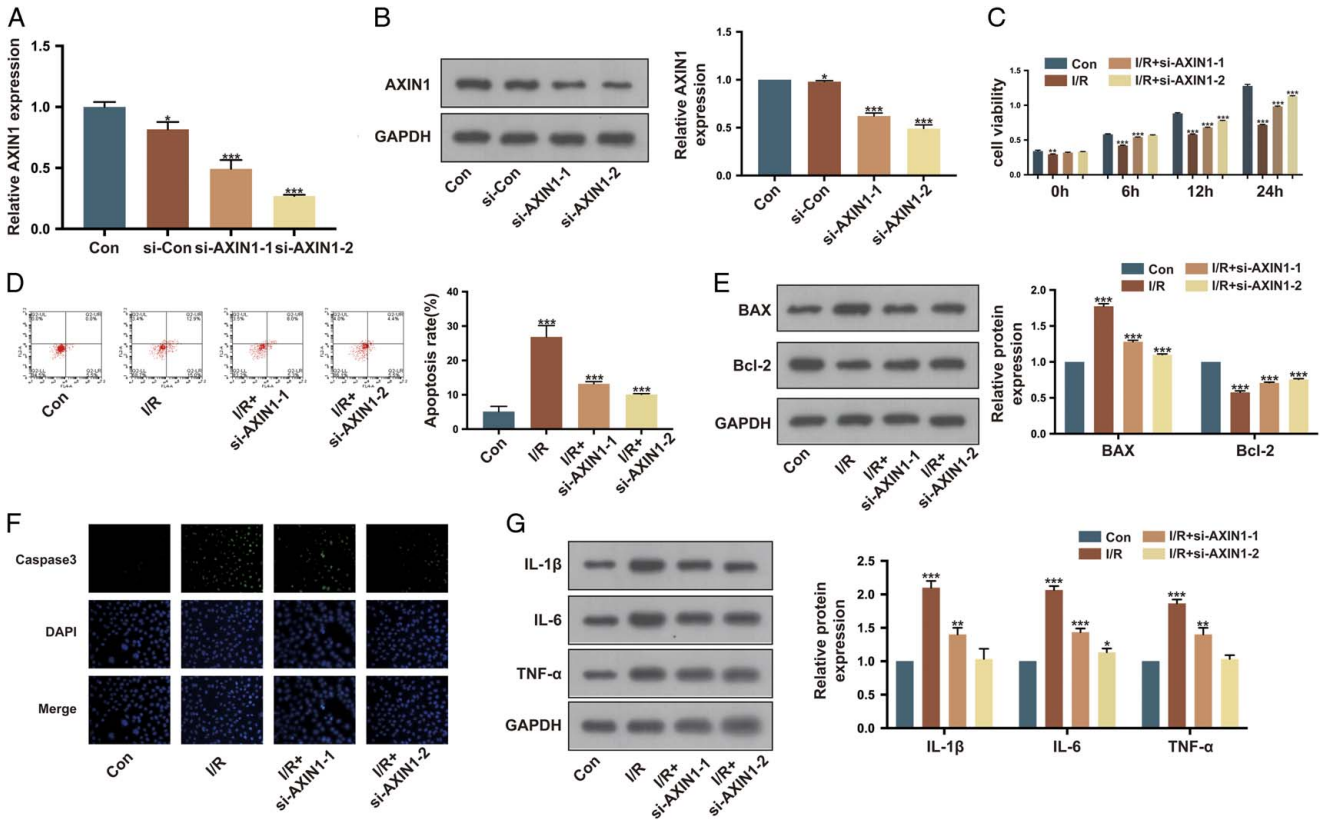

Figure 2. Knocking down AXIN1 alleviates I/R injury in AC16 cells. AC16 cells were transfected with or without si-NC or si-SNHG15and then exposed to a control condition or a condition of oxygen glucose deprivation followed by reperfusion (OGD/R). (A) AXIN1 expression was gauged by qRT-PCR in treated AC16 cells. (B) AXIN1 expression was gauged by Western Blot in treated AC16 cells. (C) Viability of treated AC16 cells was assessed by CCK-8 assay. (D) Annexin V/PI assay tested cell apoptosis. (E) Western blot showed the levels of Bax and Bcl-2 in treated AC16 cells. (F) The caspase3 expression in AC16 cells was verified by Immunofluorescence. (G) The production levels of IL-6and TNF-α in treated AC16 cells were tested by Western Blot. $n = 3$ independent biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. I/R, ischemia-reperfusion.

damage, at least in part. Also, the highly cytoplasmic restriction of AXIN1 in AC16 cells could give the likelihood of the associations between AXIN1 and c-Myc.

The basic function of c-Myc in human illnesses has been broadly revealed[6,20,21]. For instance, dysregulation of c-Myc is tightly associated with the development of various cancers, such

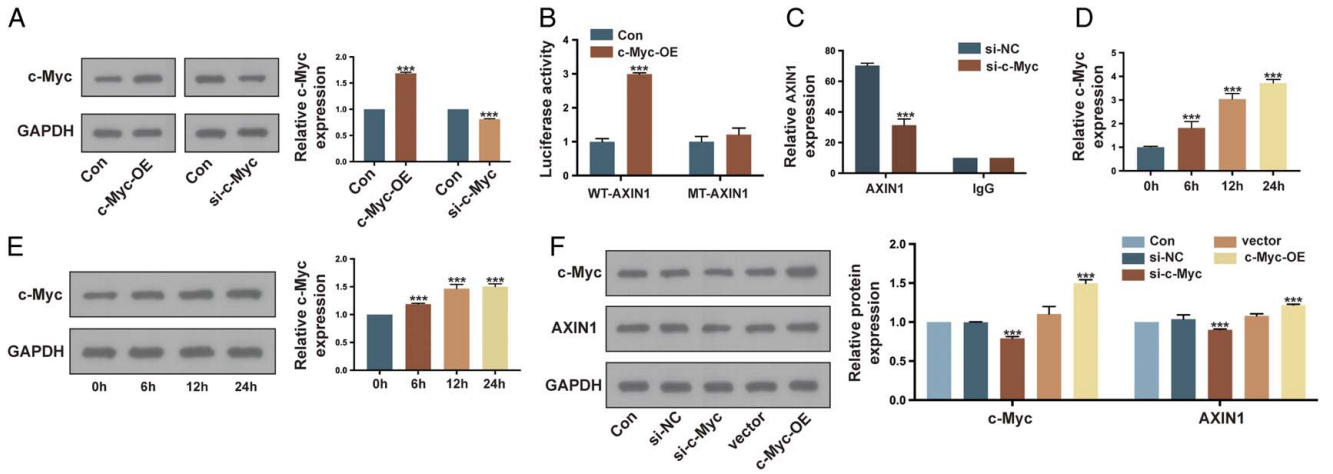

Figure 3. AXIN1 targets c-Myc. (A) c-Myc expression was gauged by Western Blot in treated AC16 cells. (B) Relative luciferase activity of AXIN1 wild-type (WT-AXIN1) or mutant (MUT-AXIN1) luciferase reporter was detected in AC16 cells transfected with c-Myc mimic. (C) AC16 cells were transfected with si-c-Myc and then lysed. CHIP experiment was performed to assess AXIN1 enrichment level using an antibody against c-Myc or IgG. (D) c-Myc expression was gauged by qRT-PCR in treated AC16 cells. (E) c-Myc expression was gauged by Western Blot in treated AC16 cells. (F) The expression of AXIN1 and c-Myc in treated AC16 cells. $n = 3$ independent biological replicates.$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

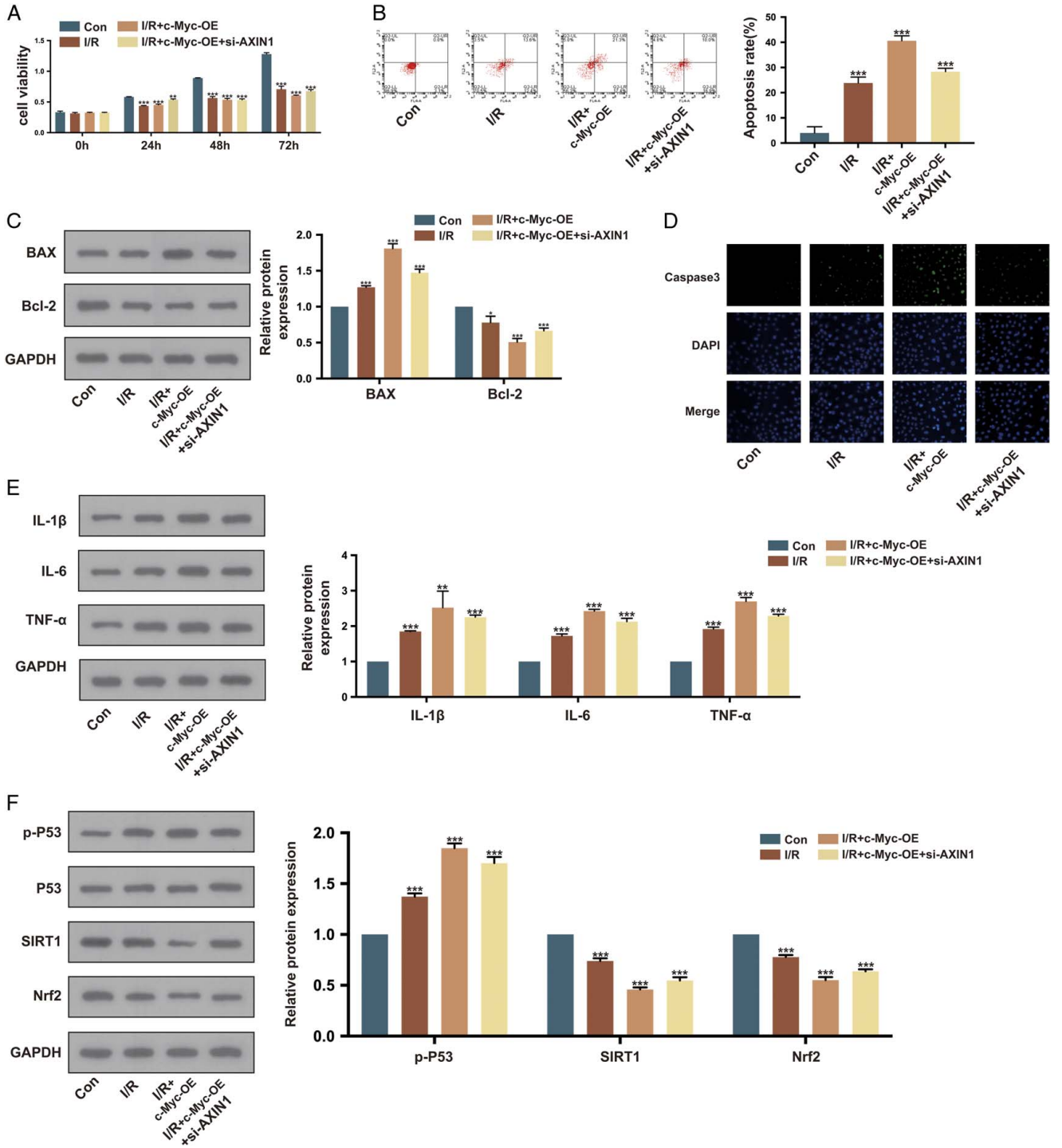

Figure 4. c-Myc is a downstream mediator of AXIN1 function. (A–F): AC16 cells were transfected with or without c-Myc-OE, c-Myc-OE + si-AXIN1, then exposed to a control condition or a condition of oxygen glucose deprivation followed by reperfusion (OGD/R). (A) Viability of treated AC16 cells was assessed by CCK-8 assay. (B) Annexin V/PI assay tested cell apoptosis. (C) Western blot showed the levels of Bax and Bcl-2 in treated AC16 cells. (D) The caspase3 expression in AC16 cells was verified by Immunofluorescence. (E) The production levels of IL-6and TNF-α in treated AC16 cells were tested by Western Blot. (F) The expression of p53, SIRT1 and Nrf 2 in in treated AC16 cells were tested by Western Blot. $n = 3$ independent biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

as breast cancer[22], nonsmall cell lung cancer[23], gastric cancer[24], and prostate cancer[20]. Our findings show that AXIN1 managed myocardial I/R injury *in vitro*, most likely via c-Myc, a controller in cerebral I/R injury. The correlation between c-Myc and AXIN1 levels in I/R-stimulated AC16 cells suggests that AXIN1 may act by binding to c-Myc. Furthermore, up-regulation or down-regulation of c-Myc did not affect AXIN1 expression in I/R-stimulated AC16 cells (Fig. 3F), suggesting that AXIN1 up-regulation may be the main event. In I/R-stimulated AC16 cells, transfected si-AXIN1 markedly reverses the effects of

c-Myc-OE cell viability enhancement, inflammation inhibition, apoptosis defect, and proliferation promotion. The above experimental data indicates that c-Myc is downstream of AXIN1.

Based on the effect of si-AXIN1 in AC16 cells with or without I/R injury compared to si-NC controls, we speculated that AXIN1 knockdown might be more important in AC16 cells with I/R playing a more significant role. Furthermore, our results show that AXIN1 and c-Myc are upregulated in I/R-stimulated human AC16 cardiomyocytes. Furthermore, AXIN1 can participate in the dysfunction of various human cells, such as endothelial cells[25], neurons[26,27], and cancer cells[28–31]. However, we failed to validate the role of AXIN1 *in vivo*, which is one of our limitations. In follow-up, we will construct cardiomyocyte-specific AXIN1-deficient mice to investigate its role *in vivo*.

In summary, our discoveries in this study showed a defensive capacity of AXIN1 overexpression on I/R-animated AC16 cells. The defensive activities may be delivered by focusing on c-Myc and the regulation of SIRT1/p53/Nrf2 pathways. This study gives an original insight into the role of c-Myc in myocardial I/R injury.

Ethical approval

This topic does not involve animal or human research.

Consent

This topic does not involve animal or human research.

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Author contribution

J.L. and J.X.: provided the experimental design; J.L. and H.W.: performed the experiments; L.C.: analyzed the data; J.Z. and J.W.: prepared all figures; J.L. and J.X.: wrote the draft of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest disclosure

The authors declare that they have no conflicts of interest.

Research registration unique identifying number (UIN)

1. Name of the registry: not applicable.
2. Unique identifying number or registration ID: not applicable.
3. Hyperlink to your specific registration (must be publicly accessible and will be checked): not applicable.

Guarantor

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Data availability statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Provenance and peer review

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