



HHS Public Access

Author manuscript

Genesis. Author manuscript; available in PMC 2015 September 04.

Published in final edited form as:

Genesis. 2013 July ; 51(7): 515–522. doi:10.1002/dvg.22397.

Generation of *Shox2-Cre* allele for tissue specific manipulation of genes in the developing heart, palate, and limb

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Abstract

Shox2 is expressed in several developing organs in a tissue specific manner in both mice and humans, including the heart, palate, limb, and nervous system. To better understand the spatial and temporal expression patterns of *Shox2* and to systematically dissect the genetic cascade regulated by *Shox2*, we created *Shox2-LacZ* and *Shox2-Cre* knock-in mouse lines. We show that the *Shox2-LacZ* allele expresses beta-galactosidase reporter gene in a fashion that recapitulates the endogenous *Shox2* expression pattern in developing organs, including the sinoatrial node (SAN), the anterior portion of the palate, and the proximal region of the limb bud. Conditional deletion of *Shox2* in mice carrying the *Shox2-Cre* allele yielded SAN phenotypes that resemble conventional *Shox2* knockout mice. Our results indicate that the *Shox2-Cre* allele offer a useful tool for tissue specific manipulation of genes in a number of developing organs, particularly in the developing SAN.

Keywords

Shox2; Cre; gene deletion; heart; palate; limb

During development, the vertebrate heart develops from a tubular structure with a single circulation to a four chambered structure with a dual circulation. This correlates with change of the heart beating pattern from a peristaltic motion to a sophisticated synchronous contraction due to the maturation of the cardiac conduction system (CCS). The CCS initiates and conducts electric signals to stimulate the contraction of atrial and ventricular working myocardium in a coordinated manner. This conduction system is comprised of the following components: the sino-atrial node (SAN) the atrio-ventricular node (AVN), the Bachmann's bundle (in the atrium), and ventricular conduction sites including the His bundle, right bundle branch, left bundle branch and Purkinje fibers.

The SAN is the first site of CCS components to become functional and the origin of myocardial stimulating electrical current. The SAN is located at the junction between the vena cava and the right atrium. At E10.5, the SAN is fully functional and can be identified histologically (van Mierop and Gessner, 1970, Christoffels et al., 2010). During embryogenesis from E11.5 onward, the SAN appears to be a bulging structure at the dorsal

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side of the right atrium wall. Previous studies revealed several SAN specific molecular markers including *Tbx3* and the *hyperpolarization-activated cyclic nucleotide-gated channel 4 (Hcn4)* (Ludwig et al., 1999; Hoogaars et al., 2004). These markers are expressed distinctively in CCS and are important to maintain SAN identity and functions during cardiogenesis.

Heart disease is the leading cause of the deaths in the developed countries. (Xu et al., 2010; Kochanek et al., 2011). Various types of heart diseases from the most common atrial fibrillation to the lethal sudden cardiac death have been associated with pacemaker defects (Tsai et al., 2000; Marsman et al., 2011). Recent studies have established a casual relationship between certain genetic defects and SAN abnormalities, though the genetic regulatory network is still not well understood (Puskaric et al., 2010; Aanhaanen et al., 2011). Understanding such genetic regulatory mechanism may shed light on therapeutic approaches for both congenital and acquired CCS defects.

The *Short stature homeobox 2 (Shox2)* gene has been shown to be expressed in a number of developing organs in a tissue specific manner (Yu et al., 2005; Blaschke et al., 2007). In the developing palate, *Shox2* expression is restricted in the anterior portion of the mesenchyme; and in the developing limb, its expression is only found in the proximal region of the limb (Yu et al., 2005; 2007; Cobb et al., 2006). In the developing heart, *Shox2* is expressed specifically in the inflow tract region where the SAN derives and later in the developing SAN (Blaschke et al., 2007; Espinoza-Lewis et al., 2009). Targeted inactivation of *Shox2* leads to severe defects in multiple organs including anterior clefting of the palate, elimination of the stylopod, and defective differentiation of the SAN (Yu et al., 2007; 2007; Cobb et al., 2006; Blaschke et al., 2007; Gu et al., 2008a; Espinoza-Lewis et al., 2009). In order to reveal a fine and real time expression pattern of *Shox2* in developing mouse embryos, we generated a *Shox2-LacZ* knock-in allele by gene targeting in ES cells. In this case, the beta-galactosidase protein coding sequence and a *FRT* flanked *PGK-neo* cassette were introduced to replace *Shox2*'s exon1, intron 1 and a small proportion of exon 2 (Fig. 1A and B). Southern blotting and a PCR screen confirmed the successful targeting of the knock-in alleles in ES cells (Fig. 1C and D). The PCR screening strategy and probe used for Southern blotting are indicated in Fig. 1A. The numbers of positive ES cell clones were 9 out of 180 for *Shox2-LacZ* allele. Germline transmitted heterozygous mice were genotyped using a PCR based method as described in Materials and Methods. 10 agouti (indicates SV129 CJ-7 cell lineage) pups out of 13 (76.9%) F1 mice were observed in two litters from *Shox2-LacZ* chimeras.

After removal of the *PGK-Neo* cassette by crossing F1 heterozygous mice to FLP deleter mice, *Shox2^{LacZ}* mice were used to examine *Shox2* expression patterns by X-gal staining in developing embryos at selected stages. As shown in Fig. 2, X-gal staining indeed revealed *Shox2* expression patterns identical to that was reported previously by in situ hybridization in developing embryos at E9.0, E10.5, and E11.5 (Yu et al., 2005; 2007; Blaschke et al., 2007; Espinoza-Lewis et al., 2009). At E9.0, beta-galactosidase activity was detected in the sinus venosus of the developing heart (Fig. 2A). At 10.5 and E11.5, Robust expression of beta-galactosidase activity persisted in the SAN and surrounding superior vena cava tissue (Fig. 2B–D). We observed beta-galactosidase activity in the SAN region and tissue on top of

the ventricular septum in the atrioventricular junction at E18.5 (Fig. 2E) as we predicted from previous report (Blaschke et al., 2007; Hahurij, 2011). We identified that this group of cells contribute to the developing atrioventricular conduction system (Data not shown). *Shox2* expression was restricted in the atrium and was not found in the ventricular proportion of the heart. In addition, beta-galactosidase activity was observed in the proximal region of the limb, the anterior portion of the palatal shelves, and the temporomandibular junction in E10.5 and E11.5 embryos (Fig. 2B, C, F and G), similar to the *Shox2* mRNA expression patterns reported previously (Yu et al., 2005, 2007; Gu et al., 2008b). We also observed beta-galactosidase activity in the dorsal root ganglia and brain region (Fig. 2B and C), consistent with previous reports (Scott et al., 2011). These observations indicate that the knock-in strategy we used does not affect the regulatory elements required for the tissue specific expression of *Shox2*, allowing recapitulation of endogenous *Shox2* expression.

The unique expression pattern of *Shox2* in the developing SAN, palatal shelves, and the limb in *Shox2-LacZ* knock-in mice prompted us to generate *Shox2-Cre* knock-in mice, which would provide a valuable research tool for studying gene function in the developing SAN, the palate and limb. Using the same targeting strategy for the creation of *Shox2-LacZ* allele (Fig. 1B), we generated the *Shox2-Cre* knock-in allele in ES cells and *Shox2-Cre* mice, as confirmed by PCR genotyping (Fig. 1F–G). The *Shox2-Cre* construct was targeted into G4 ES cell line (George et al. 2007). The numbers of positive ES cell clones were 7 out of 144 for *Shox2-Cre* allele. 8 *Cre* positive F1 mice out of 14 were identified in two litters from chimeras.

To evaluate the Cre activity in *Shox2-Cre* knock-in mice, *R26R* reporter mice were crossed to *Shox2-Cre* knock-in mice to generate *Shox2^{Cre/+};R26R* double heterozygous embryos for X-gal staining (Fig. 1I). At E9.5, beta-galactosidase activity was first detected in the developing sinus venosus (Fig. 3A). Compared to the earlier expression of beta-galactosidase in *Shox2-LacZ* embryos, such delay of about a half day in *Shox2^{Cre/+};R26R* double heterozygous embryos is likely attributed by the time needed for accumulation of Cre recombinase protein and transcription/translation of the activated *R26R* allele. At E10.5 and E11.5, in whole mount stained embryos, beta-galactosidase was robustly expressed in all early *Shox2*-expressing tissues including the SAN, the proximal domain of the limb, the anterior region of the palatal shelves, the temporomandibular junction, as well as the dorsal root ganglia and brain (Fig. 3B–F). These expression domains correlate with that was observed in *Shox2-LacZ* mice. To determine the efficiency of Cre recombinase in inactivating floxed gene, we crossed *Shox2-Cre* mice to *Shox2^{fllox}* mice (Cobb et al., 2006) to generate *Shox2^{Cre/F}* embryos. In *Shox2^{Cre/F}* embryos, we observed hypoplastic SAN phenotype similar to that found in *Shox2^{-/-}* embryos at E11.5 (Fig. 4A–C). At E12.5, We also found that both *Shox2^{Cre/LacZ}* and *Shox2^{Cre/F}* exhibited similar phenotypes in the SAN as seen in *Shox2*-null mice (Fig. 4D–F). Similar to that was found in *Shox2*-null mutants (Yu et al., 2005; Blaschke et al., 2007), *Shox2^{LacZ/LacZ}*, *Shox2^{Cre/LacZ}* and *Shox2^{Cre/F}* embryos began to exhibit embryonic lethality at E11.5 (Supplementary data). To confirm that the *Shox2-Cre* line is a null allele, reverse transcription PCR was performed using methods described previously (Yu et al., 2005), *Shox2* mRNA was undetectable in *Shox2^{Cre/Cre}* homozygous mice (Fig. 4G). This proved that *Shox2* transcription was

successfully inactivated in *Shox2-Cre* knock-in alleles demonstrating that the *Shox2-Cre* allele can be used as an efficient tool for tissue specific inactivation of targeted genes.

While several *Cre* lines have been created previously for manipulating gene expression in the CCS including the SAN (Hoesl et al., 2011; Arnolds et al., 2011), our *Shox2-Cre* allele provide another unique *Cre* line to manipulate gene function in SAN development. It has been demonstrated that heterogeneity, at both the cellular and molecular levels, exists in the developing palatal shelves along the anteroposterior (A-P) axis (Hilliard et al., 2005; Okano et al., 2006; Gritli-Linde, 2007). A number of genes including *Shox2* exhibit differential expression and play different role in regulating development of different portion of the palate along the A-P axis (Zhang et al., 2002; Yu et al., 2005; He et al., 2008; Liu et al., 2008; Xiong et al., 2009; Li et al., 2011). The vertebrate limb grows and lengthens along the proximodistal axis, and is patterned into the stylopod, zeugopod, and autopod by differential expression of genes such as *Hox* genes (Davis et al., 1995; Wellik and Capecchi, 2003; Boulet and Capecchi, 2004). The unique *Cre* expression in the anterior portion of the developing palatal shelves and the proximal domain of the limb bud in the *Shox2-Cre* mice suggest that *Shox2-Cre* mice could be a valuable tool for gene manipulation in the specific domain of the developing palate and limb to study gene function in these two organs.

MATERIALS AND METHODS

Mouse Strains

The FLP deleter and *R26R* mice (Soriano, 1999) were obtained from the Jackson laboratory (Bar Harbor, ME). The *Shox2^{lox}* mice have been described previously (Cobb et al., 2006). A set of primers (5'-AAAGTCGCTCTGAGTTGTTAT-3' and 5'-GCGAAGAGTTTGTCTCAACC-3') is used to identify the *R26R* allele.

Generation of *Shox2-lacZ* and *Shox2-Cre* knock-in mice

A 15 kb *EcoRI* genomic DNA fragment comprising all six exons of the *Shox2* gene was cloned and used in generation of *Shox2-lacZ* and *Shox2-Cre* allele, respectively. To insert the *LacZ* or *Cre* expression cassette into the *Shox2* locus, we constructed a targeting vector containing a 5.6 kb homologous arm and a 2.9 kb homologous arm flank the *lacZ/Cre-FRT-neo-PGK-FRT* cassette (Fig. 1A and B). Additionally, a diphtheria toxin expression cassette was placed in the 5' flanking region of the 5' homologous arm to rule out random insertion. The targeting vector was digested by *AscI* restriction endonucleases for linearization and subsequently electroporated into mouse ES cells. The *Shox2-LacZ* construct was targeted in CJ7 ES cells (Swiatek and Gridley, 1993), while the *Shox2-Cre* construct was targeted in G4 ES cells (George et al. 2007). To obtain ES cell clones with correct homologous recombination, Neomycin (G418) resistant colonies were selected and screened by Southern blotting assay (Fig. 1C), or by a long range PCR screening strategy (Fig. 1D). For *Shox2-LacZ* targeted allele, the 5' PCR primer located in the *neo-PGK* cassette (5'-CGAAACGATCCTCATCTGT-3') and the 3' PCR primer located in 3' flanking region of 3' homologous arm (5'-GGACCAGCCTCTGTATTGGA-3') (Fig. 1A) amplified a 3.6 kb product from the genuine *Shox2-lacZ* allele (Fig. 1D). As to the identification of 5' homologous recombination, a forward primer (5'-

AGTTTGGGAGATGAGGGAAAACCATCATCAGAATAT-ATTGTATACAG-3') located in 5' flanking region of 5' homologous arm and a reverse primer (5'-GACAGTATCGGCCTCAGGAA-3') located in the *LacZ* cassette were used and a PCR product of 7.1 kb indicated targeted *Shox2* locus (Fig. 1D). For *Shox2-Cre* allele, long range PCR on both 5' end and 3' end of the targeted *Shox2* locus was performed to identify correctly targeted allele using two pairs of primers. The first set of primers is identical to that used to identify 3' homologous recombination of *Shox2-lacZ* allele, and the second set of primers, with the same forward primer used in *Shox2-LacZ* 5' homologous recombination screening and the reverse primer (5'-GCAAACGGACAGAAGCATTT-3') located in the *Cre* cassette, identified 5' homologous recombination by amplifying a product of 6.8 kb (Fig1. F). For both lines, ES cells from two different positive clones were injected in to blastocysts with C57BL/6 background. Chimeras were crossed with C57BL/6 females (Charles River) to generate F1 mice and they were genotyped by long ranged PCR of tail DNA. Mice and embryos, maintained on C57BL/6 and CD-1 background separately, were routinely genotyped by PCR analysis of tail DNA or yolk sacs of embryos. CD-1 mice with knock-in alleles are used for subsequent analysis in this study. For *Shox2-LacZ* allele, a set of three primers (Common: 5'-GCCCCATTGATGTGTTATTA-3'; Mutant: 5'-GACAGTATCGGCCTCAGGAA-3'; WT: 5'-GATAAGGGAAGGCAGTAAGG-3') produced a product of 650bp from the targeted allele and a product of 515 bp from the wild type allele (Fig.1A and E). PCR genotyping of *Shox2-Cre* allele was conducted using a set of three primers (Common: 5'-GCCCCATTGATGTGTTATTA-3'; Mutant: 5'-GCAGTTTCCAGGTATGCCAG-3'; WT: 5'-GATAAGGGAAGGCAGTAAGG-3') produced a product of 277bp from the targeted allele and a product of 515 bp from the wild type allele (Fig. 1B and G). After crossing F1 mice with FLP deleter mice, to confirm the removal of *neo-PGK* cassette, a set of primers (5'-AGAGGCTATTCGGCTAT-GACTG-3' and 5'-ATACTTTCTCGGCAGGAGCA-3') was used to amplify a 312 bp product from *neo-PGK* fragment. F1 mice are positive for *neo-PGK* detection, in contrast, the *neo-PGK* cassette is not detectable in WT mice and offspring from F1 mice and FLP deleter mice (Fig. 1H). The *Shox2-LacZ* and *Shox2-Cre* alleles will be available upon request to the research community.

Long Range PCR

DNA sample was prepared following DNA extraction procedure for southern blot assay described previously (Alexandra, 2000). TaKaRa LA Taq enzyme was used for long range PCR. Annealing temperature was set to 58°C for 45 seconds and the time for elongation was set to 8 minutes for 5' homologous recombination screening and 5 minutes for 3' homologous recombination screening. Cycle number was set to 35.

X-Gal Staining

X-gal staining on cryosections and whole embryo was carried out following the procedures described previously (Hogan et al., 1994). After X-gal staining, sections were counterstained with nuclear fast red subsequently.

Immunohistochemistry Assay

Immunohistochemistry assay using antibody against Nkx2.5 (abcam ab35842) on paraffinsections was carried out following the procedures available online (Invitrogen). After diaminobenzidine color development, sections were counterstained with methyl green subsequently.

Reverse Transcription PCR

E11.5 mouse heart with sinus venosus were used to isolate mRNA by TRIzol reagent (Invitrogen). Subsequent first-strand cDNA synthesis was performed using a SuperScript kit (Invitrogen). A set of primers (5-ACCAGCAAGAACTCCAGCAT-3 and 5-GCCCACTCCTTTGTCCAGT-3) that cover exon 4 to exon 6 of *Shox2* amplified a 371 bp cDNA product to detect *Shox2* transcript in the *Shox2^{Cre/Cre}* mice. As the positive control, a pair of primers (5-TTCCGCAAGTTCACCTACC-3 and 5-CGGGCCGGCCATGCTTTACG-3) that amplify a 361 bp sequence of S15 RNA was included.

Acknowledgements

This work was supported by NIH grant R01 DE17792 to Y.C.

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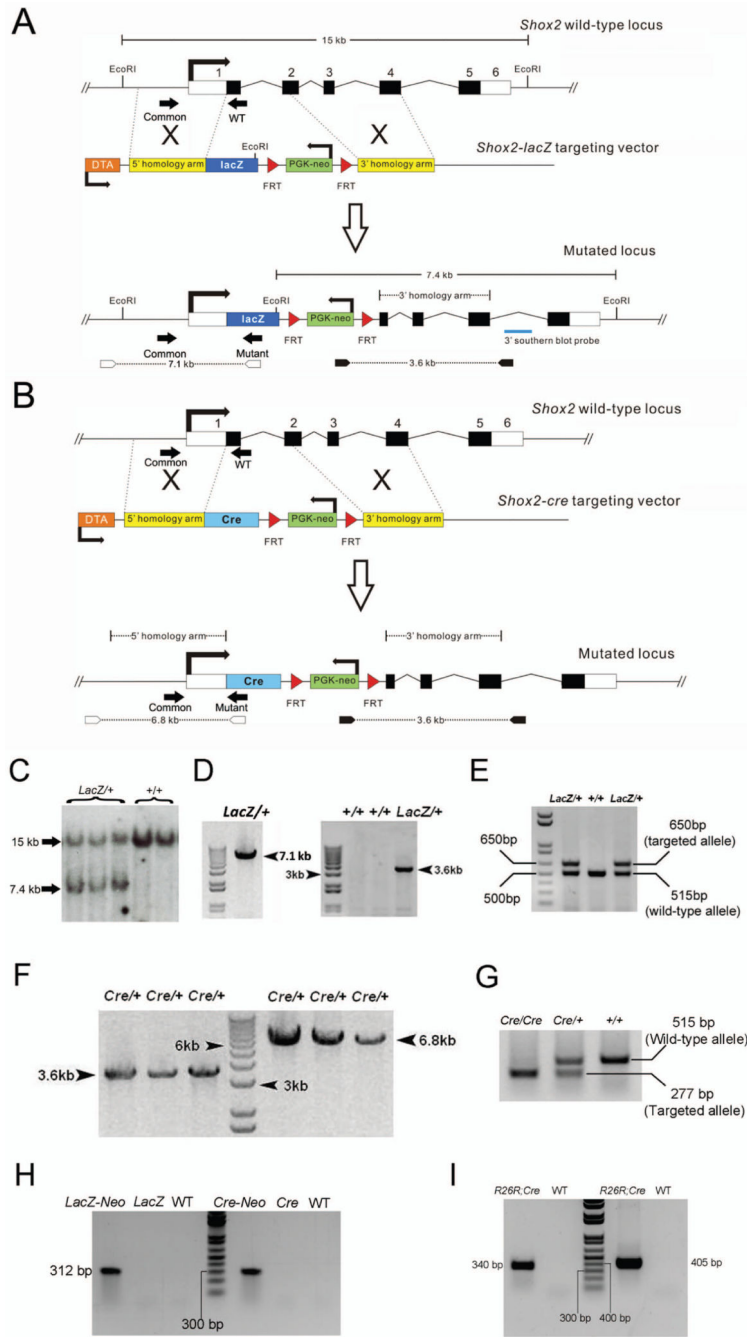


Figure 1. Targeting strategy of generating *Shox2-LacZ* and *Shox2-Cre* mice. (A and B) Targeted *Cre* or *LacZ* replacement of the *Shox2* gene in mice. The mouse *Shox2* genomic structure comprises an 8.3 kb region, and contains 6 exons, as indicated by numbered blocks. Black blocks indicate coding regions. The targeting vector contains genomic fragments flanking the *lacZ/Cre-FRT-neo-PGK-FRT* cassette which replaces exon 1 and part of exon 2 of *Shox2* when correct homologous recombination occurs in ES cells. (C) Southern blot analysis for *Shox2-LacZ* targeted allele (digested with *EcoRI* and probed with 3' southern blot probe).

(D) PCR product for *Shox2-LacZ* knock-in allele in ES cells. 7.1 kb and 3.6 kb PCR products indicate the correct homologous recombination at 5' and 3' of the *Shox2* locus respectively. Primer sets are labeled by arrow heads in A. (E) PCR analysis of DNA extracted from yolk sac of E10.5 embryos reveals the wild-type and *LacZ* targeted alleles. Primer sets are labeled by arrow heads in B. (F) PCR screen showing correct homologous recombination of *Shox2-Cre* targeted vector in ES cells. A set of common, WT and Mutant primers are used and labeled by black arrows in A. (G) PCR analysis of DNA extracted from yolk sac of E10.5 embryos reveals the *Shox2-Cre* alleles. The primer sets are shown by black arrowheads in B. (H) Verification of *neo-PGK* deletion by PCR analysis. The *neo-PGK* cassette is detected in F1 mice (*LacZ-Neo* and *Cre-Neo*) and undetectable in WT mice and offspring from F1 mice and FLP deleter mice (*LacZ* and *Cre*). (I) Identification of *R26R;Shox2^{Cre/+}* double heterozygous mice by PCR assay. A 340 bp PCR product indicates the *R26R* allele in double heterozygote. The 405 bp product indicates *Cre* allele.

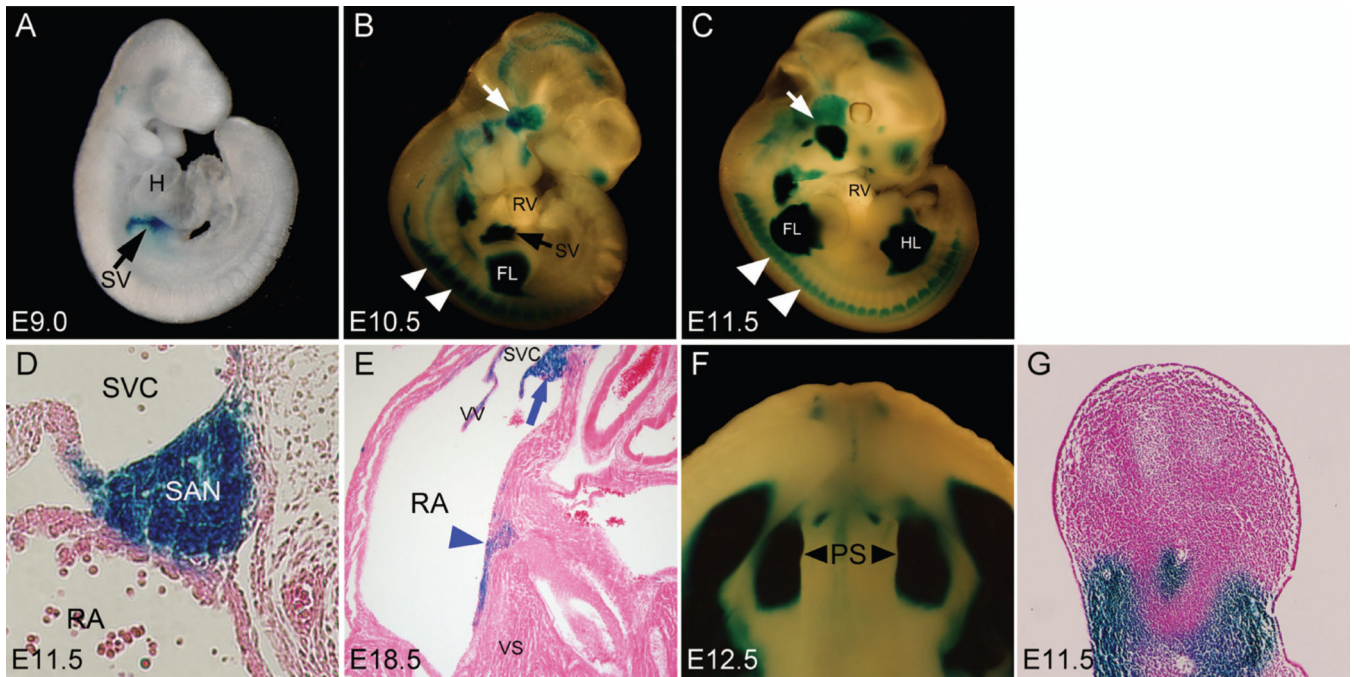


Figure 2.

Expression of beta-galactosidase in *Shox2^{lacZ/+}* embryos revealed by X-gal staining. (A) beta-galactosidase expression in the sinus venosus (black arrow) was detectable as early as E9.0. (B) At E10.5, beta-galactosidase expression was seen, besides the sinus venosus (SV), in other developing organs and tissues including the proximal parts of the limb buds, the dorsal root ganglion (white arrowheads), several craniofacial structures including the temporomandibular junction (white arrow), and the brain region. (C) At E11.5, beta-galactosidase was continuously expressed in the developing organs and tissues observed at E10.5. (D) X-gal staining replicated *Shox2* expression pattern in the sinoatrial node (SAN) at E11.5. (E) beta-galactosidase expression was detected the SAN and on top of the ventricular septum at E18.5. (F) At E12.5, beta-galactosidase was strongly expressed in the anterior portion (black arrow heads) of the palatal shelves (PS). (G) Section of an E11.5 *Shox2^{LacZ/+}* limb showing beta-galactosidase expression in the mesenchymal cells of the proximal limb bud. H, heart; SV, sinus venosus; RV, right ventricle; RA, right atrium; FL, forelimb bud; HL, hindlimb bud; SAN, sinoatrial node; SVC, superior vena cava; PS, palatal shelves; VS, ventricular septum.

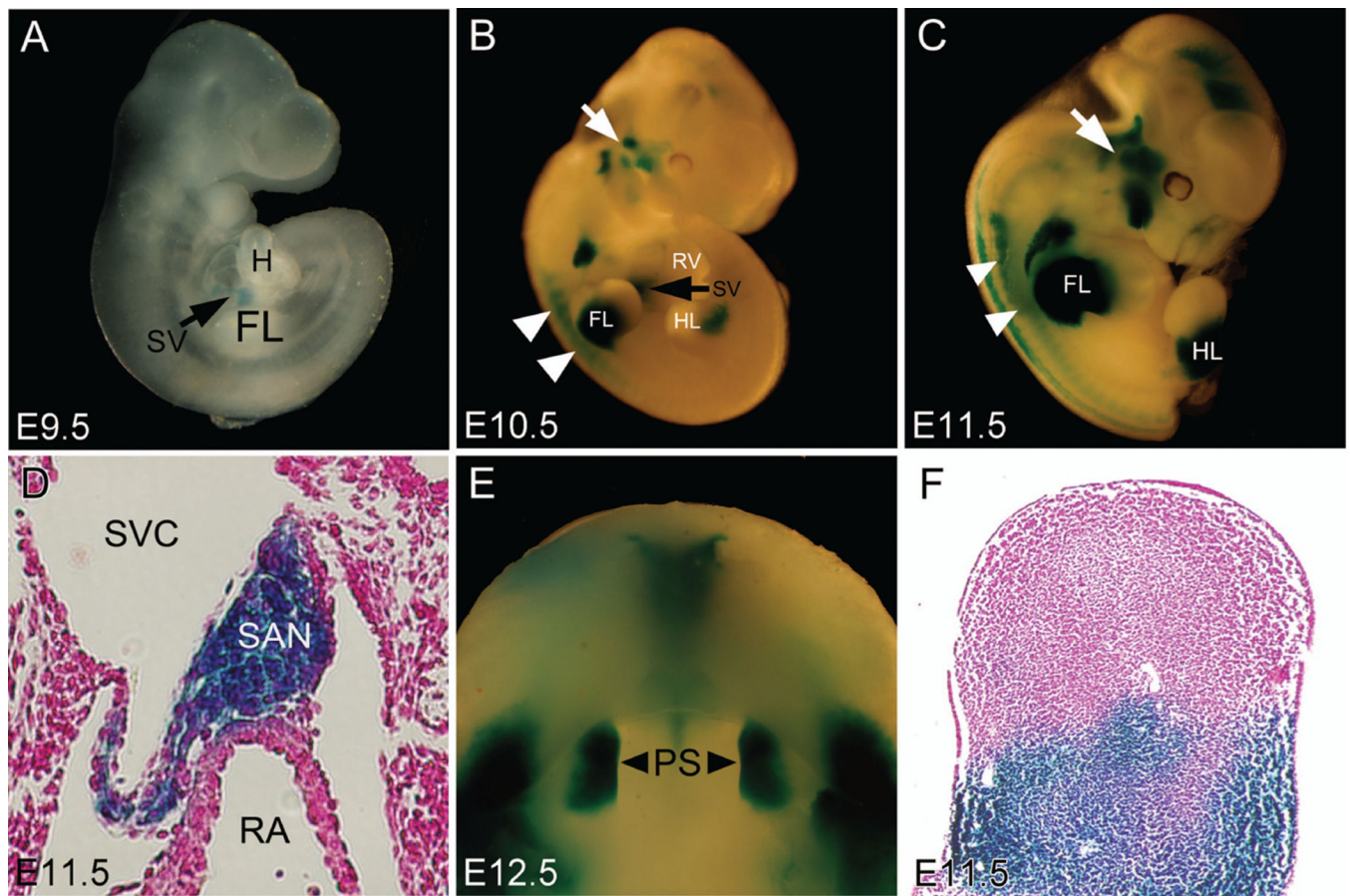
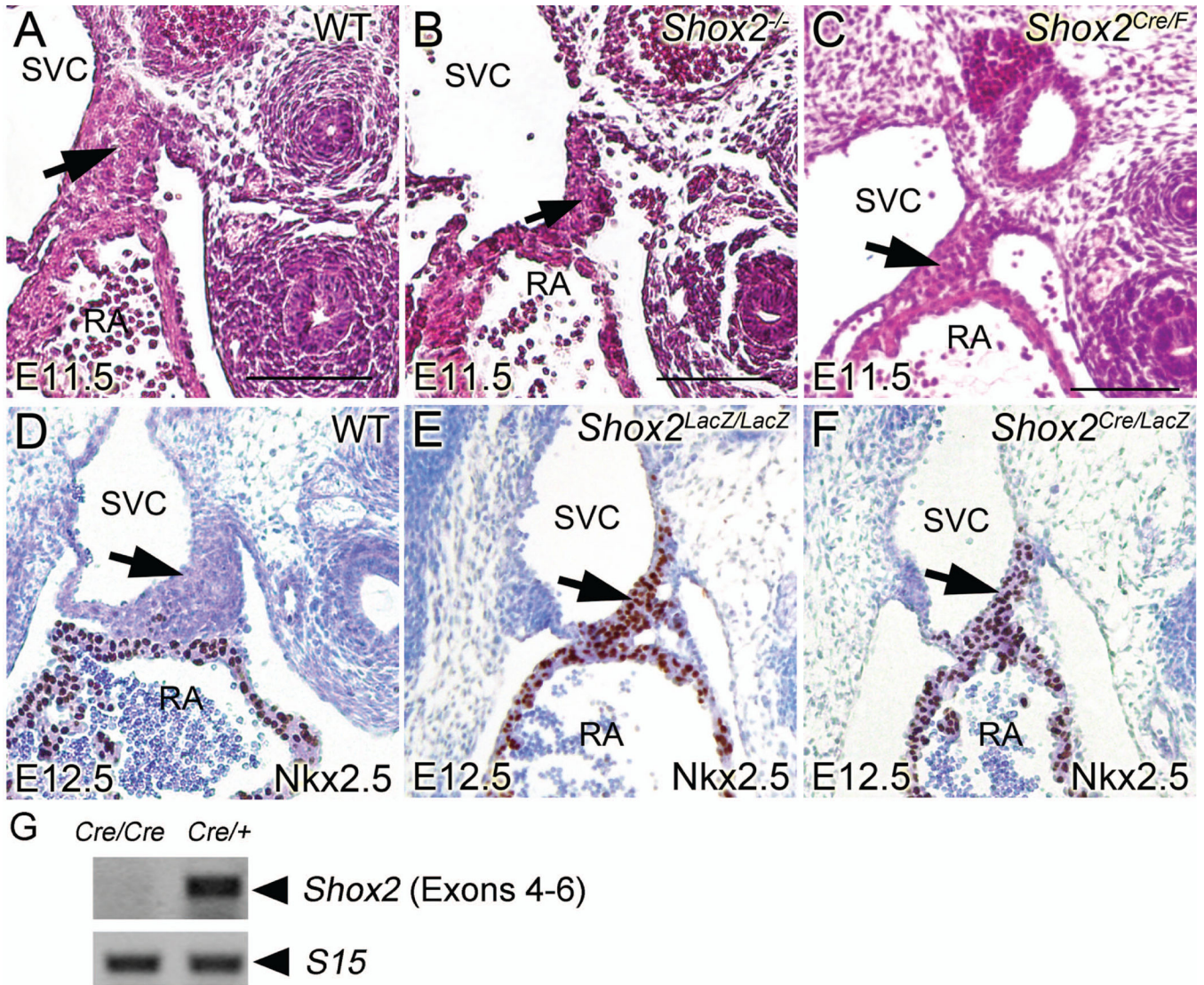


Figure 3.

Detection of Cre activity in *Shox2^{cre/+};**R26R* double heterozygous embryos. (A) Cre-mediated beta-galactosidase expression (blue) in the sinus venosus (black arrow) was detectable at E9.5. (B) At E10.5, beta-galactosidase expression in *Shox2^{cre/+};**R26R* double heterozygous embryos persisted in the sinus venosus (SV), and was also found in the proximal domain of the limb buds, the dorsal root ganglion (white arrowheads), and the temporomandibular junction (white arrow). (C) An E11.5 *Shox2^{cre/+};**R26R* embryo showing beta-galactosidase activity in the same organs and tissues as described in E10.5 embryo. (D) X-gal staining was seen in the SAN of an E11.5 *Shox2^{cre/+};**R26R* embryo. (E) Beta-galactosidase expression was detected in the anterior domain (black arrowheads) of the palatal shelves (PS). (F) Beta-galactosidase expression was found restrictedly in the proximal region of the limb bud at E11.5.

**Figure. 4.**

Similar hypoplasia phenotype was observed in the SAN of *Shox2*^{Cre/F}, *Shox2*^{LacZ/LacZ} and *Shox2*^{Cre/LacZ} mice as seen in *Shox2*-null mice. (A–C) representative H&E staining pictures of the developing SAN in E11.5 WT (A), *Shox2* conventional knockout (B) and *Shox2*^{Cre/F} embryos (C). Arrows denote SAN region. (D–F) representative IHC pictures of the developing SAN in E11.5 WT (D), *Shox2*^{LacZ/LacZ} (E) and *Shox2*^{Cre/LacZ} embryos (F). Arrows denote SAN region. Note the ectopic expression of Nkx2.5 in the SAN region of *Shox2*^{LacZ/LacZ} and *Shox2*^{Cre/LacZ} embryos. (G) RT-PCR assays demonstrating the absence of *Shox2* transcript in E11.5 *Shox2*^{Cre/Cre} mice. Scale bar: 100 μ m.

Table 1
Embryonic lethality in *Shox2*^{LacZ/LacZ}, *Shox2*^{Cre/LacZ} and *Shox2*^{Cre/F} mice

Embryonic lethality was observed at different developmental stages. This table shows the number of dead and survived mutant embryos. Total embryo numbers include WT embryos.

Mutant Allele	Stage	Dead Mutants	Survived Mutants	Total Embryos
<i>Shox2</i> ^{LacZ/LacZ}	E11.5	3	6	33
	E12.5	7	3	42
	E14.5	12	1	58
<i>Shox2</i> ^{Cre/LacZ}	E11.5	2	3	21
	E12.5	6	3	35
	E14.5	1	0	10
<i>Shox2</i> ^{Cre/F}	E11.5	2	4	13
	E12.5	1	1	6
	E14.5	2	0	7

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