Phosphorylation dependent mitochondrial translocation of Nr4a3 provokes cardiomyocyte death

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Abstract

Immediate early gene-nuclear receptor subfamily 4, group A, member 3 (Nr4a3) is implicated in a variety of cellular processes. However, its role and underlying mechanisms in ischemia reperfusion (IR) remain unknown. Here, we show that Nr4a3 expression is upregulated in the heart after IR and that cardiac specific Nr4a3 deficiency protects against myocardial ischemic injury and improves cardiac function. The overexpression of Nr4a3 in both neonatal and adult mouse hearts is sufficient to cause left ventricular dilation and contractile dysfunction without additional ischemic stress. Mechanistically, ischemia or hypoxia triggers Nr4a3 phosphorylation and translocation from the nucleus to mitochondria, where it interacts with and promotes Bnip3 integration into the mitochondrial membrane. This integration leads to mitochondrial permeability transition pore (mPTP) opening and decreased mitochondrial permeability transition pore ($\Delta\Psi_m$), which causes cardiomyocyte apoptotic and necrotic death. Our findings describe an Nr4a3-Bnip3 mitochondrial pathway underlying cell death.

Introduction

Myocardial infarction (MI) remains a major source of mortality worldwide ¹. Although immediate primary percutaneous coronary intervention (PPCI) can rapidly restore blood flow in occluded coronary arteries, many patients in remote areas cannot access PPCI quickly. It is well known that reperfusion itself can initiate a cascade of events that accelerate and extend post-ischemic injury, which accounts for up to 50% of post-infarction sequelae ². During continuous ischemia and reperfusion, cell death pathways including necrosis, apoptosis, and autophagy are activated and extensive cardiomyocyte cell death leads to their loss, myocardium fibrosis, and heart failure ³. Early regulation of cell death pathways that reduce cardiomyocyte loss and improve MI prognosis might be useful therapeutic targets after myocardial ischemia reperfusion (IR) injury.

Mitochondria-mediated cardiomyocyte death (mainly including apoptosis and necrosis) is at the intersection of mechanical and molecular mechanisms underlying post-infarction remodeling and therefore might be an attractive therapeutic target to break the cycle leading to heart failure ⁴. Bcl2 family members including Bax, Bak, Nix, and Bnip3 have been implicated in programmed cell death by permeabilizing the outer mitochondrial membrane and subsequently initiating the caspase cascade ^{5, 6}, whereas the pharmacological inhibition of caspase or Bcl2 family proteins can be limited due to nonspecific systemic side effects ^{7, 8}, making it advantageous to identify and target specific upstream mediators of ischemia-induced cardiomyocyte death.

Immediate early genes (IEGs) can be upregulated rapidly after stress, hypoxia, and others stimulus ⁹. They regulate cell growth, differentiation and cell death signals at early stage. The orphan nuclear receptor Nr4a subfamily was reported to comprise IGEs that are involved in various processes including inflammation regulation, neurological diseases, cell death regulation, metabolic diseases, and carcinogenesis ⁹. The Nr4a subfamily comprises three members, Nr4a1 (Nur77), Nr4a2 (Nurr1), and

Nr4a3 (Nor1)^{9,10}. These proteins have organ- and tissue-specific functions due to their differential expression in various organs. Although no ligands have been identified as Nr4a receptors, they play important roles through protein–protein interactions^{10,11,12}. However, the contribution of Nr4a proteins to cardiomyocyte death during myocardial ischemic injury and remodeling remains poorly defined.

Here, we demonstrated that *Nr4a3*, but not *Nr4a1* and *Nr4a2*, is significantly increased in the ischemic myocardium and hypoxic cardiomyocytes. Cardiac specific *Nr4a3* deficiency protects against ischemia- and hypoxia-triggered cardiomyocyte necrotic and apoptotic death, resulting in alleviated myocardial remodeling and heart failure. Meanwhile, forced overexpression of *Nr4a3* in both neonatal and adult mouse hearts was sufficient to cause cardiomyocyte death in normal hearts and produce LV (left ventricular) dilation and contractile dysfunction without added ischemic stress. Mechanistically, ischemia or hypoxia induced Nr4a3 phosphorylation and translocation from the nucleus to the mitochondria where it triggered cell death by interacting with Bnip3 and increasing permeability transition pore (mPTP) opening and decreasing mitochondrial transmembrane potential ($\Delta\Psi_m$). Collectively, these findings identified Nr4a3 as an important mediator of ischemia-induced cardiomyocyte death and demonstrated a novel therapeutic target to reduce cardiomyocytes loss, myocardial injury, and heart failure.

Results

Nr4a3 expression is selectively induced in cardiomyocytes following myocardial IR injury

To elucidate the involvement of the Nr4a subfamily in myocardial ischemic injury, we first investigated *Nr4a* expression levels in the myocardium at different time points after myocardial ischemia reperfusion (IR) injury. Among Nr4a subfamily members, mRNA expression levels of *Nr4a3*, but not *Nr4a1* and *Nr4a2*, were significantly increased in the ischemic myocardium (Fig. 1A) and hypoxic cardiomyocytes (Fig. 1B), although overall *Nr4a1* mRNA expression was much higher than *Nr4a3*. Next, we examined the kinetics of *Nr4a3* expression after IR or hypoxia, *Nr4a3* mRNA and protein expression levels were upregulated in the early phase after IR or hypoxia, and maintained at relatively higher levels than those at baseline (Fig. 1, C to F). To identify the cellular expression of *Nr4a3*, we separated fibroblasts, endothelial cells, CD45⁺ leukocytes and cardiomyocytes from the ischemic hearts on day 1 post-IR. *Nr4a3* was selectively induced in cardiomyocytes, but not in other cell types (Fig. 1G).

Cardiac specific Nr4a3 deficiency leads to improved myocardial necrosis and cardiac function after myocardial IR injury

Next, we generated cardiac specific Nr4a3 knockout mice (abbreviated as Nf/f Cre⁺) by crossing *Nr4a3*^{flox/flox} (abbreviated as Nf/f) mice with transgenic mice expressing a tamoxifen-inducible Cre recombinase protein fused to a mutant estrogen-receptor ligand binding domain driven by α -myosin heavy chain promoter (Myh6-CreERT2, abbreviated as Myh6-Cre) (fig. S1, A and B). Initially, we confirmed

the decrease of *Nr4a3* expression in Nf/f Cre⁺ mice after tamoxifen administration (fig. S1C). There were no obvious differences in heart, liver, spleen, lung, and kidney tissue histologies between Nf/f Cre⁺ and Nf/f Cre⁻ mice under unstressed conditions (fig. S1D). Thus, cardiac specific Nr4a3 knockout mice are indistinguishable from control mice under basal physiological conditions.

Nf/f Cre⁺ and Nf/f Cre⁻ mice with tamoxifen administration were subjected to 2 hours of ischemia and 24 hours of reperfusion. With similar size of area at risk (AAR), the infarct size/AAR ratio was shown to be significantly lower in Nf/f Cre⁺ mice, compared with those of control mice ($25.05 \pm 1.62\%$ vs. $51.13 \pm 2.36\%$) (Fig. 2A). Notably, Nf/f Cre⁺ hearts were resistant to IR-induced myocardial necrosis, as evidenced by reduction in Evans blue dye (EBD) penetration (Fig. 2B), suggesting that the protective effect of Nr4a3 ablation is attributable to its inhibitory effect on IR-induced myocardial necrosis.

To determine the effects of Nr4a3 on cardiac function after IR and to demonstrate the clinical relevance, we measured the left ventricular (LV) end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), and ejection fraction (EF) by echocardiography 1 d after IR. Echocardiographic parameters in Nf/f Cre⁺ mice were comparable to those in control mice after the sham operation. While Nr4a3 knockout mice had significantly decreased LVEDV and LVESV, as well as increased EF ($33.98 \pm 2.83\%$ vs. $49.72 \pm 3.91\%$, $P = 0.003$), which was accompanied by other improvements in LV diameters (LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and fractional shortening (FS)), compared to those measured in control mice after IR (Fig. 2, C and D).

Besides, in the chronic IR experimental setting (2h ischemia followed by 4 weeks of reperfusion), Masson trichrome staining of the hearts showed that infarct size was significantly smaller in Nf/f Cre⁺ mice than in control mice ($28.97 \pm 1.51\%$ vs. $42.18 \pm 1.88\%$, respectively), and Nf/f Cre⁺ mice had a decreased ratio of HW/BW (heart weight/body weight) and increased wall thickness at the infarct area compared to those in control mice (Fig. 2, E and F).

Next, we performed TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining of LV sections obtained from Nf/f Cre⁺ and Nf/f Cre⁻ mice at 24 hours after myocardial IR. As shown in Fig. 2G, the number of TUNEL-positive cardiomyocytes was significantly decreased in the Nf/f Cre⁺ mice after IR, compared with the control mice. In line with these findings, the protein levels of the proapoptosis molecules cleaved caspase-3 was concomitantly downregulated in the LV of Nf/f Cre⁺ mice after the IR injury (Fig. 2H).

These above results indicated that Nr4a3 deficiency in cardiomyocytes effectively blocked IR-induced cardiac dysfunction, remodeling and heart failure.

Forced cardiac Nr4a3 overexpression induces ventricular remodeling and heart failure

To further confirm the functional role of Nr4a3 in the heart, we generated α -myosin heavy chain-driven (α -MHC-driven) Nr4a3-transgenic mice (abbreviated as Tg-Nr4a3) (fig. S2). Neonatal mice overexpressing

Nr4a3 were viable; however, many individuals died before reaching adulthood. The survival rate was significantly lower for *Nr4a3*-transgenic mice than for WT mice at 12 w of age (23.08% vs. 100%, respectively; log-rank test $P = 0.0005$; Fig. 3A). Mice overexpressing *Nr4a3* developed progressive LV dilation and corresponding diminished systolic performance, which was assessed by echocardiography at 12 w of age (Fig. 3, B and C). Cardiac enlargement was confirmed by gravimetric measurements of HW/BW, which was 6.90 ± 0.17 mg/g in *Nr4a3*-overexpressing mice vs. 4.83 ± 0.03 mg/g in control mice ($P = 0.017$; Fig. 3D). Necropsy and Masson trichrome staining of the hearts showed that *Nr4a3*-transgenic mice had dilated hearts, massive cardiomyocyte loss, and large-scale fibrosis (Fig. 3, E and F). Consistently, the number of apoptotic cells in *Nr4a3*-transgenic mice was significantly higher than that in WT mice when measured at 12 w of age (Fig. 3, G and H).

To obtain *Nr4a3*-overexpressing mice with stable reproductive capacity and temporally-controlled overexpression of cardiac specific *Nr4a3* in adult hearts, we created conditional *Nr4a3*-knock-in mice (abbreviated *Nr4a3* KI or NKI) and then crossed this line with *Myh6*-CreERT2 (abbreviated as *Myh6*-Cre) (fig. S3, A and B). There were no obvious differences in the histologies of the heart, liver, spleen, lung, and kidney tissues between *Nr4a3* KI-*Myh6*-CreERT2 (NKI Cre⁺) and NKI Cre⁻ mice before tamoxifen treatment (fig. S3C). Both NKI Cre⁺ and NKI Cre⁻ mice were administered tamoxifen for 5 d at 10 w of age. We first confirmed that NKI Cre⁺ mice had much higher expression of *Nr4a3* than NKI Cre⁻ animals after tamoxifen treatment (fig. S3D). As shown in Fig. 4A, most NKI Cre⁺ mice died within 1 month after tamoxifen treatment, and the survival rate of NKI Cre⁺ mice was significantly lower than that of control (NKI Cre⁻) mice (30.77% versus 100%, respectively, log-rank test $P = 0.0107$). Similar to that in *Nr4a3*-transgenic mice, echocardiographic analysis demonstrated that NKI Cre⁺ mice had LV dilation and contractile dysfunction compared to those in NKI Cre⁻ mice after tamoxifen administration (Fig. 4, B and C). Moreover, histological analysis of heart sections revealed that myocardial fibrosis was significantly increased in NKI Cre⁺ mice compared to that in the control group (Fig. 4, D and E). Besides, NKI Cre⁺ but not NKI Cre⁻ mice showed a large number of apoptotic cells (Fig. 4, F and G) and a high abundance of swelling and dysmorphic mitochondria in the heart after tamoxifen administration (Fig. 4, H and I). Thus, these results indicate that forced overexpression of *Nr4a3* in both neonatal and adult mouse hearts is sufficient to cause LV dilation and contractile dysfunction without additional ischemic stress.

***Nr4a3* is critically involved in cardiomyocyte necrotic and apoptotic cell death**

Experimental evidence suggests that both ischemia- and hypoxia-triggered cardiac injury induces myocardial cell death through necroptosis and apoptosis, leading to maladaptive remodeling and heart failure^{13,14,15}. To further examine the essential role *Nr4a3* in hypoxia induced cell death, PI (propidium iodide) and TUNEL staining were performed to detect cardiomyocyte necrotic and apoptotic cell death in vitro. We showed that *Nr4a3* knockdown significantly reduced the rate of necrotic and apoptotic cell death after hypoxia reoxygenation (HR) injury (Fig. 5, A to D and fig. S4A). We also observed concomitant downregulation of *cleaved-caspase-3* expression in hypoxic cardiomyocytes after siRNA knockdown of *Nr4a3* (Fig. 5E). Inversely, overexpression of *Nr4a3* by adenovirus vector (Ad-*Nr4a3*) in cultured

cardiomyocytes led to robust necrotic and apoptotic cell death in both normoxic and hypoxic conditions (Fig. 5, F to I and Fig. S4B). Thus, these results indicate that Nr4a3 is required for hypoxia-induced cell death in cardiomyocytes.

Nuclear Nr4a3 translocates to mitochondria where it interacts with and promotes Bnip3 integration into mitochondrial membranes in response to hypoxia

Next, we tried to elucidate the mechanisms linking nuclear receptor-Nr4a3 and cardiomyocyte death. Increasing evidence suggests that some nuclear receptors translocate towards mitochondria to potentially influence various mitochondrial functions in a noncanonical manner¹⁶. This finding prompted us to examine the subcellular localization of Nr4a3 in response to hypoxia. First, immunofluorescence staining was performed to detect the subcellular localization of Nr4a3 in neonatal myocytes and heart sections, and this unexpectedly revealed that Nr4a3 is exclusively located in the nucleus in the unstressed conditions, while it co-localizes with Hsp60, a mitochondrial marker after HR or IR injury (Fig. 6, A and B). To further confirm our findings, we separated the proteins from the nuclei and mitochondria of cardiomyocytes or hearts, and examined Nr4a3 quantity after hypoxia or IR injury. The results revealed that Nr4a3 translocates from the nucleus to mitochondria in response to hypoxia (Fig. 6, C and D). Collectively, these data suggest that mitochondrial targeting of Nr4a3 might mediate cardiomyocyte death independent of its nuclear transcriptional function.

It was reported that the B-cell lymphoma (Bcl)-2 family of proteins is mainly localized to the mitochondrial membrane and is at the center of mitochondrial cell death regulation^{3, 5}. We therefore examined whether the pro-apoptotic and necrotic function of Nr4a3 was achieved through an interaction with Bcl2 family proteins. We found that Nr4a3 could interact with Bcl2, Nix, and Bnip3 (fig. S5). Interestingly, hypoxia dramatically increased the binding capacity of Nr4a3 to Bcl2 and Bnip3, but not Nix. Bcl2 is a cell survival protein best known for its roles in inhibiting apoptosis via interactions with the proapoptotic Bax and Bak⁵. Bnip3 (Bcl2/adenovirus E1B 19KD interacting protein 3) is another proapoptotic Bcl2 family member that is upregulated in the ischemic heart⁶. Bnip3-knockout mice exhibit decreased hypoxia-induced cardiomyocyte death, preserved LV systolic performance, and diminished LV dilation, whereas the cardiac specific overexpression of *Bnip3* increases cardiomyocyte apoptosis in unstressed mice, causing progressive LV dilation and diminished systolic function⁶. These phenotypes in Bnip3 mutant mice were very similar to those in Nr4a3-mutant mice, which allowed us to focus on Bnip3 as an Nr4a3 downstream effector molecule.

First, we demonstrated that Nr4a3 binds Bnip3 and that both proteins are significantly increased and colocalize in the mitochondria under hypoxic stress (Fig. 6, E to G). Bnip3 is associated with the mitochondria in normoxic cells and integrated into mitochondrial membranes in hypoxic cells, which is critically required for the Bnip3-mediated cell death pathway^{17, 18}. Bnip3 can be eluted from the mitochondria by alkali or DTT treatment under associated but not integrated conditions. Therefore, to examine if Nr4a3 is required for Bnip3 integration, we separated the proteins from the cytoplasm and mitochondria of neonatal cardiomyocytes to examine levels of Bnip3 (Fig. 6H). We observed that hypoxia

induced Bnip3 expression and targeting to mitochondria, whereas Nr4a3 knockdown did not affect the expression levels of Bnip3 and mitochondrial localization under hypoxic conditions. However, Bnip3 mitochondrial integration was significantly decreased in Nr4a3-knockdown cells after alkali treatment under hypoxic stress conditions (Fig. 6I). Thus, Nr4a3 is required for Bnip3 integration into the mitochondrial membrane in response to hypoxic stress.

Mitochondrial targeting of Nr4a3 triggers mitochondrial permeability transition pore opening and decreased mitochondrial membrane potential in response to hypoxia

Bnip3 induces cardiomyocyte death by triggering mPTP opening, causing a collapse in $\Delta\Psi_m$, mitochondria dysfunction, and cell death pathway activation^{6, 18, 19, 20, 21}. Therefore, we pinpointed mPTP as an essential downstream event in Nr4a3-activated cell death signaling. Calcein-AM and tetramethylrhodamine methyl ester (TMRM) were used to detect mPTP and $\Delta\Psi_m$, respectively²². Calcein-AM is a membrane-permeable fluorophore that diffuses freely into the mitochondria and can be quenched by cobalt chloride when mPTPs open. TMRM is a cationic fluorescent dye that accumulates within the mitochondrial matrix depending on membrane potential, whereas Mitotracker fluorescence is mitochondria-specific and potentially independent. As shown in Fig. 6J–M, there was no statistically significant difference in Calcein and TMRM fluorescence between control and Nr4a3-knockdown cells under normoxic conditions. However, the reduction in Calcein and TMRM fluorescence in Nr4a3-knockdown cells was significantly less than that in control cells under hypoxic conditions, suggesting that $\Delta\Psi_m$ was preserved in Nr4a3-knockdown cells due to a decrease in mPTP opening during hypoxia. In addition, we demonstrated that Bnip3 knockdown significantly reduced the rate of necrotic and apoptotic cell death induced by Nr4a3 overexpression in both normoxic and hypoxic conditions (Fig. 5, F to I and fig. S4C). Collectively, these results indicate that ischemia or hypoxia triggers Nr4a3 translocation from the nucleus to mitochondria, where it interacts with and promotes Bnip3 integration into mitochondrial membrane, leading to mPTP opening, loss of $\Delta\Psi_m$ and cardiomyocyte death.

LBD and AF1 domains of Nr4a3 are essential for its binding to Bnip3

Next, to further elucidate which domain of Nr4a3 is essential for Bnip3 binding, we constructed full-length and three truncated *Nr4a3* adenovirus (Δ AF1, Δ DBD, and Δ LBD) with flag tags at their C-terminal ends. These constructs were transfected into neonatal cardiomyocytes and could be recognized by anti-flag and anti-Nr4a3 antibodies that targeted amino acids 2–95 (Fig. 7, A and B). We showed that deletion of AF1 or LBD domain greatly reduced the mitochondrial translocation of Nr4a3 (Fig. 7C). And immunoprecipitation revealed that Δ AF1 and Δ LBD Nr4a3 had significantly lower binding capacity to Bnip3 under hypoxic conditions than full-length and Δ DBD Nr4a3 (Fig. 7D). Moreover, mitochondrial integration of Bnip3 was significantly suppressed after deletion of AF1 or LBD, but not DBD domain in response to hypoxia (Fig. 7E). Lastly, we demonstrated that mPTP opening and loss of $\Delta\Psi_m$ were greatly improved in Δ AF1 and Δ LBD Nr4a3 expressing cardiomyocytes in both normoxic and hypoxic conditions (Fig. 7, F to I). Thus, these results indicate that the LBD and AF1 domains of Nr4a3 are required for Bnip3 binding, and that Bnip3 assists in the mitochondrial targeting of Nr4a3.

Phosphorylation of Nr4a3 is essential for its targeting to mitochondria

It was reported that phosphorylation of a conserved sequence (RGRLP(phospho-S)KPKSP) in Nr4as by RSK (ribosomal S6 kinase) is crucial for Nr4as nuclear export and cell death^{23,24}. We first showed that the motif is highly conserved among different species and Nr4as (Fig. 8A). As no p-Nr4a3 (Ser376) antibody was available, a phospho-specific antibody that recognized the peptide CGRLP(phospho-S)KPKQP was used to detect the phosphorylation of Nr4a3 after immunoprecipitation^{23,24}. We first demonstrated that phosphorylation of Nr4a3 was significantly increased in response to hypoxia (Fig. 8B). To further examine the functional significance of phosphorylated Nr4a3 in cardiomyocytes, constitutively active (Adenovirus-Nr4a3(S376D), abbreviated as Ad-Nr4a3(S376D)) and dominant negative (Adenovirus-Nr4a3(S376A), abbreviated as Ad-Nr4a3(S376A)) mutants of Nr4a3 were generated. Ectopic expression of the activated Nr4a3 mutant (a phosphorylation simulation) in cardiomyocytes facilitated its translocation to mitochondria (Fig. 8C), where it binds Bnip3 (Fig. 8D) and promotes its integration into the mitochondrial membrane (Fig. 8E), resulting in mPTP opening (Fig. 8F), loss of $\Delta\Psi_m$ (Fig. 8G) and necrotic/apoptotic cell death (Fig. 8, H and I) in both normoxic and hypoxic conditions. Inversely, expression of the dominant negative Nr4a3 mutant (an unphosphorylation simulation) caused its retention in nucleus, a lower binding capacity to Bnip3, decreased mPTP opening and preserved $\Delta\Psi_m$, leading to attenuated cell death. These results indicate that phosphorylation of Nr4a3 is required for its nuclear export and involved in mitochondria-mediated cell death.

Discussion

Our findings showed that Nr4a3, but not Nr4a1 or Nr4a2, is increased significantly during the early stages of ischemia and hypoxia and plays a crucial role in the regulation of necrotic and apoptotic cell death with or without ischemia. We also show that hypoxia triggers Nr4a3 phosphorylation and translocation to mitochondria, where it binds Bnip3 and promotes its integration into the mitochondrial membrane, leading to mPTP opening, cardiomyocyte loss, and adverse myocardial remodeling (Fig. 8J). Therefore, manipulation of the Nr4a3–Bnip3 pathway might assist in the treatment of ischemic heart disease.

Nr4a family members are immediate early response genes that sense and respond to changes in the cellular environment¹⁰. Many stimuli such as endoplasmic reticulum (ER) stress, platelet growth factor (PGF), epidermal growth factor (EGF), and dietary long-chain free fatty acids, have been shown to induce Nr4a mRNA transcription in many cells, exerting its diverse effects including influencing cell proliferation and apoptosis¹⁰. Another recent study reported that hypoxia upregulates Nr4a3 expression and neuronal cell apoptosis through activation of a CREB transcriptional factor²⁵. Our study did not examine the upstream regulator of Nr4a3 expression in the context of ischemia or hypoxia; however, it is proposed that a variety of cytokines and ER stress after IR might be responsible for downstream Nr4a3 expression through different signaling pathways. Thus, further studies are needed to elucidate the exact molecular mechanisms underlying Nr4a3 regulation.

It has been reported that the double abrogation of Nr4a1 and Nr4a3 in mice leads to severely lethal acute myeloid leukemia, which does not occur in Nr4a1- or Nr4a3-single-knockout mice, suggesting that these

two proteins do not simply overlap in function ²⁶. An additional report suggests that *Nr4a3* mRNA expression in the heart and skeletal muscles is higher than that of *Nr4a1* ²⁷, however, our findings showed that even though *Nr4a3* mRNA expression was lower than that of *Nr4a1*, only *Nr4a3* expression increased significantly in the early stages of IR and hypoxia, indicating that *Nr4a3* might be critically involved in ischemia- and hypoxia-induced myocardial injury. Although a previous study demonstrated that *Nr4a1* mitochondrial translocation in cardiomyocytes induces cytochrome c release and cardiomyocyte apoptosis under oxidative stress and hypoxia ¹¹, detailed mechanisms and key regulatory proteins in this pathway are largely unknown, especially with respect to the *in vivo* functional role of *Nr4a1* in the context of ischemia. However, the present study provided strong evidence that the orphan nuclear receptor *Nr4a3* interacts with *Bnip3* resulting in translocation to the mitochondria in the pathologically-stressed myocardium. The use of multiple animal models (*Nr4a3*-conditional knockout, *Nr4a3*-conditional overexpression, and *Nr4a3*-transgenic mice) added molecular precision.

In contrast to our findings, a recent study demonstrated that lentiviral overexpression of *Nr4a3*, 7 days before MI, suppressed post-MI inflammation responses by inhibiting the translocation of p65 to the nucleus in a Stat3-dependent manner ²⁸. The opposite results between these two studies can be explained by the following reasons. First, these two studies used different systems (lentivirus vs. genetic mutant mice). Second, lentiviral overexpression of *Nr4a3* in the healthy heart prior to MI might act as a preconditioning stimulus, which attenuates the following ischemic insults. Finally, the period and quantity of *Nr4a3* expression during ischemia might determine the functional outcome after MI, indicating that fine tuning of *Nr4a3* is crucial for its proper function *in vivo*.

Mitochondrial dysfunction acts as an irreversible step in both necrotic and apoptotic cell death ⁴. Ruptured mitochondria release proapoptotic proteins such as cytochrome c into cytoplasm. Mitochondrial dysfunction leads to Ca^{2+} overload and ROS overproduction, resulting in cell death ^{3,4}. Our *in vivo* and *in vitro* data demonstrate that *Nr4a3* localizes to the nucleus of cardiomyocytes under normal conditions, and translocates to the mitochondria under ischemia or hypoxia conditions. During hypoxia, the mitochondrial $\Delta\Psi\text{m}$ was preserved in *Nr4a3*-knockdown cells due to diminished mPTP opening. The mPTP is a channel in the inner mitochondrial membrane, and its opening leads to decreased mitochondrial $\Delta\Psi\text{m}$ and increased mitochondrial membrane permeability, which causes mitochondrial swelling and rupture ⁴. Previous studies showed that *Nr4a*s proteins cannot directly localize to the mitochondria due to a lack of classical mitochondrial localization signals ²⁹. However, *Nr4a1* can target the mitochondria through its interaction with Bcl-2 family proteins such as Bcl2 and Nix, owing to the presence of a transmembrane sequence in Bcl-2 ^{12,29}. *Bnip3* is another proapoptotic Bcl2 family member and is upregulated in the ischemic heart. *Bnip3* has been reported to mediate mitochondrial dysfunction and cell death via opening of the mPTP ^{30,31}, whereas *Bnip3*-deficiency or conditionally-overexpressed *Bnip3* in the heart lead to similar phenotypes as those observed in *Nr4a3*-gene-modified mice ⁶. Further analysis revealed that *Nr4a3* interacts with *Bnip3* and promotes *Bnip3* integration into mitochondrial membranes after hypoxia but does not affect its expression or mitochondrial localization. In addition, we found that full-length and three truncated *Nr4a3* constructs had differential affinity for

Bnip3, although no mutant completely eliminated the binding of the two proteins. Collectively, these findings support targeting the Nr4a3–Bnip3 mitochondrial pathway to alleviate cardiomyocyte death. Further studies are needed to examine whether interference with Nr4a3 and Bnip3 binding patterns can reduce cell death and rescue myocardial ischemic injury.

Phosphorylation is an important and common way to regulate the function of proteins. The Ras–MAPK pathway is crucial for regulating proliferation, survival, and differentiation^{32, 33, 34}. The 90-kDa ribosomal S6 kinases (RSKs) are downstream members of the Ras–MAPK (MEK–ERK) cascade, which phosphorylates a conserved Arg-X-Arg-X-X-pSer/Thr (R-X-R-X-X-pS/T) motif of its substrates. RSK also phosphorylates many IEG products including FOS, Nr4a1, and Nr4a3. Phosphorylation of Nr4a1 by the MEK-ERK-RSK cascade is necessary for Nr4a1 mitochondrial translocation and apoptosis^{23, 32}. In line with these findings, we demonstrated that phosphorylation of Nr4a3 is required for its targeting to mitochondria and contributes to mitochondria-mediated cell death. Meanwhile, nuclear receptor signaling is integral to dynamic changes in the cardiac mitochondrial phenotype via the transcriptional regulation of gene expression in the nucleus in response to diverse pathophysiological conditions^{16, 35}. However, in the present study, we revealed that ischemia or hypoxia promotes Nr4a3 phosphorylation and nuclear export, which indirectly excludes the possibility of Nr4a3 nuclear transcriptional function. In summary, our findings revealed the cell death-promoting role of Nr4a3 during IR and suggest new compounds and therapeutic targets for ischemic heart disease.

Methods

Mice

Nr4a3 conditional knockout mice in a C57BL/6 background were generated by CRISPR/Cas-mediated genome engineering (fig. S1). The Nr4a3 gene (NM_015743.3) is located on mouse chromosome 4. Nr4a3 has identified 8 exons, among which exon 3 is the ATG start codon and exon 8 is the TGA stop codon. Exon 4 and flanking sequences on both sides of it were replaced by the "exon 4-8-2A-LacZ-polyA-loxP intron 3-CDS loxP endogenous SA" box. To engineer the targeting vector, BAC clones were used as templates to generate homology arms and cKO regions by PCR. Then Cas9, gRNA and targeting vector were co-injected into fertilized eggs to produce F0 Nr4a3^{fllox/+} (abbreviated as Nf/+) mice. Nf/+ mice were crossed to obtain Nf/f mice. After mating Nf/f mice with transgenic mice expressing a tamoxifen-inducible Cre recombinase protein fused to a mutant estrogen-receptor ligand binding domain driven by α -myosin heavy chain promoter (Myh6-CreERT2, abbreviated as Myh6-Cre), Nf/+^{Myh6-Cre+} and Nf/+^{Myh6-Cre-} mice were obtained. Thereafter, Nf/+^{Myh6-Cre+} mice were crossed to Nf/f mice to obtain Nf/f^{Myh6-Cre+} (we brief called Nf/f Cre⁺) mice. Nr4a3 was knockout specifically in cardiomyocytes of Nf/f Cre⁺ mice by tamoxifen treatment. Nf/f Cre⁻ littermates were used as control mice.

We generated α -MHC-Nr4a3 transgenic mice by using the PiggyBAC transposase system (fig. S2). A targeting vector comprises right arm, α -MHC promoter, Nr4a3 cDNA, P2A, BGH polyA and left arm sequences. The target fragment was cloned into the PiggyBAC transposon plasmid and injected into a

fertilized mouse egg together with the transposase. Under the action of the transposase, the target fragment was integrated into the TTAA site in the genome to obtain the transgenic mouse.

To generate conditional *Nr4a3* overexpressing mice, we first created *Nr4a3* flox/flox mice using CRISPR/Cas9 technology to insert the CAG promoter-loxp-stop-loxp-*Nr4a3*-flag-WPRE-polyA expression cassette at the *Rosa26* locus by homologous recombination (fig. S3). A targeting vector was constructed using In-Fusion cloning, which contains a 3.3 kb 5' homology arm, a CAG promoter, a loxp-stop-loxp, a *Nr4a3*-flag-WPRE-polyA and a 3.3 kb 3' homology arm. The Cas9 mRNA, gRNA and donor vector were microinjected into fertilized eggs of C57BL/6J mice to obtain F₀ mice. The loxp-stop-loxp expression cassette prevents transcription of the downstream target gene, *Nr4a3*. After mating this line with transgenic mice expressing a tamoxifen-inducible Cre recombinase protein fused to a mutant estrogen-receptor ligand binding domain driven by α -myosin heavy chain promoter (Myh6-CreERT2, abbreviated as Myh6-Cre), high expression of *Nr4a3* was achieved specifically in cardiomyocytes by tamoxifen treatment. All three mutant mice were generated by Shanghai Biomodel Organism Science & Technology Development Co. Ltd. in Shanghai, China.

This study and all animal procedures conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and were approved by the Animal Care Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine.

In vivo myocardial ischemia reperfusion (IR) protocol

Surgical induction of myocardial IR was performed as previously described³⁶. Briefly, mice were lightly anesthetized with diethyl ether, intubated, and fully anesthetized with 1.0–1.5% isoflurane gas, and the mechanical ventilation was performed with a rodent respirator. Left thoracotomy was performed, and the left anterior descending (LAD) coronary artery was visualized using a microscope and ligated at the site of its emergence from the left atrium, using 8-0 silk suture around fine PE-10 tubing with a slipknot. Complete occlusion of the vessel was confirmed by the presence of myocardial blanching in the perfusion bed. Mice underwent 2-hour long LAD ischemia followed by different periods of reperfusion. Sham-operated animals were subjected to the same surgical procedures, except that the suture was passed under the LAD artery, but not tied.

Infarct size and infarct wall thickness determination

Heart tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μ m-thick sections. Hematoxylin and eosin (H&E) and Masson's trichrome staining of the paraffin-embedded sections were performed to determine the morphological effects and infarct size, with the latter calculated as total infarct circumference divided by total LV circumference $\times$ 100, as described previously^{13, 36}. The wall thickness of the scars was measured as well. Myocardial fibrosis was quantified by morphometric analysis with Masson's trichrome stained sections; mean fibrotic area was calculated from 10 - 12 areas per heart section, which were analyzed at 200X magnification.

Infarct size assessment

To assess infarct size by TTC (2,3,5-triphenyltetrazolium chloride) staining, the LAD artery was re-occluded at the previous ligation, and 1 mL of 1% Evans blue (Sigma-Aldrich, St. Louis, MO, USA) was injected into the LV cavity. The heart was quickly excised, washed twice, immediately frozen, and sliced at a 1-mm thickness. These sections were incubated in 1% TTC (Sigma-Aldrich) solution and digitally photographed. LV area and infarct area were determined by computerized planimetry and comprehensively analyzed in serial sections for each mouse. These data were analyzed using Image J software (version 1.38x, National Institutes of Health).

Echocardiographic analysis of cardiac function

Transthoracic echocardiography was performed with a Vevo 2100 instrument (VisualSonics, Toronto, Ontario, Canada) equipped with an MS-400 imaging transducer. Mice were slightly anesthetized with isoflurane in a box and then were gently fixed on the echo pad in a supine position without additional anesthesia. Mice were kept awake during the echocardiographic examination to minimize data deviation and heart rate was maintained at approximately 550–650 bpm in all mice. B-mode tracings of the LV endocardial border in a parasternal long axis were conducted to directly evaluate LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV). The LV ejection fraction (EF) was calculated following the formula: $EF = [(LVEDV - LVESV) / LVEDV] \times 100$. M-mode tracings were recorded through the anterior and posterior LV walls of the papillary muscle to measure LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVESD). LV fractional shortening (FS) was calculated according to the following formula: $FS = [(LVEDD - LVESD) / LVEDD] \times 100$. Echocardiographic acquisition and analysis were performed by a technician who was blinded to the treatment groups.

Isolation neonatal and adult cardiomyocytes and non-myocyte fraction cells

Neonatal cardiomyocytes were isolated from 1-day old neonatal rats by enzymatic digestion³⁷. Briefly, ventricles were extracted, cut into pieces and then dissociated digested in calcium and magnesium-free Hanks buffer containing Collagenase II (1mg/ml, 37 °C water bath) under gentle agitation. Digestion was performed in 10-min steps, collecting the supernatant to equal amount of DMEM with 10% FBS after each step. The collected supernatant was passed through a cell strainer (70 µm, BD Falcon) and then centrifuged to separate the cells, which were then resuspended in DMEM with 5% FBS and with penicillin and streptomycin. The collected cells seeded onto uncoated 100-mm plastic dishes for 2 h at 37 °C in 5% CO₂ and humidified atmosphere. The supernatant composed mostly of cardiomyocytes, was then collected, pelleted and resuspended in DMEM with 10% FBS, counted and plated at the appropriate density.

Adult cardiac myocytes and non-myocyte fraction cells from mouse heart were isolated according to a Langendorff-free procedure³⁸. Non-myocyte fraction cells were incubated on ice for 30 min with the appropriate combination of fluorochrome-conjugated antibodies diluted in FACS buffer: CD45-BV421 (1:300, Biolegend, Cat:103134), CD31-PE (1:300, Biolegend Cat:102419), gp38-APC (1:100, Biolegend

Cat:127410), Zombie Aqua™ Fixable Viability Kit (1:100, Biolegend Cat:423101) was used to distinguish live/dead cells. Cells were sorted with the FACS Aria III 4L (BD Biosciences): CD45⁺ population were leukocyte, the endothelial cells were CD45⁻CD31⁺ and fibroblasts were defined as a CD45⁻CD31⁻gp38⁺ population.

Quantitative real-time PCR

Total RNA samples from cultured cells and tissues were prepared using an RNeasy Mini Kit (Qiagen) or Trizol reagent (Invitrogen) according to the manufacturer's instructions. A first-strand cDNA synthesis kit (Invitrogen) was used for cDNA synthesis. Quantitative real-time PCR was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). Predesigned gene-specific primer and probe sets were used for q RT-PCR amplification and detection (TaqMan Gene Expression Assays, Applied Biosystems) and 18S ribosomal RNA levels were used as an internal control. Each reaction was performed in duplicate and the changes in relative gene expression normalized to the internal control levels were determined using the relative threshold cycle method.

Western blotting

Heart tissue samples or cultured cells were homogenized in lysis buffer containing proteinase and a phosphatase inhibitor cocktail. The protein concentration was determined with a BCA protein assay kit (Bio-Rad). Nr4a3 (H7833, R&D), Caspase-3 (#9662, CST), Cleaved-caspase3 (#9661, CST), Bnip3 (#3769, CST) were detected with specific antibodies. Histone-H3 (4499, CST) was used as a nuclear protein control, VDAC (#4866, CST) was used as a mitochondrial protein control, GAPDH (60004-1-1g, Proteintech) and α -tubulin (T9026, Sigma) levels were used as total protein internal controls.

Assessment of necrotic and viable cardiomyocytes

Necrotic cells were labeled by Evans Blue Dye (EBD) due to increased membrane permeability, as described previously¹⁵. Evans Blue was dissolved in PBS (10 mg/ml) and intraperitoneally injected into mice (100 μ g/g body weight). 12 hours later, the mice were subjected to 2-hour long LAD ischemia followed by 24 hours reperfusion. Then, mice were sacrificed, and heart was harvested and embedded in OCT (Sakura), snap frozen in liquid nitrogen and cut into 8- μ m cryosections. Immunohistochemistry was performed with anti-CaV3 antibody (Abcam, ab2912). The necrotic cells were labeled by Evans blue dye and viable cardiomyocytes were labeled by caveolin 3 (CaV3) antibody. The Images were acquired with Zeiss Axio Scan.Z1.

Immunohistochemistry

Immunofluorescence analysis was performed on the paraffin-embedded sections of heart tissue or cultured cells fixed with 4% paraformaldehyde as described previously³⁶. The sections were incubated with primary mouse monoclonal anti-Nr4a3 (#H7833, R&D), goat polyclonal anti-Hsp60 (AP22882PU-N, OriGene), rabbit monoclonal anti-Bnip3 (ab109362, Abcam), and mouse monoclonal anti- α -actinin

(A7811, Sigma-Aldrich) antibodies overnight at 4°C. The sections were then incubated with the appropriate Alexa-Fluor-coupled secondary antibodies for 1 h at room temperature and counterstained with DAPI. Images were acquired with ZEISS LSM710 confocal and Olympus BX61 microscopes, and analyzed using ImageJ software (version 1.38×, National Institutes of Health).

Calcein-am/PI and TUNEL staining

Calcein-am/PI staining was performed with Live/Dead Cell Double Staining Kit (04511-1KT-F, Merck) following the manufacturer's instructions. Calcein-AM only stains viable cells. PI reaches the nucleus by passing through disordered areas of dead cell membrane, and intercalates with the DNA double helix of the cell to emit red fluorescence. TUNEL staining was performed with In Situ Cell Death Detection Kit-Fluorescein (Cat. 11684795910, Roche) following the manufacturer's instructions. Sections or cell slides were counterstained with DAPI. Images were acquired with an ZEISS LSM710 confocal microscope and analyzed using ImageJ (version 1.38×, National Institutes of Health).

Mitochondrial transmembrane potential and mPTP opening detection

Neonatal rat ventricular myocytes (NRVM) were cultured on glass-bottomed culture dishes with or without hypoxia/reoxygenation. Mitochondrial transmembrane potential ($\Delta\Psi_m$) was measured with the tetramethylrhodamine methyl ester (TMRM) fluorescence method. NRVM were stained with TMRM (50 nM, Invitrogen) and Mitotracker Green (200 nM, Invitrogen) in DMEM for 30 min at 37°C in the dark. Then, the cells were washed twice with PBS. Mitochondrial PTP opening was assessed by the quenching of Calcein-AM fluorescence by cobalt. The cells were stained with Calcein-AM (1 μ M, Invitrogen) and Mitotracker Red (150 nM, Invitrogen) at 37°C in the dark for 30 min, and then CoCl_2 (1 mM, Sigma) in HBSS 1X (Gibco) was added and the samples were incubated at 37°C for another 10 min. Then, cells were washed three times in 1X HBSS. Live images of the cells were captured with a Zeiss LSM710 confocal microscope and analyzed using ImageJ software (version 1.38×, National Institutes of Health).

Lentivirus and Adenovirus

For lentiviral based endogenous NR4A3 and Bnip3 gene knockdown, three NR4A3 shRNA and plasmid DNA constructs in hU6-MCS-CBh-gcGFP-IRES-puromycin-shRNA lentiviral vectors complementary to rat NR4A3 gene coding sequences were purchased from Genechem (Shanghai, China). The shRNA sequences are 5'-CCGGGCAGACTTATGGCTCGGAATACTCGAGTATTCCGAGCCATAAGTCTGCTTTTT-3', 5'-CCGGCCTCCGATCTGTATGATGAACCTCGAGATTCATCATAACAGATCGGAGGTTTTT-3' and 5'-CCGGCGGCCTTTGATCAAGATGGAACCTCGAGTTCCATCTTGATCAAAGGCCGTTTTT-3'. The Bnip3 Rat shRNA Lentiviral Particle (CAT#: TL711845V, OriGene) and Lentiviral Packaging Kits (CAT#: TR30037) were purchased from OriGene (Beijing, China). The recombinant shRNA lentiviral plasmid or scrambled shRNA control vector was transfected into HEK-293 cells to generate lentiviruses. Thereafter, shRNA lentiviruses were transduced into NRVM.

Adenoviral carrying full-length, three truncated (Δ AF1, Δ DBD, and Δ LBD) and two mutant (S376D, S376A) human Nr4a3 cDNA clones were constructed, packed, and purified by GeneChem (Shanghai, China). NRVM cells were transfected with Adv-*Nr4a3* cDNA clones.

Nuclear/mitochondria/cytosol isolation

The nuclear and cytosolic fractions were isolated from cells treated with or without hypoxia/reoxygenation using Nuclear/Cytosol Fractionation Kit (Cat.K266, Biovision). The mitochondrial and cytosolic fractions were isolated from cells with or without hypoxia/reoxygenation using a mitochondria/cytosol isolation kit (Cat.K256, Biovision).

Alkali extraction

Mitochondrial and cytosolic fractions were isolated using an isolation kit (Cat.K256, Biovision). To analyze whether Bnip3 integrated into mitochondrial membranes during hypoxia/reoxygenation, the mitochondria fraction was treated with ice cold alkali solution (0.1M Na₂CO₃, pH 11.5) on ice for 20 min. Mitochondrial fractions were recovered by centrifugation for 45 min at 20,000 x g, 4°C. The supernatant was discarded and cell pellets were lysed for western blot analysis.

Immunoprecipitation

Cells were cultured with or without hypoxia/reoxygenation and then lysed using IP Lysis buffer (Thermo Scientific). Target proteins were extracted using Protein A/G magnetic beads (Cat.B23202, Bimake). Cell lysates were incubated with Beads-Ab complex for 10 min at room temperature. Then, Beads-Ab-Ag complex were washed with wash buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40, pH 7.5) and eluted with elution buffer (0.1 M-0.2 M Glycine, 0.5% NP-40, pH 2.5-3.1). Finally, solutions were neutralized with neutralize buffer (1 M Tris, pH 8.0). Western blotting was used to detect proteins that interacted with the target protein. All antibodies and kits used in this study are listed in Table, S1.

Statistical analysis

Data were presented as box-and-whisker plots with all points. Comparisons between two groups were made using the Mann-Whitney U test, whereas the data obtained from multiple groups were compared using the Kruskal-Wallis test with Dunn's multiple comparison test. Two-way ANOVA followed by Bonferroni post hoc analysis was performed to analyze data with two factors. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism 7.0 (Graph Pad Prism Software Inc, San Diego, CA) and SPSS 15.0 for Windows (SPSS, Inc, Chicago, IL)

Declarations

Data availability

All data needed to evaluate the conclusions are available in the main text or the supplementary materials. The data, analytical methods, and study materials will be available to other researchers by contacting the corresponding authors upon reasonable request.

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Author contributions

Z.H. and H.Y.X. carried out experiments and analyzed the data. J.H., Z.B.Z., L.F.Z. and Q.J.C. contributed to data analysis. W.F.S, M.S. and K.F. contributed to manuscript discussion. Z.H. wrote the first draft. R.Y.Z., L.L. and X.X.Y. designed the study, supervised the experiments, and wrote the manuscript. All co-authors read the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information for this article is available in the Online Supplementary Data.

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Figures

Figure 1

Figure 1

Nr4a3 levels increase after ischemia reperfusion injury a Quantitative PCR analysis of Nr4a family mRNA expression in the mouse heart subjected to 2 hours of ischemia followed by 1 day of reperfusion (IR) (n = 5), and b in NRVMs after 24 hours of hypoxia followed by 24 hours of reoxygenation (HR) (n = 5). c Temporal dynamics of Nr4a3 mRNA expression in the heart after ischemia reperfusion (IR) injury (n = 5), and d in vitro NRVMs after hypoxia reoxygenation (HR) (n = 5). e Temporal dynamics of Nr4a3 protein

expression in the heart after myocardial IR injury (n = 5), and f in vitro in NRVMs after HR (n = 5). g Nr4a3 mRNA expression in four types of cells isolated from the heart after myocardial IR injury (2 hours of ischemia followed by 1 day of reperfusion) (n = 4). NRVMs, neonatal rat ventricular myocytes. Data are expressed as box and whisker plot with all points. Data presented in panels a, b, and g were analyzed by Mann-Whitney U tests. Data in panels c, d, e and f were analyzed by Kruskal-Wallis tests with Dunn's multiple test.

Figure 2

Figure 2

Cardiac specific Nr4a3 deficiency protects against myocardial ischemic reperfusion injury Control (Nr4a3^{flox/flox} Myh6-CreERT2⁻, abbreviated as Nf/f Cre⁻) and cardiomyocyte specific Nr4a3 deficiency (Nr4a3^{flox/flox} Myh6-CreERT2⁺, abbreviated as Nf/f Cre⁺) mice were subjected to 2 hours of ischemia followed by 1 day of reperfusion (IR). a Tissue sections of both Nf/f Cre⁻ and Nf/f Cre⁺ mice stained with Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) at day 1 after IR, in order to delineate the area at risk (AAR) and the infarcted region (n = 8), scale bar, 1mm. b Necrotic area evaluated by Evans blue dye (EBD) uptake and viable cardiomyocytes labeled by caveolin 3 (CaV3) antibody in Nf/f Cre⁻ and Nf/f Cre⁺ mice after myocardial IR injury (n = 7), scale bar, 1 mm. c and d Echocardiographic analysis of left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), ejection fraction (EF), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD) and fractional shortening (FS) 1 d after IR or sham operation (n = 5–15). e Representative Masson trichrome staining of cardiac tissue obtained from Nf/f Cre⁻ and Nf/f Cre⁺ mice 28 d after IR, and quantitative analysis of infarct size (left) and wall thickness (right) 28 d post-IR in Nf/f Cre⁻ and Nf/f Cre⁺ mice (n = 8). f Body weight-adjusted heart weights 28 d post-IR in Nf/f Cre⁻ and Nf/f Cre⁺ mice (n = 4-8). g TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling) staining of heart tissue sections from Nf/f Cre⁻ and Nf/f Cre⁺ mice 1 d post-IR or sham operation. scale bar, 100 μ m. h Western blot analysis of cleaved caspase-3 and caspase-3 from Nf/f Cre⁺ and Nf/f Cre⁻ mice hearts 1 d after IR or sham operation (n = 5). Data are expressed as box and whisker plot with all points. Data presented in panel a and e were analyzed by Mann-Whitney U tests. Data in panels b, d, f, g and h were analyzed using two-way ANOVA followed by a Bonferroni post hoc analysis.

Figure 3

Figure 3

Overexpression of Nr4a3 in neonatal mouse hearts leads to ventricular remodeling and heart failure. Data in a–h are from WT and α -myosin heavy chain-driven (α -MHC-driven) Nr4a3-transgenic mice (Tg-Nr4a3). a Kaplan–Meier survival analysis of WT and Tg-Nr4a3 mice. b and c Echocardiographic analysis of left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), ejection fraction (EF), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD) and

fractional shortening (FS) at 12 w of age (n = 5–6). d Heart weight adjusted for body weight in 12-w-old WT and Tg-Nr4a3 mice (n = 4). e and f, Representative photographs and quantitative data of Masson trichrome staining of cardiac tissue indicates massive fibrosis in the Tg-Nr4a3 mice compared to that in WT mice at 12 w of age (n = 4). Scale bar, 100 μ m. g Representative photographs and h quantitative data for TUNEL staining of heart tissue sections from WT and Tg-Nr4a3 mice. scale bar, 100 μ m. Data are expressed as box and whisker plot with all points. a Survival distributions were estimated by the Kaplan–Meier method and compared using log-rank tests. Data in panels c, d, f and h were analyzed by Mann–Whitney U tests.

Figure 4

Figure 4

Overexpression of Nr4a3 in adult mouse hearts leads to ventricular remodeling and heart failure Data in a–i are from conditional Nr4a3-knock-in mice. a Kaplan–Meier survival analysis of Nr4a3 KI-Myh6 Cre– (NKI Cre–) and Nr4a3 KI-Myh6 Cre+ (NKI Cre+) after tamoxifen injection. b and c Echocardiographic analysis of left ventricular systolic function 7 d after tamoxifen intraperitoneal injection (n = 6). d and e representative photographs and quantitative data for Masson trichrome staining of cardiac tissue

indicates massive fibrosis in NKI Cre+ mice than in the NKI Cre- ones, 14 d after tamoxifen treatment (n = 4). Scale bar, 100 μ m. f Representative photographs and g quantitative data for TUNEL staining of heart tissue sections from NKI Cre- and in NKI Cre+ mice. scale bar, 100 μ m. h and i Transmission electron microscope (TEM) images and quantitative data of mitochondrial area in the hearts from NKI Cre+ and NKI Cre- mice 7 d after tamoxifen intraperitoneal injection (n = 5). Scale bar, 2 μ m. Data are expressed as box and whisker plot with all points. a Survival distributions were estimated by the Kaplan–Meier method and compared using log-rank tests. Data in panels c, e, g and i were analyzed by Mann-Whitney U tests.

Figure 5

Figure 5

Nr4a3 contributes to cardiomyocyte necrotic and apoptotic cell death a and c Representative Calcein-AM/PI (propidium iodide) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling) staining of control and Nr4a3-knockdown NRVMs that were cultured under hypoxia-reoxygenation (24 hours hypoxia and 6 hours reoxygenation (HR)) or normoxic conditions. Scale bar, 100 μ m. b and d Quantitative analysis of PI-positive cells and TUNEL-positive cells (n = 5). e Western blot analysis of caspase-3 and cleaved caspase-3 protein expression in control and Nr4a3-knockdown NRVMs that were cultured under HR or normoxic conditions (n = 5). f Representative photographs and g quantitative data for Calcein-AM/PI double staining of control and Nr4a3-overexpressing NRVMs with or without Bnip3-knockdown that were cultured under HR or normoxic conditions (n = 5). Scale bar, 100 μ m. h Representative photographs and (i) quantitative data for TUNEL staining of control and Nr4a3-overexpressing NRVMs with or without Bnip3-knockdown that were cultured under HR or normoxic conditions (n = 5). Scale bar, 100 μ m. Data are expressed as box and whisker plot with all points. Data in panels b, d, e, g and i were analyzed by two-way ANOVA followed by Bonferroni post hoc analysis.

Figure 6

Figure 6

Ischemia/hypoxia induces Nr4a3 translocation from the nucleus to the mitochondria, where it interacts with Bnip3 and promotes its integration into mitochondrial membranes, leading to mitochondrial permeability transition pore (mPTP) opening and decreased mitochondrial membrane potential. Double immunofluorescence staining for Nr4a3 and Hsp60 in NRVMs cultured under normoxic conditions or hypoxia reoxygenation (24 hours hypoxia and 6 hours reoxygenation (HR)) conditions (n = 5). Scale bar,

100 μ m. b Nr4a3 was increased and co-localized with Hsp60 in mouse hearts 1 d after IR or sham operation (n = 5). Scale bar, 100 μ m. c Western blot analysis of Nr4a3 subcellular location in NRVMs under normoxic or HR conditions (n = 5). d Western blot analysis of Nr4a3 subcellular location in mouse hearts 1 d after IR or sham operation (n = 5). e NRVMs were cultured under normoxic or HR conditions, and analyzed by immunoprecipitation using an anti-Nr4a3 antibody followed by western blotting using either anti-Nr4a3 or anti-Bnip3 antibodies (n = 5). Representative double immunofluorescence staining of Bnip3 and Hsp60 f or Nr4a3 and Bnip3 g in NRVMs under normoxic and hypoxic conditions (n = 5). Scale bar, 100 μ m. h Western blot analysis of Bnip3 protein in subcellular fractions of control and Nr4a3-knockdown NRVMs cultured under normoxic or HR conditions (n = 5). i Western blot analysis of mitochondrial-integrated Bnip3 in control and Nr4a3-knockdown NRVMs cultured under normoxia or HR conditions (n = 5). j Representative Calcein-AM (1 mM, green) and Mitotracker Red (150 nM, red) staining in control and Nr4a3-knockdown NRVMs cultured under normoxic or HR conditions. Mitotracker red confirmed the localization of calcein fluorescence in mitochondria. Co_2^+ (1 mM) quenched calcein fluorescence (green) in the mitochondria when mPTP open. Scale bar, 100 μ m. k Quantitative analysis of Calcein-AM fluorescence intensity with these treatments (n = 5). l Representative tetramethylrhodamine methyl ester (TMRM; 50 nM, red) and Mitotracker Green (200 nM, green) staining in control and Nr4a3-knockdown NRVMs cultured under normoxic or HR conditions. The intensity of TMRM reflects the mitochondrial membrane potential ($\Delta\Psi_m$), whereas the intensity of Mitotracker Green was not affected by altered transmembrane potential. Scale bar, 100 μ m. m Quantitative analysis of TMRM fluorescence intensity in response to these treatments (n = 5). Data are expressed as box and whisker plot with all points. Data in panels a to g were analyzed by Mann-Whitney U tests. Data in panels h, i, k and m were analyzed by two-way ANOVA followed by a Bonferroni post hoc analysis.

Figure 7

Figure 7

LBD and AF1 domains of Nr4a3 are required for its binding to Bnip3 a Diagram of constructed full-length and truncated cDNA clones of Nr4a3. b Western blot analysis of adenovirus-mediated full-length and truncated Nr4a3 expression in NRVMs using anti-Nr4a3 and anti-Flag antibodies (n = 4). c Representative photographs and quantitative data for subcellular location of full-length and truncated Nr4a3 in NRVMs under normoxic or hypoxia reoxygenation (24 hours hypoxia and 6 hours reoxygenation (HR)) conditions

using an anti-Flag and anti-Hsp60 antibody (n = 5). Scale bar, 100 μ m. d Immunoprecipitation for analysis of adenovirus-mediated Nr4a3 full-length and truncated cDNA clones expression and relative Nr4a3 bound Bnip3 level in NRVMs using an anti-Flag antibody followed by western blotting using either anti-Bnip3 or anti-Flag antibodies (n = 5). e Western blot and quantitative data analysis of mitochondrial-integrated Bnip3 in NRVMs transfected with Nr4a3 full-length and truncated cDNA clones (n = 5). f Representative photographs and g quantitative data for Calcein-AM (1 mM, green) and Mitotracker Red (150 nM, red) staining in NRVMs transfected with adenovirus-mediated full-length and truncated Nr4a3 cDNA clones cultured under normoxic or HR conditions (n = 5). Scale bar, 100 μ m. h Representative photographs and i quantitative data for tetramethylrhodamine methyl ester (TMRM; 50 nM, red) and Mitotracker Green (200 nM, green) staining in NRVMs transfected with Nr4a3 full-length and truncated cDNA clones cultured under normoxic or HR conditions (n = 5). Scale bar, 100 μ m. Data are expressed as box and whisker plot with all points. Data presented in panels c, d, e, f and h were analyzed by Kruskal-Wallis tests with Dunn's multiple test.

Figure 8

Figure 8

Phosphorylation of Nr4a3 induces its mitochondrial translocation in response to hypoxia a Sequence around Ser376 in Nr4a3_Human is highly conserved in the related nuclear orphan receptors, Nr4a1_Human (Ser405) and Nr4a2_Human (Ser347) and sequence conservation of the Arg-X-Arg-X-X-pSer/Thr (GRLP(phospho-S)KPKSP) motif in Nr4a3 among different species. b Immunoprecipitation for analysis of the phosphorylated Nr4a3 in NRVMs cultured under normoxic or hypoxia reoxygenation (24

hours hypoxia and 6 hours reoxygenation (HR)) conditions by using an anti-Nr4a3 antibody, followed by western blotting using either an anti-P-(Ser/Thr) antibody or anti-Nr4a3 antibodies (n = 5). c Representative photographs and quantitative data for double immunofluorescence staining of Nr4a3 and Hsp60 in NRVMs transfected with adenovirus-mediated S376D and S376A mutant Nr4a3 cDNA clones under normoxic and HR conditions (n = 5). Scale bar, 100 μ m. d Immunoprecipitation for analysis of relative Nr4a3 bound Bnip3 level in NRVMs transfected with adenovirus-mediated S376D and S376A mutant Nr4a3 cDNA clones using an anti-Flag antibody followed by western blotting using either anti-Bnip3 or anti-Flag antibodies (n = 5). e Western blot and quantitative data analysis of mitochondrial-integrated Bnip3 in NRVMs transfected with adenovirus-mediated S376D and S376A mutant Nr4a3 cDNA clones (n = 5). f Representative photographs and quantitative data for Calcein-AM (1 mM, green) and Mitotracker Red (150 nM, red) staining in NRVMs transfected with adenovirus-mediated S376D and S376A mutant Nr4a3 cDNA clones cultured under normoxic or HR conditions (n = 5). Scale bar, 100 μ m. g Representative photographs and quantitative data for tetramethylrhodamine methyl ester (TMRM; 50 nM, red) and Mitotracker Green (200 nM, green) staining in NRVMs transfected with S376D and S376A mutant Nr4a3 cDNA clones cultured under normoxic or HR conditions (n = 5). Scale bar, 100 μ m. h and i Representative photographs and quantitative data for Calcein-AM/PI (propidium iodide) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling) staining of NRVMs with S376D and S376A mutant Nr4a3 cDNA clones under normoxic or HR conditions (n = 5). Scale bar, 100 μ m. j Schematic diagram of Nr4a3 mediating cardiomyocyte necrotic and apoptotic death. Data are expressed as box and whisker plot with all points. Data presented in panel b and d was analyzed by Mann-Whitney U tests, Data in panels c, e, f, g, h and i were analyzed by two-way ANOVA followed by Bonferroni post hoc analysis.

Supplementary Files

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