Foxo3a prevents apoptosis by regulating calcium through the apoptosis repressor with caspase recruitment domain

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Running title: Foxo3a regulates cardiac apoptosis
Key words: apoptosis; Foxo3a; ARC; calcium

Background: Foxo3a plays a pivotal role in regulating apoptosis, however, its role in controlling cardiac apoptosis remains to be fully elucidated.

Results: Foxo3a prevents cardiac apoptosis by regulating calcium through ARC.

Conclusion: Foxo3a and ARC constitute an anti-apoptotic pathway that regulates calcium homeostasis in the heart.

Significance: Our results provide important information for exploring the beneficial effects of this pathway on apoptosis-related cardiac diseases.

Summary
Apoptosis can occur in the myocardium under a variety of pathological conditions, including myocardial infarction and heart failure. The forkhead family of transcription factor Foxo3a plays a pivotal role in apoptosis, however its role in regulating cardiac apoptosis remains to be fully elucidated. We showed that enforced expression of Foxo3a inhibits cardiomyocyte apoptosis, whereas knockdown of endogenous Foxo3a sensitizes cardiomyocytes to undergo apoptosis. The apoptosis repressor with caspase recruitment domain (ARC) is a potent anti-apoptotic protein. Here we demonstrate that it attenuates the release of calcium from sarcoplasmic reticulum and inhibits calcium elevations in the cytoplasm and mitochondria provoked by oxidative stress in cardiomyocytes. Furthermore, Foxo3a is shown to maintain cytoplasmic and mitochondrial calcium homeostasis through ARC. We observed that Foxo3a knockout mice exhibited enlarged myocardial infarction sizes upon ischemia/reperfusion and ARC transgenic mice demonstrated reduced myocardial...
infarction and a balanced calcium levels in mitochondria and sarcoplasmic reticulum. Moreover, we showed that Foxo3a activates ARC expression by directly binding to its promoter. Our present study reveals that Foxo3a maintains calcium homeostasis and inhibits cardiac apoptosis through trans-activation of the ARC promoter. These findings provided novel evidence that Foxo3a and ARC constitute an anti-apoptotic pathway that regulates calcium homeostasis in the heart.

Introduction

Apoptosis can occur in the myocardium under a variety of pathological conditions(1,2). For example, myocyte apoptosis is increased in myocardium from patients with myocardial infarction and heart failure, and from experimental models of hypertrophy and heart failure. Thus, it necessitates the identification of the molecules that are able to regulate cardiac apoptosis.

The forkhead family of transcription factors participate in regulating diverse cellular functions such as apoptosis, differentiation, metabolism, proliferation, and survival(3). Foxo3a can either induce or prevent apoptosis. For example, its activation in haematopoietic(4,5) and neuronal cells(6) results in the induction of apoptosis. In contrast, Foxo3a is necessary for the maintenance of neutrophils survival. Such a differential cellular response of Foxo3a activation can be related to the cell type-specific regulation of pro- and anti-apoptotic genes. Foxo3a is expressed in the heart and skeletal muscle(7-9). We and others have shown that Foxo3a can negatively regulate cardiac hypertrophy(8,10,11). It is not yet clear whether Foxo3a participates in the regulation of cardiac apoptosis.

Mitochondrial Ca²⁺ homeostasis plays a critical role for maintaining cell survival. Its disruption by Ca²⁺ overload can lead to apoptosis(12). For example, Ca²⁺ overload promotes the opening of the mitochondrial permeability transition pore (MPT) and agents that block MPT pore opening inhibit apoptosis(12). Oxidative stress induces a significant change in mitochondrial Ca²⁺ flux leading to mitochondrial destabilization and apoptosis(13). Post-ischemic dopamine treatment of contractile dysfunction activates pro-apoptotic signal cascades via a Ca²⁺-dependent mitochondrial damage(14).

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Sarcoplasmic reticulum (SR) and mitochondria locate close to each other in cardiomyocytes(15). There is a tight coupling of Ca²⁺ signaling between SR release sites and nearby mitochondria, because the focal SR Ca²⁺ release results in Ca²⁺ microdomains sufficient to promote local mitochondrial Ca²⁺ uptake(16,17). Hitherto, it remains unknown as to whether Foxo3a can regulate mitochondrial Ca²⁺ homeostasis.

The heart has evolutionarily developed a highly expressed anti-apoptotic protein, ARC(18,19). It was originally identified to be a caspase-inhibiting protein and can specifically inhibit the activation of caspase-2 and -8, thereby blocking apoptosis induced by a variety of stimuli requiring the engagement of these caspases(18). Further studies revealed that ARC also may elicit its anti-apoptotic function by other means. It can interact with Fas, FADD, and Bax(20,21), inhibit cytochrome c release(22) and maintain mitochondrial membrane potential(23,24). In spite of ARC’s abundant expression and blocking apoptosis through multiple manners,
cardiomyocytes still undergo apoptosis under pathological conditions such as oxidative stress (23,25) and hypoxia (26-28). Apoptosis is controlled by a complex interplay between pro- and anti-apoptotic factors. The occurrence of apoptosis under pathological conditions indicates that this interplay is imbalanced. It remains to be further elucidated as to how ARC is dysregulated under the pathological conditions. Also, it is not yet clear whether ARC is involved in the maintenance of mitochondrial Ca²⁺ homeostasis.

The present work aimed to elucidate the role of Foxo3a in cardiac apoptosis. Our results show that Foxo3a can inhibit apoptosis induced by oxidative stress. Foxo3a knockout mice exhibit accelerated myocardial infarction upon ischemia/reperfusion. Furthermore, ARC can maintain calcium homeostasis, and Foxo3a regulates calcium through ARC. Finally, our results revealed that ARC is a transcription target of Foxo3a. Taken together, our study revealed a novel anti-apoptotic pathway in which Foxo3a regulates calcium through transactivating ARC.

**Experimental Procedures**

**Cell culture and treatment**

Cardiomyocytes were isolated from 1-2 days old Wistar rats as we described (10,29). In brief, after dissection hearts were washed, minced in HEPES-buffered saline solution contained: 130 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L NaH₂PO₄, 4 mmol/L glucose and 20 mmol/L HEPES (pH adjusted to 7.35 with NaOH). Tissues were then dispersed in a series of incubations at 37°C in HEPES-buffered saline solution containing 1.2 mg/ml pancreatin and 0.14 mg/ml collagenase (Worthington). After centrifugation, cells were re-suspended in Dulbecco’s modified Eagle medium/F-12 (GIBCO) containing 5% heat-inactivated horse serum, 0.1 mmol/L ascorbate, insulin-transferring-sodium selenite media supplement, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1 mmol/L bromodeoxyuridine. The dissociated cells were pre-plated at 37°C for 1 h. The cells were then diluted to 1x10⁶ cells/ml and plated in 10 µg/ml laminin-coated different culture dishes according to the specific experimental requirements. Neonatal mice cardiomyocytes were cultured as described elsewhere (30,31). In brief, the hearts from wild type and Foxo3a knockout mice were harvested, minced and dispersed by 1.2 mg/ml pancreatin and 0.625 mg/ml collagenase (Worthington). Myocytes and nonmyocytes were separated by preplating for 1 h. Treatment of cells with hydrogen peroxide was carried out as we described (32). 10 μM Ca²⁺ chelator BAPTA-AM(1,2-bis(o-Aminophenoxy)-ethane-N,N',N'-tetraacetic acid, Invitrogen) was administrated 1 h before hydrogen peroxide or anoxia/reoxygenation treatment. Anoxia/reoxygenation was performed as described elsewhere (33).

**ARC transgenic mice**

For creating the ARC transgenic mice, rat ARC coding sequence (accession number NM_053516) was cloned to the vector, pαMHC-clone26 (kindly provided by Dr. Zhongzhou Yang), under the control of the α-myosin heavy chain promoter. Microinjection was performed following standard protocols. The primers for genotyping ARC transgenic mice include, forward primer in the α-MHC promoter,
5'-CACATAGAAGCCTAGCCCACA-3'; the reverse primer in the ARC coding sequence, 5'-TTAGGTGTTCACACACCTTC-3'.

Genotyping of Foxo3a knockout mice

Foxo3a knockout (KO) mice were purchased from Mutant Mouse Regional Resource Center, USA). Foxo3a+/− mice were interbred to give knockout mice (Foxo3a−/−), which were used for further studies. Mice were genotyped by multiplex PCR (primers and conditions are available from Mutant Mouse Regional Resource Center, USA). All experiments were performed on Foxo3a−/− mice and their wild type littermates (Foxo3a+/-), and were approved by government authorities.

Adenoviral vector construction and infection.

The adenoviruses harboring ARC, wild type Foxo3a (wtFoxo3a) and the constitutively active form of human Foxo3a (caFoxo3a) were as we described(10,34). The adenoviruses harboring rat Foxo3a and ARC RNAi were constructed using pSilencer™ adeno 1.0-CMV System. The rat Foxo3a RNAi target sequence is 5'-CAAGTACACCAAGAGCCGA-3'. A non-related, scramble RNAi without any other match in the rat genomic sequence was used as a control (5'-TCAGACAGACAGACAGACC-3'). The rat ARC RNAi target sequence is 5'-ACTGTGAGCATGCCAGACC-3', the scramble RNAi sequence is 5'-GTCATCAGACTCACAGGC-3'. All viruses were amplified in HEK-293 cells. Cells were infected at the indicated multiplicity of infection (moi) for 60 min. After washing with phosphate-buffered saline (PBS), culture medium was added and cells were cultured until the indicated time.

Preparations of subcellular fractionation

Mitochondria-enriched heavy membranes and cytosolic fractions were prepared as described (35). Briefly, cells were washed twice with PBS and the pellet was suspended in 0.5 ml of buffer (20 mmol/L HEPES pH 7.5, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, 10 mg/mL each of leupeptin, aprotinin and pepstatin A) containing 250 mmol/L sucrose. The cells were homogenized by 10 strokes in a Dounce homogenizer. The homogenates were centrifuged twice at 750 g for 5 min at 4°C. The supernatants were centrifuged at 10,000 g for 15 min at 4°C to collect mitochondria-enriched heavy membranes. The final supernatants are referred to as cytosolic fractions. The isolation of sarcoplasmic reticulum (SR)-enriched membrane fractions were carried out as previously described (36,37). In brief, the cells were homogenized, centrifuged at 12,000 × g for 10 min. The supernatants were centrifuged at 100,000 × g for 45 min. The pellets are referred to as SR fractions.

Immunoblotting analysis.

Cells were lysed for 1 h at 4°C in a lysis buffer (20 mmol/L Tris pH 7.5, 2 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L dithiothreitol [DTT], 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonly fluoride, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Samples were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Equal protein loading was controlled by
Ponceau Red staining of membranes. Blots were probed using the following primary antibodies: the anti ARC antibody (Millipore, Billerica, MA, 1:1000), the anti Foxo3a antibody (Cell Signaling, Boston, MA, 1:1000), the anti COXIV antibody (Abcam, Cambridge, UK, 1:1000), the anti calnexin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:500), the anti cytchrome c antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000). After four washes with PBS-Tween-20, horseradish peroxidase-conjugated secondary antibodies were added. Antigen antibody complexes were visualized by enhanced chemiluminescence.

Construction of rat ARC promoter and luciferase assays

ARC promoters were amplified from rat genome using PCR. The full length fragment containing three Foxo3a potential binding sites (ARC promoter-1, 3138bp) was amplified using the forward primer 5’-CAACCAAAGATCACTAGAGTCGCG-3’. The other two shorter fragments were amplified using the forward primers: 5’-AACAGATTGGGCAGAATCCTGGGC-3’ (ARC promoter-2, 2448bp) and 5’-GGGTGGAGTGTGTGAGAAGTACTC-3’ (ARC promoter-3, 1440bp), respectively. All fragments were amplified using the reverse primer: 5’-CATTTGGGCTATATCAAGAAGGAG-3’. These promoters were cloned into the reporter plasmid pGL4.17 (Promega).

Luciferase activity assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Cells were lysed and assayed for luciferase activity 24 h after transfection. 20 μl of protein extracts were analyzed in a luminometer. Firefly luciferase activities were normalized to Renilla luciferase activity.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed as we described (32). In brief, total RNA was isolated using Trizol (Invitrogen). RNA was reverse transcribed using Oligo(dT) and amplified using a TaqMan Assays kit (TOYOBO). The samples were run in triplicate using the Applied Biosystems (ABI) 7000 sequence detector according to manufacturer’s instructions. The results were standardized to control values of GAPDH. The sequences of ARC primers were Forward: 5’-ATGGGTAACATGCAGGAGCGC-3’; Reverse: 5’-GTCCAGCAGCAACCCAGAGTC-3’. GAPDH forward primer: 5’-GCTAACATCAAATGGGGTGATGCTG-3’; Reverse: 5’-GAGATGATGACCCTTTTGGCCCCAC-3’. PCR was run under the following conditions: 95 °C for 60 s for 1 cycle; 95 °C for 15 s, 55 °C for 15 s, 72 °C for 45 s for 40 cycles. The specificity of the PCR amplification was confirmed by agarose gel electrophoresis.

Chromatin immunoprecipitation (ChIP) analysis

ChIP was performed as we described elsewhere(10). PCRs were performed with the primers that encompass the three Foxo3a potential binding sites in the rat ARC promoter. The oligonucleotides were as follows: BS1 (corresponding to a 317bp fragment), BS1-Forward: 5’-CAAGTGTTCCAGATGTTCCGAC-3’, and
BS1-Reverse: 5'-TCACTTGTCCAGCCCTCTCTC-3';
BS2 (corresponding to a 338bp fragment),
BS2-forward: 5'-GGCTTGTCCACTGATGCACTG-3', and
BS2-reverse: 5'-CCATGTCTATCCCAATTCCTG-3';
BS3 (corresponding to a 329bp fragment),
BS3-forward: 5'-GGGGTGCACTGAAGTATGTGTG-3',
and BS3-reverse: 5'-TTTCAGAGCTGGGGACCGAAC-3'.

Immunofluorescence

Immunofluorescence staining was performed as we and others described previously (35,38,39). Cells were fixed in methanol (-20°C), washed, permeabilized (PBS/0.3% Triton X-100) and blocked. Specimens were incubated overnight (4°C) with primary antibodies: ARC (Chemicon, 1:100) or cytochrome c (Santa Cruz Biotechnology, 1: 200). Immunoreactive antigens were visualized after staining with Alexa Fluor® 594-conjugated goat anti-mouse IgG (1:500) or Alexa Fluor® 488-conjugated goat anti-rabbit IgG (1:500). Nuclear staining was achieved using DAPI (1:1000 in PBS; 5 min). Images from fluorescence-labeled cells were obtained with a Zeiss LSM510 confocal laser scanning microscope.

Measurement of [Ca^{2+}]_c

Cytosolic calcium was measured as previously described (40-42). In brief, the cardiomyocytes cultured on a glass coverslip were loaded with 10 μM Fluo-3-AM (Molecular Probes), in the HEPES solution without Ca^{2+} at 37°C for 30 min, then washed twice with dye-free HEPES solution and placed in chamber on the stage of a laser scanning confocal microscope (Zeiss LSM510). Fluo-3 in cells was excited with light at 488 nm and emitted fluorescence was detected at >500 nm. For fluorescence analysis, the regions of interest (ROIs) were identified in the average fluorescence intensities over 20 ROIs minus background were calculated for each frame and normalized for comparative purposes. The [Ca^{2+}]_c values were calculated by Mn^{2+} quenching.

Detections of [Ca^{2+}]_m

The mitochondrial calcium indicator Rhod-2 AM (Molecular Probes) was used to measure changes of [Ca^{2+}]_m as previously described(43,44). Briefly, cardiomyocytes were loaded with 5 μM Rhod-2 AM for 2 h at 4°C and further incubated for 2 h at 37°C in the culture medium. This 2-step cold loading/warm incubation protocol achieves exclusive loading of Rhod-2 into the mitochondria. The Rhod-2 images were captured with excitation at 543 nm and detected at >560 nm. Rhod-2 fluorescence intensities (F) in each experiment were normalized to the average baseline fluorescence for the same region (F0).

Detections of [Ca^{2+}]_SR

The low-affinity calcium indicator, Fluo-5N AM (Molecular Probes), was used to assess changes in the intraluminal SR Ca^{2+} concentration as previously described(45,46). In brief, the cardiomyocytes were loaded with 5 μM Fluo-5N AM at 37°C for 2 h, rinsed, and incubated in culture medium for a further 1.5 h at 37°C to allow deesterification and outward leak of cytosolic indicator. Fluo-5N was excited with light at 488 nm and emitted
fluorescence was detected at >500 nm. Fluo-5N fluorescence intensities (F) in each experiment were normalized to the average baseline fluorescence for the same region (F0).

Detection of mitochondrial membrane potential (ΔΨm)

ΔΨm was measured using tetramethylrhodamine methyl ester (TMRM) as previously described(39). In brief, the cells were stained with 10 nM TMRM (Molecular Probes) at 37℃ for 30 min. TMRM images were captured using a laser scanning confocal microscope (Zeiss LSM510) with excitation at 543 nm and emission at >560 nm.

Ischemia/reperfusion (I/R), hemodynamic assessment, LDH release assay and analysis of infarction sizes

Mice were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (5mg/kg), and fixed in the supine position and tracheotomy was performed to provide artificial ventilation (0.2 ml tidal volume, 110 breaths/min) with a rodent ventilator supplemented with 100% oxygen. The left anterior descending coronary artery (LAD) was identified and ligated with a slipknot using 8-0 silk suture at the inferior border of the left auricle. Myocardial ischemia was confirmed by the obvious cyanotic appearance of the left ventricle and S-T segment elevation on the electrocardiogram. After 30 min of ischemia the knot was released to permit reperfusion of the heart confirmed also by obvious S-T segment change. After removal of air and blood, the chest was closed and the animal was removed from the respirator and transferred back to its cage. Sham-operated mice were prepared identically without undergoing the occlusion of the silk suture. 24 hours after reperfusion the mice were anesthetized as described above and the LV hemodynamic measurements were conducted by an experienced investigator who is blind to the treatment through a closed-chest catheterization. A 1.4 F microtipped catheter (SPR-839, Millar Instruments, Houston, Texas) was inserted into the right carotid artery and advanced into the left ventricle. Hemodynamic parameters such as left ventricular end-diastolic pressure (LVEDP), left ventricular maximum first derivative of pressure (LV dP/dtmax), minimum first derivative of pressure (LV dP/dtmin) and left ventricular eject fraction (LV EF) were computed. The Heparin-blood at the end of the hemodynamic assessment was collected from all groups, the concentration of LDH in the plasma was assayed using an LDH ELISA kit (ADL). To determine myocardial infarct sizes the thoracotomy was reopened, the suture was reoccluded and 2% of Evans blue (Sigma-Aldrich, St. Louis, MO) was injected into the left ventricular cavity to delineate the ischemic zone from the nonischemia zone. The heart was immediately removed and flushed with ice-cold saline and frozen in -80℃ freezer. Each heart was then horizontally cut into 5 slices which were incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) for 15 min at 37℃ for demarcation of the viable and nonviable myocardium within AAR. Infarct myocardium appears yellowish white and viable myocardium stains brick red. The staining was stopped by ice-cold PBS and the slices were fixed in 10% neutral buffered formaldehyde and individually weighed. Both sides of each slice were photographed. The
areas of infarction (INF), area at risk (AAR), and left ventricle (LV) were assessed by computerized planimetry, and the ratio of AAR/LV, INF/AAR and INF/LV were analyzed by an observer blinded to the sample identity.

Evaluation of apoptosis
To determine apoptosis in the heart sections, we used In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics). The detections were performed as we described elsewhere (32,47).

Detection of caspase 3 activity
Caspase 3 activity was detected using an assay kit (R&D Systems). The assay procedures were according to the kit instructions.

Statistical analysis
Paired data were evaluated by Student’s t test. A one-way analysis of variance was used for multiple comparisons. A P value of <0.05 was considered significant.

Results
Foxo3a can suppress cytosolic Ca^{2+} elevations and apoptosis
Reactive oxygen species play an important role in mediating apoptosis in cardiomyocytes. In this study, we observed that hydrogen peroxide or anoxia/reoxygenation treatment leads to the increase of cytosolic Ca^{2+} levels [Ca^{2+}]_{c} in cardiomyocytes (Fig.1A). Administration of the Ca^{2+} chelator, BAPTA-AM, inhibits apoptosis (Fig.1B), suggesting that Ca^{2+} is necessary for the initiation of apoptosis.

Foxo3a can trigger or inhibit apoptosis depending on the cell types or cellular context (6,48). We tested whether Foxo3a can influence apoptosis in cardiomyocytes. Enforced expression of the constitutively active form of Foxo3a (caFoxo3a) itself did not induce apoptosis, however, it could prevent apoptosis triggered by hydrogen peroxide (Fig.1C). Because Ca^{2+} is necessary for the initiation of apoptosis as shown in Fig. 1B, we thus tested whether Foxo3a can influence [Ca^{2+}]_{c} levels in cells treated with hydrogen peroxide. Enforced expression of caFoxo3a could attenuate [Ca^{2+}]_{c} elevations induced by hydrogen peroxide (Fig.1D). We then tested whether endogenous Foxo3a participates in the regulation of [Ca^{2+}]_{c} and apoptosis in cardiomyocytes. Hydrogen peroxide at a low dose of 10 µM induced a slight alteration of [Ca^{2+}]_{c} levels. In contrast, knockdown of endogenous Foxo3a led to a significant elevation of [Ca^{2+}]_{c} in response to the treatment with the same low dose of hydrogen peroxide (Fig.1E). Concomitantly, a significant amount of cells underwent apoptosis (Fig.1F). Furthermore, We isolated cardiomyocytes from Foxo3a knockout mice and observed a higher level of [Ca^{2+}]_{c} in these cells upon treated with hydrogen peroxide or anoxia/reoxygenation, and noticeably, re-expression of caFoxo3a attenuated [Ca^{2+}]_{c} levels (Fig.1G). Hydrogen peroxide or anoxia/reoxygenation induced more cardiomyocytes from Foxo3a knockout mice to undergo apoptosis, and this can be inhibited by re-expression of caFoxo3a (Fig.1H). Finally, we further characterized whether apoptosis indeed occurred under our experimental condition. Enforced expression of caFoxo3a suppressed the activation of caspase 3 in neonatal rat cardiomyocytes (Figs.2A, 2B) or cardiomyocytes from...
Foxo3a deficient mice (Fig.2C). Pre-treatment of the cells with caspase inhibitor zVAD-FMK suppressed both apoptosis (Figs.2D, 2E) and caspase 3 activation (Figs.2F, 2G) triggered by hydrogen peroxide or anoxia/reoxygenation. Taken together, these data indicate that Foxo3a is able to suppress $[\text{Ca}^{2+}]_c$ elevations and apoptosis.

**Foxo3a knockout mice exhibit an enlarged myocardial infarction size**

Apoptosis is a kind of death form in myocardial infarction. We tested whether Foxo3a plays a role in regulating myocardial infarction. Foxo3a deficient mice exhibited a larger infarction size upon ischemia/reperfusion (I/R) (Fig. 3A), and a more severe injury in cardiac function (Fig. 3B). We analyzed apoptosis and observed more apoptotic cells in Foxo3a deficient mice upon I/R (Fig. 3C). These results suggest that Foxo3a participates in inhibiting apoptosis and myocardial infarction in the animal model. Furthermore, our data shows that LDH release was significantly increased in hearts from Foxo3a deficient mice (Fig. 3D), which suggests that loss of Foxo3a exacerbated myocardial injury induced by I/R.

**ARC attenuates cytosolic Ca$^{2+}$ elevations**

To understand the relationship between ARC and Ca$^{2+}$ in the apoptotic program of cardiomyocytes, we tested whether ARC can influence $[\text{Ca}^{2+}]_c$. ARC levels were decreased upon hydrogen peroxide treatment (Fig. 4A). Knockdown of endogenous ARC could sensitize hydrogen peroxide to induce $[\text{Ca}^{2+}]_c$ elevations (Fig. 4B), apoptosis (Fig. 4C) and the activation of caspase 3 (Fig.4D). Enforced expression of ARC attenuated $[\text{Ca}^{2+}]_c$ elevations induced by hydrogen peroxide (Fig. 4E). Concomitantly, apoptosis (Fig. 4F) and caspase 3 activation could be inhibited (Fig. 4G). Furthermore, we isolated the cardiomyocytes from ARC transgenic mice, and observed that $[\text{Ca}^{2+}]_c$ increases evoked by stimulation with hydrogen peroxide were significantly less than that from wild type mice (Fig. 4H). Thus, it appears that ARC is able to attenuate cytoplasmic calcium elevation induced by hydrogen peroxide in cardiomyocytes.

**ARC participates in the control of Ca$^{2+}$ release from sarcoplasmic reticulum**

How can ARC lead to a reduction of $[\text{Ca}^{2+}]_c$? Sarcoplasmic reticulum release of Ca$^{2+}$ has been proved to contribute to $[\text{Ca}^{2+}]_c$ elevations during apoptosis. We tested whether ARC can influence sarcoplasmic reticulum release of Ca$^{2+}$. ARC has been previously shown to be localized in the cytoplasm and mitochondria (20,35), but it is not yet clear whether ARC is distributed in sarcoplasmic reticulum. We analyzed ARC distributions in the subcellular organelles and observed that a portion of ARC was distributed in the sarcoplasmic reticulum as analyzed by immunoblotting and immunofluorescence (Fig. 5A). Subsequently, we investigated whether ARC can influence Ca$^{2+}$ within the sarcoplasmic reticulum ($[\text{Ca}^{2+}]_{SR}$). Hydrogen peroxide induced a reduction of $[\text{Ca}^{2+}]_{SR}$. Enforced expression of ARC elevated $[\text{Ca}^{2+}]_{SR}$ upon treatment with hydrogen peroxide (Fig. 5B). We attempted to understand whether endogenous ARC plays a role in regulating $[\text{Ca}^{2+}]_{SR}$, and observed that knockdown of ARC could sensitize the reduction of $[\text{Ca}^{2+}]_{SR}$ upon treatment with 10 µM hydrogen peroxide (Fig. 5C). These data indicate that ARC is able to regulate
sarcoplasmic reticulum Ca\(^{2+}\) release into cytoplasm.

**ARC attenuates mitochondrial Ca\(^{2+}\) uptake**

It has been well documented that the imbalance in mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{m}\)) leads to apoptosis. We tested whether ARC is involved in the maintenance of [Ca\(^{2+}\)]\(_{m}\) homeostasis. Enforced expression of ARC reduced [Ca\(^{2+}\)]\(_{m}\) upon treatment with hydrogen peroxide (Fig. 6A). Knockdown of ARC sensitized the elevation of [Ca\(^{2+}\)]\(_{m}\) upon treatment with 10 µM hydrogen peroxide (Fig. 6B). We isolated cardiomyocytes from ARC transgenic mice, and they exhibited a low level of [Ca\(^{2+}\)]\(_{m}\) than those from the wild type mice upon hydrogen peroxide treatment (Fig. 6C), suggesting that ARC serves to inhibit the elevation of [Ca\(^{2+}\)]\(_{m}\).

To further understand the role of ARC in regulating [Ca\(^{2+}\)]\(_{m}\), we made a construct in which ARC was fused to mitochondria-targeted GFP, and termed this construct as M/GFP/ARC which was specifically localized in mitochondria (Fig. 6D). The empty mitochondria-targeted GFP was termed as M/GFP. M/GFP/ARC was able to attenuate [Ca\(^{2+}\)]\(_{m}\) elevations induced by hydrogen peroxide (Fig.6E). Also, it could inhibit the collapse of mitochondrial membrane potential (Fig.6F). We analyzed cytochrome c distributions in cells expressing M/GFP/ARC. In the control cells without treatment, cytochrome c distribution pattern was coincident with that of M/GFP. Upon treatment with hydrogen peroxide, cytochrome c and M/GFP showed a differential pattern. However, in cells expressing M/GFP/ARC, the cytochrome c distribution pattern was not significantly altered (Fig. 6G).

**Foxo3a regulates Ca\(^{2+}\) through ARC**

Foxo3a is a transcription factor, how can it influence Ca\(^{2+}\) machinery in cardiomyocytes? Because of the ability of ARC to regulate Ca\(^{2+}\), we tested whether ARC can be a downstream mediator of Foxo3a to target Ca\(^{2+}\). Knockdown of ARC could abolish the effect of Foxo3a on attenuating [Ca\(^{2+}\)]\(_{c}\) elevations (Fig.7A) and apoptosis (Fig. 7B) induced by hydrogen peroxide. The effects of Foxo3a on attenuating caspase 3 activation (Fig.7C), [Ca\(^{2+}\)]\(_{m}\) increases (Fig. 7D) and cytochrome c release (Fig. 7E) were also abolished upon ARC knockdown. These data indicate that ARC is a downstream mediator of Foxo3a in regulating calcium homeostasis.

**ARC is a transcriptional target of Foxo3a**

We explored the relationship between ARC and Foxo3a. An elevated level of ARC mRNA in caFoxo3a-expressed cardiomyocytes could be observed (Fig.8A). Also, ARC protein levels were elevated upon caFoxo3a stimulation (Fig.8B). We further tested whether endogenous Foxo3a participates in the regulation of ARC. Knockdown of endogenous Foxo3a by RNAi led to a reduction in mRNA (Fig.8C) and protein (Fig.8D) levels of ARC.

The influence of Foxo3a on ARC expression led us to consider whether ARC is a transcriptional target of Foxo3a. To address this consideration, we analyzed the rat ARC promoter region, and found that the promoter region of ARC contains three potential Foxo3a binding sites (Fig. 8E). We first tested whether Foxo3a can regulate ARC promoter activity. Luciferase assay revealed that although enforced expression of wild type
Foxo3a (wtFoxo3a) and caFoxo3a could stimulate ARC promoter activity, caFoxo3a had a stronger effect. Furthermore, only the full-length ARC promoter could be activated by Foxo3a, indicating that the BS1 was responsible for the luciferase activity (Fig. 8F). Knockdown of endogenous Foxo3a by RNAi significantly reduced ARC promoter activity in cardiomyocytes (Fig. 8G). To determine Foxo3a binding sites in ARC promoter in vivo, we performed ChIP assays using primers directly against the BS1, BS2 and BS3, respectively. Foxo3a bound to the BS1 but not BS2 and BS3 in ARC promoter (Fig. 8H). Finally, we analyzed ARC levels in Foxo3a knockout mice and observed that these mice had a low level of ARC in comparison with the wild type mice (Fig. 8I). Taken together, we demonstrate that ARC is a transcriptional target of Foxo3a.

**Discussion**

Although Foxo3a plays a role in regulation of apoptosis in a variety of cell types, its role in cardiac apoptosis remains to be fully understood. Our present work demonstrated that Foxo3a inhibits apoptosis in cardiomyocytes and the cardiac anti-apoptotic protein-ARC attenuates the release of calcium from sarcoplasmic reticulum and inhibits calcium elevations in the cytoplasm and mitochondria under oxidative stress. Foxo3a is shown to maintain calcium homeostasis and inhibit apoptosis in cardiomyocytes through ARC. Furthermore, ARC is a direct transcriptional target of Foxo3a. Thus, our results provide novel evidence that Foxo3a regulates cardiac apoptosis by maintaining calcium homeostasis through ARC.

The role of Foxo3a in apoptosis is dependent on the cell types and the cellular contexts. It can provoke apoptosis in haematopoietic (4,5) and neuronal cells (6) but can prevent apoptosis in neutrophils. This discrepancy is probably due to the multiple targets of Foxo3a, and its final transcriptional output is determined by the equilibrium of the pro- and anti-apoptotic factors. The identified downstream targets of Foxo3a include Fas ligand (FasL) (6), Bim (49), Mn-superoxide dismutase (50) and catalase (51). FasL activates the extrinsic apoptotic pathway by associating with Fas and consequently leading to the formation of death inducing signaling complex and caspase-8 activation. Bim is a member of Bcl-2 family and counteracts Bcl-2 and Bcl-xL thereby inducing apoptosis (52). Mn-superoxide dismutase and catalase can scavenge reactive oxygen species that are important apoptotic stimuli. Our present work revealed that Foxo3a is able to prevent apoptosis in cardiomyocytes. Noticeably, we have found that ARC is a transcriptional target of Foxo3a and necessary for Foxo3a to exert its anti-apoptotic function.

It has been shown that ARC is a calcium-binding protein and can suppress Ca\(^{2+}\)-mediated apoptosis (53). Our present study demonstrated that ARC is present in the sarcoplasmic reticulum and participates in the regulation of mitochondrial Ca\(^{2+}\) homeostasis by attenuating the release of [Ca\(^{2+}\)]\(_{\text{SR}}\) induced by oxidative stress. ARC requires the C-terminus to bind to calcium, and the deletion of the C-terminus leads to the inability of ARC to bind to calcium (53). Intriguingly, the subcellular localization of ARC is controlled by the C-terminus. The mutation in threonine-149 in the C-terminus results in the alteration of ARC subcellular...
localization and loss of ARC anti-apoptotic function(54). Thus, it appears that the C-terminus is important for ARC function. Our data shed new light on understanding the novel molecular mechanism by which ARC inhibits apoptosis.

Sarcoplasmic reticulum (SR) and mitochondria locate close to each other in cardiomyocytes (15). There is a tight coupling of Ca\(^{2+}\) signaling between SR release sites and nearby mitochondria, because the SR Ca\(^{2+}\) release results in Ca\(^{2+}\) microdomains sufficient to promote local mitochondrial Ca\(^{2+}\) uptake(16,17). In this study, we have shown that the increase in [Ca\(^{2+}\)]\(_{m}\) may result from the reduction of [Ca\(^{2+}\)]\(_{SR}\). The membrane-permeable Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) is highly selective for Ca\(^{2+}\) and can be used to control the level of intracellular Ca\(^{2+}\). The efficiency of mitochondrial Ca\(^{2+}\) uptake depends on the upstroke velocity of cytosolic Ca\(^{2+}\) transients (55). It would be interesting to test whether BAPTA-AM can affect the effect of ARC on regulating calcium homeostasis in the future study.

ARC is involved in the control of myocardial infarction. ARC transgenic mice exhibit less myocardial infarction sizes(56), whereas ARC knockout mice demonstrate accelerated myocardial infarction(47). ARC levels are significantly decreased in cardiomyocytes upon treatment with hydrogen peroxide and hypoxia (20,23). Furthermore, ARC levels are reduced upon heart failure(47) or ischemia(57). The expression reduction of a protein can be due to its decrease in synthesis and/or increase in degradation. ARC degradation is upregulated upon apoptotic stimulation(57). Our present work has revealed that Foxo3a can transactivate ARC, and the reduced expression of Foxo3a contributes to ARC downregulation. Thus, the dysregulation of ARC expression can be related to Foxo3a.

In order to maintain the heart intact in both structure and function, it is necessary to prevent apoptosis so that the heart does not lose cardiomyocytes. Therefore, the development of anti-apoptotic strategies may proof useful as a means to prevent apoptosis-related cardiac diseases leading to heart failure. Our present work provides novel evidence that Foxo3a and ARC constitute an anti-apoptotic pathway that participates in the maintenance of calcium homeostasis. Our results can provide important information for exploring the beneficial effects of this pathway on apoptosis-related cardiac diseases.
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Conflict of Interest
The authors declare no conflict of interest.
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**Figure Legends**

**Fig.1. Foxo3a suppresses Ca^{2+} elevations and apoptosis.** A. Hydrogen peroxide or anoxia/reoxygenation induces an elevation of [Ca^{2+}]_{c}. Neonatal rat cardiomyocytes were treated with hydrogen peroxide or anoxia/reoxygenation. The confocal images show representative images. The histograms show the summary of [Ca^{2+}]_{c} levels. *P<0.05. B. Ca^{2+} chelator BAPTA-AM inhibits apoptosis. Neonatal rat cardiomyocytes were exposed to Ca^{2+} chelator BAPTA-AM (10 μM, 1 h) followed by H_{2}O_{2} (24 h) or anoxia/reoxygenation treatment. *P<0.05. C. Foxo3a prevents apoptosis triggered by hydrogen peroxide. Neonatal rat cardiomyocytes were infected with the adenoviruses harboring constitutively active Foxo3a (caFoxo3a), or β-galactosidase cDNA (β-gal) at a moi of 80. 24 h after infection cells were treated with hydrogen peroxide. The upper panel shows Foxo3a levels analyzed by immunoblotting. Apoptosis was detected 24 h after hydrogen peroxide treatment (low panel). *P<0.05. D. caFoxo3a attenuates [Ca^{2+}]_{c} elevations triggered by hydrogen peroxide. Neonatal rat cardiomyocytes were treated as described for (C). The histograms show the summary of [Ca^{2+}]_{c} levels. *P<0.05. E and F. Inhibition of endogenous Foxo3a sensitizes [Ca^{2+}]_{c} elevations (E) and apoptosis (F) in response to hydrogen peroxide treatment. Neonatal rat cardiomyocytes were infected with the adenoviral Foxo3a RNAi or its scramble form (Foxo3a-S-RNAi) at a moi of 50. 24 h after infection cells were treated with 10 μM H_{2}O_{2}. The upper panel in Fig. 1E shows Foxo3a levels analyzed by immunoblotting. [Ca^{2+}]_{c} levels are shown in the low panel of Fig. 1E. Apoptosis is shown in Fig. 1F. *P<0.05. G and H. The cardiomyocytes from Foxo3a knockout mice show a higher level of [Ca^{2+}]_{c} (G) and apoptosis (H) in response to hydrogen peroxide stimulation. Cardiomyocytes isolated from Foxo3a knockout mice were infected with adenoviral caFoxo3a or β-gal for 24 h, and then treated with hydrogen peroxide. *P<0.05. Data are expressed as mean ± SEM from 3 independent experiments.

**Fig.2. Caspase 3 activation is inhibited by Foxo3a or zVAD-FMK.** A and B. Enforced expression of caFoxo3a attenuates caspase 3 activation triggered by hydrogen peroxide (A) or anoxia/reoxygenation (B). Neonatal rat cardiomyocytes were infected with the adenoviruses harboring constitutively active Foxo3a (caFoxo3a), or β-galactosidase cDNA (β-gal) at a moi of 80. 24 h after infection cells were subjected to hydrogen peroxide or anoxia/reoxygenation. Caspase 3 catalytic activity was measured after stimulation. *P<0.05. C. The cardiomyocytes from Foxo3a knockout mice show a higher level of caspase 3 activity in response to anoxia/reoxygenation. Cardiomyocytes isolated from Foxo3a knockout mice were infected with adenoviral caFoxo3a or β-gal for 24 h, and then subjected to anoxia/reoxygenation. *P<0.05. Data are expressed as mean ± SEM from 3 independent experiments. D and E. Analysis of cell death triggered by hydrogen peroxide (D) or anoxia/reoxygenation (E). The neonatal rat cardiomyocytes were pre-treated with or without caspase inhibitor zVAD-FMK (100 μM, 1h) and subjected to hydrogen peroxide or anoxia/reoxygenation treatment. Apoptosis was detected 24 h after stimulation. *P<0.05. F and G. Analysis of caspase 3 activation in cells stimulated with hydrogen peroxide (F) or anoxia/reoxygenation (G). The neonatal rat cardiomyocytes were
treated as described for D and E. The caspase 3 activity was analyzed. *P<0.05. Data are expressed as mean ± SEM from 3 independent experiments.

**Fig. 3. Foxo3a knockout mice demonstrate an enlarged myocardial infarction size.** A. Mice deficient in Foxo3a show an enlarged myocardial infarction size upon I/R injury. Foxo3a knockout mice and wild type (WT) littermates were subjected to 30 min of ischemia and 24 h of reperfusion. The ratio of area-at-risk (AAR)/left ventricle (LV), infarction myocardium (INF)/AAR and INF/LV were calculated. (n=6). *p<0.05. B. Mice deficient in Foxo3a present worsened hemodynamics in response to I/R injury. Mice were treated as described for (A). 24 h after reperfusion the LV hemodynamic assessments were performed. (n=6). *p<0.05. C. TUNEL assay illustrates more apoptosis in Foxo3a knockout mice than the wild type littermates. Representative myocardial sections stained by TUNEL method are shown in left panel. Apoptotic nuclei are shown by the bright green nuclear fluorescence. Sections were counterstained with anti-α-actinin antibody and DAPI to identify cardiac myocytes and nuclei. Bar = 20 µm. Foxo3a and ARC levels in the hearts were analyzed by immunoblotting (right lower panel). Quantification of apoptosis by the TUNEL method (right upper panel, n=5). *P<0.05. D. Foxo3a null mice show increased LDH release induced by I/R injury. Plasma LDH levels at the end of 24 h reperfusion in vivo were measured. (n=5). *p<0.05. Data are expressed as mean ± SEM from 3 independent experiments.

**Fig. 4. ARC attenuates [Ca^{2+}]_{c} elevations.** A. Hydrogen peroxide reduces Foxo3a and ARC levels. Neonatal rat cardiomyocytes were treated with hydrogen peroxide. Foxo3a and ARC were analyzed by immunoblotting. The protein loading was illustrated by actin. A representative result of three independent experiments is shown. B, C and D. Knockdown of endogenous ARC sensitizes hydrogen peroxide to induce [Ca^{2+}]_{c} elevations (B), apoptosis (C) and caspase 3 activation (D). Neonatal rat cardiomyocytes were infected with the adenoviral ARC RNAi or its scramble form (ARC-S-RNAi) at a moi of 50. 24 h after infection cells were treated with hydrogen peroxide. ARC levels analyzed by immunoblotting are shown in the upper panel in Fig. 3B, *P<0.05. E, F and G. Enforced expression of ARC suppresses [Ca^{2+}]_{c} rises (E), apoptosis (F) and caspase 3 activation (G) induced by hydrogen peroxide. Neonatal rat cardiomyocytes were infected with the adenoviral ARC or β-gal at a moi of 80. 24 h after infection cells were treated with hydrogen peroxide. *P<0.05. H. The cardiomyocytes from ARC transgenic mice show a lower level of [Ca^{2+}]_{c} in response to hydrogen peroxide stimulation. The cardiomyocytes from ARC transgenic mice were treated with hydrogen peroxide. *P<0.05. Data are expressed as mean ± SEM from 3 independent experiments.

**Fig. 5. ARC reduces Ca^{2+} release from sarcoplasmic reticulum.** A. Analysis of ARC intracellular localizations. The left panel shows ARC in the subcellular fractions of cardiomyocytes as analyzed by immunoblotting. The cytochrome oxidase subunit IV (COX IV) is a mitochondrial marker. SERCA2 is a marker of sarcoplasmic reticulum. Endogenous ARC
and SERCA2 were visualized by immunofluorescence (right panel). Bar = 20 µm. B. ARC inhibits the reduction of \([\text{Ca}^{2+}]_{\text{SR}}\) induced by hydrogen peroxide. Neonatal rat cardiomyocytes were infected with the adenoviral ARC or β-gal at a moi of 80. 24 h after infection cells were treated with hydrogen peroxide. *P<0.05. C. Knockdown of ARC sensitizes \([\text{Ca}^{2+}]_{\text{SR}}\) reduction upon treatment with hydrogen peroxide. Neonatal rat cardiomyocytes were infected with the adenoviral ARC RNAi or its scramble form (ARC-S-RNAi) at a moi of 50. 24 h after infection cells were treated with hydrogen peroxide. *P<0.05. Data are expressed as mean ± SEM from 3 independent experiments.

Fig.6. ARC attenuates the elevation of \([\text{Ca}^{2+}]_{\text{m}}\). A. Enforced expression of ARC reduces \([\text{Ca}^{2+}]_{\text{m}}\) rises induced by hydrogen peroxide. Neonatal rat cardiomyocytes were infected with the adenoviral ARC or β-gal at a moi of 80. 24 h after infection cells were treated with hydrogen peroxide. The left panel shows the representative confocal image. The right upper panel shows ARC levels in mitochondria-enriched heavy membranes analyzed by immunoblotting. The right low panel shows \([\text{Ca}^{2+}]_{\text{m}}\). *P<0.05. B. Knockdown of endogenous ARC sensitizes hydrogen peroxide-induced \([\text{Ca}^{2+}]_{\text{m}}\) elevations. Neonatal rat cardiomyocytes were infected with the adenoviral ARC RNAi or its scramble form (ARC-S-RNAi) at a moi of 50. 24 h after infection cells were treated with hydrogen peroxide. The upper panel shows ARC levels in mitochondria-enriched heavy membranes analyzed by immunoblotting. The low panel shows \([\text{Ca}^{2+}]_{\text{m}}\). *P<0.05. C. The cardiomyocytes from ARC transgenic mice show a lower level of \([\text{Ca}^{2+}]_{\text{m}}\) in response to hydrogen peroxide stimulation. The upper panel shows ARC levels in ARC transgenic mice (Tg) and wild type mice (WT). \([\text{Ca}^{2+}]_{\text{m}}\) levels are shown in the low panel. *P<0.05. D. Fluorescent analysis of M/GFP/ARC. Neonatal rat cardiomyocytes were transfected with the construct of M/GFP/ARC. Mitochondria were visualized by Mitotracker red. Bar = 20 µm. E. ARC attenuates \([\text{Ca}^{2+}]_{\text{m}}\) accumulation induced by hydrogen peroxide. Neonatal rat cardiomyocytes were transfected with the constructs of the empty vector (M/GFP) or M/GFP/ARC, and subjected to hydrogen peroxide. *P<0.05. F. ARC attenuates mitochondrial membrane potential depolarization. Neonatal rat cardiomyocytes were treated as described for (E). Mitochondrial membrane potential was analyzed by TMRM (red). GFP positive cells exhibit green color (left panel). Bar = 20 µm. The right panel shows the summary of TMRM fluorescence. G. Subcellular distributions of cytochrome c. Neonatal rat cardiomyocytes were treated as described for (E), then fixed and stained for cytochrome c. Bar = 20 µm. Data are expressed as mean ± SEM from 3 independent experiments.

Fig.7. Foxo3a regulates \(\text{Ca}^{2+}\) through ARC. A, B and C. Knockdown of ARC abolishes the effect of caFoxo3a on \([\text{Ca}^{2+}]_{\text{c}}\) (A), apoptosis (B) and caspase 3 activation (C). Neonatal rat cardiomyocytes were co-infected with the adenoviral caFoxo3a at a moi of 80, ARC RNAi or ARC-S-RNAi at a moi of 50. The cells were treated with hydrogen peroxide 24 h after adenoviral infection. The upper panel in Fig. 7A shows Foxo3a and ARC levels analyzed by immunoblotting. The low panel in Fig. 7A shows \([\text{Ca}^{2+}]_{\text{c}}\). *#P<0.05. D and E. Knockdown of ARC abolishes the effect of caFoxo3a on \([\text{Ca}^{2+}]_{\text{m}}\) (D) and cytochrome c distributions (E).
Neonatal rat cardiomyocytes were treated as described for (A). *p<0.05. Cytochrome c distributions in mitochondria-enriched heavy membranes (HM) and cytosol were analyzed by immunoblotting. Data are expressed as mean ± SEM from 3 independent experiments.

Fig. 8. ARC is a transcriptional target of Foxo3a. A. Foxo3a stimulates ARC mRNA expression. Neonatal rat cardiomyocytes were infected with the adenoviral caFoxo3a or β-gal at a moi of 80. Cells were harvested for the analysis of ARC mRNA by quantitative real-time RT-PCR (qRT-PCR). The values were normalized to that of GAPDH. *P<0.05 vs control. B. Foxo3a upregulates ARC protein levels. Neonatal rat cardiomyocytes were infected as described for (A). Cells were harvested for the analysis of ARC protein by immunoblotting. The blots shown here are the representative blots from 3 independent experiments. Numbers above immunoblots show the ratios of the band intensity of ARC to that of actin. C. Knockdown of Foxo3a leads to a reduction in ARC mRNA levels. Neonatal rat cardiomyocytes were infected with adenoviral Foxo3a-RNAi or its scramble form (Foxo3a-S-RNAi) at a moi of 100. Cells were harvested 48 h after infection for the analysis of ARC mRNA levels by qRT-PCR, *P<0.05 vs control. D. Knockdown of Foxo3a leads to a reduction in ARC protein levels. Neonatal rat cardiomyocytes were infected as described for (C). Cells were harvested 48 h after infection for the analysis of ARC protein levels by immunoblotting. The blots shown here are the representative blots from 3 independent experiments. Numbers above immunoblots show the ratios of the band intensity of ARC to that of actin. E. ARC promoter region has three potential Foxo3a binding sites. Rat ARC promoter contains three potential Foxo3a binding sites (BS) indicated as BS1, BS2 and BS3. Three fragments of ARC promoter were synthesized and linked to luciferase (Luc) reporter vector, respectively. F. Foxo3a stimulates the activity of ARC promoter containing BS1. HEK293 cells were infected with adenoviral β-gal, wild type Foxo3a (wtFoxo3a) or caFoxo3a at a moi of 80. 24 h after infection cells were transfected with the constructs of the empty vector (pGL-4) or ARC promoter constructs, respectively. Firefly luciferase activities were normalized to Renilla luciferase activities. G. Inhibition of endogenous Foxo3a leads to a reduction of ARC promoter activity. Neonatal rat cardiomyocytes were infected with adenoviral Foxo3a-RNAi or its scramble form (Foxo3a-S-RNAi) at a moi of 100. 24 h after infection cells were transfected with the constructs of the empty vector (pGL-4) or the pGL-4-ARC promoter-1, respectively. Firefly luciferase activities were normalized to Renilla luciferase activities. *p<0.05 vs pGL-4-ARC promoter-1 alone. H. Foxo3a directly binds to BS1 of ARC promoter as analyzed by ChIP assay. Neonatal rat cardiomyocytes were infected with adenoviral β-gal, wtFoxo3a or caFoxo3a at a moi of 80. Cells were harvested 24h after infection for ChIP analysis. Chromatin-bound DNA was immunoprecipitated with the anti-Foxo3a antibody. The anti actin antibody was used as a negative control (Neg). Immunoprecipitated DNA was analyzed by PCR using a primer combination that encompassed the 3 potential Foxo3a binding sites in ARC promoter (BS1, BS2 and BS3), respectively. I. ARC protein levels in hearts from Foxo3a knockout mice were less than that from wild type mice. The hearts from Foxo3a knockout mice and their wild type littermates were harvested and ARC levels were analyzed by immunoblotting. Data are expressed as mean ± SEM from 3 independent experiments.
Caspase-3 activity (arbitrary units)

A

200µM H₂O₂ - - + + +
β-gal - - - + +
caFoxo3a - - - - +

B

A/R - - + + +
β-gal - + - - +
caFoxo3a - - - + +

C

A/R - - + + +
WT - - - - -
Foxo3a−/− - - + + +
β-gal - - - + -
caFoxo3a - - - - +

D

TUNEL positive cells (%)

200µM H₂O₂ - - + +
zVAD-FMK - + - +

E

TUNEL positive cells (%)

A/R - - + +
zVAD-FMK - + - +

F

Caspase-3 activity (arbitrary units)

200µM H₂O₂ - - + +
zVAD-FMK - + - +

G

Caspase-3 activity (arbitrary units)

A/R - - + +
zVAD-FMK - + - +
A. TUNEL, DAPI, α-actinin, Overlay images for WT and Foxo3a<sup>−/−</sup> under Sham and I/R conditions.

B. Graphs showing LVEDP, dP/dt, and LVEF for WT and Foxo3a<sup>−/−</sup> under Sham and I/R conditions.

C. TUNEL images for WT and Foxo3a<sup>−/−</sup> under Sham and I/R conditions, with bar graphs showing the percentage of apoptotic myocytes.

D. Graph showing the level of plasma LDH for WT and Foxo3a<sup>−/−</sup> under Sham and I/R conditions.
Fig. 5

A

Cyto Mito SR
ARC
SERCA2
COX IV

ARC
SERCA2
DAPI
Overlay

B

ARC
Calnexin

ARC
Calnexin

200μM H₂O₂
β-gal
ARC

ARC-S-RNAi
ARC RNAi

C

[Ca²⁺]_{SR} (F/F₀)

10μM H₂O₂

*
Figure 7

(A) Foxo3a, ARC, Actin

[B] TUNEL positive cells (%)

200µM H$_2$O$_2$ - + + + + + +
β-gal - - + - - - -
cFoxo3a - - - + - + +
ARC-S-RNAi - - - + + + +
ARC RNAi - - - - + + -

[C] Caspase-3 activity (arbitrary units)

200µM H$_2$O$_2$ - + + + + + +
β-gal - - + - - - -
cFoxo3a - - - + - + +
ARC-S-RNAi - - - + + + +
ARC RNAi - - - - + + -

[D] [Ca$^{2+}$]$_m$ (F/F0)

200µM H$_2$O$_2$ - + + + + + +
β-gal - - + - - - -
cFoxo3a - - - + - + +
ARC-S-RNAi - - - + + + +
ARC RNAi - - - - + + -

(E) Cyto c, COX IV, Cytosol, Cyto c, Actin

200µM H$_2$O$_2$ - + + + + + +
β-gal - - + - - - -
cFoxo3a - - - + - + +
ARC-S-RNAi - - - + + + +
ARC RNAi - - - - + + -
**A**

![Graph showing ARC mRNA levels (Arbitrary units)](image)

| β-gal | 48 | - | - | - |
|---|---|---|---|---|
| caFoxo3a | - | 12 | 24 | 36 | 48 |

![Graph showing Foxo3a RNAi and Foxo3a-S-RNAi effects](image)

**B**

![Western blot analysis of ARC levels](image)

- **Foxo3a RNAi**
- **Foxo3a-S-RNAi**
- **Fas**
- **Actin**

**C**

![Graph showing ARC mRNA levels (Arbitrary units)](image)

| Foxo3a-S-RNAi | - | + | - |
|---|---|---|---|
| Foxo3a RNAi | - | - | + |

**D**

![Schematic map of Foxo3a binding sites](image)

**E**

**Schematic map of Foxo3a binding sites**

**Foxo consensus sequence**

GATAAAATA

**BS1**

| 500bp | 250bp |
|---|---|
| wtFoxo3a | - | + |
| caFoxo3a | - | - |

**F**

![Graph showing luciferase activity (Fold induction)](image)

| pGL4 empty | - | - | - |
|---|---|---|---|
| pGL4-ARC promoter-3 | - | + | - |
| pGL4-ARC promoter-2 | - | - | + |
| pGL4-ARC promoter-1 | - | - | - |

**G**

![Graph showing luciferase activity (Fold induction)](image)

- **pGL4-ARC promoter-1**
- **pGL4-ARC promoter-2**
- **pGL4-ARC promoter-3**

**H**

![ChIP assay results](image)

| M | Neg |
|---|---|
| 500bp | 250bp |
| 500bp | 250bp |
| 500bp | 250bp |
| 500bp | 250bp |

| β-gal | - | + | - | - |
|---|---|---|---|---|
| wtFoxo3a | - | - | + | - |
| caFoxo3a | - | - | - | + |

**I**

- **WT Foxo3a**
- **Foxo3a**
- **ARC**
- **Actin**
Foxo3a prevents apoptosis by regulating calcium through the apoptosis repressor with caspase recruitment domain
Daoyuan Lu, Jinping Liu, Jianqin Jiao, Bo Long, Qian Li, Weiqi Tan and Peifeng Li

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