New approaches to prevent LEOPARD Syndrome-associated cardiac hypertrophy by specifically targeting Shp2-dependent signaling*

Christine Schramm, Michelle A. Edwards, and Maike Krenz1

From the Department of Medical Pharmacology & Physiology / Dalton Cardiovascular Research Center
University of Missouri-Columbia, Columbia MO 65211

*Running title: Targeting pro-hypertrophic signaling in LEOPARD Syndrome

To whom correspondence should be addressed: Maike Krenz, Dalton Cardiovascular Research Center, 134 Research Park Dr, Columbia, MO 65211. Phone: 573-884-8761; Fax: 573-8844232; Email: krenzm@missouri.edu

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Background: In LEOPARD Syndrome, hypertrophic cardiomyopathy develops due to hyperactivated pro-hypertrophic signaling.

Results: Pharmacological inhibition of Shp2, focal adhesion kinase, Akt, or mammalian target of rapamycin counteracts the disease mechanism.

Conclusion: Interventions at multiple levels of the signaling cascade effectively prevent cardiomyocyte hypertrophy.

Significance: Identification of these novel targets will facilitate the development of highly specific therapies for LEOPARD Syndrome.

SUMMARY
In LEOPARD Syndrome (LS) patients, mutations in the protein tyrosine phosphatase Shp2 cause hypertrophic cardiomyopathy (HCM). The pro-hypertrophic effects of mutant Shp2 are mediated downstream by hyperactivation of mammalian target of rapamycin (mTOR). Our goal was to further define the signaling cascade that is essential for the underlying pathomechanism, thus expanding the list of potential future therapeutic targets.

Using cultured neonatal rat cardiomyocytes (NRCM) with adenoviral gene delivery and pharmacological inhibitors, we found that hypertrophy induced by a particularly aggressive LS mutation in Shp2 depends on hyperactivation of Akt and focal adhesion kinase (FAK) as well as mTOR. Dissecting domain-specific functions of Shp2 using double and truncation mutants, we determined that the hypertrophic effects of mutant Shp2 depend on the two SH2 domains and on an intact catalytic center. The latter finding prompted us to test the efficacy of a Shp2 inhibitor targeted directly at the catalytic pocket. This compound, PHPS1, effectively prevented mutant Shp2-induced hypertrophy.

In summary, we identified three novel targets for pharmacological therapy of LS-associated cardiac hypertrophy. Of particular importance is the finding that intervention directly at the mutant Shp2 protein is effective, since this would facilitate custom-tailored therapeutic approaches for patients carrying LS mutations in Shp2.

Despite many recent advances, hypertrophic cardiomyopathy (HCM) in pediatric patients poses a particular therapeutic challenge since none of the current pharmacological approaches have been shown to prevent, attenuate, or reverse HCM in humans or impact the prognosis (1). Additionally, childhood-onset HCM is often associated with other congenital malformations, which may further complicate the treatment. We have focused our research efforts on unraveling the disease mechanism of HCM associated with LEOPARD Syndrome (LS), which can lead to severe heart failure symptoms already in infants. LEOPARD is an acronym for the manifestations

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of this syndrome: multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and deafness (2).

Clinically and histologically, LS-associated HCM resembles severe forms of classic HCM (3). However, sarcomeric protein mutations could not be found in the affected families. Instead, mutations in the protein tyrosine phosphatase Shp2 (encoded by the gene PTPN11) are responsible in 90% of LS patients (4,5). This suggests that the LS-specific disease mechanisms may be very different from those in classic HCM. Therefore, custom-tailored therapeutic approaches for such ‘phenocopy’ forms of HCM as seen in LS could be much more effective than standard treatment protocols.

Recently, substantial progress has been made in unraveling LS-specific signaling mechanisms that promote the associated HCM. Using various in vitro and in vivo models, multiple groups including ourselves discovered in parallel that LS mutations in Shp2 result in hyperactivation of signaling through Akt or mammalian target of rapamycin (mTOR) (6-9). In two independent mouse models, administration of rapamycin rescued the HCM phenotype. However, the rescue was only complete in the milder model with late-onset HCM (7). In the more severe model with neonatal-onset HCM, cardiac hypertrophy was effectively reversed by rapamycin, but the functional deficit could not be improved (6).

Therefore, benefits from rapamycin treatment may be very limited in severely affected pediatric patients with LS. Furthermore, the use of rapamycin or its derivatives may be impeded by the ensuing immunosuppression and other side effects, in particular negative inotropic effects (10). Consequently, the first goal of this study was to test whether other signaling elements upstream of mTOR could be equally effective at inhibiting LS-associated cardiac hypertrophy. Secondly, we wanted to dissect the structure-function relationship of mutant Shp2. Detailed knowledge of the roles of individual domains of the protein would facilitate the future design and development of pharmaceutical compounds to target the mutant Shp2 protein directly and therefore potentially have the highest efficacy with the least side effects.

To accomplish these goals, we used cultured neonatal rat cardiomyocytes (NRCM) with adenoviral vectors to express various Shp2 mutants and several pharmacological inhibitors. We focused on the LS mutation Q510E-Shp2, which is associated with a particularly aggressive form of biventricular HCM in pediatric patients and therefore is ideally suited for proof-of-principle studies of disease mechanisms and treatment efficacy (11,12). Biochemically, the Q510E mutation confers dominant-negative effects, similar to the effects of other LS mutations (6,13). We previously used the NRCM model system for in vitro assessment of the pro-hypertrophic effects of the LS mutation Q510E in Shp2 (6). As expected, we found that Q510E-Shp2 overexpression resulted in a robust increase in NRCM size (6). Since this approach increases total Shp2 protein levels, we excluded potential gene dose effects by also overexpressing wild type (WT)-Shp2 (6). WT-Shp2 overexpression at equivalent levels did not alter NRCM size, consistent with our previous finding that transgenic WT-Shp2 overexpression in the mouse ventricle also does not induce HCM or any other cardiac abnormalities (14).

For this study, we first validated the NRCM model by confirming that the pro-hypertrophic effects of Q510E-Shp2 were mediated by mTOR hyperactivation as previously seen in our mouse model (6). We then tested the roles of signaling proteins upstream of mTOR for promoting hypertrophy. We found that targeting focal adhesion kinase (FAK) or Akt effectively counteracts the pro-hypertrophic effects of Q510E-Shp2. Furthermore, we found that targeting either the SH2 domains or the catalytic center of Shp2 directly prevents hypertrophy induced by overexpression of Q510E-Shp2. This has not only important implications for the design of future therapies but also advances our insight into the molecular interactions underlying the pathogenesis of LS-associated HCM.
EXPERIMENTAL PROCEDURES

Cardiomyocyte Isolation and Culture—NRCM (day 1-3) were isolated (neonatal cardiomyocyte isolation kit; Worthington) and grown on gelatin-coated polystyrene plates in serum-free M199 medium with 1% penicillin/streptomycin. For conditioned media (CM) experiments, cardiac fibroblasts obtained during pre-plating were passaged twice to remove all cardiomyocytes prior to adenovirus infection. After infection, fibroblasts were washed twice and then kept in serum-free medium for 48 – 72 hrs. CM were collected and stored frozen for up to two weeks before being added to cardiomyocyte cultures. 4-Amino-5-(4-chlorophenyl)-7-((t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3), rapamycin, Akt inhibitor VIII, cyclosporin A, and PHPS1 (PTP inhibitor V) were obtained from EMD Millipore and dissolved in dimethyl sulfoxide as vehicle. Phenylephrine hydrochloride (PE) and U0126 were obtained from Sigma-Aldrich and dissolved in water or dimethyl sulfoxide, respectively. To induce strong tyrosine phosphorylation of focal adhesion kinase, NRCM were stimulated for 30 min with 10% fetal bovine serum in the presence of 0.1 mM pervanadate (PV) (generated by adding hydrogen peroxide to sodium orthovanadate stock solution (Sigma-Aldrich)).

Plasmid and Adenovirus Construction—FVB/N mouse cardiac mRNA was used as template for WT-Shp2 cDNA. Truncation mutants and point mutations were generated using standard polymerase chain reaction methodology. In the truncation mutants, the 211 N-terminal amino acids of Shp2, which comprise the two SH2 domains of the protein, were eliminated. After full length sequencing of the constructs and comparison to the published C57/BL6 Shp2 mRNA sequence (NCBI database NM_011202.3, Swiss-Prot: P35235.2), the cDNAs were inserted into the pAdTrack-CMV vector and subsequently used for adenovirus generation (AdEasy System, Stratagene). Adenovirus encoding β-galactosidase (β-gal) was obtained from Cell Biolabs. Adenovirus encoding green fluorescent protein (GFP)-FAK related nonkinase (FRNK) was a generous gift from Dr. Alan Samarel (15). As in our previous study, we confirmed that protein levels of the various Shp2 constructs were equivalent across groups Western blot (6). GFP-FRNK protein expression was confirmed by the presence of intracellular fluorescent signals as well as by Western blots (data not shown).

Protein Tyrosine Phosphatase (PTP) Activity Assay—Shp2 proteins were immunoprecipitated from NRCM lysates, incubated with src phosphopeptide (TSTEPQ-pY-QPGENL; Upstate), and inorganic phosphate production quantified as described (16).

mTOR Kinase Activity Assay—The enzymatic activity of mTOR was determined using an ELISA-based assay (K-LISA mTOR Activity Kit, EMD Chemicals). In short, mTOR protein was immunoprecipitated from NRCM lysates. Purified proteins were then incubated with a p70S6kinase (p70S6K)-GST fusion protein as substrate together with ATP. The phosphorylated substrate was detected with anti-phospho-p70S6K (T389) antibody, followed by detection with horseradish peroxidase-conjugated antibody and 3,3′,5,5′-tetramethylbenzidine substrate. Relative activity was determined by reading the absorbance at dual wavelengths of 450/540 nm.

Western Blotting, Immunofluorescent Staining, and Cell Area Measurements—The following antibodies were used: Akt, phospho-Akt S473, p70S6K, phospho-p70S6K T389, S6, phospho-S6 S240/244, mTOR, phospho-mTOR S2481, p44/42 mitogen-activated protein kinase (extracellular signal-regulated kinase, ERK1/2), phospho-ERK1/2 T202/Y204 (all from Cell Signaling Technologies), Shp2 (C-18, Santa Cruz), Flag (Sigma-Aldrich), troponin I (clone C5, Millipore), FAK (A17, Santa Cruz), phospho-FAK Y397 (Invitrogen), and paxillin (BD Transduction Laboratories). Signal intensities in Western blots were quantified using Quantity One Software (Bio-Rad). For cell surface area measurements, NRCM were stained with anti-troponin I / Alexa-conjugated fluorescent secondary antibody (Invitrogen) and multiple random fields per well photographed (Olympus IX51). All cells in each field were planimetered using Image J software to obtain one average per field. For each experiment, data from 2-3 independent isolates with 2-4 random fields per group and isolate were summarized, n
numbers given in figure legends denote the number of random fields. To semi-quantitatively assess sarcomeric organization, a blinded observer scored high-resolution images of troponin I-stained NRCM (1=low, 2=intermediate, 3=high level of organization as judged by density and continuity of myofibrillar staining).

Protein content, protein synthesis rate, and mRNA expression—Protein content in NRCM lysates was determined colorimetrically (Bradford method, Bio-Rad) and expressed as total soluble protein yield per 1 million NRCM. Protein synthesis rates were measured using $^{35}$S-methionine incorporation as described in (17). Quantitative reverse transcription-polymerase chain reactions (qRT-PCR) were conducted as previously described (6). Primer sequences were: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward agc ttg tca tca acg gga ag; GAPDH reverse ttt gat gtt agt ggg gtc tgc; $\alpha$-skeletal actin forward aat gag cgt ttc cgt tgc; $\alpha$-skeletal actin reverse atc ccc gca gac tcc ata c.

Data Normalization and Statistical Analysis—Data from mTOR activity assays, cardiomyocyte surface areas, and Western blot signal quantifications were normalized as follows: All measurements obtained from the respective control group were averaged, and then all data (including individual measurements from the control group) divided by this average. In contrast, activities obtained from the WT group in the PTP activity assays (Fig. 7A) were set as 1.0 for each individual run. Unpaired Student t tests were used for comparisons of two groups. One-way ANOVA followed by Scheffé posthoc test was used for comparing multiple groups (StatPlus software). All values are given as average ± SEM, * denotes $P<0.05$ vs. the respective $\beta$-gal control group at the same conditions unless otherwise specified in the figure legend.

RESULTS

Characterization of the NRCM in vitro model. NRCM are a well-established and frequently used system for hypertrophic signaling studies (18). However, such cultures do represent a reductionist approach. Therefore, we wanted to confirm that increases in cell surface area are indeed accompanied by other classic characteristics of cell hypertrophy. Fig. 1A shows representative images of NRCM immunostained for troponin I (Tnl) expressing Q510E-Shp2 or Y279C-Shp2, a second LS-associated mutation in Shp2. For comparison, NRCM were also exposed to 50 µM PE. NRCM cell surface areas were significantly increased by expression of the LS mutants as well as by PE (for quantification, please refer to Figs. 2-7).

Next, NRCM were scored for sarcomeric organization by a blinded observer. Scores were consistently higher in NRCM expressing LS mutants or exposed to PE (Fig. 1B). Next, we tested whether NRCM expressing LS mutants or treated with PE contained increased amounts of protein. Protein concentrations were significantly increased in lysates obtained from NRCM expressing Q510E-Shp2 or Y279C-Shp2, as well as from PE-treated NRCM (Fig. 1C). Consistently, rates of protein synthesis as reflected in $^{35}$S-methionine incorporation were increased by Q510E-Shp2 or Y279C-Shp2 expression and PE treatment (Fig. 1D). Lastly, we determined mRNA expression levels of the $\alpha$-skeletal actin isoform as a marker of the fetal gene program. Similar to PE treatment, both Q510E-Shp2 and Y279C-Shp2 expression increased $\alpha$-skeletal actin expression (Fig. 1E).

mTOR hyperactivation is necessary for Q510E-Shp2-induced hypertrophy. To further support that mTOR is hyperactivated in the presence of Q510E-Shp2, Western blots were used. Representative blots and signal quantifications are shown in Fig. 2B,C. Phosphorylation levels of mTOR as well as its downstream effectors p70S6K and S6 were significantly increased by Q510E-Shp2.
expression. Furthermore, we confirmed that mTOR is a necessary mediator of Q510E-Shp2’s effects on NRCM size using pharmacological inhibition. Rapamycin prevented Q510E-Shp2-induced hypertrophy in a dose-dependent manner (Fig. 2D). At 0.2 μM rapamycin, cell surface areas of Q510E-Shp2-expressing NRCM were completely normalized.

**Akt1/2 inhibition ameliorates Q510E-Shp2-induced hypertrophy.** Next, we tested to which extent signaling proteins known to be upstream activators of mTOR were involved in the pro-hypertrophic effects of Q510E-Shp2. In particular, we tested the role of Akt activation. As shown in Fig. 3A,B, the level of Akt phosphorylation was significantly increased in Q510E-Shp2-expressing NRCM. To test whether Akt hyperactivation is required for the pro-hypertrophic effects of Q510E-Shp2, we used Akt inhibitor VIII, which targets both Akt1 and Akt2. Akt1 and Akt2 may have partially redundant functions in the heart (19-21), therefore we did not use an isoform-specific Akt inhibitor. At 2 and 4 μM, Akt inhibitor VIII prevented Q510E-Shp2-induced hypertrophy (Fig. 3C).

**FAK hyperactivation is essential for Q510E-Shp2-induced hypertrophy.** Our next step was to test the role of protein kinases further upstream of Akt, in particular FAK. First, we used immunohistochemistry to assess the level of FAK activation in NRCM. FAK phosphorylation was increased in Q510E-Shp2-expressing NRCM (Fig. 4A,B), whereas total FAK and paxillin remained unchanged (Fig. 4E,F,I,J). To confirm that the punctate increase in signal intensity correctly reflected FAK hyperactivation, we used a pharmacological approach. In the presence of the FAK/src inhibitor PP2, phospho-FAK signal intensities in Q510E-Shp2 expressing NRCM were similar to those of β-gal-expressing controls (Fig. 4C,D). Expression of total FAK or paxillin was not altered by PP2 (Fig. 4G,H,K,L). Secondly, we used Western blots to quantify the level of FAK activation (Fig. 4M). Q510E-Shp2 expression increased the ratio of phospho-to-total FAK signal by more than 2-fold (Fig. 4N).

Subsequently, we measured NRCM cell sizes in the presence of PP2. As seen with rapamycin and Akt inhibitor VIII, we observed dose-dependent effects. At doses ≥ 0.5 μM, PP2 prevented the Q510E-Shp2-induced cell size increase (Fig. 4O). As an additional negative control, we used PP3, a very similar compound that does not inhibit Src family protein tyrosine kinases. PP3 did not affect cell sizes in β-gal or Q510E-Shp2 expressing NRCM (Fig. 4O).

For a more specific test, the endogenous FAK inhibitor FRNK was used in a separate set of experiments. GFP-tagged FRNK was co-expressed with Q510E-Shp2, which resulted in normal NRCM surface areas (Fig. 4P). Together with the PP2 data, this indicates that FAK is indeed a pro-hypertrophic signaling mediator activated by Q510E-Shp2 expression.

Furthermore, we tested whether the presence of PP2 normalized signaling further downstream using Western blots. As shown in Fig. 4Q, PP2 decreased the levels of Akt, mTOR, and p70S6K phosphorylation to baseline levels or below, indicating that Akt and mTOR are likely to be mediators of the pro-hypertrophic signal downstream of FAK.

**Calcineurin activation do not play a role for Q510E-Shp2-induced hypertrophy.** Since NRCM cultures may be contaminated with cardiac fibroblasts, we also wanted to exclude that the hypertrophy induced was due to secondary effects. Therefore, we exposed normal NRCM to CM harvested from cardiac fibroblasts that had been infected with β-gal, WT-Shp2, or Q510E-Shp2 adenoviruses. In all groups, the presence of CM increased NRCM size, but there were no quantitative differences between groups (Fig. 5A). This indicated that fibroblast-specific effects were unlikely to play a role for the pro-hypertrophic effects of Q510E-Shp2.

Furthermore, we tested to which extent calcineurin inhibition could ameliorate Q510E-Shp2-induced NRCM hypertrophy. The calcineurin signaling pathway is not directly linked to FAK/Akt/mTOR signaling, and therefore less likely to be involved in the underlying pathomechanism. However, a recent study has shown that calcineurin does play a role in hypertrophy caused by mutations in RAF1 that were identified in a subset of LS patients (22). In our model, the presence of the calcineurin inhibitor cyclosporin A did not affect Q510E-Shp2-induced hypertrophy (Fig. 5B),
indicating that calcineurin signaling is unlikely to play a major role.

Role of ERK signaling in Q510E-Shp2-induced hypertrophy. Since ERK hyperactivation has recently been shown to play a role in other LS models (23,24), we quantified the levels of ERK1/2 activation in NRCM expressing Q510E- or Y279C-Shp2. In our serum-free cultures, no significant differences were detected, but the presence of Q510E- or Y279C-Shp2 did result in non-significant reductions in ERK1/2 activation (Fig. 5C,D). We also wanted to include a gain-of-function mutation in Shp2 for comparison and chose the Noonan Syndrome-associated Q79R-Shp2 mutation. This mutation renders the Shp2 protein constitutively active and thereby stimulates ERK signaling (14,16). As shown in Fig. 5C and D, NRCM expressing Q79R-Shp2 displayed a clear trend towards ERK1/2 hyperactivation. This is consistent with a number of previous studies including our own (6,13,16,25) demonstrating that under conditions of serum or specific growth factor stimulation, cultured cells expressing LS mutants of Shp2 exhibit reduced ERK activation, whereas cells expressing gain-of-function mutants of Shp2 display increased ERK activation.

We also tested whether ERK inhibition affected NRCM size in our model. Fig. 5E shows that the mitogen-activated protein kinase / ERK kinase 1 (MEK1) inhibitor U0126 partially reduced the size of Q510E-Shp2-expressing NRCM. However, Q510E-Shp2-expressing NRCM remained significantly larger in size compared to the β-gal group.

Taken together, our data therefore indicate that ERK signaling is unlikely to play a major role in the hypertrophic response to Q510E- or Y279C-Shp2 expression. We suspect that the partial effect of U0126 on NRCM size may be due to crosstalk between the ERK and Akt pathways.

Role of SH2 domains for the pro-hypertrophic effects of Q510E-Shp2. Since we found multiple feasible therapeutic targets upstream of mTOR, we considered the possibility that intervention directly at the level of Shp2 could also be effective. How this could be accomplished, has not yet been explored. To better define the structure-function relationship of Shp2, we examined the relative contributions of the different Shp2 protein domains to the pro-hypertrophic effects. First, we tested the necessity of the presence of the two SH2 domains for the pathogenic effects. New cDNA constructs were generated encoding only the PTP domain and the linker region between the C-terminal SH2 domain and the PTP domain, but lacking both SH2 domains. One construct had no other mutations introduced (termed WT-trunc), whereas the other construct also contained the Q510E mutation (termed Q510E-trunc).

The truncated proteins were expressed in NRCM at levels equivalent to the levels of all other Shp2 constructs. The truncated Shp2 proteins were extracted by immunoprecipitation using an antibody against the C-terminal Flag tag. The purified proteins were incubated with a tyrosine-phosphorylated src peptide to determine in vitro phosphatase activity. PTP activity of WT-trunc was close to normal, and the activity of Q510E-trunc was significantly reduced and not different from that of full-length Q510E-Shp2 (Fig. 6A).

Subsequently, NRCM surface areas were determined in a new set of cultures. Expression of WT-trunc had no effect on NRCM size, similar to full-length WT-Shp2 overexpression. However, deletion of the two SH2 domains of the Q510E mutant abolished the pro-hypertrophic effect (Fig. 6B). Lastly, we tested the level of Akt activation in the presence of the two truncation mutants. Neither expression of WT-trunc nor of Q510E-trunc affected the degree of Akt phosphorylation, consistent with the NRCM size data (Fig. 6C,D).

Role of the catalytic center for the pro-hypertrophic effects of Q510E-Shp2. Next, we evaluated the role of the catalytic center for the pro-hypertrophic effects of mutant Shp2. In order to completely destroy any possibly remaining enzymatic activity of the Q510E-Shp2 protein, we generated a double mutant containing both the C459S and the Q510E mutations. The C459S mutation completely abolishes Shp2 phosphatase activity by replacing the cysteine residue essential for nucleophilic attack of the substrate’s phosphate group. To ensure that the C459S substitution itself did not have any hypertrophic effects, we also
introduced the C459S mutation into the full-length WT sequence.

As before, we first assessed PTPase activities of the mutant proteins in vitro (Fig. 6A). As expected, the C459S mutation significantly reduced enzymatic activity of the Shp2 protein. The double mutant C459S-Q510E displayed similarly low phosphatase activity.

Next, NRCM surface areas after infection with the respective constructs at equivalent levels were determined. Expression of C459S-Shp2 did not affect NRCM size (Fig. 6B). Notably, combination of the C459S substitution with the Q510E mutation completely abolished the pro-hypertrophic effects of Q510E-Shp2 (Fig. 6B).

It has been suggested that C459S-Shp2 may adopt a closed conformation (13). Therefore, it could be possible that introducing the C459S mutation abolished the pro-hypertrophic effects of Q510E-Shp2 simply by preventing any interactions with other proteins. To test this, we combined the Q79R and C459S mutations in order to promote an open conformation. The Q79R-Shp2 mutation is thought to activate Shp2 by disrupting interactions between the PTP and SH2 domains, thereby promoting unfolding of the protein (26). We previously found in vitro as well as in mouse models that Q79R-Shp2 does not alter cardiomyocyte cell size (6,14). Consistent with our previous findings, PTPase activity of the Q79R-Shp2 mutant was significantly increased (Fig. 6A). As expected, combination of the C459S and Q79R mutations lowered the resulting protein’s enzymatic activity to close to zero (Fig. 6A). Surprisingly, after infection with the Q79R-C459S double mutant, NRCM surface areas remained normal (Fig. 6B).

We also determined the levels of Akt activation in NRCM expressing C459S-, C459S-Q510E-, and Q79R-C459S-Shp2. Consistent with the cell size data, the degree of Akt phosphorylation was unchanged compared to control whereas only expression of Q510E-Shp2 resulted in upregulation of Akt activity (Fig. 6C,D).

Pharmacological PTP inhibition prevents Q510E-Shp2-induced hypertrophy. Since the C459S mutation abolished the pro-hypertrophic effects of Q510E-Shp2, we tested whether a pharmacological inhibitor that is specific for the catalytic center of Shp2 would affect Q510E-Shp2-induced hypertrophy. NRCM were infected with Q510E-Shp2 or β-gal control viruses and incubated with either vehicle or 10 μM PHPS1 (Shp2 inhibitor V). PHPS1 was designed to bind at the catalytic site and thereby disrupt interactions between Shp2 and all of its substrates (27). As shown in Fig. 7A, the presence of PHPS1 completely abolished the pro-hypertrophic effects of Q510E-Shp2. To confirm that this effect was not limited to the Q510E mutation, we also tested the combination of PHPS1 with Y279C-Shp2 expression. As expected, incubation with PHPS1 normalized the size of NRCM expressing Y279C-Shp2 (Fig. 7B). To further establish that the signaling mechanism in Y279C-Shp2 expressing NRCM closely resembles that of Q510E-Shp2 expression at other levels of the pathway as well, we extended our studies to FAK and mTOR.

Both PP2 and rapamycin effectively reversed the Y279C-Shp2-induced increase in NRCM size (Fig. 7C,D). This indicates that the two LS mutations Q510E-Shp2 and Y279C-Shp2 both induce NRCM hypertrophy through similar mechanisms.

Lastly, we assessed the effects of PHPS1 on downstream signaling. In NRCM expressing Q510E-Shp2 and incubated with PHPS1, the levels of Akt and mTOR activation were reduced to close to normal levels (Fig. 7E).

DISCUSSION

In this study, Q510E-Shp2-induced cardiomyocyte hypertrophy depended on hyperactivation of FAK, Akt, and mTOR signaling. Hypertrophy was effectively counteracted not only by inhibition of mTOR as shown before in mice (6), but also by the FAK/src inhibitor PP2, the endogenous FAK inhibitor FRNK, and by the Akt1/2 inhibitor VIII. Dissecting domain-specific functions of Shp2 using double and truncation mutants, we found that the pro-hypertrophic effects of Q510E-Shp2 require the presence of the two SH2 domains and a functional catalytic center. Consistent with the cell size data, Akt hyperactivation was also only noted when the SH2 domains or the catalytic center, respectively, remained intact. The latter finding
suggested that eliminating catalytic activity by preventing access to the pocket could also abolish Q510E-Shp2’s pro-hypertrophic effects. We therefore tested the Shp2-specific phosphatase inhibitor PHPS1, which had been specifically designed to bind Shp2’s catalytic site. Importantly, PHPS1 effectively prevented hypertrophy induced by Q510E-Shp2 as well as by another LS mutant.

To our knowledge, this is the first study to test these various pharmacological inhibitors in the context of LS-associated HCM. Importantly, our novel findings demonstrate that pharmacological inhibition of particular signaling steps upstream of mTOR could become feasible therapeutic approaches in the clinical arena. Our results complement the recent findings of two other groups showing that inhibition of phosphoinositide 3-kinase with LY294002 or wortmannin is also effective against the pro-hypertrophic effects of LS mutations in Shp2 (8,9). This is not surprising since PI3K is an important activator of Akt, and in turn is regulated by FAK (28,29). Therefore, it appears that targeting any step of this signaling pathway could be considered for new treatment strategies for LS-associated HCM.

However, inhibitors acting upstream of mTOR are also likely to cause substantial side effects. In particular, FAK and Akt are essential regulators of normal growth and development. Mouse models with genetic manipulations to specifically disrupt FAK activity revealed severe developmental phenotypes (30-32). In contrast, deletion of one Akt isoform does not affect baseline cardiac function in rodent models (33,34). However, lack of Akt1 diminishes somatic growth and deletion of Akt2 causes insulin resistance and diabetes (35,36). Furthermore, Akt1 deletion exacerbates cardiac hypertrophy after pressure overload, indicating that Akt1 is essential for cardiac adaptation to such stresses (34). Therefore, long-term administration of Akt inhibitors may not be advisable and this may limit the clinical applicability of our findings. However, in the case of LS patients, the therapeutic goal would be normalization, not a complete blockade of Akt signaling. Therefore, low-dose administration of Akt inhibitors could be of great benefit for affected patients.

In recent years, specific Shp2 inhibitors have become available for research applications. The fact that a Shp2-specific phosphatase inhibitor prevents LS-associated hypertrophy in our model is particularly exciting. This inhibitor was originally developed with the goal to treat an entirely different class of Shp2 mutations, i.e. gain-of-function mutations as found in patients with Noonan Syndrome or juvenile myelomonocytic leukemia (27). Our data suggest that such inhibitors might also be beneficial for LS patients, thus increasing the number of patients that could benefit from the use of Shp2-specific inhibitors. In terms of side effects, Shp2-specific inhibitors may have additional advantages compared to FAK and Akt inhibitors. In case of the Shp2 inhibitor NSC-87877, it has been shown that the inhibitor preferentially binds Shp2 protein if the gain-of-function mutation E76K is present, although the reasons for this are still unclear (37). If PHPS1 also demonstrated preferential binding to mutant Shp2, the possibility of off-target effects would be greatly reduced, but this has not yet been tested.

At first sight, our finding that a pharmacological inhibitor reverses the effects of two different dominant-negative mutations in Shp2 appears to be a paradox. However, our data are consistent with two other studies in which inhibition of enzymatic activity of the respective LS-associated Shp2 mutant by replacing an essential arginine in the catalytic pocket with a methionine also abolished the phenotypic effects of mutant protein expression (23,24).

To explain this apparent paradox, we need to consider the possibility that the C459S alteration simply interferes with the dominant-negative effects of the resulting protein, thereby allowing the endogenous intact Shp2 protein to function normally. However, studies in other tissues such as endothelial cell cultures or 293T cells have found that C459S does indeed exert dominant-negative effects in those systems (13,38,39). Therefore, loss of dominance in the C459S-Q510E double mutant is in our opinion an unlikely explanation. On the other hand, our truncation mutants lack both SH2 domains and therefore most likely also lost their ability to interact with Shp2’s regular binding partners.
such as receptor tyrosine kinases. Consequently, loss of dominance could very well explain why the Q510E-trunc mutant failed to promote NRCM hypertrophy in our studies.

An alternative explanation for the paradox could lie in our limited understanding of the molecular interactions between mutant Shp2 and its various substrates. Recent studies have demonstrated that *in vitro* phosphatase activities greatly depend on the substrate chosen for the assay and therefore individual assays with only one substrate may be misleading (40). Consequently, it is possible that Q510E-Shp2, although unable to de-phosphorylate the src peptide, may have retained substantial activity for other substrates. It is feasible that the putative loss-of-function mutations in fact induce gain-of-function effects regarding certain signaling pathways, which would explain why the presence of a PTP inhibitor as well as the introduction of the C459S mutation confer protection against the pro-hypertrophic effects of Q510E-Shp2. However, whether or not Q510E-Shp2 is able to dephosphorylate certain substrate(s) remains unclear. This issue is currently impossible to address because many substrates of Shp2 are still unknown as suggested by unbiased screening approaches (41). Substrate trapping mutants have been employed to identify some of Shp2’s substrates such as EGFR (42,43), but have the potential of changing substrate specificities themselves which might skew the results. Identification of all Shp2 substrates would most likely also lead to further insight into how exactly Q510E-Shp2 increases FAK activation. FAK is thought to be a substrate of Shp2 (44,45), but the effects seen in this study may not be due to direct interaction. To fully understand how disease-causing mutations such as Q510E alter Shp2’s functionality, comprehensive assessments of substrate affinities and enzymatic activities for individual substrates will be needed.

Another explanation for the paradox could be that Q510E-Shp2 binds and traps certain substrates, thus reducing their availability. The phosphatase inhibitor orthovanadate competes with the substrates at the binding site of Shp2 and thereby interferes with substrate trapping (43). Presumably, PHPS1 would also compete with the regular substrates and prevent trapping in a similar fashion, so this concept would be consistent with our data. However, substrate trapping can not explain why introduction of the C459S mutation abolishes the pathogenic effects. In fact, substrate-trapping effects have been described for C459S-Shp2 itself (43). Importantly, C459S-Shp2 as well as the Q79R-C459S double mutant did not have any hypertrophic effects in our study, which would therefore argue against substrate trapping being the key event of the molecular pathomechanism.

Our finding that the Q79R-C459S double mutant did not have any hypertrophic effects also suggests that the complex molecular underpinnings of the pathogenic effects of LS mutations may be insufficiently described by current concepts. It has been proposed that LS mutations may preferentially adopt ‘open-inactive’ conformations, whereas C459S-Shp2 would be ‘closed-inactive’ (13). The Q79R-C459S double mutant would presumably also be ‘open-inactive’, and should therefore have induced NRCM hypertrophy. Why the double mutant did not have the expected effects is difficult to explain since crystal structures of mutant Shp2 proteins are not yet available. But our findings do indicate that deeper insight into the structure and functionality of normal and mutant Shp2 is urgently needed and will most likely lead to refinement of the current concepts of the three-dimensional structure of various Shp2 mutants.

In summary, we found that pharmacological reduction of signaling through Shp2 / FAK / Akt / mTOR at all levels of the signaling cascade effectively counteracts the pro-hypertrophic response to Q510E-Shp2 expression. Importantly, this greatly broadens the range of tools that could be used to treat patients with such mutations in the future. Our discovery that interventions targeted directly at the mutant Shp2 protein could be highly effective is particularly exciting, because already available compounds such as PHPS1 are likely to be suitable, thus facilitating translation into the clinic. With such new custom-tailored approaches, morbidity and mortality of severely affected LS patients may substantially improve in the future.
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FOOTNOTES
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1 To whom correspondence should be addressed: Maike Krenz, Dalton Cardiovascular Research Center, 134 Research Park Dr, Columbia, MO 65211. Phone: 573-884-8761; Fax: 573-8844232; Email: krenzm@missouri.edu
2 The abbreviations used are: β-gal, β-galactosidase; CM, conditioned media; CsA, cyclosporin A; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FRNK, FAK-related nonkinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPF, green fluorescent protein; HCM, hypertrophic cardiomyopathy; LS, LEOPARD Syndrome; MEK1, mitogen-activated protein kinase / ERK kinase 1; mTOR, mammalian target of rapamycin; NRCM, neonatal rat cardiomyocytes; p70S6K, p70S6kinase; PE, phenylephrine hydrochloride; PP2, 4-Amino-5-(4-chlorophenyl)-7-(t-buty)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; p, phospho; PTP, protein tyrosine phosphatase; PV, pervanadate; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; WT, wild type.

FIGURE LEGENDS

FIGURE 1. Expression of the LS mutants Q510E-Shp2 and Y279C-Shp2 induces hypertrophy in NRCM. A, Representative images of NRCM 48 hrs after adenoviral infection with vectors encoding β-gal, Q510E-, or Y279C-Shp2 and immunostained with anti-TnI. The fourth panel shows uninfected NRCM treated with 50 µM PE. B, Random images of NRCM after anti-TnI staining were scored for sarcomeric organization by a blinded observer (1=low, 2=intermediate, 3=high level of sarcomeric organization as judged by density and continuity of myofibrillar staining). Expression of Q510E- or Y279C-Shp2 or PE significantly increased the level of organization compared to β-gal, n=10-12 images per group. C, NRCM expressing either β-gal, Q510E-, Y279C-Shp2, or treated with PE were lysed and the total soluble protein quantified. Protein content per cell number was increased in the three hypertrophied groups compared to the β-gal control group, n=8-12 per group. D, Protein synthesis rates were determined using 35S-methionine. NRCM expressing Q510E-, Y279C-Shp2, or treated with PE incorporated significantly more radiolabel compared to β-gal, n=10 per group. E, Expression of α-skeletal actin was quantified using qRT-PCR. NRCM expressing Q510E-, Y279C-Shp2, or treated with PE demonstrated increased mRNA expression of α-skeletal actin, n=4 per group.

FIGURE 2. Q510E-Shp2 induces hypertrophy by hyperactivating mTOR signaling. A, mTOR kinase activity was determined in NRCM extracts. Q510E-Shp2 expression significantly increased the enzymatic activity of purified mTOR, n=4 per group. B, Representative Western blots showing levels of phospho (p)-mTOR, p-p70S6K, and p-S6 together with the respective total protein levels. C, Western blot signal quantification, n=4-5 per group. Q510E-Shp2 expression significantly increased the ratio of phospho-to-total protein signal intensity for mTOR, p70S6k, and S6. D, NRCM surface areas were determined in cultures expressing Q510E-Shp2 or β-gal as control. Rapamycin abolished the Q510E-Shp2-induced increase in NRCM size in a dose-dependent manner, n=4 per group.

FIGURE 3. Q510E-Shp2 expression results in hyperactivation of Akt signaling, which promotes hypertrophy. A, Representative Western blots showing increased levels of phospho-Akt whereas total
Akt protein remained unchanged by Q510E-Shp2 expression. B, Western blot signal quantification, n=4 per group. Q510E-Shp2 expression significantly increased the ratio of phospho-to-total protein signal intensity for Akt. C, NRCM surface areas were determined in cultures expressing Q510E-Shp2 or β-gal as control. Akt inhibitor VIII prevented the Q510E-Shp2-induced NRCM size increase, n=12 per group.

FIGURE 4. Q510E-Shp2 expression causes hyperactivation of FAK, which is required to mediate the pro-hypertrophic effects of this LS mutation. We used immunohistochemistry to assess the level of FAK activation in Q510E-Shp2-expressing NRCM. Scale bars in panels A-L: 20 µm. A, B, The degree of FAK phosphorylation was increased in Q510E-Shp2 expressing NRCM (arrows point at activated FAK complexes). E, F, I, J, Total FAK and paxillin remained unchanged. C, D, G, H, K, L, Addition of the pharmacological inhibitor of FAK/src activation PP2 abolished this increase without affecting total FAK or paxillin staining. M and N, Western blots for quantitative assessment of FAK activation. Uninfected NRCM incubated with pervanadate (PV) and stimulated with FBS were used as positive control. Expression of Q510E-Shp2 increased the level of phospho-FAK, n=3 per group. O, The presence of the FAK/src inhibitor PP2, but not of the control compound PP3, normalized cell areas of Q510E-Shp2-expressing NRCM, n=5 per group. P, Co-infection with GFP-FRNK similarly reversed Q510E-Shp2’s effects on NRCM size depending on GFP-FRNK multiplicity of infection (MOI), n=8 per group. Q, Evaluation of downstream signaling using Western blots. The levels of Akt, mTOR, and p70S6K phosphorylation were reduced to baseline or below in NRCM expressing Q510E-Shp2 and treated with PP2, n=5-7 per group.

FIGURE 5. Fibroblast-specific effects or calcineurin-dependent signaling do not contribute to the hypertrophic mechanism, whereas MEK1 inhibition results in a partial effect on NRCM size. A, Non-infected NRCM were exposed to CM harvested from independently cultured cardiac fibroblasts. These fibroblasts had previously been infected with β-gal, WT-Shp2, or Q510E-Shp2. 50 µM PE in fresh serum-free medium served as a positive control, n=7-8 per group. * P<0.05 vs. control, # P<0.05 vs. vehicle. B, The presence of the calcineurin inhibitor cyclosporin A (CsA) did not reduce cell surface areas of Q510E-Shp2-expressing NRCM, n=4 per group. C and D, Quantification of ERK1/2 phosphorylation using Western blots. Since NRCM were cultured in serum-free conditions, no significant differences compared to WT were found in the groups expressing Shp2 mutants, n=4-6 per group. E, Effects of MEK1 inhibition on NRCM size. U0126 partially reduced NRCM size in the Q510E-Shp2-expressing groups, n=8-12 per group.

FIGURE 6. Q510E-Shp2 pro-hypertrophic effects depend on the SH2 domains and on the catalytic center. A, Various Shp2 proteins were expressed in NRCM and the respective PTPase activities determined after immunoprecipitation. For comparison, the activity of full-length WT-Shp2 was set to 1.0. The activity of WT-trunc was close to normal and the activity of Q79R-Shp2 was increased by more than 3-fold. In contrast, all other single or double Shp2 mutants displayed activities close to zero. N=4-7 per group, #P<0.05 vs. C459S-Shp2. B, NRCM cell surface areas after infection with various Shp2 constructs. Only the Q510E-Shp2 mutant increased NRCM size, n=8-12 per group. C, Representative Western blots showing levels of phospho-Akt and total Akt in NRCM expressing various Shp2 constructs. D, Western blot signal quantification, n=4 per group. Only Q510E-Shp2 expression increased the ratio of phospho-to-total protein signal intensity for Akt.

FIGURE 7. PHPS1 inhibits both Q510E- and Y279C-Shp2 induced NRCM hypertrophy. A, Q510E-Shp2 was expressed in NRCM in the presence of 10 µM PHPS1 or vehicle, n=12 per group. PHPS1 reduced NRCM size in the Q510E-Shp2 expressing group to close to normal levels. B, Y279C-Shp2 was expressed in NRCM in the presence of 10 µM PHPS1 or vehicle, n=8 per group. Again, PHPS1 prevented Y279C-Shp2-induced hypertrophy. C, The FAK/src inhibitor PP2 normalized cell surface areas of Y279C-Shp2-expressing NRCM, n=7-8 per group. D, Rapamycin abolished the Y279C-Shp2-induced
NRMC size increase in a dose-dependent manner, n=7-8 per group. E, Western blot signal quantification to evaluate downstream mediators. In the presence of PHPS1, the levels of Akt and mTOR phosphorylation were normalized in Q510E-Shp2-expressing NRMC, n=5 per group.

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Figure 1
Figure 2
Figure 3

A

B

p-Akt (473)

Akt

β-gal

Q510E

C

relative cell area

vehicle 2 μM 4 μM

Akt inhibitor VIII

ratio of phospho / total protein signal intensity

β-gal

Q510E

*
Figure 4
Figure 5

A

B

C

D

E

relative cell area

relative cell area

vehicle CsA 1 μM

vehicle 5 10 μM U0126

β-gal Q510E

β-gal Q510E

β-gal WT Q510E Y279C Q79R

p-ERK

ERK

ratio of phospho / total protein signal intensity

#
Figure 7

A, B, C, D, E represent different experiments or conditions with bar graphs showing relative cell area.

- **A**: Comparison of relative cell area between vehicle and 10 μM PHPS1 with β-gal and Q510E conditions.
- **B**: Similar comparison with β-gal and Y792C conditions.
- **C**: Relative cell area comparison with vehicle, 0.5, and 1.0 μM PP2 conditions.
- **D**: Relative cell area comparison with vehicle, 0.1, and 0.2 μM rapamycin conditions.
- **E**: Diagram comparing the ratio of phospho-total protein signal intensity between β-gal, vehicle, Q510E, vehicle, and 10 μM PHPS1 conditions.
New approaches to prevent LEOPARD Syndrome-associated cardiac hypertrophy by specifically targeting Shp2-dependent signaling
Christine Schramm, Michelle A. Edwards and Maike Krenz

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