Functional Redundancy between Human SHOX and Mouse Shox2 Genes in the Regulation of Sinoatrial Node Formation and Pacemaking Function*

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The homeodomain transcription factor Shox2 plays a crucial regulatory role in the development of sinoatrial node (SAN) by repressing the expression of Nkx2.5, as demonstrated by failed differentiation of SAN in Shox2 null mice. The SHOX (short stature homeobox) gene family consists of two closely related members, SHOX and SHOX2 in humans, but a SHOX ortholog does not exist in the mouse genome. These two genes exhibit overlapping and distinct expression patterns in many developing organs but whether they share functional redundancy is not known. In this study, we set to investigate possible functional redundancy between SHOX and SHOX2 in vitro and in vivo. We first showed that human SHOX and SHOX2 and mouse Shox2 possess similar transcriptional repressive activities in cell cultures, particularly the repressive effects on the Nkx2.5 promoter activity. We further created an SHOX/Shox2 knock-in mouse line (replacement of Shox2 with SHOX, referred as Shox2K1/K1). Mice carrying the hypomorphic Shox2K1+Neo/K1+Neo allele exhibit bradycardia and arrhythmia and die a few days after birth. However, mice carrying the Shox2K1/K1 allele grow to adulthood. Physiological, histological, and molecular analyses demonstrate a fully developed SAN and normal pacemaking function in Shox2K1/K1 mice. Our results demonstrate a functional redundancy between human SHOX and mouse Shox2 in the regulation of SAN formation and pacemaking function in addition to several other organs. The SHOX/Shox2 dose appears to be critical for normal pacemaking function.

The sinoatrial node (SAN) serves as the primary pacemaker and is responsible for the rest of the electrical activity of the heart. It consists of a group of modified cardiomyocytes that are positioned on the wall of the right atrium, near the entrance of the superior caval vein (1), and regulates the rhythm of the heart beat. Defects in such pacemaking functions lead to arrhythmia and, in severe cases, cause sudden cardiac death (2). In the developing heart, all the cardiomyocytes initially possess pacemaking properties. However, only a small portion of cardiomyocytes forms the SAN and other conduction tissues, and the majority of them differentiate into working myocardium. Although the exact molecular mechanisms underlying the pacemaker formation remain to be elucidated, recent studies have demonstrated essential roles of a genetic cascade involving multiple transcriptional factors in the SAN formation by suppressing working myocardial differentiation (3). Among these factors, Shox2 has been identified as a key regulator in pacemaker differentiation and functions (4–6).

Shox2 belongs to the SHOX (short stature homeobox) gene family, having been shown to be expressed in the developing heart particularly in the SAN in a very restricted and specific manner in the mouse and human embryos (4, 5, 7, 8). The Shox2 null mutation causes severe developmental defects in multiple organs and results in embryonic lethality at around E11.5 due to cardiovascular defects, specifically a failed differentiation of the SAN cells and a reduced pacemaker function (4, 5, 9–12). Shox2 mutation disrupts the SAN genetic network resulting in the down-regulation of Tbx3 and Hcn4, and the ectopic expression of Nkx2.5, Cx40, and Nppa in this region (5). On the other hand, overexpression of Shox2 in cell cultures as well as in Xenopus embryos results in the down-regulation of Nkx2.5 expression and the Nkx2.5 promoter activity (5), indicating regulation of Nkx2.5 expression by Shox2. It was reported recently that Shox2 mediates Tbx5 activity in the pacemaker region by regulating Bmp4 expression (6). In humans, Shox2 has not yet been linked to a disease, but SHOX haploinsufficiency is responsible for Turner syndrome and Leri-Weill dyschondrosteosis, and complete SHOX deficiency causes Langer mesomelic dysplasia syndrome (13–17). The fact that SHOX and SHOX2 exhibit temporally and spatially overlapping but distinct expression patterns in the human embryos (18), share 83% homology at the amino acid level, and have an identical homeodomain (8, 13) raises the possibility of functional redundancy between SHOX and SHOX2 during human embryogenesis.

The SHOX gene encodes two alternatively spliced transcripts named SHOXa and SHOXb. Both transcripts are identical at the

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4 The abbreviations used are: SAN, sinoatrial node; E, embryonic day; P1, postnatal day 1; TMJ, temporomandibular joint.
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5’ end but differ at the 3’ end in the last exon (15) and are translated into two protein isoforms of 292 (SHOXa) and 225 (SHOXb) amino acids, the latter lacks the C-terminal portion, which harbors a 14-amino acid motif known as the OAR domain that was thought to be important for transcription activity and be involved in protein-protein interactions or DNA binding (15, 19). Nucleotide sequence analysis suggests that the human and mouse SHOX2 shares 99% identity at the amino acid level and generates two isoforms by alternative splicing, SHOX2a and a shorter version SHOX2b (7, 20). Both isoforms contain the sequence encoding the homeodomain, an SH3 binding domain, and the OAR domain. However, SHOX2b lacks 363 nucleotides corresponding to the N terminus of SHOX2a and 36 nucleotides corresponding to the C terminus of SHOX2a. However, functional significance of these isoforms remains elusive.

Because the mouse does not have a SHOX ortholog in its genome, and the mouse Shox2 shares 99% identity at the amino acid level with its human counterpart and exhibit an similar expression pattern during mouse embryogenesis to that of human SHOX2 (18), creation of a knock-in mouse line with a replacement of mouse Shox2 with human SHOX (referred as Shox2<sup>KI/KI</sup>) would provide an excellent model to study the functional relationship between human SHOX and SHOX2.

**EXPERIMENTAL PROCEDURES**

**Generation of SHOXa/Shox2 Knock-in Mice**—Genomic DNA isolated from 129 ES cells was used to amplify homologous sequences by PCR. The targeting vector was constructed by placing a diphtheria toxin A cassette, a 5.6-kb 5’ homologous fragment, a full-length human SHOXa cDNA with transcription terminator, and a 2.9-kb 3’ homologous arm into the pSV-FLP vector, in which the PGK-neo cassette is flanked by two Flippase Recognition Target sites. Correct targeting of the Shox2 locus with this vector results in the expression of SHOXa-PGK-neo DNA fragment replacing exon 1, which includes the start codon of Shox2, and exon 2. The targeting vector was linearized by Ascl and electroporated into C57 ES cells (21). Clones were selected with G418 and screened by Southern blotting analysis with both a 5’ probe and a 3’ probe outside of the flanking homologous sequences. Targeted ES cells were injected into C57BL/6 blastocysts to produce chimeras. Chimeric mice that were derived from two independently targeted ES cell clones were mated with C57BL/6 mice to generate Shox2<sup>KI/KI+Neo</sup> heterozygous animals, whose genotype was further confirmed by Southern blotting of tail DNA. Homozygous Shox2<sup>KI/KI</sup> mice from both clones exhibit identical phenotypes. Animals maintained on different backgrounds were genotyped routinely by PCR analysis of tail DNA or yolk sacs of embryos. The three primers (LHB89, 5’-CGTCCTCCTCTCCTCTCTTAC-3’; LHB62, 5’-TTTCAGCTTGGTCTGCC-3’; and LHB258, GAACGCCGTAAGTTCTTCCA) amplified a 510-bp product from the knock-in allele and a 245-bp product from the wild type Shox2 allele. All animal experiments were approved by the Tulane University Institutional Animal Care and Use Committee.

**TaqMan Real-time PCR**—Tissues from E12.5 mouse embryos were subjected to mRNA isolation with TRIzol (Invitrogen). The isolated mRNA was treated by DNase I (Invitrogen) prior to reverse transcription using a SuperScript kit (Invitrogen). TaqMan real-time PCR was used to quantify SHOXa expression level with GAPDH as endogenous control for normalization. The comparative Ct (2<sup>-ΔΔCt</sup>) method (22) was used for calculation. SHOXa Custom TaqMan Expression Assay (A1X0ZZE) was designed based on the specific mRNA sequence after SHOXa knock-in. The forward primer, AIX02ZFE (5’-CTTGAGGCCGAGTTGAC-3’), and the reverse primer, AIX0ZFF_R (5’-GATAAAAGCGCTGAGCTCTTC-3’), were chosen to amplify an 89-bp fragment in the fusion region of Shox2 promoter and SHOXa sequence. The internal TaqMan probe (5’-ATGGGACC-GTTAATTAA-3’) was designed and provided by the manufacturer.

**Electrocardiogram Recordings**—Postnatal day 1 (P1) mice of wild type, Shox2<sup>KI+Neo/KI+Neo</sup>, and Shox2<sup>KI/KI</sup> were fixed by tape to built-in ECG electrode-contact pads (THM100, Indus Instruments). Standard Lead II ECGs were acquired using DATA Q DI-158U at 1 kHz. ECG recordings were obtained during 3–5 min periods.

**Histology, In Situ Hybridization, and Immunohistochemistry**—Mouse embryos were dissected in cold PBS and fixed in 4% paraformaldehyde in PBS at 4 °C overnight. Surgically and medically terminated human embryos, provided by the Hospital for Women and Children of Fujian Province, were staged and fixed in 4% paraformaldehyde, with the permission of the Ethics Committee of Fujian Normal University. Samples were then dehydrated through a graded ethanol series, embedded, and processed for paraffin serial sections at 10 μm. For histological analysis, sections were subjected to standard hematoxylin/eosin staining or Azan Red/Aniline Blue staining (23). In situ hybridization and probes used were similar to that described previously (5). At least two identical samples of mutant and wild type samples were used for in situ hybridization for each probe. Full-length human SHOXa and SHOX2a cDNAs were cloned by PCR into TOPO vector and confirmed by DNA sequencing, and used as probes. Immunohistochemical staining was performed using polyclonal antibody against Nkx2.5 (Santa Cruz Biotechnology) according to the manufacturer’s instruction.

**Plasmid Constructs, Cell Culture, and Luciferase Assays**—HEK-293T or H9c2 cells (ATCC number: CRL-1446<sup>TM</sup>) cells were cultured at 37 °C in DMEM medium containing high glucose, supplemented with 10% fetal calf serum and antibiotics. To generate constructs expressing fusion proteins with the GAL4 DNA-binding domain, we subcloned the following DNA fragments, respectively, into the pBXGI vector that contains the GAL4 DNA-binding domain under the control of the SV40 enhancer/promoter (24): full-length mouse Shox2a, full-length Shox2a lacking the OAR domain (Shox2a-ΔT), full-length mouse Shox2b, and full-length human SHOXa and SHOX2a. To test potential transcription activities, the resultant expression vectors were co-transfected into cells with L8G5-Luc reporter plasmid that contains eight LexA operators with five Gal4-binding sites upstream of a TATA box.
box and the luciferase (Luc) reporter gene. The CMV-β-gal plasmid was included as internal control for transfection efficiency. Each experiment was performed in triplicate and repeated twice using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s instruction. Forty eight hours after transfection, luciferase activities were determined and normalized to β-galactosidase activity.

RESULTS

Mouse Shox2, Human SHOX, and SHOX2 Possess Similar Transcription Activities—We and others (12, 25) have previously mapped the transcriptional domain of human SHOXα and mouse Shox2a and Shox2b to the C-terminal region (from the amino acid immediately after the homeodomain to the last amino acid). To determine whether mouse Shox2 and human SHOX and SHOX2 possess similar transcription activity and to test the functional significance of the OAR domain, we generated a serial constructs and performed reporter gene expression assays in cell culture. The schematic structure of the constructs was shown in Fig. 1A, including mouse Shox2α and Shox2α lacking the OAR domain (Shox2α-T), Shox2b, and mouse SHOXα and SHOX2α. Alignment of the predicted full-length amino acid sequences of these proteins has been reported previously (7). These constructs were fused in frame to the DNA-binding domain of yeast GAL4 under the control of the SV40 enhancer/promoter and were co-transfected into HEK-293T cells, respectively, with the L8G5-Luc reporter plasmid to determine transcription potentials. The results demonstrate that mouse full-length Shox2α and Shox2b, and human SHOXα and SHOX2α all exhibit transcriptional repressive potential in the HEK-293T cells, with mouse Shox2α and human SHOX2α showing almost identical repression activity and human SHOXα being more potent (Fig. 1B). Although mouse Shox2b exhibited relatively lower repressive activity, mouse Shox2α-T lost repressive activity completely, indicating an essential role for the OAR domain in the transcriptional activity of these homeodomain proteins (Fig. 1B).

We have reported previously that Shox2α acts as a transcriptional repressor on Nkx2.5 in vitro and in vivo (5). To further determine whether human SHOXα has the same function as Shox2α to repress the expression of Nkx2.5, we carried out reporter gene expression assays in the H9c2 embryonic rat-heart derived cell line using Nkx2.5-Luc, a reporter construct harboring a 3.3-kb mouse Nkx2.5 promoter in H9c2 cell cultures. The activity of the Nkx2.5 promoter co-transfected with empty pCDNA3.1 was included as control, and the expression level after normalization was set as 1. Error bars, S.D. D, in situ hybridization shows expression of SHOX2 in the SAN (arrows) of a 7-week-old human embryo. A, origin of the thoracic aorta; RA, right atrium; SVC, superior vena cava.
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is expressed specifically in the embryonic SAN was not known. We performed in situ hybridization and indeed detected restricted SHOX2 expression in the SAN of human embryo of 7 weeks post-conception (Fig. 1D), implicating a similar role of SHOX2 as mouse Shox2 in the SAN development. However, we failed to detect SHOXa expression in the human embryonic SAN (data not shown).

Shox2KI/Neo Represents a Hypomorphic Allele and Causes Bradycardia and Arrhythmia—Our in vitro studies suggest a functional equivalency between Shox2/SHOX2 and SHOX. To address whether these two genes function equivalently in vivo, we used gene targeting approach in ES cells to replace the Shox2 coding region with the human SHOXa isoform cDNA together with an FRT-flanked PGK-neo expression cassette (Fig. 2A). This is because the mouse developing heart expresses Shox2a isoform but not Shox2b (Ref. 5 and data not shown). Chimeric mice were generated from correctly targeted ES cell clones (Fig. 2B and C) and germ line transmission of the targeted allele was confirmed with Southern blotting of F1 mouse progeny tail DNA samples (Fig. 2D). The targeted Shox2 allele containing the PGK-neo cassette is referred as Shox2KI/Neo. F1 mice heterozygous for the Shox2+/KI/Neo allele were fertile and indistinguishable phenotypically from wild type mice after four to five generations on either 129, C57, or CD-1 background. RT-PCR analysis of E11.5 embryos demonstrated the presence SHOXa mRNA in heterozygous (Shox2+/KI) and homozygous (Shox2KI/KI) knock-in mice. Note the lack of SHOXa mRNA in wild type embryo and the absence of Shox2 in Shox2KI/KI sample. The KI allele shown in this figure contains the PGK-neo cassette.

FIGURE 2. Targeted replacement of the mouse Shox2 locus with human SHOXa cDNA. A, the Shox2 gene contains six exons that span ~8.3 kb of genomic DNA. Exons 1–5 are shown as blue boxes. The position of the translation start codon (ATG) is indicated. The targeting vector used the 5.6-kb fragment upstream to the ATG codon as the 5' homology arm and a 2.9-kb fragment after exon 2 as the 3' arm, as indicated by the dashed lines. The human SHOXa cDNA and a FRT-flanked PGK-neo cassette were inserted in between the arms and a diphtheria toxin A (DTA) expression cassette was cloned 5' to the 5' arm for negative selection. The 5' and 3' probes used for Southern blotting were indicated by short lines. B, a representative of Southern blot analysis of genomic DNA from ES cell clones using the 5' probe showed correct targeting of the Shox2 locus. The 15-kb EcoRI fragment corresponds to the wild type allele and the 6.4 kb to the targeted allele. E, EcoRI site. C and D, Southern blot analysis of genomic DNA from ES cell clones (C) and F0 mice (D) using the 3' probe showed correct targeting of the Shox2 locus. The 15-kb EcoRI fragment corresponds to the wild type allele and the 8.5 kb to the targeted allele. E, PCR analysis of tail DNA from newborn F1 mice showed a 245-bp Shox2 band and a 510-bp SHOXa knock-in band. F, RT-PCR analysis of E11.5 embryos demonstrated the presence SHOXa mRNA in heterozygous (Shox2+/KI) and homozygous (Shox2KI/KI) knock-in mice. Note the lack of SHOXa mRNA in wild type embryo and the absence of Shox2 in Shox2KI/KI sample. The KI allele shown in this figure contains the PGK-neo cassette.
survive the embryonic lethality of Shox2 deficiency. However, Shox2<KI>Neo/<KI>Neo mice were born much smaller (1.25 ± 0.21 g), as compared with their wild type littermates (1.84 ± 0.31 g) (Fig. 3, A and B), and none of them could survive beyond 3 days after birth. We have reported previously that Shox2 deficiency leads to anterior clefting of the secondary palate (11). We wondered whether the perinatal lethality of Shox2<KI>Neo/<KI>Neo mice could result from a cleft palate defect. However, morphological and histological analyses indicated a full rescue of the cleft palate defect (data not shown). In contrast, ECG recording of P1 mice revealed a significantly reduced heartbeat rate (140 beats/min) and arrhythmia in Shox2<KI>Neo/<KI>Neo mice (n = 11), as compared with their wild type littermates (248 beats/min) (n = 9) (Fig. 3, E and G). Although the SAN and sinus valves in Shox2<KI>Neo/<KI>Neo mice exhibit comparable morphology to the wild type controls (data not shown), indicating a rescue of SAN development in the absence of Shox2, the physiological defects suggest a malfunctioned pacemaker, which could be the causative of perinatal death. We assumed that the presence of the PGK-<i>neo</i> cassette likely produces a hypomorphic SHOXa allele.

The FRT-flanked PGK-<i>neo</i> cassette was removed by crossing Shox2<sup>KI</sup>/<I>KI</I> mice to homozygous ROSA26FLpo mice (Jackson Research Laboratories). After segregation of the ROSAFLpo allele from the Shox2<sup>KI</sup>/<I>KI</I> mice, the latter were intercrossed to produce homozygous Shox2<sup>KI</sup>/<I>KI</I> mice that were indistinguishable from their wild type littermates at the birth (Fig. 3D). ECG recording demonstrated that the heartbeat rate of homozygous SHOXa knock-in mice without the PGK-<i>neo</i> cassette (referred as Shox2<sup>KI</sup>/<I>KI</I>) (n = 7) was almost the same as that of wild type mice (Fig. 3E). We further performed real-time RT-PCR using mRNAs isolated from the heart and fore limbs of E12.5 embryos to determine whether the Shox2<sup>KI</sup>/<I>KI</I> allele represents a hypomorphic allele. As shown in Fig. 3F, the level of SHOXa expression in the heart of Shox2<sup>KI</sup>/<I>KI</I>Neo/<I>KI</I>Neo mice is ~56% and ~58% in the limb buds as compared with that of the heart and limbs from Shox2<sup>KI</sup>/<I>KI</I> embryos, respectively, indicating that the presence of the PKG-<i>neo</i> cassette indeed causes a reduced expression level of SHOXa.

**Shox2<sup>KI</sup>/<I>KI</I> Mice Develop a Severe Wasting Syndrome**—The Majority (72%, n = 29) of Shox2<sup>KI</sup>/<I>KI</I> mice that were monitored were able to survive beyond 3 days after birth but developed a severe wasting syndrome and died gradually. Only 14% of
controls and P21) showed a comparable body weight for both wild type (P0, P7, P14, and P21; Shox2) showed a normal palate in adulthood but appeared much smaller than their littermates (4/29 mice) survived to weaning time (P21) and then to adulthood if liquid foods were provided (10). To determine the cause of weight loss in Shox2-deficient mice, we examined phenotype of the palate and TMJ histologically and found ankylosis of the stylopod in limbs, in addition to the anterior clefting in the cranial neural crest cells causes the temporoman-dibular joint (TMJ) ankylosis in addition to the anterior clefting of the secondary palate (10). These mutant mice also developed TMJ ankylosis in addition to the anterior clefting (Fig. 4, A and B). Growth rate analysis at selected time points (P0, P7, P14, and P21; n = 10 for P0, P7, and P14; n = 4 for P21) showed a comparable body weight for both wild type controls and Shox2KI/KI mice at the birth but became significantly different at P7 and at other time points measured (Fig. 4C).

We have reported previously that conditional deletion of Shox2 in the cranial neural crest cells causes the temporomandibular joint (TMJ) ankylosis in addition to the anterior clefting of the secondary palate (10). These mutant mice also developed a severe wasting syndrome and died within 2 weeks but could survive to adulthood if liquid foods were provided (10). To determine the cause of weight loss in Shox2KI/KI mice, we examined phenotype of the palate and TMJ histologically and found a normal palate in Shox2KI/KI mice. However, although an ankylosis phenotype was not identified in the TMJ of Shox2KI/KI mice, these mice developed a new type of TMJ defect, premature wear out of the articulating disc (Fig. 5). At P0, the TMJ and articulating disc appeared comparable between controls and Shox2KI/KI mice (Fig. 5, A and B). However, at P5 and P10, the Shox2KI/KI articulating disc became much thinner, as compared with the controls (Fig. 5, C–F). At P21, the Shox2KI/KI articulat-ting disc had almost worn out (Fig. 5H, and inset). In addition, the condyle of Shox2KI/KI mice appeared smaller at P10 and P21, most likely due to retarded growth of the mice. The defective articulating disc could affect food intake and attribute to the wasting syndrome. This conclusion is further supported by the fact that Shox2KI/KI mice could slowly catch up growth rate if they were fed with liquid foods (data not shown). The molecular mechanism of the premature wear out of the articulating disc warrants future studies.

Shox2KI/KI Mice Form Normal SAN and Sinus Valves—Shox2-deficient mice die at mid-gestation stage due to cardiovascular defects, including bradycardia and hypoplastic SAN and sinus valves (4, 5). In addition, the SAN cells in Shox2 mutants also failed to differentiate, as evidenced by the absence of Hcn4 and Tbx3 expression and ectopic expression of Nkx2.5, Nppa, and Cx40. We set to examine the development of SAN in Shox2KI/KI mice at histological and molecular levels. We found that at E11.5, in contrast to severe hypoplasia of the SAN and sinus valves in Shox2 mutants, Shox2KI/KI embryo formed a SAN and sinus valves comparable to the wild type control (Fig. 6, A and B). This was also true for Shox2KI/KI mice at E15.5 and P0 (Fig. 6, C–F). Molecular analyses further revealed the expression of Hcn4 and Tbx3 and exclusion of Nkx2.5 expression in the SAN of Shox2KI/KI embryos at E11.5 and E12.5 (Fig. 7), indicating normal development of the SAN. Together with the ECG results, we conclude that SHOXa exerts equivalent function as Shox2 during SAN development by regulating a genetic cascade through the repression of Nkx2.5, consistent with SHOXa repressive function on the Nkx2.5 promoter activity in vitro (Fig. 1C).

DISCUSSION

In the present study, we investigated functional redundancy between the two members of the SHOX gene family, SHOX and SHOX2, during embryogenesis, particularly in SAN development and function. Previously, we mapped the transcriptional activity to the C-terminal domain of human SHOXa and mouse Shox2a and Shox2b, respectively (12). Here, we further demonstrated that full-length mouse Shox2a and Shox2b and human SHOXa and SHOX2a possess similar transcriptional repressive potential in the HEK-293T cell line. In addition, both mouse Shox2a and human SHOXa exhibit identical repressive effect on the mouse Nkx2.5 promoter. Our studies also demonstrated that the OAR domain is essential for the transcriptional activity of these homeodomain proteins, which is consistent with the previous report (25). These in vitro studies implied a functional redundancy between SHOX2 and SHOX, which prompted us to investigate whether these two genes have equivalent or distinct functions during embryogenesis in vivo using a gene knock-in approach.

Despite that SHOX2 has not been linked to any known syndrome in humans, null mutation in Shox2 leads to a spectrum of developmental defects, including the cleft palate formation, TMJ ankylosis, virtual deletion of the stylopod in limbs, in addition to embryonic lethality at the mid-gestation stage due to failed differentiation of SAN cells and dysplasia of the sinus valves (4, 5, 9–12). We found that the expression of SHOXa in the Shox2 locus appears to exert similar functions as Shox2 at a

FIGURE 4. Shox2KI/KI mice develop a severe wasting syndrome. A, a wild type mouse at P41. B, a Shox2KI/KI mouse at P41. C, growth curves of Shox2KI/KI mice and littermate controls.
large extent, as evidenced by the overcame of embryonic lethality and the rescue of the developmental defects of several organs in the absence of Shox2. Shox2<sup>KI/KI</sup> mice form normal palate and are able to survive to adulthood. However, whereas the TMJ ankylosis is completely rescued, Shox2<sup>KI/KI</sup> mice develop a new TMJ defect, premature wear of the articulating disc, which appears to contribute to the wasting syndrome. This observation indicates a distinct function of SHOXa, at least in a tissue-specific manner, during embryogenesis. The different function exerted by SHOXa and Shox2a in a tissue-specific manner could be best exemplified by the limb development in Shox2<sup>KI/KI</sup> mice in which the fore limbs form normally but the hind limb defect persists (data not shown). We are currently investigating this differential function of SHOXa in the development of fore limb versus hind limb. Indeed, in our <em>in vitro</em> transcriptional activity assay, SHOXa appears to be more

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**FIGURE 5.** Histological analyses reveal premature wear out of the articulating disc in the TMJ of Shox2<sup>KI/KI</sup> mice. Coronal sections through the TMJ of wild type (A, C, E, and G) and Shox2<sup>KI/KI</sup> mice (B, D, F, and H) at different ages. Note that the articulating disc in the knock-in mice appears comparable with that in the control at P0 (A and B), but becomes almost complete wear out at P21 (H and inset). C, condyle; d, disc; 7gf, glenoid fossa; lsc, lower synovial cavity;usc, upper synovial cavity. Scale bar, 500 μm.
potent than Shox2a in terms of transcriptional repressive activity (Fig. 1B).

Shox2 has been demonstrated to play an essential role in SAN development and the sinus valve formation (4, 5). In the absence of Shox2, the expression of a set of genes in the genetic network that regulates SAN development is dysregulated, leading to a failure in the differentiation of SAN cells and causing bradycardia and eventually embryonic lethality. These cardiac defects are apparently absent in Shox2<sup>KI/KI</sup> mice, as assessed by the analyses at histological, molecular, and physiological levels. Morphologically, Shox2<sup>KI/KI</sup> mice form indistinguishable SAN and sinus valves as compared with the wild type controls; molecularly, normal expression of the Shox2-regulated genetic network genes was observed in the SAN of Shox2<sup>KI/KI</sup> embryo, including the expression of the SAN markers Hcn4 and Tbx3 and the exclusion of Nkx2.5. Lastly at physiological level, Shox2<sup>KI/KI</sup> mice exhibit a heartbeat rate comparable with their wild type littermates. These lines of evidence demonstrate a complete rescue of the cardiac defects in the knock-in mice. It has been demonstrated that Shox2 regulates SAN development by repressing the expression of Nkx2.5 to prevent SAN cells from becoming working atrial myocardium (5). The identical repressive effect of SHOXa and Shox2a on the mouse Nkx2.5 promoter in the reporter gene expression assay strongly suggests that SHOXa substitutes Shox2 in the regulation of SAN development by repressing Nkx2.5 expression. This is also true in the in vivo situation, as evidenced by the exclusion of Nkx2.5 expression in the SAN of Shox2<sup>KI/KI</sup> embryo. We therefore conclude that SHOX plays a similar role as Shox2 in the SAN formation and pacemaking function by controlling a genetic cascade through the repression of Nkx2.5.

We found that the SHOXa/Trmt2 knock-in allele containing the PGK-neo expression cassette (Shox2<sup>KI+Neo</sup>) represents a hypomorphic allele, expressing the SHOXa gene at a level about half of that by the knock-in allele without the PGK-neo cassette. Interestingly, although the Shox2<sup>KI+Neo/KI+Neo</sup> mice formed normal SAN and sinus valves morphologically and overcame...
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Given the fact that SHOX2 is expressed in the SAN of human embryo, we would predict that a haploinsufficient mutation in SHOX2 could likely cause bradycardia and arrhythmia in humans.

In sum, we conclude that mouse Shox2 and human SHOX possess similar transcriptional activity. Human SHOXa is able to substitute for Shox2 in regulating the development of several organs. In the heart, SHOXa exerts a similar function as Shox2 in regulating the formation of SAN and the pacemaking function, indicating a functional redundancy between these two genes. However, given the fact that Shox2<sup>Ki/Ki</sup> mice develop premature wear out defect in the articulating disc of the TMJ and differential rescue of the defect in the fore limb <i>versus</i> the hind limb, SHOXa appears to exert its redundant function with Shox2 in a tissue-specific manner. Because the mouse Shox2 shares 99% identity at the amino acid level with its human counterpart, we predict that such functional redundancy between SHOX and SHOX2 must exist, but in a tissue-specific manner, during human embryonic development. Because the mouse does not have an SHOX ortholog in the genome, mouse Shox2 likely plays a broader function than human SHOX2 during embryogenesis.

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FIGURE 7. <i>Shox2<sup>Ki/Ki</sup></i> mice exhibit normal expression patterns of the SAN genetic cascade genes. A–D, in situ hybridization reveals specific expression of Shox2 in the wild type SAN and sinus valves and SHOXa in the Shox2<sup>Shoxa</sup> SAN and sinus valves at E11.5. E and F, immunohistochemical staining shows exclusion of Nkx2.5 expression in the SAN of both wild type (E) and Shox2<sup>Shoxa</sup> (F) embryos at E11.5. G–N, in situ hybridization shows specific expression of Tbx3 (G, H, K, and L) and Hcn4 (I, J, M, and N) Hcn4 in the SAN of wild type (G, I, K, and M) and Shox2<sup>Shoxa</sup> (H, J, L, and N) embryos at E11.5 (G–J) and E12.5 (K–N). Arrowheads point to the SAN.
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