Rho-Kinase Inhibition During Early Cardiac Development Causes Arrhythmogenic Right Ventricular Cardiomyopathy in Mice


Objective—Arrhythmogenic right ventricular cardiomyopathy (ARVC) is characterized by fibrofatty changes of the right ventricle, ventricular arrhythmias, and sudden death. Though ARVC is currently regarded as a disease of the desmosome, desmosomal gene mutations have been identified only in half of ARVC patients, suggesting the involvement of other associated mechanisms. Rho-kinase signaling is involved in the regulation of intracellular transport and organizes cytoskeletal filaments, which supports desmosomal protein complex at the myocardial cell–cell junctions. Here, we explored whether inhibition of Rho-kinase signaling is involved in the pathogenesis of ARVC.

Approach and Results—Using 2 novel mouse models with SM22α- or αMHC-restricted overexpression of dominant-negative Rho-kinase, we show that mice with Rho-kinase inhibition in the developing heart (SM22α-restricted) spontaneously develop cardiac dilatation and dysfunction, myocardial fibrofatty changes, and ventricular arrhythmias, resulting in premature sudden death, phenotypes fulfilling the criteria of ARVC in humans. Rho-kinase inhibition in the developing heart results in the development of ARVC phenotypes in dominant-negative Rho-kinase mice through 3 mechanisms: (1) reduction of cardiac cell proliferation and ventricular wall thickness, (2) stimulation of the expression of the proadipogenic noncanonical Wnt ligand, Wnt5b, and the major adipogenic transcription factor, PPARγ, and inhibition of Wnt/β-catenin signaling, and (3) development of desmosomal abnormalities. These mechanisms lead to the development of cardiac dilatation and dysfunction, myocardial fibrofatty changes, and ventricular arrhythmias, ultimately resulting in sudden premature death in this ARVC mouse model.

Conclusions—This study demonstrates a novel crucial role of Rho-kinase inhibition during cardiac development in the pathogenesis of ARVC in mice. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305872.)

Key Words: cytoskeletal filaments ■ desmosomes ■ myocardial fatty change ■ PPARγ ■ Rho-kinase ■ Wnt signaling pathway

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a genetically determined myocardial disease characterized by fibrofatty replacement, predominantly affecting the right ventricle (RV), ventricular arrhythmias, and an increased risk of sudden death, particularly in young people and athletes. The recognition of biventricular or isolated left ventricular (LV) forms as part of the disease spectrum has recently encouraged the adoption of the broader term arrhythmogenic cardiomyopathy. Mutations causing ARVC have been identified mostly in genes that encode the 5 major components of the cardiac desmosome: namely, desmoplakin, junction plakoglobin, plakophilin-2, desmoglein-2, and desmocollin-2. Thus, ARVC is currently recognized as a disease of the desmosome. Molecular genetic studies have identified mutations in ≥1 of the desmosomal genes in only approximately half of the ARVC patients, suggesting that other...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ARVC</td>
<td>arrhythmogenic right ventricular cardiomyopathy</td>
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<tr>
<td>DN-RhoK</td>
<td>dominant-negative Rho-kinase</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
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<td>RV</td>
<td>right ventricle</td>
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Disease-related genes may be involved. Despite considerable research advances for the genetic and molecular backgrounds of ARVC, the pathophysiological mechanisms still remain to be fully elucidated.3

Rho-kinase, the major downstream effector of the small GTPase Rho, is a protein kinase that has recently attracted much attention in the cardiovascular research field.6 Abnormalities of the Rho/Rho-kinase pathway have been implicated in the pathogenesis of several cardiovascular disorders in human and animal studies.7 Rho-kinase regulates a wide range of cellular functions, including actin cytoskeleton assembly, cell contractility, proliferation and differentiation, and gene expression.8,9 In addition, the Rho/Rho-kinase system plays an important role in the regulation of adipogenesis.10 Indeed, the Rho/Rho-kinase system has been shown to negatively regulate adipogenesis through interacting with Wnt signaling11 and, in part, by controlling the expression of pro- and antiadipogenic Wnt genes.10 Activation of canonical Wnt/β-catenin signaling is known to inhibit adipogenesis.10 The less well-characterized noncanonical β-catenin–independent pathway, which involves activation of small G proteins and their downstream effectors, including the Rho/Rho-kinase system, plays a more complex role.12 Interestingly, downregulated Wnt signaling has been recently implicated in the development of ARVC in mice.13,14 Because systemic Rho-kinase disruption in mice results in perinatal lethality, it has been difficult to examine the specific role of Rho-kinase in the cardiovascular system in mouse models. Thus, in the present study, we developed a novel mouse model in which Rho-kinase deficiency was targeted to the cardiovascular system during development. We here show that these Rho-kinase–deficient mice spontaneously develop unique phenotypes fulfilling the criteria of ARVC in humans associated with altered desmosome structure and aberrant Wnt signaling, indicating a novel and crucial role for Rho-kinase inhibition during development in the pathogenesis of ARVC in mice.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Inhibition of Rho-Kinase Activity in the Developing Heart Through Overexpression of Dominant-Negative Rho-Kinase

To study the role of Rho-kinase inhibition in the cardiovascular system, we generated a novel mouse model in which dominant-negative Rho-kinase (DN-RhoK)16 was overexpressed using the Cre-loxP system (Figure 1A and 1B).

DN-RhoK consists of the carboxy-terminal fragment of Rho-kinase, which serves as an autoregulatory inhibitor of the amino-terminal kinase domain.17 In which 2 point mutations were introduced to suppress Rho-binding activity.16 DN-RhoK has been shown to inhibit intrinsic Rho-kinase activity when overexpressed in vivo.18 Overexpression of DN-RhoK in our mouse model successfully reduced the activity of Rho-kinase in the embryonic day (E) 12.5 embryonic hearts (Figure 1C) and in the postnatal aortas (Figure 1D) of DN-RhoK mice compared with controls. We noted some sudden deaths of several pups before they were weaned at the age of 4 weeks. The breeding strategy used was expected to give rise to a 50% DN-RhoK offspring (Figure 1E). The genotype frequencies observed on E12.5 and until 1 week after birth in DN-RhoK mice were comparable to the expected frequency, indicating the absence of embryonic or neonatal lethality in DN-RhoK mice. However, the genotype frequency of DN-RhoK mice was reduced to 30% at 4 weeks of age (Figure 1E). The heart weight to body weight ratio and the ventricular weight to body weight ratio were both significantly higher in DN-RhoK mice compared with controls (Figure 1E). Systolic blood pressure was significantly lower in DN-RhoK mice compared with controls (Figure 1G). In addition, the aortas of DN-RhoK mice were thinner than those of controls (Figure 1H).

Crucial Role of Rho-Kinase in Cardiac Development

We first examined the sections of E12.5 embryos. Interestingly, we observed a marked thinning of the ventricular walls and interventricular septum of the hearts of DN-RhoK embryos (Figure 2A). Similar changes were noted in E14.5 hearts (Figure 2B) when the ventricular septation is normally completed and the heart has acquired the definite prenatal form.19 To explore the mechanisms leading to ventricular thinning in DN-RhoK embryos, we examined cell proliferation and apoptosis in E12.5 hearts. TUNEL assay showed a comparable extent of apoptosis between DN-RhoK and control hearts (Figure 2C). On the contrary, BrdU analysis showed a marked reduction in the number of proliferating cardiomyocytes in the ventricular walls and interventricular septum (both in compact layer and trabeculated layer) of DN-RhoK embryos and adult hearts compared with controls (Figure 2D).

Frequent Sudden Death and Cardiovascular Dysfunction in DN-RhoK Mice

Survival curves demonstrated a significantly higher mortality rate for DN-RhoK mice compared with controls, and by 1 year of follow-up, 34.6% of the DN-RhoK mice died suddenly (Figure 3A). Telemetry ECG recordings revealed significantly prolonged P-R intervals and QRS durations in DN-RhoK mice, suggesting the presence of cardiac conduction disturbances (Figure 3B). Interestingly, frequent spontaneous ventricular arrhythmias were detected in all the DN-RhoK mice examined, but not in the controls (Figure 3C). These spontaneous ventricular arrhythmias were evident as long frequent runs of ventricular extrasystoles characterized by widened QRS complexes with no apparent association with the P waves (Figure 3D). Interestingly, one of the DN-RhoK mice spontaneously developed sustained ventricular tachycardia...
We further examined the effects of Rho-kinase inhibition on cardiovascular performance in DN-RhoK mice. Echocardiographic evaluation of DN-RhoK mice revealed dilated ventricular chambers, especially the RV (Figure 4A). Furthermore, DN-RhoK mice showed increased RV and LV dimensions and reduced LV ejection fraction and fractional shortening compared with controls (Figure 4B), suggesting the involvement of the LV in the cardiac phenotypes of DN-RhoK mice.

**Myocardial Fibrofatty Changes in DN-RhoK Mice**

We next examined the morphological changes in the heart after birth (Figure 4C). Interestingly, the hearts of DN-RhoK mice were markedly dilated as early as postnatal day 3. The dilatation was more prominent in the RV and was progressive with age (Figure 5A). Cross sections at the level of the ventricles showed a progressive ventricular dilatation and thinning in DN-RhoK hearts, more prominent in the RV, evident from postnatal day 3 (Figure 5A). In contrast, the hearts from controls showed no evidence of ventricular dilatation or thinning (Figure 5B). Myocardial fibrotic changes, though minimal, were evident as early as postnatal day 3 in both the LV and RV in DN-RhoK hearts (Figure 5C). The fibrosis was initially noted in epicardial and perivascular regions of the ventricles in DN-RhoK mice, progressing to the adjacent myocardium with advancing age and involving the entire thickness of the RV free wall in some areas at week 19 (Figure 5C). In contrast, by the age of 19 weeks, control hearts did not show...
Abnormal Myocardial Cell–Cell Junctions in DN-RhoK Mice

ARVC is now widely accepted as a disease of the cardiac desmosome. Interestingly, the Rho/Rho-kinase pathway has been shown to interact with desmosomal proteins possessing intracellular signaling functions. Thus, we were interested in examining the ultrastructure of myocardial cell–cell junctions, intercalated discs, where the desmosome resides. Transmission electron microscopy showed widening of the gaps at the intercalated discs in the myocardium of 25-week-old DN-RhoK mice (Figure 5G). In addition, the desmosomes of DN-RhoK mice were less electron-dense compared with the controls (Figure 5G; arrows). The gap width at the desmosomes of DN-RhoK hearts was significantly increased compared with that of control hearts (Figure 5H). These results suggest that normal Rho-kinase function may be necessary for the integrity of cardiac desmosomes.

To further examine the desmosomal abnormalities demonstrated in our mouse model, we next examined the distribution of the desmosomal proteins in ventricular sections by immunofluorescence staining. The expression of plakoglobin was confined to the myocardial cell–cell junctions in the RVs and LVs of control mice (Figure 6). On the contrary, the RVs of DN-RhoK mice displayed a markedly disorganized myocardial structure with poorly defined cell junctional areas. The plakoglobin expression was visibly reduced and, interestingly, was mostly situated at the cardiomyocyte nuclei, rather than at junctional areas (Figure 6). The LV myocardial organization of DN-RhoK heart was more orderly, and though the plakoglobin expression was reduced in intensity compared with control LVs, the signal was mostly present at the myocardial cell–cell junctions (Figure 6). We also stimulated neonatal rat cardiomyocytes with a selective Rho-kinase inhibitor hydroxyfasudil for 24 hours and examined the expression of plakoglobin. However, the expression of plakoglobin did not significantly change by the treatment with hydroxyfasudil (unpublished data). This will be because the impact of Rho-kinase inhibition needs to be chronic during the cardiac development for changing the expression of plakoglobin in heart tissues. Additionally, the expression of Connexin43 was also reduced in DN-RhoK hearts compared with control hearts; the signal was mostly present at the myocardial cell–cell junctions (Figure I in the online-only Data Supplement).

Significantly Altered Gene Expression Profiles in DN-RhoK Hearts

Based on the hypothesis that the Rho-kinase inhibition in DN-RhoK hearts may change the gene profiles responsible for the phenotypes of ARVC, we performed microarray analysis using the Agilent SurePrint G3 Mouse GE microarray kit. The statistical analysis by the statistical computing software R revealed that DN-RhoK hearts had significant changes in 3653 genes (1909 increased and 1744 decreased; \( P<0.05 \)) compared with age-matched controls (E12.5 embryonic hearts). Hierarchical clustering analysis (Figure II in the online-only Data Supplement) and volcano plots analysis (Figure III in the online-only Data Supplement) revealed the significantly altered gene expression profiles in DN-RhoK hearts. Genes with significant changes were further subjected to the pathway analysis using Ingenuity Pathway Analysis software (Ingenuity) to identify gene sets representing specific biological processes or functions. Ingenuity Pathway Analysis software revealed the list of genes significantly

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upregulated or downregulated in DN-RhoK hearts (Table) and the list of top 25 gene networks (Table I and Figures III–XV in the online-only Data Supplement). Interestingly, among the top up- or downregulated genes, we found many genes associated with the formation of cytoskeleton, cell-to-cell connection, or extracellular matrix (Table), all of which are related with the Rho-kinase signaling. Moreover, several networks were related with the pathways associated with cell morphology, cellular assembly, cell-to-cell interaction, metabolism, connective tissue disorder, and organismal development (Figure IV in the online-only Data Supplement), all of which are consistent with the effects by Rho-kinase inhibition. Consistent with our hypothesis, many genes in the downstream of RhoA signaling were suppressed in the DN-RhoK hearts (Figure V in the online-only Data Supplement). Importantly, cofilin and profilin, both of which are actin-binding proteins and regulate assembly/disassembly of actin filaments, were significantly downregulated in the DN-RhoK hearts. This may cause dysregulation of actin filament, inducing dysfunction of cytoskeletal structure, which may partially explain the histological changes in the hearts of DN-RhoK mice (Figure XVII in the online-only Data Supplement).

Abnormal Wnt Signaling in DN-RhoK Mice

Wnt signaling abnormalities have been recently implicated in the adipogenic phenotype and the pathogenesis of ARVC in mice.\textsuperscript{13–15} The Rho-kinase pathway has been shown to regulate adipogenesis through interactions with Wnt signaling.\textsuperscript{10,11,23} Thus, we examined the mRNA levels of several genes related to Wnt signaling and adipogenesis. In DN-RhoK mice, the expression levels of PPARγ (\textit{Pparg}), the master regulator of adipogenesis,\textsuperscript{24} and Wnt5b (\textit{Wnt5b}), a proadipogenic noncanonical Wnt ligand\textsuperscript{25} were significantly increased in E12.5 hearts compared with control hearts (Figure 7A). In addition, the mRNA level of β-catenin (\textit{Ctnnb1}) and its downstream signaling Axin2 (\textit{Axin2}), the central transcriptional regulator of canonical Wnt/β-catenin signaling,\textsuperscript{26} was significantly increased in the E12.5 hearts and in the RV of 3-week-old DN-RhoK mice (Figure 7A; Figure XVIII in the online-only Data Supplement). We further examined the mRNA expression of cardiac stress markers. The expression of connective tissue growth factor (\textit{Ctgf}), one of the earliest growth factors induced in cardiomyocytes in response to hypertrophic stimuli\textsuperscript{27} and a powerful profibrotic cytokine,\textsuperscript{28} was increased in the 3-week-old RV (Figure 7B). The increased expression of connective tissue growth factor was consistent with the...
fibrotic changes observed in the hearts of DN-RhoK mice. In addition, the expression of cardiac stress markers, such as atrial natriuretic peptide (Nppa) and b-type natriuretic peptide (Nppb), were increased in both the embryonic hearts and the 3-week-old RVs (Figure 7B).

Because inhibition of Wnt/β-catenin signaling has been demonstrated in several mouse models of ARVC,13–15 we further examined the protein levels of Wnt5b, PPARγ, and β-catenin in the DN-RhoK hearts. The protein level of PPARγ in the LV and IVS was comparable between the DN-RhoK hearts and controls (Figure 7C; Figure XVI in the online-only Data Supplement). In contrast, PPARγ levels in the RV were significantly increased in the DN-RhoK hearts compared with controls, pointing toward adipogenic phenotypic change (Figure 7C; Figure XVI in the online-only Data Supplement). Consistently, plakoglobin protein levels were almost half in the DN-RhoK hearts compared with controls (Figure 7D; Figure XVI in the online-only Data Supplement), which is consistent with the immunostaining that revealed a markedly disorganized myocardial structure with poorly defined cell junctional areas (Figure 6). Finally, the downregulation of Rho-kinase activity in DN-RhoK postnatal hearts was mild, probably because of considerable involvement of fibrofatty tissues in DN-RhoK hearts (Figure 7E).

**No Myocardial Fibrofatty Change in αMHC-Directed DN-RhoK Mice**

To further discriminate the roles of Rho-kinase inhibition before (SM22α-directed) and after (αMHC-directed) the birth, we also developed mice with αMHC (Myh6)-directed expression of DN-RhoK. Interestingly, the hearts of 18-week-old αMHC-directed DN-RhoK mice showed no ventricular thinning, RV dilatation, or fibrosis in contrast to the SM22α-directed DN-RhoK mice (Figure XIXA in the online-only Data Supplement). Moreover, these mice developed normally with normal life spans and cardiac structure and function comparable to littermate controls (Figures XIXB and XIXC in the online-only Data Supplement). Because αMHC is mainly a postnatal ventricular gene,29 intrinsic Rho-kinase activity is most probably inhibited after birth in the hearts...
of αMHC-directed DN-RhoK mice. In contrast, SM22α is strongly expressed in the developing heart, especially in the RV, thereby inhibiting Rho-kinase activity during embryogenesis in SM22α-directed DN-RhoK mice. Thus, the present findings suggest that Rho-kinase inhibition during early cardiac development promotes abnormal phenotypic changes in the heart, resulting in ARVC.

Discussion

The present study is the first report to implicate Rho-kinase inhibition in the development of ARVC in mice. We have generated a novel mouse model in which Rho-kinase activity is inhibited in the developing heart as early as E7.5. Our DN-RhoK mainly caused cardiac dilatation and dysfunction, myocardial fibrofatty changes, ventricular arrhythmias, and sudden death, fulfilling the criteria for ARVC in humans. Several mouse models of ARVC involving desmosomal gene alterations have been previously developed, including desmoplakin, junction plakoglobin, desmoglein-2, and plakophilin-2. These models have provided additional support to the currently accepted concept that ARVC is a disease of the desmosome. However, approximately half of the ARVC patients do not harbor any known causative gene mutations, suggesting the involvement of other associated mechanisms. Indeed, the present study suggests that diminished activation of a Rho-kinase signaling pathway could contribute to disease development of ARVC in mice. The recent recognition of the involvement of aberrant Wnt signaling in ARVC pathogenesis in mice support our findings because the Rho/Rho-kinase system are intracellular regulators involved in Wnt signaling pathways. However, there are no previous reports that suggested the involvement of Rho-kinase in the pathophysiology of ARVC in humans. Nevertheless, a recent study aiming at characterizing the ARVC-specific myocardial transcriptome demonstrated the altered regulation of several genes related to Rho-signaling, suggesting that modulation of the Rho/Rho-kinase system may be potentially involved in the pathogenesis of ARVC in humans. The present mouse model provides evidence for the involvement of Rho-kinase inhibition in the pathogenesis of ARVC in mice and may serve as a novel model for ARVC.

Figure 5. Structural and ultrastructural changes in dominant-negative Rho-kinase (DN-RhoK) hearts. A–D, Representative Elastica–Masson staining of the hearts from DN-RhoK mice (postnatal day 3 and weeks 3, 5, 10, and 19) and control mice (week 19). Bar, 1 mm (A, B) and Bar, 200 μm (C, D). E, Representative Masson–trichrome of the right ventricles (RV) from DN-RhoK mice showing adipogenic deposits next to fibrotic areas at week 19. Bar, 200 μm. F, Representative Oil red O staining of the RV confirming the adipogenic changes in DN-RhoK hearts. Bar, 200 μm. G, Transmission electron microscopy analysis of the ventricular myocardium showed less electron-dense desmosomes with widened gaps at the intercalated disks (white arrows) in the hearts from DN-RhoK mice at 25 weeks of age. Bar, 100 nm. H, Gap width at the myocardial desmosomes in DN-RhoK mice vs controls. Data are presented as the means±SEM. n=7 intercalated disc areas/mouse. *P<0.05 by Student’s t test.
as well as in mouse models of ARVC. These changes most probably result from mechanical disruption of desmosomes secondary to mutated or defective desmosomal proteins. We observed intercalated disc changes in the hearts of our DN-RhoK mice, including less electron-dense desmosomes and widened gaps at the intercalated discs, in which Rho-kinase will organize cytoskeletal filaments and supports desmosomal protein complex. Consistent with our findings, the Rho/Rho-kinase pathway has been shown to be necessary for normal desmosomal assembly. In addition, recent studies have demonstrated a cross talk between the Rho/Rho-kinase pathway, on the one hand, and the intracellular signaling pathways involving the desmosomal proteins of the armadillo family, including plakophilin-2 and plakoglobin, on the other hand. Interestingly, plakoglobin was reduced at the myocardial cell–cell junctions and the intercalated discs and was translocated to the nucleus in the RV of DN-RhoK mice. Loss of plakoglobin at the intercalated discs and reduction of protein levels may offer an explanation for the structural changes observed by electron microscopy. The nuclear relocalization of plakoglobin was similar to that observed in the mouse models developed by Garcia-Gras et al and Lombardi et al, where plakoglobin inhibits Wnt/β-catenin signaling through competing with β-catenin for its transcriptional targets, thereby stimulating adipogenic programming of the cardiomyocyte. In those studies, the trigger for the nuclear translocation of plakoglobin was the presence of desmosomal protein mutation. In the absence of desmosomal mutation in our mouse model, the nuclear translocation of plakoglobin might be explained by the recently uncovered role for RhoA signaling in the assembly of desmosome protein complex (Figure 8). Godsel et al have demonstrated that the RhoA-mediated actin reorganization is necessary for directing desmosomal precursors enriched in desmplakin to sites of cell–cell contacts to be incorporated into newly forming desmosomes. In that study, pharmacological inhibition
of Rho-kinase inhibited desmoplakin border accumulation. In our DN-RhoK model, microarray analysis demonstrated significant alterations in genes responsible for actin fiber assembly and cytoskeletal reorganization. Thus, considering the early embryonic Rho-kinase inhibition in our mouse model, the direction of desmoplakin-enriched desmosomal precursors to the cell border and the subsequent incorporation into newly formed desmosomes might have been impaired. Plakoglobin, which anchors desmoplakin to the intermediate filament network, will consequently fail to incorporate into the desmosome and is available to translocate to the nucleus (Figure 8). Nuclear plakoglobin competes with β-catenin for its transcriptional factors, thereby inhibiting Wnt/β-catenin signaling. Inhibition of Wnt/β-catenin signaling will promote adipogenesis and is believed to be responsible for a developmental myogenic–adipogenic switch in cardiac progenitor cells. Those studies have also demonstrated increased expression levels of the proadipogenic noncanonical Wnt ligand, Wnt5b, and have suggested Wnt5b as a potential mediator in ARVC pathogenesis. As mentioned earlier, there is an established role for the Rho/Rho-kinase pathway in the regulation of adipogenesis. Inhibition of the Rho-kinase system in vitro, both pharmacologically and genetically, has been shown to promote adipogenesis. In addition, the Rho-kinase system plays an important role in regulating the adipogenesis–myogenesis cell fate decision. Several studies have shown that Rho-kinase regulates adipogenesis through interaction with Wnt signaling and, in part, by regulating the expression of pro- and antiadipogenic Wnt genes, where Rho-kinase inhibition stimulates the expression of proadipogenic Wnt genes, whereas Rho-kinase activation stimulates the expression of antia adipogenic Wnt genes. In addition, recent studies have suggested that Rho-kinase is also involved in adipogenesis through regulating the expression of PPARγ, the master

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Adipogenic replacement of the myocardium is an important pathological feature of ARVC. The hearts of DN-RhoK mice exhibited marked myocardial fatty change, which is interesting because fat tissue is normally rare in the mouse heart. In addition, the adipogenic phenotype has been displayed only in a few desmosomal mouse models of ARVC, with aberrations of Wnt signaling found to be involved. These studies have shown that, in response to desmosomal
regulator of adipogenesis, and β-catenin, the central transcriptional regulator of canonical Wnt/β-catenin signaling, where Rho-kinase inhibition promotes adipogenesis through upregulating PPARγ and downregulating β-catenin. In the present study, we have demonstrated that Rho-kinase inhibition was associated with a significant increase in the expression levels of Wnt5b, PPARγ, and β-catenin in the heart of DN-RhoK mice. 

The disease process started prenatally in our mouse model, as reflected by the cardiac structural changes. Prenatal onset has been reported in some desmosomal mouse models of ARVC. In human ARVC, the diagnosis might be difficult before the age of 10 years, although cases of ARVC in infants, and even in the embryo, have also been reported. In the present study, we have demonstrated that Rho-kinase deficiency in the embryonic heart resulted in abnormal cardiac development, involving ventricular thinning and reduced cardiac proliferation. These findings are consistent with the results of the previous studies that have used pharmacological inhibition of Rho-kinase in cultured mouse embryos, starting from E7.5 to E8.0. Those studies implicated Rho-kinase in the regulation of cardiac cell proliferation through controlling the expression of cell cycle proteins. It has been shown that Rho-kinase inhibition downregulates the expression of β-catenin and its downstream target cyclin-D1, suggesting that the reduced cardiac cell proliferation mediated by Rho-kinase inhibition may be partially caused by reduced

Figure 7. Molecular alterations in the dominant-negative Rho-kinase (DN-RhoK) heart. A and B, Relative gene expression of Wnt signaling and adipogenesis genes (Wnt5b, PPARγ, and β-catenin) and cardiac stress genes (CTGF, ANP, and BNP) in embryonic day (E) 12.5 hearts and the right ventricle (RV) of 3-week-old (w) mice. Relative expression levels are normalized to Gapdh. E12.5 hearts, n=4 for control and n=6 for DN-RhoK mice. RV 3w, n=6 for control and n=8 for DN-RhoK mice. Data are presented as the mean±SEM. *P<0.05, **P<0.001 by Student t test. C, Western blots of Wnt signaling and adipogenesis genes (Wnt5b, PPARγ, and β-catenin) of the adult hearts (20 weeks old) showing increased expression of Wnt5b, PPARγ, and β-catenin especially in the RV of DN-RhoK hearts. n=5 per group. D, Western blots of adult hearts (20 weeks old) showing reduced expression of plakoglobin, especially in the RV and IVS of DN-RhoK hearts. n=5 per group. *P<0.05 by Student t test. E, Western blots of adult hearts (20 weeks old) showing slightly reduced activities in DN-RhoK hearts. n=5 per group. ANP indicates atrial natriuretic peptide; BNP, B-type natriuretic peptide; and CTGF, connective tissue growth factor.
Wnt/β-catenin signaling. Our microarray data also stress the involvement of Rho-kinase functional networks related to pathways associated with cell morphology, cellular assembly, and organismal development. Among the top downregulated genes, α-actinin has been shown to colocalize with the proteins in the adherens junctions at the intercalated disc,\(^4^7\) where it acts as the linkage with the actin filament. Thus, reduction of α-actinin may have induced the detachment of actin filaments at the intercalated disc and dysfunction of cell-to-cell attachment of cardiac myocytes. The Ingenuity Pathway Analysis network with the highest score showed the downregulation of transforming growth factor-β, an important growth factor for the development of various organs (Figure VI in the online-only Data Supplement). Transforming growth factor-β3 (encoded by TGFB3) is known as one of the causative genes for the development of familial ARVC.\(^4^8\) These reports and our present findings suggest that the downregulation of transforming growth factor-β may have promoted the development of fibrofatty changes and the phenotypes of ARVC in DN-RhoK hearts.

Finally, we have performed experiments to examine the role of Rho-kinase in the regulation of calcium currents in myocytes of the RV. Here, it was difficult to obtain intact isolated myocytes from DN-RhoK hearts because of its fibrofatty changes and fragile structure of the RV. Thus, we used trabeculae from the RV of rats to confirm the role of Rho-kinase inhibition in the RV. Interestingly, Rho-kinase inhibition by selective Rho-kinase inhibitor, hydroxysasudil, did not significantly reduce force nor [Ca\(^{2+}\)] in the RV trabeculae of rats ex vivo (unpublished data).
To summarize the role of Rho-kinase inhibition in our DN-RhoK mice, we have shown that Rho-kinase inhibition in the developing heart results in the development of ARVC through the following 3 mechanisms (Figure 8). First, Rho-kinase inhibition in the developing heart reduces cardiac cell proliferation and ventricular wall thickness. We consider that the attenuated myocardium fails to withstand the growing circulatory demands of the developing cardiovascular system, eventually resulting in cardiac dilatation and dysfunction. The cardiac stress is reflected by the increased expression of the profibrotic cytokine, connective tissue growth factor, resulting in a progressive myocardial fibrosis. Second, Rho-kinase inhibition possibly stimulates the expression of Wnt5b and PPARy, which may be responsible for the myocardial fatty change in our mouse model. Third, inhibition of Rho-kinase is responsible for the abnormalities of the intercalated discs and desmosomes detected. The myocardial fibrofatty changes and the aberrant cell–cell communication may explain the ventricular arrhythmias that ultimately resulted in the sudden premature death in this ARVC model. Thus, Rho-kinase inhibition may offer an explanation for the development of the seemingly unrelated cardiac changes observed in ARVC and may justify Rho-kinase as a novel target to be explored in ARVC. Interactions between the Rho-kinase pathway and the Wnt signaling pathway would offer a plausible new insight into the pathogenesis of the important but yet incompletely understood disorder, ARVC.

Our present findings support a crucial role for Rho-kinase in cardiac development and may warn against the use of Rho-kinase inhibitors during pregnancy. Rho-kinase plays a crucial role in the development of cardiovascular diseases. The beneficial effects of Rho-kinase inhibition for the treatment of cardiovascular disease have been demonstrated in various animal models and in humans. However, based on the present study, Rho-kinase inhibitor may adversely affect the fetus, which is similar to the inhibitors of tenin—angiotensin system. The renin—angiotensin system plays a crucial role during the development of ARVC. Interactions between the Rho-kinase pathway and the Wnt signaling pathway would offer a plausible new insight into the pathogenesis of the important but yet incompletely understood disorder, ARVC.

The maximal change in our mouse model. Third, inhibition of Rho-kinase is responsible for the abnormalities of the intercalated discs and desmosomes detected. The myocardial fibrofatty changes and the aberrant cell–cell communication may explain the ventricular arrhythmias that ultimately resulted in the sudden premature death in this ARVC model. Thus, Rho-kinase inhibition may offer an explanation for the development of the seemingly unrelated cardiac changes observed in ARVC and may justify Rho-kinase as a novel target to be explored in ARVC. Interactions between the Rho-kinase pathway and the Wnt signaling pathway would offer a plausible new insight into the pathogenesis of the important but yet incompletely understood disorder, ARVC.

Acknowledgments
Alia Ellawindy is a recipient of a scholarship from the Honjo International Scholarship Foundation and received the first place of the 2013 Young Investigator Award for International Students by the Japanese Circulation Society for the present study. We are grateful to the laboratory members in the Department of Cardiovascular Medicine at Tohoku University for valuable technical assistance, especially Hiromi Yamashita, Ai Nishihara, and Yumi Watanabe.

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Disclosures
None.

References
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Significance

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a genetically determined heart disease that is currently regarded as a disease of the cardiac desmosome. In this article, we suggest Rho-kinase as a new mediator in the development of ARVC. Using a novel mouse model, with Rho-kinase deficiency directed to the embryonic heart, we have demonstrated the development of a remarkable cardiac phenotype, recapitulating ARVC in humans. The cardiac desmosomal changes are of particular interest because they were not related to desmosomal gene mutations or over/underexpression, as in previous ARVC mouse models. We were also able to demonstrate a link between Rho-kinase deficiency and aberrant Wnt signaling, which has recently been implicated in the pathogenesis of ARVC. We think that Rho-kinase inhibition offers an explanation for the development of the seemingly unrelated cardiac changes observed in ARVC and justifies Rho-kinase as a novel target to be explored in ARVC.
Rho-Kinase Inhibition During Early Cardiac Development Causes Arrhythmogenic Right Ventricular Cardiomyopathy in Mice

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SUPPLEMENTAL MATERIAL

Rho-kinase Inhibition during Early Cardiac Development Causes Arrhythmogenic Right Ventricular Cardiomyopathy in Mice

Alia Ellawindy,1 Kimio Satoh,1 Shinichiro Sunamura,1 Nobuhiro Kikuchi,1 Kota Suzuki,1 Tatsuro Minami,1,2 Shohei Ikeda,1 Shin-ichi Tanaka,1,2 Toru Shimizu,1 Budbazar Enkhjargal,1 Satoshi Miyata,1 Yuhto Taguchi,3 Tetsuya Handoh,3 Kenta Kobayashi,4 Kazuto Kobayashi,4 Keiko Nakayama,5 Masahito Miura,3 Hiroaki Shimokawa1

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Supplemental Tables I-II

Supplemental Figures I-XVII and its Figure Legends
Supplemental Figure Legends

**Supplemental Figure I.** Connexin43 in the Right Ventricles of DN-RhoK Mice

(\textit{RV}) Immunofluorescence staining showing the distribution of Connexin43 in the right ventricle (RV) of DN-RhoK mice as compared with littermate controls (20 weeks). Note that the Connexin43 signal is situated at the myocardial cell-cell junction in control hearts, whereas the signal is significantly reduced in DN-RhoK hearts. (\textit{LV}) Immunofluorescence staining showing the distribution of Connexin43 in the left ventricle (LV) of DN-RhoK mice as compared with littermate controls. Note that the Connexin43 signal is reduced in DN-RhoK hearts as compared with control hearts. Bar, 200 μm (×100), 50 μm (×400).

**Supplemental Figure II.** Hierarchical Clustering Analysis

Microarray analysis using the Agilent SurePrint G3 Mouse GE microarray kit revealed a significantly changed gene profiles in DN-RhoK hearts compared with age-matched controls (E12.5 embryonic hearts). All the relevant genes were grouped according to their expression values (log ratios). Each row corresponds to one gene, each column to the different 8 microarray experiments. The quantitative gene expression changes across all the samples are shown by different colors (red indicates up-regulated genes; green indicates down-regulated genes). The top labels indicate the different experiments.

**Supplemental Figure III.** Volcano Plots Analysis

The DN-RhoK hearts showed significant changes in 3,653 genes (1,909 increased; 1,744 decreased, \(P<0.05\)) as compared with the control hearts. Volcano plots analysis revealed the significantly altered gene expression profiles in DN-RhoK hearts. Significantly increased top 5 genes (DN-RhoK/control ratio>2.0, \(P<0.05\)) and significantly reduced top 16 genes (DN-RhoK/control ratio<0.5, \(P<0.05\)) are presented as a table.

**Supplemental Figure IV.** Heat Map Analysis

Heat map analysis showed that many genes are associated with the pathways of cell morphology, cellular assembly, cell-to-cell interaction, metabolism, connective tissue disorder and organismal development, all of which are related with the Rho-kinase signaling. Red panels show the up-regulated genes in DN-RhoK hearts. Blue panels show the down-regulated genes in DN-RhoK hearts.

**Supplemental Figure V.** The RhoA Signaling in the Hearts of DN-RhoK Embryos

Using microarray, the gene expression profiles of the hearts of embryonic day (E) 12.5 DN-
RhoK embryos were compared with that of littermate controls. Genes are displayed in the network as nodes. The relationships among the genes are represented with lines according to the Ingenuity Pathway Analysis (IPA). Up-regulated and down-regulated genes in DN-RhoK mice compared with control mice are shown as red and green spots, respectively.

Supplemental Figure VI-XV. IPA Functional Analysis and Top 10 Networks Associated with Rho-kinase Inhibition in the Hearts of DN-RhoK Embryos
Using microarray, the gene expression profiles of the hearts of embryonic day (E) 12.5 DN-RhoK embryos were compared with that of littermate controls. Genes are displayed in the network as nodes. The relationships among the genes are represented with lines according to the Ingenuity Pathway Analysis (IPA) software. Up-regulated and down-regulated genes in DN-RhoK mice compared with control mice are shown as red and green spots, respectively.

Supplemental Figure VI.
No.1 Associated Network in DN-RhoK Cardiac Tissue: Molecular Transport, Drug Metabolism, Lipid Metabolism

Supplemental Figure VII.
No.2 Associated Network in DN-RhoK Cardiac Tissue: Cellular Compromise, Cell Cycle, Cellular Assembly and Organization

Supplemental Figure VIII.
No.3 Associated Network in DN-RhoK Cardiac Tissue: Cell Morphology, Cellular Movement, Digestive System Development and Function

Supplemental Figure IX.
No.4 Associated Network in DN-RhoK Cardiac Tissue: Connective Tissue Disorders, Developmental Disorder, Hereditary Disorder

Supplemental Figure X.
No.5 Associated Network in DN-RhoK Cardiac Tissue: RNA Post-Transcriptional Modification, Cell Morphology, Cellular Assembly and Organization

Supplemental Figure XI.
No.6 Associated Network in DN-RhoK Cardiac Tissue: Developmental Disorder,
Hematological Disease, Hereditary Disorder

**Supplemental Figure XII.**
No.7 Associated Network in DN-RhoK Cardiac Tissue: Molecular Transport, RNA Trafficking, Cancer

**Supplemental Figure XIII.**
No.8 Associated Network in DN-RhoK Cardiac Tissue: Cell Morphology, Cellular Assembly and Organization, Developmental Disorder

**Supplemental Figure XIV.**
No.9 Associated Network in DN-RhoK Cardiac Tissue: Protein Degradation, Protein Synthesis, Carbohydrate Metabolism

**Supplemental Figure XV.**
No.10 Associated Network in DN-RhoK Cardiac Tissue: Post-Translational Modification, DNA Replication, Recombination, and Repair, Organismal Development

**Supplemental Figure XVI.**
Representative Western blotting of Wnt5b, PPARγ, β-catenin, Plakoglobin.

**Supplemental Figure XVII.**
TUNEL assay showed a significant increase of apoptotic cells (shown as brown nuclei) in the postnatal hearts from DN-RhoK and control mice. Bar, 100 μm. Data are presented as the mean ± SEM. n=5 per group. *P<0.05 by Student’s t test.

**Supplemental Figure XVIII.**
Relative gene expression of Axin2 signaling in embryonic day (E) 12.5 hearts and the right ventricle (RV) and left ventricle (LV) of 3-week-old mice. Relative expression levels are normalized to Gapdh. Data are presented as the mean ± SEM.

**Supplemental Figure XIX.**
Normal cardiac structure and function in mice with αMHC-directed DN-RhoK mice. (A) Normal heart morphology in mice with αMHC-directed DN-RhoK over-expression. Elastica-Masson staining of αMHC-directed DN-RhoK mice (αMHC-RhoK+/−) and littermate controls (αMHC-RhoK+/−) showed no signs of ARVC-like phenotype. Bar, 2 mm. (B)
Mean values of body weight (BW), heart weight (HW)/BW, right ventricular (RV) weight/BW and left ventricular (LV) weight/BW in 10-week-old mice. $n=3$ per group. Data are presented as the mean ± SEM. Student’s $t$ test showed no statistically significant differences between the 2 groups. (C) Echocardiographic parameters in 10-week-old mice with αMHC-directed DN-RhoK over-expression compared with littermate control mice, including interventricular septum thickness at end-diastole (IVSTd), LV end-systolic and end-diastolic diameters (LVESD and LVEDD, respectively), LV posterior wall diastolic thickness (LVPWTd), LV ejection fraction (LVEF) and fractional shortening (LVFS), and RV end-diastolic diameter (RVEDD). Data are presented as the mean ± SEM. $n=3$ per group. Student’s $t$ test showed no statistically significant differences between the 2 groups.
### Supplemental Table I. Primers and probes used for mouse genotyping and RT-PCR.

<table>
<thead>
<tr>
<th>PCR primers for genotyping</th>
<th>RT-PCR primers</th>
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<tr>
<td>DN-RhoK-1</td>
<td>Ctnnb1-F 5’-CTG AGC TGG TGG ACT GCA GAA-3’</td>
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<tr>
<td>DN-RhoK-2</td>
<td>Ctnnb1-R 5’-CAC CAC TGG CCA GAA TGA TGA-3’</td>
</tr>
<tr>
<td>IL-2 F</td>
<td>Wnt5b-F 5’-CTC TCA TGA ACC TAC AGA ACA ACG A-3’</td>
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<tr>
<td>IL-2 R</td>
<td>Wnt5-R 5’-TGG AGC CAG CAG GTC TTG A-3’</td>
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<tr>
<td>Cre-F</td>
<td>Nppa-F 5’-TGA CAG GAT TGG AGC CCA GA-3’</td>
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<tr>
<td>Cre-R</td>
<td>Nppa-R 5’-GAC ACA CCACAA GGG CTT AGG A-3’</td>
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<tr>
<td>RT-PCR probes</td>
<td>RT-PCR probes</td>
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<tr>
<td>Rock1</td>
<td>5’-GGA GAC CTT CAA GCA CGA ATT ACA T-3’</td>
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<tr>
<td>Rock2</td>
<td>5’-GTT GTG AAC GGA TTT GGC CGT ATT G-3’</td>
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<tr>
<td>Gapdh</td>
<td>5’-GTT CTG TTG AAG TCG CAG GAG-3’</td>
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### Supplemental Table II.

#### Top 25 Gene Networks Significantly Changed in DN-RhoK Hearts

<table>
<thead>
<tr>
<th>No.</th>
<th>Top Diseases and Functions</th>
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<tbody>
<tr>
<td>1</td>
<td>Molecular Transport, Drug Metabolism, Lipid Metabolism</td>
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<tr>
<td>2</td>
<td>Cellular Compromise, Cell Cycle, Cellular Assembly and Organization</td>
</tr>
<tr>
<td>3</td>
<td>Cell Morphology, Cellular Movement, Digestive System Development and Function</td>
</tr>
<tr>
<td>4</td>
<td>Connective Tissue Disorders, Developmental Disorder, Hereditary Disorder</td>
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<tr>
<td>5</td>
<td>RNA Post-Transcriptional Modification, Cell Morphology, Cellular Assembly and Organization</td>
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<tr>
<td>6</td>
<td>Developmental Disorder, Hematological Disease, Hereditary Disorder</td>
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<tr>
<td>7</td>
<td>Molecular Transport, RNA Trafficking, Cancer</td>
</tr>
<tr>
<td>8</td>
<td>Cell Morphology, Cellular Assembly and Organization, Developmental Disorder</td>
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<td>9</td>
<td>Protein Degradation, Protein Synthesis, Carbohydrate Metabolism</td>
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<tr>
<td>10</td>
<td>Post-Translational Modification, DNA Replication, Recombination, and Repair, Organismal Development</td>
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<td>11</td>
<td>RNA Post-Transcriptional Modification, Infectious Disease, Organismal Injury and Abnormalities</td>
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<tr>
<td>12</td>
<td>Embryonic Development, Organismal Development, Tissue Development</td>
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<tr>
<td>13</td>
<td>DNA Replication, Recombination, and Repair, Energy Production, Nucleic Acid Metabolism</td>
</tr>
<tr>
<td>14</td>
<td>Cell Morphology, Connective Tissue Development and Function, Organismal Development</td>
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<td>15</td>
<td>Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry</td>
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<td>16</td>
<td>Developmental Disorder, Hereditary Disorder, Metabolic Disease</td>
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<td>Embryonic Development, Tissue Development, Cell Cycle</td>
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<td>Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Cycle</td>
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<td>RNA Post-Transcriptional Modification, Tissue Morphology, Carbohydrate Metabolism</td>
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<td>Cancer, Cellular Assembly and Organization, Connective Tissue Development and Function</td>
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<tr>
<td>25</td>
<td>Gene Expression, DNA Replication, Recombination, and Repair, Dermatological Diseases and Conditions</td>
</tr>
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</table>

Top 10 networks are shown in detail in the Supplementary Figures 8-17.
Supplemental Figure Ib

- **Control**
  - LV wall
  - Connexin43
  - α-actinin
  - DAPI

- **LV**
  - LV wall
  - Connexin43 /Tubulin

- **DN-RhoK**
  - LV wall
  - Connexin43 /Tubulin

- (Western blots)
Hierarchical Clustering Analysis

Control

DN-RhoK

Down regulated genes in DN-RhoK Hearts (Green)

Up regulated genes in DN-RhoK Hearts (Red)

A_51_P360715
A_55_P2448654
A_55_P2167339
A_51_P347862
A_51_P196509
A_52_P3864140
A_55_P1990038
A_51_P346272
A_65_P16688
A_55_P2029533
A_55_P2165234
A_55_P2253195
A_55_P2064695
A_55_P1959648
A_55_P2012051
A_65_P101026364
A_55_P183524
A_55_P2039454
A_50_P1017560
A_55_P2032770
A_51_P128876
A_55_P2067221
A_55_P2048867
A_51_P318830
A_50_P1023314
A_55_P2031999
A_55_P1960735
A_55_P2009988
A_51_P207591
A_51_P227222
Volcano Plot Analysis

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<th>p-value</th>
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<td>0.020</td>
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<td>GDF15</td>
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<td>TRIB3</td>
<td>tribbles homolog 3 (Drosophila)</td>
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Changes in the RhoA/Rho-kinase Pathway in DN-RhoK Embryonic Heart

Supplemental Figure V

Up regulated genes
Down regulated genes

Cytoskeletal reorganization
Cytokinesis
Actin nucleation and polymerization

IPA Node Types
- Chemical or Drug
- Cytokine
- Enzyme
- G-protein Coupled Receptor
- Group or Complex

IPA Edge Types
- Transporter
- Other
- binding only
- acts on
- inhibits AND acts on
- leads to
- direct interaction
- indirect interaction

Membrane trafficking
Cytoskeletal reorganization
Cytokinesis
Actin nucleation and polymerization
No. 1 Associated Network in DN-RhoK Cardiac Tissue: Molecular Transport, Drug Metabolism, Lipid Metabolism

Supplemental Figure VI

Up regulated genes

Down regulated genes
No.2 Associated Network in DN-RhoK Cardiac Tissue: Cellular Compromise, Cell Cycle, Cellular Assembly and Organization

Supplemental Figure VII

Up regulated genes
Down regulated genes
No.3 Associated Network in DN-RhoK Cardiac Tissue:
Cell Morphology, Cellular Movement, Digestive System Development and Function

Supplemental Figure VIII

Up regulated genes

Down regulated genes
No. 4 Associated Network in DN-RhoK Cardiac Tissue: Connective Tissue Disorders, Developmental Disorder, Hereditary Disorder

Supplemental Figure IX

Up regulated genes

Down regulated genes
No.5 Associated Network in DN-RhoK Cardiac Tissue:
RNA Post-Transcriptional Modification, Cell Morphology, Cellular Assembly and Organization

Up regulated genes
Down regulated genes
No. 6 Associated Network in DN-RhoK Cardiac Tissue: Developmental Disorder, Hematological Disease, Hereditary Disorder

Supplemental Figure XI

Up regulated genes

Down regulated genes
No. 7 Associated Network in DN-RhoK Cardiac Tissue:
Molecular Transport, RNA Trafficking, Cancer

Supplemental Figure XII

Up regulated genes
Down regulated genes
No.8 Associated Network in DN-RhoK Cardiac Tissue: Cell Morphology, Cellular Assembly and Organization, Developmental Disorder

Supplemental Figure XIII

Up regulated genes

Down regulated genes

IPA Node Types
- Chemical or Drug
- Cytokine
- Enzyme
- G-protein Coupled Receptor
- Growth Factor
- Ion Channel
- Peptidase
- Transcription Regulator
- Transporter
- Other

IPA Edge Types
- Binding only
- Acts on
- Inhibits AND acts on
- Direct interaction
- Indirect interaction
No.9 Associated Network in DN-RhoK Cardiac Tissue: Protein Degradation, Protein Synthesis, Carbohydrate Metabolism

Supplemental Figure XIV
No. 10 Associated Network in DN-RhoK Cardiac Tissue:
Post-Translational Modification, DNA Replication, Recombination, and Repair,
Organismal Development

Supplemental Figure XV
Supplemental Figure XVII

LV

Control

DN-RhoK

TUNEL-positive nuclei / filed

RV

TUNEL-positive nuclei / filed

*
Supplemental Figure XVIII

Embryonic hearts
Axin2/GAPDH

P=0.09 (n=5 each)

Adult heart (LV)
Axin2/GAPDH

P=0.68 (LM n=4, DN n=6)

Adult heart (RV)
Axin2/GAPDH

P=0.09 (LM n=4, DN n=6)
Materials and Methods

Transgenic Mice
All animal experiments were performed in accordance with the guidelines of the Tohoku University Ethics Committee on Animal Experiments (2012 Koudo-001). We used the SM22α promoter to target Rho-kinase activity during the development. Although SM22α is commonly known as a vascular smooth muscle cell-specific promoter, it has been increasingly used for cardiac muscle-selective expression as well.1-3 SM22α is transiently expressed in the developing murine heart, starting in the heart field on E7.5,4 making it a very early marker of the cardiovascular system. Importantly, once SM22α-Cre-promoter is activated, the switch of the Cre-loxP system continues to inhibit Rho-kinase in the heart. Interestingly, Rho-kinase expression is detectable in the murine heart from E7.5,5 coinciding with the expression of SM22α. The SM22α (transgelin, Tgln) promoter was used to target DN-RhoK expression in the developing murine heart. Although SM22α is expressed in smooth muscle cells,6 it is expressed in the developing mouse heart between embryonic days E7.5 and E12.5.4,6 Mice containing a dominant-negative Rho-kinase mutant (CAT-Rho-K DN/3-1, BRC_No 01294) were obtained from the Riken (Tsukuba, Japan). They were crossed with SM22α-Cre mice [Tg(Tagln-cre)1Her/J, Jackson Laboratory, Bar Harbor, U.S.A] or αMHC-Cre mice [Tg(Myh6-cre)1Jmk/J, Jackson Laboratory, Bar Harbor, U.S.A.] to obtain DN-RhoK mice (SM22α-Cre+/−/DN-RhoK+/− and αMHC-Cre+/−/DN-RhoK+/−), respectively. Transgenic littermates lacking the DN-RhoK mutant (SM22α-Cre+/−/DN-RhoK−/− and αMHC-Cre+/−/DN-RhoK−/−, respectively) were used as controls for all experiments. The genotype was confirmed by PCR amplification of tail DNA. The primers used are listed in the Supplemental Table 1.

Preparation of Mouse Embryos
Male SM22α-Cre mice were mated with female CAT-Rho-K DN/3-1 mice. For accurate timing of the pregnancy, female mice were examined the next morning for the presence of a vaginal plug. At noon on the day when vaginal plug was detected, the embryos were aged as embryonic day (E) 0.5. The pregnant mother was sacrificed by cervical dislocation and the embryos were dissected at noon on the 12th day (E12.5) and the 14th day (E14.5) after detection of the vaginal plug. Embryos were genotyped using DNA from tail biopsies.

Histology and Transmission Electron Microscopy (TEM)
Sections of paraffin-embedded whole embryos were examined by hematoxylin and eosin (H&E). The bromodeoxyuridine (5-bromo-2’-deoxy-uridine, BrdU) labeling and detection kit II (Roche, Basel, Switzerland) was used to examine cardiac cell proliferation in embryo sections. TUNEL assay was performed using the in situ apoptosis detection kit (Takara,
Otsu, Japan) to examine cardiac cell apoptosis in embryo sections. Paraffin sections of isolated post-natal hearts were examined with H&E, Masson and Elastica-Masson trichrome stains. Oil red O staining was used to detect adipogenic deposits in the heart. For TEM, isolated mouse hearts were fixed in 2% glutaeraldehyde plus 2% paraformaldehyde/0.1 M phosphate buffered saline (pH 7.4) and processed for sectioning. TEM was performed with the aid of the electron microscopy laboratory of the Tohoku University Graduate School of Medicine, Sendai, Japan.

**Echocardiography and Blood Pressure Measurements**

Transthoracic echocardiography was performed using the Vevo 2100 system (Visualsonics, Toronto, Canada). Mice were anesthetized with isofluorane using 1.5% for induction and 1% for maintenance of anesthesia. Blood pressure was measured by the tail-cuff system (Muromachi Kikai Co., Ltd., MK-2000ST NP-NIBP Monitor, Tokyo, Japan) in conscious mice.

**Telemetry ECG Monitoring**

Mice were anesthetized with 1:1 pentobarbital (4 mg/ml) and xylasine hydroxychloride (5 mg/ml) in 0.1 ml/10 g body weight intraperitoneally. The telemetric ECG transmitter (Data Sciences International, TA10ETA-F20, St. Paul, USA) was implanted into the abdominal cavity with the electrodes placed subcutaneously, as previously described. One week was allowed for convalescence after implantation. ECG was continuously recorded in freely mobile mice. Notocord-hem software (Notocord Systems S.A.S. Version 0.15, Croissy Sur Seine, France) was used for data acquisition.

**Quantitative Real-Time PCR (qRT-PCR)**

Total RNA was extracted from mouse hearts using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Venlo, the Netherlands). Reverse transcription was performed using PrimeScript RT Master Mix (Perfect Real Time) (Takara, Otsu, Japan). qRT-PCR was performed using the CFX96 Real-Time System (BioRad, Hercules, USA), with both SYBR Green I and TaqMan gene expression assays. Relative expression levels were normalized to those of the house-keeping gene Gapdh. The primers used are listed in Supplemental Table.

**Western Blot Analysis**

Total protein was prepared from the aortas and the embryonic hearts of mice for Western blots using previously described protocols. Briefly, proteins were separated via SDS-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with primary antibodies against total (t) myosin phosphatase (MYPT) (BD Transduction Laboratories, 612165, Franklin Lakes, U.S.A.), phosphorylated (p) MYPT (Millipore, ABS45, Billerica, USA), α-tubulin (Sigma-
Aldrich, T5168, St. Louis, USA), and β-actin (Abcam, ab6276, Cambridge, UK). Rho-
kinase activity was assessed by measuring the extent of phosphorylation of MYPT and was expressed as a relative blot density ratio, which was standardized to the positive control (PC) blot as (pMYPT sample density/tMYPT PC)/(tMYPT sample density/tMYPT PC density) 9.

Cell Protein Fractionation
Nuclear and membrane proteins were extracted from the ventricular tissues of mice using the Qproteome Cell Compartment Kit (Qiagen, Venlo, the Netherlands). Proteins were used for Western blotting and the PVDF membranes were probed with primary antibodies against β-catenin (Sigma-Aldrich, C7082, St. Louis, USA). Membranes were stripped in WB Stripping Solution (Nacalai Tesque, Kyoto, Japan) and re-probed with antibodies against α-tubulin (Sigma-Aldrich, T5168, St. Louis, USA) and lamin A/C (BD Transduction Laboratories).

Immunofluorescence
The hearts were perfused with PBS and perfusion-fixed with 4% phosphate-buffered paraformaldehyde at physiological pressure for 5 min. The whole heart was harvested, fixed for 6 hours, embedded in OCT (Tissue-Tek, Miles Inc., Elkhart, Illinois, USA) and snap-frozen, and cross-sections (10 μm) were prepared. The sections were stained with primary antibodies for plakoglobin (γ-catenin; Santa Cruz Biotechnology, sc-7900, Dallas, USA), plakophilin 2 (Cat. No. 651167, Progen Biotechnik GmbH, Heidelberg, Germany), desmoglein 1+2 (Cat. No. 61002, Progen Biotechnik GmbH, Heidelberg, Germany), α-actinin (Abcam, ab9465, Cambridge, UK), β-catenin (Sigma-Aldrich, C7082, St. Louis, USA), and actin (Santa Cruz Biotechnology, sc-1616, Dallas, USA). The secondary antibodies used were Alexa Fluor® 488 goat anti-rabbit IgG (H+L) antibody (Life Technologies, A-11008, Carlsbad, California, USA) and Alexa Fluor® 594 goat anti-mouse IgG (H+L) antibody (Life Technologies, A-11005, Carlsbad, California, USA). The sections were mounted with ProLong® Gold Antifade Reagent with DAPI (Life Technologies, P-36931, Carlsbad, California, USA) and examined under fluorescence microscopy.

Microarray
RNA was extracted from the whole heart of E12.5 mouse embryo and was quantified using NanoDrop 2000C (Thermo Scientific, Wilmington, USA). For microarray expression profiling, samples (n=4 in each group) were processed using the Agilent SurePrint G3 Mouse GE Microarray Kit (Agilent Technologies, G4852A Palo Alto, USA). The statistical computing software R (version 3.0.2.) was used for preprocessing and statistical analysis. Differentially expressed genes were considered significant at P values of <0.05. Genes with significant changes were further subjected to pathway analysis using Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com) to identify gene sets representing specific
biological processes or functions.

Statistical Analysis
The log-rank test was used to determine the P value for Kaplan-Meier survival curves. Other data were presented as mean ± SEM. Student’s t test was used to calculate the P values. P values of <0.05 were considered to be statistically significant.

References


