Interferon Regulatory Factor 9 Protects Against Cardiac Hypertrophy by Targeting Myocardin

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Abstract—Pathological cardiac hypertrophy is a major risk factor for heart failure. In this study, we identified interferon regulatory factor 9 (IRF9), a member of the IRF family, as a previously unidentified negative regulator of cardiac hypertrophy. The level of IRF9 expression was remarkably elevated in the hearts from animals with aortic banding–induced cardiac hypertrophy. IRF9-deficient mice exhibited pronounced cardiac hypertrophy after pressure overload, as demonstrated by increased cardiomyocyte size, extensive fibrosis, reduced cardiac function, and enhanced expression of hypertrophy markers, whereas transgenic mice with cardiac-specific overexpression of murine IRF9 exhibited a significant reduction in the hypertrophic response. Mechanistically, IRF9 competes with p300 for binding to the transcription activation domain of myocardin, a coactivator of serum response factor (SRF). This interaction markedly suppresses the transcriptional activity of myocardin because IRF9 overexpression strongly inhibits the ability of myocardin to activate CARG box–dependent reporters. These results provide compelling evidence that IRF9 inhibits the development of cardiac hypertrophy by suppressing the transcriptional activity of myocardin in the heart. (Hypertension. 2014;63:00-00.) ●

Online Data Supplement

Key Words: cardiomegaly ■ fibrosis ■ IRF9 protein, mouse ■ myocardin

Cardiac hypertrophy is an essential adaptive or maladaptive response of myocytes to diverse pathophysiological stimuli (eg, hypertension, ischemia, valvular insufficiency and stenosis, or sarcomeric gene mutations). The underlying pathological process of cardiac hypertrophy is complex and involves a variety of signaling pathways and regulators. At the cellular level, cardiac hypertrophy is characterized by increased cardiomyocyte size, elevated protein synthesis, and altered gene expression. Although hypertrophic responses are initially compensatory, continuous stress leads to deleterious changes in the left ventricle and increases the risk of heart failure, arrhythmia, and sudden death. During the development of cardiac hypertrophy, transcription factors, such as GATA4, myocyte enhancer factor 2, nuclear factor of activated T cells, and serum response factor (SRF), play crucial roles by recruiting cofactors (coactivators or corepressors) to regulate the expression of a series of hypertrophic genes. The association between a transcription factor and a regulator brings specificity and diversification to gene regulation, a critical component of many biological processes. During the past decade, much progress has been made in deciphering this type of cooperation in cardiac hypertrophy. However, the mechanism of transcription factor regulation and the effect of this phenomenon on cardiac hypertrophy remain largely unknown, in particular the counter-regulatory pathway, which has the potential to inhibit the cardiac hypertrophic response.

Interferon regulatory factor 9 (IRF9, also known as p48 or ISG3γ), a member of the interferon regulator factor (IRF) family, is a subunit of the interferon-stimulated gene factor 3 protein and is essential for the activity of type I interferons. In addition to mediating innate immunity, IRF9 participates in the regulation of oncogenesis, cell proliferation, and apoptosis. We recently reported that IRF9 binds to peroxisome proliferator-activated receptor α (PPARα) to activate PPARα target gene expression in the liver, thus attenuating hepatic insulin resistance and steatosis. These results suggest that in addition to acting as a transcription factor, IRF9 also cooperates with other transcription factors to regulate downstream gene expression. This feature allows IRF9 to function in the regulation of a wide range of genes. However, the role of IRF9 in the heart, particularly in response to stress stimuli, has not been well studied.

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been investigated. Therefore, determining whether a deficiency in or the overexpression of IRF9 in the myocardium could affect the process of heart disease is important.

In the present study, we first identified IRF9 as an endogenous negative regulator of cardiac hypertrophy and observed that IRF9 was significantly activated in an aortic banding (AB)-induced animal model of cardiac hypertrophy. Compared with the controls, the IRF9-deficient mice developed more severe hypertrophy upon stimulation, whereas IRF9 overexpression in the heart suppressed this pathological process. In addition, we provided evidence that myocardin is an important target of IRF9 because its transcriptional activity is strongly suppressed in the presence of IRF9.

Materials and Methods

All animal experimental protocols were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. An expanded Methods section is available in the online-only Data Supplement, including Reagents, Animal Models and Procedures, Histological Analysis, Cultured NRCMs and Recombinant Adenoviral Vectors, Immunoprecipitation and the GST Pull-down Assay, Western Blotting, and Quantitative Real-Time PCR, Plasmid Constructs, Immunoprecipitation and the GST Pull-down Assay, Luciferase Reporter Assays, EMSA, and Statistical Analysis.

Results

IRF9 Levels Are Elevated During Cardiac Hypertrophy

To investigate the role of IRF9 in the development of cardiac hypertrophy and heart failure, we first measured IRF9 expression in murine hypertrophic hearts and neonatal rat cardiomyocytes (NRCMs). Both Western blot analysis and reverse transcription polymerase chain reaction demonstrated that IRF9 levels were significantly upregulated in the hearts of mice that underwent AB for 2 or 4 weeks (Figure 1A; Figure S1 in the online-only Data Supplement). As shown in Figure 1A, compared with the sham-operated control hearts, IRF9 protein levels were ≈3-fold and 5-fold higher in the experimental mouse hearts after 2 or 4 weeks of AB, respectively; this increase was also observed at the mRNA level (Figure S1). In addition, the increase in IRF9 correlated with an increase in the hypertrophy markers β-myosin heavy chain (β-MHC) and atrial natriuretic peptide (ANP) (Figure 1A). Furthermore, the stimulation of NRCMs with isoproterenol (ISO; 10 μmol/L) or angiotensin II (Ang II; 1 μmol/L) for 48 hours to induce hypertrophy resulted in the upregulation of IRF9, β-MHC, and ANP (Figure 1B). These results indicate that IRF9 is markedly activated by hypertrophic stress in vivo and in vitro, which suggests that IRF9 may be involved in cardiac hypertrophy.

IRF9-Deficient Mice Exhibit an Increased Susceptibility to Pathological Cardiac Hypertrophy

We then examined the potential function of IRF9 during hypertrophy in vivo, subjecting global homozygous IRF9 gene knockout (IRF9−/−) mice to 4 weeks of AB; these mice did not produce IRF9 protein in the heart (Figure S2A) and did not display apparent phenotypic abnormalities at baseline (Table S1). After 4 weeks of AB, we observed a higher mortality rate in the IRF9−/− mice than in the wild-type littermates (IRF9+/+; Figure S2B). The IRF9−/− mice also exhibited enhanced hypertrophy compared with the IRF9+/+ mice for 4 weeks of AB, as indicated by the higher ratios of heart weight to body weight (HW/BW), lung weight to BW, and HW to tibia length in the knockout mice (Figure 2A). No comparable differences were noticed in the sham-operated IRF9−/− and IRF9+/+ mice (Figure 2A). Hematoxylin-eosin and wheat germ agglutinin staining showed a greater ventricular cross-sectional area in the IRF9−/− mice than in the controls (Figure 2B and 2C), further confirming that IRF9 deficiency promoted the development of AB-induced cardiac hypertrophy. We also performed echocardiography and hemodynamic measurements to monitor the cardiac function of the IRF9−/− and IRF9+/+ mice after hypertrophic stimuli. After 4 weeks of AB, the IRF9−/− mice exhibited reduced cardiac function (Table S2). In response to AB, the expression levels of ANP, B-type natriuretic peptide, and β-MHC were dramatically higher in the IRF9−/− mice than in the controls (Figure 2B and 2C). Histological analysis with picrosirius red staining revealed more extensive perivascular and interstitial fibrosis in the hearts of IRF9−/− mice at 4 weeks after AB (Figure 2E and 2F). Subsequently, these results were corroborated by detecting the mRNA levels of fibrotic markers such as connective tissue growth factor, collagen I,
and collagen III (Figure S2D). Thus, IRF9 is an endogenous negative regulator of cardiac hypertrophy.

**IRF9 Overexpression Suppresses AB-Induced Cardiac Hypertrophy**

To further confirm the inhibitory effect of IRF9 on cardiac hypertrophy, cardiac-specific transgenic (TG) mice that overexpress murine IRF9 under the control of the α-MHC promoter were generated (Figure S3A), and 4 independent lines of IRF9-TG mice were established. Western blot analysis indicated that cardiac IRF9 expression in these TG mice was 2- to 6-fold higher than that in their non-TG (NTG) littermates (Figure S3B). However, at baseline, the IRF9-TG mice developed normally, with no apparent structural or functional deficits (Table S1). The 4 IRF9-TG mouse lines exhibited a similar phenotype after 4 weeks of AB (data not shown), and TG line No. 9 mice and their littermates were used for further experiments. As shown in Figure 3A, AB induced a 51% increase in the HW/BW ratio, indicating the development of cardiac hypertrophy in the NTG mice, whereas the TG mice exhibited significantly blunted hypertrophic growth, with an increase of ≈18% in the HW/BW ratio (Figure 3A).

Figure 2. Interferon regulatory factor 9 (IRF9) deficiency in the heart exaggerates aortic banding (AB)–induced cardiac hypertrophy. A, The ratios of heart weight to body weight (HW/BW), lung weight (LW)/BW, and HW/tibia length (TL) in IRF9+/+ and IRF9−/− mice after sham treatment or AB for 4 weeks. n=15 for each group. B, Sections of hearts from IRF9+/+ and IRF9−/− mice subjected to AB or sham treatment were stained with hematoxylin-eosin (H&E) and wheat germ agglutinin (WGA) to analyze heart and cardiomyocyte size (n=10 mice per group). Top, middle, and bottom scale bars, 100 μm, 20 μm, and 20 μm, respectively. C, Quantification of cardiomyocyte cross-sectional area in sham-treated and AB-treated IRF9+/+ and IRF9−/− mice. D, Expression levels of the indicated hypertrophic markers in the hearts of sham– and AB-treated IRF9+/+ and IRF9−/− mice. Western blot analyses were performed, and the results are representative of 3 independent experiments. n=4 mice for each group. E and F, Picrosirius red (PSR) staining of histological sections of hearts from sham– and AB-treated IRF9+/+ or IRF9−/− mice (n=10 mice per group). Scale bars, 20 μm. Fibrotic areas of individual sections were measured using Image-Pro Plus 6.0 software (F). n>25 for each group. For A, C, D (right), and F, the data are presented as the mean±SD, *P<0.05 vs IRF9+/+ sham; #P<0.05 vs IRF9−/− sham. ANP indicates atrial natriuretic peptide; and LV, left ventricle.
results indicate that IRF9 overexpression is capable of suppressing the development of cardiac hypertrophy induced by AB in vivo.

**IRF9 Inhibits ISO-Induced Cardiac Hypertrophy**

The above findings clearly demonstrate that IRF9 protects the heart from AB-induced cardiac hypertrophy, but different hypertrophic stimuli have been reported to act on different receptors and induce different molecular responses. Therefore, we used another classic method for inducing hypertrophy, ISO infusion, to determine whether IRF9 is a general regulator of the development of cardiac hypertrophy. Consistent with the results observed during AB, after ISO infusion (30 mg/kg per day) for 4 weeks, the ratios of heart weight to body weight (HW/BW), lung weight (LW/BW, and HW/tibia length (TL) determined in non-TG (NTG) and IRF9-TG mice 4 weeks after sham or AB treatment. n=15 for each group. B. Hematoxylin-eosin (H&E) and wheat germ agglutinin (WGA) staining showing hypertrophic growth in the indicated mice (n=10 mice per group). Top, middle, and bottom scale bars, 100 μm, 20 μm, and 20 μm, respectively. C. Cardiomyocyte cross-sectional area quantified in sham- and AB-treated NTG or IRF9-TG mice. D. Western blot analysis of atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC) expression in the hearts of sham- and AB-treated NTG and IRF9-TG mice. E and F. PSR staining for detecting fibrosis in NTG and IRF9-TG mice subjected to sham or AB (n=10 mice per group). Scale bars, 20 μm. The fibrotic area is quantified in F. n>25 for each group. For A, C, D (right), and F, the data are presented as the mean±SD; *P<0.05 vs NTG/sham; #P<0.05 vs NTG/AB. LV indicates left ventricle.

**IRF9 Inhibits the Ang II–Induced Hypertrophic Response in Cardiomyocytes**

We next tested whether IRF9 contributes to the hypertrophy of cardiomyocytes in vitro. NRCMs were infected with AdshIRF9, AdIRF9, or control vectors (Figure 4A) and by a greater ventricular cross-sectional area and increased cardiac fibrosis (Figure S4B–S4D), whereas the hearts of the IRF9-TG mice were smaller and exhibited less cardiac fibrosis in response to ISO than the control mice (Figure S4H–S4J). Echocardiography of the IRF9−/− mice revealed significantly reduced cardiac function on ISO infusion; however, the IRF9-TG mice exhibited mild ventricular dilatation and cardiac dysfunction (Table S4). Furthermore, ISO induced the expression of fetal genes, and fibrotic markers were elevated in the hearts of the IRF9−/− mice but significantly reduced in the hearts of the TG mice (Figure S4E, S4F, S4K, and S4L). Similar results from ISO infusion further confirmed that IRF9 is generally required to protect the heart from pathological hypertrophy.
further stimulated with Ang II (1 μmol/L) for 48 hours. Immunostaining with the α-actinin antibody indicated that AdshIRF9 treatment enhanced Ang II–induced increase in cell size, whereas overexpression of IRF9 (AdIRF9), but not the control vector, suppressed the hypertrophic response to Ang II stimulation (Figure 4B and 4C). Furthermore, the ANP and β-MHC mRNA levels were significantly higher in the NRCMs infected with AdshIRF9 after exposure to Ang II than in controls (Figure 4D). However, IRF9 overexpression attenuated the elevation of ANP and β-MHC levels induced by Ang II (Figure 4E). Taken together, these observations indicate that IRF9 suppresses the hypertrophic response in cardiomyocytes.

IRF9 Interacts With Myocardin and Represses Its Transcriptional Activity

Once we found that IRF9 is capable of inhibiting pathological hypertrophy, we investigated the mechanism by which this effect occurs. A dual-luciferase reporter analysis was performed to identify the IRF9-regulated target using a Cignal 45-Pathway Reporter Array kit. We found that the activity of the luciferase reporter linked to the CArG box (which serves as the SRF-binding site) was dramatically inhibited by IRF9, and this result was further confirmed in NRCMs (Figure S5A). Our results showed that IRF9 overexpression significantly suppressed Ang II activation of a luciferase reporter linked to 3 tandem copies of a CArG box (Figure S5A). The CArG box is found in the ANP promoter and regulates its promoter activity;20,25 thus, we also examined the effect of IRF9 on ANP activity. Consistent with the CArG box results, IRF9 overexpression also suppressed the luciferase activity of ANP induced by Ang II in NRCMs (Figure S5A). To determine whether the inhibitory effect of IRF9 on the expression of CArG box–dependent reporters resulted from influencing SRF expression, we tested the activity of a luciferase reporter linked to the promoter of SRF. The results showed that IRF9 overexpression did not alter the Ang II–induced luciferase activity controlled by the SRF promoter in the NRCMs (Figure S5B). Furthermore, a coimmunoprecipitation assay performed in HEK293T cells showed that IRF9 did not directly interact with SRF (data not shown). On the basis of the above findings, we next determined the effect of IRF9 on the activity of myocardin, a well-characterized coactivator of SRF that potently transactivates the CArG box.26,27 As shown in Figure S5C, IRF9 overexpression strongly...

Figure 4. Interferon regulatory factor 9 (IRF9) suppresses angiotensin II (Ang II)–induced cardiomyocyte hypertrophy. 

A. NRCMs infected with AdIRF9, AdshIRF9, or their respective controls (adenoviral vectors expressing green fluorescent protein [AdGFP] and adenoviral vectors expressing short hairpin ribonucleic acid [AdshRNA]) were analyzed by Western blotting. This result is quantified to the right. The data are presented as mean±SD, *P<0.05 vs AdGFP/AdshRNA. 

B. Representative images of cardiomyocytes (immunostained with the α-actinin antibody) infected with AdshIRF9 or AdIRF9 after treatment with Ang II (1 μmol/L) for 48 hours (n=4 independent experiments). Scale bars, 20 μm.

C. Quantification of the cardiomyocyte cross-sectional area as determined using Image-Pro Plus 6.0 software. The data are presented as mean±SD, n>40 cells, *P<0.05 vs AdGFP/AdshRNA.

D and E. Atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC) mRNA levels in NRCMs after Ang II treatment. The cells were treated as in B, and real-time polymerase chain reaction analysis was performed. The data are presented as the mean±SD and are representative of 3 independent experiments. n=3, *P<0.05 vs AdGFP or AdshRNA/phosphate buffered solution (PBS), #P<0.05 vs AdGFP or AdshRNA/Ang II.
suppressed the ability of myocardin to activate the CArG box–dependent reporters (Figure S5C). In addition, the IRF9−/− mice exhibited enhanced myocardin transcriptional activity compared with the IRF9+/+ mice for 4 weeks of AB (Figure S5D). This effect was specific to myocardin and independent of SRF as demonstrated by the fact that the activation of the GAL4-dependent reporter by myocardin that is linked to the GAL4 DNA-binding domain was also markedly suppressed by IRF9 (Figure S5E). To define the mechanism responsible for the IRF9 suppression of myocardin activity, we performed a glutathione S-transferase (GST) pull-down assay to determine whether IRF9 interacts directly with myocardin. As shown in Figure 5A, IRF9 did bind to myocardin. This interaction was further confirmed with a coimmunoprecipitation assay (Figure 5B). Subsequently, we mapped the domains of IRF9 and myocardin that are responsible for this interaction. Both the N terminus (residues 1–120) and the proline-rich (pro) domain (residues 120–220) of IRF9 contributed to myocardin binding (Figure 5C). However, the transcription activation domain (TAD; residues 738–938) of myocardin specifically interacted with IRF9 (Figure 5D). To determine whether the inhibitory effect of IRF9 on myocardin activity depends on this direct interaction, we tested the effect of an IRF9 mutant that lacks the myocardin-binding domain and found that this IRF9 mutant did not affect the activation of the CArG box–dependent reporters by myocardin (Figure S5F).

The IRF9 association domain of myocardin, the TAD, is also bound by p300. p300 enhances myocardin activity in African green monkey simian virus 40 transformed kidney cell (COS cells) by interacting directly with the TAD of myocardin, and this effect was also observed in NRCMs and H9C2 cells (Figure S5G and S5H). IRF9 and p300 bind to the same region of myocardin and have opposite effects on myocardin activity; thus, we cotransfected H9C2 cells with different doses of IRF9 and p300 to determine the relationship between these 2 regulators. As shown in Figure S5I, IRF9 competed with p300 to modulate the transcriptional activity of myocardin in H9C2 cells. Furthermore, IRF9 significantly inhibited the p300-mediated activity of myocardin in the NRCMs (Figure S5G). To determine whether the binding activity of myocardin is truly required for IRF9 function, we tested the luciferase activities of 2 mutated ANP promoters lacking 1 or 2 CArG box sequences, as previously reported. As shown in Figure S5J, myocardin activated the luciferase reporter that was linked to the wild-type ANP promoter (ANP-1000), and this effect was further enhanced after knocking down IRF9.

![Figure 5](http://hyper.ahajournals.org/figure/)

**Figure 5.** Interferon regulatory factor 9 (IRF9) interacts with myocardin and represses its transcriptional activity. A, Glutathione S-transferase (GST) pull-down assay to detect the direct interaction between IRF9 and myocardin. B, Lysates of HEK293T cells overexpressing enhanced green fluorescent protein (EGFP)-myc-IRF9 and Flag-Myocardin were subjected to immunoprecipitation (IP) with antibodies specific for myc (left) and Flag (right). The results were analyzed by Western blotting. C, Top, A schematic diagram of the IRF9 vectors used to map the myocardin-binding domain. Bottom, Western blots of IRF9 deletion mutant proteins or myocardin after IP from lysates of HEK293T cells overexpressing the indicated vector products. D, Top, A schematic diagram of the myocardin vectors used to map the IRF9-binding domain. Bottom, Western blots of myocardin deletion mutant proteins or IRF9 after IP from lysates of HEK293T cells overexpressing the indicated vector products. *P<0.05 vs AdGFP, #P<0.05 vs Admyocardin. Three independent experiments were performed. IB indicates immunoblot.
Furthermore, deletion of the distal CArG box from the ANP promoter (ANP-236) led to a small increase in the transcriptional activity of myocardin after interfering IRF9 expression, and IRF9 was not able to alter the luciferase activity of ANP-121, which lacks both CArG boxes in the ANP promoters (Figure S5J). Therefore, the role of IRF9 depends on myocardin binding to the target gene promoter. Taken together, these data indicate that IRF9 interacts directly with myocardin and thereby suppresses its transcriptional activity.

Discussion

The results of this study are compelling evidence of the critical role of IRF9 in pathological cardiac hypertrophy. We observed that the level of IRF9 was elevated by hypertrophic stimuli. Subsequently, we showed that IRF9 protected against pressure overload–induced cardiac hypertrophy: IRF9−/− mice exhibited an augmented hypertrophic response after 4 weeks of AB or ISO infusion, whereas hypertrophic growth was reduced in the cardiac-specific IRF9 TG mice. Furthermore, we identified that IRF9 exerted an antihypertrophic effect by binding to the TAD of myocardin, thus suppressing its transcriptional activity. Notably, this interaction also inhibited p300-mediated activation of myocardin.

Members of the IRF family participate in the regulation of many biological processes, including the innate and adaptive immune responses, antiviral defense, cell growth and apoptosis, oncogenesis, and hematopoietic development.13–30 However, little is known about the functions of IRFs in the cardiac system. Recently, we have demonstrated that IRF3 and IRF4 are involved in regulating cardiac hypertrophy.14,15 Only IRF3 acts as a negative regulator of cardiac hypertrophy by blocking the extracellular regulated protein kinases 1/2 (ERK1/2) signaling pathway,15 and IRF4 aggravates pathological cardiac hypertrophy by activating the cAMP response element–binding domain.13 It is not surprising that both IRF3 and IRF4 function in cardiac hypertrophy because different IRFs have many features in common.13 For example, most IRFs regulate cell growth. The specific function of each member of the IRF family must be investigated because both common and specific features enable members of the IRF family to receive, integrate, and transmit signals arising from a diverse array of stresses. Like IRF3 and IRF4, IRF9 is expressed in the heart; however, IRF9 exhibits a different expression pattern than either IRF3 or IRF4 in response to hypertrophic stress. IRF3 expression increased to maximal levels in the hearts of mice subjected to AB at day 14 and then decreased to basal levels at day 28,15 whereas IRF4 was downregulated during cardiac hypertrophy.14 In contrast, the IRF9 level was markedly elevated in pathological hypertrophic hearts. This phenomenon suggests that IRF9 may play a specific role in cardiac hypertrophy that is different from the roles of IRF3 and IRF4.

The mechanism by which hypertrophic stress elevates the IRF9 levels has not yet been studied. Nevertheless, a mechanism can be hypothesized based on the studies of other negative regulators of cardiac hypertrophy. One feature of hypertrophy is enhanced protein synthesis, including that of numerous positive and negative factors. Several endogenous molecules such as inducible cAMP early repressor (ICER), myocyte-enriched calcineurin–interacting protein 1 (MCIP1), and suppressor of cytokine signaling-3 (SOCS3) have been relatively well characterized and exhibit increased expression on hypertrophic stimulation.32–35 The results of these studies are similar to our observations of IRF9, and this increased expression may be a compensatory cardiac response to the detrimental effects of hypertrophy. Another mechanism may contribute to the increased IRF9 level because a negative-feedback pathway related to IRF9 may exist. Consistent with our results, the expression of IRF9 in cardiomyocytes at baseline is low,48 and the increased level of IRF9 induced by hypertrophic stress may in turn inhibit upstream pathways. Further studies are needed to understand the mechanisms by which IRF9 expression is regulated during stress.

After hypertrophic stimulation, IRF9 overexpression strongly inhibits the expression of a luciferase reporter linked to the CArG box, which is the binding site of SRF and is required for SRF-target gene expression;27 however, SRF is not the direct target of IRF9 because the luciferase activity controlled by SRF promoter did not change after IRF9 overexpression, and no interaction between IRF9 and SRF was detected. As a transcription factor, SRF is a relatively weak transcriptional activator and must recruit cofactors for transcriptional activity.27 Thus, we further investigated the effect of IRF9 on the cofactors of SRF. Subsequently, myocardin was identified as the target of IRF9. As an important coactivator of SRF, myocardin participates in cardiac and smooth muscle differentiation and mediates cardiac development and hypertrophy.27–30 The mapping experiment determined that both the N terminus and the pro-domain of IRF9 are responsible for the interaction with myocardin. The N terminus of IRF9 is the DNA-binding domain, which is characterized by 5 tryptophan repeats and is highly conserved within the IRF family.30 The pro-domain corresponds to a linker region,13,40 which is reported to be the PPARα-binding domain.31 Only the TAD of myocardin contributes to the association with IRF9. The TAD of myocardin, as its name indicates, possesses powerful transcriptional activity and acts through CArG boxes in genes.27 Furthermore, when the TAD is absent, myocardin fails to induce hypertrophy in cardiomyocytes.30 The binding of IRF9 to the TAD of myocardin results in a marked inhibition of its transcriptional activity, thereby influencing the development of cardiac hypertrophy.

Intriguingly, p300 also interacts directly with the TAD of myocardin, but this interaction activates myocardin.25 IRF9 and p300 bind to the same region of myocardin, and we subsequently discovered that IRF9 competes with p300 to modulate the activity of myocardin. In other words, the opposing effects of IRF9 and p300 on myocardin activity seem to the result of direct competition for the same site on myocardin. The repressive influence of IRF9 seems to be dominant because p300 overexpression was unable to completely abrogate the inhibitory effect of IRF9 on the transcriptional activity of myocardin in H9C2 cells, and IRF9 overexpression significantly suppressed the effect of p300 on myocardin activity in NRCMs. Although our results might not exclude other mechanisms by which IRF9 suppresses cardiac hypertrophy, the inhibitory effect of IRF9 on the development of cardiac hypertrophy seems to be largely dependent on the repression of myocardin activity by IRF9.
In summary, we demonstrated that IRF9 suppresses the development of cardiac hypertrophy both in vitro and in vivo. Furthermore, we identified myocardin as a new target of IRF9 and demonstrated that the transcriptional activity of myocardin is strongly inhibited by IRF9. Thus, targeting IRF9 might be a useful therapeutic strategy to prevent cardiac hypertrophy and heart failure.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Interferon regulatory factor 9 (IRF9) is upregulated in murine hypertrophic hearts.
- IRF9 is a negative regulator of aortic banding or isoproterenol-induced cardiac hypertrophy, fibrosis, and cardiac dysfunction.
- IRF9 binds to myocardin and suppresses its transcriptional activity, thus regulating the development of cardiac hypertrophy.

**What Is Relevant?**

- Many hypertrophic activators have been investigated, but relatively little is known about the negative modulators of cardiac hypertrophy.
- The functions of IRF9 in the heart are unclear.

**Summary**

This study demonstrates that the absence of IRF9 promotes aortic banding or isoproterenol-induced cardiac hypertrophy, fibrosis, and cardiac dysfunction, whereas cardiac-specific overexpression of IRF9 inhibits the hypertrophic response by suppressing the transcriptional activity of myocardin. These findings suggest that IRF9 might be a useful therapeutic target for preventing cardiac hypertrophy and heart failure.
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Running title: IRF9 protects against cardiac hypertrophy

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Materials and Methods

Reagents
GAPDH (MB001, 1:10000 dilution) antibodies were purchased from Bioworld Technology (Harrogate, UK). Antibodies against ANP (sc20158, 1:200 dilution), β-MHC (sc53090, 1:200 dilution) and IRF9 (sc10793, 1:200 dilution) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). ISO (I5627), and antibodies against Flag (F3165, 1:1000 dilution) and HA (H6908, 1:1000 dilution) were purchased from Sigma (St. Louis, MO, USA). Antibodies against Myc (11814150001, dilution at 1:1000) were obtained from Roche (Mannheim, Germany). The BCA protein assay kit was obtained from Pierce (Rockford, IL, USA). IRDye® 800CW-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used for visualization. Fetal calf serum (FCS) was obtained from Hyclone (Waltham, MA, USA). Cell culture reagents and all other reagents were obtained from Sigma (St. Louis, MO, USA).

Animal Models and Procedures
All the animal experimental protocols were approved by the Animal Care and Use Committee of Renmin Hospital Wuhan University. A full-length mouse IRF9 cDNA was ligated into the α-MHC promoter expression vector. This DNA construct was microinjected into fertilized mouse embryos (C57BL/6J background) and the resulting transgenic mice (IRF9-TG) were PCR-genotyped using the tail genomic DNA and the following primers: forward: 5'-ATCTCCCCCATAAGAGTTTGAGTC-3' and reverse: 5'-GTTGAGGGCACAGCGTAG-3'. Global IRF9 knockout mice (IRF9-/-, C57BL/6J background) were obtained from Dr. Taniguchi Tadatsugu (The University of Tokyo). The IRF9-/- and the IRF9-TG mice as well as their wild-type littermates (C57BL/6J background) were analyzed at 8 to 10 weeks.

A pressure overload was produced by aortic banding (AB) as previously described1-5. Briefly, thoracic aortas were located following dissection at the second intercostal space after mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Sigma, St. Louis, MO, USA) and did not display any foot reflex. The thoracic aortas were banded against a 27G (for body weights of 24-25 g) or 26G (for body weights of 26-27 g) needle that was removed before closing the thoracic cavity with a 7-0 silk suture. In addition, Doppler analysis was used to verify adequate constriction of the aortas. A sham-operated group underwent a similar procedure without aortic constriction. For ISO infusion, ISO was dissolved in 50 mM ascorbic acid and administered for 4 weeks (30 mg/kg/day) by subcutaneously implanting Alzet osmotic minipumps (Alzet model 2004).

Echocardiography and Hemodynamic Measurements
After the indicated time, the surviving mice were anesthetized with 1.5-2% isoflurane and subjected to echocardiography and hemodynamic measurements to examine cardiac function and structure as previously described2,6,7. To measure the left ventricle (LV) end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD) and LVFS [(LVFS (%) = (LVEDD-LVESD/LVEDD) × 100%], we
performed echocardiography with a Mylab30CV (ESAOTE) machine equipped with a 15-MHz probe. M-mode tracings were recorded from the short axis of the left ventricle, and the LV internal diameter was measured based on at least three beats. The hemodynamic parameters were determined using a 1.4-French Millar catheter-tip micromanometer catheter (SPR-839; Millar Instruments) inserted through the right carotid artery into the left ventricle. An Aria pressure-volume conductance system coupled with a Powerlab/4SP A/D converter was used to continuously record and store the pressure and dp/dt, which were then displayed on a personal computer.

**Histological Analysis**
The animals were sacrificed 4 weeks after AB or sham surgery. The hearts were arrested with a 10% potassium chloride solution at end-diastole and then fixed in 10% formalin. Paraffin-embedded hearts were cut transversely into 4-5 µm sections. Heart sections were stained with HE and PSR (to detect collagen). Cross-sectional areas of the myocytes were visualized with FITC-conjugated WGA (Invitrogen) staining, and the cell size was measured using a quantitative digital image analysis system (Image-Pro Plus 6.0).

**Cultured NRCMs and Recombinant Adenoviral Vectors**
Primary cultures of NRCMs from 1- to 2-day-old Sprague-Dawley rats were prepared as described previously. Briefly, the ventricles were enzymatically dissociated into individual cardiomyocytes in PBS containing 0.03% trypsin and 0.04% type II collagenase. After removing the fibroblasts using a differential attachment method, NRCMs were plated at a density of 1×10^6 cells/well onto gelatin-coated culture dishes in cardiomyocyte culture medium (DMEM/F12 medium supplemented with 20% fetal calf serum, 0.1 mM BrdU and penicillin/streptomycin). To induce hypertrophy, cardiomyocytes were maintained in serum-free DMEM/F12 for 12 hours and treated with ISO (10 µM) and Ang II (1 µM). AdIRF9, a recombinant adenovirus that expresses rat IRF9, was generated by subcloning full-length rat IRF9 cDNA under the control of the cytomegalovirus (CMV) promoter into a replication-defective adenoviral vector. AdIRF9 was modified to express green fluorescent protein (GFP). Three rat shIRF9 constructs were obtained from SABiosciences (KR48866G). Subsequently, AdshIRF9 adenoviruses were generated, and the construct that led to the most significant decrease in IRF9 levels was selected for further experiments. AdshRNA was used as the control. NRCMs were infected with recombinant adenoviruses at a multiplicity of infection (MOI) of 25 particles per cell for 24 hours.

**Immunofluorescence Analysis**
Immunofluorescence analysis was performed using standard immunocytochemical techniques. Briefly, NRCMs cultured on gelatin-coated cover slips were infected with the indicated recombinant adenoviruses for 24 hours and then treated with hypertrophic stimuli for 48 hours. Subsequently, the cardiomyocytes were fixed with 3.7% formaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 in PBS for 40 minutes, blocked with a 10% BSA solution for 1 hour at room temperature and
then incubated with an anti-α-actinin antibody (1:100 dilution). The surface areas were measured using Image-Pro Plus 6.0 software.

**Western Blotting and Quantitative Real-Time PCR**

Whole-cell lysates were obtained by homogenizing the hearts or NRCMs in RIPA lysis buffer. The proteins (50 μg) were resolved via SDS-PAGE (Invitrogen) and transferred to a PVDF membrane (Millipore). The membrane was blocked with 10% non-fat milk and then incubated with the indicated primary antibodies overnight at 4°C. The membrane was then incubated with a secondary IRDye® 800CW-conjugated antibody (Li-Cor Biosciences), and an Odyssey Imaging System (Li-Cor Biosciences) was used for signal detection. Total RNA was isolated from heart tissues or NRCMs using TRIzol Reagent (Invitrogen), and the Transcriptor First Strand cDNA Synthesis Kit (Roche) was used to synthesize cDNA. The mRNA levels of the indicated genes were quantified with real-time PCR using SYBR Green (Roche). The real-time PCR primers that were used are shown in Table S4.

**Plasmid Constructs**

EGFP-myc-IRF9, GST-IRF9 and IRF9 fragments consisting of residues 1 to 120, 210 to 393 and 120 to 220 were generated as previously reported. Flag-myocardin and pCherry-myocardin constructs were generated by amplifying the coding region of the myocardin gene with the primers MyoD-5’ and MyoD-3’ from human cDNAs and cloning the gene into psi-Flag-C1 and psi-Cherry-C1. To obtain the myocardin fragments comprising residues 1 to 738 and 738 to 938, Flag-myocardin was amplified via PCR with MyoD-5’ and MyoD-N-3’ or MyoD-C-5’ and MyoD-3’, respectively. The products were digested with XhoI and ligated into psi-Flag to create an in-frame fusion with the Flag tag. The primers used to generate these constructs are provided in Table S3. All plasmids were verified by sequencing.

**Immunoprecipitation and the GST Pull-down Assay**

EGFP-myc-IRF9 and Flag-myocardin were co-transfected into 293T cells for 48 hours. The cells were lysed at 4°C in immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche), and then sonicated. The resulting lysates were incubated for 20 minutes at 4°C and then centrifuged at 13,000×g for 15 minutes at 4°C. For each IP, 500 μl of the supernatant was incubated with 1 μg of the indicated antibodies and 10 μl of Protein A/G-agarose beads (11719394001, 11719386001, Roche) on a rocking platform overnight at 4°C. The beads were washed 5–6 times with cold IP buffer, and the proteins were subsequently analyzed by Western blotting.

The GST-IRF9 plasmid was transformed into Rosetta (DE3) E. coli. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG) for 4-6 hours, 10 ml of the culture was harvested, and the GST fusion protein was purified using glutathione-sepharose 4B beads (GE Healthcare Bio-Sciences AB). Immobilized GST-IRF9 fusion proteins were incubated with Flag-Myocardin -transfected 293T cell
lysates in IP buffer for 4 hours at 4 °C. The beads were washed four times with IP buffer before the loading buffer was added and then analyzed by Western blotting. The GST tag was used as a negative control under the same conditions.

**Luciferase Reporter Assays**

The SRF-luc plasmid was obtained by cloning approximately 1,500 bp of the SRF promoter (-1354~+184) into the pGL3-basic vector (Promega). The CArG-luc plasmid was produced by inserting three CArG consensus sequences into the pGL3-promoter vector (Promega). The ANP-luc plasmid was constructed as previously reported. The primers used to make this construct are listed in Table S3. NRCMs and H9C2 cells were cultured in 24-well plates. NRCMs were infected with the indicated recombinant adenoviruses, and the plasmids encoding myocardin, p300 and IRF9 together with 3xCArG-luc or ANP-luc were co-transfected into H9C2 cells. The cells were harvested 48 hours later and lysed with 100 µl of passive lysis buffer (PLB, Promega). After removing cell debris by centrifugation, the supernatant was used for luciferase assays performed using a Single-Mode SpectraMax® Microplate Reader according to the manufacturer’s instructions. Luciferase activities were normalized based on protein content.

A Cignal 45-Pathway Reporter Array (SABiosciences, CCA-901L) was used to simultaneously test 45 different pathways in H9C2 cells according to the manufacturer’s protocol. Briefly, 200ng of GFP or IRF9 plasmid diluted in 50µl Opti-MEM® was added to each well of the Cignal Finder Array plate, and then resuspend the reporter assay constructs resident in the well. After incubating at room temperature for 5 minutes, 0.6 µl of Attractene Transfection Reagent (QIAGEN) diluted in 50 µl of Opti-MEM® was added to each well. Cells were suspended in Opti-MEM® containing 10% of fetal bovine serum and then 50 µl of the cell suspension was added into each well and mixed with the constructs-Attractene complexes. After 16 hours of transfection, the medium was replaced by complete growth medium (DMEM with 10% FBS, 0.1mM NEAA, 1mM Sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin). After transfection for 48 hours, the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

**Electrophoretic mobility shift assays (EMSA)**

The EMSA assay was performed as our previously described. Sequence of the DNA oligonucleotide was 5’-CCGAACTGATAACTTTAAAAGGGCATCTTCG C-3’. 1µL 32P-labeled oligonucleotide probe was incubated with10µg nuclear protein of mouse hearts for 30 min at room temperature in binding buffer (20 mM Hepes, 25 mM KCl, 10% glycerol, 2 mM MgCl2 and 10 mM DTT).Reaction was stopped by adding 1µL of loading buffer and the mixture was subjected to 5% polyacrylamide gel electrophoresis in 0.5 x TBE buffer. After electrophoresis (390V, 1hour), the gel was autoradiographed.

**Statistical analysis**
The data are presented as the mean ± SD. Statistical analysis was performed using SPSS 13.0 software to conduct an unpaired 2-tailed Student’s \( t \) test (for two groups) and a one-way or two-way ANOVA test followed by the Bonferroni post hoc test (for more than two groups). \( P \) values of less than 0.05 were considered statistically significant.

References


Supplemental Tables

Table S1 Anatomic and Echocardiographic Analysis in 10-12 Week Old Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IRF9-/- mice (n=11)</th>
<th>IRF9+/- mice (n=10)</th>
<th>IRF9 TG mice (n=8)</th>
<th>IRF9 NTG mice (n=10)</th>
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<tbody>
<tr>
<td>BW (g)</td>
<td>27.17±1.68</td>
<td>27.58±1.31</td>
<td>27.23±1.18</td>
<td>27.11±1.26</td>
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<td>HW/BW(mg/g)</td>
<td>4.21±0.34</td>
<td>4.08±0.22</td>
<td>4.05±0.10</td>
<td>4.10±0.24</td>
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<tr>
<td>LW/BW(mg/g)</td>
<td>5.24±0.35</td>
<td>5.32±0.35</td>
<td>5.23±0.27</td>
<td>5.24±0.27</td>
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<tr>
<td>HW/TL(mg/mm)</td>
<td>6.17±0.32</td>
<td>6.21±0.42</td>
<td>6.04±0.27</td>
<td>6.07±0.36</td>
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<tr>
<td>HR(bpm)</td>
<td>549±33</td>
<td>540±28</td>
<td>567±48</td>
<td>551±54</td>
</tr>
<tr>
<td>LVEDd(mm)</td>
<td>3.50±0.10</td>
<td>3.50±0.09</td>
<td>3.54±0.11</td>
<td>3.58±0.11</td>
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<tr>
<td>LVESd(mm)</td>
<td>1.94±0.11</td>
<td>1.90±0.10</td>
<td>1.98±0.04</td>
<td>1.98±0.18</td>
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<tr>
<td>LVEF (%)</td>
<td>81.6±4.3</td>
<td>83.2±2.0</td>
<td>81.4±3.0</td>
<td>82.0±3.7</td>
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<tr>
<td>LVFS (%)</td>
<td>44.4±4.2</td>
<td>45.6±1.5</td>
<td>44.0±2.4</td>
<td>45.0±3.7</td>
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</table>

All values are presented as the mean ± SD.

BW=body weight; HW=heart weight; LW=lung weight; TL=tibial length; HR=heart rate; LVEDd=left ventricular end-diastolic diameter; LVESd=left ventricular end-systolic diameter; LVEF=Left ventricular ejection fraction; LVFS=Left ventricular fractional shortening.
Table S2 Parameters in IRF9+/− and IRF9+/+ mice 4 weeks after sham or AB operation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham 4W</th>
<th>AB 4W</th>
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<tbody>
<tr>
<td>IRF9+/+ (n=10)</td>
<td>IRF9−/− (n=10)</td>
<td>IRF9+/+ (n=10)</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>523±37</td>
<td>524±32</td>
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<tr>
<td>LVEDd (mm)</td>
<td>3.50±0.07</td>
<td>3.47±0.07</td>
</tr>
<tr>
<td>LVESd (mm)</td>
<td>1.85±0.05</td>
<td>1.80±0.13</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>84.8±1.8</td>
<td>85.0±2.8</td>
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<tr>
<td>LVFS (%)</td>
<td>47.3±2.1</td>
<td>48.0±3.1</td>
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<tr>
<td>ESP (mmHg)</td>
<td>112.09±4.67</td>
<td>106.60±4.44</td>
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<tr>
<td>dp/dt max (mmHg/sec)</td>
<td>10390.01±1251.38</td>
<td>10063.63±1190.16</td>
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<tr>
<td>dP/dt min (mmHg/sec)</td>
<td>-8494.54±806.92</td>
<td>-8183.06±692.44</td>
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*P<0.05 versus IRF9+/− sham operation. †P<0.05 versus IRF9+/+ AB after 4 weeks.
All values are presented as the mean ± SD.
LVESP=Left ventricular End-systolic Pressure.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham 4W NTG (n=10)</th>
<th>Sham 4W TG (n=10)</th>
<th>AB 4W NTG (n=10)</th>
<th>AB 4W TG (n=10)</th>
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<td>HR (bpm)</td>
<td>538±43</td>
<td>537±32</td>
<td>495±32</td>
<td>525±53</td>
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<td>LVEDd (mm)</td>
<td>3.43±0.05</td>
<td>3.46±0.05</td>
<td>4.42±0.06*</td>
<td>4.19±0.06*†</td>
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<td>LVESd (mm)</td>
<td>1.83±0.08</td>
<td>1.80±0.08</td>
<td>3.13±0.07*</td>
<td>2.79±0.12*†</td>
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<td>LVEF (%)</td>
<td>84.0±2.3</td>
<td>85.3±1.9</td>
<td>63.4±3.2*</td>
<td>70.0±2.9*†</td>
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<tr>
<td>LVFS (%)</td>
<td>46.6±2.4</td>
<td>48.1±2.2</td>
<td>29.2±1.6*</td>
<td>33.4±2.4*†</td>
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<tr>
<td>ESP (mmHg)</td>
<td>102.71±2.76</td>
<td>114.41±8.59</td>
<td>163.93±14.66*</td>
<td>168.99±17.51*</td>
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<tr>
<td>dp/dt max</td>
<td>10810.14±1056.14</td>
<td>10724.71±2092.15</td>
<td>7961.38±877.86*</td>
<td>10305.00±1781.31*†</td>
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<tr>
<td>dP/dt min</td>
<td>-9192.43±690.48</td>
<td>-9198.50±1526.85</td>
<td>-6850.50±702.91*</td>
<td>-8810.29±880.34*†</td>
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</tbody>
</table>

*P<0.05 versus NTG sham operation. †P<0.05 versus NTG AB after 4 weeks.
All values are presented as the mean ± SD.
Table S4 Parameters in IRF9\(^{+/+}\), IRF9\(^{-/-}\), IRF9 NTG, IRF9 TG mice after treatment with ISO or saline for 4 weeks.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Saline 4W</th>
<th>ISO 4W</th>
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<th>ISO 4W</th>
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<tbody>
<tr>
<td></td>
<td>IRF9(^{+/+}) (n=10)</td>
<td>IRF9(^{-/-}) (n=10)</td>
<td>IRF9(^{+/+}) (n=10)</td>
<td>IRF9(^{-/-}) (n=10)</td>
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<tr>
<td>HR (bpm)</td>
<td>492±56</td>
<td>518±52</td>
<td>537±57</td>
<td>477±33</td>
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<tr>
<td>LVEDd (mm)</td>
<td>3.54±0.08</td>
<td>3.50±0.08</td>
<td>4.44±0.05*</td>
<td>5.12±0.08*†</td>
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<tr>
<td>LVESd (mm)</td>
<td>1.86±0.12</td>
<td>1.85±0.10</td>
<td>3.10±0.01*</td>
<td>3.96±0.08*†</td>
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<td>LVEF (%)</td>
<td>84.8±2.8</td>
<td>84.5±2.7</td>
<td>65.8±1.0*</td>
<td>49.9±2.5*†</td>
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<tr>
<td>LVFS (%)</td>
<td>47.5±2.8</td>
<td>47.2±2.9</td>
<td>30.4±0.5*</td>
<td>22.8±1.0*†</td>
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</table>

\(^*^P<0.05\) versus IRF9\(^{+/+}\) or NTG Saline operation. \(\dagger P<0.05\) versus IRF9\(^{+/+}\) or NTG ISO after 4 weeks.

All values are presented as the mean ± SD.
Table S5 The primers for vector construct.

<table>
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<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
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<tr>
<td>MyoD-5'</td>
<td>CCGCTCGAGATGACACTCCTGGGGTGCTGAG</td>
</tr>
<tr>
<td>MyoD-3'</td>
<td>CCGCTCGAGCTACCACTGCTGCAAGTGAGAAG</td>
</tr>
<tr>
<td>MyoD-N-3'</td>
<td>CCGCTCGAGCTAATCCATCTGCTGACTCCGGGT</td>
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<td>MyoD-C-5'</td>
<td>CCGCTCGAGCGACAGATGGATGAACCTCCTG</td>
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<tr>
<td>SRF-promoter-5'</td>
<td>GCCAGATTTGTGCTACTTGGTTCCAGGGA</td>
</tr>
<tr>
<td>SRF-promoter-3'</td>
<td>GCCAGTTCTAGTGCCGCTATCGCTG</td>
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The underlined sequences are the sites for restriction enzyme

Table S6 The primers for Real-Time PCR.

<table>
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<th>Primer name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>IRF9-Mouse</td>
<td>ACAACTGAGGGCCACCATTAGAGA</td>
<td>CACCACCTCGGCCACCATAAG</td>
</tr>
<tr>
<td>ANP-Mouse</td>
<td>ACCTGCTAGACCACCTGGAG</td>
<td>CTTTGCTGTATCTTCGCTACCGG</td>
</tr>
<tr>
<td>β-MHC-Mouse</td>
<td>CCGAGTCCCAGGTCAACAA</td>
<td>CTTCAACGGGCAACCTTGGGA</td>
</tr>
<tr>
<td>BNP-Mouse</td>
<td>GAGGTCACTCCTATCTCTCTGG</td>
<td>GCCATTTGCCTCGACTTCTCTC</td>
</tr>
<tr>
<td>CTGF-Mouse</td>
<td>TGACCCCTCAGCACCACA</td>
<td>TACACCCGACCCAGAACACACAG</td>
</tr>
<tr>
<td>Collagen I-Mouse</td>
<td>AGGCTTCATGGGTGGTGATG</td>
<td>CAACGACAGCACCACAGTTA</td>
</tr>
<tr>
<td>Collagen III-Mouse</td>
<td>CCAACCCAGAGATCCCATT</td>
<td>GAAGCAGGAGGCAGGGTGATAG</td>
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Supplemental Figures

Figure S1. IRF9 is induced by hypertrophic stimuli. Real-time PCR analysis of IRF9 in AB-induced cardiac hypertrophy at the indicated time. n=4 mice per group, *P<0.05 vs. sham. Data are presented as the mean ± SD.

Figure S2. IRF9 deficiency in the heart exaggerates AB-induced cardiac hypertrophy. (A) Western blot analysis for IRF9 in whole-cell lysates from the hearts of wild-type (IRF9+/+) and knockout (IRF9-/−) mice. (B) The cumulative survival rate of IRF9+/+ and IRF9-/− mice after Sham or AB surgery. (C) Expression levels of the indicated hypertrophic markers in the hearts of sham- and AB-treated IRF9+/+ and IRF9-/− mice. Real-time PCR analyses were performed, and the results are representative of three independent experiments. n=4 mice for each group. (D) mRNA levels of CTGF, collagen I and collagen III in the heart samples from IRF9+/+ and IRF9-/− mice subjected to AB or sham treatment. n=4 mice per group. Data are presented as the mean ± SD; *P<0.05 vs. IRF9+/+ sham; #P<0.05 vs. IRF9+/+ AB.
Figure S3. IRF9-TG mice are protected from AB-induced cardiac hypertrophy.
(A) A schematic diagram depicting the construction of IRF9-TG mouse lines. (B) IRF9 expression analysis of heart extracts from TG mice and their WT littermates. (C) The cumulative survival rate of NTG and IRF9-TG mice after Sham or AB surgery. (D) CTGF, collagen I and collagen III mRNA levels in heart samples from NTG and IRF9-TG mice subjected to sham or AB. n=4 mice per group. (E) Real-time PCR analysis of the indicated hypertrophic markers in sham- or AB-treated NTG and IRF9-TG mice, n=4 mice per group. Data are presented as the mean ± SD; *P<0.05 vs. NTG sham; #P<0.05 vs. NTG AB.
Figure S4. IRF9 attenuates ISO-induced cardiac hypertrophy. (A) HW/BW, LW/BW and HW/TL ratios in IRF9+/+ and IRF9-/- mice treated with saline or ISO. n=15 for each group. (B) Histological analysis of the hearts sections from IRF9+/+ and IRF9-/- mice after ISO or saline infusion (30 mg/kg/day for 4 weeks)(n=10 mice per group). Heart cross-sections were stained with H&E, indicating hypertrophic growth (top row: scale bars represent 100 μm; second row: scale bars represent 20 μm); WGA staining was performed to determine cell boundaries (third row: scale bars represent 20 μm); fibrosis was detected by PSR staining (fourth and fifth rows: scale bars represent 20 μm). (C and D) Quantification of cardiomyocyte cross-sectional and fibrotic areas in saline or ISO-induced IRF9+/+ and IRF9-/- mice hearts. n=100+ for each group. For B-D, data are presented as the mean ± SD; *P<0.05 vs. IRF9+/+ saline; #P<0.05 vs. IRF9+/+ ISO. (E and F) mRNA levels of the indicated fibrotic markers and hypertrophic fetal genes in saline or ISO-treated IRF9+/+ and IRF9-/- mice. n=4 for each group. Data are presented as the mean ± SD; *P<0.05 vs. IRF9+/+ mice with saline; #P<0.05 vs. IRF9+/+ with ISO. (G) HW/BW, LW/BW and HW/TL ratios in NTG and IRF9-TG mice induced by saline or ISO. n=15 for each group. (H) Histological analysis of the hearts sections from NTG and IRF9-TG mice after ISO or saline infusion. Detection was as described in A. (I and J) Quantification of cardiomyocyte cross-sectional (I) and fibrotic areas (J) in saline or ISO-treated NTG and IRF9-TG hearts. n=25+ for each group. For F-H, data are presented as the mean ± SD; *P<0.05 vs. NTG/saline; #P<0.05 vs. NTG/ISO. (K and L) Real-time PCR
analysis of the indicated fibrotic markers and hypertrophic fetal genes in NTG and IRF9-TG mice after saline or ISO infusion. n=4 mice for each group. Data are presented as the mean ± SD; *P<0.05 vs. NTG saline; #P<0.05 vs. NTG ISO.

Figure S5. IRF9 regulates the transcriptional activity of myocardin. (A) Luciferase activity (fold change) of reporter linked to the CArG box (left) or the ANP promoter (right) after overexpression of IRF9 in Ang II-stimulated NRCMs. (B) Luciferase activity (fold change) of SRF after overexpression of IRF9 in Ang
II-stimulated NRCMs. (C) Luciferase activity (fold change) of reporter linked to the CArG box (left) or the ANP promoter (right) after overexpression of myocardin and IRF9 in NRCMs. (D) The myocardin transcriptional activity determined by EMSA assay in IRF9+/+ and IRF9-/− mice after Sham or AB operation. (E) Luciferase activity (fold change) of UAS after overexpression of GAL4-myocardin and IRF9 in NRCMs. (F) Luciferase activity (fold change) of reporter linked to the CArG box (left) or ANP promoter (right) after overexpression of the IRF9 mutant, IRF9 and myocardin in NRCMs. (G) Luciferase activity (fold change) of reporter linked to the CArG box (left) or ANP promoter (right) after overexpression of myocardin, p300 and IRF9 in NRCMs. (H) H9C2 cells were transfected with expression vectors for myocardin (1 μg) and p300 (0.5 and 1 μg) as well as luciferase activity (fold change) of reporter linked to the CArG box (left) and ANP promoter (right), respectively, and then assayed for luciferase activity. *P<0.05 vs. control. (I) Luciferase activity (fold change) of reporter linked to the CArG box (left) and ANP promoter (right) after overexpression of myocardin, p300 and IRF9 in H9C2 cells. (J) NRCMs were infected with the indicated adenoviruses (AdGFP, Admyocardin or AdshIRF9) and the luciferase reporters of ANP (wild type and CArG box mutant ANP promoters) for 48 hours and assayed for luciferase activity (fold change). ANP-1000: luciferase reporter linked to 1,000 bp of the ANP promoter; ANP-236: luciferase reporter linked to 236 bp of the ANP promoter; ANP-121: luciferase reporter linked to 121 bp of the ANP promoter. Data are presented as the mean ± SD and are representative of three independent experiments.