Targeted Mutation Reveals Essential Functions of the Homeodomain Transcription Factor Shox2 in Sinoatrial and Pacemaking Development

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Background—Identifying molecular pathways regulating the development of pacemaking and coordinated heartbeat is crucial for a comprehensive mechanistic understanding of arrhythmia-related diseases. Elucidation of these pathways has been complicated mainly by an insufficient definition of the developmental structures involved in these processes and the unavailability of animal models specifically targeting the relevant tissues. Here, we report on a highly restricted expression pattern of the homeodomain transcription factor Shox2 in the sinus venosus myocardium, including the sinoatrial nodal region and the venous valves.

Methods and Results—To investigate its function in vivo, we have generated mouse lines carrying a targeted mutation of the Shox2 gene. Although heterozygous animals did not exhibit obvious defects, homozygosity of the targeted allele led to embryonic lethality at 11.5 to 13.5 dpc. Shox2−/− embryos exhibited severe hypoplasia of the sinus venosus myocardium in the posterior heart field, including the sinoatrial nodal region and venous valves. We furthermore demonstrate aberrant expression of connexin 40 and connexin 43 and the transcription factor Nkx2.5 in vivo specifically within the sinoatrial nodal region and show that Shox2 deficiency interferes with pacemaking function in zebrafish embryos.

Conclusions—From these results, we postulate a critical function of Shox2 in the recruitment of sinus venosus myocardium comprising the sinoatrial nodal region. (Circulation. 2007;115:1830-1838.)

Key Words: arrhythmia ▪ genes ▪ heart defects, congenital ▪ heart rate ▪ immunohistochemistry

Shox2 encodes a member of a small subfamily of paired, related homeodomain transcription factors1,2 that has been identified by virtue of its sequence similarity to the short-stature homeobox gene SHOX3, causing various short-stature syndromes. Human phenotypes caused by SHOX2 deficiency have not been identified so far. According to the complex embryonic expression pattern of Shox2, it has initially been implicated in craniofacial, limb, brain, and heart development.1,2 This assumption has recently been confirmed by analyses of independently generated knockout mouse models that revealed crucial functions of Shox2 in palate formation3 and chondrocyte maturation during bone development.4 In the present study, we describe the expression of Shox2 and its crucial role in the developing venous pole of the embryonic mouse heart.

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The development of the vertebrate heart comprises multiple cell fate decisions that are necessary to create the diverse cell types required for an integrated function of the mature organ. Recently, new insights have shown that during cardiac development new myocardium is added at the arterial and venous poles of the primary heart tube. The
venous pole of the heart, also known as sinus venosus, is the location where blood drains into the heart. It is suggested that the sinus venosus myocardium in which the sinus venosus is incorporated derives predominantly from a second lineage of cardiomyocytes. We refer to this population, in contrast to the anterior heart field at the arterial pole, as the posterior heart field (PHF). The sinus venosus myocardium compromises at the borderline of the right cardinal vein and the right atrium, a sinoatrial nodal (SAN) region that can be both morphologically and immunohistochemically defined.

Nkx2.5 is an early precardiac marker. We have investigated its expression in the development of the PHF. Nkx2.5, the vertebrate homolog of the Drosophila tinman, encodes a homeodomain transcription factor that, among other functions, is essential for normal development of the cardiac conduction system (CCS). Patients diagnosed with Nkx2.5 haploinsufficiencies exhibit several progressive heart defects, including atrial and ventricular septal defects and atrioventricular conduction system abnormalities.

To substantiate our hypothesis that Shox2 has an essential role in heart development, we have established the detailed expression pattern within the developing heart, examined the effects of Shox2 depletion in zebrafish embryos, and generated mouse lines carrying a targeted mutation of Shox2. Furthermore, myosin light chain (MLC-2a), Nkx2.5, connexin 40 (Cx40), and connexin 43 (Cx43) were used to investigate the differentiation and possible aberrant formation of the PHF-derived sinus venosus myocardium compromising the SAN region of Shox2<sup>−/−</sup> embryos. The results were evaluated with 3-dimensional reconstruction techniques.

**Methods**

All animal experiments were conducted according to German animal protection laws and approved by the regional board of Baden Württemberg (permission No. 35-9185.81/G–64/05). A short summary of the techniques, which is supported by online data for routine procedures, is provided.

We generated chimeras and mutant mice (Department of Human Genetics, Heidelberg, Germany) by replacing the targeting vector aiming at the Shox2 locus. Genomic DNA was prepared from tail biopsies and yolk sac as previously described. Details for constructing the targeting vector and the primer design of both DNA and RNA are provided in the online Data Supplement.

Whole-mount and sectioned mouse embryos (9.5 dpc, n=10; 10.5 dpc, n=2; 11.5 dpc, n=3; 12.5 dpc, n=2; 13.5 dpc, n=1) were studied by in situ hybridization using previously described protocols, and sections were subjected to immunohistochemistry using antibodies against MLC-2a and Nkx2.5 and markers for the developing CCS Cx40 and Cx43. The standard procedures for immunohistochemistry are provided in the online Data Supplement. For 3-dimensional visualization, we generated reconstructions of the developing sinus venosus area using AMIRA software (Mercury/TGS, Berlin, Germany).

To establish a possible hypoplastic development of the SAN region, we performed volume measurements of this region in wild-type (n=3) and Shox2<sup>−/−</sup> (n=3) embryos of 11.5 dpc according to the Cavalieri method. Statistical analysis was performed with an independent-sample t test (P<0.05) with the SPSS 11.0 (SPSS Inc, Chicago, Ill) software program.

For zebrafish studies, we isolated the full-length zebrafish Shox2 cDNA sequence, which was followed by whole-mount RNA in situ hybridization as described previously and elaborated on in the online Data Supplement.

To compare the heart rate in age-matched resting conscious animals, ECGs were recorded with a custom-made mouse jacket and silver electrode clips attached to the paws (Förh Medical Instru-
Shox2 expression had expanded and included the SAN expression of myocardium–derived venous valves showed strong expression at 10.5 dpc, both leaflets of the sinus venosus myocardium is formed. Although the atrial and ventricular myocardium itself was negative for ventricular region that resembled the ventricular expression observed at 11.5 dpc was retained at later stages up to 13.5 dpc.

Results

Shox2 Expression During Heart Development
Initial insight into the possible functions of Shox2 during heart development was derived from expression analyses by antisense in situ hybridization on wild-type whole-mount embryos, isolated embryonic hearts, and serial sections. These analyses revealed Shox2 transcripts as early as 8.5 dpc in the posterior region of the primitive heart tube. At 9.5 dpc, Shox2 expression was restricted to the inflow tract (Figure 1A), particularly to the mesenchyme of the transitional zone between the sinus venosus and the common atrium (Figure 1B), where the sinus venosus myocardium is formed. Although the atrial and ventricular myocardium itself was negative for Shox2 expression at 10.5 dpc, both leaflets of the sinus venosus myocardium—derived venous valves showed strong expression of Shox2 at this stage (Figure 1C). At 11.5 dpc, Shox2 expression had expanded and included the SAN region and the venous valves, 2 parallel bundles spanning the longitudinal axis of the atria (Figure 1D and 1E). These structures have previously been demonstrated to constitute an integral part of the developing conduction system by analyses of lacZ expression in the CCS-LacZ mouse.22,23 In addition, we observed a distinct staining in the upper ventricular region that resembled the ventricular expression of CCS-LacZ and the primitive stages of left and right bundle-branch formation (Figure 1D and 1E). This pattern of expression observed at 11.5 dpc was retained at later stages up to 13.5 dpc.

Homologous Loss of Shox2 Functions Is Lethal
To investigate the functions of Shox2 in vivo, we have inactivated its mouse equivalent by gene targeting. Targeted mutation of Shox2 was performed in a classic homologous recombination approach using the replacement vector depicted in Figure 2A. In the targeted locus, a PGK-neo cassette replaces 2282 bp of genomic DNA, including the entire second exon of the Shox2 gene that encodes most of the homeodomain. This strategy allows concomitant disruption of both Shox2 isoforms, Shox2a and Shox2b, that have been described so far.1,2 Although expression from the Shox2a promoter produces compromised mRNA missing the major part of the homeodomain, use of the Shox2b promoter results in a noncoding mRNA missing the Shox2b ATG start codon. The resulting locus is therefore likely to represent a null allele. Targeted alleles were generated in 2 different embryonic stem cell lines (RI and E14); their presence was confirmed by Southern blot analyses and germ-line transmission verified by polymerase chain reaction analysis of chimera offspring (Figure 2B and 2C). Furthermore, reverse-transcription
polymerase chain reaction analysis using total RNA from 10.5 dpc hearts revealed that both described isoforms, Shox2a and Shox2b, are affected in heterozygous embryos and could not be detected in homozygous embryos (Figure 2D).

Heterozygous mouse lines were bred into and maintained on both C57BL/6 and CD1 backgrounds. In both genetic backgrounds, heterozygous animals were fertile, had comparable body weight, and did not exhibit any obvious abnormalities. Furthermore, genotype analysis of 276 animals from 3 generations revealed frequencies of 51.8% and 48.2% for the Shox2<sup>+/−</sup> and Shox2<sup>+/+</sup> alleles, respectively (Data Supplement Table I). We therefore concluded that Shox2<sup>+/−</sup> animals do not carry any gross abnormalities affecting overall integrity and body growth. Furthermore, assessment of resting heart rates by ECG recordings revealed no difference between wild-type and heterozygous animals. We observed some PR variability but no differences between groups in PQ intervals, QRS duration, or QT intervals (Data Supplement Figure I).

Although Shox2<sup>+/−</sup> animals did not exhibit any obvious phenotype, heterozygote intercrosses yielded 38% Shox2<sup>+/+</sup> and 62% Shox2<sup>−/−</sup> animals but no Shox2<sup>−/−</sup> offspring (n=87), although Shox2<sup>−/−</sup> embryos dissected at 9.5 dpc were viable and did not exhibit any obvious abnormalities. However, analyses of embryos at different developmental stages revealed that homozygous mutants died between 11.5 and 13.5 dpc. A normal mendelian frequency for Shox2<sup>−/−</sup> embryos was observed up to 10.5 dpc (29%). At 12.5 dpc, this frequency dropped to 13%, with all Shox2<sup>−/−</sup> embryos exhibiting distinctive phenotypic features, and at 14.5 dpc, only 2 homozygous embryos could be recovered (4.3%), both of which were dead at the time of dissection. This embryonic lethality, combined with the highly restricted Shox2 expression in the developing heart, suggested a heart defect as the most likely cause of death.

**Shox2-Deficient Embryos Exhibit Heart Defects**

Consistent with the observed Shox2 expression in the developing heart, we saw several signs of cardiovascular failure in homozygous Shox2<sup>−/−</sup> embryos compared with wild-type embryos of similar age (Figure 2E through 2H). These included pericardial edema (Figure 2F). Immunohistochemical staining analyses with MLC-2a-, Nkx2.5-, Cx40-, and Cx43-specific antibodies were performed to reveal specific differences between wild-type and Shox2<sup>−/−</sup> embryonic hearts from 9.5 to 11.5 dpc.

**Wild-Type Embryos**

**9.5 dpc**

At this stage, the shape of the primary heart tube was still clearly discernible, and the looping process of the heart was not yet completed (data not shown).

**10.5 dpc**

The embryonic heart clearly showed the common atrium and primitive left and right ventricles (Data Supplement Figure IIa). The atrial myocardium showed strong expression of MLC-2a, whereas the expression of MLC-2a in the ventricles was less strong (Data Supplement Figure IIc). Expression of Nkx2.5 was observed in both the common atrium and primitive ventricles. The developing venous valves were positive for MLC-2a and Nkx2.5 (data not shown).

The sinus venosus was located caudodorsally to the common atrium and formed the inflow tract of the heart. At this stage, 2 large vessels drain into the left and right horns of the sinus venosus, called the left and right common cardinal veins, respectively. The pulmonary vein, which drains into the common atria, was clearly discernible but not enclosed by myocardium yet (Data Supplement Figure IIa).

A U-shaped band of myocardium that formed the wall of the sinus venosus adjacent to the atrial myocardium was positive for MLC-2a and showed negative Nkx2.5 expression. This comprises the SAN region, which is located in the medial wall of the right cardinal vein (Data Supplement Figure IIa).

**11.5 dpc**

The embryonic heart showed further maturation; the septation of the atria and ventricles was still not completed at this stage. The future left and right atria and the future left and right ventricles were clearly discernible. Both the atrial and ventricular myocardium was positive for MLC-2a and showed expression of Nkx2.5 (Figure 3C and 3G). The more developed venous valves were positive for MLC-2a and showed expression of Nkx2.5 (Figure 3C and 3I). Compared with earlier stages, the intensity of the expression of MLC-2a and Nkx2.5 appeared to be stronger at 11.5 dpc. The sinus venosus myocardium was positive for MLC-2a but negative for Nkx2.5 (Figure 3D and 3H) and for Cx40 and Cx43 (Figure 4A, 4C, and 4E).

A 3-dimensional reconstruction of the embryonic heart showed that the MLC-2a- and Nkx2.5- negative sinus venosus myocardium formed a U-shaped structure, which is situated caudodorsally to the atria (Figure 3A, 3M, and 3N). In addition, the pulmonary vein showed further maturation; however, it was not yet enclosed by myocardium (Figure 3A).

**Shox2<sup>−/−</sup> Embryos**

**9.5 dpc**

Compared with wild-type hearts of similar age, the region of the common atrium seemed to be slightly enlarged. Besides this atrial enlargement, no major abnormalities were observed (data not shown).

**10.5 dpc**

In a comparison of 3-dimensional reconstructions of wild-type and Shox2<sup>−/−</sup> embryonic hearts, the morphologies appeared comparable, with only the exception being a slightly dilated common atrium (compare Data Supplement Figure IIa and IIb). As in wild-type embryos, MLC-2a expression was found in the common atrium and primitive left and right ventricles, with strongest expression in atrial myocardium (Data Supplement Figure IIc). In addition, the atrial and...
ventricular myocardium showed expression of Nkx2.5. The sections showed that the venous valves in Shox2−/− embryos were hypoplastic, were still MLC-2a positive, and showed expression of Nkx2.5.

The MLC-2a–positive and Nkx2.5-negative myocardium lining the sinus venosus and cardinal veins did not differ essentially from that seen in the wild-type embryo. The SAN region also was MLC-2a positive but Nkx2.5 negative and did not show any malformations in development (Data Supplement Figure IIB, IIE, and III).

11.5 dpc

Three-dimensional reconstructions of Shox2−/− embryonic hearts showed that the morphology of these hearts was altered dramatically. Compared with wild-type hearts, the left and right ventricles were markedly dislocated in Shox2−/− embryos (compare Figure 3A and 3B). The atria were severely dilated, and the myocardial wall of the atria seemed to be much thinner. The atrial and ventricular myocardium was positive for MLC-2a (Figure 3F) and showed expression of Nkx2.5 (Figure 3L). Here also, the atria showed the strongest expression of MLC-2a. The venous valves were severely hypoplastic and, in some embryos, even completely absent. Remnants of these valves were positive for MLC-2a and Nkx2.5 (Figure 3F and 3K).

In Shox2−/− embryos, the myocardium of the sinus venosus appeared less well developed. At a few locations where myocardium was lining the sinus venosus, the myocardium was positive for MLC-2a and showed expression of Nkx2.5 (Figure 3O and 3P). Interestingly, compared with wild-type embryos, not only was the size of the SAN region markedly decreased (P=0.018; power of 81.9%) (compare Figure 3D and 3E), but this hypoplastic SAN region in contrast to wild-type embryos was positive for Nkx2.5 (Figure 3J). In addition, an aberrant expression of connexins was observed in the myocardium of the SAN region, which turned out to be positive for Cx40 (Figure 4D) and moderately positive for Cx43 (Figure 4F). Furthermore, 3-dimensional reconstructions revealed that the U-shaped MLC-2a–positive and Nkx2.5-negative myocardial structure was almost absent (Figure 3B).

Shox2 Antisense Morpholino-Injected Zebrafish Embryos Develop Severe Sinus Bradycardia

Because the loss of Shox2 functions directly affects the developing sinus venosus myocardium, which includes the SAN region, we addressed the possibility that a loss of Shox2 functions may lead to pacemaking and conduction deficiencies using an antisense morpholino-based approach in zebrafish embryos. The zebrafish Shox2 gene encodes a protein that exhibits a similarity of 82% to the mouse protein (Data Supplement Figure III), and expression of the zebrafish Shox2 gene mirrors the expression in human and mouse central and peripheral nervous systems, the pectoral fin buds, and the inflow tract of the heart (data not shown). Injection of morpholino-modified antisense oligonucleotides specifically targeting the exon 3 splice site of Shox2 (Data Supplement Figure IV) into early embryos led to severe cardiac dysfunction, with a pronounced sinus bradycardia (70±15 versus 165±25 bpm) and intermittent sinus exit block after 72 hours of development, when the embryo is still able to survive on passive diffusion of oxygen and nutrients (Data Supplement Movies I and II). Identical results were obtained with a morpholino-oligo targeting the ATG start codon.

Discussion

In the present study, we have investigated the expression pattern of Shox2 during cardiogenesis and its crucial function in sinus venosus myocardial development. Shox2 belongs to a small subfamily of homeodomain transcription factors and has previously been suggested to play a role in early heart development.1 Reevaluation of the embryonic expression pattern showed that the initial description of Shox2 expression in the outflow tract region of the developing heart could not be confirmed.1 The detailed analyses presented here clearly demonstrate that Shox2 is expressed in the myocardium surrounding the developing sinus venosus and in a myocardial band on top of the ventricular septum. This sinus venosus myocardium is added to the venous pole of the heart and is recruited after formation of the primary heart tube.4 We refer to the sinus venosus area as the PHF located at both the right and left sides of the developing atrium.7 For this study, we concentrated on the right-side Shox2 expression, which is prominent in the sinus venosus myocardium compromising the developing SAN region and the venous valves within the right atrium.

To elucidate the predicted functions of Shox2 in sinus venosus myocardium development, we have generated mice carrying a targeted mutation of the Shox2 gene and observed embryonic lethality in Shox2−/− embryos at 11.5

Figure 3. Shox2-deficient mice exhibit a different expression pattern of Nkx2.5 in the myocardium of the developing sinus venosus (SV), including the SAN region. The 3-dimensional reconstructions show the dorsal view of a wild-type (WT) (A) and a Shox2−/− (B) embryonic heart of 11.5 dpc, in which the MLC-2a– and Nkx2.5-positive myocardium of the atria and ventricles is indicated in brown and gray, respectively (A, B). The MLC-2a–positive myocardium of the SV is indicated in lime green where there is Nkx2.5 negativity (A, B) and black where Nkx2.5 is aberrantly positive (B). The blue arrow (A) indicates the SAN region in a WT embryo; the red arrow (B) indicates the same region in a Shox2−/− embryo. Immunohistochemical analysis demonstrates that the SAN region in both WT (D detail of box in C) and Shox2−/− hearts (D detail of box in F) is positive for MLC-2a. G, Section of a WT heart in which the box indicates the SAN region, which is negative for Nkx2.5 (H enlargement of box in G). In WT hearts, the venous valves (arrows in L, enlargement of dotted box in L) are positive for Nkx2.5. Remarkably, in Shox2−/− embryos, the hypoplastic SAN region (J enlargement of box in L) is positive for Nkx2.5. Furthermore, the Nkx2.5-positive venous valves in Shox2−/− embryos appeared to be severely hypoplastic (arrowheads in K, enlargement of dotted box in L). In WT embryonic hearts, the MLC-2a–positive myocardium in which the SV is incorporated (M) is negative for Nkx2.5 (N). Conversely, in Shox2−/− embryos, this specific myocardium is positive for both MLC-2a (O) and Nkx2.5 (P). In the 3-dimensional reconstruction, gray shows the region negative for myocardium surrounding the pulmonary veins; blue transparent, lumen of the SV; and pink, pulmonary veins. LA indicates left atrium; RA, right atrium; LV, left ventricle; and RV, right ventricle. Scale bars: C, F, G, L=300 μm; all others=60 μm.
to 13.5 dpc resulting from cardiovascular failure. We have shown that there is a functional relationship between Shox2 and the formation of the myocardium of the PHF, which is evident from the observation that the PHF myocardial areas that normally express Shox2 are severely hypoplastic in Shox2−/− embryos. This leads to diminished myocardium surrounding the cardinal veins and a marked hypoplasia of the venous valves and the SAN region. As it is not known exactly where and to what degree the sinus venosus myocardium is incorporated into normal atrial development, it is possible that the observed thin dilated atrial wall is also a result of the insufficient recruitment of sinus venosus myocardium or improper replication and differentiation of the existing or newly added myocardium. Alternatively, it also can be explained by ultimately failing heart functions. The latter could be attributed to a deficient function of the hypoplastic and abnormally differentiated SAN region.

Identification of regulatory pathways involved in CCS formation has been impeded mainly by an insufficient definition of the developmental origin of structures involved in these processes and the unavailability of animal models specifically targeting these structures. The transition from descriptive to molecular analyses of CCS development has recently gained momentum with the generation of minK-lacZ and CCS-lacZ transgenic mice.22–24 Interestingly, the specific pattern of expression observed for Shox2 strongly correlates with the lacZ expression in the SAN region and venous valves in these transgenic animals. Shox2 therefore represents an example of a homeodomain transcription factor with an expression pattern that overlaps part of the developing CCS.

It is therefore appealing not only to show a role for Shox2 in the formation of the PHF myocardium but also to postulate a function in the differentiation of the SAN region, the future pacemaking area. We were able to verify this hypothesis by analyzing the physiological effects of a specific downregulation of Shox2 expression by antisense morpholino injections into zebrafish embryos. Indeed, anti-Shox2–injected embryos exhibited severe bradycardia and intermittent sinus exit blocks, suggesting severe sinus arrhythmia and/or pacemaking system malfunction as the primary cause of death in Shox2−/− deficient animals.

To gain more insight into the cellular and molecular mechanisms underlying the proposed Shox2–related SAN region malfunction, we have investigated the expression of Nkx2.5, Cx40, and Cx43 in this area in wild-type and knockout embryos. In our model, we confirm that Nkx2.5 expression is absent in the cardiomyocytes of the normal developing SAN region7,25 that are destined to acquire pacemaking properties. It can be postulated that the aberrant Nkx2.5 positivity in the SAN region of the Shox2 knockout mouse interferes with its normal pacemaking function. This result is supported by recent data showing that transgenic mice ectopically expressing Nkx2.5 under the control of an α-MHC promoter present with sinus bradycardia and prolonged PR intervals.26 It seems tempting to postulate that the molecular pathway underlying the observed sinus bradycardia in our zebrafish is identical in Shox2−/− and α-MHC-Nkx2.5 transgenic animals. It can furthermore be presumed that this mechanism acts in a stringent regional manner because Nkx2.5−/− driven pathways have recently been shown to be indispensable for maturation and maintenance of other conduction system components, including atrioventricular nodal cardiomyocyte lineage specification.27 The aberrant spatiotemporal Nkx2.5 regulation in Shox2−/− embryos was observed only within the hypoplastic SAN region, not within other regions exhibiting high levels of Shox2 expression, including the venous valves that in normal development do not show a lack of Nkx2.5 expression. In addition, Nkx2.5 expression is present in the myocardium of the atrioventricular conduction system in both normal and Shox2−/− embryos. Interestingly, this phenomenon also sheds light on the observation that patients diagnosed with Nkx2.5 haploinsufficiencies exhibit atrioventricular but not sinoatrial node dysfunction.12–18 With our model, we can for the first time sufficiently explain both aspects of this phenotype. Although the absent or abrogated Nkx2.5 expression explains atrioventricular conduction problems, these patients may not exhibit sinoatrial node disease because the devel-
oping SAN region is not primarily hampered because this area normally does not express Nkx2.5.

Cx40 and Cx43 have been used mainly to characterize the adult SAN but also have value as markers for earlier differentiation stages. We have shown aberrant expression of these markers in the mutant SAN region. Because Nkx2.5 has been reported to be involved in the regulation of connexins, the aberrant expression of Nkx2.5 might be linked to the abnormal expression patterns of Cx40 and Cx43 and thus might explain a postulated disturbed pacemaking function in Shox2−/− embryos.

It is not clear at this stage whether Nkx2.5 expression is directly regulated by Shox2 in the SAN region or if this is a downstream event within a more elaborate pathway. It is evident, however, that a complete understanding of the molecular mechanisms underlying the observed Nkx2.5 regulation requires identification of additional regulatory molecules specifically expressed within the SAN region and investigation of their potential to interact with and modulate the functional properties of Shox2.

In summary, our data demonstrated the essential role of Shox2 in the developing embryonic heart. We have shown that Shox2 is necessary for the normal anlage of the PHF myocardium, which is uniquely Nkx2.5 negative. Furthermore, we have established a functional link between Shox2 and the expression of Nkx2.5, which itself was shown to play an important role in the development and maturation of the SAN region. This observation provides a working hypothesis to further investigate the recruitment of sinus venosus myocardium and the molecular pathways underlying critical cell fate decisions that are required for pacemaking differentiation.

Acknowledgments
With deep regret, we announce that our first author, R.J. Blaschke, at the age of 43, passed away earlier this year.

Sources of Funding
This work was supported in part by the Deutsche Forschungsgemeinschaft and by the Gisela Thier Fonds (to N.D. Hahurij).

Disclosures
None.

References
23. Blaschke et al Cardiac Malformations in Shox2 Knockout Embryos 1837
We have shown that in early cardiac development the venous pole of the heart is subjected to extensive remodeling. In addition, recruitment of second heart field sinus venosus myocardium is seen. This myocardium forms a U-shaped band lining the base of the left and right cardinal veins through the area of the dorsal mesocardium. This sinus venosus myocardium is unique in that it does not express the precardiac marker Nkx2.5. Pacemaking activity is already functional in the sinus venosus myocardium during development and is restricted in adult life to the sinoatrial node. Shox2, a homeobox gene highly homologous to SHOX, which is involved in short-stature syndrome in humans, is a novel marker for the sinus venosus myocardium. To unravel the role of Shox2 in heart development, Shox2 knockout mice were made. These mice showed embryonic lethality between 11.5 and 13.5 dpc, and sinus venosus myocardium was markedly hypoplastic, including the sinoatrial nodal region. This latter region also showed abnormal differentiation in that genes that are normally negative in the developing node (Nkx2.5, connexin 40, and connexin 43) were now aberrantly positive. Shox2 downregulation in zebrafish resulted in marked bradycardia because of a diminished pacemaking function. Therefore, we assume that the embryonic lethality in the Shox2 mutant mice might result from a comparable process. The hypoplasia and abnormal differentiation of the sinoatrial nodal region could lead to a disturbed pacemaking function and resultant heart failure.
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\textit{Circulation}. 2007;115:1830-1838; originally published online March 19, 2007;
doi: 10.1161/CIRCULATIONAHA.106.637819
\textit{Circulation} is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/115/14/1830

Data Supplement (unedited) at:
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