Heart

Rnd3/RhoE Modulates Hypoxia-Inducible Factor 1α/Vascular Endothelial Growth Factor Signaling by Stabilizing Hypoxia-Inducible Factor 1α and Regulates Responsive Cardiac Angiogenesis

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Abstract—The insufficiency of compensatory angiogenesis in the heart of patients with hypertension contributes to heart failure transition. The hypoxia-inducible factor 1α–vascular endothelial growth factor (HIF1α–VEGF) signaling cascade controls responsive angiogenesis. One of the challenges in reprogramming the insufficient angiogenesis is to achieve a sustainable tissue exposure to the proangiogenic factors, such as HIF1α stabilization. In this study, we identified Rnd3, a small Rho GTPase, as a proangiogenic factor participating in the regulation of the HIF1α–VEGF signaling cascade. Rnd3 physically interacted with and stabilized HIF1α, and consequently promoted VEGFA expression and endothelial cell tube formation. To demonstrate this proangiogenic role of Rnd3 in vivo, we generated Rnd3 knockout mice. Rnd3 haploinsufficient (Rnd3+/−) mice were viable, yet developed dilated cardiomyopathy with heart failure after transverse aortic constriction stress. The poststress Rnd3+/− hearts showed significantly impaired angiogenesis and decreased HIF1α and VEGFA expression. The angiogenesis defect and heart failure phenotype were partially rescued by cobalt chloride treatment, a HIF1α stabilizer, confirming a critical role of Rnd3 in stress-responsive angiogenesis. Furthermore, we generated Rnd3 transgenic mice and demonstrated that Rnd3 overexpression in heart had a cardioprotective effect through reserved cardiac function and preserved responsive angiogenesis after pressure overload. We assessed the expression levels of Rnd3 in the human heart and detected significant downregulation of Rnd3 in patients with end-stage heart failure. We concluded that Rnd3 acted as a novel proangiogenic factor involved in cardiac responsive angiogenesis through HIF1α–VEGFA signaling promotion. Rnd3 downregulation observed in patients with heart failure may explain the insufficient compensatory angiogenesis involved in the transition to heart failure. (Hypertension. 2016;67:597-605. DOI: 10.1161/HYPERTENSIONAHA.115.06412.) • Online Data Supplement

Key Words: angiogenesis factor ■ G-protein ■ heart failure ■ hypertension ■ hypertrophy ■ vascular endothelial growth factor A

Hypertension is one of the most prevalent myocardial stress factors for the development of heart failure. Neovascularization is a compensatory mechanism in the heart in response to hemodynamic stress. The compounding effect of the increased cardiac demand for oxygen and nutrients and responsive cardiac angiogenesis contributes to the transition into pathological adaption, eventually leading to heart failure.1–4 Activation of the cardiac angiogenesis program is a promising therapeutic strategy validated in multiple forms of cardiac disease animal models.5–8 The hypoxia-inducible factor 1α–vascular endothelial growth factor (HIF1α–VEGF) molecular signaling cascade is the most powerful and well-studied signaling pathway in ischemia/hypoxia-induced angiogenesis. Preclinical animal studies using proangiogenic factors such as VEGF, HIF1α, and fibroblast growth factor have achieved remarkable success.7–12 However, the translation of these animal studies to clinical therapy is intermittent, and the results are far from satisfactory.5,6,13,14 Several important lessons have
been demonstrated in clinical trials. One of the challenges in clinical practice is to achieve sustainable tissue exposure to proangiogenic factors to achieve a prolonged stimulation. One possible solution is through the stabilization of HIF1α, which is unstable and quickly degraded by ubiquitin proteasome system under normoxic conditions. Another challenge is to develop new patient-relevant animal models for basic research. Therefore, identifying a novel factor to stabilize key proangiogenic factors as well as establishing a new animal model closely related to human heart failure are critical strategies for the resolution of the serious deficiency in currently available information pertaining to translational studies of angiogenesis involvement in heart failure.

Our recent study discovered that Rnd3, a small Rho GTPase also called RhoE, was a cardiac protective factor and protected the heart from pressure overload–induced heart failure. Mice with Rnd3 haploinsufficiency developed severe heart failure after pressure overload challenge. However, the associated molecular mechanism remains largely unknown. In this study, we demonstrated Rnd3 as an important proactive factor for adaptive myocardial angiogenesis in response to pressure overload. We revealed the molecular mechanism of Rnd3-mediated angiogenesis regulation through the stabilization of HIF1α, a key proangiogenic transcriptional factor. The findings uncovered a new function of this small GTPase in the refinement of the HIF1α–VEGFA regulatory pathway, and provided a potential new target for pharmacological manipulation of HIF1α–VEGF signaling. The assessment of Rnd3 expression levels in patients could be a new reference biomarker for human heart failure.

In summary, this study uncovered a proangiogenic function of Rnd3 in the regulation of the HIF1α–VEGFA signaling pathway. The decrease in Rnd3 expression levels contributed to the insufficiency of adaptive coronary angiogenesis in the mouse heart in response to hemodynamic stress. The discovery of a significant downregulation in Rnd3 levels in myocardia of patients with heart failure implicated the translational and clinical significance of these findings.

**Methods**

Methods and associated references were available in detail in the online-only Data Supplement of the article, which included the approval of animal protocol and human heart tissue usage, generation of the mutant mouse lines, and assessments of cardiac function and angiogenesis.

**Statistics**

Data are expressed as means±SD. In multiple group comparisons, 1-way ANOVA followed by Student–Newman–Keuls method was used. In 2 group comparisons, unpaired, 2-tailed student’s t test was used. All the analyses were conducted by SigmaPlot 11.0 (Systat, San Jose, CA). A value of P<0.05 was considered statistically significant.

**Results**

**Expression of Rnd3 Was Downregulated in End-Stage Human Failing Hearts**

The study of the role of Rnd3 in heart failure was initiated by analyzing human heart tissue samples. Rnd3 protein levels were measured in 12 normal and 51 human failing hearts.

In patients with end-stage heart failure, we found a 57.9% decrease in the Rnd3 expression levels (Figure 1). This clinical observation suggested that Rnd3 may act as a potential new regulator in the pathogenesis of the transition to heart failure. Systemic and comprehensive experiments based on genetic Rnd3 knockout mice were conducted for the understanding of the underlying molecular mechanisms.

**Rnd3 Deficiency Resulted in an Angiogenesis Defect in the Mouse Heart in Response to Pressure Overload**

Our recent study found that Rnd3 haploinsufficient (Rnd3+/−) mice were hypersensitive to pressure overload and developed heart failure after transverse aortic constriction (TAC) challenge. However, the severity of cardiac fibrosis and hypertrophy showed no significant differences between the wild-type mice and Rnd3 mutant mice (Figure S1). To investigate the underlying molecular mechanisms, we conducted a series of experiments. We first analyzed cardiac capillary generation in the mouse hearts after TAC. Compensatory increases in the capillary area and capillary number were found in WT mice in response to TAC stress as expected. However, we failed to detect the responsive angiogenesis in Rnd3+/− mice (Figure 2A and 2B). Significantly smaller capillary areas and fewer capillary numbers were observed in the Rnd3 haploinsufficient mouse heart compared with the WT control after TAC (Figure 2A and 2B). The impaired angiogenesis was further indicated by the decrease in capillary/cardiomyocyte ratio (Figure S2). In line with the morphological changes, the upregulation of endothelial-specific receptor tyrosine kinase 2 was detected in the WT mouse heart in response to TAC but not in the Rnd3+/− heart (Figure 2C). To assess coronary microvascular circulation, coronary flow reserve was measured. Rnd3+/− hearts displayed no abnormality in the microvascular function compared with WT hearts before stress (Figure S3, Sham); however, a 20.8% reduction of coronary flow reserve was detected in the Rnd3+/− heart after TAC (Figure S3, TAC). Cardiac tissue hypoxia staining displayed a 5.9-fold increase in hypoxic...
areas in the Rnd3+/− heart compared with the WT control heart (Figure 2D). These data strongly suggested that the adaptive coronary angiogenesis was severely impaired in the Rnd3+/− heart in response to hemodynamic challenge.

**Rnd3 Deficiency Led to the Downregulation of Key Proangiogenic Factors, HIF1α and VEGFA, in the Mouse Heart in Response to Pressure Overload**

HIF1α and its downstream target VEGFA are critical for responsive angiogenesis in the heart after stress. These factors were evaluated to better understand the effect of Rnd3 deficiency in angiogenesis. As expected, the responsive increase in levels of HIF1α and VEGFA proteins was detected in WT animal hearts after TAC (Figure 3A). However, the responsive increase in levels of HIF1α and VEGFA proteins was not detected in Rnd3-deficient hearts after pressure overload (Figure 3A), which was consistent with the observation of impaired angiogenesis. Because VEGFA was transcriptionally regulated by transcription factor HIF1α, we measured VEGFA transcripts in the hearts. Again, the responsive increase in VEGFA by TAC was attenuated by Rnd3 deficiency (Figure 3B).

Cardiomyocytes are the major source of VEGFA as a paracrine effector for angiogenesis in the heart. To investigate the role of Rnd3 in relation with proangiogenic factors, we conducted human umbilical vein cell (HUVEC) tube formation experiments by treating the cells with different cardiomyocyte culture media. We found that fewer tubes were developed in the culture media from Rnd3+/− cardiomyocytes, whereas the tube formation was stimulated by the media from the WT cardiomyocytes (Figure 3C and 3D). Significantly lower levels of VEGFA were detected in the Rnd3+/− cardiomyocyte culture media compared with the WT group, indicating downregulation of the HIF1α–VEGFA pathway in cardiomyocytes with Rnd3 deficiency (Figure 3E).

**Rnd3 Stabilized HIF1α Protein and Facilitated VEGFA Expression**

To investigate if HIF1α protein levels could be directly regulated by Rnd3, we knocked down and overexpressed Rnd3 in cell cultures. We found that HIF1α protein levels were decreased when Rnd3 was knocked down (Figure 4A). Meanwhile, gradual increases in the expression levels of HIF1α were detected corresponding with incremental increases in Rnd3-deficient hearts after pressure overload (Figure 3A), which was consistent with the observation of impaired angiogenesis. Because VEGFA was transcriptionally regulated by transcription factor HIF1α, we measured VEGFA transcripts in the hearts. Again, the responsive increase in VEGFA by TAC was attenuated by Rnd3 deficiency (Figure 3B).

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Next, because HIF1α is a transcription factor, we conducted a luciferase experiment to determine if Rnd3 had any effects on its transcriptional activity. The luciferase reporter was driven by a VEGFA promoter containing HIF1α response elements. Hypoxic conditions were applied to induce HIF1α expression. Relative luciferase activity was measured under both normoxic and hypoxic conditions. Rnd3 knockdown resulted in an ~50% decline in luciferase activity relative to the control level in response to hypoxia (Figure 4F). Consistent with the change in the HIF1α transcriptional activity, a responsive decrease in the VEGFA mRNA levels was detected as well (Figure 4G), indicating that Rnd3 regulated responsive HIF1α transcriptional activity. Downregulation of Rnd3 weakened HIF1α transcriptional activity, leading to a decrease in its target gene transcripts in response to hypoxia.

Hydroxylation of HIF1α at 2 conserved proline residues by HIF1α prolyl hydroxylases is the key step to control HIF1α protein levels. On hydroxylation of HIF1α, it interacts with von Hippel–Lindau tumor suppressor protein, therefore leading to HIF1α protein ubiquitination and degradation under normoxic conditions. We next evaluated the effects of Rnd3 on HIF1α hydroxylation. Because hydroxylated HIF1α degradation is dynamic and extremely fast, cell cultures with Rnd3 overexpression (Myc-Rnd3) and Rnd3 knockdown by siRNA (siRnd3) were first treated with MG132, a cell-permeable proteasome inhibitor, to block degradation of ubiquitin-conjugated proteins. Then hydroxylated HIF1α (hydroxy-HIF1α) levels were analyzed by Western blot. Lower levels of hydroxy-HIF1α were detected in cells with Rnd3 overexpression (Figure 4H). Consistently, increased hydroxy-HIF1α levels were observed under Rnd3 knockdown conditions (Figure 4I). These data strengthened our hypothesis and suggested that Rnd3 stabilized HIF1α by preventing its hydroxylation.

CoCl2 Treatment Rescued Rnd3 Deficiency–Mediated Angiogenesis Defects and Dilated Cardiomyopathy in Rnd3 Haploinsufficient Mice After Hemodynamic Stress

CoCl2 is an inhibitor of prolyl hydroxylase domain-containing protein, which is responsible for the hydroxylation of HIF1α, promoting its eventual degradation. Application of CoCl2 prevents HIF1α protein from degradation. To test if HIF1α down-regulation was responsible for the Rnd3 deficiency–induced cardiac dysfunction and angiogenic defects, we treated mice with CoCl2. Improvement in cardiac function was observed in Rnd3+/− mice after the administration of CoCl2. Echocardiographic assessment revealed partial recoveries in the ejection fractions and fractional shortenings of Rnd3+/− mice with CoCl2 treatment compared with the mice without CoCl2 treatment (Figure 5A). Consistent with the improved cardiac function, hematoxylin and eosin staining (Figure 5B) and cardiac echo analysis (Figure 5C) both displayed less dilation in Rnd3+/− hearts with CoCl2 treatment compared with the nontreated group of animal hearts. The post-TAC survival rate of Rnd3+/− mice was also significantly improved by CoCl2 treatment (Figure 5D).

Finally, we analyzed protein expression levels of HIF1α and VEGFA, along with cardiac capillaries and cardiac coronary flow reserve without CoCl2 treatment in both WT and Rnd3+/− mouse hearts. As expected, TAC stress induced the upregulation of HIF1α and VEGFA in WT animal hearts; however, the Rnd3 haploinsufficient mice failed to respond to pressure overload and displayed no increase in HIF1α and VEGFA protein levels. The treatment of CoCl2 partially recovered the adaptive response to TAC in the Rnd3+/− animal with obvious increases in HIF1α and VEGFA protein levels (Figure 5G). Isolectin staining showed improved capillary densities after CoCl2 applications (Figure 5D and 5E). The impaired coronary flow reserve after TAC was recovered by 13.6% because of CoCl2 administration (Figure 5F).

Rnd3 Overexpression Attenuated the Transition to Heart Failure by Preserving Responsive Angiogenesis

We generated the transgenic Rnd3 overexpression mice (myosin heavy chain [MHC]–Rnd3) to further demonstrate the critical role of Rnd3 in angiogenesis and the hypoxic response of the heart. Elevated cardiac Rnd3 protein levels in the MHC-Rnd3 mice were confirmed by Western blot analysis (Figure 6A). MHC-Rnd3 mice exhibited reserved cardiac function, and they were more resistant to the development of heart
failure after TAC stress compared with the WT mice. In comparison with the WT mice from the Sham group, MHC-Rnd3 mice showed only slight decreases in the ejection fraction and fractional shortening percentages after TAC (Figure 6B). Furthermore, the MHC-Rnd3 mice displayed a conserved LVID in systole detected by echocardiography compared with the WT mice after TAC surgery, indicating a resistance to the development of dilated cardiomyopathy because of Rnd3 overexpression (Figure 6C). Higher coronary flow reserves were measured in the MHC-Rnd3 mice compared with the WT mice after TAC and showed no significant differences compared with the measurements from the WT Sham group, demonstrating preserved capillary function (Figure 6D). In addition, more cardiac capillaries and larger capillary areas were visualized in the MHC-Rnd3 mice after TAC compared with the WT controls (Figure 6E and 6F). These findings suggested that Rnd3 upregulation can prevent the transition to heart failure, and Rnd3 overexpression had multiple beneficial effects under cardiac hypoxic conditions.

**MHC-Rnd3 Cardiomyocytes Exhibited HIF1α-VEGFA Pathway Stimulation**

Elevated HIF1α and VEGFA protein levels were observed, as expected, in the control WT mouse heart in response to TAC by Western blot. However, further increases in these 2 proteins were detected in the MHC-Rnd3 transgenic mouse heart (Figure 7A). To determine the angiogenic effect of Rnd3 overexpression in the animal cardiomyocytes, HUVEC cells were treated with the media from Rnd3+/− and MHC-Rnd3 cardiomyocyte cultures. A significant promotion of HUVEC cell tube formation was observed in the culture treated with media from the MHC-Rnd3 cardiomyocytes. In addition, the previously observed tube formation deficiency of the cells cultured with Rnd3+/− cardiomyocyte media was partially rescued (Figure 7B). The cell tube formation was quantified and analyzed by WimTube software, revealing more loops and branching points in the MHC-Rnd3 culture. Significant improvement in the total number of loops and branching points in the MHC-Rnd3/Rnd3+/− co-culture was also observed.
Finally, VEGFA levels in the media from MHC-Rnd3 and WT cardiomyocyte cultures were measured by ELISA. MHC-Rnd3 cardiomyocyte media revealed significantly higher VEGFA levels compared with the WT control (Figure 7D). Taken together, cardiomyocytes from the MHC-Rnd3 mouse heart released more VEGFA compared with the WT control, indicating increased VEGFA paracrine activity and augmented angiogenesis.

A schematic illustration of the molecular mechanism involving Rnd3 and HIF1α-VEGFA signaling was depicted in Figure S5. Rnd3 prevented HIF1α from hydroxylation by stabilizing it through direct interaction, which then led to increased activation of the VEGF gene. Without the presence of Rnd3, increased HIF1α hydroxylation occurred, which facilitated HIF1α protein degradation through ubiquitin proteasome system and resulted in impaired angiogenesis.

Discussion

The HIF1α-VEGFA signaling pathway is the best studied molecular mechanism in the regulation of hypoxia/ischemia-induced angiogenesis. VEGFA, mainly secreted by cardiomyocytes in the heart, is the major driving force promoting endothelial cell proliferation during angiogenesis. HIF1 works as an αβ-heterodimer that induces transcriptional activation by binding to hypoxia response elements in response to hypoxia. HIF1β is a constitutive nuclear protein, whereas HIF1α is dynamically regulated. In normoxic conditions, HIF1α can be hydroxylated by prolyl hydroxylase domain, and then quickly degraded by von Hippel–Lindau tumor suppressor protein-mediated proteolysis and results in an accumulation of HIF1α protein. The latter translocates into the
nuclei to form a transcriptional complex with HIF1β, p300, and CREB (cAMP response element)-binding protein, and initiates the transcription of many genes including the VEGF family of genes (Figure S5). In this study, we demonstrated that Rnd3 functioned as a new mediator in the angiogenesis process by interacting with and stabilizing HIF1α, adding a new dimension to the regulation of the HIF complex function, which secured HIF1α–VEGFA–mediated angiogenesis progression during stress (Figure S5). Deficiency of Rnd3 compromised the HIF1α–VEGFA signaling and impaired the responsive angiogenesis. Application of CoCl2, a chemical that inhibits prolyl hydroxylase domain and stabilizes HIF1α, partially rescued the Rnd3 haploinsufficient mouse phenotype and resulted in improvement of cardiac functions and coronary flow reserve (Figure S5).

Because drugs can have pleiotropic effects in vivo, we verified the critical role of Rnd3 in the heart through the use of the transgenic Rnd3 overexpression mice. Forced expression of Rnd3 partially rescued the Rnd3+/− angiogenic defect both in vitro and in vivo. Interestingly, a recent study showed Rnd3 as a transcriptional target of HIF1α in gastric cancer cells. If the same regulation existed in the heart, there would be a forward feedback mechanism in which Rnd3 would stabilize HIF1α and the latter would enhance Rnd3 gene transcription.

![Cardiac-specific overexpression of Rnd3](image)

**Figure 6.** Cardiac-specific overexpression of Rnd3 protected the heart from the transition to heart failure by preserving responsive angiogenesis. **A**, Representative Rnd3 protein expression levels in the hearts of wild-type (WT) and Rnd3 transgenic (MHC-Rnd3) mice by Western blot analysis. **B**, MHC-Rnd3 mice have reserved cardiac function and developed less severe heart failure after transverse aortic constriction (TAC). Only slight decreases in the cardiac ejection fractions and fractional shortenings were detected in MHC-Rnd3 mice after TAC compared with WT mice from the Sham group. **C**, No significant increase in the left ventricular internal dimension (LVID) in systole was detected by echocardiography in MHC-Rnd3 mice after TAC compared with the Sham WT mice. **D**, MHC-Rnd3 mice have preserved capillary function after TAC compared with WT mice. **E**, More capillaries were observed in the post-TAC MHC-Rnd3 mouse heart by isolectin staining (green). **F**, Larger capillary areas and more capillary numbers were observed in the MHC-Rnd3 mouse heart compared with the WT control after TAC. Cardiac capillaries were quantified by LAS V4.0 software (Germany). The numbers in the columns represented the number of mice in each group.
Pathological stimuli such as hypertension, ischemia, or chronic neurohormonal hyperactivation are known to promote capillary growth within the myocardium. However, the compensatory enhancement of angiogenesis in response to these pathological stimuli seems to be insufficient, and this insufficiency is one of the critical factors responsible for the transition of the heart from compensated hypertrophy to heart failure.

In this study, we revealed a critical role of Rnd3 in the responsive angiogenesis process in the heart. We detected a 57.9% reduction of Rnd3 protein levels in end-stage human failing hearts. Although the pathogenic meaning of Rnd3 downregulation in patients with heart failure remains unknown, understanding the pathological consequence of Rnd3 down-regulation is clearly important and has clinical implications. The Rnd3 haploinsufficient mouse recapitulated the situation observed in patients with heart failure, and it is a patient-relevant mouse model. Importantly, the mutant mice displayed impaired adaptive coronary angiogenesis that has not previously been linked to Rnd3 function. Given the critical role of HIF1α in proangiogenic signaling, identification of its endogenous stabilizer, Rnd3, offers a new positive regulator toward to the HIF1α–VEGFA signaling pathway. The findings have basic and clinical significance. The Rnd3 mutant mice provide a patient-relevant animal model for further mechanistic investigation.

Rnd3 was originally identified as a repressor of Rho signaling by either directly binding to Rho-associated coiled-coil protein kinase 1 (ROCK1) or indirectly targeting RhoA through p190-B RhoGAP. The importance of Rho kinase ROCK 1 in the heart has been investigated by our laboratory as well as by other groups. We demonstrated that ROCK1-null mice were resistant to pressure overload–induced injury and fibrotic response. In human patients, we first found that ROCK1 was a caspase-3 target. The cleavage of ROCK1 by caspase-3 generated a constitutively active isoform of Rho kinase fragment that was accumulated in human failing hearts. The transgenic mice with forced expression of this cleaved ROCK1 isoform led to fibrotic cardiomyopathy. Treatment with Rho kinase inhibitor reversed the cardiac phenotype and improved cardiac functions. Therefore, it was logical to assume that the Rnd3 deficiency–mediated cardiac dysfunction was through the augmentation of ROCK1 activity. However, by taking the approaches of genetic deletion of ROCK1 and the use of a Rho kinase inhibitor, we recently found that Rho kinase activation was partially, but not entirely, responsible for Rnd3 deficiency–induced cardiomyopathy, suggesting that there was a Rho kinase–independent mechanism regulated by Rnd3.

**Perspectives**

This study extended the previous observation and uncovered a new function of Rnd3, in which Rnd3 participated in the regulation of responsive angiogenesis. The downregulation of Rnd3 resulted in an angiogenic response defect in the animal heart, contributing to the transition to heart failure. The associated molecular mechanism was conceived that Rnd3 bound to HIF1α, and stabilized HIF1α through the inhibition of its hydroxylation and prevented the protein from degradation. Downregulation of Rnd3 resulted in an increase in HIF1α hydroxylation and a subsequent decrease in its protein levels; which in turn impaired HIF–VEGF signaling and attenuated responsive angiogenesis progression. The findings uncovered a new function of this small GTPase in the refinement of the HIF1α–VEGF regulatory pathway, and provided a potentially new target of pharmacological manipulation for HIF1α–VEGF signaling. The assessment of Rnd3 expression levels in patients could be a new reference biomarker for human heart failure.

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**Disclosures**

None.
References


Novelty and Significance

What Is New?

• This study uncovered previously undescribed function of Rnd3 and the associated molecular mechanism. Rnd3 was a proangiogenic factor involved in cardiac responsive angiogenesis through hypoxia-inducible factor 1α–vascular endothelial growth factor signaling promotion.

• A significant decrease level of Rnd3 protein was detected in human failing myocardia.

• Genetic deletion of Rnd3 in mice impaired angiogenic response in heart in response to pressure overload.

What Is Relevant?

• Neovascularization is a compensatory mechanism in heart in response to hypertension.

• The insufficiency of compensatory angiogenesis in heart in patients with hypertension contributes to the transition to heart failure.

Summary

This study demonstrated a proangiogenic role of Rnd3 through the regulation of the hypoxia-inducible factor 1α–vascular endothelial growth factor signaling pathway. The decrease in Rnd3 expression levels contributed to the insufficiency of adaptive coronary angiogenesis in the mouse heart in response to pressure overload. The finding of the significant downregulation of Rnd3 in myocardia of patients with heart failure implicated the translational and clinical significance of this discovery.
Rnd3/RhoE Modulates Hypoxia-Inducible Factor 1α/Vascular Endothelial Growth Factor Signaling by Stabilizing Hypoxia-Inducible Factor 1α and Regulates Responsive Cardiac Angiogenesis

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Rnd3/RhoE Modulates HIF1α/VEGF Signaling by Stabilizing HIF1α and Regulates Responsive Cardiac Angiogenesis

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Short Title: Rnd3, Responsive Angiogenesis, HIF1α/VEGF Signal

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Supplemental Methods

Human failing heart tissues
Failing myocardial samples were obtained from 51 patients with end-stage heart failure at the time of transplantation. Normal heart tissues were obtained from 12 patients who died of non-cardiac causes as a normal heart group. Left ventricular ejection fraction was <20% in all heart failure patients. The procurement of human heart tissue usage for the study was obtained with written patient-informed consent and approval by the Institutional Review Board of Methodist Hospital and Texas A&M University Health Science Center.

Generation and verification of Rnd3 knockout (KO) and Rnd3 overexpression transgenic mouse lines
The establishment of the Rnd3 KO mouse line was described previously. Human full-length Rnd3 cDNA was subcloned into the α-myosin heavy chain (α-MHC) promoter expression vector. The DNA fragment including α-MHC-V5-β1-Rnd3-SV40p(A) was cut out by Pacl and EcoRV for pronuclear microinjection with a C57/B6 background. Genomic DNA isolated from the tails of the mice was used for genotyping purposes. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Texas A&M University Health Science Center-Houston.

Transverse aortic constriction (TAC) surgery and CoCl2 treatment
TAC was conducted in 10-15 week-old adult male mice for 3 weeks as described previously followed by cardiac function analysis and molecular assessments. For the CoCl2 rescue experiments, mice were allowed access to drinking water containing 2 mM CoCl2 (Alfa Aesar, 12303, MA, USA) for 3 weeks starting immediately after TAC surgery.

Assessments of cardiac function and coronary flow reserve (CFR)
Cardiac function was evaluated by Vevo770 High-Resolution Micro-Imaging System (VisualSonics, Toronto, ON, Canada) as described previously. CFR was measured by Doppler with MM3 system (World Precision Instruments, FL, USA) and a Pulsed Doppler probe (Indus Instruments, TX, USA) as described previously. Peak left main coronary flow velocity was recorded at baseline (1% isoflurane) and hyperemic conditions (2.5% isoflurane), respectively. The ratio of hyperemic/baseline coronary velocity was calculated as the index of CFR. Duplicate measurements were performed.

Immunoblotting, immunoprecipitation, ELISA, hematoxylin & eosin staining, fluorescence staining, Sirius Red staining, and hypoxyprobe-1 staining
Protein samples for Western blot analysis were extracted and separated as described previously, and the immunoblotting densitometry was quantified by Gel Logic 6000 PRO Imaging System (Carestream Health, Inc.). Antibodies were from the following sources: anti-Rnd3 (customer designed and made by Cocalico Biologicals, PA, USA); anti-VEGF (ab1316, Abcam, MA, USA); anti-HIF1α (H1alpha67, Novus Biologicals, CO, USA); anti-Tie2 (sc-7403), anti-c-Myc (sc-40) and anti-HA (sc-805) from Santa Cruz, TX, USA. Equal protein loading was verified by the intensity of the GAPDH blot (Santa Cruz, sc-20357, TX, USA). The recombinant GST-Rnd3 protein was synthesized and incubated with the cell lysates with HA-HIF1α expression. The mutual co-immunoprecipitations were conducted in HeLa cells. VEGFA concentration was
assessed by the VEGF Mouse ELISA kit (ab100751, Abcam, MA, USA) using a microplate reader at the wavelength of 450 nm. Paraffin sections of the whole heart were used for hematoxylin & eosin, wheat germ agglutinin (WGA) (W11262, Thermo Fisher Scientific, NY, USA), isolectin GS-IB4 (I21411, Invitrogen, NY, USA), and picrosirius red staining (26357-02; Electron Microscopy Sciences, Hatfield, PA, USA). The hypoxia regions in the heart were evaluated by Hypoxyprobe™-1 Kit (HP1-100Kit, Hypoxyprobe Inc, MA, USA). Pictures were taken under the 40x microscope objective. A total of 20 staining pictures from each group were quantified by Leica Application Suite Imaging Software (Version 4.0, Germany). Nuclei were visualized by DAPI staining. The images were acquired by fluorescence microscopy.

**Cardiomyocyte isolation, cell culture, tube formation assay, and gene transient transfection**

Mouse cardiomyocytes were isolated from 10-15 week-old adult male mice by enzymatic digestion with Langendorff perfusion system (120108, Radnoti, CA, USA) followed by calcium reintroduction. The collagenase cocktail isolation perfusion buffer contained 0.15 mg/mL Liberase TM (05401127001, Roche, IN, USA). The cells were cultured onto the laminin-coated dishes with 5% fetal bovine serum (FBS) for 16 hours, then the culture media were used for the tube formation experiments in human umbilical vein endothelial cells (HUVECs).\(^8\) Briefly, HUVECs were seeded onto BME gel (3433-005-01, Trevigen, MD, USA) in precoated dishes with EBM culture media (CC-3156, Lonza, NJ, USA). After 10 min of cell attachment, the EBM media was replaced by the mouse cardiomyocytes conditional media. Images were acquired after 6 hours of cell culture under a 5x microscope objective. The tube formation images were analyzed by the software WimTube Image Analysis (ibidi GmbH, Germany). Hypoxia cell culture was performed in the hypoxia chamber (MIC-101, Billups-Rothenberg Inc, CA, USA) with 1% O\(_2\).

The human HA-HIF1α-pcDNA3 construct was purchased from Addgene (plasmid #18949). The GST-Rnd3 construct was generated with pGEX-6P-1 backbone (GE Healthcare). The myc-Rnd3 expression vector and the siRNA specific for Rnd3 were described in our previous study.\(^1\)

**Quantitative PCR analysis**

Transcripts were quantified by quantitative PCR (qPCR) analysis as described previously.\(^5\) Total RNA was prepared by TRIzol extraction (Life Technologies). The forward and reverse PCR primers (5’ to 3’) were as follows: VEGFA (mouse): CTGTGCAGGCTGCTGTAACG/GTTCCCGAAACCCTGAGGAG; GAPDH (mouse): GAGTCAACGGGATTTGGTTCGT/TTGATTTTGGAGGGATCTCG. GAPDH expression levels were used for qPCR normalization. Expression levels were determined by the 2\(^{-\Delta\Delta Ct}\) threshold cycle method.

**Luciferase assay**

Luciferase reporter vector with the promoter containing three hypoxia response elements (24-mers) was purchased from Addgene (HRE-luciferase, plasmid #26731). The luciferase assay was conducted as described earlier.\(^9\) Each sample was measured three times. All results were normalized to co-transfected Renilla luciferase enzyme activity (E1960, Promega).
References


Figure S1. Cardiac fibrosis and hypertrophy were detected in both wild-type (WT) and Rnd3 haploinsufficient mice after 3-week TAC. Heart sections were stained with Sirius Red (a) for collagen deposition (red) and WGA (b) for cardiomyocyte size. Noticeable collagen deposition and enlarged cardiomyocytes were observed. No significant difference between the WT and mutant mice was observed. Scale bar represents 250 µm in a and 20 µm in b. Heart weight over tibial length (HW/TL) (c), heart weight over body weight (HW/BW) (d), and body weight (e) were summarized. The upregulation of hypertrophic markers were assessed by qPCR and presented in f and g. The qPCR data were pooled from 3 mice/group with analyses for each mouse in triplicates. WGA: wheat germ agglutinin.
Figure S2. Angiogenesis was assessed by capillary/cardiomyocyte ratio. (a) Representative images showed capillaries (green, by isolectin), cardiomyocytes (red, by WGA), and nuclear counterstaining (blue, by DAPI). Scale bar represents 20 µm. * indicates the overlap of green and red staining. (b) The capillary/cardiomyocyte ratio in the left ventricle was quantified. A total of 20 staining pictures from 3 mouse hearts in each group were quantified for the capillary/cardiomyocyte ratio.
Figure S3. A reduction in coronary flow reserve was detected in WT mice and an even more profound reduction was measured in Rnd3+/− mice after TAC.
Figure S4. CoCl₂ treatment significantly increased the survival rate of Rnd3⁺/- mice after TAC. No significant difference was observed in the survival rate of the Rnd3⁺/- mice treated with CoCl₂ compared to the WT mice after TAC surgery. Rnd3⁺/- mice without CoCl₂ treatment had a lower survival rate compared to the WT mice post-TAC. P-values were calculated by two-tailed, chi-square Fisher’s test.
Figure S5. Proposed model outlining the molecular mechanism of the Rnd3 deficiency-mediated angiogenic defect. Rnd3 bound to and prevented HIFα protein from hydroxylation, and therefore enhanced its stability; enhancing VEGF secreted by cardiomyocytes. Downregulation of Rnd3 resulted in increased HIF1α UPS-mediated protein degradation, which in turn weakened the expression of its target genes, including VEGFA, and led to a myocardial angiogenesis defect and cardiac dysfunction. The impaired cardiac phenotype can be rescued by CoCl2 treatment in animals. UPS: ubiquitin proteasome system.