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SHP-2 is required for the maintenance of cardiac progenitors

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The isolation and culturing of cardiac progenitor cells has demonstrated that growth factor signaling is required to maintain cardiac cell survival and proliferation. In this study, we demonstrate in *Xenopus* that SHP-2 activity is required for the maintenance of cardiac precursors in vivo. In the absence of SHP-2 signaling, cardiac progenitor cells downregulate genes associated with early heart development and fail to initiate cardiac differentiation. We further show that this requirement for SHP-2 is restricted to cardiac precursor cells undergoing active proliferation. By demonstrating that SHP-2 is phosphorylated on Y542/Y580 and that it binds to FRS-2, we place SHP-2 in the FGF pathway during early embryonic heart development. Furthermore, we demonstrate that inhibition of FGF signaling mimics the cellular and biochemical effects of SHP-2 inhibition and that these effects can be rescued by constitutively active/Noonan-syndrome-associated forms of SHP-2. Collectively, these results show that SHP-2 functions within the FGF/MAPK pathway to maintain survival of proliferating populations of cardiac progenitor cells.

KEY WORDS: SHP-2, Cardiac, *Xenopus*, Cell cycle, Survival, FGF

INTRODUCTION

Cells of the cardiac lineage are among the first mesodermal cells to be allocated to a specific tissue type in vertebrates. By the onset of gastrulation, the cells that will give rise to cardiac tissue are located in two regions at the anterior edge of the mesoderm. Extirpation, explantation and tissue isolation studies in amphibian and avian embryos are all consistent with the cells of the cardiac lineage being specified and committed to the heart lineage during these early stages of development (Dehaan, 1963; Sater and Jacobson, 1989; Warkman and Krieg, 2007). Once cells are committed to the cardiac lineage, the cells migrate laterally and anteriorly, and subsequently fuse at the ventral anterior midline to form the bilaminar heart tube, comprised of an outer myocardial layer and an inner endocardial layer (van den Hoff et al., 2004). It is during this period that the vertebrate heart expresses the first molecular markers of cardiac development *Tbx5*, *Gata4*, *Tbx20* and *Nkx2.5*, the homolog of the *Drosophila* gene *tinman* (Fishman and Chien, 1997; Harvey et al., 2002). It is also during this time that the cardiac precursors begin a period of rapid proliferation (Goetz and Conlon, 2007; Pasumarthi and Field, 2002).

The isolation and culturing of cardiac progenitor cells has strongly implied the requirement for growth factor function to maintain cardiac cell survival. Collectively these studies have shown that survival and proliferation of cardiac progenitor populations requires either the aggregation of clonal colonies, that the cells be co-cultured with heart tissue, or that the cultures be supplemented with a mixture of growth factors and cytokines (Goetz and Conlon, 2007; Kattman et al., 2006; Kouskoff et al., 2005; Moretti et al., 2006; Parmacek and Epstein, 2005; Srivastava, 2006; Wu et al., 2006). However, the precise nature of the endogenous growth factors and the downstream signaling pathways required for cardiac survival or proliferation remain unidentified.

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SHP-2, also known as SH-PTP2, Ptpn11, PTP1D or PTP2C, is the vertebrate homolog of the *Drosophila* gene *corkscrew* (*csw*), a widely expressed non-receptor protein tyrosine phosphatase (PTP) known to function genetically and biochemically downstream of a number of growth factors including epidermal growth factors (EGFs), platelet derived growth factor (PDGF), insulin and fibroblast growth factors (FGFs) (Delahaye et al., 2000; Feng, 1999; Pawson, 1994; Qu, 2000; Van Vactor et al., 1998; Zhang et al., 2000). The sequence, expression pattern and function of SHP-2 are highly conserved throughout evolution, with genetic studies in a number of animal models all suggestive of a crucial role for SHP-2 in early development. For example, mice homozygous for a null mutation in *Shp-2* (*Ptpn11*) die at implantation, owing to a failure in the development of the extra-embryonic trophectodermal lineage, while introduction of a dominant-negative form of SHP-2 in *Xenopus* can completely block mesoderm formation in response to the FGF/MAPK pathway and leads to gastrulation arrest (Tang et al., 1995; Yang et al., 2006).

Studies have also suggested a role for SHP-2 in heart development. Noonan syndrome, a relatively common autosomal dominant disorder that leads to a number of cardiac abnormalities, including atrial septal defects, ventricular septal defects, pulmonary stenosus and hypertrophic cardiomyopathy, is associated with mutations in *SHP-2* (*PTPN11*) in approximately half of affected individuals (Noonan and O'Connor, 1996; Tartaglia et al., 2001). All SHP-2-associated Noonan syndrome mutations are mis-sense mutations and occur within one of the two SRC-homology 2 (SH2) domains, regions required for protein-protein interactions, or within the phosphatase domain. These mutations are thought to be involved in switching SHP-2 between its inactive and active states, and to act in a constitutively active fashion (Allanson, 2002; Maheshwari et al., 2002; Schollen et al., 2003; Tartaglia et al., 2002; Tartaglia et al., 2001). However, the precise requirement for SHP-2 in heart development remains to be established.

In this study, we have bypassed the early embryonic requirements for SHP-2 by means of a cardiac explant assay. Using this assay, we define a requirement for SHP-2 in maintaining cardiac precursor populations in vivo. In the absence of SHP-2 signaling, all early cardiac makers are downregulated and cardiac cells fail to initiate cardiac differentiation. We further show that SHP-2 is required for cardiac progenitor populations that are actively proliferating, but not

those that have exited the cell cycle. We show that SHP-2 functions directly downstream of FGF in this process and that inhibiting FGF phenocopies SHP-2 inhibition. Furthermore, SHP-2 is directly phosphorylated on specific residues in vivo in response to FGF signaling, and co-immunoprecipitates with FRS, a component of the FGF pathway. Most crucially, we can rescue the cardiac lineage and the downstream signaling pathways in FGF-inhibited tissues by the expression of a constitutively active/Noonan syndrome version of SHP-2.

MATERIALS AND METHODS

DNA constructs

FL-SHP-2 was generously provided by Nikola Pavletich (Georgescu et al., 2000). *Shp-2* N308D and N308D-PTP were generated by site-directed mutagenesis (Stratagene) according to the manufacturer's protocol. Primer sequences available upon request. For epitope labeling, each construct was subcloned into an HA-modified pcDNA3.1(+) vector kindly provided by Da-Zhi Wang (UNC-Chapel Hill, NC).

Embryo injections

Xenopus embryos were obtained by in vitro fertilization (Smith and Slack, 1983), cultured in 0.1× modified Barth's saline (MBS) and staged according to the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1975). RNA for injection was synthesized using the mMessage in vitro transcription kit (Ambion) according to the manufacturer's instructions. Embryos were injected at the one-cell stage with 2 ng RNA dissolved in 10 nl water unless otherwise stated.

Cardiac explants

Tissue posterior to the cement gland and including the heart field was excised at stage 22 in a manner similar to that described by Raffin et al. (Raffin et al., 2000). The explants include overlying pharyngeal endoderm and some foregut endoderm. Explants were cultured at 23°C in either 2.5 mM DMSO, 500 μM NSC-87877 (Sigma), 50 μM SU5402 (Pfizer), 150 μM aphidicolin (Sigma), 20 mM hydroxyurea (Sigma), or 50 μM colchicine (Sigma) in 1× MBS (Chemicon) (Chen et al., 2006; Dasso and Newport, 1990; Harris and Hartenstein, 1991; Mason et al., 2002). Explants were cultured until specified stages and fixed for 2 hours in MEMFA at room temperature.

Immunoblotting

To detect endogenous SHP-2, five embryos per condition were homogenized in lysis buffer (100 mM NaCl, 20 mM NaF, 50 mM Tris pH 7.5, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% NP40 and 1% sodium deoxycholate) with the addition of complete protease inhibitor cocktail (Roche) and PMSF (Sigma) and processed according to standard protocols. In vitro translation of SHP-2 was performed using wheatgerm TNT coupled transcription/translation (Promega) according to the manufacturer's instructions.

Western blots were probed with anti-mouse total SHP-2 antibody PTP1D/SHP-2 (BD Transductions Laboratories) at 1:2500. Heart explant western blots were probed with antibodies against phospho-ERK1/2 and total ERK1/2, each used at 1:1000 (Cell Signaling). Whole heart immunoblots were prepared from 70 dissected hearts as described and probed with antibodies against total SHP-2. Calf intestinal phosphatase (CIP) treatment was carried out by incubating whole embryo lysate or heart lysate with 5 U of CIP, CIP buffer, and EDTA-free complete protease inhibitors at 37°C for 1.5 hours before western blot analysis. Loading levels of tissue were standardized in pilot runs of western blots assayed by densitometry.

Whole-mount antibody staining and in situ hybridization

Whole-mount antibody staining of whole embryos and explants were performed as described (Kolker et al., 2000) with anti-tropomyosin (1:50; Developmental Studies Hybridoma Bank), anti-myosin heavy chain (1:500 Abcam), and phosphohistone H3 (1:200 Upstate) to mark cells in M phase (Goetz et al., 2006) and visualized on a Leica MZFLIII microscope. Immunostaining of histological sections was performed according to protocols and procedures described previously (Goetz et al., 2006). For these studies, phospho-SHP-2 (Tyr542; Cell Signaling) and phospho-SHP-2 (Tyr580; Cell Signaling) were used at 1:1000. Whole-mount in situ hybridizations were performed with *Nkx2.5* (Tonissen et al., 1994), *Tbx5* (Brown et al., 2003; Horb and Thomsen, 1999), *Tbx20* (Brown et al., 2003), *Gata4* (Jiang and Evans, 1996), *Gata5* (Jiang and Evans, 1996), *Gata6* (Gove et al., 1997), *MLC1v'* (IMAGE clone 4408657, GenBank accession no. BG884964), *Sox2* (Lu et al., 2004), *Endocut* (Costa et al., 2003), *Ami* (Inui and Asashima, 2006), *Xmsr* [*Xenopus* EST clone XL327k24ex (Mills et al., 1999)] and *Shp-1* (IMAGE clone 5513271, GenBank accession no. BC09538), using protocols as previously described (Harland, 1991). In situ hybridization of sectioned *Xenopus* hearts was performed on 14 μm cryostat sections using DIG-labeled antisense RNA probes followed by enzymatic detection according to the manufacturer's protocols (Roche). The following probes were used: *Shp-2* (cloned from stage 19-26 *X. laevis* pCS2+ cDNA library) using a forward *Shp-2* primer sequence of CGCCCTAAA-GAATCGCAC and a reverse *Shp-2* primer sequence of ACACT-GTAGAGATGAAGATGCCTC resulting in a 1.8 kb insert, *Shp-1* (IMAGE clone 5513271, GenBank accession no. BC09538) and *Tbx20* (Brown et al., 2003). Embryos were cleared using 2:1 benzyl benzoate:benzyl alcohol.

Whole-mount TUNEL staining

Apoptotic cells were detected by TUNEL staining as previously described (Hensey and Gautier, 1998), with the chromogenic detection of DIG-dUTP incorporation carried out with BCIP (175 μg/ml, Roche) and nitro blue tetrazolium (337 μg/ml, Roche).

Immunoprecipitation

For immunoprecipitations from hearts, embryos were injected, as described above, with 2 ng HA-tagged full-length *Shp-2* RNA. One thousand four hundred hearts were dissected at stage 35 and homogenized in lysis buffer [50 mM Tris 7.6, 150 mM NaCl, 10 mM EDTA, 1% Surfact-Amps Triton-100, 25 mM PMSF supplemented with complete protease inhibitor mini tablet (Roche)]. Supernatants were pre-cleared with protein A/G beads for 2 hours at 4°C. HA beads (20 μl; Covance) or *Shp-2* agarose beads (30 μl; Santa Cruz Biotechnology) was added to the supernatant and rotated overnight at 4°C. Immunoblotting was performed using anti-HA (Covance) at 1:1000, anti-FRS-2 (Santa Cruz) at 1:200, anti-SHP-2 (BD Transduction Labs) at 1:2500 and anti-phospho Y-542 SHP-2 (Cell Signaling) at 1:1000. For immunoprecipitations from explants, endogenous SHP-2 was immunoprecipitated from 100 explants per condition, and immunoprecipitations were carried out as described above.

RESULTS

SHP-2 is required for MHC expression in cardiac tissue

To begin to elucidate the molecular pathways involved in cardiac cell survival, we have focused on the role of SHP-2 during the early stages of heart development. Clinical studies in humans and genetic studies in mice are all consistent with a role for SHP-2 in early heart development. However, it remains unclear whether SHP-2 acts directly or indirectly in the cardiac lineage. Western blot analysis with an antibody specific for total SHP-2, as well as section in situs, shows SHP-2 to be present throughout stages of early *Xenopus* embryogenesis, including in isolated embryonic heart tissue (see Fig. S1A-C, Fig. S2B in the supplementary material).

Having established that SHP-2 is expressed in early embryos, we next tested the requirement for SHP-2 in early heart development. To bypass the early embryonic requirement for SHP-2, we used a cardiac explant assay. Based on anatomical and gene expression studies in *Xenopus*, at late neurulation (stage 22) the cardiac precursors exist in two cell populations, which lie directly posterior to the cement gland along the anterior-ventral aspects of the embryo (Dale and Slack, 1987; Moody, 1987; Raffin et al., 2000; Sater and Jacobson, 1989). When dissected and cultured in isolation, this tissue forms a ridge of cardiac tissue on top of developing endoderm

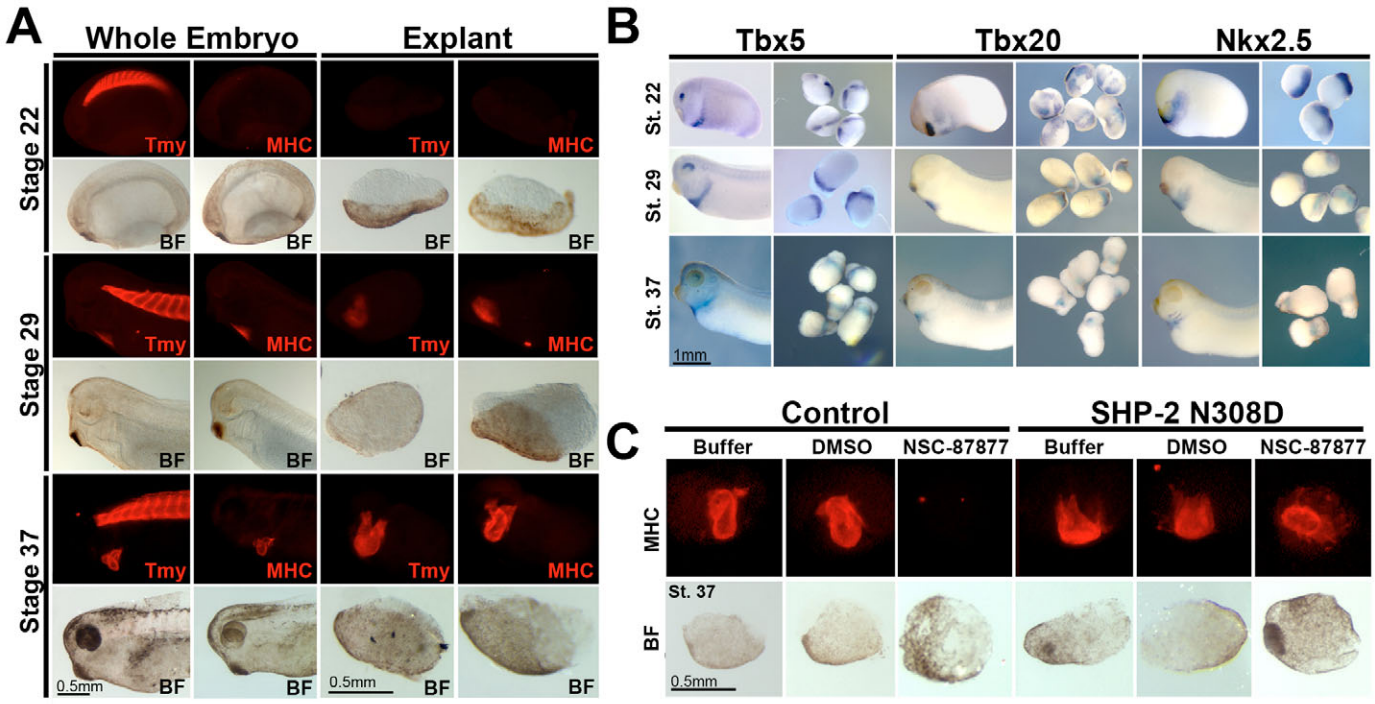

Fig. 1. Inhibition of SHP-2 activity results in loss of MHC expression. Tissue explants show identical cardiac expression profiles as intact *Xenopus* embryos. (**A**) Whole-mount antibody staining of cardiac differentiation with tropomyosin (Tmy; red) or myosin heavy chain (MHC; red) antibodies as indicated, in whole embryos and cardiac explants at stages 22 (neurula), 29 (tailbud) and 37 (tadpole). (**B**) Whole-mount in situ hybridization for early heart markers *Tbx5*, *Tbx20*, *Nkx2.5* in whole embryos and cardiac explants at stage 22, 29 and 37, as indicated. (**C**) MHC expression is dependent on SHP-2 activity. Whole-mount antibody staining for MHC (red) in cardiac explants taken from uninjected (control) embryos or embryos injected with *Shp-2* N308D and treated with either buffer or DMSO carrier as controls or with NSC-87877 as indicated. BF, bright field.

(stages 22-33) and will eventually form a beating heart (stage 33/34), whereas the donor embryo completely lacks any cardiac tissue (*Xenopus* can develop to late tadpole stage in the absence of a functioning heart or circulation). We have carried out an extensive analysis of these explants using early, mid- and late molecular markers of heart tissue and show that the explants display a temporal and spatial expression of cardiac genes that faithfully recapitulates that of control (unmanipulated) embryos (Fig. 1A,B). Therefore, the use of explants for studying the requirement for SHP-2 allows us to bypass secondary morphogenesis and tissue interactions that may complicate the analysis of the role of SHP-2 in early heart development. To determine the requirement for SHP-2 in developing heart tissue, explants were treated with DMSO or the SHP-2-specific inhibitor NSC-87877 (Chen et al., 2006). We observed a dramatic downregulation of myosin heavy chain (MHC) expression in NSC-87877-treated explants compared with controls, suggesting that SHP-2 is required for the maintenance of MHC expression (Fig. 1C).

Previous studies have shown that NSC-87877 can also inhibit SHP-1 activity. To ensure the downregulation of MHC is not due to interference with SHP-1, we identified a full-length *Xenopus Shp-1* EST (Image Clone 5513271, GenBank accession no. BC097538) and performed in situ analysis on early stage *Xenopus* embryos. Consistent with work in mouse, we never detected the presence of *Shp-1* in developing *Xenopus* heart tissue between stages 22-37 (see Fig. S2 in the supplementary material); therefore, the defects we observe are most likely due to the inhibition of SHP-2. To confirm the specificity of NSC-87877, we show that its effect on MHC can be rescued by injection of the Noonan-syndrome-associated constitutively active form of human SHP-2, N308D (*Shp-2* N308D) but not a phosphatase-dead version of N308D (Fig. 1C and see Fig. S3 in the supplementary material; data not shown). To ensure that N308D is not inducing ectopic cardiac tissue in explants, we further tested the effects of *Shp-2* N308D on heart development by injecting mRNA encoding *Shp-2* N308D and performing whole-mount in situ hybridization with markers of early heart development, including *Nkx2.5* (Tonissen et al., 1994), *Tbx5* (Brown et al., 2003; Horb and Thomsen, 1999) and *Tbx20* (Brown et al., 2003). Consistent with the results shown in Fig. 1, there were no alterations in the expression of any of these markers between control and N308D-derived embryos (see Fig. S4 in the supplementary material). Taken together, these data indicate that SHP-2 signaling is required to induce or maintain expression of MHC.

SHP-2 signaling is required for the maintenance of cardiac progenitors

As we observed that SHP-2 is required for MHC expression in cardiac tissue, we addressed whether this effect is specific for MHC or reflects a general requirement for SHP-2 signaling in heart development. To establish the role of SHP-2 in heart development and to determine how rapidly SHP-2 inhibition effects cardiac gene expression, we assayed cardiac explants for expression of *Nkx2.5*, *Tbx5*, *Tbx20* and the cardiac differentiation marker *MLC1v′* at time points corresponding to: stage 22, the stage when the cardiac precursors are two distinct lateral populations of cells; stage 26, the period when the two cardiac precursors populations are positioned at the anterior, ventral region of embryo flanking the midline; stage

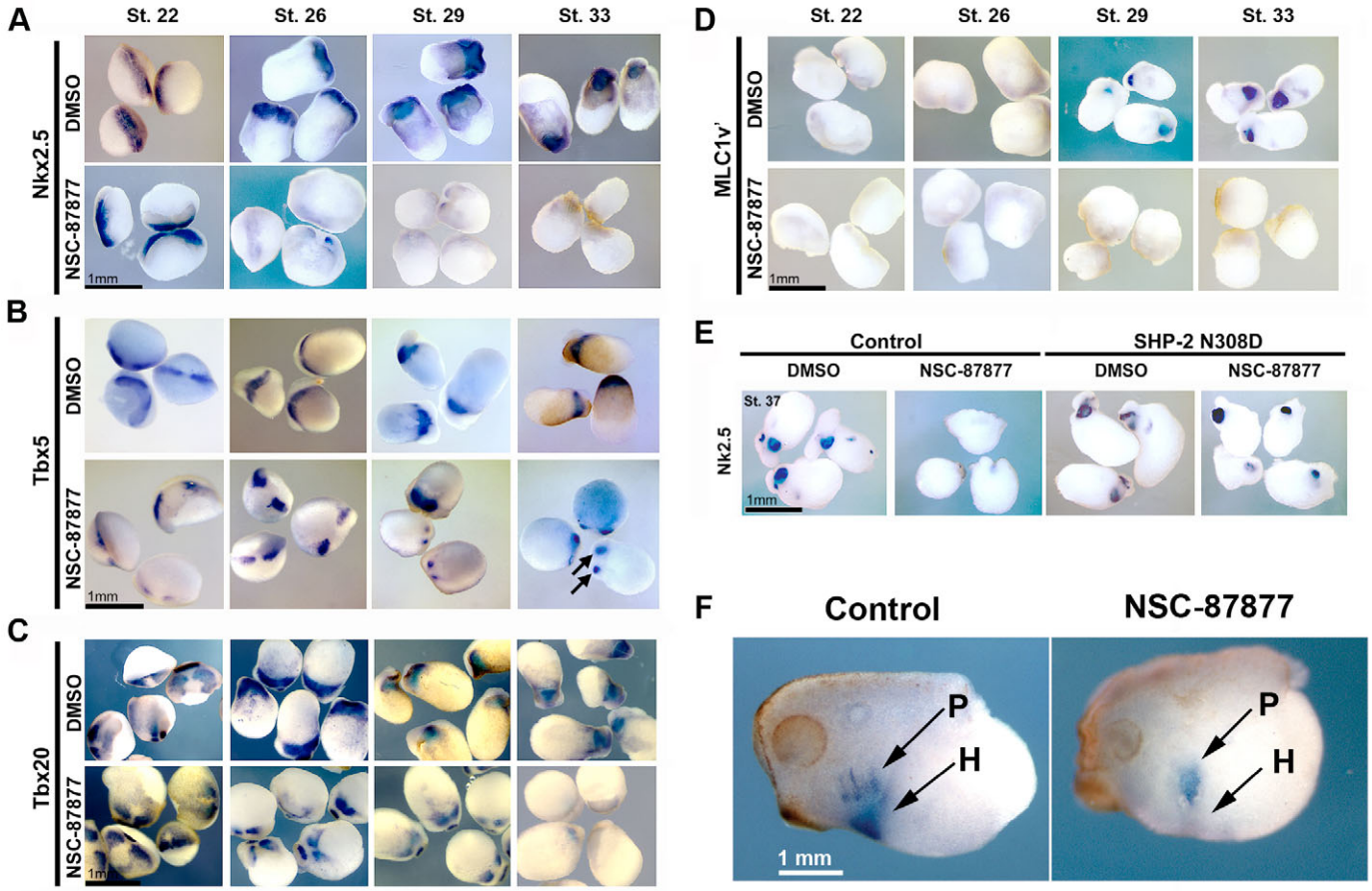

Fig. 2. SHP-2 activity is required for the maintenance of expression of cardiac markers. (**A-D**) Cardiac explants isolated and cultured in DMSO or NSC-87877 beginning at stage 22. In situ hybridization performed on explants with probes specific for *Nkx2.5* (A), *Tbx5* (B), *Tbx20*-(C) and *MLC1v'* (D) at stages 22, 26, 29 and 33, as indicated. Black arrows denote *Tbx5* expression at the leading edge of the cardiac ridge at stage 33. (**E**) In situ hybridization of *Nkx2.5* in uninjected (control) or *Shp-2* N308D-injected explants treated with DMSO or NSC-87877 beginning at stage 22, and assessed at stage 37. (**F**) In situ with *Nkx2.5* of anterior region of whole *Xenopus* embryos cultured in DMSO (control) or NSC-87877. H, heart; P, pharyngeal arches.

29, when the cardiac fields fuse across the ventral midline; and stage 33, when the bilaminar heart tube initiates cardiac looping. These studies show that there is a progressive loss of all three early markers with increasing length of SHP-2 inhibition. We observed that controls and tissue treated for 1 hour were indistinguishable at stage 22 (Fig. 2A-C); however, by early tailbud stage (St. 26), cardiac precursors in treated explants remained in two bilateral populations, whereas the cardiac precursors in controls had migrated toward the midline (Fig. 2A-C). At stage 29, when the hearts in control explants had formed a linear heart tube, the cardiac fields in SHP-2 inhibited explants remained unfused and displayed reduced expression of *Nkx2.5*, *Tbx5* and *Tbx20*. Similarly, at stage 33, *Nkx2.5*, *Tbx5* and *Tbx20* expression appeared to continue to be restricted to a subset of tissue at the leading edge of the cardiac field or was absent entirely. The expression of the cardiac differentiation marker *MLC1v'* was never initiated in SHP-2-inhibited explants (Fig. 2D). Thus overall there appears to be a progressive and rapid loss of early cardiac marker expression in SHP-2-inhibited explants and markers of cardiac differentiation fail to be expressed (Fig. 2A-D). We did note, however, that cardiac cells at the leading edge continued to express *Tbx5* until at least stage 33 (Fig. 2B, arrows), suggesting that in these cells *Tbx5* expression is regulated in an SHP-2-independent fashion.

To confirm and extend these findings, we tested explants for expression of the early cardiac/endoderm markers *Gata4*, *Gata5* and *Gata6*. Similar to *Nkx2.5*, *Tbx5* and *Tbx20*, we detected a dramatic downregulation of *Gata4*, *Gata5* and *Gata6* (see Fig. S5 in the supplementary material). The loss of cardiac markers is not due to dedifferentiation, because the explants did not express markers of undifferentiated mesoderm (data not shown). Moreover, the effects of SHP-2 were dose-dependent, with the NSC-87877 dose that affected early cardiac marker expression being the same dose that in cardiac explants blocks MAPK signaling, a downstream mediator of SHP-2 signaling (see Fig. S6 in the supplementary material). However, we cannot formally exclude the possibility that other SHP-2 like molecules may also be involved in the same signaling process. Consistent with published reports showing that NSC-87877 affects the phosphatase activity of SHP-2, we are able to rescue the expression of molecular markers of early cardiac development in SHP-2-inhibited explants by the expression of a constitutively active SHP-2 N308D and to a lesser extent wild-type SHP-2 (Fig. 2E and see Figs S3, S6 in the supplementary material; data not shown).

As NSC-87877 cannot be absorbed by whole *Xenopus* embryos, to determine whether the effects we observe with our tissue culture explants is reflective of a requirement for SHP-2 in developing embryos we cultured the anterior third of stage 22 embryos in

media containing NSC-87877 at the same dose used in our explant studies. Identical to the cardiac explants, treatment with NSC-87877 specifically inhibited expression of *Nkx2.5* in the developing heart but had no effects on its expression in the developing pharyngeal endoderm (Fig. 2F). Collectively, these results suggest that SHP-2 is required to maintain the expression of early cardiac markers in most of the cardiac field and for the onset of cardiac differentiation.

SHP-2 signaling is required for pharyngeal mesoderm but is not required for the induction and/or maintenance of endodermal or endothelial tissue types

To determine whether the requirements for SHP-2 are cardiac-specific, we assayed the effects of SHP-2 inhibition on the additional cell types present in tissue explants: endoderm, endothelial cells and overlying pharyngeal mesoderm (Fig. 3A). Similar to our findings with cardiac-specific markers, we found that SHP-2 signaling is required for the maintenance of the pharyngeal mesoderm-associated genes *Fgf8*, *Tbx1* and *Isl1*, as inhibition of SHP-2 signaling resulted in loss of expression of these genes in explanted tissue (Fig. 3B and data not shown). By contrast, results show that SHP-2 signaling is not required for the expression of genes associated with the deep endoderm (*Edd*- and *Endocut*-positive tissue) or pharyngeal endoderm (*Sox2* positive; Fig. 3C). Similarly, we observed that SHP-2 signaling is not required for the onset of expression of the endothelial cell markers *Xmsr* and *Ami* (Fig. 3C). We note however, that *Xmsr* and *Ami* were expressed in SHP-2-inhibited tissue in unbranched patterns verses control explants, suggesting that SHP-2 is required either directly or indirectly for the proper development and growth of endothelial tissues. Collectively, these results show that SHP-2 is required for the maintenance of early markers of cardiac and pharyngeal mesoderm but is not required for the maintenance of endodermal or endothelial cell types, and further imply that the requirement for SHP-2 in cardiac tissue is not an indirect effect of alterations in endodermal induction or patterning.

Inhibition of SHP-2 results in a progressive increase in cardiac cell death

To determine whether the loss of cardiac tissue in response to SHP-2 inhibition is due to defects in cardiac cell survival or proliferation, we repeated our analysis examining programmed cell death in control explants and explants in which SHP-2 signaling was inhibited. Explants were again treated with the SHP-2 inhibitor beginning at stage 22, and then analyzed at stages 22, 26, 29 and 33. TUNEL staining of cardiac explants revealed that at stage 22 there is no apparent difference in cardiac cell death in the ridge of mesodermal tissue, which contained the cardiac tissue in both control and SHP-2-inhibited explants (Fig. 4); however, by stage 26 we began to detect an increase in TUNEL-positive cells in SHP-2-inhibited cardiac tissue (Fig. 4). By stages 29 and 33, the number of apoptotic cells in the SHP-2-inhibited explants had further expanded in the more lateral regions of the cardiac ridge (Fig. 4). To further ensure that the cells undergoing programmed cell death were cardiac cells, we performed double

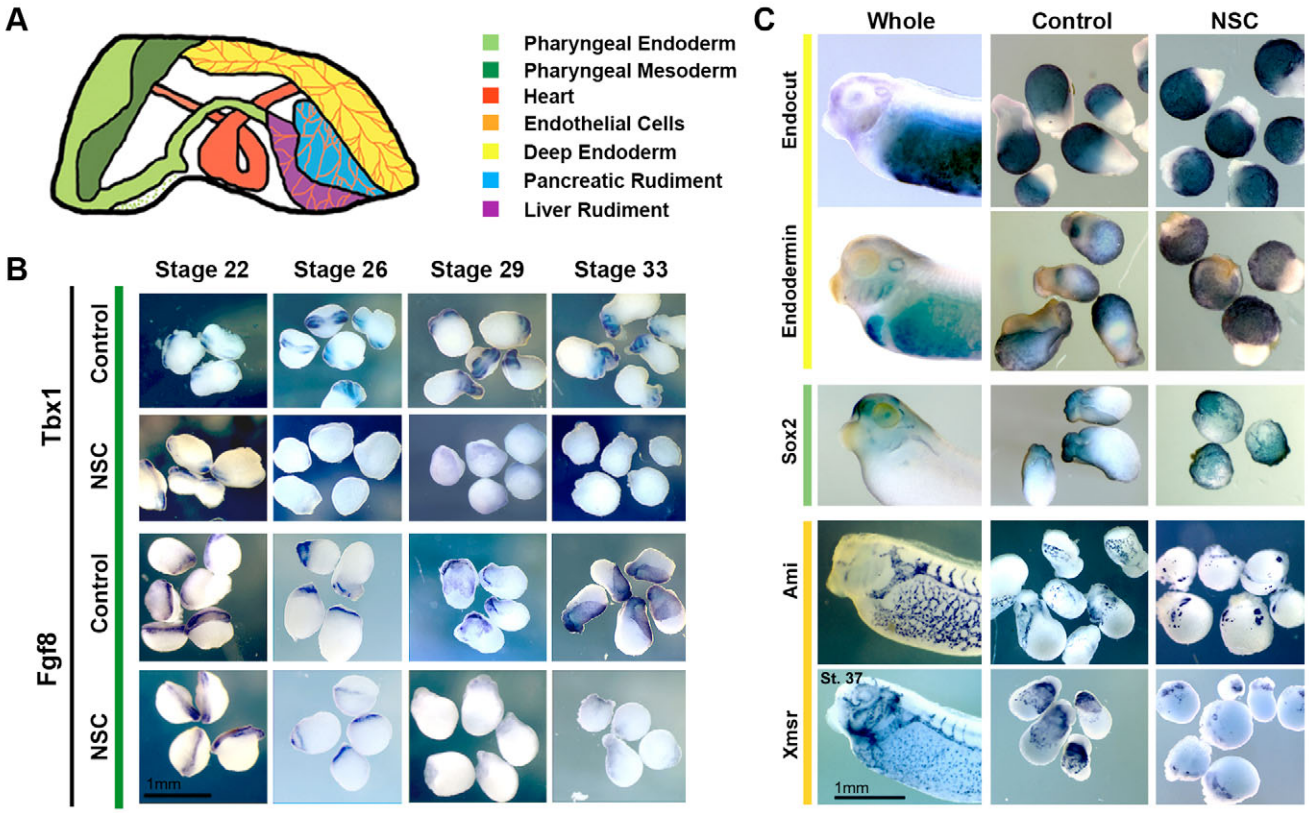

Fig. 3. SHP-2 activity is required for pharyngeal mesoderm. (**A**) Schematic of tissues and rudimentary organ structures in *Xenopus* tissue explants. (**B**) Cardiac explants isolated and cultured in DMSO or NSC-87877 (NSC) beginning at stage 22. In situ hybridization performed on explants with *Tbx1*, *Fgf8*, at stages 22, 26, 29 and 33. (**C**) Whole-mount in situ hybridization of *Endocut*, *Endodermin*, *Sox2*, *Ami* and *Xmsr* at stage 37; whole embryos and cardiac explants treated with DMSO (control) or NSC-87877, as indicated.

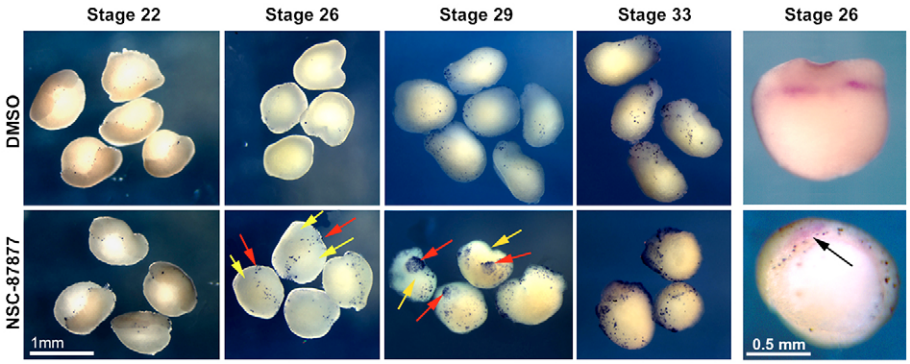

Fig. 4. SHP-2 is required for cardiac cell survival. TUNEL staining of control cardiac explants (DMSO) and explants treated with NSC-87877. Cell death examined in explants at stages 22, 26, 29 and 33, as indicated. Red arrows denote cardiac cells, yellow arrows endodermal cells. Double in situ (red)/TUNEL (dark blue) using a *Tbx5*-specific probe on stage 26 explants that were cultures in DMSO or NSC-87877 from stage 22-26. Black arrow points to *Tbx5*-expressing cells in NSC-87877-treated explants.

in situ-TUNEL staining on cardiac explants in which SHP-2 was inhibited. Results from these studies show that the cells undergoing programmed cell death were adjacent to those expressing TBX5, which was only expressed in the cardiac tissue in the explants (Fig. 4). Therefore, in the absence of SHP-2 signaling, cardiac cells cease development and undergo programmed cell death initiated by stage 26.

SHP-2 is required in proliferating cardiomyocytes

As studies have implied a role for SHP-2 in cell cycle progression (Guillemot et al., 2000; Yuan et al., 2003; Yuan et al., 2005), we tested whether withdrawal from the cell cycle could account for the observed loss of cardiac marker expression and programmed cell death in SHP-2-inhibited explants. Therefore, we treated explants with cell cycle inhibitors and determined the effects on the expression of early and late heart markers. As expected, the explants cultured in media containing aphidicolin (Aph), which blocks S-phase progression, led to a dramatic reduction in the number of mitotic cells (Fig. 5A). Surprisingly, results show that Aph led to a loss of the early cardiac markers *Nkx2.5*, *Tbx5*, *Tbx20* and *Gata6* (Fig. 5B,C); however, G1/S interphase arrest by Aph had no effect on the expression of other early heart markers, including *Gata4* and *Gata5* (Fig. 5C), and in contrast to SHP-2 inhibition, had no effect on markers of cardiac differentiation, including *Hsp27* (Brown et al., 2007), *MLC1v′*, MHC and tropomyosin (Fig. 5D,E). However, as predicted from cell cycle arrest, we observed a reduction in the size of the hearts in the Aph-treated explants. These results were not due to treatment with Aph per se, as identical results were obtained with M-phase arrest by treatment with colchicine (Fig. 5F). Thus, these findings suggest that the lack of cardiac differentiation in SHP-2-inhibited tissue is not the result of cell cycle arrest. Moreover, these observations are consistent with studies demonstrating that genetic

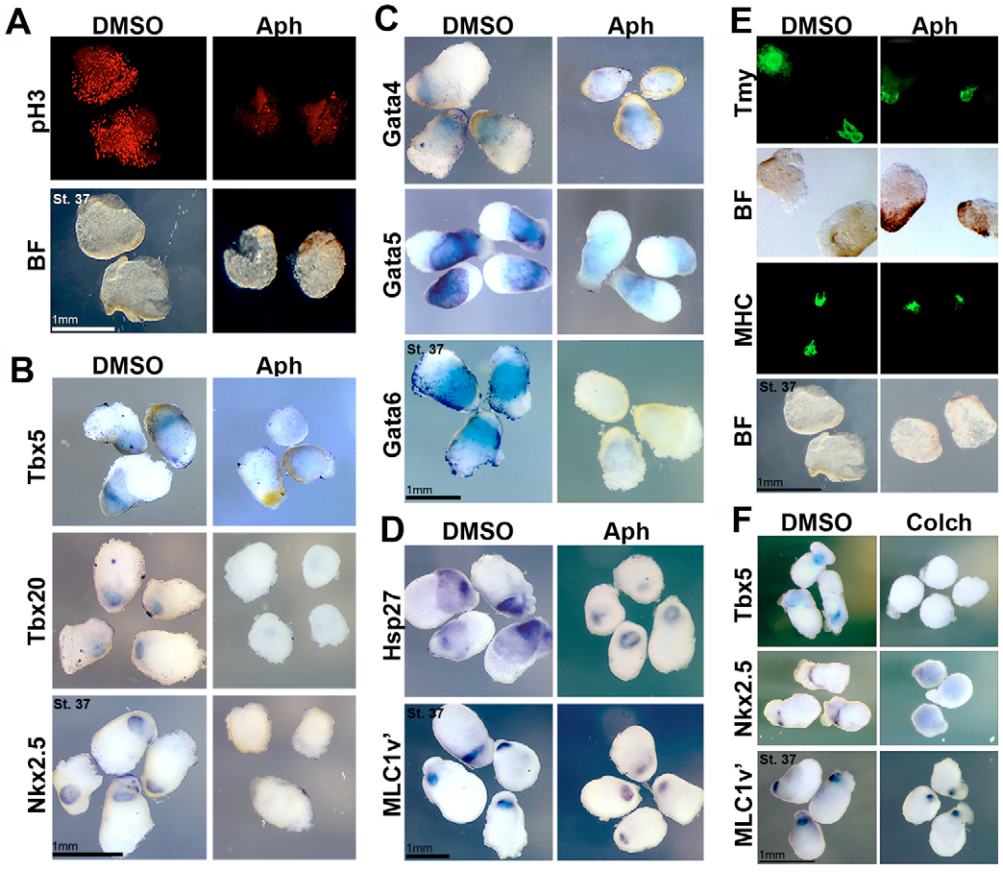

Fig. 5. Blocking the cardiac cell cycle results in loss of early, but not late, cardiac markers. (**A**) Cardiac explants isolated and cultured in DMSO or aphidicolin to block cells in S phase beginning at stage 22 and fixed at stage 37. Whole-mount immunostaining of explants with phosphohistone H3-specific antibody (red). In situ hybridization on explants with the early cardiac markers (**B**) *Tbx5*, *Tbx20*, *Nkx2.5*, (**C**) *Gata4*, *Gata5* and *Gata6*, (**D**) with the cardiac differentiation markers *Hsp27* and *MLC1v′*, and (**E**) by whole-mount immunostaining with the cardiac differentiation markers Tmy and MHC. (**F**) Explants were treated with DMSO or colchicine (Colch) to block cells in M phase of the cell cycle. In situ hybridization was performed to examine expression of *Tbx5*, *Nkx2.5* and *MLC1V′*, as indicated. Aph, aphidicolin; BF, bright field; pH3, phosphohistone H3-specific antibody.

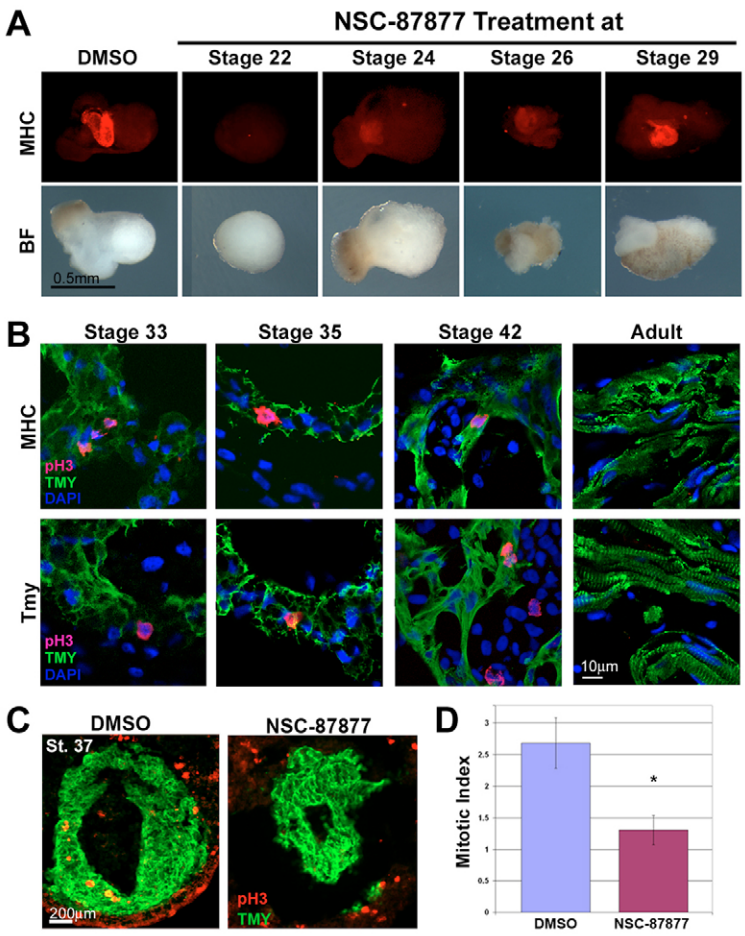

Fig. 6. SHP-2 is required for the survival of proliferating cardiac cells. (**A**) *Xenopus* cardiac explants were treated with the SHP-2 inhibitor NSC-87877 beginning at stage 22, 24, 26 or 29 as indicated, cultured to stage 37 and stained with an MHC antibody (red). (**B**) Representative transverse sections through stage 33, 35, 42 and adult *Xenopus* hearts with antibodies against MHC (green) or Tmy (green), phosphohistone H3 (red) and DAPI (blue). (**C**) Representative transverse sections through the cardiac tissue of a DMSO-treated and an explant treated with NSC-87877 beginning at stage 29, and stained with antibodies against phosphohistone H3 (red) and Tmy (green), as assessed at stage 37. (**D**) Cardiac mitotic index of explants treated beginning at stage 29 with DMSO (blue bar) or NSC-87877 (magenta bar) and assessed at stage 37. Bars represent the mean mitotic index of four explants per condition. Error bars denote the standard deviation. *, a statistically significant reduction in mitotic index of NSC-87877-treated explants (P=0.0021). BF, bright field; pH3, phosphohistone H3.

mutations or protein depletion of *Nkx2.5*, *Tbx5* and/or *Tbx20* has no effect on cardiac differentiation and further implies that cell cycle arrest and cardiac differentiation are independently regulated in vivo (Brown et al., 2005; Bruneau et al., 2001; Cai et al., 2005; Goetz et al., 2006; Lyons et al., 1995; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005).

To determine the developmental period at which SHP-2 functions to maintain survival of cardiomyocytes, SHP-2 signaling was blocked in cardiac explants beginning at a series of developmental stages [late neurula (St. 22 and 24), early tailbud (St. 26), and late tailbud (St. 29)] and cultured to tadpole stage (St. 37; Fig. 6A). Results from these studies show a decreasing requirement for SHP-2 between stages 22 and 33: treatment at stage 22 showed no MHC expression; treatment starting at stages 24 and 26 showed a marked reduction in MHC expression by stage 37; and treatment beginning at stage 29 resulted in hearts with high levels of MHC expression, but that were reduced in size by stage 37 (Fig. 6A).

Our results demonstrate that SHP-2 signaling is required during late neurula stages, a period when increasing numbers of cardiomyocytes begin to exit the cell cycle and undergo terminal differentiation (Goetz et al., 2006). To further examine the correlation between the requirement for SHP-2 and cell cycle exit, we analyzed cardiac cell proliferation and terminal differentiation between stages 33 and adult. Taken together with our past studies (Goetz et al., 2006), these results demonstrate that there is a gradual reduction in cycling cardiomyocytes during early and mid-tadpole stages and that proliferation persists in terminally differentiated cardiomyocytes until late tadpole stage (Fig. 6B).

To directly determine whether SHP-2 signaling is required for the maintenance of proliferating cardiac cells, we inhibited SHP-2 signaling, beginning at a stage when there are two populations of cardiomyocytes [one that is undergoing active division and a second that has exited the cycle and undergone terminal differentiation (stage 29)] and allowed the explants to mature to stage 37. Results from these studies show that SHP-2 is required at this later stage; inhibited explants had a mitotic index that was approximately half that of control explants (Fig. 6C,D), suggesting that SHP-2 signaling is required for the maintenance and survival of proliferating cardiac cells.

SHP-2 functions downstream of the FGF pathway to regulate cardiac cell survival

The phosphorylation state of SHP-2 has been demonstrated to be reflective of its function within a specific receptor tyrosine kinase (RTK) pathway (e.g. Bjorbaek et al., 2001). For example, SHP-2 has been shown to be phosphorylated on tyrosine residues 542 and 580 in response to FGF or PDGF stimulation but not EGF stimulation (Araki et al., 2004). To determine the phosphorylation state of SHP-2 in heart tissue in vivo, we immunoprecipitated SHP-2 from embryonic and adult hearts and performed western blots with a phospho-Y542 SHP-2 antibody. Results show that SHP-2 was phosphorylated at residue Y542 in cardiac tissue during the same period when SHP-2 functions to maintain cardiac cell survival (Fig. 7B; Fig. 6). Consistent with these results, immunohistochemistry shows that both phospho-Y542 SHP-2 and phospho-Y580 SHP-2 were expressed in the developing myocardium (Fig. 7A).

Collectively these results demonstrate that SHP-2 is present in its phosphorylated state in developing myocardial tissue, and therefore most likely acting within the FGF and/or PDGF pathways.

In tissue culture, SHP-2 interacts with the docking protein FRS within the FGF but not the PDGF signaling pathway (Kouhara et al., 1997). To test if SHP-2 is functioning downstream of FGF in embryonic heart tissue in vivo, we carried out co-immunoprecipitation experiments from isolated embryonic heart tissue. Results show that in isolated embryonic heart tissue SHP-2 directly interacted with FRS (Fig. 7B). To our knowledge this is the first demonstration that SHP-2 interacts with FRS in vivo.

As the decrease in *Nkx2.5* expression in SHP-2-inhibited explants is similar to that reported in embryos that genetically lack *Fgf8* (Ilagan et al., 2006) or those in which the endoderm adjacent to the cardiac mesoderm has been surgically removed (Alsan and Schultheiss, 2002), and as we observe phosphorylation of SHP-2 on tyrosine residues 542 and 580 and direct association of SHP-2 with FRS in embryonic heart tissue, we reasoned that FGF acts through SHP-2 to maintain the cardiac lineage. To investigate this possibility, we tested the effects of inhibiting FGF signaling in cardiac explants. Results from these assays show that similar to SHP-2 inhibition, treatment of cardiac explants with the FGFR inhibitor SU5402 led to a decrease in expression of early and late cardiac markers (Fig. 8A). However, we note that in contrast to SHP-2 inhibition, FGF inhibition led to a reduction but not loss of *Tbx5* (Fig. 8A,B). Consistent with the weaker *Tbx5* phenotype, we observed the persistence of the SHP-2-FRS interaction and a reduction but not loss of phospho-Y542 SHP-2 in FGF-inhibited explants (Fig. 8E). Taken together, these results imply that SHP-2 functions in both the FGF pathway and an additional unidentified SHP-2-FRS pathway in the developing heart.

To determine if SHP-2 functions within the FGF pathway to maintain survival of proliferating cardiomyocytes, we first determined whether the alteration in cardiac gene in response to FGF inhibition expression temporally mimics that seen with SHP-2 inhibition. As observed with SHP-2 inhibition, the cardiac explants responded to FGF inhibition between stages 22 and 26 (data not shown) and western blots of cardiac explants lacking SHP-2 activity or FGF signaling showed a dramatic decrease in phospho-ERK (threefold or more in response to inhibition as assayed by densitometry; Fig. 8C,D). Consistent with SHP-2 acting downstream of FGF, injection of a constitutively active SHP-2 (N308D) in FGFR-inhibited explants rescued expression of the early heart markers *Nkx2.5* and resulted in full expression of *Tbx5* (Fig. 8B). Taken together these studies demonstrate that SHP-2 functions in the FGF pathway to regulate cardiac progenitor survival.

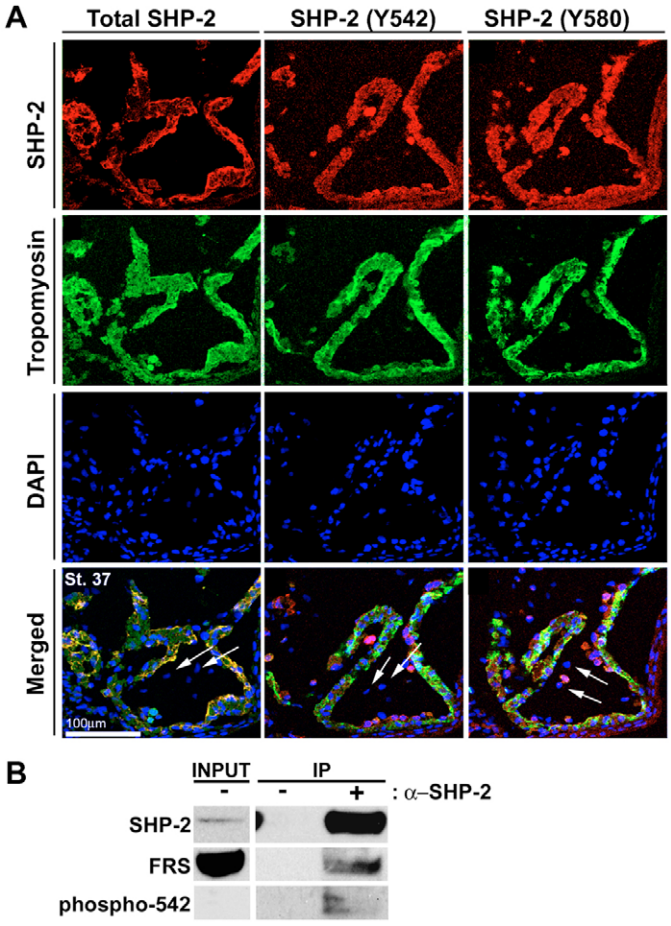

Fig. 7. SHP-2 is phosphorylated and interacts with FRS in vivo. (**A**) Transverse cryosections through the heart of control *Xenopus* embryos stained with tropomyosin to mark cardiac tissue (Tmy; green), DAPI to mark cell nuclei (blue), and anti-total SHP-2, anti-phospho-542 SHP-2 or anti-phospho-580 SHP-2 (all shown in red). Arrows denote endocardial cells that are negative for SHP-2. All samples from stage 37. (**B**) SHP-2 interacts with FRS in vivo. Hearts from FL-HA-SHP-2-derived embryos were dissected at stage 35 and immunoprecipitated with an anti-SHP-2 antibody (+) or beads with no antibody (–). Western analysis was then performed using antibodies specific for total SHP-2, phospho-542 SHP-2 and FRS-2. Note that the level of SHP-2 phopho-542 in the input was below levels of detection.

DISCUSSION

In recent years, there has been great clinical interest in identifying cardiac progenitor cells from various sources; however, little effort has been expended to understand the precise nature of the endogenous growth factor signaling pathways required for survival or proliferation of cardiac cells (Goetz and Conlon, 2007; Kattman et al., 2006; Kouskoff et al., 2005; Moretti et al., 2006; Parmacek and Epstein, 2005; Srivastava, 2006; Wu et al., 2006). To date, studies of early cardiac tissue have implied a requirement for growth factors to maintain cardiac cell survival, with survival and proliferation of cardiac progenitor populations requiring either the aggregation of clonal colonies, that cardiac progenitors be co-cultured with heart tissue, or that the cultures be supplemented with a mixture of growth factors and cytokines. However, neither the endogenous growth factor nor the signaling cascade required for cardiac progenitor survival has been identified (Parmacek and Epstein, 2005; Srivastava, 2006). To address these issues, we have characterized the endogenous role for SHP-2, a non-receptor protein phosphatase disrupted in the congenital heart disease Noonan syndrome, and have demonstrated that SHP-2 functions in the FGF pathway to maintain the survival of proliferating cardiomyocytes in vivo.

SHP-2 and cardiac cell cycle

The time at which SHP-2 is required for the maintenance of cardiac progenitor cells corresponds with a period of rapid cardiac proliferation (Fishman and Chien, 1997; Goetz and Conlon, 2007; Goetz et al., 2006; Pasumarthi and Field, 2002). In many tissues, such as muscle and nerves, the withdrawal of cells from the cell cycle is tightly associated with the onset of terminal differentiation (Alexiades and Cepko, 1996; Dyer and Cepko, 2001; Lathrop et al.,

1985; Li and Vaessin, 2000; Walsh and Perlman, 1997). By contrast, relatively little is known about the relationship between the cell cycle progression and terminal differentiation in the heart.

Our previous work has demonstrated that cardiac cells that initiate terminal differentiation retain the ability to divide (Goetz and Conlon, 2007; Goetz et al., 2006). In the current study, we have extended these findings to demonstrate that while cardiomyocytes of the adult frog ultimately exit the cell cycle, cells expressing markers of terminal differentiation are still undergoing cell division at stage 42. By this stage, cardiac morphogenesis is largely complete and all cardiac cells, including those still dividing, possess the anatomical and molecular hallmarks of differentiation, suggesting that in the heart the onset of terminal differentiation does not require cell cycle exit. Our findings are broadly consistent with recent work showing that cell cycle exit and terminal differentiation are mechanistically separable processes (Goetz and Conlon, 2007; Grossel and Hinds, 2006; Nguyen et al., 2006; Vernon and Philpott, 2003). As a corollary to these experiments, we have also examined here the consequences of induced cell cycle arrest on cardiac differentiation and found that blocking the cell cycle in S phase with aphidicolin, or in M phase with colchicine, does not result in a block in cardiac differentiation. Interestingly, however, we have found that cell cycle arrest results in reduced expression of the early cardiac markers *Tbx5*, *Tbx20* and *Nkx2.5*. Thus, these findings are consistent with the observation that none of these early cardiac proteins are required for cardiac differentiation, and further imply that the expression of these early cardiac transcription factors may be cell-cycle-dependent.

Coinciding with programmed cell death, we also observe that blocking SHP-2 activity leads to a failure of early cardiac cells to fuse at the ventral midline. At present we cannot distinguish between a role for SHP-2 mediating a trophic factor response and/or a role for SHP-2 in cell adhesion. However, genetic studies in zebrafish and mouse strongly imply that the inability of the cardiac fields to

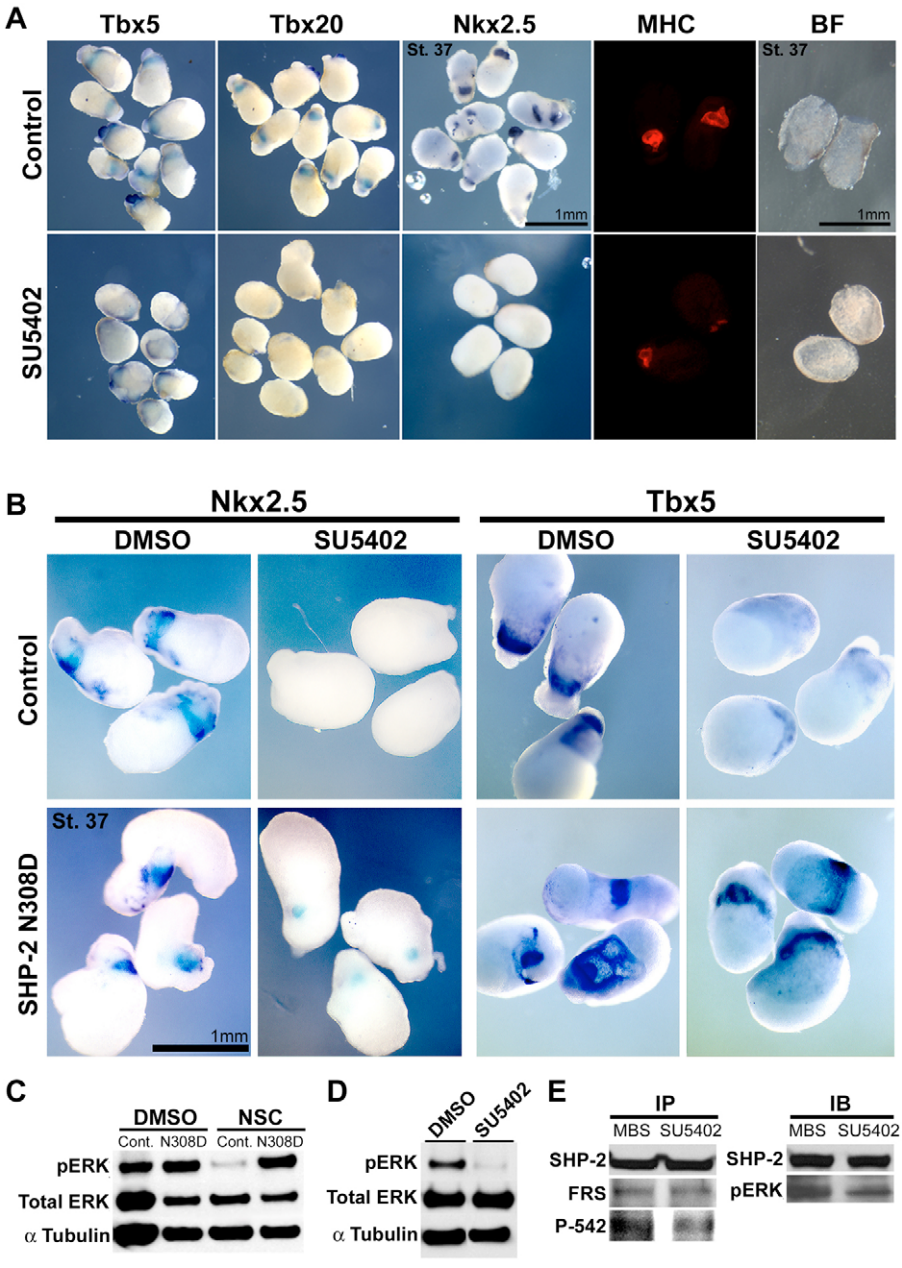

Fig. 8. FGF functions through SHP-2 to maintain the cardiac lineage. (**A**) Whole-mount in situ hybridization for *Tbx5*, *Tbx20* and *Nkx2.5* or whole-mount immunostaining for MHC (red) performed on explants treated with DMSO or the FGFR1 inhibitor SU5402. (**B**) Explants isolated from uninjected (control) or SHP-2 N308D-injected *Xenopus* embryos cultured in DMSO or SU5402 and analyzed by in situ hybridization for the cardiac markers *Nkx2.5* and *Tbx5*. (**C**,**D**) Western blot analysis of DMSO-, NSC-87877- (C) or SU5402- (D) treated explants for phosphorylated and total ERK; α-tubulin was used as a loading control. (**E**) Explants were cut at stage 22 and then incubated in either modified Barth's solution (MBS) or SU5402 until stage 35. Either endogenous SHP-2 was immunoprecipitated (IP) or explants were lysed (IB) and western analysis performed as in Fig. 7B.

fuse is not the primary cause of the downregulation of early cardiac markers or the failure of SHP-2 inhibited explants to initiate cardiac differentiation. For example, genetic mutations resulting in cardiac bifida, such as *gata5*, *hand2*, *casanova* (*sox32* – ZFIN), *bonnie and clyde* and *miles apart* (*edg5* – ZFIN) in zebrafish (Alexander et al., 1999; Kupperman et al., 2000; Reiter et al., 1999) or *Gata4* and *Mesp1* (Molkentin et al., 1997; Saga et al., 1999) in mouse, as well as genetic mutations in cardiac cell adhesion proteins (Trinh and Stainier, 2004), show no alteration in the expression of early cardiac markers such as *Nkx2.5* or of markers associated with terminal differentiation. Therefore, it is most likely that the failure of cardiac cells to migrate is a secondary consequence of cell survival or it may be that SHP-2 has two temporally distinct roles in heart development, in regulating cell adhesion and in cell survival.

SHP-2 and the FGF pathway

In this study we show that SHP-2 is phosphorylated on tyrosines 542 and 580 in the embryonic heart and that it co-immunoprecipitates with FRS-2, demonstrating an in vivo interaction between SHP-2 and FRS-2 for the first time. Given that we have shown inhibitors of both SHP-2 and FGFR to cause comparable cardiac phenotypes, and that a constitutively active form of SHP-2 can rescue formation of cardiac tissue in FGF-inhibited explants, we conclude that SHP-2 participates in the FGF signal transduction pathway in *Xenopus* embryonic hearts.

Recent work examining the role of FGFs in response to cardiac damage or injury lends further support for the direct role of SHP-2 in cardiac cell survival. The overexpression of both FGF-1 and FGF-2 have been shown to promote the survival of adult cardiomyocytes in response to ischemic injury in vivo (House et al., 2005; Jiang et al., 2002; Jiang et al., 2004; Palmen et al., 2004), and the cardioprotective effects of FGF-2 in the adult myocardium are mediated through the MAPK pathway (House et al., 2005), the same branch of the FGFR signaling cascade that we have shown in cardiac tissue functions through SHP-2. Interestingly, the specific function of FGF-2 in preventing programmed cell death in response to ischemic insult was shown to be independent of its mitogenic or angiogenic functions, suggesting that FGF-2 is functioning specifically to promote cardiomyocyte cell survival (Jiang et al., 2004). Together with our data showing that SHP-2 activity downstream of FGFR is required for the maintenance of proliferating cardiac progenitor cells, these data suggest that the FGF/MAPK pathway functions in promoting cardiac progenitor cell survival during development and further suggests that the FGF/SHP-2/MAPK pathway must be maintained to promote survival of cardiac progenitor cells in vitro. Intriguingly, the FGF/SHP-2 pathway has also recently been shown to be required for the survival of trophectoderm stem cells and for the ability of hematopoitetic stem cells to self-renew (Chan et al., 2006; Yang et al., 2006), thus raising the possibility that the FGF/SHP-2 pathway is a common pathway for progenitor cell survival.

What are the mechanisms by which SHP-2 acts to activate the MAPK pathway and promote cell survival? Studies have shown that SHP-2 acts as a positive regulator in the FGF pathway in at least two ways, the first by acting as a scaffold to recruit GRB2, which in turn recruits SOS, the guanine nucleotide exchange factor for RAS, this leads to the activation of the ERK cascade, potentially resulting in the destabilization of the pro-apoptotic protein BIM (Yang et al., 2006). Alternatively, or concomitantly, SHP-2 may act as a positive regulator in RAS signaling by inhibiting Sprouty, a key FGF/RTK inhibitor (Christofori, 2003; Kim and Bar-Sagi, 2004; Tsang and Dawid, 2004). Consistent with the later possibility, Sprouty has recently been shown to be a direct substrate of SHP-2, and studies have shown that one of the four mammalian sproutys, Sprouty 1, is expressed in the heart and is upregulated upon cardiac insult (Hanafusa et al., 2004; Huebert et al., 2004; Jarvis et al., 2006). However, it remains unknown if any of the Sprouty family has an endogenous role in early heart development or, if like in *Drosophila*, Sprouty acts as an endogenous substrate of SHP-2 in vivo (Jarvis et al., 2006).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/22/4119/DC1

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