Deletion of Ptpn11 (Shp2) in Cardiomyocytes Causes Dilated Cardiomyopathy via Effects on the Extracellular Signal–Regulated Kinase/Mitogen-Activated Protein Kinase and RhoA Signaling Pathways

Maria I. Kontaridis, PhD; Wentian Yang, MD, PhD; Kendra K. Bence, PhD; Darragh Cullen, MA; Bo Wang, MD; Natalya Bodyak, PhD; Qingen Ke, MD; Aleksander Hinek, MD, PhD, DSc; Peter M. Kang, MD; Ronglih Liao, PhD; Benjamin G. Neel, MD, PhD

Background—Heart failure is the leading cause of death in the United States. By delineating the pathways that regulate cardiomyocyte function, we can better understand the pathogenesis of cardiac disease. Many cardiomyocyte signaling pathways activate protein tyrosine kinases. However, the role of specific protein tyrosine phosphatases (PTPs) in these pathways is unknown.

Methods and Results—Here, we show that mice with muscle-specific deletion of Ptpn11, the gene encoding the SH2 domain–containing PTP Shp2, rapidly develop a compensated dilated cardiomyopathy without an intervening hypertrophic phase, with signs of cardiac dysfunction appearing by the second postnatal month. Shp2-deficient primary cardiomyocytes are defective in extracellular signal–regulated kinase/mitogen-activated protein kinase (Erk/MAPK) activation in response to a variety of soluble agonists and pressure overload but show hyperactivation of the RhoA signaling pathway. Treatment of primary cardiomyocytes with Erk1/2- and RhoA pathway–specific inhibitors suggests that both abnormal Erk/MAPK and RhoA activities contribute to the dilated phenotype of Shp2-deficient hearts.

Conclusions—Our results identify Shp2 as the first PTP with a critical role in adult cardiac function, indicate that in the absence of Shp2 cardiac hypertrophy does not occur in response to pressure overload, and demonstrate that the cardioprotective role of Shp2 is mediated via control of both the Erk/MAPK and RhoA signaling pathways. (Circulation. 2008;117:1423-1435.)

Key Words: cardiomyopathies • heart diseases • molecular biology • myocardium • myocytes • signal transduction

Heart failure is an emerging epidemic and a leading cause of death in the United States.1 Delineating the signaling pathways that regulate normal survival, proliferation, and growth of cardiomyocytes and determining how these processes are deregulated are essential for us to understand the pathogenesis of cardiomyopathy and heart failure.

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Cardiomyocytes respond to a variety of soluble endocrine, paracrine, and autocrine factors, as well as solid-state signals, which in turn stimulate an array of membrane-bound receptors. Soluble agonists include classic growth factors such as heregulin, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1), which signal via receptor tyrosine kinases (RTKs), and cytokines and ligands for G-protein–coupled receptors,2 which signal, at least in part, via nonreceptor protein tyrosine kinases (PTKs). Solid-state signals (eg, ligands for integrins) also can activate nonreceptor PTKs. Upon receptor activation, PTK-associated receptors activate a number of downstream signaling cascades, including the Ras/extracellular signal–regulated kinase (Erk) mitogen-activated protein kinase (MAPK) pathway, other MAPKs (eg,
p38, jun N-terminal kinase [JNK]), the PI3K/Akt pathway, and in some cases the RhoA, Stat, and/or calcineurin/nuclear factor of activated T cells (NFAT) pathways. The integrated output of these pathways determines whether cardiomyocytes survive, hypertrophy, or proliferate. Consequently, different types of stimuli can result in distinct patterns of heart growth.

Although many cardiomyocyte signaling pathways involve PTKs, the role of specific protein tyrosine phosphatases (PTPs) in normal cardiac development and disease remains largely obscure. Shp2, a nonreceptor PTP containing 2 SH2 domains, a PTP domain, and a C-terminal tail with 2 tyrosine phosphorylation sites, is a key component of multiple RTK, cytokine receptor, and integrin signaling cascades. In most, if not all, PTK-signaling pathways, active Shp2 is required for Ras/Erk MAPK pathway activation, although the underlying mechanism remains controversial. Several studies have implicated Shp2 as a regulator of the small GTPase RhoA, and effects on JNK, Stat, and NFAT activation.

Human genetic studies implicate SHP2 in cardiac developmental defects. Missense gain-of-function mutations in PTPN11, the gene encoding SHP2, cause ~50% of cases of Noonan syndrome (NS), a fairly common (1 in 1000 to 1 in 2500 live births) autosomal-dominant disorder characterized by multiple, variably penetrant cardiac defects, including pulmonary valvular stenosis with dysplastic leaflets, septal defects, and hypertrophic cardiomyopathy (HCM). NS mutants are believed to cause disease primarily, if not all, PTK-signaling pathways, active Shp2 is required for Ras/Erk pathway activation, although the underlying mechanism remains controversial. Several studies have implicated Shp2 as a regulator of the small GTPase RhoA, and effects on JNK, Stat, and NFAT activation.

Mice bearing a floxed Shp2 allele (Shp2 fl/fl) were generated as described and maintained on a mixed 129SvJ/C57BL6/J background. Shp2 fl/fl mice were crossed with muscle creatine kinase (MCK)–Cre or α-myosin heavy chain (α-MHC)–Cre (M. Schneider, Baylor College of Medicine, Houston, Tex) transgenic mice (both maintained on a C57BL6/J background) to obtain MCK-Cre/Shp2 fl/fl (MCK-Shp2-null) or α-MHC-Cre/Shp2 fl/fl (α-MHC-Shp2-null) mice. Mice were genotyped using tail DNA; details are available (from M.I.K.) on request. Unless otherwise indicated, Shp2 fl/fl littermates were used as controls, and analyses were performed on 6- to 8-week–old male mice on a mixed 129SvJ/C57BL6/J background.

**Histology, Immunohistochemistry, and Morphometry**

Hearts were flushed with PBS, perfusion fixed in formalin or Bouin’s reagent, and paraffin embedded. Sections were stained with hematoxylin and eosin or reticulin at the Rodent Histopathology Core at Harvard Medical School (Boston, Mass). Apoptosis was assessed in paraffin-embedded heart slices by immunohistochemistry using cleaved caspase-3 antibodies or by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay, as previously described, at the Brigham and Women’s Hospital Histopathology Core Facility (Boston, Mass). Control immunohistochemistry for Shp2 expression was performed with a commercial polyclonal Shp2 antibody (C-18; Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Cardiomyocyte area, length, and width measurements were obtained on an average of 200 to 500 cardiomyocytes per animal with Sigma ScanPro 4.0 Software.

**Echocardiography**

Transhoracic echocardiography was conducted on nonanesthetized animals as described previously with a VisualSonics Vevo 770 high-frequency ultrasound rodent imaging system (VisualSonics, Toronto, Ontario, Canada). VisualSonics Vevo 770 software was used for data acquisition and subsequent analysis. Hearts were imaged in the 2-dimensional parasternal short-axis view, and an M-mode echocardiogram of the midventricular region was recorded at the level of papillary muscles.

**Quantitative Real-Time Polymerase Chain Reaction**

RNA was isolated from whole hearts with TRIzol (Invitrogen, Carlsbad, Calif), and quantitative real-time polymerase chain reaction was performed with SYBR-Green (Applied Biosystems, Foster City, Calif) and an Applied Biosystems 7900. Primer sequences and conditions are available (from M.I.K.) on request. Data were quantified with the comparative C_T method (ΔΔC_T) with Gapdh expression as a control.

**Electron Microscopy**

Electron microscopy was performed at the Harvard Medical School Facility. Briefly, mouse hearts were perfused in PBS and fixed with a mixture of paraformaldehyde (2%) and glutaraldehyde (1.5%) in cacodylate buffer (0.1 mol/L, pH 7.4). Pieces (1 mm^3) were dissected from the left ventricle, postfixed in 1% OsO_4, dehydrated in a series of alcohols, and embedded in Epon Araldite (SPI Supplies, West Chester, Pa). Ultrathin sections (silver to gold) were obtained with a Reichert Ultracut E microtome, stained with uranyl acetate and lead citrate, and observed in a Tecnai G^2 Spirit BioTWIN (Tecnai, Verona, Italy) with an AMT 2k charge-coupled device camera.

**Biochemical Analyses**

Mouse tissues were dissected, perfused in PBS, and immediately frozen in liquid N_2. Whole-cell lysates were prepared by homogenizing the tissue in radioimmunoprecipitation buffer (10 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 0.1% SDS, 1% Triton-X100, 1% sodium deoxycholate, 5 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, and a protease cocktail) at 4°C, followed by clarification at 14 000g.

Primary cardiomyocytes were isolated and cultured as previously described. Briefly, adult cardiomyocytes were obtained by heart Langendorff perfusion with Ca^2+-free Tyrode buffer (135 mmol/L NaCl, 4 mmol/L KC1, 1 mmol/L MgC12, 0.33 mmol/L NaH2PO4, 10 mmol/L HEPES) and 10 mmol/L glucose (pH 7.4), 10 mmol/L 2,3-butanediol monoxime (Sigma, St Louis, Mo), and 5 mmol/L
Taurine (Sigma) for 3 to 5 minutes. Perfusion was continued for 7 to 10 minutes with recirculating Tyrode solution containing collagenase D (0.3 mg/g body weight; Roche, Indianapolis, Ind), collagenase B (0.4 mg/g body weight; Roche), and protease XIV (0.05 mg/g body weight, Sigma). Ventricular tissue was then minced in Tyrode solution containing 2% bovine serum albumin (Sigma), incubated for 15 minutes at 37°C, and then filtered through a 250-μm nylon mesh. The cell suspension was centrifuged at 420 g for 2 minutes and then gradually subjected to Tyrode solution with increasing concentrations of calcium and decreasing concentrations of EDTA/H9262 at 4°C, followed by clarification at 14 000 rpm for 10 minutes with recirculating Tyrode solution containing collagenase D (0.3 mg/g body weight; Roche), papain (0.4 mg/g body weight; Sigma), proteinase XIV (0.05 mg/g body weight, Sigma). Typical yields were 1.5 to 2.5×10^6 cells per heart with 70% to 80% of the cells retaining their rod-shaped morphology. After 12 hours, cardiomyocytes were stimulated with 10^6 cells/mL, 10^8 cells/mL, or 10^9 cells/mL of either the Mek inhibitor UO126 (Calbiochem, San Diego, Calif) or the Rho kinase inhibitor Y27632 (Calbiochem) for 4 hours and then either were fixed with 3.7% paraformaldehyde and photographed or were lysed for biochemical assays, as described above.

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed with an anti-Erk1, anti-Akt, anti–Stat 3, anti-JNK, anti-NFATc3, anti–p-MLC, anti–MCL-2, or anti–Shp2 (Santa Cruz Biotechnology), anti-p38 (Cell Signaling Technology, Danvers, Mass), or anti-pAkt473, anti–pErk1/2, anti–p-p38, or anti–p-JNK (Cell Signaling), following the manufacturer’s instructions. Bands were visualized with enhanced chemiluminescence and quantified by densitometry (Molecular Dynamics/Amersham Biosciences Corp, Piscataway, NJ).

For RhoA assays, primary cardiomyocytes were harvested or whole hearts were homogenized and lysed in 50 mmol/L Tris (pH 8.0), 350 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mmol/L MgCl_2, 1 mmol/L sodium vanadate, 0.2 mmol/L phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin for 30 minutes at 4°C. Lysates were cleared by centrifugation at 20 800g for 10 minutes at 4°C and incubated for 45 minutes at 4°C with 50 μL of a 50% slurry of Rhotekin coupled to glutathione-Sepharose beads (Upstate Biotechnology) to precipitate GTP-bound Rho. Bound proteins were washed 3 times in cold wash buffer (50 mmol/L Tris [pH 8.0], 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L MgCl_2, 0.2 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin), eluted with loading buffer, and separated by 10% SDS-PAGE. RhoA was visualized by immunoblotting with 0.5 μg/mL of mouse monoclonal antibody (Santa Cruz Biochemicals).

**Aortic Banding**

Short- and long-term aortic banding experiments were performed as described previously. Briefly, mice were anesthetized with ketamine (300 mg/kg), Cardiac function was evaluated by measuring the maximum rate of increase (dP/dtmax) and decline (dP/dtmin) in left ventricular pressure with a micromanometer catheter (Millar 1.4F, SPR 671, Millar Instruments, Houston, Tex) positioned in the left ventricle via a right common carotid artery cannulation. Mechanical stress was imposed on the left ventricle through ascending aortic coarctation. A separate group of mice (sham) underwent the same surgical procedures but were not banded.

**Statistical analysis**

Results are expressed as mean±SEM. Comparisons between groups were made by unpaired 2-tailed Student t test, with values of P<0.05 considered statistically significant.
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

Generation of Muscle-Specific Shp2-Null Mice

We generated an inducible (“floxed”) allele of Ptpn11 (Shp2fl/fl) by flanking exon 11 with loxP sites33 and then bred these mice to mice expressing Cre recombinase under the control of the MCK promoter (Figure 1A)34 to characterize the effects of Ptpn11 deletion in skeletal muscle.12 MCK also is expressed in cardiomyocytes, and the MCK-Cre line can sometimes catalyze excision of floxed alleles in the heart.34 In the course of our initial studies, we noticed that MCK-Cre:Shp2 fl/fl (MCK-Shp2–null) mice showed substantial cardiac deletion of Shp2 (Figure 1B). In normal (Shp2 fl/fl) hearts, Shp2 levels were easily detectable at birth and remained present throughout adulthood (Figure 1B). Although Shp2 levels at birth were unaffected in MCK-Shp2–null mice, they declined by 50% by 2 weeks and were undetectable at 2 months, giving us the opportunity to study the functional role of Shp2 in mature adult cardiomyocytes. Shp2 levels were unaffected in the liver (Figure 1C) and other tissues (data not shown) of MCK-Shp2–null mice.

Lack of Shp2 Causes Dilated Cardiomyopathy

As reported previously, MCK-Shp2–null mice were born at the expected Mendelian ratio and displayed no overt phenotype.12 However, although mutant hearts appeared normal at birth, they were grossly enlarged by 8 weeks (Figure 2A). Histological analysis of hematoxylin and eosin–stained longitudinal sections of 6- to 8-week–old hearts revealed dramatically dilated ventricular chambers without evidence of hypertrophy (Figure 2B). The ratio of heart weight to body weight, heart DNA content, and the ratio of heart protein to DNA content were unaffected in MCK-Shp2–null mice compared with littermate Shp2 fl/fl or MCK-Cre:Shp2+/+ mice (Figure 2C and 2D and data not shown).
Echocardiography confirmed that the ventricular cavities of MCK-Shp2–null mice were markedly dilated (Figure 3A). Left ventricular posterior wall thickness was decreased substantially, and left ventricular chamber diameter had a significant increase. Concomitant with these anatomic abnormalities, left ventricular function, as assessed by ejection fraction and fractional shortening, was severely impaired in MCK-Shp2–null mice at various ages. The "wild-type groups" comprised 3 Shp2 fl/fl mice for each of the age categories, with the remainder of each group made up of Mck-Cre:Shp2+/– mice. No statistical difference in cardiac function between the Shp2 fl/fl mice and Mck-Cre:Shp2+/– littermates was found (These 2 groups were then analyzed as 1 group against the Mck-Shp2–null mice of similar ages). *P < 0.05, 6- to 8-week-old MCK-Shp2–null mice vs Shp2 fl/fl and Mck-Cre:Shp2+/– mice. No other age group revealed any statistical differences. C, Total RNA from Shp2 fl/fl and MCK-Shp2–null mice (n=8 of each) at 8 weeks of age was used to perform quantitative reverse-transcription polymerase chain reaction. The ratio of ∆∆Ct was analyzed with Gapdh as a control. BW indicates body weight; HR, heart rate; LVPW th, left ventricular posterior wall thickness; LVID-d, left ventricular chamber dimension in diastole; EF, ejection fraction; and FS, fractional shortening. *P<0.05, significant differences in sarco(endo)plasmic reticulum calcium pump, atrial natriuretic factor, and brain natriuretic peptide gene expression between MCK-Shp2-null and Shp2 fl/fl control mice.

End-stage HCM can transition to DCM. Although MCK-Shp2–null mice clearly had DCM at 6 to 8 weeks, it remained formally possible that they first develop HCM, which then rapidly converts to a dilated state. To test this possibility, we carefully monitored cardiac morphology, histology, and function (as assessed by echocardiography) in younger mice (from 1 to 8 weeks of age) yet found no evidence of antecedent hypertrophy (Figures 2C, 3A, and 3B). We conclude that MCK-Shp2–null mice dilate directly, with kinetics consistent with the complete (or nearly complete) deletion of Shp2 in adult cardiomyocytes (Figure 1B).

### Structural Effects of Shp2 Deletion on Cardiomyocytes

Histological examination indicated that, although Shp2 deficiency did not affect cardiomyocyte number, MCK-Shp2–null cardiomyocytes were longer and thinner and their nuclei were enlarged compared with those from control Shp2 fl/fl mice (Figure 4A and 4B). Masson’s trichrome and periodic acid–Schiff staining showed no evidence of fibrosis or abnormal glycogen, respectively (data not shown). Also, no evidence existed of increased apoptosis, as assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay or immunohistochemistry for cleaved (active) caspase-3 in MCK-Shp2–null hearts (online-only data not shown).
Data Supplement Figure II). However, electron microscopy of MCK-Shp2–null hearts showed defective demarcation of sarcomeric Z lines (Figure 4C, lower right, arrow). The structure of the intercalated disks also was altered, with the presence of lacunae, widened spaces at the site of myofibril attachment to the intercalated disks (Figure 4C, lower right, arrowhead). Individual myofibrils were longer and mitochondria were larger (Figure 4C, lower right panel, asterisk) in MCK-Shp2–null hearts compared with Shp2 fl/fl controls (Figure 4C, lower left). Morphometric analysis confirmed that MCK-Shp2–null cardiomyocytes were longer and thinner than their Shp2 fl/fl littermates with no difference in total number of cells or area per cardiomyocyte (Figure 4D and data not shown). In concert, these data show that cardiac Shp2 deficiency causes a primary DCM.

**Loss of Shp2 in the Heart Disrupts Erk/MAPK Activation in Multiple Receptor Signaling Pathways**

Multiple types of receptors contribute to the survival and growth of cardiomyocytes, and Shp2 is required for normal activation of the Ras/Erk pathway downstream of most, if not all, RTKs, cytokine receptors, G-protein–coupled receptors, and integrins. To assess the functional consequences of loss of Shp2 on downstream signaling, we isolated primary cardiomyocytes from 4- and 8-week–old MCK-Shp2–null

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**Figure 4.** Effects of Shp2 deletion on cardiomyocytes. A, Hematoxylin and eosin–stained or (B) reticulin-stained cross section of paraffin-embedded heart slices from 8-week–old Shp2 fl/fl (left) and MCK-Shp2–null (right) hearts (magnification ×20). C, Electron microscopy of cardiomyocytes (CM). Top, Sarcomeric structures (magnification ×4800). Bottom, MCK-Shp2–null mice have aberrant Z lines (arrow), less dense myofibrils with lacunae (arrowhead), and larger mitochondria (asterisk) (magnification ×13 000). D, Representative photomicrograph (magnification ×40) of Shp2 fl/fl vs MCK-Shp2–null cardiomyocytes. Cardiomyocyte length, width, and area were quantified in 200 to 500 cardiomyocytes (n=4 mice each). Results are given as mean±SEM. *P<0.05, significant difference in length and width but not total area of the MCK-Shp2–null cardiomyocytes.
Figure 5. Absence of Shp2 in cardiomyocytes disrupts Erk/MAPK activation in multiple receptor signaling pathways. Primary cardiomyocytes from 4- or 8-week-old Shp2 fl/fl or MCK-Shp2–null hearts were (A) stimulated with heregulin (1 μg/mL), IGF-1 (10 nmol/L), EGF (25 ng/mL), angiotensin II (100 nmol/L), or IL-6 (10 ng/mL), lysed, and immunoblotted with phospho–Erk1/2 antibodies, followed by anti-Erk1 antibodies to control for loading or (B) stimulated with heregulin (1 μg/mL), lysed, and immunoblotted with phospho-Akt, -p38, -JNK, and -Stat 3 antibodies. Immunoblots for total Akt, p38, JNK, and Stat 3 antibodies were used to control for loading. No statistical differences were observed in activation of p38, JNK, or Stat 3, and Akt was hyperactivated in MCK-Shp2–null cardiomyocytes. C, Primary cardiomyocytes were stimulated with IGF (10 nmol/L), lysed, immunoblotted with phospho-Akt, and reprobed with total Akt antibodies to control for loading. Results are representative of 3 experiments and given as mean ± SEM (P < 0.05 is significant).

and littermate Shp2 fl/fl mice and examined their responses to various agonists, including those that activate growth factor (heregulin, IGF-1, EGF), cytokine (IL-6), and G protein–coupled (angiotensin II) receptors (Figure 5A and online-only Data Supplement Figure III). These times were chosen to enable comparison of cardiomyocytes (4 weeks old) with residual Shp2 protein expression (Figures 1B and 5A) and no overt DCM (Figure 3B) with those (8 weeks old) lacking Shp2 (Figures 1B and 5A) and exhibiting DCM (Figures 2B and 3B). We found no significant difference in Erk activation between MCK-Shp2–null and Shp2 fl/fl cardiomyocytes from 4-week–old mice (Figure 5A and online-only Data Supplement Figure III, left). However, in cardiomyocytes of 8-week–old mice, Erk1/2 activation was either decreased or abrogated in response to heregulin, IGF-1, EGF-1, and IL-6 (Figure 5A and online-only Data Supplement Figure III, right). Each of these ligands also activates other downstream signaling cascades, some of which are affected by Shp2 deficiency in other cell systems.5,35 Indeed, heregulin-stimulated Akt activation was enhanced in Shp2-deficient cardiomyocytes (8-weeks) (Figure 5B). In contrast, Akt activation in response to IGF-1 was minimally affected by Shp2 deficiency (Figure 5C), and we detected no significant increase in Akt activation on IL-6, EGF, or angiotensin II stimulation (data not shown). No consistent differences between MCK-Shp2–null and Shp2 fl/fl control cardiomyocytes were detected in any of the other downstream signaling pathways assessed, including the p38 and JNK MAPKs,
NFAT, Stat 1, Stat 3, and Stat 5 (Figure 5B and data not shown) at either 4 or 8 weeks.

**Shp2 Deficiency Also Affects the Response to Pressure Overload**

In addition to soluble agonists, cardiomyocytes respond to stretch. To test the possible involvement of Shp2 in stretch-activated signaling, we subjected MCK-Shp2–null and littermate control mice to pressure overload by carrying out short-term (10 minutes) and long-term (7 day) ascending aortic banding experiments (Figure 6A and 6B). As expected from previous studies, short- and long-term banding evoked an increase in Erk activation compared with sham-operated controls in Shp2 fl/fl mice, but this response was dramatically impaired in MCK-Shp2–null mice (Figure 6A). In contrast, Akt activation was enhanced in MCK-Shp2–null hearts after long-term banding (Figure 6A), suggesting a potentially compensatory cardioprotective effect to pressure overload.

Pressure overload caused by aortic constriction normally results in hypertrophy. To determine whether Shp2 is necessary for this presumably cardioprotective response, systolic left ventricular blood pressure measurements were assessed after short-term (baseline) and long-term ascending aortic constriction. MCK-Shp2–null mice, unlike Shp2 fl/fl mice, failed to significantly compensate by increasing their intraventricular pressures (Figure 6B). The ratio of heart weight to body weight increased markedly only in Shp2 fl/fl mice, indicating that MCK-Shp2–null mice failed to hypertrophy (Figure 6B). Taken together, these results show that cardiac-specific Shp2 deficiency results in impaired Erk activation in response to both soluble and solid-state stimuli.

**Loss of Shp2 in the Heart Increases RhoA Activity and Downstream Signaling**

Activated RhoA induces hypertrophic cell growth and gene expression in cultured cardiomyocytes. In vivo, however, RhoA expression leads to DCM and heart failure. We measured Rho-GTP levels in basal and heregulin-stimulated Shp2 fl/fl and MCK-Shp2–null cardiomyocytes. Strikingly, RhoA activity was basally upregulated and sustained on heregulin stimulation in MCK-Shp2–null compared with Shp2 fl/fl cardiomyocyte lysates (Figure 7A). Likewise, the RhoA effector Rho kinase (ROCK) was hyperactivated in MCK-Shp2–null cardiomyocytes, as indicated by increased phosphorylation of the ROCK substrate myosin light chain (MLC) (Figure 7B).

**Shp2 Regulates the Cardiomyocyte Phenotype via the Erk/MAPK and RhoA Signaling Pathways**

To assess the role of defective Erk activation on the phenotype of MCK-Shp2–null cardiomyocytes, we treated normal (Shp2 fl/fl) cardiomyocytes with the Mek1/2 inhibitor UO126 (Figure 7B). As expected, UO126-treated cardiomyocytes showed impaired Erk1/2 phosphorylation (Figure 7B), and remarkably, these cells became longer and thinner than dimethyl sulfoxide (DMSO)–treated wild-type cardiomyocytes (Figure 7C and 7D), resembling MCK-Shp2–null cardiomyocytes, albeit to a lesser extent. Similarly, to assess the physiological significance of RhoA upregulation, we treated primary MCK-Shp2–null cardiomyocytes with the ROCK inhibitor Y-27632 (Figure 7B). Notably, ROCK inhibition in Shp2-null cardiomyocytes led to decreased MLC phosphorylation, increased cell width, and shortened cell length compared with DMSO-treated MCK-Shp2–null cells (Figure 7D).
Cardiac-Specific Deletion of Shp2 Recapitulates the MCK-Shp2–Null Phenotype

Because MCK-Shp2 mice lack Shp2 in skeletal muscle and cardiomyocytes, we wanted to ensure that the cardiac defects in these mice were not the consequence of secondary effects caused by skeletal muscle deletion of Shp2. Therefore, we crossed Shp2 fl/fl mice with mice that express Cre under the control of the cardiac-specific α-MHC promoter.39 Like Mck-Shp2–null mice, 6-week–old α-MHC-Cre/fl/fl Shp2 (αMHC-Shp2–null) mice displayed overt DCM as determined by echocardiography and histology (Figure 8A through 8D). In addition, total heart lysates taken from 12- to 14-day–old α-MHC-Shp2–null mice revealed increased RhoA activity and enhanced MLC phosphorylation. Expression of α-MHC occurs earlier40 than MCK; consequently, we were able to assess the involvement of physiological Erk activation in Shp2-deleted hearts during the early postnatal proliferative phase.41 Although there was some variability between α-MHC-Shp2–null heart lysates (Figure 8E), overall Erk1/2 activation was decreased compared with Shp2 fl/fl controls (Figure 8E). Together, these data suggest that Shp2, by virtue of its ability to regulate RhoA and Erk/MAPK signaling, is essential for maintenance of normal adult myocardium.

Discussion

The roles of specific PTPs in the heart remain obscure. Shp2 is a required positive signaling component necessary for Erk/MAPK pathway activation downstream of RTKs, cytokine receptors, G-protein–coupled receptors, and integrins.5 In addition, several studies have implicated Shp2 as a critical regulator of RhoA,6–8 although the mechanisms remain controversial. Furthermore, missense mutations in PTPN11, which encodes SHP2, cause human genetic syndromes (NS, LS) associated with cardiac developmental defects. Thus, understanding the role of SHP2 in normal cardiac development and physiology is of obvious interest. Here, using conditional mutagenesis, we show that Shp2, through regulation of Erk/MAPK and RhoA signaling pathways, is essential to maintain normal function of the postnatal heart and postnatal Shp2 deficiency in mice results in the rapid development of DCM. Shp2 is the first PTP known to be required for adult cardiac function.

Several lines of evidence implicate defective Erk activation in the pathogenesis of DCM in Shp2-deficient hearts. First, Erk activation is globally impaired in response to every agonist tested, including classic growth factors, cytokines, and G-protein–coupled receptor ligands. Second, Erk activation was impaired in response to pressure overload. Third, most compellingly, treatment of primary cardiomyocytes with Erk1/2 and RhoA pathway–specific inhibitors was sufficient to induce a partially dilated phenotype in Shp2 fl/fl cells and to nearly reverse the DCM in MCK-Shp2–null cardiomyocytes, respectively, suggesting that both Erk/MAPK and RhoA signaling pathways are critical regulators in adult myocardium.

Several previous studies are consistent with defective Erk activation as a significant cause of cardiomyopathy in MCK-Shp2–null mice. For example, deletion of the transcription factor serum response factor, which acts in concert with Ets factors that are Erk substrates, causes DCM.42 Moreover, overexpression of activated Mek in the heart promotes physiological hypertrophy.6 In mice, cardiac-specific deletion of ErbB2,31 β1 integrin,37 or both the insulin and IGF-1 receptors44 results in DCM; all of these receptors signal, at least in part, through the Erk/MAPK pathway. The cardiomyopathy seen in MCK-Shp2–null mice shares multiple features with these mouse models of DCM, including an increase in cardiomyocyte length compared with width, myocardial remodeling without an increase in overall heart weight, increased atrial natriuretic factor transcripts, and a
characteristic alteration in sarcomeric morphology, as revealed by electron microscopy. However, although the cardiomyopathy evoked by Shp2 deficiency is functionally significant (as indicated by the decrease in ejection fraction and fractional shortening), unlike mutations in other signaling molecules (eg, ErbB2 and β1 integrin), it is compensated for and does not result in overt cardiomyocyte cell death, fibrosis, cardiac failure, and death. The simplest explanation for this difference is that receptors such as ErbB2 and β1 integrin activate multiple other downstream signaling cascades, in addition to the Ras/Erk pathway regulated by Shp2, and thus their deletion might be expected to have more severe consequences on cardiac function. In any event, our finding that treatment of primary cardiomyocytes with U0126 results in a “dilated” phenotype ex vivo supports a role for Erk activation in maintaining normal cardiomyocyte function.

On the other hand, our results are, at first glance, at odds with those of 2 other studies. Cardiac-specific deletion of Raf-1 causes DCM by increasing cardiomyocyte apoptosis without affecting endothelin-1–stimulated Mek1/2 or Erk1/2 activities. Because Shp2 is believed to lie upstream of Ras in the Erk/MAPK signaling cascade, it is formally possible that Shp2-dependent effects on other Ras effector pathways can account for the less severe effects of Shp2 deletion. In addition, while this article was in revision, Purcell et al reported on the effects of inactivation of Erk1/2 in the heart by means of inducible overexpression of the MAPK phosphatase DUSP6 (MAPK phosphatase 3), which dephosphorylates Erk1/2. This group found that although dephosphorylation of Erk1/2 sensitized mice to stress-induced apoptosis and heart failure, unlike Shp2 deletion, overexpression of DUSP6 alone was not sufficient to cause DCM. Conceivably, DUSP6, when overexpressed, has additional targets or non-catalytic effects. However, the simplest explanation for such apparent differences between their results and ours is that the combined effects of Shp2 on the Erk/MAPK and RhoA pathways account for the DCM in MCK-Shp2–null mice.

Indeed, several lines of evidence suggest that abnormal RhoA signaling contributes to DCM caused by Shp2 deficiency. Shp2 mutant fibroblasts show increased stress fibers and focal adhesions attributable to increased Rho activity, suggesting that Shp2-mediated RhoA activation is required for normal cytoskeletal architecture and function. Likewise, upregulation of Shp2, leading to downregulation of RhoA, is necessary for nitric oxide–induced cell motility in differentiated aortic smooth muscle cells. Transgenic mice expressing constitutively activated RhoA were shown to have significant left ventricular dilatation and associated decreases in left ventricular contractility, consistent with our findings. As in these other systems, Shp2 activity is inversely correlated with RhoA signaling in Shp2-deficient cardiomyocytes and hearts, and this results in the predicted downstream effects on normal cardiomyocyte function.

Figure 8. Deletion of Shp2 by expression of the cardiac-specific promoter α-MHC recapitulates the MCK-Shp2–null phenotype. A, Representative echocardiography of Shp2 fl/fl and α-MHC-Shp2–null mice at 6 weeks of age. Two-headed arrow indicates left ventricular chamber size. B, Cardiac anatomic and functional parameters as assessed by echocardiography in Shp2 fl/fl and α-MHC-Shp2–null mice at various ages. *P < 0.05, 6-week-old α-MHC-Shp2–null mice vs Shp2 fl/fl mice. C, Representative photomicrograph (magnification ×20) of Shp2 fl/fl and α-MHC-Shp2–null cardiomyocytes. D, Cardiomyocyte (CM) length, width, and area were quantified in 200 to 500 cardiomyocytes from 6-week-old Shp2 fl/fl and MCK-Shp2–null mice (n = 4 each). Results are given as mean ± SEM. *P < 0.05, significant difference in length and width but not total area of the α-MHC-Shp2–null cardiomyocytes. E, Total heart lysates were made from 12- to 14-day-old Shp2 fl/fl control or α-MHC-Shp2–null mice, and GST-Rhotekin–bound RhoA and total RhoA in cell lysates were detected by immunoblotting with anti-RhoA antibodies or immunoblotted with phospho-

Figure 8 (Continued). Erk1/2 or -MLC antibodies, followed by anti-Erk1 or -MLC-2 antibodies, respectively, to control for loading. Immunoblotting with anti-Shp2 antibodies detected deletion of Shp2 in the α-MHC-Shp2–null hearts at 12 to 14 postnatal days. Each lane represents 1 mouse heart lysate from the indicated experimental group.
the RhoA effector ROCK. Most compelling, however, is our finding that ROCK inhibition in Shp2-null cardiomyocytes appears to rescue the dilated phenotype of MCK-Shp2–null cardiomyocytes ex vivo.

Although our results clearly implicate enhanced RhoA activity in the cardiac pathogenesis, it is important to note some clear differences with the effects of skeletal muscle Shp2 deficiency. In skeletal muscle, active Shp2 promotes p190-B RhoGAP dephosphorylation, thereby stimulating the RhoA pathway, and promotes NFAT activation in myoblasts. MCK-Shp2–null mice have reduced skeletal myofiber size and expression of IL-4, an NFAT-regulated cytokine known to stimulate myofiber growth. We observed no effect of Shp2 deficiency on NFATc activation, and notably, the effects of Shp2 deficiency on RhoA activation in cardiomyocytes are exactly the opposite of those in skeletal muscle. The molecular basis for these tissue-specific differences in Shp2 action on the RhoA and NFAT pathways remains an important topic for future research.

Deletion of Shp2 in postnatal cardiomyocytes also inhibits hypertrophy in response to pressure overload. Our biochemical data suggest that this results from impaired Erk activation, potentially compensated for, at least in part, by enhanced Akt activity after long-term banding. Increased Akt activation might help prevent apoptosis in Shp2-deficient cardiomyocytes, as evidenced by lack of fibrosis and cleaved caspase-3.

Although our data implicate abnormal Erk/MAPK and RhoA activation as critical for the DCM phenotype, the precise pathways by which this occurs remain unclear. Conceivably, given the effects of Shp2 deficiency on the response to pressure overload, Shp2-deficient hearts may not even be able to respond to the normal postnatal increase in systemic blood pressure. Alternatively, Shp2-deficient hearts might fail because of defective trophic signaling through receptors such as ErbB2, which has a known cardioprotective role postnatally. Most likely, defective signaling from multiple receptors contributes to DCM in MCK-Shp2–null mice, and in this regard, the fact that cardiac function in these mice remains well compensated for is perhaps surprising and a testament to the power of homeostatic feedback pathways.

Finally, our results have interesting, if somewhat unexpected, implications for the pathogenesis of NS and LS. Biochemical, cell biological, and genetic studies indicate that the PTPN11 alleles associated with NS are hypermorphs that enhance Erk pathway activation. Given that cardiac-specific expression of activated Mek promotes HCM, along with our finding that the absence of Shp2 in cardiomyocytes causes DCM, one might expect NS-associated PTPN11 mutations to cause HCM. But, although HCM is found in many NS patients, it is rare in those with PTPN11 mutations. Furthermore, our mouse model of NS generated by “knock-in” of the NS allele Ptn11 D61G exhibits valvuloseptal defects similar to those seen in NS but with no evidence of HCM; indeed, rare D61G/+ and all D61G/D61G embryos exhibit abnormally thin myocardium. On the other hand, LS is caused almost exclusively by PTPN11 mutations, and HCM is the most common cardiac abnormality in this syndrome. Yet, LS mutations impair SHP2 activity, and LS alleles can interfere with RTK-evoked Erk activation, at least in transfection assays. From our results with MCK-Shp2–null mice, the simplest model would predict that LS should be associated with DCM rather than HCM.

Several potential explanations exist for these apparent paradoxes. Conceivably, postnatal Shp2 deficiency (as in α-MHC– and MCK-Shp2–null mice) causes DCM, whereas impaired Shp2 function during embryogenesis (eg, as expected in LS patients) results in HCM. Alternatively, the complete absence of Shp2 activity could cause DCM, whereas a reduction in activity to levels between 0% and 50% of wild type (as expected in LS) causes HCM. A variant of this model could hold that some degree of increased Shp2 activity (potentially caused by specific NS alleles) also might lead to HCM. Third, the complete absence of Shp2 may have consequences different from the expression of a dominant-negative mutant. For example, the latter may have some adapter functions and could prevent increased/gratuitous binding of other SH2 domain-containing proteins to what are normally Shp2 binding sites. Finally, the possibility of significant interspecies differences in the cardiac effects of Shp2 mutants cannot be excluded. Resolution of these intriguing paradoxes requires analyzing the effects of expressing LS mutants and other NS alleles on mouse cardiac development and function.

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Disclosures

None.

References


1 receptor signaling in cardiac development and function. *Mol Cell Biol.*

45. Yamaguchi O, Watanabe T, Nishida K, Kashiwase K, Higuchi Y, Takeda
T, Hikosso S, Hirotani S, Asahi M, Taniike M, Nakai A, Tsujimoto I,
Matsumura Y, Miyazaki I, Chien KR, Matsuzawa A, Sadamitsu C, Ichijo
H, Baccarini M, Horii M, Otsu K. Cardiac-specific disruption of the

46. Purcell NH, Wilkins BJ, York A, Saba-El-Leil MK, Meloche S,
Robbins J, Molkentin JD. Genetic inhibition of cardiac ERK1/2
promotes stress-induced apoptosis and heart failure but has no effect
on hypertrophy in *vivo*. *Proc Natl Acad Sci U S A.* 2007;104:
14074–14079.

47. Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL,
Chen J, Kahn R, Condorelli G, Ross J Jr, Chien KR, Lee KF. ErbB2 is

Y, Karayama H, Ohyama K, Onishi T, Hanew K, Okuyama T,
Horikawa R, Tanaka T, Ogata T. Protein-tyrosine phosphatase, non-
receptor type 11 mutation analysis and clinical assessment in 45
patients with Noonan syndrome. *J Clin Endocrinol Metab.*
2004;89:3359–3364.

49. Nishikawa T, Ishiyama S, Shimomo T, Takeda K, Kasajima T, Momma K.


**CLINICAL PERSPECTIVE**

The role of protein tyrosine phosphatases in the heart is still unknown. Our results are the first to reveal that the SH2
domain–containing protein tyrosine phosphatase Shp2 is essential in the adult heart and that postnatal deletion of Shp2
causes dilated cardiomyopathy without an intervening hypertrophic phase. Our findings indicate that Shp2 is required to
mediate activation of Erk1/2 in response to several agonist stimulations and is a required pathway for regulation of normal
cardiac function. In addition, our data also reveal that wild-type cardiomyocytes treated with the Mek1/2 inhibitor UO126
cause the cells to appear longer and thinner, resembling Shp2-deficient cardiomyocytes, albeit to a lesser extent. In
addition, Shp2 deficiency in cardiomyocytes causes a significant upregulation in RhoA activation, and most strikingly,
inhibition of this pathway with the Y-27632 RhoA effector Rho kinase inhibitor can reverse the dilated cardiomyocyte
phenotype in culture. Shp2-deleted hearts also cannot hypertrophy in response to aortic banding, suggesting that Shp2 plays
an integral role in cardiac remodeling. These data are especially important because Shp2 mutations have recently been
implicated in human genetic disorders affecting the heart. Together, our data suggest that Shp2, by virtue of its actions on
the extracellular signal–regulated kinase/mitogen-activated protein kinase and RhoA signaling pathways, serves a
cardioprotective role and is essential for normal cardiac function.
Deletion of Ptpn11 (Shp2) in Cardiomyocytes Causes Dilated Cardiomyopathy via Effects on the Extracellular Signal–Regulated Kinase/Mitogen-Activated Protein Kinase and RhoA Signaling Pathways

Maria I. Kontaridis, Wentian Yang, Kendra K. Bence, Darragh Cullen, Bo Wang, Natalya Bodyak, Qingen Ke, Aleksander Hinek, Peter M. Kang, Ronglih Liao and Benjamin G. Neel

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In the article by Kontaridis et al, “Deletion of Ptpn11 (Shp2) in Cardiomyocytes Causes Dilated Cardiomyopathy via Effects on the Extracellular Signal–Regulated Kinase/Mitogen-Activated Protein Kinase and RhoA Signaling Pathways” (Circulation. 2008;117:1423–1435), the information on the method of isolation of the cardiomyocytes (page 1424, last paragraph) was incorrect.

The beginning of that paragraph (until the sentence that begins, “After 12 hours”) should read:

Primary cardiomyocytes were isolated and cultured as previously described. Briefly, adult cardiomyocytes were obtained by heart Langendorff perfusion with Ca^{2+}-free Tyrode buffer (135 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl2, 0.33 mmol/L NaH2PO4, 10 mmol/L HEPES) and 10 mmol/L glucose (pH 7.4), 10 mmol/L 2,3-butanedione monoxime (Sigma, St Louis, Mo), and 5 mmol/L Taurine (Sigma) for 3 to 5 minutes. Perfusion was continued for 7 to 10 minutes with recirculating Tyrode solution containing collagenase D (0.3 mg/g body weight; Roche, Indianapolis, Ind), collagenase B (0.4 mg/g body weight; Roche), and proteinase XIV (0.05 mg/g body weight; Sigma). Ventricular tissue was then minced in Tyrode solution containing 2% bovine serum albumin (Sigma), incubated for 15 minutes at 37°C, and then filtered through a 250-μm nylon mesh. The cell suspension was centrifuged at 420 g for 2 minutes and then gradually subjected to Tyrode solution with increasing concentrations of calcium and decreasing concentrations of 2,3-butanedione monoxime (final: 1.2 mmol/L CaCl2, no 2,3-butanedione monoxime). Typical yields were 1.5 to 2.5×10^6 cells per heart with 70% to 80% of the cells retaining their rod-shaped morphology.

In addition, the original reference 30 should be replaced with the following reference:


These changes have been made to the online version of the article.

The authors regret these errors.

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Supplementary Figure 1. Older hearts from Shp2 deleted mice also exhibit DCM.
Cardiac anatomic and functional parameters, as assessed by echocardiography in Shp2 fl/fl, Mck-Cre:Shp2+/+ and MCK-Shp2 null mice at 5-8 months. There was no statistical difference in cardiac function between the Shp2 fl/fl mice and the Mck-Cre:Shp2+/+ littermates (these two groups were then analyzed as one group against the Mck-Shp2 null mice of similar ages). *P<0.05 comparing difference between the 5-8 month old MCK-Shp2 null mice from the Shp2 fl/fl and Mck-Cre:Shp2+/+. (BW=body weight, HR=heart rate, LVPW th=left ventricular posterior wall thickness, LVID-d=left ventricular chamber dimension in diastole, LV Mass= weight of left ventricle, EF%=ejection fraction, FS%=fractional shortening).
Supplementary Figure 2. **No increase in apoptosis in MCK-Shp2 null hearts.** (A) IHC staining using Shp2 polyclonal antibodies (red) to assess deletion efficiency in the MCK-Shp2 null hearts. (B) TUNEL staining (red) on histological sections from 8-week old Shp2 fl/fl and MCK-Shp2 null hearts. Tonsil, a highly apoptotic tissue, was used as a positive control. No TUNEL positive staining was detected in either the Shp2 fl/fl controls or in the MCK-Shp2 null hearts. (C) IHC staining using caspase 3 antibodies (red) to detect enhanced apoptotic activity in Shp2 fl/fl or MCK-Shp2 null hearts. No detectable difference was observed.
Supplementary Figure 3. **Absence of Shp2 in cardiomyocytes disrupts Erk/MAPK activation in multiple receptor signaling pathways.** Phosphorylation activity is shown after phosho-Erk/Erk protein normalization from lysates of 3 separate experiments using various agonist stimulations for the indicated times. Results are the mean ± SEM and the *P<0.05 values for MCK-Shp2 null lysates are generated by comparing these levels of activation by the various agonists to the Shp2 fl/fl activities from the same group; All 4-week p-Erk/Erk ratios are not statistically significant; however, at 8 weeks, activation of p-Erk by HRG, EGF, AngII and IL-6 are all statistically significant (p<0.05); IGF-1 is significant only at a p<0.1.