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Shox2 is essential for the differentiation of cardiac pacemaker cells by repressing *Nkx2-5*

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Abstract

The pacemaker is composed of specialized cardiomyocytes located within the sinoatrial node (SAN), and is responsible for originating and regulating the heart beat. Recent advances towards understanding the SAN development have been made on the genetic control and gene interaction within this structure. Here we report that the *Shox2* homeodomain transcription factor is restrictedly expressed in the sinus venosus region including the SAN and the sinus valves during embryonic heart development. *Shox2* null mutation results in embryonic lethality due to cardiovascular defects, including an abnormal low heart beat rate (bradycardia) and severely hypoplastic SAN and sinus valves attributed to a significantly decreased level of cell proliferation. Genetically, the lack of *Tbx3* and *Hcn4* expression, along with ectopic activation of *Nppa*, *Cx40*, and *Nkx2-5* in the $Shox2^{-/-}$ SAN region, indicates a failure in SAN differentiation. Furthermore, *Shox2* overexpression in *Xenopus* embryos results in extensive repression of *Nkx2-5* in the developing heart, leading to a reduced cardiac field and aberrant heart formation. Reporter gene expression assays provide additional evidence for the repression of *Nkx2-5* promoter activity by *Shox2*. Taken together our results demonstrate that *Shox2* plays an essential role in the SAN and pacemaker development by controlling a genetic cascade through the repression of *Nkx2-5*.

Keywords

Shox2; *Nkx2-5*; Sinoatrial node; Pacemaker; Sinus valves; Heart development

Introduction

During embryonic heart development, the fused linear primitive heart tube contracts spontaneously, randomly and irregularly beginning at embryonic day 8.0 (E8.0). By E9.0 the heart beat becomes organized and regular with a caudal to cranial directionality (van Mierop, 1967; Nishii and Shibata, 2006). The regularity of the heart beat coincides with the "maturation" of the cardiac pacemaker located in the caudal region of the linear heart tube, the sinus venosus (van Mierop, 1967) and eventually becomes restricted to the right dorsal wall

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of the right atrium, structurally known as the sinoatrial node (SAN) (van Mierop, 1967; van Mierop and Gessner, 1970). Deficiencies in such pacemaking function lead to cardiac defects specially arrhythmias and in severe cases to cardiac arrest and sudden death (Durham and Worthley, 2002).

Genetically, the SAN is characterized by the expression or lack thereof of several molecular markers. The hyperpolarization-gated cyclic nucleotide cation-activated channel, *Hcn4*, a specific molecular marker for the pacemaker (Santoro and Tibbs, 1999) is expressed as early as E7.5 in the cardiac crescent, becoming restricted to the sinus horns of the sinus venosus and eventually to the SAN region by E12.5 (García-Frigola et al., 2003). *Hcn4* mutant mice die at mid-gestation stage due to the lack of formation of a "mature" pacemaker, while its overexpression mimics pacemaker properties in cell cultures (Santoro and Tibbs, 1999; Santoro et al., 2000; Moosmang et al., 2001; Stieber et al., 2003; Liu et al., 2007). The T-box transcription factor *Tbx3*, has been reported to be expressed in the cardiac conduction system including the SAN (Hoogaars et al., 2004). Mutation of the *Tbx3* gene causes limb and mammary deformities and embryonic lethality due to cardiovascular defects (Davenport et al., 2003), however, does not impair the early formation of the SAN in mice (Hoogaars et al., 2007). Moreover, *Tbx3* was shown to repress the expression of *Nppa* (*ANF*) and *Cx40* by directly binding to the promoter of each gene in cultured cells (Hoogaars et al., 2004). Indeed, the expression of *Nppa* and *Cx40* is mutually exclusive to that of *Tbx3* in the heart (van Kempen et al., 1996; Gaussin, 2004; Soufan et al., 2004; Stennard and Harvey, 2005). The early cardiac differentiation marker *Nkx2-5* is expressed as early as the formation of the cardiac crescent (Komuro and Izumo, 1993; Moses et al., 2001). However, it is not expressed in the SAN or in the venous valves (Kasahara et al., 1998). Mice deficient in *Nkx2-5* are embryonically lethal due to disruption in the differentiation of cardiac tissue (Lyons et al., 1995; Tanaka et al., 1999), while its overexpression induces nodal myocytes characteristics in rat myocardial cultures (Wang et al., 2007). Additionally, haploinsufficiency and point mutations in the human *Nkx2-5* gene are related to arrhythmias and atrioventricular conduction deficiencies (Schott et al., 1998; Benson et al., 1999; Watanabe et al., 2002). *Nkx2-5*, *Tbx3* and *Hcn4* have been integrated in a genetic network involved in the development and patterning of the SAN (Hoogaars et al., 2007; Mommersteeg et al., 2007).

Shox2, formerly known as *Og12* in mice (Rovescalli et al., 1996), *Prx3* in rats (van Schaick et al., 1997) and *SHOT* in humans (Blaschke et al., 1998), is closely related to the short stature homeobox gene *SHOX*, both being present only in vertebrates; however, a *SHOX* ortholog is absent in the mouse. Mutations in either *SHOX* or *Shox2* cause skeletal and other abnormalities, particularly in the long bones (Ellison et al., 1997; Rao et al., 2001; Belin et al., 1998; Shears et al., 1998; Zinn et al., 2002; Yu et al., 2005, 2007; Cobb et al., 2006; Gu et al., 2008). *Shox2* has been shown to be expressed in the SAN; its null mutation causes embryonic lethality and ectopic expression of *Nkx2-5* and *Cx40* in the SAN region, suggesting a possible role in the differentiation of this structure (Blaschke et al., 2007). However the detailed relationship between *Shox2* and the development of the SAN is still not complete and the *Shox2* molecular niche in such a process is still not clear.

In this study we show that *Shox2* expression is restricted to the sinus venosus region, and eventually to the SAN, including the pacemaker and sinus valves in the developing mouse heart. *Shox2* null mutation leads to cardiac edema, vascular defects, hypoplasia of the SAN and sinus valves as well as severe bradycardia. Gene expression analyses of molecular markers reveal failure in the differentiation of pacemaker cells and absence of a defined SAN in the *Shox2* mutant hearts. *Shox2* overexpression represses the expression of *Nkx2-5* in cell cultures, and in *Xenopus* embryos leads to cardiac defects similar to those seen in the *Nkx2-5*-deficient *Xenopus*, zebrafish and mouse models.

Materials and methods

Animals and embryos

Generation of *Shox2* mutant mice has been described previously (Yu et al., 2005). *Shox2* heterozygotes were maintained on both C57BL/6 and CD-1 genetic backgrounds. Histological analyses and comparisons were performed using wild type (WT), and $Shox2^{-/-}$ mice on both genetic backgrounds. Embryos were collected at different embryonic stages for examination of death rate. Embryonic age was determined by counting the appearance of vaginal plug as embryonic day 0.5 (E 0.5). Genotyping was performed by PCR using genomic DNA extracted from tail biopsies or from the amniotic membrane as described previously (Yu et al., 2005). *Xenopus laevis* eggs were obtained from hormone-stimulated females, fertilized *in vitro*, and cultured following a standard procedure (Sive et al., 2000). Embryos were reared in 0.1× Marc's Modified Ringer's solution (MMR) at 25 °C until desired stages. Embryo staging was determined according to Nieuwkoop and Faber (1967). Microinjection was performed in 3% Ficoll solution; mRNA encoding GR-*Shox2* (50 pg) was injected into the marginal zone of two dorsal blastomeres at the 4-cell stage. At the early neurula stage, control and injected embryos were divided into two groups, with one group cultured in 0.2× MMR, while the other group cultured in 0.2× MMR containing 10 μM dexamethasone (Sigma) (Kolm and Sive, 1995). Embryos were harvested at the tadpole stage, processed and analyzed for gene expression by *in situ* hybridization.

Histology, in situ hybridization, detection of cell proliferation and apoptosis

Wild type and $Shox2^{-/-}$ embryos on C57BL/6 or CD-1 backgrounds were collected in DEPC-treated PBS, fixed in 4% paraformaldehyde (PFA) in DEPC-treated PBS overnight at 4 °C, dehydrated in a series of alcohol, cleared in xylene and embedded in paraffin wax. Embryos were sectioned at 7 μm–10 μm and subjected to either Hematoxilin/Eosin staining or *in situ* hybridization after deparaffination and rehydration as described previously (Alappat et al., 2005). *Xenopus* embryos were similarly treated; however, dehydration in methanol series was used for whole mount *in situ* hybridization. Samples for *in situ* hybridization were hybridized with antisense RNA probes for mouse *Tbx3* (provided by Dr. Ken Muneoka, Tulane University), mouse *Nkx2-5* and *Nppa* (provided by Dr. Eric Olson's laboratory, Southwestern Medical Center, University of Texas), *Xenopus Nkx2-5* and *cTnI* (provided by Dr. Frank Conlon's laboratory, University of North Carolina-Chapel Hill). Other probes used were cloned by RT-PCR in our laboratory and sequence confirmed. Cell proliferation rate was detected by *in vivo* BrdU labeling using a BrdU Labeling and Staining Kit following the manufacturer's instruction (Roche Applied Sciences, IN), and cell apoptosis was detected by using a Cell Death Detection Kit (Roche Applied Sciences, IN), as previously described (Yu et al., 2005; Alappat et al., 2005). To obtain consistent results, all the experiments, including *in situ* hybridization, BrdU labeling, and TUNEL assays were repeated at least three times. For BrdU labeling studies, BrdU-positive cells were counted and presented as percentage of total cells within the selected areas. Nine sections from three individual samples of either wild type or mutant were counted and applied to statistical calculation. The Student's *t*-test was used to determine significant statistical difference.

Measurement of heart beat rate

For measurement of heart beat rate, embryonic hearts were isolated and cultured according to a standard method as previously described (Stieber et al., 2003). Briefly, embryos at E9.5 and E10.5 were dissected in pre-warmed ADS buffer containing glucose. Hearts were excised and cultured in DMEM containing 10% FBS and 1% antibiotics at 37 °C and 10% CO_2 for 24 h. Heart beats were counted under inverted microscope for 10 s three times for each heart before the genotype was known, thus assuring a blind heart beat count. Heart beat counts were

converted to heart beats per minute and statistical analysis was performed using the Student's *t*-test for determination of statistical difference.

Plasmid constructs

The *Shox2a* coding sequence was amplified by PCR using *Pfu* high fidelity enzyme (Strategene) and inserted into the pcDNA3 vector (Invitrogen) containing a myc tag in order to create the myc-*Shox2* construct. To make the dexamethasone inducible fusion protein version of *Shox2* (GR-*Shox2*), the *Shox2a* coding sequence was cloned into the pCS2+_GR vector, which contains the glucocorticoid ligand-binding domain (amino acids 513–777). The 3.3-kb mouse *Nkx2-5* upstream sequence driving the expression of the Luciferase gene (*Nkx2-5-Luc*) (Liberatore et al., 2002) was provided by Dr. Katherine Yutzey (Cincinnati Children's Hospital Medical Center) and utilized in subsequent experiments. Site-directed mutagenesis was carried out on the *Nkx2-5-luc* construct by PCR using *Pfu* high fidelity enzyme following the manufacturer's instructions (Stratagene) and the following mutagenesis primers (forward sequence shown): BS-I, 5′-GGGCTGATCGCTTTTCAATCAAGAAGAAGTTATTTACGCAGGATGCGC-3′, BS-II, 5′-GCAGCTTATCTTTCACTTCCTCATATATACTTTTCGCGGC-3′; BS-III, 5′-GACGTCTCCCCGGCGCATTCCTGACATTCCGGGTGATAGTTG-3′; BS-IV, 5′-CAATATAGCTCCCCCAATTCAACGGTCCTATTTCAGGCGTCAGC-3′. Truncated derivatives of the *Nkx2-5-luc* construct were obtained by digesting plasmid DNA with KpnI and BstEII (2.9-kb) or NruI (2.1-kb) restriction endonucleases, filled in by Klenow fragment and re-ligated using *T4* ligase. The resulting constructs were verified by sequence analysis.

Cell cultures and reporter expression assays

Rat cardiomyocytes were isolated from neonatal Sprague–Dawley rat hearts using a cardiomyocyte isolation kit (Worthington Biochemical, NJ) following the manufacturer's instructions. H9c2 embryonic rat-heart derived cell line was obtained from American Tissue Culture Collection (ATCC — Manassas, VA). Cell cultures were maintained in DMEM supplemented with 10%FBS, with or without antibiotics, at 37 °C and 5% CO_2. H9c2 cell cultures were not allowed to reach confluency to avoid differentiation and myoblast formation. Transfections were performed in triplicate in each experiment using Fugene6 (Roche) or Lipofectamine (Invitrogen) reagents following the manufacturer's instructions. Cell cultures were lysated 48 h post-transfection with Luciferase Reporter lysis buffer (Promega). *CMV-LacZ* reporter vector was used as an internal control and LacZ activity was used for normalizing transfection results. Experiments were repeated twice.

Results

Shox2 expression in the developing heart

Shox2 expression has been reported to be restricted to the SAN, sinus valves and branches of the central conduction system in mouse and embryonic heart and (Blaschke et al., 1998, 2007; Semina et al., 1998; Clement-Jones et al., 2000). We analyzed a detailed *Shox2* expression pattern in the early developing mouse heart by *in situ* hybridization. *Shox2* expression was not detectable in the heart at E8.0 (data not shown), but was found in a restricted pattern at the junction formed by the common cardinal vein and the common atrial chamber, the sinus venosus, at E8.5 (Fig. 1A). Subsequently, cardiac heart looping and remodeling brings the sinus venosus to the right side of the heart and *Shox2* expression extends to the sinus valves originating from the sinus node at E10.5 (Fig. 1B). At E11.5 the sinus valves develop to a definite structure, *Shox2* expression becomes specifically restricted to the SAN and to the venous layers of the sinus valves (Fig. 1C). At E12.5 and later in development the expression pattern remains consistently in the SAN region and the sinus valves (Figs. 1D, E). Nucleotide sequence analysis suggests that the *Shox2* gene generates two isoforms by alternative splicing,

Shox2a and a shorter version *Shox2b* (Rovescalli et al., 1996; Blaschke et al., 1998). Both isoforms contain the sequence coding for the homeodomain, an SH3 binding domain, and P-loop binding domain. However, *Shox2b* lacks 363 nucleotides which correspond to the N-terminus unique to *Shox2a*. By using this sequence as a *Shox2a* specific probe, we were able to detect *Shox2a* expression in the sinus venosus region (Fig. 1F). It is noteworthy to mention that our analysis showed a very restricted *Shox2* expression pattern, but could not confirm the presence of *Shox2* in the primitive bundle branches of the cardiac conduction system, as reported by Blaschke et al. (2007).

Shox2-deficient mice die at mid-gestation and exhibit morphological abnormalities

We have previously reported that on C57BL/6 background, 63% of *Shox2* homozygous embryos died between E11.5 and E12.5, while few embryos survive up to E17.5 (Yu et al., 2005). Interestingly, we have found that on CD1 background, about 78% of the embryos die between E11.5 and E12.5 and no surviving embryos were found beyond E13.5. Embryonic mortality is attributable to cardiovascular defects evidenced by cardiac and vascular edema (Figs. 2A, B arrows). Histological analyses demonstrate that at E10.5 the *Shox2*$^{-/-}$ hearts from both genetic backgrounds show no obvious structural differences when compared to wild type embryos (WT). However, the mutant forming sinus valves show a slight difference (data not shown). At E11.5, the *Shox2*$^{-/-}$ hearts from both genetic backgrounds exhibit severe hypoplasia of the sinus valves, thinner atrial walls and enlargement of the right atrium in some specimens (Figs. 2C, D; and data not shown). In the most severe cases, the sinus valves were almost absent (Fig. 2D). In addition, the SAN region is also hypoplastic, as evidenced by its markedly reduced size (Figs. 2C, D, arrows). The cardiac defect observed in *Shox2*$^{-/-}$ mice on C57BL/6 background is relatively milder in comparison to the *Shox2*$^{-/-}$ hearts on CD-1 background (data not shown), which possibly accounts for the survival of some mutant embryos up to late gestation stages. For those *Shox2*$^{-/-}$ embryos on C57BL/6 background that survived to late developmental stages, severe cardiac abnormalities were observed, including defective myocardial wall and significantly enlarged right atrium (data not shown). For all subsequent experiments we chose to use *Shox2*$^{-/-}$ embryos on CD-1 background which exhibit more severe cardiac abnormalities.

Shox2$^{-/-}$ mice show reduced growth in the SAN region

Shox2 expression in the developing heart is restricted to the sinus venosus, SAN and sinus valves. Histological analyses demonstrate severe abnormalities in these structures of *Shox2* mutants, consistent with *Shox2* expression pattern. To identify the cellular defects that contribute to the formation of the hypoplastic SAN and sinus valves, we examined the levels of both cell proliferation and apoptosis in the heart of wild type and *Shox2*$^{-/-}$ embryos at E11.5 by BrdU labeling and TUNEL assays. As a control region, BrdU-positive cells were counted in the interventricular septum myocardium, which does not express *Shox2* and does not present obvious structural differences when the control and *Shox2*$^{-/-}$ mutant hearts were compared. Although the level of apoptosis was not altered (data not shown), a dramatically reduced level of cell proliferation was observed in the mutant SAN and sinus valves, as compared to their wild type controls (Figs. 2E, F, arrows). Cell counting for BrdU-positive cells and total number of cells in the mentioned regions show that the percentage of proliferating cells is significantly different in the mutant hearts (9.9%±3.6%) when compared to the wild type controls (18.9% ±1.0%). Similar analysis show no significant difference in the percentage of proliferating cells in the interventricular septum myocardium (wt=31.9%±5.5%; *Shox2*$^{-/-}$ =3.4%) (Fig. 2G).

Shox2$^{-/-}$ hearts present a slower heart beat rate

Morphological defects observed in the SAN region of the *Shox2*$^{-/-}$ hearts strongly suggest that the cardiac pacemaker is dysfunctional. We examined the heart beat rate, an index of the

pacemaker function (Stieber et al., 2003) in isolated hearts individually cultured *in vitro* for 24 h. The heart beat rate of each sample was counted before the genotype was determined. Analysis on embryonic hearts of E9.5 embryos yielded no significant difference in heart beat rates observed in *Shox2* mutants as compared to those of wild type and *Shox2* heterozygotes (data not shown). However, analysis on embryonic hearts of E10.5 embryos revealed this defect in pacemaker function in the mutant. Although the cultured hearts contracted regularly, *Shox2*$^{-/-}$ hearts (n=8) were contracting slower than wild type (n=10) and heterozygote hearts (n=36) (Fig. 2H). The heart beat rate in the mutant (69.75±12.40 beats per minute—bpm) was less than 50% of that in the wild type (165±31.65 bpm) and heterozygote hearts (154±35.61 bpm), respectively. This cardiac abnormality observed in *Shox2*$^{-/-}$ hearts exhibit similar characteristics to human bradycardia cases (Watanabe et al., 2002; Milanesi et al., 2006) and to those found in the mouse *Hcn4*$^{-/-}$ model (Stieber et al., 2003).

Shox2 is a key regulator in pacemaker differentiation

Genetically the SAN and pacemaker region in the developing embryo have been generally determined by a combination of the expression or lack thereof of several molecules to define its boundaries. Thus, to investigate the role of *Shox2* in the development of the SAN, we examined the expression of several genes that have been used to identify the SAN or known to play a role in its formation and function (Soufan et al., 2004). These include *Hcn4*, a specific molecular marker for the pacemaker; *Tbx3*, a molecular marker for the cardiac conduction system including the SAN; the early cardiac differentiation marker *Nkx2-5*, expressed in the myocardium, but not in the SAN (Kasahara et al., 1998), *Nppa* (also known as atrial natriuretic factor—*ANF*), a chamber differentiation marker that is absent in the pacemaker; and *Cx40* whose high expression represents areas of conductivity outside the SAN region. Our results reveal that expression of *Hcn4* in the SAN, while remaining unchanged at E10.5 (Figs. 3A, B), was severely reduced at E11.5 in *Shox2*$^{-/-}$ hearts (Figs. 3C, D). *Tbx3* was specifically down-regulated in the SAN region and the sinus valves in the *Shox2*$^{-/-}$ hearts (Figs. 3F, H), as compared to the wild type counterparts at both stages (Figs. 3E, G). *Nkx2-5* was not expressed in the SAN of wild type embryos (Figs. 3I, K), but was ectopically expressed in the *Shox2*$^{-/-}$ SAN at E10.5 and E11.5 (Figs. 3J, L), in concordance to results obtained by Blaschke et al. (2007). *Nppa* and *Cx40*, the direct *Tbx3* transcription targets, and whose expression patterns are mutually exclusive to that of *Tbx3* (Rovescalli et al., 1996; van Kempen et al., 1996), were ectopically activated in the mutant SAN and sinus valves at low levels at E10.5 (Figs. 3N, R) and markedly expressed at E11.5 (Figs. 3P, T), as compared to the wild type controls (Figs. 3M, Q, O, S). In contrast, expression of the pan-marker for differentiated cardiomyocytes cardiac Troponin T (*cTnt*) (Wang et al., 1994; Jiao et al., 2003; Wang et al., 2001), was unaltered in the *Shox2*$^{-/-}$ hearts, as compared to the wild type samples (Figs. 3U–X). We also analyzed the expression of two other cardiac markers, *MLC2a*, a marker for differentiated atrial myocardium (Yutzey et al., 1994; Doevendans et al., 2000), and *MLC2v*, a marker for differentiated ventricular myocardium (O'Brien et al., 1993; Kubalak et al., 1994). These two genes showed no changes in expression in the mutants (data not shown). These observations demonstrate that cardiomyocyte differentiation is not affected in the absence of *Shox2*. The reduced expression of *Hcn4* at E10.5 and the absence of *Hcn4* expression at E11.5 in *Shox2* mutants, along with the fact that *Hcn4* is required for the regulation of a normal heart beat (Stieber et al., 2003) appear to account for the slow firing of the SAN which leads to an aberrant heart function, as evidenced by the bradycardia phenotype.

Histological, cellular and molecular results indicate failure in the differentiation process of pacemaker cells and lack of a defined SAN. Moreover, the ectopic *Nkx2-5* expression and the down-regulated *Tbx3* expression, along with the ectopic expression of *Nppa* and *Cx40*, suggest that the SAN in the mutant adopts an atrial working myocardial fate.

Shox2 acts upstream of Nkx2-5 to regulate SAN differentiation

The early cardiac transcription factor *Nkx2-5* is vital for the differentiation of primary heart field (PHF) cardiomyocytes (Komuro and Izumo, 1993; Tanaka et al., 1999; Moses et al., 2001) and the cardiac conduction system (Schott et al., 1998; Benson et al., 1999; Watanabe et al., 2002). Recent studies suggest that the absence of *Nkx2-5* expression is necessary for the SAN formation (Mommersteeg et al., 2007; Blaschke et al., 2007). The fact that the lack of *Shox2* causes an ectopic expression of *Nkx2-5* in the SAN (Blaschke et al. 2007; and this study) prompted us to test if *Shox2* functions to repress *Nkx2-5*. We took a gain-of-function approach by overexpressing *Shox2* driven by the rat *cTnT* promoter in developing mouse embryos. This rat *cTnT* promoter has been shown to drive gene expression specifically in the developing heart as early as E7.5 (Wang et al., 1994, 2001; Jiao et al., 2003). Unexpectedly, of 65 total potential transient transgenic embryos that were collected at E8.5 and E9.5, only 6 were positive for the transgene but none of them exhibited expression of the transgene in the heart (data not shown), suggesting that the earlier transgenic expression of *Shox2* might be lethal to the embryos. To overcome this obstacle, we carried out the over-expression experiment in *Xenopus* embryos using an inducible system. Frog embryos were collected and injected with mRNA synthesized from the GR-*Shox2* construct. Nuclear translocation of the fusion protein was induced by addition of Dexamethasone (10 μM) into the embryo culture buffer. Induction was initiated at stage 14, and embryos were harvested at stage 27 (early cardiac formation) or stage 41 (late cardiac formation). At stage 27 injected but un-induced embryos resembled control ones, while injected induced embryos show morphological abnormalities including a reduced size of the head (data not shown). Whole mount *in situ* hybridization for *XNkx2-5* in control embryos showed the laterally extended expression resembling wings connected at the ventral midline by two small masses which will form the heart tube (Fig. 4A). In contrast, *XNkx2-5* expression in the injected induced embryos maintained the lateral extension; however, the expression at the midline was absent (Fig. 4B). Expression of cardiac troponin I (*cTnI*) in control embryos shows an overlapping expression pattern with that of *XNkx2-5* at the midline indicating cardiac formation in this region (Fig. 4E). In the injected induced embryos, expression of *cTnI* at the midline was absent, but nevertheless it persisted in regions adjacent to the midline (Fig. 4F). Histological sections of *Xenopus* embryos clearly revealed that the loss of *XNkx2-5* in the ventral midline section correlates to the loss of a population of cells which corresponds to mesodermal cardiac progenitors (Figs. 4A′–D′). This observation is consistent with previous results obtained with over-expression of *XNkx2-5* dominant negative repressor derivatives in *Xenopus* embryos (Fu et al., 1998) and morpholino knockdown against *nkx2.5* in zebrafish (Targoff et al., 2008). A summary of the number of normal and abnormal embryos collected at stage 26 after injection and *in situ* hybridization is shown in Table 1. Later in development, at stage 41, injected induced embryos exhibited a smaller tubular or slightly chambered heart as compared to control samples and very slow and irregular heart beat was observed at the moment of collection. *XNkx2-5* expression was absent in these embryonic hearts, while *cTnI* expression was unaltered (Figs. 4C, D, G, H). These results resemble and are consistent with the phenotype of *Nkx2-5* null mice that also present cardiac arrest after cardiac looping and poor cardiac development (Tanaka et al., 1999).

Shox2 acts as a transcriptional repressor on the Nkx2-5 promoter

To determine if *Shox2* has a direct or indirect effect on the expression of *Nkx2-5*, Luciferase reporter gene expression assays were carried out. A reporter construct harboring a 3.3-kb mouse *Nkx2-5* upstream fragment linked to the luciferase reporter gene (*Nkx2-5-Luc*) was co-transfected with different concentrations of myc-*Shox2* into neonatal rat cardiomyocytes or into H9c2 rat cardiogenic cells. H9c2 cells were induced to differentiate 24 h after transfection by exposure to DMEM culture medium supplemented with 1% horse serum. The induction of differentiation is necessary to induce the expression of the *Nkx2-5* promoter (Lim et al., 2008). The reporter gene expression assays clearly demonstrated a concentration-dependent

repression of the *Nkx2-5* promoter activity by *Shox2* in both types of cells (Fig. 5A). While the expression of reporter gene in H9c2 cells was markedly lower than that in rat cardiomyocytes, the results are consistent in both cell lines. Base on the criteria for homeobox genes binding sites, *Shox2* might bind to a consensus "P" site, defined as a palindromic "ATTA$(N)_n$TAAT" sequence in which "N" is any nucleotide and "n" is the number of nucleotides which separate the ATTA palindromic sequence (Rao et al., 2001), or to a consensus "TAATTA" site (Berger et al., 2008). Four putative *Shox2* binding sites within the *Nkx2-5* promoter were identified and labeled BS-I–IV. To determine if any of these sites mediates the repressive effect of *Shox2* on the *Nkx2-5* promoter, we eliminated these potential binding sites by site-directed mutagenesis, and by promoter deletion to engineer two shorter promoters, a 2.9 kb promoter which does not contain putative binding sites I–III and a 2.1 kb promoter which does not contain any putative binding site. Co-transfection of mutated or truncated promoters with different concentrations of *Shox2* demonstrate that either mutant or truncated promoters responded in the same fashion as the 3.3-kb original promoter (Fig. 5B and data not shown). Promoter activities were down-regulated in a *Shox2* concentration-dependent manner. Taken together, our results suggest that *Shox2* indeed represses the *Nkx2-5* promoter activity. However, *Shox2* might not directly interact with the *Nkx2-5* promoter.

Discussion

Histological analyses of the *Shox2*$^{-/-}$ heart revealed early cardiac structural malformation including hypoplastic SAN and sinus valves, defective atrial myocardial wall and at later developmental stages a significantly enlarged right atrium. These results are in concordance with results obtained by Blaschke et al. (2007). Since *Shox2* expression in the heart is restricted to the sinus venosus, the SAN and the sinus valves, it indicates that the structural defects observed in other regions are most likely a secondary effect caused by the absence of a functional pacemaker and hypoplastic sinus valves. Malformation of the SAN and sinus valves is attributed to a significantly decreased level of cell proliferation, consistent with a role previously reported for *Shox2* in the developing palate, limbs and the TMJ (Yu et al., 2005, 2007; Gu et al., 2008). The sinus valves are composed of two myocardial layers that have different physical characteristics (Gallego et al., 1997) with one being continuous with the atrial working myocardium and the other continuous with the venous dorsal wall of the atrium containing the pacemaker area. Reduced growth observed in both of the sinus valves layers and the SAN region of the *Shox2*$^{-/-}$ heart suggests that growth of the myocardial layer uses the venous layer as a template to elongate and "mature" before the recession and integration of the entire valve in the dorsal wall of the right atrium.

Reduction and possibly absence of a functional SAN leads to a reduced heart beat rate and embryonic lethality in the *Shox2* mutant embryo. This phenotype resembles symptomatic sinus bradycardia related to sick sinus syndrome in humans which affects the capability of the heart to contract at a normal rate or below 55 beats per minute (Savalieva and Camm, 2008). *Hcn1-4* molecules are responsible for the generation of the "funny" current which is characteristic of the pacemaker activity (Accili et al., 2002). *Hcn4* expression in the SAN is the strongest and has been demonstrated to simulate pacemaker characteristics when overexpressed in cell cultures (Santoro and Tibbs, 1999; Santoro et al., 2000; Moosmang et al., 2001; Stieber et al., 2003; Liu et al., 2007). A mutation in the cAMP binding site of the human *HCN4* gene was identified as the cause of sinus bradycardia and sick sinus syndrome in a familial case (Milanesi et al., 2006). Moreover, mice deficient in *Hcn4* die between E9.5 and E11.5 presenting a similar electrophysiological phenotype with lower heart beat rate and slower pacemaker kinetics as compared to the wild type controls (Stieber et al., 2003). In this study we demonstrate that the expression of *Hcn4* is down-regulated in the *Shox2*$^{-/-}$ heart beginning at E10.5. Taking this into consideration along with the fact that *Hcn4* is required for the regulation of the normal intrinsic electric firing of the SAN, we conclude that the loss of

Hcn4 is the cause for the slow firing of the SAN cells and the consequent slowing of the heart beat rate in *Shox2*$^{-/-}$. This phenomenon is accompanied by the altered expression of several genes that have been used to demarcate the location of the SAN and pacemaker. *Tbx3* is expressed in the cardiac conduction system including the SAN (Hoogaars et al., 2004). In contrast, the expression of *Nkx2-5*, *Nppa* and *Cx40* is widely seen in the myocardium but not in the SAN region (Soufan et al., 2004; Liu et al., 2007). The absence of *Hcn4* and *Tbx3* expression and the ectopic expression of *Nkx2-5*, *Nppa* and *Cx40* in the sinus node of the *Shox2*$^{-/-}$ heart indicate the lack of a definable SAN region. It has been reported that *Tbx3* imposes a sinoatrial fate to myocardial cells (Hoogaars et al. 2007), however it was concluded that its function is to shield the SAN from becoming atrial working myocardium and preserve an embryonic phenotype. Additionally in *Tbx3*$^{-/-}$ hearts the formation of a primordial SAN is observed and expression of *Hcn4* is maintained beyond the embryonic stage in which *Shox2* mutation becomes lethal. These observations suggest that *Shox2* might act earlier than *Tbx3* in the formation of the SAN and the pacemaker. Alterations in gene expression pattern observed in the *Shox2*$^{-/-}$ heart are not likely attributed to a specific loss of potential SAN progenitor cells, since the mutant heart does not exhibit an increased level of apoptosis. However, it could be argued that a low level of cell proliferation might account for the lack of a definable SAN and the lack of expression of certain genes. Nevertheless, positive expression of *Hcn4* in the *Shox2*$^{-/-}$ heart at E10.5 reveal that progenitor cells are indeed present and the genetic pattern of other genes is similar at E10.5 and at E11.5 when *Hcn4* is absent. Our histological, functional and molecular results combined with observations detailed above indicate that cells within the SAN region of the *Shox2*$^{-/-}$ heart fail to differentiate into specialized pacemaker cardiomyocytes but rather adopt the fate of the atrial working myocardium which places *Shox2* as a key and upstream intrinsic factor in a very specific and important niche in the molecular network that regulates the differentiation of SAN cells. It was proposed that *Pitx2*, which is expressed in the left side of the heart (Ryan et al., 1998; Yu et al., 2001), acts to determine the asymmetrical positioning of the sinus node to the right side, thereby preventing the left atrium to assume right side characteristics (Mommersteeg et al., 2007). Thus, expression of *Pitx2* in the left side of the heart restricts *Shox2* expression to the sinus node in the right atrium. This conclusion is supported by the observation that in *Pitx2* mutants, an ectopic *Shox2* expression domain is seen in the left atrium, mirror-imaging the original one in the sinus node in the right atrium and that *Pitx2* expression in the *Shox2*$^{-/-}$ heart is unaltered (Espinoza-Lewis and Chen, unpublished observations). Together with previous studies that place *Tbx3* and *Hcn4* downstream of *Nkx2-5* (Mommersteeg et al., 2007), we present a model integrating *Pitx2*, *Shox2*, *Nkx2-5*, *Tbx3*, and *Hcn4* that operates to regulate the pacemaker development (Fig. 6). In this model, the expression of *Pitx2* leads to the inhibition of the right side program in the left side, thus the absence of *Pitx2* in the right side leads to the activation of *Shox2* expression, which in turn acts to repress *Nkx2-5*. The repression of *Nkx2-5* further allows for the activation of the differentiation program in the sinus node where *Tbx3* further inhibits the expression of *Nppa* and *Cx40*.

Early histological studies (van Mierop and Gessner, 1970; Challice and Virágh, 1973) and recent molecular analyses indicate that SAN cells are recruited into the venous pole of the heart, and that this population is *Nkx2-5* negative and *Tbx18* positive (Christoffels et al., 2006). Thus, the question pertaining to how these cells become *Nkx2-5* negative arises. Here, gain-of-function studies in *Xenopus* embryos as well as in cell cultures reveal that *Shox2* is able to repress the expression of *Nkx2-5*. In *Xenopus* embryos overexpression of *Shox2* causes the down-regulation of *XNkx2-5* in the ventral middle section, however the lateral expression remains. Later in development the resulting heart is small and tubular. These observations are consistent with several previous studies in which, 1) overexpression of dominant negative derivatives of *XNkx2-5* results in the loss of mesodermal cardiac progenitor cells; 2) anti-*nkx2.5* morpholino injection in zebrafish results in elongation abnormalities of the tubular heart, disorganized atrial cells and an enlarged ventricle; and 3) *Nkx2-5* null mutation in mice

leads to cardiac arrest after cardiac looping and the formation of an abnormal, small and tubular heart. Additionally, Luciferase reporter assays show that indeed *Shox2* represses the activity of the *Nkx2-5* promoter. Mutations at the putative *Shox2* binding sites and truncations of the promoter to eliminate these binding sites have no effect in the repressive activity of *Shox2* on the *Nkx2-5* promoter. It is known that homeobox genes have transcriptional activity either by binding directly to the DNA or by binding to other proteins and acting as co-factors (del Bene and Wittbrodt, 2005). The *Runx2* homeobox gene has been shown to regulate gene expression by direct binding to the *Osteocalcin* promoter (Ducy et al., 1997) or by acting as a co-factor to SRF (Serum Response Factor, a MADS box transcription factor) to prevent the *Myocardin*-induced myogenic genetic program and to promote the activation of the osteogenic genetic program (Tanaka et al., 2008). It is also likely that *Shox2* interacts with other factors in order to repress the expression of *Nkx2-5*. This repressive activity of *Shox2* on *Nkx2-5* facilitates the expression of a genetic program to maintain the SAN fate and shield the SAN from becoming working atrial myocardium.

Our results demonstrate that *Shox2* is essential for the proper formation and differentiation of the SAN, in addition to regulate a genetic network through the indirect repression of *Nkx2-5* to maintain a SAN muscle fate. Additionally, this study provides one more insight for future genetic therapeutic approach against deficiencies in the SAN function in humans.

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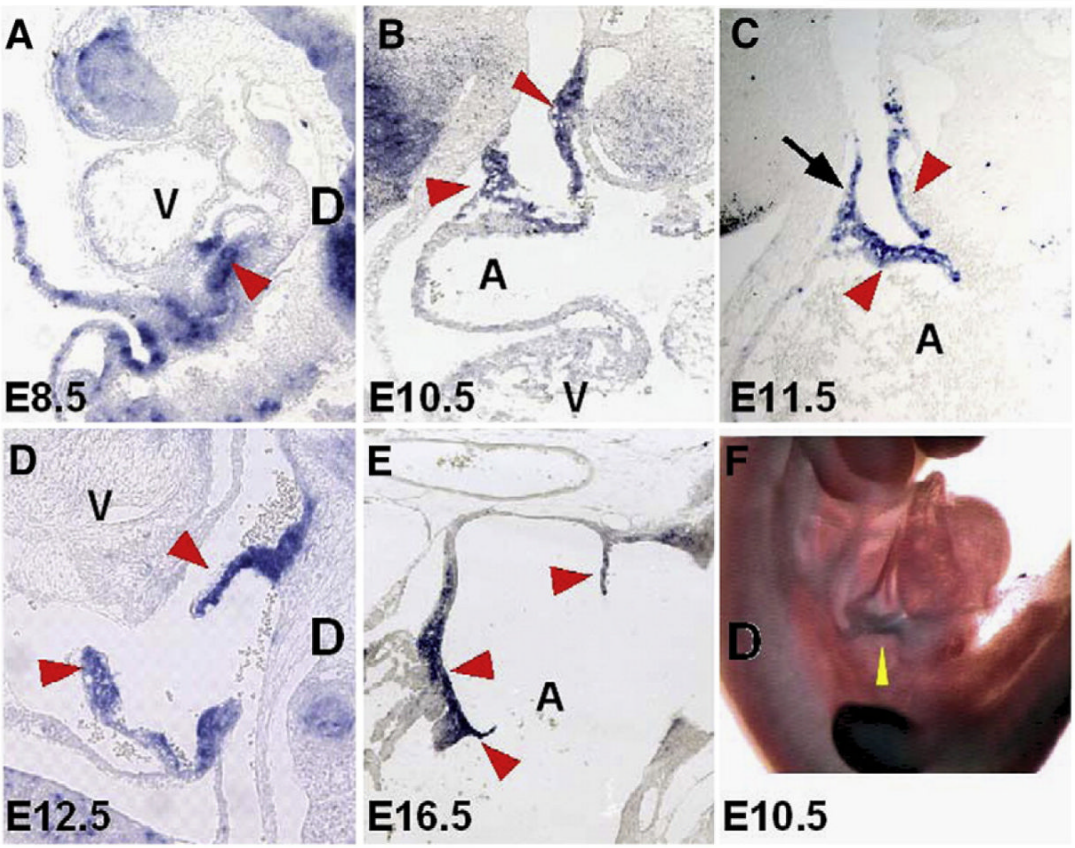

Fig. 1.

Shox2 expression in the murine developing heart. (A) *Shox2* expression is initially detected in the sinus venosus region (arrowhead) at E8.5 embryo. At E10.5 (B) and E11.5 (C), *Shox2* expression gradually becomes restricted to the right side of the heart to the dorsal wall of the right atrium, the SAN region (arrow, C) and sinus valves (arrowheads, B, C). At E12.5 (D), restricted *Shox2* expression in the SAN and sinus valves (arrowheads) is clearly observed. (E) *Shox2* expression at E16.5 follows the same pattern as previous stages. (F) Whole mount *in situ* hybridization showing the expression of *Shox2a* isoform in the sinus venosus region (yellow arrowhead). A, atrium; V, ventricle; D, dorsal side.

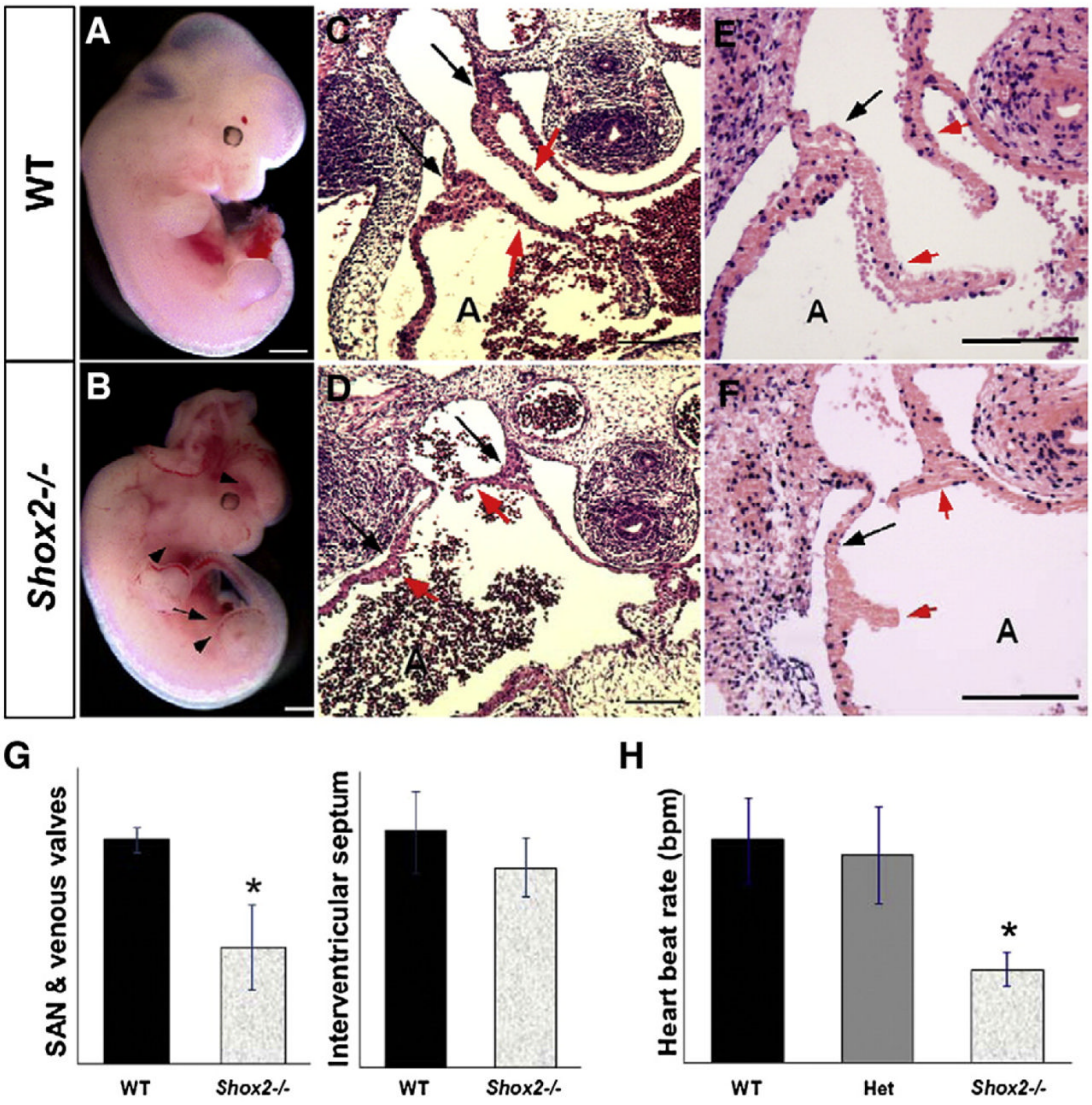

Fig. 2.

Shox2 deficiency causes embryonic lethality and cardiac morphological and functional abnormalities. (A, B) Panoramic comparison between a wild type (A) and a *Shox2*$^{-/-}$ (B) embryos at E11.5. The mutant embryo exhibits cardiac edema (arrow) and vascular defects (arrowheads). Brain defects are observed in some specimens. (C, D) Histological comparison shows hypoplastic SAN and sinus valves in the *Shox2*$^{-/-}$ mutant heart (red arrowheads in panel D), as compared to the wild type controls (C), evidenced by the length of the sinus valves (red arrows in panels C and D) and the difference in size of the SAN region (black arrows in panel D). Additionally, a thinner atrial wall and an enlarged atrial chamber were observed in the *Shox2*$^{-/-}$ heart (D). (E, F) BrdU labeling shows a significantly decreased level of cell proliferation in the *Shox2*$^{-/-}$ SAN and sinus valves (F), as compared to the wild type (E). (G) Comparison of the percentage of BrdU-positive cells present in the SAN and sinus valves demonstrate a significant difference between wild type and *Shox2*$^{-/-}$ hearts (*: $P<0.05$); the interventricular septum region was chosen as a control region shows no significant difference. (H) Measurement of the contraction rate in isolated hearts at E10.5 after 24 h of culture shows a significantly slower beating rate in the *Shox2*$^{-/-}$ samples, as compared to the wild type (wt)

and *Shox2* heterozygote (het) samples (*: $P<0.001$). A, atrium; bpm=beats per minute. Scale bars represent 1 mm (A, B) or 50 μm (C–F).

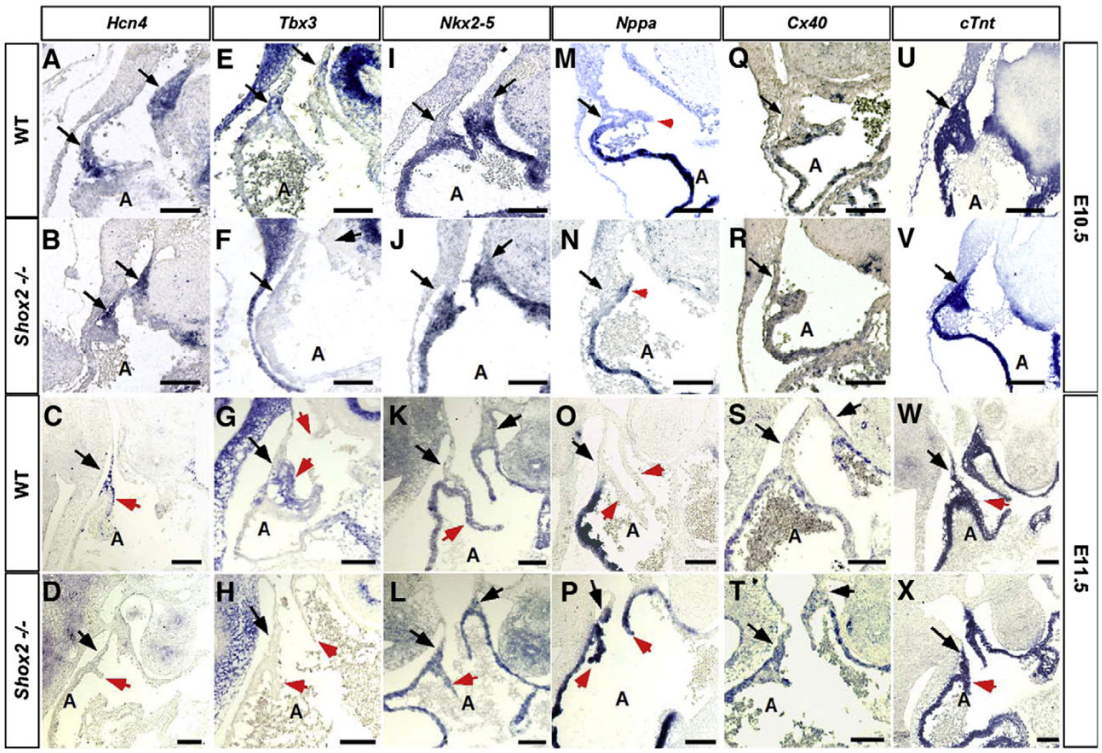

Fig. 3.

Altered gene expression in the *Shox2*$^{-/-}$ heart. Expression patterns of *Hcn4* (A–D), *Tbx3* (EH), *Nkx2-5* (I–L), *Nppa* (M–P), *Cx40* (Q–T) and *cTnt* (U–X) at E10.5 and at E11.5 in the wild type and *Shox2*$^{-/-}$ hearts are shown. *Hcn4* is expressed in the dorsal right wall of the right atrium in the wild type (A, C) and is reduced or absent in the *Shox2* mutant (B, D). *Tbx3* is expressed in the dorsal right wall of the right atrium and in the sinus valves (E, G), however is absent in the *Shox2* mutant (F, H) at both stages. *Nppa*, *Cx40*, and *Nkx2-5* are ectopically expressed in the dorsal right wall of the *Shox2*$^{-/-}$ right atrium at both stages (J, N, R, L, P, T), as compared to the wild type controls (I, M, Q, K, O, S). *cTnt* shows comparable expression level in the *Shox2*$^{-/-}$ heart (V, X) and the wild type controls (U, W). In all panels, the arrows point to the SAN region, while the arrowheads point to the sinus valves. A, atrium. Scale bars represent 50 μm.

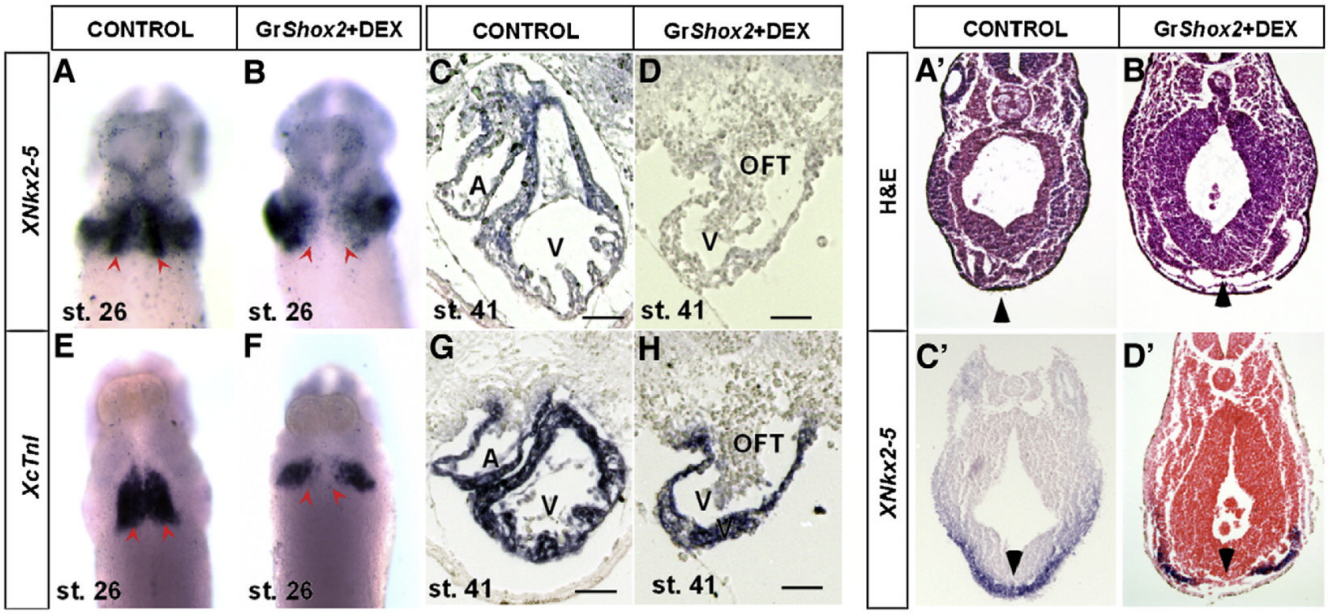

Fig. 4.

Shox2 overexpression downregulates *Nkx2-5* expression. *XNkx2-5* and *XcTnI* expression was analyzed by *in situ* hybridization in control embryos at stage 26 (A, E) and at stage 41 (C, G) and in GR-*Shox2* mRNA injected induced embryos at same stages (B, F, D, H). *XNkx2-5* expression is clearly reduced at the midline of injected induced embryos at stage 26 (B), as compared to control embryos (A). *XcTnI* expression is maintained in injected induced embryos laterally, but is absent at the midline at stage 26 (F), as compared to the controls (E). At stage 41, *XNkx2-5* expression is absent in injected induced embryos (D) compared to control embryos (C). *XcTnI* expression in injected induced embryos (D) is comparable to that of control embryos (H). Altered gene expression is accompanied by the abnormal heart morphology when comparing control and injected induced embryos (C, D, G, H). Histological transverse sections of *Xenopus* embryos showing loss of mesodermal cardiac progenitor cells in the ventral midline in injected induced embryos (B′, D′, arrowheads), as evidenced by the loss of *XNkx2-5* expression in the corresponding region when compared to control embryos (A′, C′, arrowheads). Scale bars represent 50 μm.

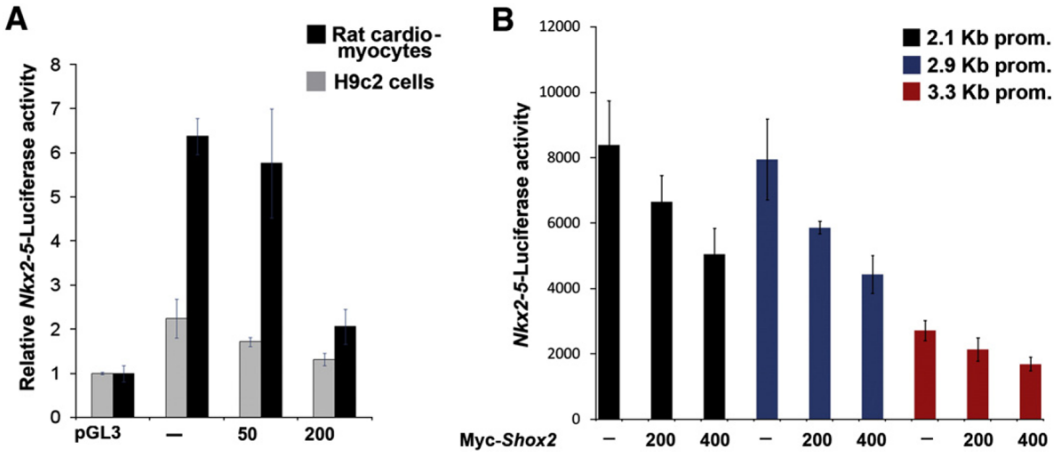

Fig. 5.

Shox2 represses the activity of the *Nkx2-5* promoter in cell cultures. (A) Neonatal rat cardiomyocytes and H9c2 cells were utilized. Co-transfection of with 100 ng of pGL3 basic vector as controls or *Nkx2-5-Luc* construct, with *Myc-Shox2* expression vector at 50 ng, and 200 ng was performed. Reporter gene expression shows a progressive significant reduction in the activity of the *Nkx2-5* promoter on both cell culture systems (* =P<0.001 at 200 ng). The Y axis represents activity of the *Nkx2-5* promoter relative to the background after normalization. B) Reporter gene expression assay shows that the activity of the truncated *Nkx2-5* promoters when co-transfected with myc-*Shox2* (0, 200 and 400 ng/μl) follows a similar trend to the 3.3-kb promoter (*, significant difference, P<0.05).

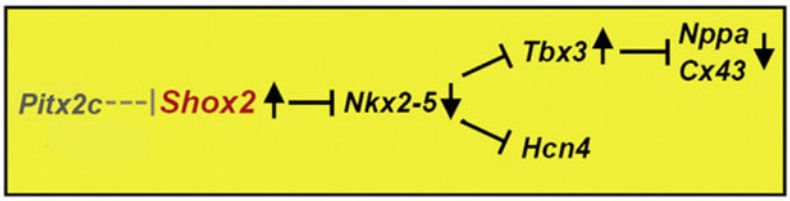

Fig. 6.

Shox2 regulates the SAN genetic program through repression of *Nkx2-5*. A genetic hierarchy model places *Shox2* downstream of *Pitx2* but upstream of *Nkx2-5* in the regulation of SAN formation. Black arrows represent the up- or downregulation of expression depending on the expression or lack thereof of the upstream gene.

Table 1

Summary of the number of normal and abnormal embryos collected at stage 26 after injection and *in situ* hybridization for *XNkx2-5* and *cTnI*

	Normal expression	Abnormal expression	Total	% Normal	% Abnormal
Control	37	0	37	100	0
Gr-*Shox2*	38	0	38	100	0
Control DEX	64	2	66	97	3
Gr-*Shox2* DEX	36	45	81	44	56