Published in final edited form as:
Circ Res. 2005 October 14; 97(8): 813–820.

The Noonan Syndrome Mutation Q79R in Shp2 Increases Proliferation of Valve Primordia Mesenchymal Cells via ERK1/2 signaling

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Abstract

The molecular pathways regulating valve development are only partially understood. Recent studies indicate that dysregulation of MAPK signaling might play a major role in the pathogenesis of congenital valvular malformations and, in this study, we explore the role of ERK1/2 activation in valve primordia expressing the Noonan syndrome mutation Q79R-Shp2. Noonan syndrome is an autosomal dominant disease characterized by dysmorphic features and cardiac abnormalities, with frequent pulmonic stenosis. The Q79R mutation of *PTPN11* previously identified in Noonan syndrome families results in a gain-of-function of the encoded protein tyrosine phosphatase Shp2. We compared the effects of wild-type- and Q79R-Shp2 on endocardial cushion development. Atrioventricular and outflow tract endocardial cushions were excised from chick embryos, infected with wild-type Shp2 or Q79R-Shp2 adenovirus and embedded in a gel matrix. Q79R-Shp2, but not wild-type-Shp2 expression resulted in increased outgrowth of cells into the gel. The dependence of the Q79R-Shp2 effect on ERK1/2 and p38 MAPK signaling was then determined. The MEK-1 inhibitor U0126, but not the p38-MAPK pathway inhibitor SB203580, abolished Q79R-Shp2's effect on cushion outgrowth. Co-infection with Q79R-Shp2 and dominant negative MEK-1 prevented enhanced endocardial cushion outgrowth, whereas expression of constitutively active MEK-1 mimicked the effect of Q79R-Shp2. Furthermore, dissociated cushion cells displayed increased BrdU incorporation when infected with Q79R-Shp2, but not with wild type-Shp2. This pro-mitotic effect was eliminated by U0126. Our results demonstrate that ERK1/2 activation is both necessary and sufficient to mediate the hyperproliferative effect of a gain-of-function mutation of Shp2 on mesenchymal cells in valve primordia.

Keywords

phosphatase; development; valve stenosis; endocardial cushion

Congenital heart defects are present in about one percent of live births and represent the most common congenital malformation in newborns. In most cases, defects in valvuloseptal development appear to be responsible. However, our understanding of the pathogenesis of such structural abnormalities is limited. The formation of the valvular apparatus is a complex event, relying on the coordinated migration and differentiation of multiple cell types. Not surprisingly, numerous signaling pathways that utilize signaling molecules such as VEGF, NFATc1, Notch, Wnt/β-catenin, BMP/TGF-β, ErbB or NF1/Ras, and regulate cell growth, migration, and differentiation, are involved.[1]

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Sources of Support: National Institutes of Health Grants HL69779, HL56370, HL074728, HH61638 and HL52318.

Conflict-of-Interest Disclosure None.

Previous studies have indicated that the ERK1/2 pathway might play a particularly important role in valve formation at certain developmental stages. We have shown recently that ERK1/2 is activated in the endocardial endothelium overlying the undifferentiated valve primordia (stage E6 in chick).[2] At later stages (E9-10), ERK1/2 activation is present at the distal ends of both the atrioventricular (AV) valve leaflets and the aortic semilunar leaflets. These data are consistent with the hypothesis that inappropriate activation of the ERK1/2 pathway could impact on endocardial cushion (EC) growth and possibly result in congenital valve stenosis. Thus, inappropriate activation of ERK1/2 signaling could be a key mechanism in the formation of pulmonary valve stenosis in hereditary diseases such as Noonan syndrome (NS).

NS is an autosomal dominant disorder characterized by short stature, facial dysmorphia, and congenital heart defects, one of the most common being pulmonary valve stenosis.[3,4] Genetic studies revealed that in about 50% of families, mutations of *PTPN11* are responsible for NS.[5–8] *PTPN11* encodes Shp2, a ubiquitously expressed non-receptor protein tyrosine phosphatase. Shp2 can be either a positive or negative modulator in multiple signaling pathways.[9] Importantly, it can augment signaling through the ERK1/2 pathway.[10–12] NMR studies revealed that, for at least a subset of NS mutants, including Q79R, the active open conformation of Shp2 protein is favored.[5] Consequently, ERK1/2 signaling in the developing valves of NS patients might be hyperactivated, leading to aberrant valve formation and subsequent dysfunction.

Recently, it was shown that the *ptpn11* mutation D61G-Shp2 introduced via gene targeting recapitulates aspects of the NS phenotype in mice.[13] Mouse embryos heterozygous for D61G-Shp2 displayed enlarged valve primordia in both the AV canal and outflow tract associated with decreased viability, whereas homozygosity was embryonic lethal at mid-gestation. The cardiac phenotype of these mice further included atrial, atrioventricular and ventricular septal defects, double-outlet right ventricle, and thinning of the myocardial wall. The authors detected increased activation of the ERK1/2 pathway in the developing valves, but whether this was a direct cause of increased cushion growth or a secondary phenomenon is unresolved.

We wished to explore the hypothesis that there is a mechanistic link between activation of the ERK1/2 pathway and increased proliferation of the EC mesenchyme during valve formation. Using chick valve explants, we studied the effects of enhanced Shp2 activity on the outgrowth of cells from explanted valve primordia. Recombinant adenoviruses were used to transiently express the NS mutation Q79R-Shp2 in whole EC explants or in single-layer cultures of dissociated cushion cells. Cushion explant outgrowth and mesenchymal cell proliferation in the presence and absence of MAPK pathway inhibition were then analyzed to explore the role of ERK1/2 signaling in these processes.

Materials and Methods

Plasmid and Adenovirus Construction

Standard techniques were used to generate the wild type (WT)-Shp2 and Q79R-Shp2 replication-deficient recombinant adenoviruses. FVB/N mouse cardiac mRNA was used as template for WT-Shp2 cDNA. The Q79R-Shp2 mutation was introduced by site-directed PCR mutagenesis. After full-length sequencing of the construct and comparison to the published C57/BL6 Shp2 mRNA sequence (NCBI database NM_011202, gi:37360980), the cDNA was inserted into the pAdTrack-CMV vector and subsequently used for adenovirus generation (AdEasy System, Stratagene). Recombinant adenoviruses that express constitutively active and dominant negative MEK-1 were provided by Dr. Jeffery Molkentin.[14]

Cardiomyocyte Infection and Immunocomplex Phosphatase Activity Assay

Rat neonatal cardiomyocytes (RNCs) were isolated (neonatal cardiomyocyte isolation kit, Worthington) and grown on gelatin-coated polystyrene plates in DMEM/F12 medium with 1% penicillin/streptomycin and 0.5% fetal bovine serum. Cells were infected with lacZ-, WT-Shp2, or Q79R-Shp2 adenoviruses at a multiplicity of infection of 50, and harvested 48 hours after infection. Adeno-lacZ infected cells stimulated with 2% fetal bovine serum for 30 minutes were used as a positive control. Lysates from adenovirus-infected RNC expressing WT- or Q79R-Shp2 were used for Shp2 immunoprecipitation (rabbit polyclonal anti-Shp2 against C-terminal epitope, Santa Cruz Biotechnology). Beads with immunocomplexes were incubated with Src phosphopeptide (TSTEPQ-pY-QPGENL, Upstate) and released inorganic phosphate was detected colorimetrically using malachite green solution (Upstate).[15]

EC Explant Cultures

All procedures were approved by the Institutional Animal Care and Use Committee. AV EC (Hamburger-Hamilton Stage 25–26) and proximal EC of the outflow tract (Hamburger-Hamilton Stage 27–28) from E4.5 - 5.0 white leghorn chicken embryos (CBT Farms) were excised with the associated myocardial layer attached. For all experiments, cushions were collected from 1–2 dozen eggs and randomly assigned to the different treatment groups. For adenovirus infection, cushions were incubated in 50 μl medium with adenovirus for 5 minutes before being plated on growth factor reduced Matrigel Matrix (BD Biosciences). Cushions were covered with 20 μl Matrigel containing the respective adenoviruses. Plated AV cushions were cultured in medium M199 (Cellgro) with 10% fetal bovine serum (Gibco BRL), 1% chick embryo extract (Sera Laboratories International), and 1% penicillin/streptomycin (Gibco BRL). Outflow tract cushions were kept in medium M199 with 1% insulin-transferrin-sodium selenite (Cellgro), 1% chick embryo extract, and 1% penicillin/streptomycin. Photographs (Olympus SZH10 microscope, Olympus DP12 digital camera) were taken daily for quantification of explant growth. Four diameter measurements differing by 45° were averaged for each individual cushion explant and time point. Data were then normalized to the respective explant size immediately after plating for each individual EC. For the MAPK inhibitor experiments, the medium contained either 50 μM U0126 or 100 μM SB203580 (Sigma) and was changed daily. For calcineurin/NFAT inhibition, 1 μg/ml cyclosporin A was used. For retrieval of cultured cushion cells, Matrigel matrix was depolymerized at 4°C using BD™ Cell Recovery Solution. Cells were pelleted, washed with cold PBS, lysed, and pooled protein preparations from 10–16 individual cushions were electrophoresed on SDS page gels and transferred to membranes for Western blotting (Shp2, Santa Cruz; MEK-1, p44/42 MAPK, phospho-p44/42 MAPK, Cell Signaling).

For adenovirus infection or BrdU labeling of dissociated EC cells, chick AV EC (Hamburger-Hamilton Stage 25–26) were isolated and the adjacent myocardial layer removed. Cushions from 10–12 embryos were pooled and dissociated with trypsin/EDTA for 2–3 minutes, passed three times through a 25 gauge needle, and plated onto chamber glass slides treated with 0.01% rat tail collagen I (Sigma). Cells were cultured in medium M199 with 10% fetal bovine serum, 1% chick embryo extract, and 1% penicillin/streptomycin. For adenovirus infection, adenovirus was added upon plating and present for 24 hours. Forty-eight hours after infection, cells were incubated with BrdU label for 2 hours and then fixed in 70% ethanol. BrdU-positive nuclei were identified using immunohistochemistry (Zymed Laboratories). The majority of cells growing on the collagen coating displayed the characteristic spindle-like shape of mesenchymal cells. Occasionally, we could observe small patches of endothelial cells growing in a cobblestone pattern, these areas were excluded from the analysis.

Statistical Analysis

All values are given as average ± S.E.M. Student's *t*-tests were used for comparisons of two groups. One-way ANOVA followed by Tukey's post hoc test was used for comparison of multiple groups.

Results

Q79R is a "Gain-of-Function" Mutation of Shp2

We first confirmed that the Q79R mutation did indeed lead to a gain of function in the protein as predicted from NMR studies.[5] For in vitro phosphatase activity assays, WT- and Q79R-Shp2 were overexpressed in RNCs and Shp2 phosphatase activity measured (Figure 1A). The phosphatase activity of mutant Q79R was 4.5 fold higher than that of the WT protein, although approximately equal levels of Q79R- or WT-Shp2 were produced (Figure 1B). Shp2 is a known positive modulator of the ERK1/2 pathway; therefore a gain-of-function mutation of Shp2 should result in increased signaling through this pathway. We analyzed the phosphorylation state of ERK1/2 in the adenovirus-infected cardiomyocytes. At low serum concentrations, both ERK1 and ERK2 were hyper-phosphorylated in Q79R-Shp2-infected RNCs, relative to cells infected with WT-Shp2 (Figure 1C). However, we did not observe hyperactivation of the ERK1/2 pathway in Q79R-Shp2-infected fibroblasts isolated from neonatal rat hearts (data not shown).

Q79R-Shp2 Expression Increases Cushion Outgrowth

To assess Q79R-Shp2's ability to influence growth of valve primordia, we used chick EC explants grown on a gel matrix and infected with adenoviruses expressing WT- or mutant Shp2. Q79R-Shp2 expression significantly increased outgrowth of cells from excised proximal outflow tract cushions into the gel matrix (Figure 2). In contrast, cultures infected with WT-Shp2-adenovirus showed no difference in diameter compared to explants infected with lacZ-containing adenovirus, when cultured for up to three days.

To determine whether Q79R-Shp2 specifically increased outgrowth from excised outflow tract cushions, or whether this effect could also be observed in AV cushions, we repeated this assay using explanted EC from the AV canal. Similar to the cultures used in the above studies, AV EC grew to significantly larger diameters if infected with Q79R-Shp2 compared to lacZ controls or WT-Shp-2 (Figure 3). In these cultures, after 4 days of growth, the WT-Shp2-infected cushions did show a slight but statistically insignificant increase in diameter. This was expected, as overexpression of the WT form of Shp2 itself for an extended period should partially mimic the effects of the gain of function Q79R-Shp2. Shp2 protein levels in the WT-Shp2 and Q79R-Shp2 infected AV cushions were comparable (Figure 3B).

ERK1/2 Inhibition Abolishes the Effects of Q79R-Shp2 on EC Outgrowth

We observed a similar response to Q79R-Shp2 expression in both explanted AV and outflow tract cushions, indicating that Q79R-Shp2 might play a fundamental role in outgrowth mechanisms in both cushion types. We chose the AV cushions for additional studies as AV EC were larger, displayed a higher rate of cell outgrowth than the outflow tract cushions and were therefore more amenable for developmental manipulation and subsequent protein analyses.

To investigate the role of ERK1/2 activation in cushion explant outgrowth, we incubated the AV cushions with the MEK-1 inhibitor U0126. As shown in Figure 3A, the vehicle DMSO had no effect on explant diameter. U0126 completely abrogated the increase in explant culture size due to Q79R-Shp2 expression (Figure 3A and 3D). Figure 3C demonstrates that the presence of U0126 effectively reduced the extent of ERK1/2 phosphorylation. Interestingly,

cushions infected with lacZ and exposed to U0126 were slightly, but not significantly smaller after 4 days of culture when compared to the untreated cultures, indicating that the ERK1/2 pathway may also be involved in normal explant expansion.

Local cell densities are somewhat difficult to quantitate in the three-dimensional gel matrix. However, we infected 10 atrioventricular cushions with either lacZ-adenovirus or with Q79R-Shp2. After 5 days, the original explants were excised, and the remaining gel with the outgrown cells dissolved. Cells were pooled from the 10 explants, pelleted and incubated with trypsin for 5 minutes to disrupt cell-cell adhesions. Cell numbers were twice as high in the Q79R group compared to the lacZ group, a result consistent with proliferation playing a role in augmenting cushion size. In order to define more precisely the role that cell proliferation played in augmenting cushion size when Q79R-Shp2 was expressed, the rate of proliferation was assessed via BrdU labeling of adenovirus-infected dissociated cushion cells grown on collagen-coated chamber slides (Figure 4). The mitotic indices did not differ between lacZ- and WT-Shp2 infected cells. However, infection with Q79R-Shp2 resulted in a significant increase in mitotic index, consistent with an increased rate of proliferation. To confirm that ERK1/2 activation was directly responsible for the increased mitotic rate in Q79R-Shp2 infected cushion cells, we incubated dissociated EC with U0126 and labeled the cells with BrdU (Figure 4). Addition of the MEK-1 inhibitor completely abrogated the increase in mitotic index induced by Q79R-Shp2, indicating that Q79R-Shp2 exerts its hyperproliferative effect mainly via ERK1/2 activation. Although we could not rigorously discount all migration effects in the cushion explant experiments, the magnitude of the effect observed with BrdU labeling in dissociated EC cells implies that increased proliferation is the major contributor to the increase in explant outgrowth in Q79R-Shp2 expressing cushions.

To confirm that the observed effects of Q79R-Shp2 on cushion explant outgrowth were not due to non-specific pharmacologic effects, we also used adenoviruses encoding MEK-1, a major upstream activator of the ERK1/2 pathway (Figure 5). Infection with constitutively active MEK-1 resulted in a significant increase in explant growth, similar to that seen in the Q79R-Shp2 infections. Conversely, double-infection with Q79R-Shp2 and dominant negative MEK-1 reversed the effect of Q79R-Shp2 alone. Similar to U0126, dominant negative MEK-1 alone also slightly reduced explant diameter after 4 days of culture. Although ERK1/2 phosphorylation was increased in EC cells expressing constitutively active MEK-1 (Figure 5C), expression of dominant negative MEK-1 did not result in a significant decrease in ERK1/2 phosphorylation, indicating that ERK1/2 pathway inhibition was not as pronounced as with the pharmacological inhibitor U0126. We think the likely explanation for this is the incomplete (<50%) infection rate. Incomplete infection is also responsible for the observation that cushions infected with Q79R-Shp2 did not show the same increase in ERK1/2 activation as seen in cardiomyocytes (Figure 1C). However, although not apparent in the phospho-ERK1/2 immunoblots, the effect of dominant negative MEK-1 expression was sufficient to abrogate Q79R-Shp2's effects on cushion cell outgrowth.

To test the specificity of these effects, we performed a similar set of experiments on an alternative MAPK pathway. Exposure of the AV cushion cultures to the p38 inhibitor SB203580 had no effect on the extent of explant growth (Figure 6A and 6B). We also examined the effect of calcineurin inhibition on Q79R-Shp2-expressing cushion explants, using in vitro assays designed to assess effects on mesenchymal cell proliferation after endothelial-mesenchymal transformation, before further maturation and elongation of the valve primordia. Calcineurin activation is critical for early stages of the endothelial-mesenchymal transformation in mice,[16] so we did not expect any substantial involvement in our in vitro studies. However, recent evidence in cardiomyocytes indicates that the calcineurin-Nfat and MEK-1-ERK1/2 pathways can constitute a co-dependent signaling module.[17] Therefore, we tested the effect of cyclosporin A on cushion explants infected with Q79R-Shp2. Not

surprisingly, cyclosporin A did not affect the diameters of the cushion explants over 4 days of culture (Figure 6), confirming the primacy of the ERK1/2 pathway on explant growth.

Discussion

To date, the complex molecular pathways regulating valve development are only partially understood with even less known about the potential dysregulation of these pathways in the pathogenesis of valvular disease. Although a number of animal models have become available that recapitulate the human phenotype of congenital valvular stenosis,[13,18–21] specifics of the molecular basis for valvular pathogenesis remain obscure. In particular, enlargement of the valve primordia at mid-gestation appears to be a common characteristic in models of NS-like phenotypes. Hyperplasia of valve primordia can not only effectively impinge upon blood flow in the developing heart, thereby causing secondary defects in cardiovascular development, but might also compromise valve maturation in later developmental stages.

With these points in mind, we focused our studies on the hyperproliferative aspect of valve disease and explored the role of the ERK1/2 pathway. We chose to study the Q79R mutation of Shp2, which was one of the first gain-of-function mutations identified in NS families.[5] Our data show that adenoviral expression of Q79R-Shp2 in explanted EC leads to hyperproliferation. During culture development, we did not observe any differences in cell density within the gel matrix. Although we can not exclude that migration of the mesenchymal cells might contribute to the effects observed by Q79R-Shp2 infection, cell proliferation clearly played the major role and our results show that ERK1/2 activation is both necessary and sufficient to create a hyperproliferative phenotype in EC expressing Q79R. In contrast, activation of the p38 MAPK and calcineurin limbs of the pathway do not play critical roles in the hyperproliferation induced by Q79R-Shp2.

A recent study examined the effects of the NS mutation, D61G-Shp2, in mice.[13] Similar to Q79R, D61G is situated on the N-SH2 domain of Shp2 facing the PTP domain, can affect conformation and activity of the Shp2 protein,[5,22] and results in a 7–8 fold increase in basal phosphatase activity of the Shp2 protein.[13] Araki et al. demonstrated that the NS-like cardiac phenotype was associated with increased BrdU incorporation, increased ERK1/2 activation, and decreased TUNEL-positive cells within the enlarged EC of the outflow tract and AV canal. However, whether or not the observed alterations of the cushion mesenchyme were an integral part of the underlying mechanism of valve malformation in NS remained unresolved. Our study reveals that ERK1/2 activation is one of the critical events during valve formation in the case of the Q79R mutation of Shp2. Considering the functional similarities of the D61G and Q79R mutations, it is likely that a similar mechanism is responsible for the valve defects in mice carrying the D61G-Shp2 mutation.

Spatial and temporal distribution of ERK1/2 activation in the embryo is tightly regulated.[2,23] These discreet patterns are striking given the broad spectrum of downstream targets of ERK1/2 signaling involved in gene transcription and translation. During valvulogenesis, increased ERK1/2 activation is present in the endothelium overlying the undifferentiated valve primordia,[2] while at later stages, double-phosphorylated ERK is found at the distal ends of the AV valve leaflets. In the present study, MEK-1 inhibition alone resulted in a trend towards smaller diameters of the explanted cushion cultures, supporting the hypothesis that ERK1/2 activation is also involved in normal valve development. In D61G-Shp2 embryos, the spatial distribution of phosphorylated ERK1/2 was identical to the pattern in controls,[13] but the degree of ERK1/2 phosphorylation in the outflow tract cushions was increased. Thus, the intensity of ERK1/2 activation in particular cell types may play an important role in valvular stenosis. Consistent with this hypothesis is the observation that cell type-selective inactivation of neurofibromin-1, a Ras-GTPase activating protein, results in enhanced Ras/ERK1/2 activation

in EC cells.[19] As in the D61G-Shp2 model, enlarged AV and outflow tract valve primordia were observed at mid-gestation. Therefore, overexpression of normal Shp2 should mimic the phenotype caused by Shp2 gain-of-function mutations. Indeed, a trend towards increased AV cushion explant diameter with WT-Shp2 infection was noted (Figure 3). However, signaling through the ERK1/2 pathway is unlikely to be the only mechanism responsible for the NS phenotype. Shp2 modulates other receptor tyrosine kinase signaling pathways involved in cell proliferation, migration, and differentiation such as p38 MAPK, Jnk kinase, Jak/STAT, phosphatidylinositol 3-kinase/Akt, and NFκB cascades.[24–28] In fact, our data show clearly that ERK1/2 inhibition does not completely abolish Q79R-Shp2's effects on AV cushion diameter: Explants infected with Q79R-Shp2 under ERK1/2 inhibition were of similar size compared to lacZ controls with intact ERK1/2 signaling, but slightly larger than lacZ controls with ERK1/2 inhibition. This trend could be observed both with pharmacological MEK-1 inhibition and with dominant negative MEK-1 adenovirus. Therefore, it is likely that the Q79R mutation also affects other signaling pathways in the developing EC.

Cardiac valvulogenesis requires signaling between at least three different cell types: endothelial cells residing on the surface of the valve primordia, cells undergoing endothelial-mesenchymal transformation, and cardiomyocytes.[1] Importantly, the gain-of-function effects of D61G-Shp2 (and most likely other NS mutations) is highly cell-context dependent.[5,13] Since D61G-Shp2 expression was driven by the endogenous Shp2-promoter and therefore ubiquitous, that model did not address whether signals in the cells undergoing the endothelial-mesenchymal transformation or inductive signals from other tissues, such as the adjacent myocardial layer, are responsible for valve formation defects. In the present study, we used dissociated cultures of EC cells that had been dissected away from the myocardium. Q79R-Shp2 infection resulted in significantly higher rates of mitosis. Importantly, the MEK-1 inhibitor U0126 prevents this pro-mitotic effect in our study. Therefore, the BrdU assay indicates that the observed increase in proliferation rate is directly related to ERK1/2 pathway activation and likely to be independent of signaling within or from the adjacent myocardium. However, as the myocardium was present and exposed to adenovirus in our EC explant experiments, we can not completely exclude that altered signaling from the myocardial layer played a role for EC explant outgrowth.

The animal models of NS remain limited in some respects. An important difference between these experimental models and human disease is the involvement of the artioventricular valve primordia. Both in avian and mouse models, hyperproliferative effects of Shp2 mutations are independent of the cushion type, whereas in NS patients, mostly pulmonary valve stenosis is found. One possible explanation for this discrepancy in the avian model is that we used non-physiologically high levels of mutant Shp2, possibly overriding negative feedback mechanisms in the AV cushions that might normally compensate for ERK1/2 hyperactivation. However, this does not explain why, in the D61G mouse, the AV valves were affected to a similar degree as the outflow tract valves, since total Shp2 protein expression levels were unchanged. It is likely that species differences in susceptibility of outflow tract versus AV cushion tissue to ERK1/2 hyperactivation exist. Furthermore, it is not known whether expression of Shp2 itself differs dependent on valve type in NS patients. Alternatively, Shp2 binding partners such as Grb2-associated binder 1 (Gab1) might dictate whether or not abnormalities develop.

The present study demonstrates that activation of the ERK1/2 pathway is both necessary and sufficient to induce increased proliferation of EC tissue in response to expression of Q79R-Shp2. Since hyperplasia of the valve primordia can be prevented by selective inhibition of the ERK1/2 pathway, this might become the basis for the development of novel therapeutic strategies. In the D61G-Shp2 mouse model, disease severity is gene-dosage dependent, indicating that moderate reduction of ERK1/2 signaling in valve primordia might prevent hemodynamically relevant valvular stenosis after birth.

Acknowledgements

This work was supported by National Institutes of Health grants HL69779, HL56370, HL074728, HL61638 and HL52318 (J.R.).

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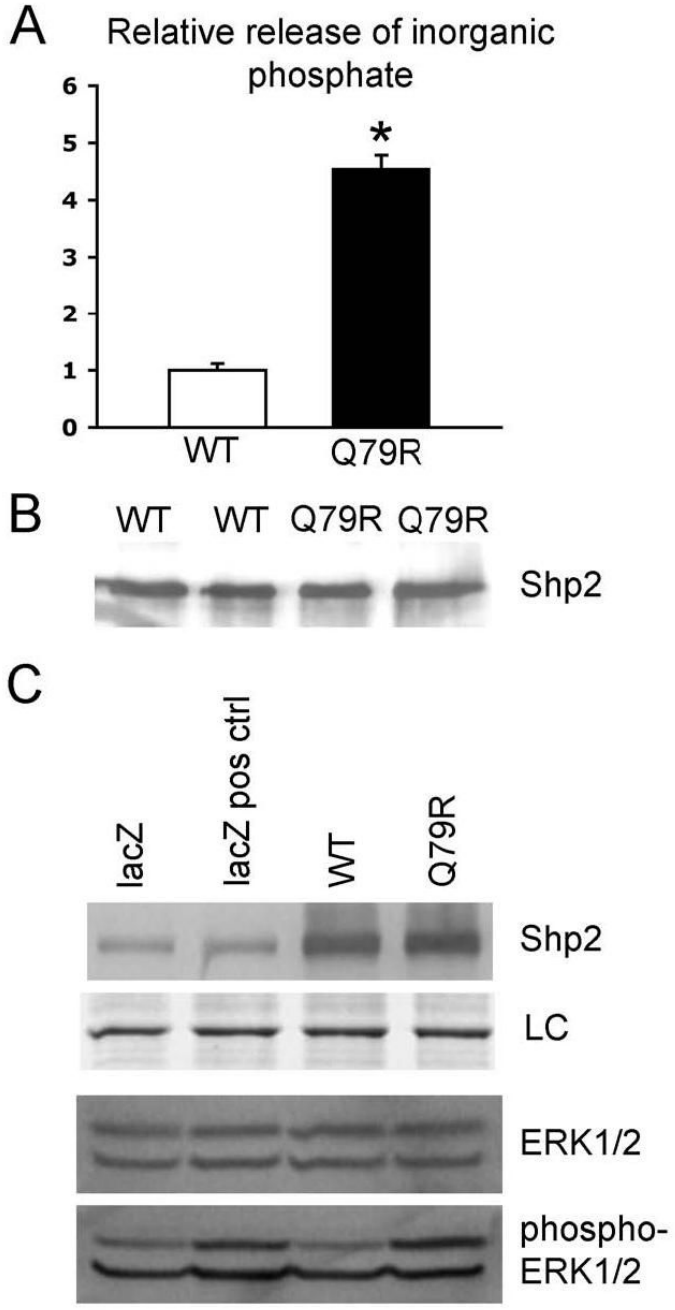

Figure 1.

Functional effects of the Q79R-mutation on Shp2 activity. A, Phosphatase assays with wild type and Q79R-Shp2 protein extracted from adenovirus-infected cardiomyocytes via immunoprecipitation. B, Representative immunoblot of immunoprecipitates used for phosphatase assays shown in panel A confirm that approximately equal amounts of protein were present. C, Immunoblots for Shp2 expression and ERK1/2 activation in cardiomyocytes 48 hours after adenovirus infection. As positive control, cells were stimulated with 2% fetal bovine serum for 30 min. LC, loading control (Coomassie stain of 65 – 75 kDa protein bands).

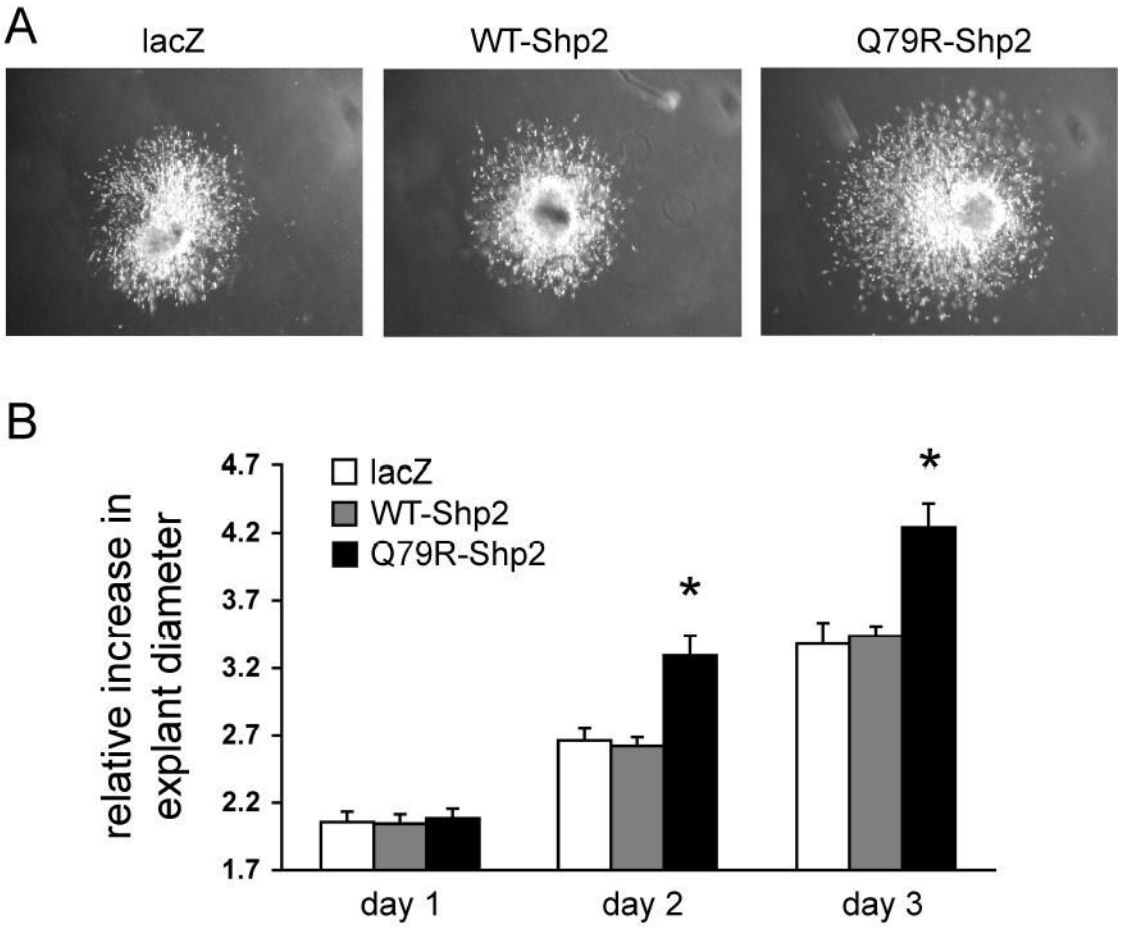

Figure 2.

Growth rates of distal outflow tract cushion explants (Hamburger-Hamilton stage 27–28) infected with either the Q79R-Shp2 or wild type Shp2 adenoviruses. A, Representative photographs of individual explants after 3 days of culture. B, Diameters of cushion explants in culture, normalized to respective explant diameter immediately after plating. n = 10 individual explants per group, *P<0.05 versus lacZ.

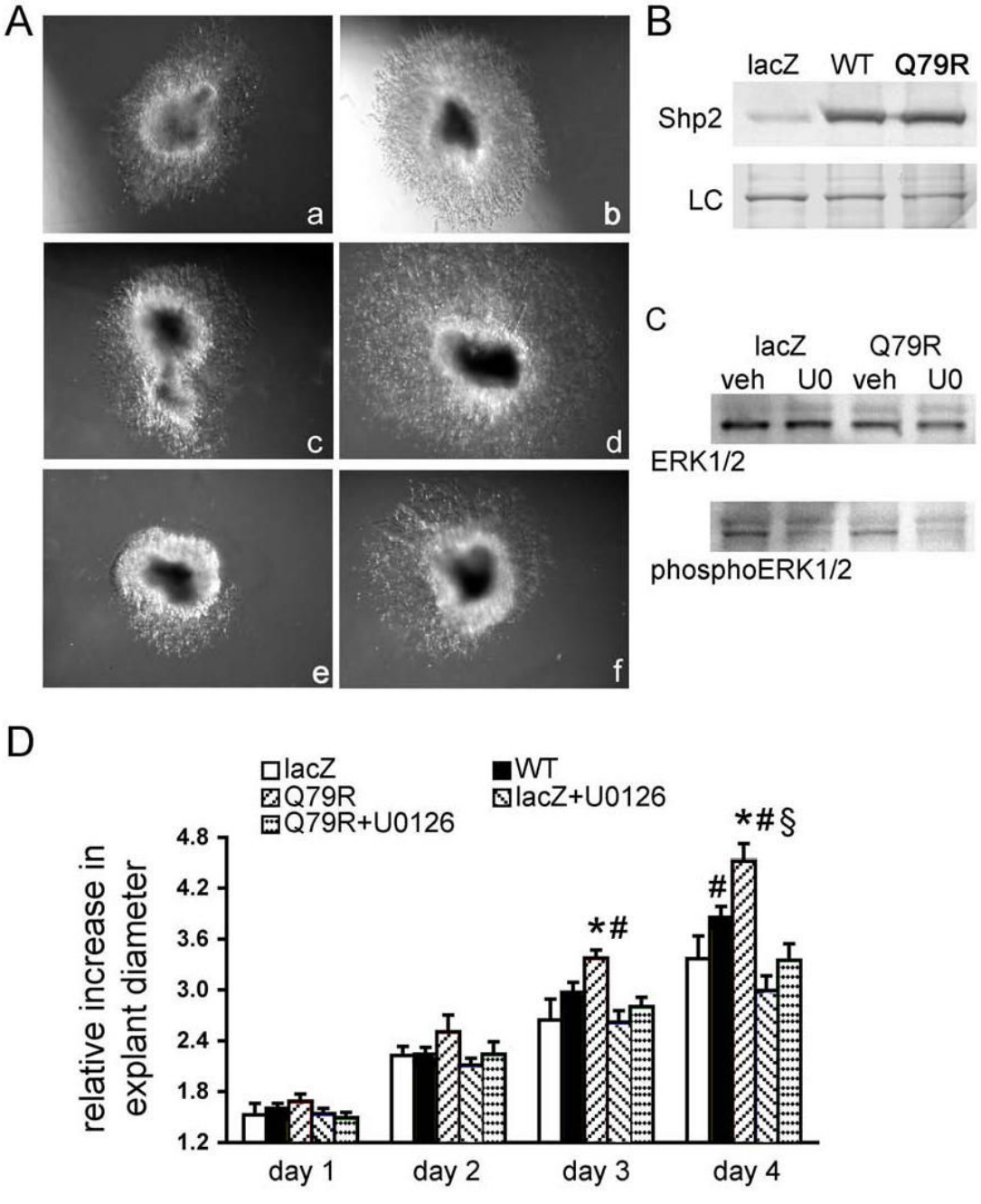

Figure 3.

Growth rates of AV cushion explants (Hamburger-Hamilton stage 25–26) infected with adenoviruses and in the presence or absence of pharmacological ERK1/2 inhibition. A, Representative photographs of individual explants after 4 days of culture. (a) lacZ, (b) WT-Shp2, (c) lacZ + DMSO, (d) Q79R-Shp2 + DMSO, (e) lacZ + U026, (f) Q79R-Shp2 + U0126. B, Representative immunoblot for Shp2 expression in AV explants after 5 days of culture. LC, loading control (Coomassie stain of 65 – 75 kDa protein bands). C, Immunoblots for ERK1/2 activation in AV explants cultured in the presence or absence of the ERK1/2 inhibitor U0126. veh, vehicle (DMSO). D, Diameters of cushion explants in culture, normalized to respective

explant diameter immediately after plating, n = 4–8 per group, *P<0.05 versus lacZ, $^{\#}P$<0.05 versus lacZ-U0126, $^{\S}P$<0.05 versus QR-U0126.

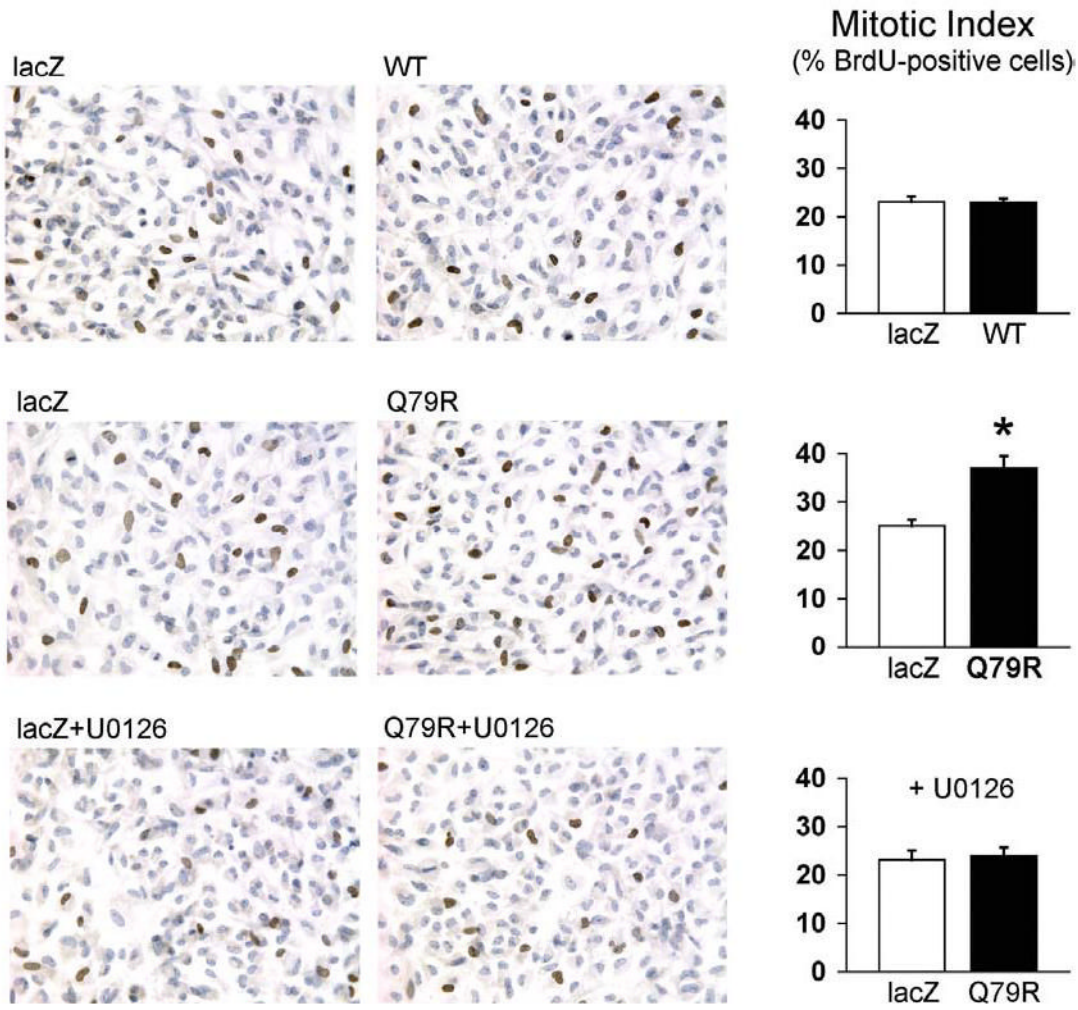

Figure 4.

BrdU-labeling of adenovirus-infected dissociated AV cushion cells grown on collagen-coated glass. Left panels, immunohistochemistry. Brown, BrdU-positive nuclei, blue, hematoxylin counterstain. Right panels, quantitative data. Counts of >2000 cells per independent experiment, n = 4 in all groups

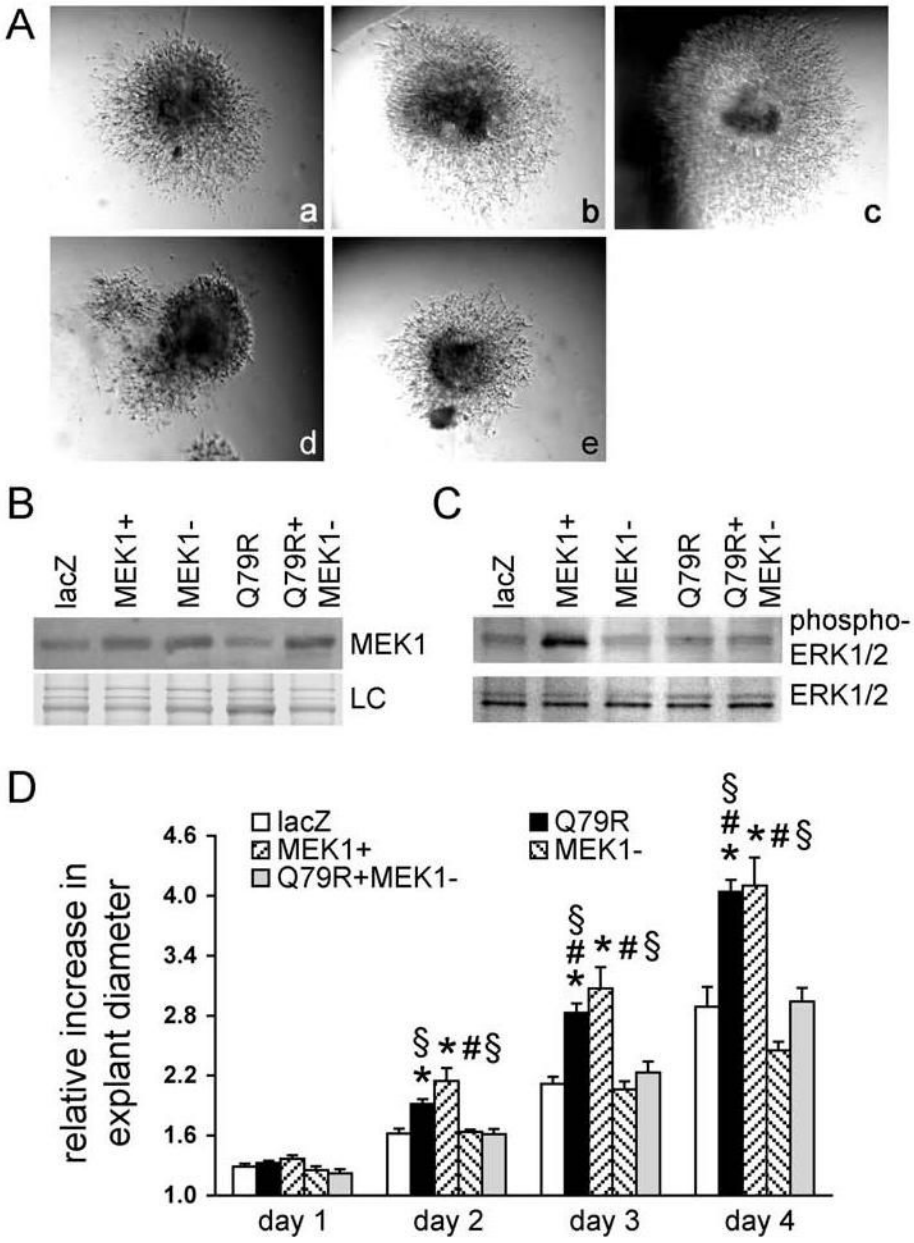

Figure 5.

Growth rates of AV cushion explants (Hamburger-Hamilton stage 25–26) infected with Q79R-Shp2 and MEK-1 adenoviruses. A, Representative photographs of individual explants after 4 days of culture. (a) lacZ, (b) Q79R-Shp2, (c) constitutively active MEK-1, (d) dominant negative MEK-1, (e) Q79R-Shp2 + dominant negative MEK-1. B, Representative immunoblot for MEK-1 expression in AV explants after 5 days of culture. LC, loading control (Coomassie stain of 65 – 75 kDa protein bands). C, Immunoblots for ERK1/2 activation in adenovirus-infected AV explants. D, Diameters of cushion explants in culture, normalized to respective explant diameter immediately after plating, n = 6–10 per group, $^{*}P<0.05$ versus lacZ, $^{\#}P<0.05$ versus MEK-, $^{\S}P<0.05$ vs QR-MEK-.

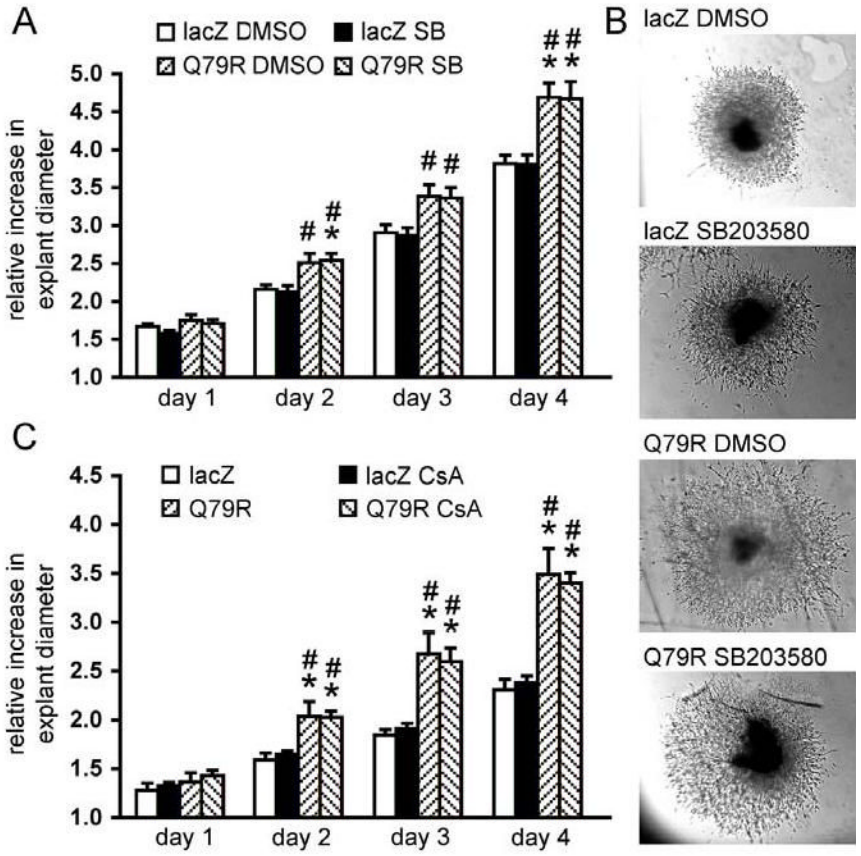

Figure 6.

Growth rates of AV cushion explants (Hamburger-Hamilton stage 25–26) infected with adenoviruses and in the presence or absence of p38 MAPK or calcineurin inhibition. A, Diameters of cushion explants treated with SB203580 in culture, normalized to respective explant diameter immediately after plating, n = 10–12 per group. B, Representative photographs of individual explants after 4 days of culture. B, C, Diameters of cushion explants treated with cyclosporine A (CsA) in culture, normalized to respective explant diameter immediately after plating, n = 5–6 per group. **P*<0.05 vs lacZ, $^{\#}P$<0.05 vs lacZ + respective inhibitor.