

Original Research

FOXO3a Induces Myocardial Fibrosis by Upregulating Mitophagy

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Abstract

Background: Atrial fibrillation is one of the most common cardiac arrhythmias. Myocardial fibrosis is closely associated with atrial remodeling, which leads to heightened risk of atrial fibrillation. This study aimed to explore whether forkhead box protein O3 (FOXO3a) impacts myocardial fibrosis incidence by regulating mitophagy. **Methods:** Cell viability was assessed by cell counting kit-8 (CCK-8) assays. The expression of vimentin and cytochrome C was detected by immunofluorescence assays. Quantitative real-time polymerase chain reaction (PCR) was used to analyze the relative mRNA level of FOXO3a. Expression of FOXO3a, phosphorylated FOXO3a, Collagen I, Collagen III, alpha-smooth muscle actin (α -SMA), matrix metalloproteinase 9 (MMP9), light chain 3 (LC3), phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1), Parkin, and sequestosome-1 (p62) proteins were determined by western blotting. 5-ethynyl-2'-deoxyuridine (EDU) incorporation was employed to measure cell proliferation. Changes in mitochondrial membrane potential were determined by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) staining. A wound healing assay was used to examine cell migration, and the levels of reactive oxygen species were determined by flow cytometry. **Results:** The expression of FOXO3a was upregulated in cardiac fibroblasts treated with angiotensin II (AngII), while the expression of phosphorylated FOXO3a was downregulated under these conditions. FOXO3a knockdown significantly inhibited the proliferation, migration and collagen secretion of cardiac fibroblasts treated with AngII. The ratio of LC3 II/I as well as expression of PINK1 and Parkin was increased, and the expression of p62 was decreased, in cardiac fibroblasts treated with AngII. Moreover, these effects were limited by FOXO3a knockdown. Finally, the mitophagy inducer everolimus (RAD001) attenuated the suppressive effect of FOXO3a knockdown on cardiac fibroblast activation. **Conclusions:** FOXO3a promotes the progress of myocardial fibrosis by triggering mitophagy in cardiac fibroblasts.

Keywords: myocardial fibrosis; FOXO3a; mitophagy; cardiac fibroblasts

1. Introduction

Atrial fibrillation (AF) is one of the most common clinical arrhythmias and is associated with the risk of stroke and heart failure [1,2]. Electrical and structural remodeling are two essential pathophysiologic hallmarks of AF [3], while myocardial fibrosis (MF) is the principal characteristic associated with structural remodeling [4,5]. Cardiac fibroblasts (CFs) are the primary cell type in the myocardial interstitium, and can differentiate into myofibroblasts under conditions of myocardial ischemia or pressure stimuli [6]. Active myofibroblasts exhibit proliferative and invasive properties to initiate tissue repair, and they also secrete more collagen fibers to remodel the interstitium [7]. However, sustained activation of myofibroblasts can lead to MF and a salient characteristic feature of MF is excessive deposition of the extracellular matrix (ECM) [8]. The abnormal deposition of ECM contributes to the development and maintenance of AF by increasing the heterogeneity of atrial conduction [9,10]. Hence, further insight into the molecular mechanism of MF may afford a new theoretical basis for novel AF therapies.

Recent research evidence suggests that alterations in mitochondrial function and metabolism play a crucial role in the differentiation of CFs into myofibroblasts [11]. Mitochondrial autophagy, also known as mitophagy, is a selective intracellular degradation process that helps eliminate dysfunctional mitochondria and maintain mitochondrial homeostasis [12]. Steps in this process include depolarization of damaged mitochondria, loss of mitochondrial membrane potential (MMP), formation of mitochondrial autophagosomes, fusion of autophagosomes with lysosomes, and degradation of the mitochondrial contents [13].

The phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1)/Parkin signaling pathway has been widely reported to be involved in mitophagy [14]. PINK1 is a serine/threonine kinase that plays a crucial role in the regulation of mitochondrial function [15]. When the MMP is lost, PINK1 accumulates on the outer membrane of mitochondria and stimulates the recruitment and activation of the E3 ubiquitin ligase Parkin. Activated Parkin is responsible for binding ubiquitin molecules to adaptor proteins such as sequestosome-1 (p62), thereby amplifying mitochondrial autophagic signaling [13]. Numerous groups



have shown that mitophagy plays a crucial role in regulating heart disease. For example, Tong *et al.* [16] observed that Ulk1-Rab9-dependent replacement of mitophagy protected the heart from obesity-related cardiomyopathy. Further, Cai *et al.* [17] demonstrated that empagliflozin blocked cardiac microvascular ischemia/reperfusion injury by activating FUN14 domain-containing 1 (FUNDC1)-dependent mitophagy. In addition, the work of Wang *et al.* [18] suggested that the adenosine monophosphate-activated protein kinase catalytic subunit alpha 2 (AMPK α 2) prevented the development of heart failure by enhancing mitophagy. However, the specific mechanism that activates the mitophagy in MF remains to be fully elucidated.

Forkhead box protein O3 (FOXO3a) is a member of the forkhead transcription factor subfamily, and family members play important roles in cell cycle arrest, apoptosis, angiogenesis, DNA repair, and metabolism [19]. Several studies have highlighted the role of FOXO3a in controlling the occurrence and progression of certain diseases by regulating mitochondrial autophagy. For example, FOXO3a has been shown to be involved in diabetic retinopathy [20] and cardiac hypertrophy [21]. Additionally, FOXO3a has been implicated in insulin-like growth factor-1 receptor-mediated myocardial fibrosis and the development and maintenance of atrial fibrillation [22]. However, whether FOXO3a influences the process of MF by regulating mitophagy remains unknown. Therefore, the principal aim of this study was to determine whether FOXO3a could affect MF by regulating mitophagy to provide new insights for the treatment of MF and AF.

2. Methods

2.1 Isolation and Identification of Rat Cardiac Fibroblasts

Neonatal (1–3 day old) rats were killed under anesthesia, disinfected with 75% alcohol for twice, then the heart was dissected. Subsequently, the atrial tissue was separated and washed with phosphate-buffered saline (PBS). Next, atrial tissue was cut into fragments and digested with trypsin and type II collagenase. The cells were collected and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 2 h. Following this, the CFs were obtained by differential adherence, and isolated CFs were identified by immunofluorescence. Specifically, CFs were validated by Vimentin-positive staining and also tested negative for mycoplasma. Cells are cultured in a humidified incubator at 37 °C with 5% CO₂.

2.2 RNA Interference and Cell Treatment

To study whether FOXO3a affected the activation of CFs by promoting mitophagy and examine study the role of FOXO3a in this process, cells were transfected with negative control (si-NC) or FOXO3a-specific (si-FOXO3a) small interfering RNAs (siRNA) using Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA).

Following this, rat CFs were divided into control (no intervention) and angiotensin II (AngII)-treated (10 μ M AngII) groups and incubated for an additional 24 h. To verify whether FOXO3a affected the activation of CFs by promoting mitophagy, the si-FOXO3a cells were treated with 5 nM everolimus (RAD001), and this was termed the si-FOXO3a+RAD001 group.

2.3 CCK-8 Assay

Cells in each experimental group were treated as outlined. After removing the medium, cells were incubated with 100 μ L of Cell Counting Kit-8 (CCK-8) (NU679, Dojindo, Tokyo, Japan) working solution at 37 °C for 4 h. The absorbance value at 450 nm was measured using a Bio-Tek microplate reader (MB-530, Heales, Shenzhen, Guangzhou, China).

2.4 Immunofluorescence (IF)

CFs were applied to glass microscope slides and fixed with 4% paraformaldehyde. Subsequently, the cells were permeated with 0.3% TritonX-100 and blocked with 5% bovine serum albumin (BSA). Next, the slides were immersed in primary antibodies against vimentin (10366-1-AP, Proteintech, Chicago, IL, USA) or cytochrome C (66264-1-Ig, Proteintech, USA) overnight at 4 °C. Subsequently, the slides were incubated in secondary antibody (anti-Rabbit/Mouse IgG (H+L), 1:200, AWS0005c/AWS0004c, Abiowell, Changsha, Hunan, China) for 90 min. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus and images were collected with a Motic microscope (BA210T, Motic, Xiamen, Fujian, China).

2.5 Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (15596026, Thermo Scientific, Portsmouth, NH, USA). An mRNA Reverse Transcription kit (CW2569, CWBIO, Taizhou, Zhejiang, China) was used to synthesize cDNA from total RNA. The relative expression of the genes was examined by quantitative real-time polymerase chain reaction (qRT-PCR) using UltraSYBR Mixture (CW2601, CWBIO, Taizhou, Jiangsu, China) and a QuantStudio1 Real-Time PCR System (A40426, ABI, Foster city, CA, USA). Relative mRNA abundance was calculated using $2^{-\Delta\Delta C_t}$ method with *GAPDH* serving as the internal standard. The sequence of primers used is outlined in Table 1.

2.6 Western Blotting (WB)

Total proteins were extracted from cells using radio immunoprecipitation assay (RIPA) lysis buffer (AWB0136, Abiowell, Changsha, Hunan, China) and quantified using a bicinchoninic acid assay (BCA) kit (AWB0104, Abiowell). The proteins were separated by sodium dodecyl sulfate-

Table 1. Primer sequences.

Gene	Sequence	Length (bp)
<i>R-FOXO3a</i>	F TCTATAACTTTGTGCTGCTGCCG	206 bp
	R TACCTCGGCTCCTTCCCTTCA	
<i>R-GAPDH</i>	F ACAGCAACAGGGTGGTGGAC	252 bp
	R TTTGAGGGTGCAGCGAACTT	

polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, USA). The primary antibodies were incubated with the membranes overnight at 4 °C. Subsequently, secondary antibodies were incubated with the membrane. Enhanced chemiluminescence (ECL) chromogenic solution (Chemscope6100, CLiNX, Shanghai, China) was used to visualize protein bands. GAPDH was served as the internal control, and information regarding the antibodies used is displayed in Table 2.

2.7 Cell Proliferation Measurements Using 5-Ethynyl-2'-Deoxyuridine (EDU)

Cell proliferation was assessed using a Cell-Light EdU Apollo567 Kit (C10310, RiboBio, Guangzhou, Guangdong, China). After incubating cells with 5-Ethynyl-2'-Deoxyuridine (EDU) (50 µM) medium overnight, the cells were fixed with 4% paraformaldehyde and then decolorated using 2 mg/mL glycine. Next, the cells were incubated with 100 µL of 1 × Apollo solution for 30 min. After successive washes with methanol and PBS, the cells were treated with 100 µL of 1 × Hoechst 33342 solution for 30 min. The images were collected using a Motic microscope (BA210T, Motic, China).

2.8 Wound Healing Assay

CFs were inoculated into 6-well plates and incubated until 100% confluent. The cell layer was then scratched in with the tip of a sterile pipette (T-200-Y, Axygen, Tewksbury, MA, USA). The cells were washed 3 times with PBS and serum free RPMI-1640 was added. Cells were subsequently examined at 0 h, 24 h and 48 h for wound healing. Cells were observed using an inverted microscope (DSZ2000X, Beijing Cnmicro Instrument Co., Ltd., Beijing, China).

2.9 JC-1 Staining

Cells were treated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) staining solution at 37 °C for 20 min. After removing the supernatant, the cells were washed twice with 1 × JC-1 dyeing buffer and subsequently collected and analyzed by flow cytometry (A00-1102, Beckman Coulter, Fullerton, CA, USA).

2.10 Reactive Oxygen Species (ROS) Measurement

Cells were incubated with 10 µM 2',7'-Diacetyldichlorofluorescein (DCFH-DA) in serum free

medium for 20 min at 37 °C. After 3 washes, the cells were digested with trypsin and collected by centrifugation. The fluorescence intensity was examined by flow cytometry.

2.11 Statistical Analysis

Data was analyzed using GraphpadPrism9.0 (Graph-Pad, La Jolla, CA, USA) and displayed as mean ± standard deviation. Student's *t* test or one-way analysis of variance (ANOVA) was carried out to assess the differences between two or more groups. *p* < 0.05 was considered statistically significant.

3. Results

3.1 FOXO3a Expression is Upregulated in an AngII-induced MF Cell Model

As shown in Fig. 1A, the shape of isolated rat CFs was, as expected, fusiform in nature when examined in a light microscopy. IF was used to confirm the identity of isolated CFs, and, as expected, the expression of vimentin was positive (Fig. 1B). Subsequently, CFs were treated with AngII to establish the MF cell model. As expected, AngII treatment significantly promoted the proliferation of CFs (Fig. 1C). Furthermore, AngII treatment resulted in the up-regulation of FOXO3a and downregulation of p-FOXO3a in cytoplasm of CFs (Fig. 1D). AngII treatment also led to increased levels of FOXO3a in nucleus (Fig. 1E). Collectively, these results suggested that FOXO3a expression was upregulated in AngII-induced MF cell model.

3.2 FOXO3a Knockdown Inhibits the AngII-Induced Activation of CFs

Compared with control cells, the mRNA level of *FOXO3a* was elevated in response to AngII treatment. Further, relative to the si-NC group, the mRNA level of *FOXO3a* was decreased in cells transfected with si-FOXO3a. Changes in the protein level, as measured by WB, were consistent with mRNA measurements (Fig. 2A).

Subsequently, the effect of FOXO3a knockdown on CF proliferation was examined. As shown in Fig. 2B, the AngII-induced proliferation of CFs was significantly blunted in response to FOXO3a knockdown (Fig. 2B) and, similarly, FOXO3a knockdown inhibited AngII-induced cell migration (Fig. 2C). FOXO3a knockdown also reversed AngII-induced downregulation of MMP9 expression. In contrast, upregulation of collagen III, collagen I and alpha-smooth muscle actin (α -SMA) was observed in CFs treated with AngII but this was blunted by FOXO3a knockdown (Fig. 2D). Taken together, the above results indicated that AngII-induced activation of CFs was reduced following FOXO3a knockdown.

3.3 FOXO3a Knockdown Inhibits AngII-Induced Mitophagy in CFs

After treatment with AngII, the red fluorescence of cells decreased, while the green fluorescence was in-

Table 2. The information of antibody.

Name	Dilution rate	Cat number	Source	Company	Country
FOXO3a	1:5000	10849-1-AP	Rabbit	Proteintech, Chicago, IL	USA
p-FOXO3a	1:1000	28755-1-AP	Rabbit	Proteintech	USA
Collagen III	1:5000	ab7778	Rabbit	Abcam, Cambridge	UK
Collagen I	1:2000	14695-1-AP	Rabbit	Proteintech	USA
α -SMA	1:2000	55135-1-AP	Rabbit	Proteintech	USA
MMP9	1:1000	ab228402	Rabbit	Abcam	UK
LC3	1:2000	14600-1-AP	Rabbit	Proteintech	USA
PINK1	1:600	23274-1-AP	Rabbit	Proteintech	USA
Parkin	1:2000	14060-1-AP	Rabbit	Proteintech	USA
p62	1:4000	18420-1-AP	Rabbit	Proteintech	USA
GAPDH	1:5000	10494-1-AP	Rabbit	Proteintech	USA
PCNA	1:5000	10205-2-AP	Rabbit	Proteintech	USA
HRP goat anti-Rabbit IgG	1:6000	SA00001-2	/	Proteintech	USA

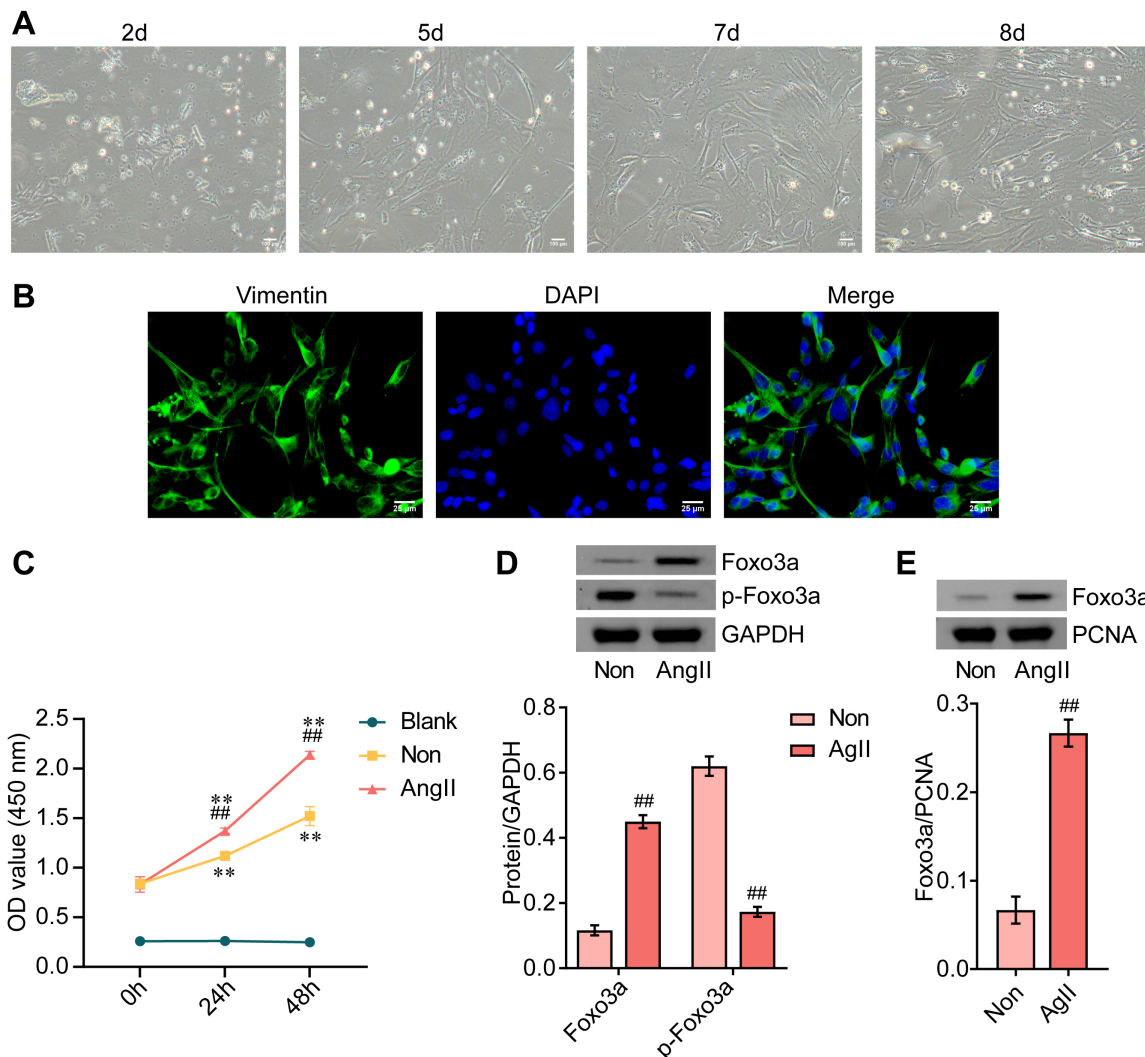


Fig. 1. Expression of FOXO3a is upregulated in AngII-induced myocardial fibrosis cell model. (A) Photomicrograph of cultured cardiac fibroblasts. Scale bar = 100 μ m. (B) IF staining of vimentin, DAPI counter-staining, and a merged image is shown. Scale bar = 25 μ m. (C) Cell viability was measured by CCK-8 assays. (D) The expression of FOXO3a and phosphorylated FOXO3a in the cytoplasm was examined by western blotting. (E) The expression of FOXO3a in nucleus was assessed by western blotting. $**p < 0.01$ vs Blank group, $^{##}p < 0.01$ vs Non group. FOXO3a, forkhead box protein O3; CCK-8, cell counting kit-8; AngII, angiotensin II; DAPI, 4',6-diamidino-2-phenylindole; OD, optical density; Non, no intervention; IF, immunofluorescence.

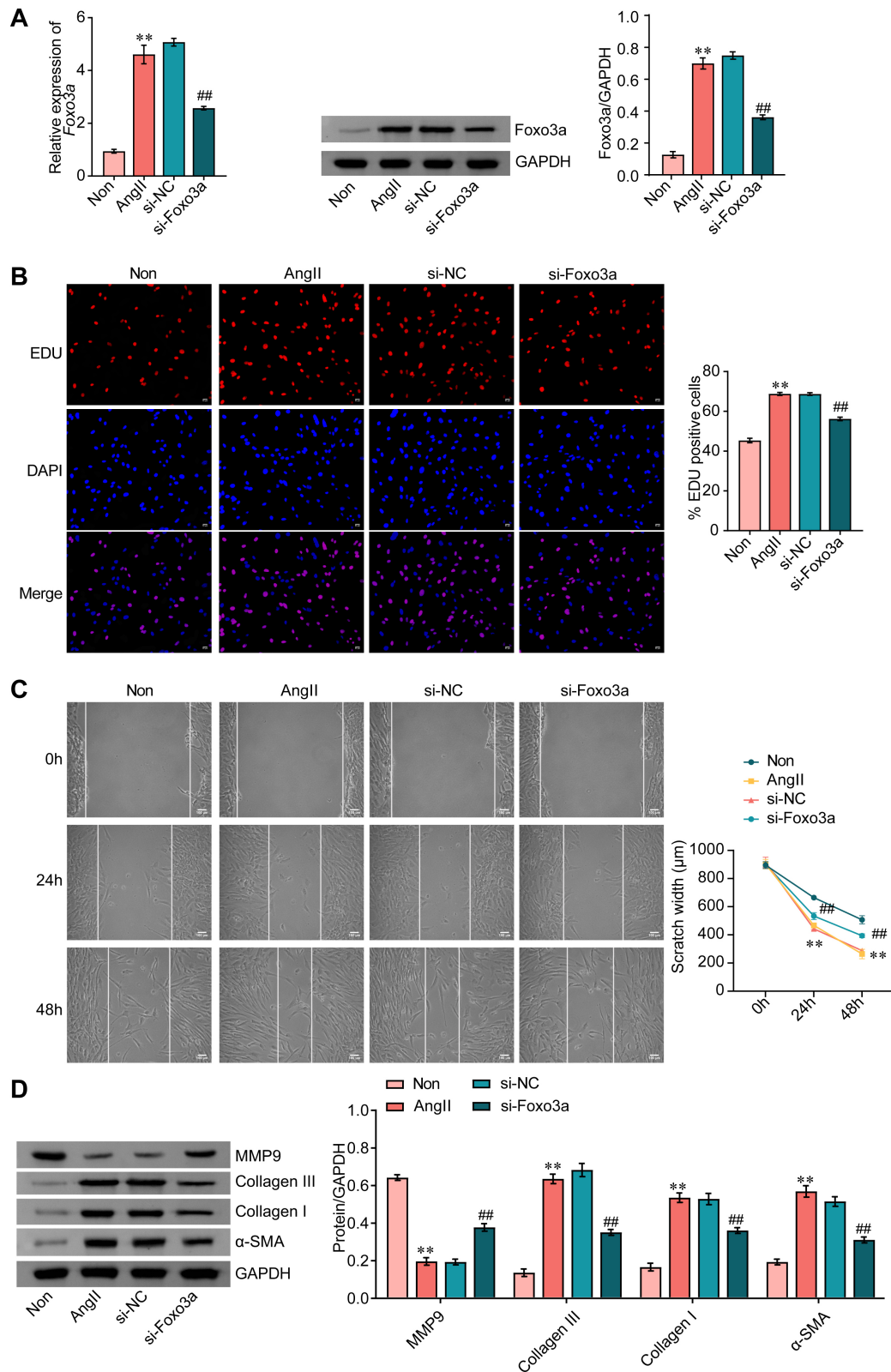


Fig. 2. FOXO3a knockdown inhibits AngII-induced activation of CFs. (A) FOXO3a expression was measured by qRT-PCR and WB. (B) The proliferation of CFs was assessed by EDU staining. Scale bar = 50 μm . (C) Wound healing assay was used to measure cell migration. Scale bar = 100 μm . (D) The expression of MMP9, collagen III, collagen I, and α -SMA was measured by WB. $**p < 0.01$ vs Non group, $^{##}p < 0.01$ vs si-NC group. CFs, cardiac fibroblasts; qRT-PCR, quantitative real-time polymerase chain reaction; WB, western blotting; EDU, 5-ethynyl-2'-deoxyuridine; MMP9, matrix metalloproteinase 9; α -SMA, alpha-smooth muscle actin; si-FOXO3a, FOXO3a-specific small interfering RNAs; si-NC, negative control-specific small interfering RNAs.

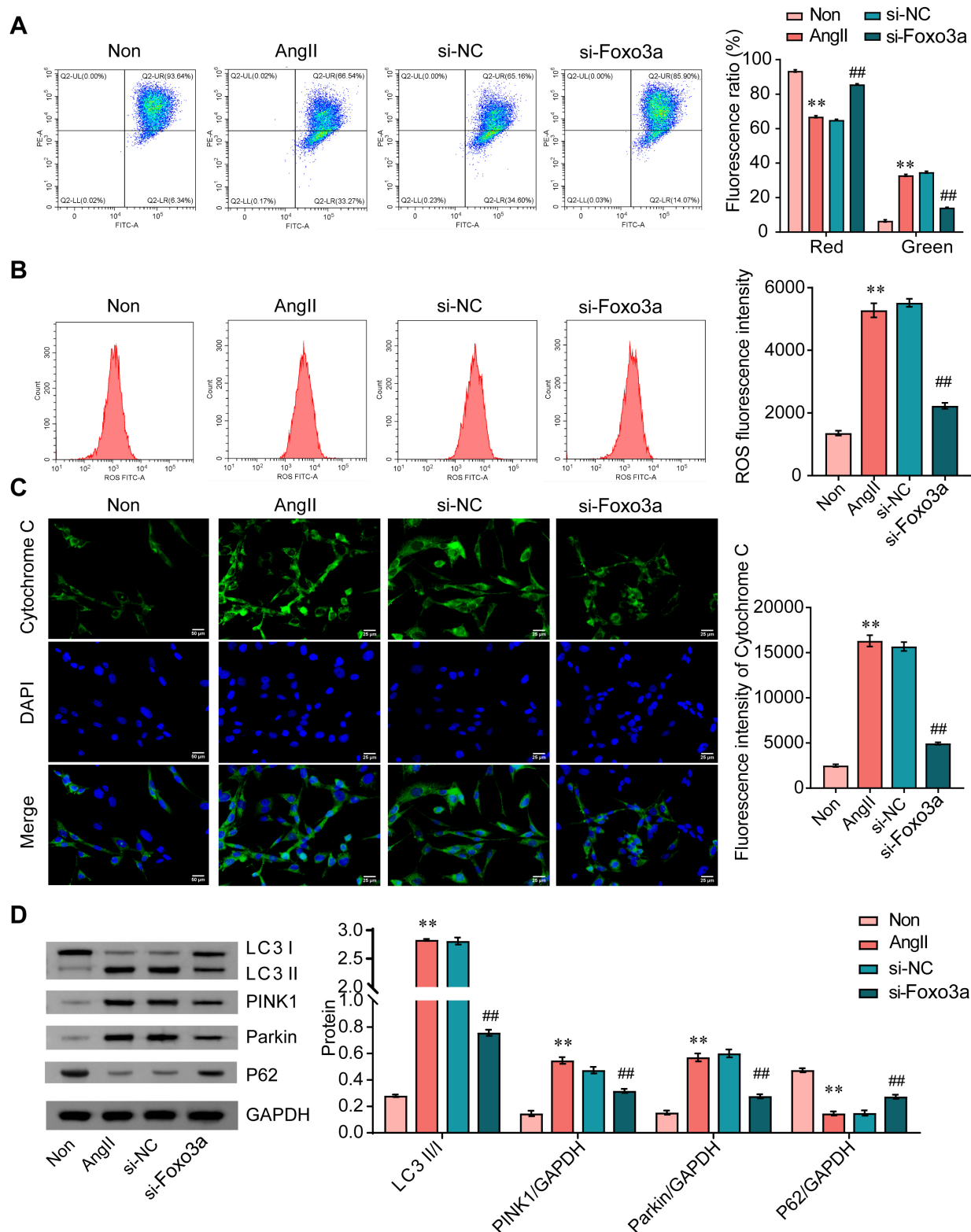


Fig. 3. FOXO3a knockdown inhibits AngII-induced mitophagy in CFs. (A) JC-1 staining was conducted to measure mitochondrial membrane potential. (B) ROS levels were measured by flow cytometry. (C) IF staining of cytochrome C. Scale bar = 25 μ m. (D) The ratio of LC3 II/I, and expression of PINK1, Parkin, and p62 was analyzed by WB. ** p < 0.01 vs Non group, ## p < 0.01 vs si-NC group. JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidazocarbocyanine iodide; ROS, reactive oxygen species; IF, immunofluorescence; LC3, light chain 3; PINK1, phosphatase and tensin homolog (PTEN)-induced kinase 1; p62, sequestosome-1.

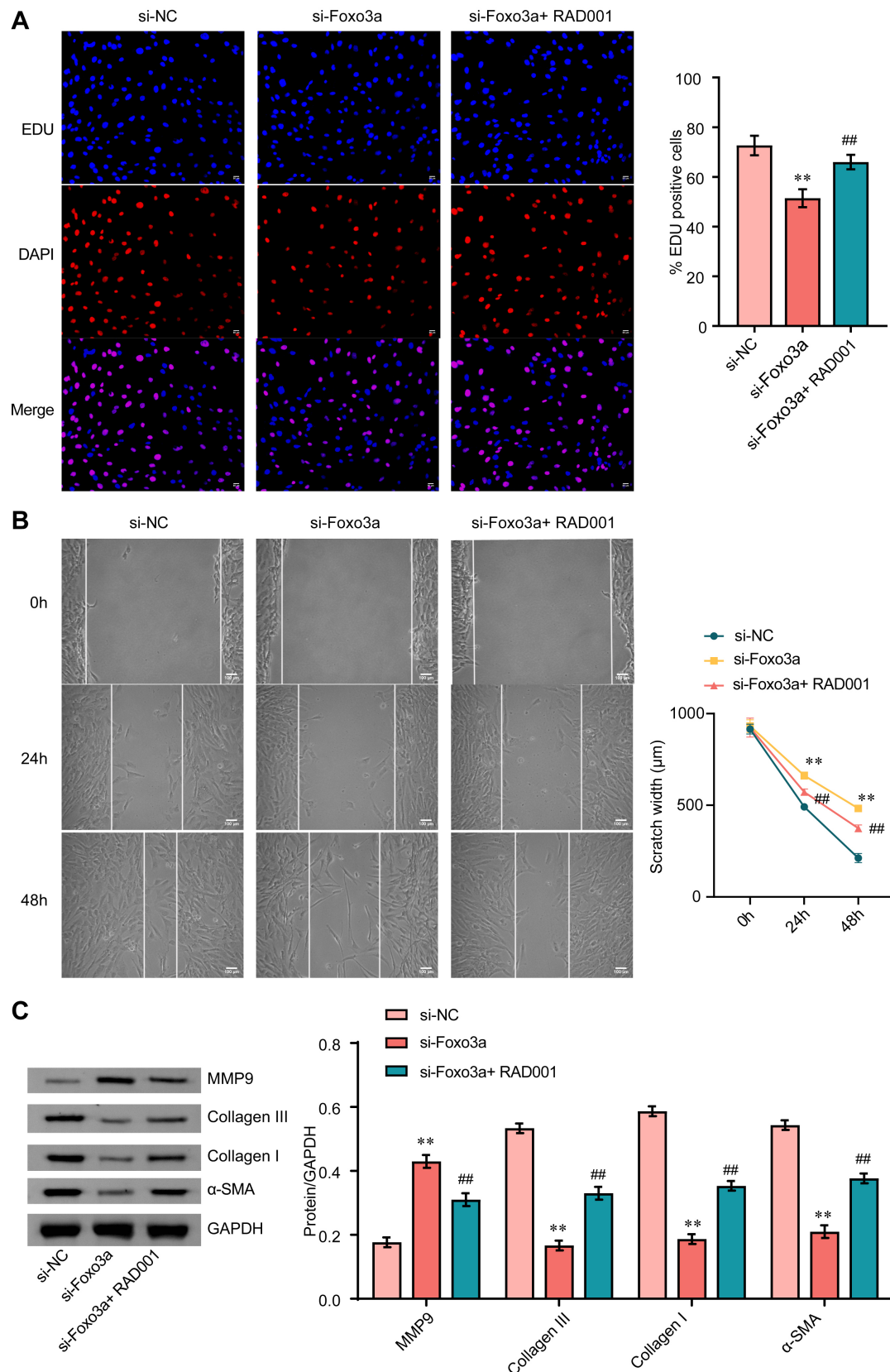


Fig. 4. FOXO3a blunts the activation of CFs by promoting mitophagy. (A) The EDU proliferation assay was used to examine the proliferation of CFs. Scale bar = 50 μm. (B) A wound healing assay was used to measure the cell migration. Scale bar = 100 μm. (C) The expression of MMP9, collagen III, collagen I, and α-SMA was determined by WB. ** $p < 0.01$ vs si-NC group, ## $p < 0.01$ vs si-FOXO3a group. RAD001, everolimus.

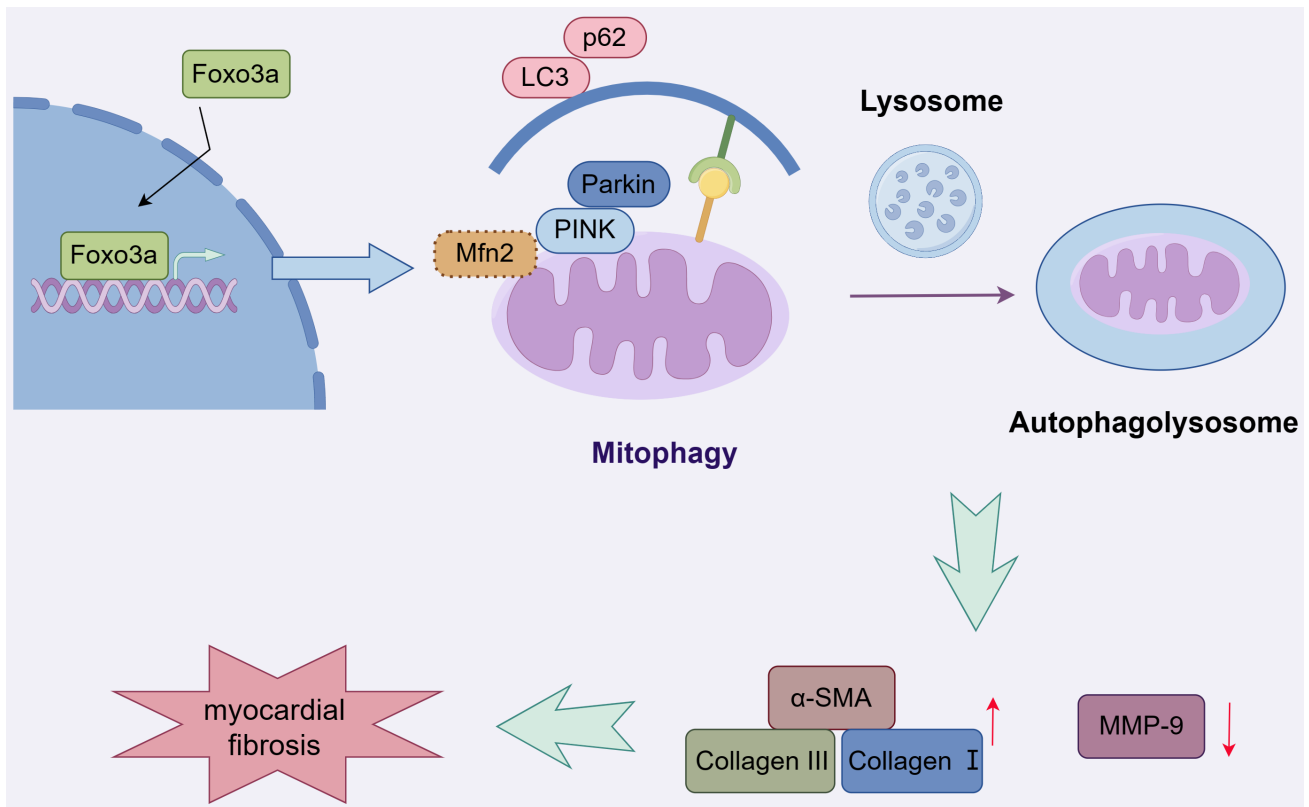


Fig. 5. A graphical abstract illustrates that Foxo3a promotes mitophagy and ultimately myocardial fibrosis *via* the PINK1/Parkin pathway. Mfn2, mitofusin-2. Fig. 5 was created by Figdraw (<https://www.figdraw.com>).

creased, indicating that the mitochondrial membrane potential of CFs decreased in response to AngII. Furthermore, FOXO3a knockdown effectively suppressed AngII-induced mitochondrial membrane potential reduction in CFs (Fig. 3A). We also noted that FOXO3a knockdown alleviated AngII-induced elevations in reactive oxygen species (ROS) (Fig. 3B). FOXO3a knockdown significantly reduced the release of mitochondrial cytochrome C in CFs following treatment with AngII (Fig. 3C). Furthermore, the ratio of mitophagy markers light chain 3 (LC3) II and I, and the expression of PINK1 and Parkin rose while expression of p62 was reduced in CFs treated with AngII, and these effects were blunted by FOXO3a knockdown (Fig. 3D). Our findings indicated that FOXO3a knockdown inhibited AngII-induced mitophagy in CFs.

3.4 FOXO3a Impacts CF Activation by Promoting Mitophagy

As shown in Fig. 4A, the mitophagy inducer RAD001 significantly attenuated the proliferation inhibition caused by FOXO3a knockdown. RAD001 also rescued CF migration inhibition in cells with FOXO3a knockdown (Fig. 4B). Finally, RAD001 reversed the upregulation of MMP9 and downregulation of collagen III, collagen I and α -SMA induced by FOXO3a knockdown (Fig. 4C). These results demonstrated that FOXO3a impacted CF activation by promoting mitophagy.

4. Discussion

MF is a critical pathological change that is associated with the development and maintenance of AF [23]. The content and distribution of fibrous tissue in patients with AF is also associated with the heightened risk of clinical complications and treatment failure [24]. Therefore, a better understanding of the molecular mechanisms that give rise to MF may provide new druggable targets and new therapeutic approaches. In this study, we demonstrated the role that FOXO3a plays in mediating MF. We provided *in vitro* evidence indicating that activated FOXO3a regulated mitophagy through the PINK1/Parkin pathway, thereby promoting ECM protein deposition and fibrosis (Fig. 5).

As a transcription factor, FOXO3a not only participates in the regulation of various cell functions such as apoptosis, proliferation, cell cycle, and DNA damage repair [25], but also contributes to organ fibrosis [26]. For example, the loss of IL-6 mitigated renal fibrosis by attenuating DNA methyltransferase 1 (DNMT1)-mediated FOXO3a inhibition [27]. 5-methoxytryptophan alleviated liver fibrosis by regulating autophagy mediated by the FOXO3a/microRNA-21 (miR-21)/autophagy-related gene 5 (ATG5) signaling pathway [28]. Additionally, resveratrol relieved renal interstitial fibrosis in rats with unilateral ureteral obstruction by inhibiting the protein kinase B (AKT)/FOXO3a pathway [29].

Recently, Zhang *et al.* [22] discovered that FOXO3a was upregulated in activated CFs and was tightly associated with MF. Consistent with this work, we also observed elevated expression of FOXO3a in the nucleus of AngII-induced CFs. AngII is widely used to induce the activation of CFs into myofibroblasts [30], and we noted that AngII also limited the phosphorylation of FOXO3a in cardiac fibroblasts, an event linked to upregulating its transcriptional activity. Further data showed that FOXO3a knockdown significantly inhibited AngII-induced proliferation and migration of CFs. The pathological changes in the extracellular matrix of cardiomyocytes are primarily caused by an imbalance between collagen synthesis and degradation, and this phenomenon is characterized by excessive collagen content and reduced MMP-mediated degradation [31]. In AngII-exposed CFs, FOXO3a knockdown also decreased the expression of collagen III, collagen I and α -SMA, but elevated the expression of MMP9. These results suggested that FOXO3a knockdown significantly inhibited the excessive activation of myofibroblasts and partially restored the excessive deposition of collagen fibers induced by AngII.

Recent studies have indicated that mitophagy may serve as a potential therapeutic target for mitigating MF [32]. Mitophagy is an important mitochondrial quality control mechanism that regulates the removal of damaged mitochondria [12]. At present, there are two principal mitophagic mechanisms: mitochondrial elimination mediated by B cell leukemia/lymphoma 2 (Bcl-2) interacting protein 3 (BNIP3)/BNIP3-like (NIX), and mitophagy mediated by PINK1/Parkin [33]. Surprisingly, both pathways are regulated by FOXO3a. Studies have validated that FOXO3a silencing dramatically prevents temozolomide-induced upregulation of BNIP3 and mitophagy [34]. Meanwhile, FOXO3a promotes mitophagy by upregulating the expression of Parkin [21]. In this study, we focused on the PINK1/Parkin mitophagic pathway. We observed that AngII induced mitophagy in CFs and that FOXO3a knockdown significantly suppressed AngII-induced increases in MMP, elevation of ROS levels, and release of cytochrome C. FOXO3a knockdown also significantly downregulated the ratio of LC3 II/I, and expression of PINK1 and Parkin, while upregulating the expression of p62. It has been found that activated Parkin drives the ubiquitination of mitochondrial fusion proteins, mitofusins (Mfn1 and Mfn2), thereby triggering mitochondrial degradation [35,36]. Studies have shown that the loss of mitofusin-2 (Mfn2) prevents the recruitment of PINK1-associated Parkin and mitophagy in depolarization-induced cardiomyocytes [37,38]. Previous research has reported that the PINK1/Parkin axis mediates mitophagy to promote cardiomyocyte ferroptosis and affect MF [39]. These findings suggested that FOXO3a promoted MF by accelerating mitophagy, and PINK1/Mfn2/Parkin signaling may be intimately involved in this process.

Interestingly, the role of mitophagy in MF has met with controversy in the literature. Zheng *et al.* [40]

validated that fucoxanthin improved MF in streptozotocin (STZ)-induced diabetic rats by restoring mitophagy. Guan *et al.* [41] also concluded that ADAM17 knockout prevented MF by triggering mitophagy. While Wang *et al.* [42] indicated that microRNA-24-3p improved MF by inhibiting mitophagy in CFs. These findings may be attributed to the varying pathogenic mechanisms of different diseases, and/or the differential expression profiles of targets in these various pathological models. Given this, we explored the role of FOXO3a-mediated mitophagy in MF. We found that the mitophagy inducer RAD001 reversed the effects of FOXO3a knockdown. Specifically, in AngII-exposed CFs, supplementation with RAD001 increased cell proliferation, migration, and accelerated ECM protein deposition compared to cells transfected with FOXO3a siRNA alone. These results further indicated that silencing FOXO3a alleviated excessive activation of AngII-induced myofibroblasts by inhibiting mitophagy. Moreover, our results support the findings outlined in the study conducted by Zhang and co-workers [42].

However, there are some limitations in our study. Our *in vitro* evidence has not been validated in animal models and clinical samples. More evidence is needed to support the regulatory role of FOXO3a in mitophagy, such as mitochondrial superoxide production and changes in mitochondrial morphology. The role of Mfn2 in FOXO3a-mediated mitophagy requires further investigation as does the determination of whether the BNIP3/NIX pathway is involved in FOXO3a-mediated activation of myofibroblasts. Additionally, the mechanism of mitophagy and its crosstalk with other signaling pathways, such as the apoptotic signaling pathway in MF, are also worthy of further exploration.

5. Conclusions

In conclusion, our study provides *in vitro* evidence that limiting FOXO3a activity inhibited mitophagy in AngII-treated CFs and attenuated the excessive transformation of CFs into myofibroblasts. We also discovered that FOXO3a activated the PINK1/Parkin pathway to mediate mitophagy. Our findings suggest that targeting FOXO3a may have potential therapeutic implications for MF and AF.

Availability of Data and Materials

All data found in this study is available upon request by contact with the first author or corresponding author.

Author Contributions

DL and QS designed the experiments, participated in data analysis and interpretation of results, and drafted the manuscript. DL, HL, BD and ZH contributed to the experimental design and procedures, participated in data analysis and interpretation. LM, ZW and XW participated in data analysis and interpretation of results. QS designed the experiments, provided guidance on experimental procedures

and data analysis, and primarily responsible for writing and revising the manuscript as the corresponding author. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Medical Ethics Committee of the First Hospital of Changsha (2022-103).

Acknowledgment

Fig. 5 was created by Figdraw (<https://www.figdraw.com>). Thank this site for providing support.

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Conflict of Interest

The authors declare no conflict of interest.

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