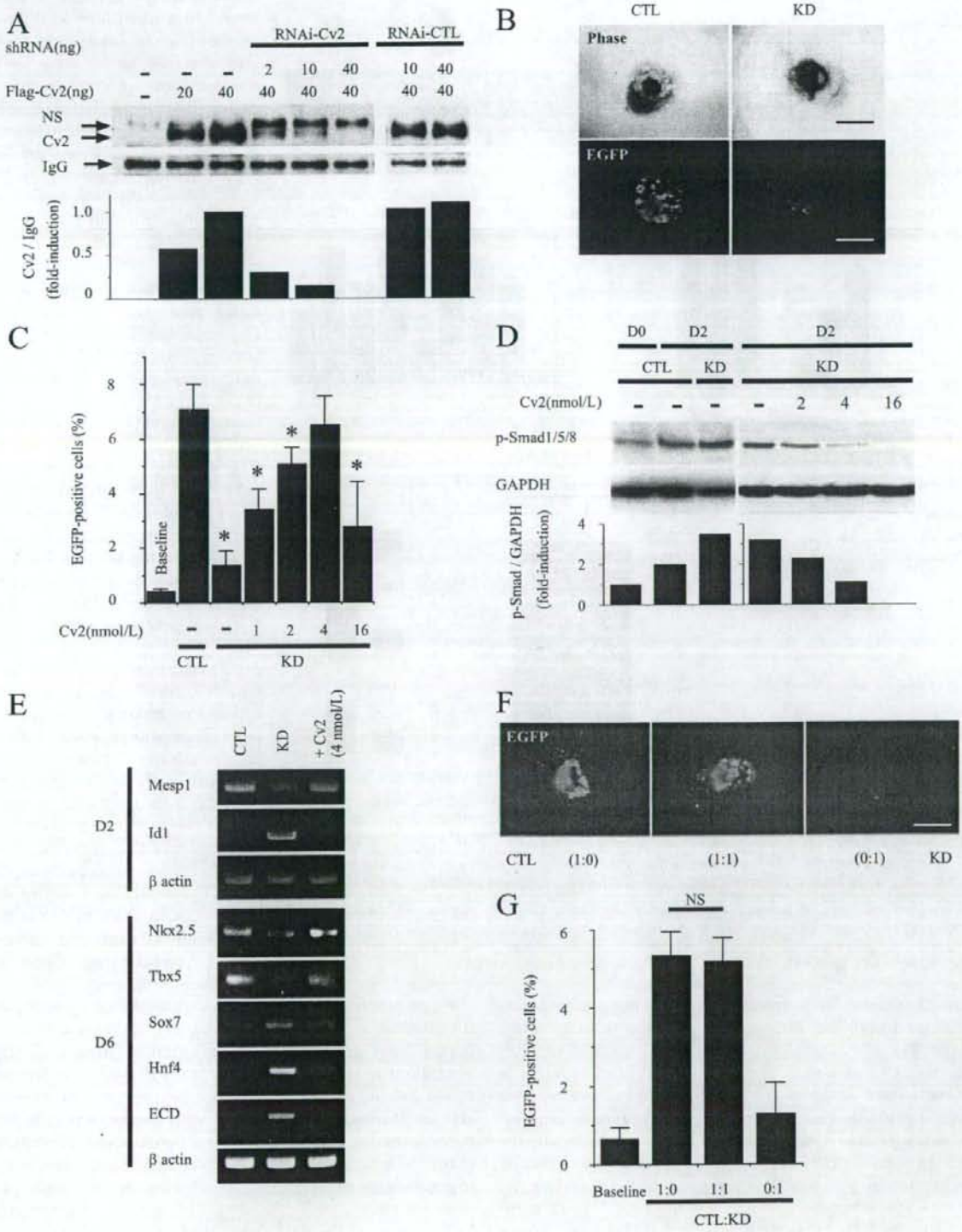

the BMP antagonists led to successive peaks of cardiomyocyte differentiation at 2 nmol/liter Cv2, 6 nmol/liter Noggin, and 2 nmol/liter BMPRIa-Fc protein (Fig. 4*D*). In contrast, treatment of three BMP antagonists with higher doses than IC_{50} independently diminished the generation of cardiac cells. These results suggest that the dose of suppression of BMP signals with Cv2 as well as Noggin and BMPRIa-Fc at this early stage is critical in effective cardiac myocytes generation.

Cv2 Regulates BMP Signaling for Cell Lineage Determination—Analogous to the dorsoventral patterning of mesoderm by BMP signal gradients in *Xenopus* (14), we hypothesized that the manipulation of BMP levels either by BMP2 or Cv2 would reproduce the fate decision of cardiac mesoderm. To gain insights into the molecular mechanisms by which the modulation of BMP2 activity plays a potential lineage determinant at the early time point of differentiation, we detected and measured pSmad1/5/8 at day 2 of differentiation as an indicator of activated BMP signaling. Exposure to BMP2 (1 nmol/liter) markedly induced the pSmad1/5/8 (Fig. 4*E*, *lane 3*) compared with no-treatment control (Fig. 4*E*, *lane 2*). Conversely, treatment with both 2 and 16 nmol/liter Cv2 reduced pSmad1/5/8 to the base-line level at day 0 and to the level beneath the base-line level at day 0, respectively (Fig. 4*E*, *lanes 4* and *5*). RT-PCR analysis of day 2 aggregates demonstrated that exposure to BMP2 completely blocked *Mesp1* expression and reduced *T* expression, whereas the *Id1* and *CK*, high-dose responders of BMPs, were remarkably up-regulated (Fig. 4*F*, *lane 2*). In addition, expression of *Oct3/4* sustained the high level relative to the others, suggesting that BMP-treated cells still sustain in the undifferentiated state. Conversely, treatment with Cv2 progressively down-regulated *Oct3/4* expression and did not induce expression of *Id1* and *CK*. Two nmol/liter Cv2 enhanced expression of *T* and *Mesp1* (Fig. 4*F*, *lane 3*), whereas 16 nmol/liter Cv2 diminished expression of mesoderm markers and advanced expression of neuronal marker *NeuroD1* (Fig. 4*F*, *lane 4*). When treated with 2 nmol/liter Cv2, real-time PCR analysis demonstrated the statistically significant increase in the mRNA levels of *Nkx2.5* and *Tbx5* (Fig. 4*G*). These results suggest the varying levels of BMP activity for priming differential genetic specification, including cardiac induction.

Loss of Cv2 Leads to Impaired Cardiomyocyte Differentiation—To further assess the involvement of endogenous Cv2 in cardiomyocyte differentiation, we performed loss-of-function experiments using short hairpin RNA specific for *Cv2* (Fig. 5*A*). RNAi-mediated knockdown (KD) of *Cv2* expression showed severely decreased cardiomyocyte differentiation at day 6 (Fig. 5, *B* and *C*). At day 2 of differentiation, increased pSmad1/5/8 and *Id1* expression were observed in KD cells but not in control cells (Fig. 5, *D* and *E*), whereas there were no differences between both cell types in the phosphorylation of ERK and p38 (data not shown). In parallel with impaired cardiomyocyte differentiation, expression of cardiac mesoderm markers was reduced, but expression of visceral endoderm markers such as *Sox7* and *Hnf4* and pan-endoderm marker E-cadherin was induced in *Cv2*-KD cells (Fig. 5*E*). These results suggest that *Cv2* KD preferentially gives rise to cells destined to the visceral endoderm through the up-regulation of BMP signals (32). These phenotypes in KD cells were reversed by the addition of recombinant Cv2 during the first 2 days of differentiation (Fig. 5*C*), and this was confirmed by mixing KD cells with control cells just before cardiac differentiation. Reduced cardiomyocyte differentiation in *Cv2*-KD cells was also reversed by co-culture with control cells KD (Fig. 5, *F* and *G*). These data suggest that *Cv2*-KD cells substituted loss of Cv2 for secreted Cv2 from control cells, and Cv2 itself can act in *trans* as an intercellular modulator in the extracellular space.

DISCUSSION

There are still many questions about the molecular mechanisms of cardiac induction of mesodermal progenitor cells into the cardiac lineage despite emerging evidence that several cardiac specific transcription factors and growth factors act as key regulators (1–3). A clear understanding about how stem cells differentiate into cardiac cells is essential for the clinical applications of stem cells and would be connected with innovation in novel medical treatments.

Evidence suggests that signaling of BMPs is crucial for regulating cardiac induction, differentiation, and development (2–7). The functional significance of BMP2/4 appears to be unique on the basis of opposite effects on cardiogenesis in the developmental stage-dependent manner, the inhibitory effect at early stage, and the promotive effect at late stage (7). Although the promotive effect of BMP signaling has been studied extensively (2–6), how the inhibitory effect of BMP signaling is regulated remains unclear. In the present study we show that distinct roles of BMP signaling for cardiomyocyte differentiation; BMP2 blocks cardiomyocyte differentiation before up-regulation of cardiac mesoderm markers, whereas BMP2 enhances cardiomyocyte differentiation when cardiac mesoderm was specified. We also show that Cv2, known as a suppressor for BMP signaling, plays a key role in the specification of cardiac cell lineage by inhibiting the anti-cardiogenic effect of BMP signaling at an early stage of cardiomyocyte differentiation.

Cardiac cell lineage is determined in the early developmental stage (such as gastrulation) when endo-, meso-, and epidermal cells differentiate and dynamically move under complex signal networks (31, 33). During the gastrulation stage, BMPs, in concert with BMP-binding proteins, specify mesoderm subdivision by demarcating the spatial extent (13, 14). Our careful stepwise

FIGURE 5. **Loss of Cv2 leads to impaired cardiac differentiation that is rescued by addition of Cv2 proteins or co-culture with parental cells.** *A*, representative western bolt shows Cv2 was successful knockdown of Cv2 by RNAi. Densitometry analysis shows significant reduction of secreted Cv2 proteins by RNAi in dose-dependent manner. *shRNA*, short hairpin RNA. *CTL*, control; *NS*, non-specific band. *B*, Cv2 RNAi clone showed impaired cardiac differentiation at day 6. *Scale bars* equal 250 μm in panels. *C*, treatment of KD cells with Cv2 during the first 2 days. Percentage of EGFP-positive cells assessed by FACS at day 7 ($n = 3$; *, $p < 0.05$ *versus* CTL). *Baseline* means no-treatment control at day 0. *D*, representative Western blot of pSmad1/5/8 at day 2. Densitometry analysis shows increased pSmad1/5/8 in KD cells more than control cells at day 2 of differentiation. Treatment of KD cells with Cv2 during the first 2 days inhibited pSmad1/5/8. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *E*, gene expression analysis by RT-PCR at day 2 and day 6 *ECD*, E-cadherin. *F*, representative figures showing that effects of co-culture on cardiomyocyte differentiation at day 6. *G*, percentage of EGFP-positive cells assessed by FACS at day 7 ($n = 3$). *NS*, not significant.

approaches recapitulating endogenous signals in the P19 cells revealed that the increase in BMP activity plays a repressive role for cardiomyocyte differentiation during the first 2 days of differentiation, at which specification of cardiac mesoderm occurs in embryos, evaluated by expression of *T/brachyury* (30) and *Mesp1* (34). Together with findings that up-regulation of *Cv2* transcription as well as *Bmp* transcription was detectable already at day 1 in the P19 system, these results suggest that the interaction between BMP and Cv2 plays a role in the decision of cardiac lineage fate.

We also found strong induction of *Id1* expression and blockade of *Mesp1* expression by administration of BMP2 during the first 2 days of cardiac differentiation, in concert with increased BMP activity. Because BMP signaling is known to suppress differentiation of stem cells and sustain self-renewal by Id1 (35), a negative regulator of basic helix-loop-helix transcription factors (36), this strong induction of *Id1* expression might block cardiac induction by suppressing differentiation itself. Alternatively, with findings that transcription of the basic helix-loop-helix transcription factor *Mesp1* but not *T* was specifically blocked, activation of BMP2 signaling at this timing might induce negative feedback repression of *Mesp1* expression by unknown factors, resulting in the blockade of differentiation into cardiac mesoderm from mesendoderm but not into endoderm lineages. In this model the level of BMP activity may be a critical determinant of the effects on cardiomyocyte differentiation, and it is also possible that timing of its signal is critical. Conversely, complete ablation of BMP signal at an early stage of development leads cells to different specification from cardiac lineage; in BMP receptor 1a null mouse embryos, the mesodermal formation is abolished at the onset of gastrulation, and no heart is formed (8). In our study, the optimized blockade of BMP activity with Cv2 (2 nmol/liter) enhanced cardiac myogenesis by promoting the specification of cardiac mesoderm but not by promoting the induction of undifferentiated mesoderm. Complete blockade of BMP activity by administration of Cv2 (16 nmol/liter) during the first 2 days of differentiation preferentially proceeded to neuronal cell lineage against cardiac cell lineage. These results support the emerging idea that early bursts of Cv2 acts to fine-tune the level of BMP activity to a dose that specifies commitment of cardiac cell fate rather than simply blocking BMPs.

The loss of Cv2 by RNAi inhibited cardiomyocyte differentiation and gave rise to cells destined to the endodermal lineage. In addition, we observed the up-regulation of *Id1* followed by predominant expression of endoderm marker but not mesoderm marker. Coupled with findings that administration of Cv2 during the first 2 days of differentiation or co-culture with wild-type cells restored impaired cardiomyocyte differentiation of *Cv2*-KD cells, these data implicate the functional importance of Cv2 in cardiac fate decision at the early stage of cardiomyocyte differentiation. Cv2 likely inhibits an unnecessary increase in BMP activity in the state of precardiac mesoderm. Currently, the genes that are regulated by this unnecessary BMP activity and may inhibit cardiomyocyte differentiation remain unknown.

Our concept that Cv2 antagonism of anti-cardiogenic BMP signaling at early cardiomyocyte differentiation seems to be similar to the results of recent studies, which found that transient inhibition by noggin significantly promoted cardiogenesis from embryonic stem cells (37). In our study, although we failed to detect transcripts of noggin at early stages of differentiation in the P19 system, biochemical analysis showed that Cv2 as well as noggin acts as an antagonist, at least, suggesting that P19 cells might substitute Cv2 for noggin in this process. Nevertheless, both might have different roles from each other in embryogenesis. Previous studies demonstrated that *in vivo* pattern of their transcriptions is quite different; noggin transcripts restrict in the node at gastrulation in the mouse embryo (38, 39), and contrary to this, Cv2 transcripts detect in the precardiac mesoderm as well as the posterior primitive streak (16, this study). In addition, based on structural characteristics (16, 19), it is supposed that Cv2 might be a bifunctional modulator of BMP activity. Recent studies have reported that Cv2 can be proteolytically cleaved and that both cleaved and uncleaved Cv2 display similar affinities to BMPs (17). Together with our findings that Cv2 is expressed autologously in the population of cardiac cells and is proteolytically cleaved in concert with cardiac differentiation, these results suggest a spatial and temporal control of Cv2 cleavage might cause context-dependent switching of a BMP antagonist to a BMP agonist at different stages of cardiomyocyte differentiation. Additionally, the functions of the long C terminus module of CV2 are still unknown (16, 19). It is likely that these domains mediate the interaction with other unknown proteins leading to further modification of BMP signals for cardiomyocyte differentiation. Elucidation of their functions may help to explain the roles of Cv2 on the complex anti- and pro-cardiogenic BMP activities in early stage of cardiomyocyte differentiation.

In summary, our results provide the evidence for the unique requirement of Cv2 antagonism of BMP signals at the early stage of cardiomyocyte differentiation in P19 cells. In addition, our results demonstrate that Cv2 acts to fine-tune levels of BMP activity for decision of differential lineages. This study will allow for the innovation of future technologies to develop cardiac cells efficiently from stem cells.

Acknowledgments—We greatly appreciate the gift of the BMP-responsive luciferase reporter plasmid from T. Katagiri (Saitama Medical School). We thank Y. Nakano and M. Kuramoto for expert technical assistance. K. H. is grateful to Christopher C. Hill for critical editing of the manuscript.

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MOLECULAR AND CELLULAR BIOLOGY, May 2008, p. 3424–3436 Vol. 28, No. 10
0270-7306/08/$08.00+0 doi:10.1128/MCB.02186-07

MURC, a Muscle-Restricted Coiled-Coil Protein That Modulates the Rho/ROCK Pathway, Induces Cardiac Dysfunction and Conduction Disturbance▽

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Received 11 December 2007/Returned for modification 10 January 2008/Accepted 4 March 2008

We identified a novel muscle-restricted putative coiled-coil protein, MURC, which is evolutionarily conserved from frog to human. MURC was localized to the cytoplasm with accumulation in the Z-line of the sarcomere in the murine adult heart. MURC mRNA expression in the heart increased during the developmental process from the embryonic stage to adulthood. In response to pressure overload, MURC mRNA expression increased in the hypertrophied heart. Using the yeast two-hybrid system, we identified the serum deprivation response (SDPR) protein, a phosphatidylserine-binding protein, as a MURC-binding protein. MURC induced activation of the RhoA/ROCK pathway, which modulated serum response factor-mediated atrial natriuretic peptide (ANP) expression and myofibrillar organization. SDPR augmented MURC-induced transactivation of the ANP promoter in cardiomyocytes, and RNA interference of SDPR attenuated the action of MURC on the ANP promoter. Transgenic mice expressing cardiac-specific MURC (Tg-MURC) exhibited cardiac contractile dysfunction and atrioventricular (AV) conduction disturbances with atrial chamber enlargement, reduced thickness of the ventricular wall, and interstitial fibrosis. Spontaneous episodes of atrial fibrillation and AV block were observed in Tg-MURC mice. These findings indicate that MURC modulates RhoA signaling and that MURC plays an important role in the development of cardiac dysfunction and conduction disturbance with increased vulnerability to atrial arrhythmias.

The heart is constantly exposed to biomechanical and neurohumoral stress, even under physiological conditions, and increased stress on the heart leads to hypertrophy and apoptosis of cardiomyocytes and ultimately heart failure (HF). Atrial fibrillation (AF) is one of the most common arrhythmias that produces substantially excess cardiovascular morbidity and mortality (13). Clinically, increased vulnerability to AF is associated with underlying heart disease, such as valvular heart disease, HF, coronary artery disease, and hypertension, particularly when left ventricular hypertrophy is present (13). The Z-disc in striated muscle constitutes an anchoring site for actin, titin, and nebulin filaments and plays a critical role in muscle structure and function, including sarcomeric assembly and organization, sarcolemmal membrane integrity, and muscle force generation and transmission (8). The Z-disc connects to the costamere, the basement membrane at periodic membrane-associated plaques, which serves to transmit force from the Z-disc to the sarcolemma and extracellular matrix (9). In addition to its role in muscle contraction, the Z-disc works as a biomechanical sensor that can respond to changes in tension in the sarcolemma. Various signaling molecules have been identified as components of the Z-disc, and a large number of the Z-disc-associated proteins have a dynamic distribution in muscle cells and shuttle between the Z-disc and other subcellular locations to transmit signals (8, 22, 35). Thus, the Z-disc is not only simply the structural border of the sarcomere but also functions in sensing and transmitting external and internal signals. Since the Z-disc is a multiprotein complex, the identification of the precise molecular mechanisms of the Z-disc and its role in signaling has become critical for understanding the regulation of cardiac function and the design of therapeutic strategies to prevent the progression to HF.

Rho GTPases are molecular switches that control a variety of signaling pathways in eukaryotic cells (10). The Rho family GTPase RhoA controls the formation of actin structures, and the RhoA-actin signaling pathway regulates serum response factor (SRF) transcriptional activity. SRF regulates serum-inducible and muscle-specific gene expression by binding to the serum response element (SRE) (also referred to as the CArG box). ROCK (Rho-kinase) is one of the downstream effectors of Rho GTPases (2, 32). In the heart, overexpression of RhoA resulted in sinus and atrioventricular (AV) nodal dysfunction, AF, and ventricular contractile failure with chamber enlargement and interstitial fibrosis (37). On the other hand, inhibition of Rho family protein activities by overexpression of Rho GDP dissociation inhibitor α resulted in an AV block with atrial enlargement and ventricular hypertrophy (47). These results suggest that fine-tuning of Rho GTPase signaling is required for maintaining cardiac rhythm, conduction, and structure.

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▽ Published ahead of print on 10 March 2008.

In this study, we sought to identify novel cardiac-restricted molecules that participate in cardiac homeostasis and pathogenesis. We identified MURC (*mu*scle-*r*estricted *c*oiled-coil protein) and characterized its function in cardiomyocytes and hearts.

MATERIALS AND METHODS

RNA extraction and quantitative reverse transcriptase (RT)-PCR. Total RNA was extracted from cells or tissues using an RNeasy Mini kit (Qiagen) or Trizol reagent (Invitrogen). Total RNA of human heart was purchased from Ambion. cDNA synthesis and kinetic real-time PCR were performed as described previously (33). Primers used were as follows: mouse MURC (mMURC) forward primer (5′-ACAGTCACACAGCAATACGGGCTA-3′) and mMURC reverse primer (5′-TTCTCGGGCAGGCTTCTGTCTTTA-3′); mouse atrial natriuretic peptide (ANP) forward primer (5′-AACCTGCTAGACCACCTGGA-3′) and mouse ANP reverse primer (5′-TGCTTTTCAAGAGGGCAGAT-3′); mouse brain natriuretic peptide (BNP) forward primer (5′-CTGAAGGTGCTGTCCC AGAT-3′) and mouse BNP reverse primer (5′-CCTTGGTCCTTCAAGAGCT G-3′); mouse serum deprivation response (mSDPR) forward primer (5′-ATGA GGAAGCCCTGGAAGAT-3′) and mSDPR reverse primer (5′-CCCAGATG ATGCTTTCTGGT-3′); mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer (5′-TTGTGATGGGTGTGAACCACGAGA-3′) and mouse GAPDH reverse primer (5′-CATGAGCCCTTCCACAATGCCAA A-3′); mouse α-myosin heavy chain (αMHC) forward primer (5′-GAGGACCA GGCCAATGAGTA-3′) and mouse αMHC reverse primer (5′-GCTGGGTGT AGGAGAGCTTG-3′); mouse β-myosin heavy chain (βMHC) forward primer (5′-TCGATTTGGGAAATTCATCC-3′) and mouse βMHC reverse primer (5′-CGCATAATCGTAGGGGTTGT-3′); mouse sarcoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) forward primer (5′-CTGTGGAGACCCTTGGTTGT-3′) and mouse SERCA2 reverse primer (5′-CAGAGCACAGATGGTGGCTA-3′); mouse transforming growth factor β1 (TGF-β1) forward primer (5′-TTGCTTC AGCTCCACAGAGA-3′) and mouse TGF-β1 reverse primer (5′-TGGTTGTA GAGGGCAAGGAC-3′); mouse TGF-β2 forward primer (5′-CAGCGCTACA TCGATAGCAA-3′) and mouse TGF-β2 reverse primer (5′-CCTCGAGCTCT TCGCTTTTA-3′); mouse TGF-β3 forward primer (5′-GATGAGCACATAGC CAAGCA-3′) and mouse TGF-β3 reverse primer (5′-ATTGGGCTGAAAGG TGTGAC-3′); mouse procollagen type I α1 (Col1a1) forward primer (5′-GAG CGGAGAGTACTGGATCG-3′) and mouse Col1a1 reverse primer (5′-GCTT CTTTTCCTTGGGGTTC-3′); mouse Col1a2 forward primer (5′-CCGTGCTT CTCAGAACATCA-3′) and mouse Col1a2 reverse primer (5′-GAGCAGCCA TCGACTAGGAC-3′); mouse Col3a1 forward primer (5′-GTCCACGAGGTG ACAAAGGT-3′) and mouse Col3a1 reverse primer (5′-GATGCCCACTTGT TCCATCT-3′); rat MURC (rMURC) forward primer (5′-ACTGAAGATGAA GACCAGGACGCA-3′) and rMURC reverse primer (5′-TGTTAACAACGTA GCCCGTGTTGC-3′); rat ANP forward primer (5′-ATACAGTGCGGTGTCC AACA-3′) and rat ANP reverse primer (5′-CGAGAGCACCTCCATCTCTC-3′); rat BNP forward primer (5′-GGAAATGGCTCAGAGACAGC-3′) and rat BNP reverse primer (5′-CGATCCGGTCTATCTTCTGC-3′); and rat GAPDH forward primer (5′-ATGGGAAGCTGGTCATCAAC-3′) and rat GAPDH reverse primer (5′-GTGGTTCACACCCATCACAA-3′).

SAGE. Serial analysis of gene expression (SAGE) was performed as described previously (33). The SAGE libraries were constructed essentially following the I-SAGE long kit protocol (Invitrogen) using total RNA extracted from adult mouse hearts. Double-stranded cDNAs were digested with NlaIII, and the restriction enzyme was replaced by MmeI after linker ligation. Ditags produced from 400 PCRs were isolated, cleaved with NlaIII, and cloned into pZErO. All sequence files were processed using SAGE2000 version 4.5 software. The extracted tags were further processed to determine the identity of associated genes through several stringent filters using the CGAP website (http://cgap.nci.nih.gov/SAGE).

Plasmid constructs. The corresponding cDNA fragments for human MURC (hMURC), mMURC, and rMURC were cloned by PCR from human heart cDNA, mouse heart cDNA, and rat cardiomyocyte cDNA templates, respectively. PCR was performed using the following primers: hMURC forward primer (5′-ATGGAACATAATGGGTCTGC-3′) and hMURC reverse primer (5′-TTA CGATGAGTGCTTTAAATCTAAC-3′); mMURC forward primer (5′-ATGG AACACAACGGATCAGCT-3′) and mMURC reverse primer (5′-CTATTTG TAGTCTGAGGACTGCTTTAGCTCCA-3′); rMURC forward primer (5′-AT GGAACACAATGGATCTGC-3′) and rMURC reverse primer (5′-CTATGAG GACTGCTTTAATTCCAAC-3′). The cDNAs encoding mMURC and hMURC with a C-terminal Flag epitope were cloned into pcDNA3 (Invitrogen) to generate pcDNA3-mMURC and pcDNA3-hMURC, respectively. The corresponding cDNA fragment for human SDPR (hSDPR) was cloned by PCR from human heart cDNA template. PCR was performed using hSDPR forward primer (5′-A TGGGAGAGGACGCTGACAGGC-3′) and hSDPR reverse primer (5′-TCAC GGCAGTCTGATCCACAT-3′). The cDNA encoding hSDPR with a C-terminal hemagglutinin (HA) epitope was cloned into pcDNA3 to generate pcDNA3-hSDPR. The cDNA encoding hMURC was cloned into pGBKT7 (Clontech) to generate a bait vector, pGBKT7-hMURC. The RNA interference (RNAi) target sequences for rMURC (5′-TTCGAGTAACCAAAGTCGAAA-3′), rat SDPR (rSDPR, 5′-GAAGCAGTGTGTACAGGTGAA-3′), and green fluorescent protein (GFP; 5′-CGTAAACGCCCACAAGTTC-3′) were cloned into the BamHI-EcoRI sites of the RNAi-Ready-pSIREN-RetroQ vector (Clontech) as an inverted repeat with a hairpin loop spacer to generate RNAi-Ready-pSIREN-RetroQ-rMURC, RNAi-Ready-pSIREN-RetroQ-rSDPR, and RNAi-Ready-pSIREN-RetroQ-GFP (used as a control), respectively.

Northern blot analysis. Total RNA was isolated from tissues with Trizol reagent (Invitrogen). Total RNA was size fractionated by electrophoresis in a 1.3% agarose gel containing 2.2 M formaldehyde and transferred to nylon membranes. A HindIII fragment of pcDNA3-mMURC (nucleotides 1 to 797 of the mMURC open reading frame) was used as a probe.

Production of polyclonal antibody. Rabbit immunization was conducted by Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan) using synthetic peptides spanning fragments of mMURC with N-terminal acetylation (GERLR QSGERFKKSISC). For immunostaining and Western blot analysis, immunoglobulin G (IgG) was purified from antisera with protein A-Sepharose beads.

Immunofluorescence microscopy. Specimens were fixed in 4% paraformaldehyde and stained with rabbit polyclonal anti-MURC antibody, mouse monoclonal anti-α-actinin antibody (Sigma), mouse monoclonal anti-α-smooth muscle actin antibody (Sigma), rat monoclonal anti-mouse CD31 antibody (BD Biosciences), rat monoclonal anti-HA antibody (Roche), mouse monoclonal anti-Flag antibody (Sigma), or rabbit polyclonal anti-Flag antibody (Sigma). Secondary antibodies were conjugated with Alexa Fluor 488, 555, or 594 (Invitrogen), and nuclei were visualized using 4′,6-diamino-2-phenylindole (DAPI; Invitrogen).

Cardiomyocyte culture. Rat neonatal cardiomyocytes, cultured from 1-day-old Sprague-Dawley rats, were prepared as described previously with slight modifications (42, 44). Briefly, ventricles were digested enzymatically, and cardiomyocytes were purified over a Percoll gradient. The culture medium was changed to serum-free medium after 24 h. Neonatal cardiomyocytes were cultured under serum-free conditions for 24 h before experiments. Adult cardiomyocytes were isolated from the hearts of male C57BL6 mice at 8 weeks of age. Ventricles were minced roughly and subsequently placed into 0.05% trypsin–EDTA (Invitrogen) at 4°C. After overnight incubation, ventricular tissue segments were put into Hanks' balanced salt solution buffer (Invitrogen) containing 0.1% (wt/vol) type 2 collagenase (Worthington Biochemical Corporation) at 37°C for 2 h. Dissociated adult cardiomyocytes were plated on 0.1% gelatin-coated slides and cultured in Dulbecco's modified Eagle's medium–F-12 medium with 5% bovine serum.

Aortic banding. Male mice were anesthetized with 2,2,2-tribromoethanol (0.25 mg/g of body weight; Aldrich). A midline abdominal incision was used to expose the suprarenal abdominal aorta. The aorta was tied with a 6-0 silk suture against a blunt needle (26 gauge). The needle was immediately removed, leaving the aortic lumen constricted to the diameter of the needle. Sham-operated mice were subjected to the same procedure without the aortic banding. Seven days after surgery, mice were sacrificed and total RNA was extracted from the heart.

Yeast two-hybrid screen. A *Saccharomyces cerevisiae* two-hybrid screen was performed using a Matchmaker Gal 4 two-hybrid system 3 (Clontech) and a Matchmaker pretransformed human heart cDNA library (Clontech) according to the manufacturer's instructions.

Replication-defective recombinant adenoviruses and gene transfer. The cDNAs encoding mMURC with a C-terminal Flag epitope and hSDPR with a C-terminal HA epitope were inserted into a pAxCAwtit cosmid vector in an adenovirus expression vector kit (Dual Version; Takara Bio Inc., Otsu, Japan). RNAi-Ready-pSIREN-RetroQ-rMURC and RNAi-Ready-pSIREN-RetroQ-luciferase (Clontech), which was used as a control, were digested with BglII and EcoRI to obtain the U6 promoter with each of the target sequences, and blunted fragments were inserted into a promoterless pAxcwit cosmid vector with an adenovirus expression vector kit (Dual Version). Recombinant adenoviruses expressing Flag-tagged mMURC (Ad-MURC), HA-tagged hSDPR (Ad-SDPR), LacZ (Ad-LacZ), MURC shRNA (Ad-rMURC shRNA), and Luc shRNA (Ad-Luc shRNA) were generated as described previously (43). Twenty-four hours after seeding, cardiomyocytes were infected with Ad-MURC, Ad-SDPR, Ad-LacZ, Ad-rMURC shRNA, or Ad-Luc shRNA diluted in the culture medium at a multiplicity of infection (MOI) of 10 or 20 and incubated at 37°C for 1 h. The

viral suspension was removed, and cardiomyocytes were cultured with serum-depleted culture medium. Phenylephrine (PE) or Y-27632 was added after infection.

Immunoprecipitation. COS cells were plated in 60-mm dishes. The following day, the cells were transfected with 1 μg of pcDNA3-hMURC and/or pcDNA3-hSDPR. The total plasmid amount was adjusted to 2.0 μg with an empty vector plasmid. Cardiomyocytes were plated in 60-mm dishes. The following day, the cells were infected with Ad-MURC and/or Ad-SDPR. The total MOI was adjusted to 20 with Ad-LacZ. Cells were cultured for another 48 h and lysed with a lysis buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.1 mM Na_3VO_4, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Cell lysates were incubated with anti-Flag M2 affinity gel (Sigma) or an anti-HA antibody and protein A-Sepharose beads (GE Healthcare) at 4°C. After the beads were extensively washed with the lysis buffer, the bound proteins were eluted by boiling the beads in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis.

Western blot analysis. Cell lysates were extracted with a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail (Pierce), 1 mM Na_3VO_4, and 1 mM NaF. Cell lysates were electrophoresed in 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were subsequently incubated with primary antibodies against Flag (Sigma), HA, and MURC. Horseradish peroxidase-conjugated anti-rabbit IgG, anti-rat IgG, and anti-mouse IgG (GE Healthcare) were used as secondary antibodies.

RhoA activation assay. RhoA activity was determined from protein isolated from adenovirus-infected neonatal cardiomyocytes or heart tissues of transgenic mice using an absorbance-based G-LISA RhoA activation assay biochemistry kit (Cytoskeleton) according to the manufacturer's instructions. Cell protein was isolated on day 2 postinfection using the provided cell lysis buffer. Heart tissue protein of mice at 6 weeks of age was also isolated by homogenizing in cell lysis buffer. Cell or tissue lysates were clarified by centrifugation at 10,000 rpm at 4°C for 2 min, and these extracts were processed rapidly on ice and snap-frozen until the time of assay. Protein concentration was determined according to the manufacturer's protocol, and extracts were equalized to contain total protein concentrations of 1 mg/ml for the assay. Signals were measured at an absorbance of 490 nm using a microplate spectrometer as suggested by the manufacturer.

Transfection and reporter assay. COS cells were plated in six-well plates. The following day, the cells were transfected with 200 ng of ANP luciferase reporter construct (−638 ANP Luc; kindly provided by Kenneth R. Chien, Massachusetts General Hospital, Boston, MA) or no-SRE1/SRE2 mutant in the ANP luciferase reporter construct (kindly provided by Andrew Thorburn, Wake Forest University School of Medicine, NC). The cells were also transfected with 1.0 μg of expression vectors containing hMURC, mMURC, RhoA Val14, hSDPR, GFP shRNA, or rSDPR shRNA, 500 ng of a C3 expression vector, and 200 ng of pTKβ-Gal using FuGene6 reagent (Roche). The total plasmid amount was adjusted to 1.9 μg with an empty vector plasmid. An expression vector containing RhoA V14 (the mutant of Gly to Val at codon 14), which is a point-mutated active form of RhoA, was kindly provided by Yoshimi Takai (Osaka University, Suita, Japan). A C3 expression vector was kindly provided by Seigo Izumo (Novartis Institutes for Biomedical Research, Cambridge, MA). Cells were cultured for another 48 h, lysed with 200 μl of reporter lysis buffer (Promega), and assayed for luciferase activity (by using a Promega assay) and β-galactosidase activity. Luciferase activity was normalized against β-galactosidase activity.

Myofibrillar organization analysis. Ad-LacZ- or Ad-MURC-infected cardiomyocytes were incubated in serum-free medium. Y-27632 was added after infection. After 96 h of 10 μM Y-27632 treatment, cells were fixed in 4% paraformaldehyde and stained with fluorescein isothiocyanate-conjugated phalloidin (Sigma) for the detection of actin filaments. Ad-Luc shRNA- or Ad-rMURC shRNA-infected cardiomyocytes were incubated in serum-free medium at 37°C for 48 h prior to stimulation with 100 μM PE. After 48 h of PE stimulation, cells were fixed and stained with fluorescein isothiocyanate-conjugated phalloidin.

Generation of transgenic mice. The cDNA encoding Flag-tagged mMURC was cloned into the third 5′-untranslated exon of αMHC promoter plasmid clone 26 (a generous gift from Jeffrey Robbins, Cincinnati Children's Hospital Medical Center, Cincinnati, OH) (16), and transgenic mice were generated as described previously (25). All of the aspects of animal care and experimentation performed in this study were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Echocardiography and electrophysiological analysis. Echocardiographic examination of mice was performed as described previously (25, 26, 39). Briefly, mice were anesthetized with 2,2,2-tribromoethanol (0.20 mg/g), and M-mode recordings of the left ventricle (LV) were obtained at the level of the papillary muscles from a parasternal window using a Hewlett-Packard (Andover) Sonos 5500 equipped with a 12-MHz probe. Mice anesthetized with 2,2,2-tribromoethanol (0.20 mg/g) by intraperitoneal injection were analyzed by multilead-surface electrocardiogram (ECG). ECG recordings were performed using an ECG-9902 (Nihon Kohden, Tokyo, Japan).

Hemodynamic measurements. For left ventricular catheterization with a 1.0-F high-fidelity micromanometer-tipped catheter (model SPR-1000; Millar Instruments), mice were anesthetized with 2,2,2-tribromoethanol (0.25 mg/g), subsequently anesthetized with isoflurane (2.5% [vol/vol] in O_2), and placed on a 36.5° table. A microtip catheter was inserted into the LV via the right carotid artery. To determine cardiac contractile and diastolic function, signals for LV pressure and the maximal rates of systolic pressure increase (maximum dP/dt) and isovolumetric relaxation (minimum dP/dt) were recorded at 1,000 Hz and analyzed by using a PowerLab system (AD Instruments).

Microarray analysis. Total RNA isolated from LV tissue was extracted using an RNeasy Mini kit and treated with the DNase I (Qiagen) according to the manufacturer's instructions. DNA microarrays used in these experiments were produced using the Mouse Genome Oligo 4.0 set of 70-mer oligonucleotides (Operon). Hybridization, processing, and the scanning process were done by Filgen Incorporated (Nagoya, Japan). Scan data images were analyzed using Microarray Data Analysis Tool version 2.0 software (Filgen).

Statistical analysis. All experiments were performed at least three times. Data are expressed as means ± standard errors and were analyzed by one-way analysis of variance with post hoc analysis. A P value of <0.05 was considered significant.

RESULTS

Cloning of MURC. To identify unknown cardiac-specific genes, we performed SAGE (45) using RNA isolated from the hearts of adult mice and searched expressed sequence tag databases for novel sequences found only in cardiac cDNA libraries. Sequences that did not correspond to known genes were then used as probes for Northern blot analysis of adult mouse tissues, in order to confirm their cardiac-specific expression. One of the cDNAs identified in this screen appeared to be a muscle-restricted gene, and the extended cDNA sequence encoded a novel protein of 362 amino acids (Fig. 1). We then isolated human and rat orthologues of this gene by using human heart and rat cardiomyocyte cDNAs. Northern blot analysis of RNA from adult mouse tissues revealed abundant expression of this transcript in the heart and skeletal muscle (Fig. 2A). Weaker signals were found in the aorta and lung, suggesting expression of this transcript in vascular smooth muscle cells. We refer to this gene as MURC (*mu*scle-*r*estricted coiled-coil protein), because the predicted open reading frame of MURC contains a putative coiled-coil motif. Database searches revealed that hMURC is encoded by a gene located on human chromosome 9q31. A cDNA encoding a MURC-like protein was also identified in the *Xenopus laevis* expressed sequence tag database (accession number NM_001097894). These findings suggest that MURC is an evolutionarily conserved protein in vertebrates.

Subcellular localization of MURC in cardiomyocytes. To identify a cell source expressing MURC in the heart, we separated cardiomyocytes from noncardiomyocytes using a rat primary culture and performed real-time RT-PCR. As shown in Fig. 2B, the MURC transcript was found to be much more abundant in cardiomyocytes than in noncardiomyocytes, indicating that the major source of MURC in the heart is cardiomyocytes. We then performed immunostaining of adult mouse heart sections with an anti-MURC antibody to investigate the subcellular localization of MURC in cardiomyocytes. MURC staining was detected in the cytoplasm with a striated and

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                                                    Coiled - coil
human   MEHNGSASNADKIHQNRLSSVTEDE-DQDAALTIVTVLDKVASIVDSVQASQKRIEERHREMENAIKSVQIDLLKLSQSHSNTGHIINKLFEKTRKVSAHI  100
mouse   MEHNGSASNAGKIHQNRLSSVTEDE-DQDAALTIVTVLDRVASVVDSVQASQKRIEERHREMGNAIKSVQIDLLKLSQSHSNTGYVVNKLFEKTRKVSAHI  100
rat     MEHNGSASNAGKIHQNRLSSVTEDE-DQDAALTIVTVLDRVATVVDSVQASQKRIEERHREMGNAIKSVQIDLLKLSQSHSNTGYVVNKLFEKTRKVSAHI  100
xenopus MDHETSSEKDRVNL-NRLSMVSEDSVDHDAALTIVAVLDKVATIVDTVQASQKRIEERHREMEDAIKTIQIELLKLAQAHSNTSYTVNKLLEKTRKVSSNV  100

human   KDVKARVEKQQIHVKKVEVKQEEIMKKNKFRVVIFQEKFRCPTSLSVVKDRNLTENQEEDDDDIFDPPVDLSSDEEYYVEESRSARLRKSGKEHIDNIKK  200
mouse   KDVKARVEKQQVRVTKVETKQEEIMKKNKFRVVIFQEDIPCPASLSVVKDRSLPENQEE-AEEVFDPPIELSSDEEYYVEESRSARLRKSGKEHIDHIKK  199
rat     KDVKARVEKQQVRVTKVETKQEEIMKKNKFRVVIFQEDVPCPASLSVVKDRSLPENEEE-AEEVFDPPIDLSSDEEYYVEESRSARLRKSGKEHIDHIKK  199
xenopus KDVKSRVDKQSDQVQKVESKQEEMLRKNKFRVVIFQEDIECPTKLTVAEQSALDGNLR---DD-LYPPDDLSSDEEYSVQESRTSRLKKSGMKRINNIKK  196

human   AFSKENMQKTRQNLDKKVNRIRTRIVTPERRERLRQSGERLRQSGERLRQSGERFKKSISNAAPSKEAFKMRSLRKGKDRTVAEGEECAREMGVDIIARS  300
mouse   AFSRENMQKTRQTLDKKVSGIRTRIVTPERRERLRQSGERLRQSGERLRQSGERFKKSISSAAPSKEAFKIRSLRKAKDPK-AEGQEVDRGMGVDIISGS  298
rat     AFSKENMQKTRQNFDKKVSGIRTRIVTPERRERLRQSGERLRQSGERLRQSGERFKKSISNATPSKEAFKIRSLRKPKDPK-AEGQEVDRGMGVDIISGS  298
xenopus AFSKESMQKTKQNFGNKVNRLRTRIVTPERRERLRQSGERL-------KTSGERFKQSIAKAAPKKEAFKINIKKKGKQRTTAEGQEGGKEDETDSEEPT  289

human   ESLGPISELYSDELSEPEHEAARPVYPPHEGREIPTPEPLKVTFKSQVKVEDDESLLLDLKHSS                                      364
mouse   LALGPIHEFHSDEFSETEKEVTKGGYSPQEGGDPPTPEPLKVTFKPQVRVEDDESLLLELKQSS                                      362
rat     LALGPIHEFHSDGFSETEKEVTKVGYIPQEGGDPPTPEPLKVTFKPQVRVEDDESLLLELKQSS                                      362
xenopus AK--PISEEVTYTEVVTVKKKKKKNEENAELLLSQEDEKPSPVPEKTEKPLLKVEKKAESDEIPLVDINLSQ                             359
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FIG. 1. Primary sequence of MURC. Alignment and amino acid sequence comparison of human MURC (GenBank accession number EU487253), mouse MURC (EU487254), and rat MURC (EU487255) and homology with a sequence from *Xenopus* (NM_001097894). Identical amino acids are shaded in gray, and gaps are represented by a dash. Positions in the amino acid sequence are given by the numbers. MURC contains a predicted coiled-coil domain.

periodic staining pattern and was partly colocalized with α-actinin in the Z-line (Fig. 3A), suggesting that MURC interacts with the Z-disc. Besides cardiomyocytes, MURC staining was also detected in vascular smooth muscle cells with a lower intensity than in cardiomyocytes, but not in other surrounding cells, including vascular endothelial cells (Fig. 3B and C), confirming the muscle-specific expression. In vascular smooth muscle cells, MURC staining was detected diffusely in the cytoplasm.

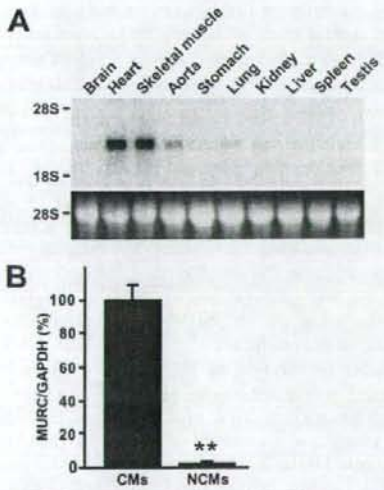


FIG. 2. MURC expression in tissues. (A) Blots were made with total RNA (20 μg) isolated from tissues of adult mice. The MURC transcript was observed in heart, skeletal muscle, aorta, and lung. 28S RNA was used as a control for assessing RNA loading. (B) Real-time RT-PCR was performed with cDNAs from cultured rat neonatal cardiomyocytes (CMs) and noncardiomyocytes (NCMs). **, $P < 0.01$ compared with CMs.

MURC expression in hearts under physiological and pathological conditions. To evaluate whether MURC expression is regulated during heart development, we analyzed mRNA expression in hearts of embryo, neonate, and adult mice. MURC mRNA expression in the heart was detected at embryonic day 10.5 (E10.5) (Fig. 4A, left). Real-time RT-PCR with RNA isolated from embryo (E10.5, E13. 5, E15.5, and E17.5), neonate, and adult hearts revealed that MURC mRNA expression increased during the developmental process from the embryonic stage to adulthood (Fig. 4A, right). We then investigated whether MURC expression might be regulated under pathological conditions in the postnatal heart. We performed abdominal aortic banding to make hypertrophied hearts in mice. Seven days after abdominal aortic constriction, the heart weight normalized to body weight ratio (HW/BW ratio) (band, 4.47 ± 0.03; sham, 4.09 ± 0.04; $P < 0.01$) and the heart weight normalized to tibial length ratio (HW/TL ratio) (band, 5.90 ± 0.06; sham, 5.21 ± 0.07; $P < 0.01$) were increased in banded mice compared with sham-operated mice, indicating that cardiac hypertrophy had indeed developed in the banded mice. As shown in Fig. 4B, MURC mRNA expression significantly increased in hypertrophied hearts (2.27-fold ± 0.12-fold; $P <$ 0.01 compared with sham-operated mice).

Identification of SDPR as a MURC-binding protein. To elucidate the molecular mechanism involving MURC, we screened over 5×10^7 clones of a human heart cDNA library with hMURC as the "bait" using the yeast two-hybrid system. In this screen, we obtained 13 independent clones. Sequence analysis showed that five isolated cDNAs encoded copper metabolism domain containing 1 (COMMD1) and two isolated cDNAs encoded leucine zipper protein 1 (LUZP1). COMMD1 and LUZP1 were excluded during verification of the protein interaction. The eight other isolated cDNAs encoded SDPR, which is a phosphatidylserine (PS)-binding protein (5, 17, 18, 30). An amino acid homology search revealed that hSDPR was 31% identical to hMURC. A database search for conserved domains revealed that hSDPR also has a predicted coiled-coil

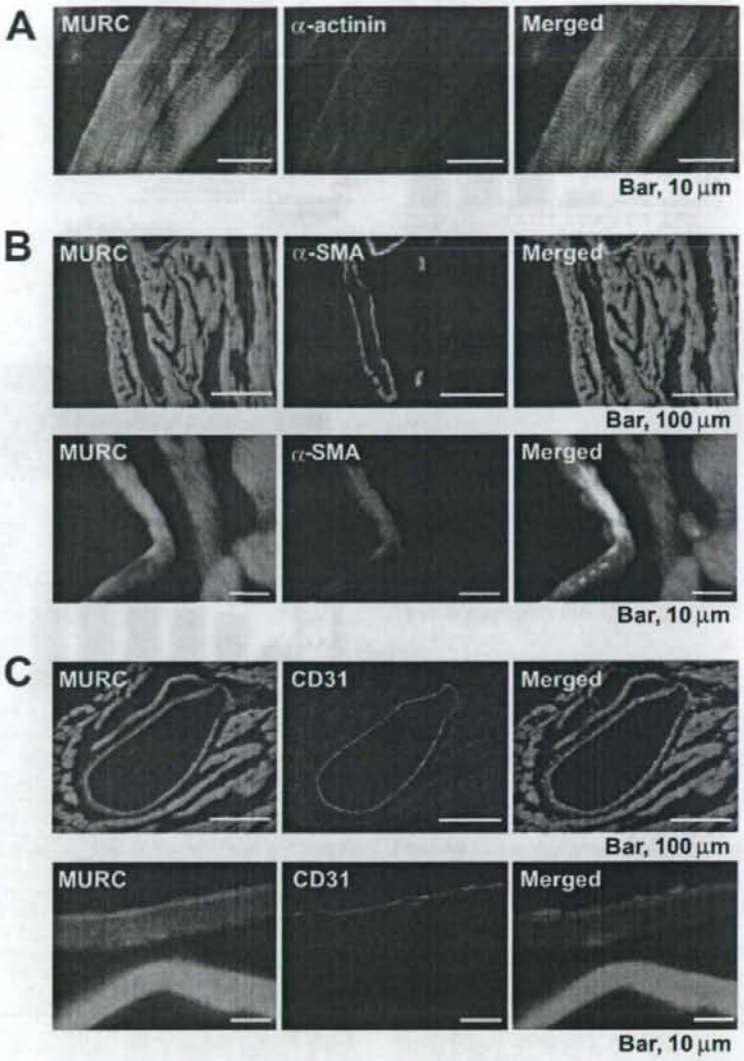


FIG. 3. Localization of MURC in the heart. (A) Immunostaining was performed using adult mouse heart sections with anti-MURC and anti-α-actinin antibodies. (B and C) Immunostaining was performed using adult mouse heart sections with an anti-MURC antibody and an anti-α-smooth muscle actin (SMA) antibody (upper panels) or an anti-CD31 antibody (lower panels). Nuclei were stained by DAPI (blue). Higher-magnification images are shown in lower panels.

domain. To examine the association between MURC and SDPR in mammalian cells, we transfected COS cells with constructs encoding Flag-tagged hMURC and HA-tagged hSDPR. Western blot analysis showed that hMURC was coimmunoprecipitated with hSDPR (Fig. 5A). Furthermore, we performed immunoprecipitation using cardiomyocytes infected with Ad-MURC and/or Ad-SDPR. As shown in Fig. 5B, Flag-tagged MURC was coimmunoprecipitated with HA-tagged SDPR in cardiomyocytes, confirming the interaction of MURC with SDPR in cardiomyocytes. In human and mouse tissues, SDPR mRNA has been shown to be highly expressed in heart and lung (17, 18). To assess the subcellular localization of SDPR, isolated adult cardiomyocytes were infected with Ad-SDPR. As shown in Fig. 5C, exogenous HA-tagged SDPR was localized to the cytoplasm, and MURC was localized to the cytoplasm and the Z-line. We then examined SDPR mRNA expression during heart development. As shown in Fig. 5D, SDPR mRNA expression in hearts gradually increased during embryonic stages and reached a maximum in neonates. Furthermore, SDPR mRNA expression increased significantly in

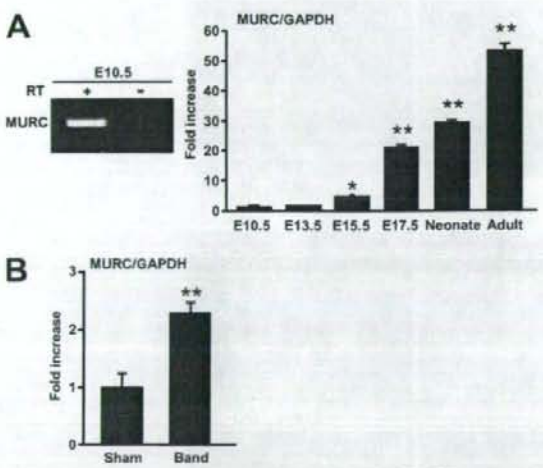


FIG. 4. MURC expression in the heart. (A) RT-PCR was performed with cDNAs from hearts at E10.5 (left). Real-time RT-PCR was performed with cDNAs from embryonic (E10.5, E13.5, E15.5, and E17.5), neonatal, and adult hearts (right). *, $P < 0.05$ compared with E10.5; **, $P < 0.01$ compared with E10.5. (B) Real-time RT-PCR was performed with cDNAs from hearts of sham-operated mice (sham) and abdominal aortic-banded mice (band). Seven days after surgery, total RNA was isolated from hearts. **, $P < 0.01$ compared with sham.

hypertrophied hearts (Fig. 5E) (1.78-fold ± 0.14-fold; $P < 0.01$ compared with sham-operated mice).

MURC activates RhoA and induces ANP mRNA expression and myofibrillar organization through the Rho/ROCK signaling pathway. SDPR is a PS-binding protein (5, 17, 18, 30). PS is implicated in signaling pathways, and PS-binding proteins contribute to a wide range of cellular process (40). Among PS-binding proteins, conventional protein kinase C (PKC) and Raf-1 have been reported to be implicated in the development of cardiac hypertrophy and heart failure (7, 31, 40). The observation of the interaction of MURC with SDPR prompted us to investigate the effect of MURC on signaling pathways in cardiomyocytes. The ANP gene is well-suited for this purpose, as it has been used as a molecular marker to explore signaling pathways that regulate cardiac gene expression during development and disease, and reactivation of ANP expression is part of a conserved adaptive change in molecular phenotypes in response to heart failure (24). Overexpression of MURC in cardiomyocytes using Ad-MURC induced ANP mRNA expression (Fig. 6A). ANP mRNA expression in cardiomyocytes is known to be regulated by the Rho/ROCK pathway (23). Furthermore, the cardiac phenotype of transgenic mice expressing Flag-tagged MURC (Tg-MURC), as described below, was reminiscent of that of transgenic mice expressing RhoA (37). Therefore, we examined whether MURC might alter RhoA activity in cardiomyocytes. RhoA was significantly activated by MURC in cardiomyocytes (Fig. 6B) (1.41-fold ± 0.07-fold; $P < 0.05$ compared with Ad-LacZ-infected cardiomyocytes). MURC-induced ANP mRNA expression was attenuated by a ROCK inhibitor, Y-27632, in a dose-dependent manner (Fig. 6C).

To further examine the effect of MURC signaling on the

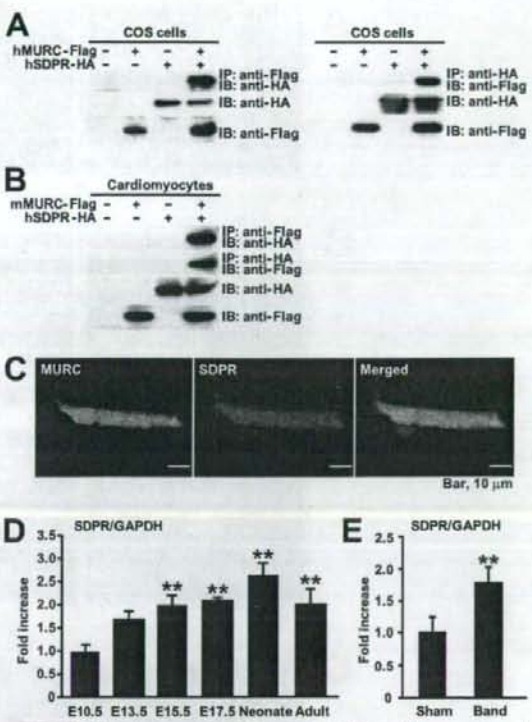


FIG. 5. Identification of SDPR as a MURC-binding protein and SDPR expression in the heart. (A) Immunoprecipitation from COS cells expressing hMURC-Flag and/or hSDPR-HA. hMURC-Flag alone, hSDPR-HA alone, or both proteins were transiently expressed in COS cells. Each cell extract was subjected to immunoprecipitation with the anti-Flag or anti-HA monoclonal antibody. The immunoprecipitate was then subjected to SDS-PAGE, followed by Western blot analysis with the anti-Flag or anti-HA monoclonal antibody. IP, immunoprecipitation; IB, immunoblot assay. (B) Immunoprecipitation from cardiomyocytes infected with Ad-MURC and/or Ad-SDPR. LacZ, mMURC-Flag with LacZ, hSDPR-HA with LacZ, or mMURC-Flag with hSDPR-HA was transiently expressed in cardiomyocytes. Each cell extract was subjected to immunoprecipitation with the anti-Flag or anti-HA monoclonal antibody. The immunoprecipitate was then subjected to SDS-PAGE, followed by Western blot analysis with the anti-Flag or anti-HA monoclonal antibody. (C) Localization of SDPR in adult cardiomyocytes. Adult cardiomyocytes were infected with Ad-SDPR at an MOI of 10. Immunostaining was performed using isolated adult mouse cardiomyocytes with anti-MURC and anti-HA antibodies. Nuclei were stained by DAPI (blue). (D) Real-time RT-PCR was performed with cDNAs from embryonic (E10.5, E13.5, E15.5, and E17.5), neonatal, and adult hearts. **, $P < 0.01$ compared with E10.5. (E) Real-time RT-PCR was performed with cDNAs from hearts of sham-operated mice and abdominal aortic-banded mice. Seven days after surgery, total RNA was isolated from hearts. **, $P < 0.01$ compared with sham.

transcription of ANP, we performed a luciferase reporter assay using the ANP promoter (−638 ANP Luc), which has two SRF-binding sites (21). hMURC transactivated −638 ANP Luc, and the extent of activation by mMURC was similar to that caused by hMURC (Fig. 6D). As shown in Fig. 6E, an activated mutant of RhoA, RhoA V14, transactivated −638

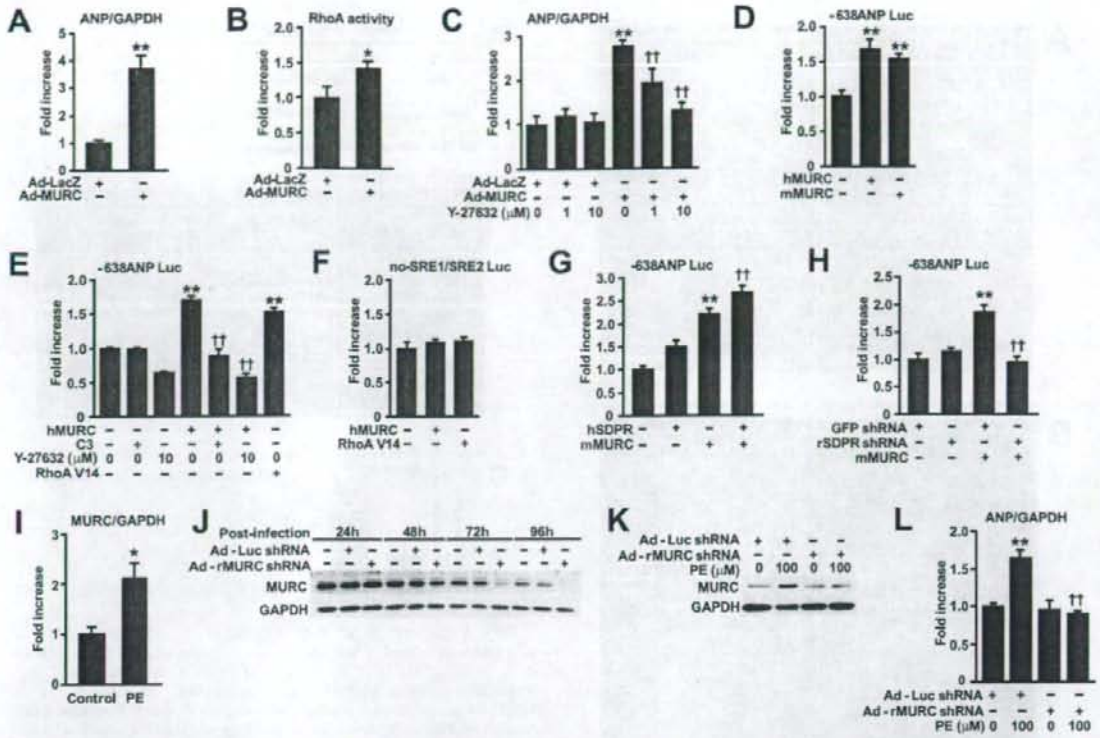


FIG. 6. Induction of ANP expression by MURC through the Rho/ROCK signaling pathway. (A and C) The bar graph shows real-time RT-PCR analysis of ANP mRNA expression in rat neonatal cardiomyocytes. Cells were infected with Ad-LacZ or Ad-MURC at an MOI of 10 and then harvested 48 h after the infection. Y-27632 was added after infection. **, $P < 0.01$ compared with Ad-LacZ; ††, $P < 0.01$ compared with Y-27632-treated Ad-LacZ. (B) The bar graph shows RhoA activity in rat neonatal cardiomyocytes. Cells were infected with Ad-LacZ or Ad-MURC at an MOI of 10 and then harvested 48 h after infection. *, $P < 0.05$ compared with Ad-LacZ. (D to F) The bar graphs show a reporter assay using −638 ANP Luc or no-SRE1/SRE2 Luc. hMURC, mMURC, C3, and/or RhoA V14 were cotransfected with −638 ANP Luc or no-SRE1/SRE2 Luc in COS cells. Cells were pretreated with Y-27632 for 30 min before transfection. Cells were harvested 48 h after transfection. **, $P < 0.01$ compared with control; ††, $P < 0.01$ compared with hMURC. (G and H) The bar graphs show a reporter assay using −638 ANP Luc. mMURC, hSDPR, GFP-shRNA, and/or rSDPR-shRNA was cotransfected with −638 ANP Luc in rat neonatal cardiomyocytes. Cells were harvested 48 h after transfection. **, $P < 0.01$ compared with control; ††, $P < 0.01$ compared with mMURC. (I) The bar graph shows real-time RT-PCR analysis of endogenous MURC mRNA expression. Rat neonatal cardiomyocytes were treated with vehicle or PE (100 μM) for 48 h and then harvested. *, $P < 0.05$ compared with vehicle. (J and K) The endogenous MURC protein level in neonatal cardiomyocytes was assessed by Western blot analysis. Cells were infected with or without Ad-Luc shRNA or Ad-rMURC shRNA at an MOI of 20. Cardiomyocytes treated with vehicle or PE were incubated for 48 h and then harvested. (L) The bar graph shows real-time RT-PCR analysis of ANP mRNA expression in neonatal cardiomyocytes. Cells were infected with Ad-Luc shRNA or Ad-rMURC shRNA at an MOI of 20. After 72 h of incubation, cells were treated with PE for another 24 h. **, $P < 0.01$ compared with Ad-Luc shRNA; ††, $P < 0.01$ compared with PE-treated Ad-LacZ shRNA.

ANP Luc. When Rho signaling was inhibited by C3, which specifically ADP-ribosylates Rho and inhibits its function (3), or Y-27632, MURC-induced transactivation of −638 ANP Luc was attenuated. We examined the requirement for the SRE sites in the ANP promoter for responsiveness to MURC signaling using no-SRE1/SRE2 Luc, which has mutations in both SREs in −638 ANP Luc (21, 41). As RhoA signaling has been reported to regulate SRF transcriptional activity (6, 14, 20, 44), the action of RhoA V14 was abolished in no-SRE1/SRE2 Luc, and the action of MURC was also abolished in no-SRE1/SRE2 Luc (Fig. 6F).

We then examined the role of SDPR in the MURC action in cardiomyocytes. When SDPR was transfected with MURC, SDPR augmented MURC-induced transactivation of −638 ANP Luc (Fig. 6G). RNAi using rSDPR shRNA was performed in neonatal rat cardiomyocytes to determine whether SDPR was required for the action of MURC. To evaluate the effect of rSDPR RNAi, we transfected COS cells with plasmids encoding Flag-tagged rSDPR and rSDPR shRNA. The protein expression of Flag-tagged rSDPR was inhibited by rSDPR RNAi (data not shown). rSDPR RNAi attenuated MURC-induced transactivation of −638 ANP Luc in cardiomyocytes (Fig. 6H). To exclude off-target effects of the rSDPR shRNA used, we used another shRNA sequence targeting SDPR and obtained similar effects on −638 ANP Luc (data not shown).

PE, an α1-adrenergic receptor agonist, induced MURC

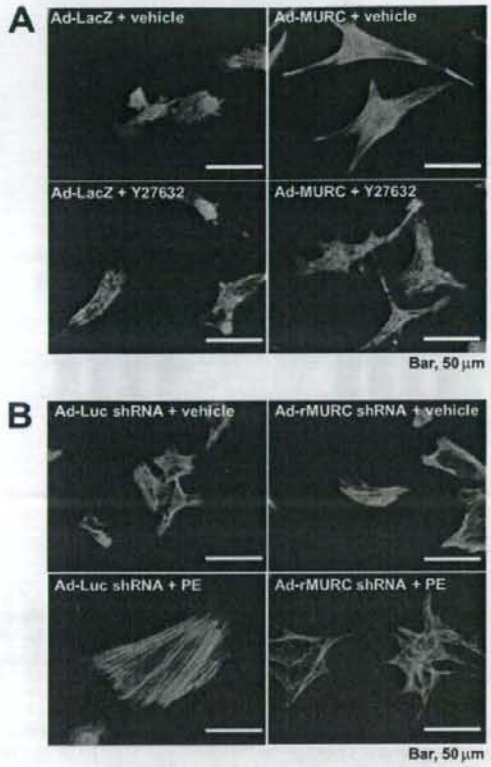


FIG. 7. Induction of myofibrillar organization by MURC through the Rho/ROCK signaling pathway. (A) Cardiomyocytes were infected with Ad-LacZ or Ad-MURC at an MOI of 10. After infection, cells were treated with vehicle or 10 μM Y-27632. Ninety-six hours later, cells were stained with fluorescein isothiocyanate-conjugated phalloidin. (B) Cardiomyocytes were infected with Ad-Luc shRNA or Ad-rMURC shRNA at an MOI of 20. After 48 h of serum deprivation, cells were treated with vehicle or 100 μM PE. Forty-eight hours later, cells were stained with phalloidin.

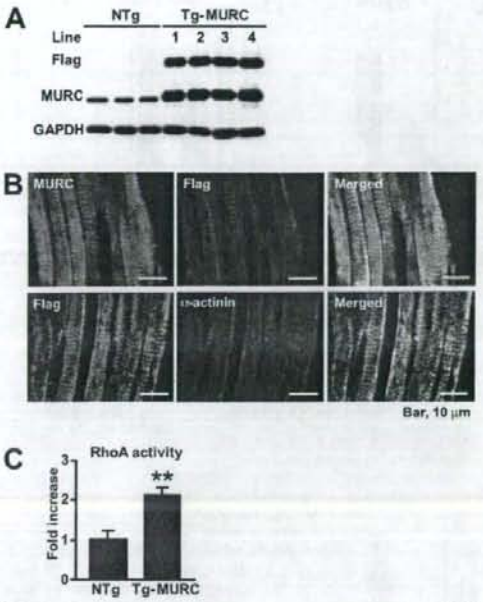


FIG. 8. Generation of a transgenic mouse expressing MURC. (A) Expression level of MURC protein in the heart. Heart lysates from NTg and Tg-MURC mice (line 1 to 4) were immunoblotted with antibodies against Flag (top panel), MURC (middle panel), or GAPDH as an internal control (bottom panel). (B) Subcellular localization of Flag-tagged MURC in cardiomyocytes of Tg-MURC mice. Immunostaining was also performed using adult mouse heart sections with an anti-Flag antibody, an anti-MURC antibody (upper panels), or an anti-α-actinin antibody (lower panels). (C) The bar graph shows RhoA activity in the hearts of NTg and Tg-MURC mice. **, $P < 0.01$ compared with NTg mice.

mRNA expression in cardiomyocytes (Fig. 6I). To directly examine the biological effects of MURC, we made a recombinant adenovirus expressing rMURC shRNA (Ad-rMURC shRNA) and examined the effect of MURC on PE-induced ANP mRNA expression. MURC protein expression was repressed in a time-dependent manner in cardiomyocytes infected with Ad-rMURC shRNA (Fig. 6J). When PE-induced upregulation of MURC protein was inhibited by Ad-MURC shRNA (Fig. 6K), PE-induced ANP mRNA expression was attenuated (Fig. 6L). We used another shRNA sequence targeting MURC and obtained a similar effect on −638 ANP Luc (data not shown).

The Rho/ROCK pathway has been also shown to mediate myofibrillar organization in cardiomyocytes (23). Therefore, we examined whether MURC could affect myofibrillar organization. Overexpression of MURC in cardiomyocytes using Ad-MURC induced myofibrillar organization, and MURC-induced myofibrillar organization was inhibited by Y-27632 (Fig. 7A). Furthermore, PE-induced myofibrillar organization was attenuated in cardiomyocytes infected with Ad-rMURC shRNA (Fig. 7B). Thus, MURC also affected myofibrillar organization in cardiomyocytes.

Generation of transgenic mice expressing MURC. To assess the functional effect of MURC in postnatal hearts, cardiac-specific transgenic mice expressing Flag-tagged MURC, Tg-MURC mice, were generated under the control of the αMHC promoter (16). Western blot analysis using an anti-Flag antibody and an anti-MURC antibody revealed that four independent lines of transgenic mice expressed comparable levels of transgenic protein (Fig. 8A). Immunostaining showed that Flag-tagged MURC was localized to the cytoplasm and the Z-line in cardiomyocytes of Tg-MURC mice (Fig. 8B). This finding supports the subcellular localization of MURC in cardiomyocytes, which was shown in Fig. 3A. We examined RhoA activity in the hearts of Tg-MURC mice. Consistent with the in vitro data, RhoA activity increased significantly in the hearts of Tg-MURC mice at 13 weeks of age (Fig. 8C) (2.11-fold ± 0.12-fold; $P < 0.01$ compared with nontransgenic [NTg] mice).

Cardiac contractile dysfunction in Tg-MURC mice. We measured heart weight at 13 weeks of age in Tg-MURC mice (Table 1). Although the HW/BW ratio in Tg-MURC mice was significantly lower than that in NTg mice, no significant differ-

TABLE 1. Morphometric analysis of NTg and Tg-MURC mice[a]

Mice (*n*)	BW (g)	TL (mm)	HW (mg)	HW/BW (mg/g)	HW/TL (mg/mm)	LW (mg)	LW/BW (mg/g)	LW/TL (mg/mm)
NTg (4)	25.00 ± 0.20	17.30 ± 0.12	120.50 ± 3.88	4.82 ± 0.19	6.97 ± 0.27	137.75 ± 2.63	5.51 ± 0.09	7.96 ± 0.16
Tg-MURC (6)	26.33 ± 0.65	17.58 ± 0.14	113.67 ± 4.74	4.31 ± 0.11[*b]	6.47 ± 0.26	140.67 ± 3.18	5.35 ± 0.14	8.00 ± 0.17

[a] BW, body weight; TL, tibial length; HW, heart weight; LW, lung weight. Values are expressed as means ± standard errors.
[b] *, $P < 0.05$ compared with sex-matched NTg mice.

ence in the HW/TL ratio was observed between Tg-MURC and NTg mice. The hearts of Tg-MURC mice at 13 weeks of age showed atrial chamber enlargement with a reduction in ventricular wall thickness (Fig. 9A and B). Histological assessment of cardiac pathology demonstrated a large organized thrombus in the left atrium (Fig. 9B). Diffuse interstitial fibrosis was detected in both atria and ventricles of Tg-MURC mice (Fig. 9C and D).

To address the functional consequences of MURC expression in vivo, echocardiography was performed on 12-week-old Tg-MURC and NTg mice, which confirmed reduced posterior wall thickness and depressed left ventricular systolic function in Tg-MURC mice compared with NTg mice (Table 2). Furthermore, we assessed systolic and diastolic LV pressures, contractility, and relaxation in Tg-MURC mice at 13 weeks of age. In Tg-MURC mice, the maximum LV *dP/dt* was significantly lower compared with that in NTg mice (Table 3). Thus, Tg-MURC mice showed cardiac contractile dysfunction associated with cardiac fibrosis.

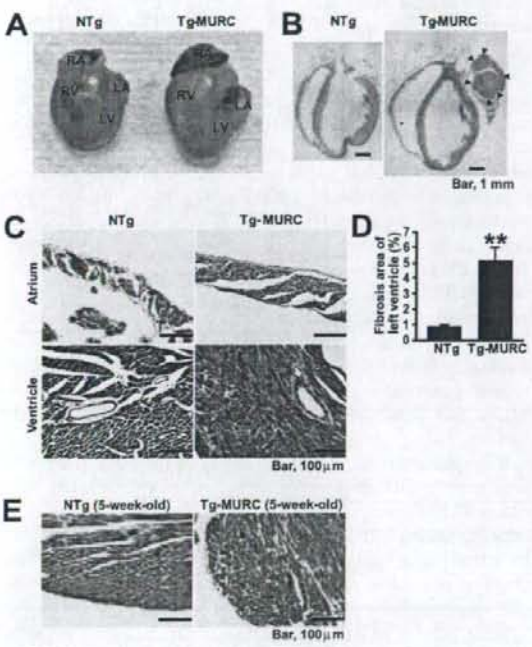


FIG. 9. Characterization of cardiac phenotypes in a transgenic mouse expressing MURC. (A) Representative hearts from 13-week-old NTg (left) and Tg-MURC (right) mice. (B) Sagittal sections of hearts from 13-week-old NTg (left panel) and Tg-MURC (right panel) mice. Arrows indicate a thrombus in the left atrium. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. (C) Histological assessment of cardiac fibrosis by Masson's trichrome staining. Representative trichrome-stained sections of cardiac atria (upper panels) and ventricles (lower panels) in 13-week-old NTg (left) and Tg-MURC (right) mice. (D) Quantification of fibrosis area (blue) from trichrome-stained cardiac histological sections in 13-week-old NTg versus Tg-MURC mice. **, $P < 0.01$ compared with NTg mice. (E) Histological assessment of the size of cardiomyocytes. Representative Masson's trichrome-stained sections of cardiac ventricles in NTg (left) and Tg-MURC (right) mice at 5 weeks of age.

We then examined the heart sections of Tg-MURC mice at 5 weeks of age to determine the earlier responses of cardiomyocytes to the MURC action in vivo. As shown in Fig. 9E, the cross-sectional area of cardiomyocytes in Tg-MURC mice was bigger than that in NTg mice (1.46-fold ± 0.02-fold; $P < 0.01$ compared with NTg mice), and various sizes of cardiomyocytes were observed in Tg-MURC mice. These findings suggest that the cardiac dysfunction in 12-week-old Tg-MURC mice is likely to be the end point of a pathological hypertrophy.

Conduction disturbances and increased vulnerability to atrial arrhythmias in Tg-MURC mice. ECGs were obtained from NTg and Tg-MURC mice at 9 and 12 weeks of age. Among 12 Tg-MURC mice analyzed by ECG at 9 weeks of age, 2 Tg-MURC mice (numbers 436 and 443) showed AF, 1 Tg-MURC mouse (429) showed complete AV block, and the other Tg-MURC mice showed AV conduction defects evidenced by prolongation of the PR interval (Fig. 10A). At 12 weeks of age, in Tg-MURC mouse 436 AF was sustained, whereas in Tg-MURC mouse 429 it showed a shift from complete AV block to AF, and Tg-MURC mouse 443 showed a shift from AF to complete AV block. The ECGs of other Tg-MURC mice with sinus rhythm (e.g., number 400) at 12 weeks of age demonstrated slow heart rate and prolongation of the PR interval (Fig. 10B). Furthermore, 16 Tg-MURC mice showed cardiac arrhythmias, including AF and AV block, among 38 Tg-MURC mice up to 20 weeks of age, whereas among 51 NTg mice, no mice showed cardiac arrhythmia.

To identify a molecular signature for the susceptibility to heart failure and atrial arrhythmias conferred by MURC, we compared microarray expression profiles of the ventricular myocardium from Tg-MURC and NTg mice and verified the findings by real-time PCR. ANP, BNP, βMHC, and fibrosis-related genes, such as TGF-β1, TGF-β2, TGF-β3, and procollagens, were upregulated in Tg-MURC mice compared with NTg mice, whereas SERCA2 was downregulated in Tg-MURC mice compared with NTg mice (Table 4).

To examine the role of MURC in ANP expression in vivo, we compared ANP mRNA expression in embryonic (E10.5), neonatal, and adult hearts of NTg and Tg-MURC mice by real-time RT-PCR. As shown in Fig. 11, ANP mRNA expres-

TABLE 2. Echocardiographic analysis of NTg and Tg-MURC mice[a]

Mice (n)	LVDd (mm)	LVDs (mm)	IVST (mm)	PWT (mm)	FS (%)
NTg (9)	3.27 ± 0.06	1.62 ± 0.05	0.58 ± 0.03	0.71 ± 0.03	50.6 ± 0.81
Tg-MURC (9)[b]	3.92 ± 0.13**	2.60 ± 0.14**	0.55 ± 0.02	0.58 ± 0.03**	34.2 ± 1.72**

[a] LVDd, left ventricular end-diastolic dimension; LVDs, LV end-systolic dimension; IVST, interventricular septum thickness; PWT, LV diastolic posterior wall thickness; FS, fractional shortening. Values are expressed as means ± standard errors.
[b] **, $P < 0.01$ compared with sex-matched NTg mice.

sion in embryonic and neonatal hearts of Tg-MURC mice was not altered compared with that in embryonic and neonatal hearts of NTg mice, respectively. However, ANP mRNA expression increased in adult hearts of Tg-MURC mice compared with NTg mice. Furthermore, although in NTg mice ANP mRNA expression decreased in adult hearts compared with neonatal hearts, which is consistent with a previous report (24), in Tg-MURC mice ANP mRNA expression increased in adult hearts compared with neonatal hearts. Since we used the αMHC promoter to generate Tg-MURC mice, most of the exogenous MURC gene driven by the αMHC promoter in the hearts of Tg-MURC mice would be expressed after birth. Taken together, our findings suggest that sustained overexpression of MURC in the postnatal heart regulates ANP expression in vivo.

DISCUSSION

We demonstrated MURC as a novel molecule localized to the cytoplasm and partly in the Z-line of the sarcomere in the heart. MURC mRNA was expressed in heart, skeletal muscle, and vascular smooth muscle. In vascular smooth muscle cells, which have no Z-disc, MURC protein was diffusely localized to the cytoplasm, suggesting that MURC is normally localized to the cytoplasm in addition to the Z-disc. We also identified SDPR as a MURC-binding protein. Exogenous SDPR was observed in the cytoplasm of cardiomyocytes. SDPR has been shown to be phosphorylated by PKC and to be localized to the cytoplasm (18). SDPR has been identified also as a PKCα-binding protein involved in the targeting of PKCα to caveolae (30). Caveolae have been suggested to be implicated in many cellular processes, including transcytosis of macromolecules, cholesterol transport, and signal transduction (15). Signaling through the caveolae is considered to be involved in the pathogenesis of the cardiovascular system (15). In cardiomyocytes, initiation and transduction of stretch-induced RhoA activation through caveloae have been reported (27). We demonstrated in the present study that MURC induced RhoA activation in vitro and in vivo. MURC also induced ANP mRNA expression and myofibrillar organization in cardiomyocytes, both of which were mediated by Rho/ROCK pathways. Furthermore, overexpression of SDPR in cardiomyocytes augmented MURC-induced transactivation of the ANP promoter, while RNAi-mediated knockdown of SDPR attenuated it. These findings suggest that in cardiomyocytes MURC functions as a molecule that shuttles between the Z-disc and other subcellular locations to transmit signals and that MURC requires SDPR to function efficiently in cardiomyocytes.

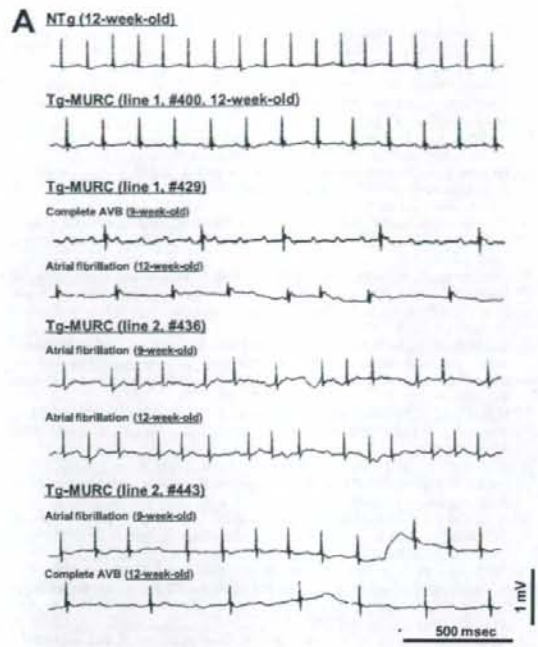
In addition to the activation of RhoA induced by MURC in vitro and in vivo, the cardiac phenotype of Tg-MURC mice was similar to that of transgenic mice expressing RhoA (37). These findings suggest that RhoA acts as one of the important downstream effectors of MURC in the heart. The Rho GTPase cycles between GDP-bound inactive and GTP-bound active forms (12). The nucleotide state of Rho GTPases is regulated by three kinds of regulators: guanine nucleotide exchange factors, GTPase-activating proteins, and Rho GDP dissociation inhibitors. Since MURC contains no recognizable protein motifs except for the coiled-coil motif, MURC probably interacts with molecules that directly or indirectly regulate the activity of upstream regulators for RhoA. The RhoA signaling pathway is activated by G protein-coupled receptor agonists angiotensin II and PE in cardiomyocytes (3, 23). The results shown here that MURC mediates PE-induced ANP mRNA expression and myofibrillar organization suggest the involvement of a MURC-regulated Rho/ROCK pathway. Taken together, our findings indicate that MURC per se causes signaling events to induce gene expression and myofibrillar organization, and MURC also serves to transmit signals from cell surface receptors.

Responsiveness to MURC signaling in the ANP promoter required the SRE sites, suggesting that MURC signaling regulates SRF transcriptional activity. Cardiac-specific transgenic mice expressing SRF develop severe heart failure accompanied by activation of the fetal gene program, cardiac hypertrophy, chamber dilatation, and interstitial fibrosis (49). Cardiac-specific overexpression of a mutant form of SRF that reduces the binding activity to SRE sites causes dilated cardiomyopathy (50), and deletion of SRF from the adult heart also shows dilated cardiomyopathy (34). These studies indicate that the function of SRF is critical for maintaining cardiac function in

TABLE 3. Hemodynamic analysis of NTg and Tg-MURC mice[a]

Mice (n)	HR (bpm)	SBP (mm Hg)	DBP (mm Hg)	LVEDP (mm Hg)	Max dP/dt (mm Hg/s)	Min dP/dt (mm Hg/s)	τ (ms)
NTg (4)	565 ± 2	87.5 ± 4.4	60.0 ± 5.6	6.6 ± 0.7	7,913 ± 572	−7,294 ± 534	8.2 ± 1.1
Tg-MURC[b] (6)	468 ± 15**	81.8 ± 3.8	54.5 ± 2.9	9.3 ± 1.2	5,599 ± 426*	−6,011 ± 599	16.1 ± 7.4

[a] HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEDP, left ventricular end-diastolic pressure; Max dP/dt, maximum LV dP/dt; Min dP/dt, minimum LV dP/dt; τ, time constant estimated from isovolumic LV pressure decay. Values are expressed as means ± standard errors.
[b] **, $P < 0.01$; *, $P < 0.05$ (compared with sex-matched NTg mice).



B

	NTg (n=9)	Tg-MURC (n=9)
HR (bpm)	516 ± 30	400 ± 17**
PR interval (msec)	43.89 ± 1.39	57.78 ± 1.47**

FIG. 10. Conduction disturbance and increased vulnerability to atrial arrhythmias in transgenic mice expressing MURC. (A) Representative ECG recordings from anesthetized NTg and Tg-MURC mice. ECG samples of Tg-MURC mice are shown at 9 and 12 weeks of age. (B) Tg-MURC mice have a slow heart rate and prolongation of the PR interval. ECG data were collected at 12 weeks of age. **, $P < 0.01$ compared with NTg mice. AVB, atrioventricular block; HR, heart rate.

the postnatal heart. Since Tg-MURC mice also exhibited a partly similar cardiac phenotype to transgenic mice expressing SRF, SRF transcriptional activation by MURC could contribute to the cardiac phenotype observed in Tg-MURC mice.

The striated muscle activator of Rho signaling (STARS) protein has been identified as a muscle-specific actin-binding protein localized to the I-band and the M-line of the sarcomere (4). STARS stimulates the transcriptional activity of SRF. STARS expression in the heart has been shown to be upregulated in mouse models of cardiac hypertrophy and in human cardiomyopathy, and cardiac overexpression of STARS sensitizes the heart to biomechanical stress induced by transverse aortic banding and calcineurin signaling (28). Since the localization of STARS abuts the Z-line on both sides (4), STARS appears not to be colocalized with MURC in cardiomyocytes. However, with regard to the Rho/ROCK pathway, MURC signaling overlaps with STARS signaling. Transgenic mice expressing STARS without biomechanical stress have been reported to show no cardiac dysfunction and no histological

TABLE 4. Changes in gene expression in hearts of Tg-MURC mice[a]

Symbol	Name	Fold increase
Nppa	Natriuretic peptide precursor type A (ANP)	39.00 ± 1.50**
Nppb	Natriuretic peptide precursor type B (BNP)	11.15 ± 1.21**
Myh6	Myosin, heavy polypeptide 6, cardiac muscle, alpha (αMHC)	0.91 ± 0.06
Myh7	Myosin, heavy polypeptide 7, cardiac muscle, beta (βMHC)	14.04 ± 2.13**
Atp2a2	ATPase, Ca transporting, cardiac muscle, slow twitch 2 (SERCA2)	0.74 ± 0.00**
Tgfb1	Transforming growth factor β1	1.50 ± 0.04*
Tgfb2	Transforming growth factor β2	27.36 ± 1.92**
Tgfb3	Transforming growth factor β3	3.30 ± 0.30**
Col1a1	Procollagen, type I α1	4.92 ± 0.92*
Col1a2	Procollagen, type I α2	3.80 ± 0.27**
Col3a1	Procollagen, type III α1	4.96 ± 0.89*

[a] Results shown are the ratios of gene expression in the hearts of Tg-MURC relative to NTg mice ($n = 3$). *, $P < 0.05$ compared with NTg mice; **, $P < 0.01$ compared with NTg mice.

abnormality (28). Tg-MURC mice spontaneously showed cardiac dysfunction and histological abnormality. The phenotypic difference between these mice may be caused by the action on the Rho/ROCK pathway and/or the functions that are dependent on their localization.

MURC expression in the heart increased during the developmental process from the embryonic stage to adulthood. Furthermore, in the postnatal heart MURC expression was upregulated in the hypertrophied heart. These results indicate that MURC expression is regulated under both physiological and pathological conditions. Tg-MURC mice showed cardiac contractile dysfunction and conduction disturbances with increased vulnerability to atrial arrhythmias, which was associated with an altered cardiac gene expression profile resembling that in pressure-overloaded mice but not in exercised mice (29). In Tg-MURC mice at 5 weeks of age, various sizes of cardiomyocytes were observed in Tg-MURC mice. These findings suggest that sustained overexpression of MURC in the postnatal heart causes pathological gene expression and that the expression level of MURC in the postnatal heart is critical for the maintenance of cardiac function and arrhythmogenesis. Increased inducibility of AF has been demonstrated in animal models of aging, congestive HF (CHF), atrial fibrosis, cardiac-specific overexpression of Rho A and constitutively active Rac1, and connexin 40 deficiency (1, 11, 19, 37). Atrial fibrosis

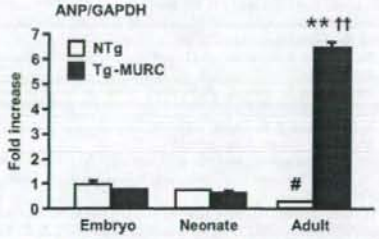


FIG. 11. ANP mRNA expression in embryonic, neonatal, and adult hearts of NTg and Tg-MURC mice. Real-time RT-PCR was performed with cDNAs from embryonic (E10.5), neonatal, and adult hearts of NTg and Tg-MURC mice. **, $P < 0.01$ compared with Tg-MURC embryo at E10.5; ††, $P < 0.01$ compared with NTg adult; #, $P < 0.05$ compared with NTg neonate.

increases with age in humans and is observed in patients with AF and in animal models of aging, CHF, and cardiac-specific overexpression of TGF-β1, tumor necrosis factor alpha, and angiotensin-converting enzyme (11, 36, 38, 46, 48). Genes involved in fibrosis, such as TGF-β and procollagens, were upregulated in the hearts of Tg-MURC mice, which also exhibited diffuse atrial fibrosis and spontaneous episodes of AF. Therefore, susceptibility to fibrosis is likely attributed to an increased vulnerability to AF in MURC-Tg mice. Our findings also suggest the critical role of MURC in the development of a vulnerable substrate for AF, especially in the setting of CHF.

In conclusion, the present study demonstrates that MURC is a Z-line-localizing molecule and it modulates the Rho/ROCK signaling pathway. Sustained overexpression of MURC facilitates functional deterioration, including cardiac function and conduction disturbances. Further investigation of the role of MURC will provide insights into the molecular mechanisms of the Z-disc that regulate cardiac homeostasis and pathogenesis.

ACKNOWLEDGMENTS

We thank M. Kuramoto, M. Nishikawa, A. Kosugi, and A. Yasui for their technical assistance. We also thank the following investigators for their kind gifts of plasmids: Jeffrey Robbins, Kenneth R. Chien, Andrew Thorburn, Yoshimi Takai, and Seigo Izumo.

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Japan Association for the Advancement of Medical Equipment, Takeda Science Foundation, and Mitsubishi Pharma Research Foundation.

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Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION
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Circulation 2007;116;1041-1051; originally published online Aug 13, 2007;
DOI: 10.1161/CIRCULATIONAHA.106.645416
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Central Role of Calcium-Dependent Tyrosine Kinase PYK2 in Endothelial Nitric Oxide Synthase–Mediated Angiogenic Response and Vascular Function

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Background—The involvement of Ca^{2+}-dependent tyrosine kinase PYK2 in the Akt/endothelial NO synthase pathway remains to be determined.

Methods and Results—Blood flow recovery and neovessel formation after hind-limb ischemia were impaired in $PYK2^{-/-}$ mice with reduced mobilization of endothelial progenitors. Vascular endothelial growth factor (VEGF)–mediated cytoplasmic Ca^{2+} mobilization and Ca^{2+}-independent Akt activation were markedly decreased in the PYK2-deficient aortic endothelial cells, whereas the Ca^{2+}-independent AMP-activated protein kinase/protein kinase-A pathway that phosphorylates endothelial NO synthase was not impaired. Acetylcholine-mediated aortic vasorelaxation and cGMP production were significantly decreased. Vascular endothelial growth factor–dependent migration, tube formation, and actin cytoskeletal reorganization associated with Rac1 activation were inhibited in PYK2-deficient endothelial cells. PI3-kinase is associated with vascular endothelial growth factor–induced PYK2/Src complex, and inhibition of Src blocked Akt activation. The vascular endothelial growth factor–mediated Src association with PLCγ1 and phosphorylation of ^{783}Tyr-PLCγ1 also were abolished by PYK2 deficiency.

Conclusion—These findings demonstrate that PYK2 is closely involved in receptor- or ischemia-activated signaling events via Src/PLCγ1 and Src/PI3-kinase/Akt pathways, leading to endothelial NO synthase phosphorylation, and thus modulates endothelial NO synthase–mediated vasoactive function and angiogenic response. (***Circulation***. **2007;116: 1041-1051.)**

Key Words: angiogenesis ■ endothelium ■ nitric oxide synthase ■ signal transduction ■ vasodilation

Nitric oxide (NO) has multiple functions in NO-mediated vascular action and angiogenic response. This was confirmed by endothelial NO synthase $(eNOS)^{-/-}$ mice exhibiting hypertension[1] or impaired vascular endothelial growth factor (VEGF) –induced angiogenesis.[2] VEGF phosphorylates eNOS, which is directly activated on the phosphorylation of ^{1177}serine in human (^{1176}serine in mouse) by Akt,[3,4] whereas the upstream molecules that activate Akt-eNOS system have not been fully clarified.

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Tyrosine kinases activate the PI3-kinase/Akt or Ca^{2+} signaling pathways, suggesting that tyrosine kinase is upstream of eNOS. Indeed, Src and VEGF receptor-2 activate eNOS through activation of the PI3-kinase/Akt pathway.[5] PYK2 (proline-rich tyrosine kinase), also known as RAFTK, CAK, and CADTK,[6,7] is the cytoplasmic tyrosine kinase and exhibits 45% amino acid sequence

Received August 9, 2005; accepted June 12, 2007.

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The online Data Supplement, consisting of an expanded Methods section and a figure, can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.106.645416/DC1.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.106.645416**

identity to focal adhesion kinase. Tyrosine phosphorylation of PYK2 and focal adhesion kinase was triggered by integrin-mediated adhesion.[8] PYK2 was stimulated by a broad range of physiological stimuli such as stimuli for G-protein–coupled receptors that elevate intracellular Ca^{2+},[6,7] phorbol ester, inflammatory cytokines, and stress signals, including ischemia.[9] PYK2 acts in concert with Src, which links Gi- or Gq-coupled receptors, leading to the MAP kinase pathway.[10] Furthermore, PYK2 binds to proteins that interact with the cytoskeleton, suggesting a role in the regulation of cellular morphology. The phenotype of PYK2-deficient mice was recently described as having macrophages that exhibit impaired migration as a result of cytoskeleton abnormality induced by diminished Ca^{2+} mobilization and reduced activation of PI3-kinase.[11] In this study, we newly generated $PYK2^{-/-}$ mice and investigated the molecular mechanism for the effects of PYK2 on NO-mediated vascular function and angiogenic response.

Methods

Statistical Analysis

Results are expressed as mean±SEM. All data were transformed by the natural logarithm before ANOVA corresponding to each experiment. Repeated-measures ANOVA was used to analyze the time course experiment. The Scheffé test was used as the multiple comparison test. For comparisons between 2 groups, a 2-sample *t* test was performed. A value of $P< 0.05$ (2 tailed) was considered statistically significant.

Materials, construction of targeting vector, generation of $PYK2^{-/-}$ mice (Figure I of the online Data Supplement), Western blotting, immunohistochemistry, transfection of DNA plasmid, measurement of GTP-Rho and GTP-Rac, migration, tubular formation, in vivo angiogenesis, preparation of endothelial progenitor cell (EPC) –like cells, hind-limb ischemia, laser Doppler perfusion image, cGMP assay, measurement of NO metabolites and NO levels, acetylcholine (Ach)- and nitroprusside-mediated vasodilatation, measurement of cytoplasmic Ca^{2+} concentration, fluorescence-activated cell sorting, and isolation of endothelial cells (ECs) from aorta and primary culture are described in the Methods section of the online Data Supplement. C57Bl/6 strain mice (SHIMIZU Laboratory Supplies, Kyoto, Japan) were used. The animal experiments were approved by our institutional review board.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Impaired eNOS/Akt Activation and Ca^{2+} Mobilization by PYK2 Deficiency

eNOS was reported to be activated by VEGF, Ach, or ischemic stress.[12] Incubation of the aorta with VEGF (100 ng/mL) phosphorylates PYK2 time dependently with a peak level around 5 minutes (Figure 1A). Ach (1 μmol/L) also caused PYK2 phosphorylation with a similar peak level, the extent of which was comparable to that in VEGF stimulation (Figure 1A). Ischemic stress time dependently increased the phosphorylation of PYK2 in the hind-limb muscle (Figure 1B). Such PYK2 phosphorylation was observed in the primary cultured aortic von Willebrand factor–positive ECs after stimulation with VEGF (100 ng/mL) and Ach (1 μmol/L) and exposure to 1% hypoxia (Figure 1C). To clarify the cell types expressing PYK2, an immunohistochemical analysis was performed. Figure 1D showed that PYK2 was present mainly in the endothelial ($CD31^+$ ECs) and medial layers (vascular smooth muscle cells) in the aorta and in the $CD31^+$ vessels in the skeletal muscle, in which a few cells in the interstitial region ($CD31^-$) also expressed PYK2.

We next examined whether the stimuli that induce PYK2 activation led to the phosphorylation of eNOS. Hind-limb ischemia causes eNOS phosphorylation in the skeletal muscle in wild-type (+/+) mice, whereas eNOS activation in the $PYK2^{-/-}$ mice was markedly inhibited (Figure 2A). Exposure of the aorta to VEGF or Ach also induced eNOS phosphorylation, whereas their activated levels were significantly diminished in the $PYK2^{-/-}$ mice (Figure 2B). Induction of eNOS phosphorylation by VEGF or Ach or exposure to 1% hypoxia also was observed (2.3- to 2.5-fold, respectively; $P<0.005$) in the wild-type aortic ECs but strongly inhibited in the PYK2-deficient ECs (Figure 2C).

The ischemia-induced Akt phosphorylation in the skeletal muscle was significantly lower in the $PYK2^{-/-}$ mice (46±3% at 2 hours, 34±3% decrease 1 day after ischemia; $P<0.01$) than the wild-type mice (Figure 2A), whereas the tyrosine phosphorylation level in VEGF receptor-2 (Flk-1) did not significantly differ between the wild-type and PYK2-deficient muscle (n=6 each; data not shown).

Akt phosphorylation in the aorta (Figure 2B) and ECs (Figure 2C) from the wild-type mice was significantly increased by VEGF stimulation (1.7±0.3-fold, $P<0.05$; and 3.0±0.7-fold, $P<0.005$, respectively), whereas Akt activation in the PYK2-deficient aorta and ECs was markedly attenuated (43±3% and 42±6% inhibition, respectively; $P<0.05$). Significant inhibition of Akt phosphorylation in ECs by PYK2 deficiency also was observed after exposure to 1% hypoxia (72±5% inhibition; $P<0.01$; Figure 2C).

We examined the involvement of AMP-activated protein kinase (AMPK)[13] and cAMP-dependent protein kinase-A (PKA),[14] known as the Ca^{2+}-independent kinase for phosphorylation of ^{1176}Ser-eNOS. The phosphorylation levels of AMPK and PKA after hind-limb ischemia did not differ significantly between $PYK2^{-/-}$ and wild-type mice (Figure 2A). Furthermore, the phosphorylation of AMPK and PKA in PYK2-deficient ECs exposed to 1% hypoxia for 18 hours also was similar to the wild-type ECs (data not shown). Akt, AMPK, and PKA showed peak phosphorylation on day 1 and at 2 hours, respectively; PKA and AMPK then reversed to baseline levels on day 7, whereas moderate activation of Akt was observed on day 7 (220±30% increase compared with basal level; Figure 2A). Neither AMPK nor PKA was activated 5 minutes after VEGF (100 ng/mL) treatment in both the wild-type and PYK2-deficient cells (data not shown), whereas dibutylic cAMP (1 mmol/L) and 5-aminoimidazole-4-carboxamide-1-β-D-riboside AICAR (1 mmol/L) apparently phosphorylated PKA and AMPK in the wild-type ECs (positive controls; data not shown). These findings suggest that Akt, rather than Ca^{2+}-independent AMPK or PKA, is involved in VEGF-mediated eNOS phosphorylation.

To prove that the reduced phosphorylation of eNOS is due directly to the loss of PYK2, we transfected GFP-tagged PYK2 plasmid to the PYK2-deficient ECs and studied by