The development of the venous pole of the heart
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Chapter 5

Molecular pathway for the localized formation of the sinoatrial node


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Abstract

The sinoatrial node, which resides at the junction of the right atrium and the superior caval vein, contains specialized myocardial cells that initiate the heart beat. Despite this fundamental role in heart function, the embryonic origin and mechanisms of localized formation of the sinoatrial node have not been defined. Here we show that subsequent to the formation of the Nkx2-5-positive heart tube, cells bordering the inflow tract of the heart tube give rise to the Nkx2-5-negative myocardial cells of the sinoatrial node and the sinus horns. Using genetic models, we show that as the myocardium of the heart tube matures, Nkx2-5 suppresses the pacemaker channel gene Hcn4 and T-box transcription factor gene Tbx3, thereby enforcing a progressive confinement of their expression to the forming Nkx2-5-negative sinoatrial node and sinus horns. Thus, Nkx2-5 is essential for establishing a gene expression border between the atrium and sinoatrial node. Tbx3 was found to suppress chamber differentiation, providing an additional mechanism by which the Tbx3-positive sinoatrial node is shielded from differentiating into atrial myocardium. Pitx2c-deficient fetuses form sinoatrial nodes with indistinguishable molecular signatures at both the right and left sinoatrial junction, indicating that Pitx2c functions within the left/right pathway to suppress a default program for sinoatrial node formation on the left. Our molecular pathway provides a mechanism for how pacemaker activity becomes progressively relegated to the most recently added components of the venous pole of the heart, and, ultimately, to the junction of the right atrium and superior caval vein.
Introduction

The sinoatrial node (SAN) is the pacemaker of the heart that initiates the heartbeat and controls its rate of contraction. It is a specialized myocardial structure positioned at the junction of the right atrium and the superior caval vein. Disease, ageing, or gene defects may cause SAN dysfunction (sick sinus syndrome), a common cardiac disorder that currently requires the implantation of a pacemaker. SAN function depends on a complex tissue architecture and the expression and function of a set of ion-channel, sarcomeric and gap junction proteins expressed at levels distinct from the atrial working myocardium. Hyperpolarization-activated pacemaker channel Hcn4 is expressed in the SAN and plays an important role in pacemaker activity in vivo. Furthermore, the SAN expresses very low levels of Connexin 40 (Cx40) and Cx43 gap-junctional subunits that are essential for the fast conduction of the electrical impulse in the atrial and ventricular working myocardium and ventricular conduction system. The T-box transcription factor, Tbx3, which, when mutated, causes ulnar-mammary syndrome of congenital malformation in human, is expressed in the developing nodal tissues of the heart of mammals and chicken and is able to suppress promoter activity of atrial chamber myocardial genes including natriuretic peptide precursor type A (Nppa, encoding atrial natriuretic factor) and Cx40 in cell culture. Despite its fundamental role in heart function, the embryonic origin of the SAN and the mechanisms of its patterning and formation are still enigmatic. In the present study we investigated the spatio-temporal patterns of genes relevant to the developing inflow tract, SAN and atrium and provide evidence for roles for Nkx2-5 (NK2 transcription factor related, locus 5 [Drosophila]), Pitx2c (paired-like homeodomain transcription factor 2), and Tbx3 in the patterning and formation of the SAN.

Materials and Methods

Mice
The R26R, Nkx2-5, Nkx2-5ires-Cre, Pitx2c, and Tbx3 transgenic mouse lines have been described previously. A cDNA fragment encoding the full-length human TBX3 protein was cloned in a construct containing a 5-kb regulatory fragment of the murine β-Mhc gene and the human growth hormone pA signal, kindly provided by Dr. J. Robbins (Children’s Hospital Research Foundation, Cincinnati, Ohio). As a control, a cDNA fragment encoding TBX3 with a point mutation in the T-box DNA binding region (L143P) was cloned in the same construct. Vector sequences of these constructs were removed and the DNA was microinjected into nuclei of FVB
zygotes, which were subsequently implanted into fosters to generate transgenic embryos. At embryonic day 9.5 (E9.5), embryos were isolated for analysis. Embryos and fetuses were dissected in PBS and fixed in 4% paraformaldehyde overnight. Amnion or tail biopsy genomic DNA was used for PCR assays to detect the Cre, lacZ, GFP or TBX3 transgenes.

**β-galactosidase activity detection and immunohistochemical analyses**
Detection of β-galactosidase activity on 20 μm cryostat sections was performed as described.\(^\text{15}\) For immunohistochemistry on 10 μm embryo sections, the following primary antibodies were used: polyclonal antibody against cardiac troponin I (cTnI) (1:1000; Hytest Ltd); polyclonal antibody against Nkx2-5 (1:250; Santa Cruz Biotechnology); polyclonal antibody against Hcn4 (1:200; Chemicon); polyclonal antibody against Cx40 (1:200; Chemicon); monoclonal antibody against desmin (1:50; Monosan).

**Non-radioactive in situ hybridization**
Non-radioactive in situ hybridization of 12 μm embryo sections was performed as described.\(^\text{16}\) The probes for Is1\(^\text{17}\) and Hcn4\(^\text{18}\) were kindly provided by S. Evans (Skaggs School of Pharmacy, University of California, San Diego) and B. Santoro (Center for Neurobiology and Behavior, Columbia University, New York). Other probes have been described previously.\(^\text{16}\) All patterns in wild-type embryos were observed in at least 3 independent embryos.

**Three-dimensional reconstructions**
Three-dimensional visualization and geometry reconstruction of patterns of gene expression determined by in situ hybridization was performed as described previously.\(^\text{19}\) Files with reconstructions are available upon request.
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Figure 1. Spatial and temporal patterns of SAN and atrial genes indicate progressive patterning and formation of the primordial SAN. A, Embryos stained whole mount show expression of Nppa in the atria and ventricles at E9.5 and E10.5. The black line indicates the level of sectioning in panel B. B, Three sister sections of an E9.5 heart show coexpression of cTnl, Nkx2-5 and Hcn4 in the inflow tract. Three sister sections of an E10.5 heart show expression of Hcn4 selectively in the sinus horns, and of Nkx2-5 excluded from the sinus horns (black arrowheads indicate the sinus horn myocardium). Three sister sagittal sections of an E10.5 heart show some overlap in expression of Hcn4 and Nkx2-5, also in the Nkx2-5-positive cells of the second heart field in the dorsal mesocardium (red arrowheads). Black arrowheads indicate the sinus horn myocardium. C, Whole mount Nppa stained E9.5 embryo indicating plane of sectioning (black line) and Cx40 expression in the inflow tract fated to become atrium. Two pairs of sister sections of an E10.5 embryo showing the expression of Nppa and Cx40 in the atria but not in the sinus horns, and Tbx3 expression in a right-sided subdomain of the Cx40-negative sinus horns (*). a indicates atrium; Cra, cranial; Cau, caudal; d, dorsal; dm, dorsal mesocardium; ift, inflow tract; l/rsh, left/right sinus horn; l/ra, left/right atrium; l/rv, left/right ventricle; v, ventral. Bars represent 100 μm.
Figure 2. Expression and lineage analysis of the venous pole. A, Three-Dimensional reconstructions of E14.5 wild-type embryos showing the lumen (orange) of the heart from dorsal (top panels) and from the right side (bottom panels). In red, Tbx3-positive myocardium; in gray, Cx40-negative myocardium. The Tbx3 expression pattern is confined to the Cx40-negative myocardium. B, sister sections of a wild-type E11.5 embryo double-stained with antibodies against desmin (blue) and Hcn4 or Nkx2-5 (pink). Nuclei are stained with SYTOX Green (green). C and D, Sister sections of E14.5 wild-type embryos showing the mutually exclusive expression patterns in the SAN and right atrium. E, Schematic representation of the patterns of expression until E9.5 (left) and between E9.5 and E14.5 (middle). Dotted lines represent borders of the expression domains of Nkx2-5 and Pitx2c. Except for the yellow-colored mesenchyme, only myocardium is depicted (see legend in right panel). san indicates sinoatrial node; l/rsh, left/right sinus horn; l/ra, left/right atrium; avc, atrioventricular canal; l/rv, left/right ventricle; vv, venous valves; ift, inflow tract; l/raka, left/right atrial appendage; as, atrial septum; pv, pulmonary vein. Bars represent 100 μm.
Results

Expression and genetic lineage analysis reveal a mechanism of SAN development

To investigate the formation of the SAN in development, we assessed the expression patterns of Hcn4, a SAN marker; Nppa and Cx40, atrial chamber myocardial markers; Tbx3, a developing conduction system marker and putative repressor of Nppa and Cx40; Nkx2-5, a key cardiac homeobox gene expressed in the first and second heart fields and essential for the activation of Cx40 and Nppa, and cTnl, desmin and myosin light chain 2a (Mlc2a), which mark all differentiated myocardium. “Inflow tract” refers to the myocardial intake at the caudal end of the heart tube until E9.5. “Venous pole” refers to the myocardial components of the systemic venous return that are added to the heart from E9.5 onward, including the SAN (this study), venous layer of the bilayered venous valves, and left and right sinus horns.

The E9.5 heart tube coexpressed myocardial marker genes and Nkx2-5. The inflow tract in addition expressed Hcn4 in a pattern that initially overlaps that of Nkx2-5 (Figure 1A and 1B). Expression of Cx40 had been initiated at this stage in the Nkx2-5-positive inflow tract (Figure 1B and 1C). After E9.5, Nkx2-5-negative myocardial sinus horns are formed from cardiac progenitor cells adjacent to the inflow tract. At E10.5, Cx40 and Nppa were expressed in the Nkx2-5-positive myocytes, which at this stage have contributed to the atrial chambers and atrial layer of the venous valves. Hcn4 expression was down-regulated in this myocardium to become confined to the newly formed Nkx2-5-negative venous pole (Figure 1B and 1C). Thus, an early pattern in which Nkx2-5 and Cx40 expression overlaps that of Hcn4 resolves into a mutually exclusive pattern by E10.5.

From E10.5 on, a thickening of myocardial cells was formed in the right sinus horn bordering but exclusive of the Nkx2-5/Cx40/Nppa-positive atrial cells, corresponding to the primordial SAN (Figure 1C). This structure also expressed Tbx3. Three-dimensional reconstructions show that the expression domains of Tbx3 and of Cx40 were mutually exclusive (Figure 2A). At the atrial side, the Tbx3 expression domain bordered the Cx40-positive domain, but neither Tbx3 nor Cx40 were expressed in the myocardium of the sinus horns (Figure 1C and 2A). Together, these data indicate the existence of 3 bordering domains at the sinuatrial junction, an atrial domain, a SAN domain and a sinus horn domain (Figure 2B through 2E).

To address whether the Nkx2-5-negative SAN primordium is derived from Nkx2-5-positive inflow tract myocardium, which subsequently loses Nkx2-5 expression, or, like the sinus horns, from adjacent Nkx2-5-negative mesenchyme, we crossed Nkx2-5ires-Cre mice with R26R mice to reveal all cells derived from Nkx2-5-positive cells. These data showed that the Tbx3-positive, Cx40-negative SAN pri-
mordium was devoid of Nkx2-5-Cre-positive cells, and therefore never expressed Nkx2-5 (Figure 3A and 3C). We conclude that the primordial SAN myocardium is formed de novo from Nkx2-5-negative cells.

LIM homeodomain transcription factor islet-1 (Isl1) is reported to be a marker of the second heart field precursors that is switched off when these mesenchymal precursors differentiate to myocardium. However, probably because of the half-life of its transcripts, Isl1 expression remains detectable shortly after differentiation of the precursors into myocardium. Isl1 expression was still observed in the Hcn4-positive primordial SAN (Figure 3B), providing support for de novo differentiation of the SAN from second heart field progenitors.

Nkx2-5 suppresses Hcn4 and Tbx3 and establishes the sinoatrial boundary
The boundary between the SAN and atrial myocardium could be established at least in part if Nkx2-5 acted as a repressor of Hcn4 and Tbx3. We therefore tested the expression of Hcn4 and Tbx3 in Nkx2-5-deficient mice. In these embryos, heart development is arrested during looping, chamber formation does not occur, and Cx40 and Nppa are not activated. We found a marked ectopic expression of both Hcn4 and Tbx3 throughout the heart tubes of Nkx2-5-deficient embryos (Figure 4A), showing that Nkx2-5 is required to repress these genes in the heart tube. These observations indicate a critical role of Nkx2-5 in the confinement of Hcn4 and Tbx3 to the Nkx2-5-negative venous pole domain (Figure 4C).

Because Nkx2-5 mutant mice die at E10, this model does not allow the analysis of the SAN region in a more advanced state of development. We investigated fetal mice compound heterozygous for the Nkx2-5ires-Cre allele and for a null allele. These mice express ~25% of the normal level of Nkx2-5 expression in wild-type, because theires-Cre insert in the 3’ untranslated region of the Nkx2-5 gene creates a hypomorphic allele (O.P, R.P.H., submitted manuscript, 2006). Nkx2-5ires-Cre / - mice die shortly after birth showing a range of cardiac malformations, including atrial and ventricular septal defects, much more severe than haploinsufficient mice. As controls, Nkx2-5ires-Cre / + mice were used which appeared completely normal. In Nkx2-5ires-Cre / - E14.5 fetuses, the Hcn4 and Tbx3 expression domains of the SAN were much broader and extended into the atrium (Figure 4B). Thus, the expression border between the SAN and atrium was blurred. Cx40 expression was reduced in these hearts, consistent with the notion that Nkx2-5 is required for Cx40 expression, but was particularly weak in the enlarged Hcn4 and Tbx3 domain around the SAN (Figure 4B). These observations indicate a critical role of Nkx2-5 in the regulation and maintenance of the atrial chamber gene program at the border with the SAN domain (Figure 4C).
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**Figure 3. SAN forms from Nkx2-5-negative precursors.** A, E12.5 Nkx2-5<sup>ires-Cre</sup>/R26R embryo stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (Xgal) (n=3) and the corresponding area in an E12.5 wild-type embryo stained for Cx40 and Tbx3. The black box in the left panel depicts the area enlarged in the right panels. Arrows depict the Xgal and Cx40-negative, Tbx3-positive SAN area. B, Sister sections of the SAN area of an E10.5 wild-type embryo. Black arrows, overlap of cTnl, Hcn4 and Isl1 expression in the Nkx2-5-negative primordial SAN. C, Simplified scheme of the development of the venous pole and the SAN. At E9.5 the inflow tract myocardium expresses both Hcn4 and Nkx2-5. After E9.5, the Hcn4-positive area becomes gradually confined to the Nkx2-5-negative myocardium venous pole that is recruited from adjacent non-cardiac progenitor cells. Black arrows indicate recruitment of mesenchyme into the Nkx2-5-negative myocardial venous pole lineage. Part of the Nkx2-5-negative myocardium initiates Tbx3 expression and thickens to form the SAN primordium. After E14.5 Hcn4 expression becomes confined to the Tbx3-positive SAN, whereas in the sinus horn myocardium Cx40 is up-regulated. ra indicates right atrium; rsh, right sinus horn; rvv, right venous valve. Bars represent 100 μm.
Figure 4. Nkx2-5 represses *Hcn4* and *Tbx3* and is required to establish the atrial-SAN boundary. A, E9.0 Nkx2-5<sup>+/−</sup> and Nkx2-5<sup>−/−</sup> embryos stained whole mount for *Tbx3* (<i>n</i>=3) and on sister sections for myocardial marker *Mlc2a* and for *Hcn4* (<i>n</i>=3). Arrow depicts the upregulation of *Tbx3* and *Hcn4* throughout the entire heart in absence of Nkx2-5. B, Sister sections of Nkx2-5<sup>ires-Cre/−</sup> (control; <i>n</i>=7) and Nkx2-5<sup>ires-Cre/−</sup> (hypomorphic; <i>n</i>=9) embryos of E14.5. Arrows depict the expression border of SAN and atrial genes. C, Scheme depicting roles and genetic interactions found in this study. Factors present in a given compartment are depicted in black, factors absent from a compartment in gray. *ev* indicates embryonic ventricle; *ift*, inflow tract; *Ish*, left sinus horn; *ra*, right atrium; *san*, sinoatrial node; *vv*, venous valves. Bars represent 100 μm.
**Tbx3 suppresses chamber myocardial differentiation and gene expression**

Our analysis revealed that *Tbx3* could be involved in the suppression of atrial chamber differentiation and repression of chamber gene expression, thus contributing to the chamber-negative phenotype of the SAN. We analysed *Tbx3* mutant embryos which die from between E11-E13. Embryos that clearly survived until E11.5 had formed a primordial SAN-like structure (Figure 5A). Moreover, *Nkx2-5*, *Cx40* and *Nppa* were not ectopically activated in this primordium, and *Hcn4* expression was maintained (Figure 5A). Notably, because *Nkx2-5* is strictly required for *Nppa* and *Cx40* gene activity, maintenance of the Nkx2-5 boundary at the junction between the atrium and SAN/venous pole at this developmental stage provides a likely explanation for the maintenance of the SAN gene expression signature in the *Tbx3* mutants.

Nonetheless, Tbx3 may provide a tuning mechanism to ensure stabilization of SAN gene expression, in particular, to ensure the absence of the atrial differentiation program. To investigate whether *Tbx3* is able to suppress chamber myocardial differentiation and *Nppa* and *Cx40* in vivo, transgenic embryos were generated that express the human *Tbx3* cDNA under the control of the β-Mhc promoter, which drives expression in the tubular heart prior to the differentiation of chamber myocardium. To evaluate possible nonspecific effects of *Tbx3* overexpression, eg, by competing for limiting cofactors, we also generated embryos that express Tbx3-LP, which contains an L to P substitution at position 143 in the T-box domain that has been found in ulnar-mammary syndrome patients. Tbx3-LP and Tbx3 protein showed comparable stability in transfected cells, but Tbx3-LP is not able to bind to its DNA binding sites. Transgenic embryos were analyzed at E9.5, after the initiation of chamber differentiation and *Nppa* and *Cx40* expression. These embryos were smaller but not obviously abnormal with respect to general body patterning (Figure 5B). Hearts of these embryos were linear, or looped to some extent, and partially or completely failed to form chambers (Figure 5B and 5C). Section in situ hybridization showed that *Nppa* and *Cx40* were not expressed in these hearts, indicating that their expression was suppressed by *Tbx3* (Figure 4C and 5C). However, *Hcn4* was not precociously activated in the Tbx3-overexpressing hearts (data not shown), supporting the data obtained in the *Tbx3* mutant suggesting that Tbx3 does not directly regulate *Hcn4* (Figure 4C). Tbx3-LP embryos were normal, and even though Tbx3-LP was robustly expressed in the hearts, the morphology and the expression of *Cx40* and *Nppa* expression were not affected (Figure 5C). We conclude that *Tbx3* is able to specifically block chamber myocardial differentiation and the expression of *Nppa* and *Cx40* in vivo in a DNA-binding dependent manner.
**Pitx2c suppresses SAN formation in the left side of the venous pole**

The Pitx2 homeobox factor is essential for late aspects of left-right asymmetric morphogenesis.\(^{25}\) Pitx2c-deficient mutant mice have right isomerism of the atria and venous pole.\(^ {11}\) In situ hybridization showed that the primordial SAN does not express Pitx2c (Figure 6A). We investigated whether Pitx2c is also involved in the placement of the primordial SAN. Pitx2c mutant embryos of E9.5, shortly before the primordial SAN is formed and the asymmetry of the sinus horns becomes apparent,\(^ {25}\) have apparently normal inflow tracts (not shown). However, after E10.5, shortly after initiation of the formation of the primordial SAN, we noted that the atria had 2 sets of venous valves, and 2 short sinus horns, indicating right isomerism of the left venous pole (Figure 6B). Moreover, a second SAN primordium was invariably observed at the left side. This left-sided SAN primordium showed an appropriate Hcn4-positive, Cx40 negative SAN gene program. At E15.5, the left-sided SAN contained the typical nodal artery, and the gene expression profile of this SAN was indistinguishable from that of the right sided SAN in the mutant, and of the SANs of wild-type littermates. We conclude that the default morphogenetic pathway of the venous pole leads to the formation of a right-sided sinus horn, SAN and venous valves, and that the Pitx2c pathway suppresses SAN formation.

**Atrialization of the venous pole**

From the above we, conclude that after establishment of an Nkx2-5-expressing heart tube, the sinus horn myocardium and SAN are formed from cardiac field cells via a novel, Nkx2-5-independent pathway and run a nodal gene program. However, from E14.5 onward, expression of Cx40 was up-regulated in the sinus horn myocardium, whereas that of Hcn4 was gradually lost from the sinus horn myocardium (Figure 7A through 7D). Furthermore, the sinus horn myocardium gradually became positive for Nkx2-5 expression (Figure 7C and 7D). Moreover, regions within the SAN initiated Nkx2-5 expression, which now for the first time overlapped with Tbx3 expression (Figure 7E through 7G). However, Tbx3 and Hcn4 were not downregulated and the SAN remained free of Cx40 expression (Figure 7F and 7G). Taken together, the data suggest that in the fetal period, the venous pole myocardium obtains an atrial gene program, except for the SAN, which, despite the up-regulation of Nkx2-5, still runs the Tbx3/Hcn4-positive, Cx40-negative nodal gene program (Figure 3C).
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Figure 5. Tbx3 suppresses chamber differentiation and Nppa and Cx40 gene expression in vivo. A, Sister sections of E11.5 Tbx3++ and Tbx3-- littermates. Arrow depicts the absence of Cx40 and presence of Hcn4 in the SAN primordium of both the wild-type and Tbx3-deficient embryo (n=2). A sinoatrial node primordium was observed in 4 of 4 mutant embryos. B, E9.5 βMhc-hTBX3 and wild-type littermate. C, Serial sections of E9.5 wild-type, βMhc-hTbx3 and βMhc-hTBX3LP embryos and a whole-mount picture of the heart region of the embryo showing the absence of Nppa and Cx40 in the Tbx3-overexpressing βMhc-hTBX3 embryo. Of βMhc-hTBX3 embryos (n=6), 3 had linear heart tubes, 1 had a looped heart tube but no chambers, and 2 showed looping and thin-walled chambers. Hearts of all βMhc-hTBX3LP embryos (n=5) were normal. Hearts of all wild-type littermates examined (n=30) showed normal looping and chamber formation. avc indicates atrioventricular canal; ht, heart; lsh/rsh, left/right sinus horn; lv, left ventricle; san, sinoatrial node. Bars represent 100 μm.
Discussion

In this study, we demonstrate that the SAN initially forms from Nkx2-5-negative precursors, and remains largely free of Nkx2-5 expression until the onset of a second phase of atrial myogenesis in the venous pole beginning at E14.5. Nkx2-5 is a cardiac homeobox gene critical for heart development in all species examined thus far and is at the core of transcriptional regulatory mechanisms that control gene expression and differentiation in the heart.\textsuperscript{21} Heterozygous mutations in Nkx2-5 in humans cause congenital atrioventricular conduction system defects.\textsuperscript{27} Moreover, the atrioventricular node, which expresses relatively high levels of Nkx2-5, is hypoplastic in heterozygous mutant mice.\textsuperscript{28} Therefore, although the SAN and the atrioventricular node share many phenotypic features, the differential expression of Nkx2-5 is a fundamental difference between the 2 nodes, implying that distinct regulatory mechanisms control their formation and function.

Figure 6. Pitx2c represses SAN gene program and morphogenesis at the left side. A, Sister sections of an E13.5 wild-type embryo stained for Nkx2-5 and Pitx2c expression. Arrows indicate lack of expression of Pitx2c in Nkx2-5-negative SAN. B, Sister sections of an E11.5 (n=5) and an E14.5 (n=11) Pitx2c\textsuperscript{-/-} embryo. Arrows indicate an Hcn4/Tbx3-positive, Nkx2-5/Cx40-negative SAN at both right and left side. avc indicates atrioventricular canal; la/ra, left/right atrium; lsh/rsh, left/right sinus horn; lv, left ventricle; san, sinoatrial node. Bars represent 100 μm.
Early localization of pacemaker activity and patterning of the venous pole

All embryonic cardiomyocytes of the early developing heart possess intrinsic molecular pacemaker activity, which is followed by contractility slightly later. However, dominant pacemaker activity is always found at the intake (inflow tract, venous pole) of the developing heart tube.\(^{29,30}\) \textit{Hcn4} is required for pacemaker activity in mouse embryos, whereas humans with a mutation in \textit{HCN4} have bradycardia.\(^{2,3}\) The steep caudo-cranial expression gradient of \textit{Hcn4} observed from E7.5 in the cardiac crescent\(^{31}\) and subsequent expression in the entire venous pole (SAN and sinus horns; Figures 1B, 2E, and 3C) may well explain the observation that dominant pacemaker activity is always localized at the intake.

The embryonic heart tube elongates by recruitment of cardiac field cells to the heart tube and inflow complex.\(^{20}\) In the inflow tract region, the atria are formed first, followed after E9.5 by the sinus horns\(^{16}\) and the primordial SAN (this study). Expression of \textit{Hcn4} initially overlaps with that of \textit{Nkx2-5} but subsequently becomes restricted to the newly recruited \textit{Nkx2-5}-negative venous pole components, indicating that \textit{Hcn4} expression is extinguished in the \textit{Nkx2-5}-positive myocardium (Figures 2E and 3C), which is fated to form the atria and atrial layer of the venous valves.\(^{16}\) In line with these patterns of expression, \textit{Nkx2-5}-deficient embryos showed a dramatic ectopic expression of \textit{Hcn4} in the heart tube, explaining previous observations that a large fraction of \textit{Nkx2-5}-deficient embryos initiate beating from the embryonic ventricular region rather than from the inflow tract.\(^{22}\) Together, these data indicate that \textit{Hcn4} expression is initiated by unknown factors in newly formed venous pole myocardium and that as the heart matures \textit{Nkx2-5} represses \textit{Hcn4}, thereby confining its expression to the \textit{Nkx2-5}-negative venous pole (Figures 3C and 4C). This repressive activity for \textit{Nkx2-5} on \textit{Hcn4} provides a mechanism for the progressively shift of \textit{Hcn4} expression as well as pacemaker activity to the newly formed venous pole components in normal embryos (Figures 3C and 4C). This mechanism provides an explanation for the longstanding observation that during heart development dominant pacemaker activity is always localized at the caudal end (intake) of the heart.\(^{29,30}\)

Patterning and formation of the SAN

\textit{Nkx2-5}-deficient embryos showed ectopic expression of \textit{Hcn4} and \textit{Tbx3} in the whole heart tube. Furthermore, in \textit{Nkx2-5} hypomorphic fetuses the otherwise sharp expression boundary between the atria and SAN was blurred. The expression of \textit{Tbx3} and \textit{Hcn4} extended into the right atrium, whereas \textit{Cx40} expression was specifically downregulated in the extended \textit{Tbx3}-positive area. These observations indicate that \textit{Nkx2-5} is required to establish the boundary between the atria and SAN and, in a dose-dependent manner, to prevent the SAN phenotype from invading the atria, or the atrial phenotype invading the SAN (Figure 3C and 4C).
Figure 7. Atrialization of the venous pole. A, Sister sections of an E14.5 heart. The Tbx3-negative left sinus horn coexpresses Cx40 and Hcn4 (red arrows). B, Section of an E17.5 wild type heart showing the downregulation of Hcn4 in the sinus horns, whereby its expression is confined to the SAN. C and D, Sister sections of an E17.5 wild-type heart. Black arrows show the up-regulation of Nkx2-5 and Cx40 in the sinus horns. The red arrows indicate also some up-regulation of Nkx2-5 in the Cx40-negative SAN region. E, E15.5 (n=2) and E17.5 (n=2) Nkx2-5ires-Cre/R26R embryos double-stained for Xgal and cTnl. Note the increase in recombination in the SAN between these stages. F, Sister sections of an E17.5 heart show upregulation of Nkx2-5 mRNA in the Tbx3-positive SAN (red arrows). G, Sister sections of an E17.5 heart show the presence of Nkx2-5 in the Cx40-negative SAN (white arrows). cg indicates cardiac ganglion; Ish/rsh, left/right sinus horn; na, nodal artery; ra, right atrium; san, sinoatrial node. Bars represent 100 μm.
A next step involves asymmetrical gene expression and morphogenesis after E9.5, which leads to the formation of a Tbx3-expressing SAN primordium at the right sinoatrial junction. Human patients and iv/iv mice with right isomerism have 2 sinoatrial nodes, whereas in patients and mice with left isomerism the sinoatrial node is hypoplastic or absent. Therefore, the SAN is an integral component of the right side-specific morphogenesis program that is repressed by Pitx2c. We found that Pitx2c-deficient embryos form 2 SANs running the SAN signature gene program. Therefore, the complete right-sided morphogenesis program, including Tbx3 expression, is the default program, which in the left sinoatrial region is masked and converted to a left-sided program without a SAN by Pitx2c. These observations also indicate that Pitx2c is a critical factor for the entire left morphogenetic program in the sinoatrial region. None of the genes studied here were deregulated in other sites of Pitx2c expression of Pitx2c mutants, including the atrioventricular canal (Tbx3) or atria (Nkx2-5, Nppa, Cx40) (Figure 6B and data not shown), indicating that Pitx2c does not directly control expression of these genes. Asymmetry of the venous pole is first observed after E9.5, when the sinus horns are being formed from their progenitors. Pitx2 was found to control asymmetric morphogenesis at least in part by regulation of cell-type specific proliferation. It is expressed in the left-sided myocardium from very early stages on and, therefore, may control proliferation of the left venous pole myocardium as soon as it is recruited from the progenitor pool.

Finally, after E14.5 the nodal gene program becomes confined to the SAN domain. The sinus horns, at the end of gestation recognizable as the myocardial sleeves surrounding the right and left caval veins and sinus venarum, gradually switch to a Cx40-positive, Hcn4-negative atrial gene program (Figure 3C). Nkx2-5 expression gradually increased toward the end of gestation, indicating that it may be responsible for the switch to the atrial gene program, although the involvement of other factors can not be excluded at this point. However, despite the up-regulation of Nkx2-5 expression in the SAN, Cx40 was not activated here, and Hcn4 was not repressed. Therefore, we hypothesize that in the SAN primordium, Tbx3 expression is, or has become, insensitive to Nkx2-5, and further that Tbx3 shields the SAN domain from atrial gene expression and differentiation once Nkx2-5 is being activated in the SAN (Figure 4C). Although this putative function of Tbx3 remains to be demonstrated, it is consistent with the found ability of Tbx3 to suppress Nppa and Cx40, and to block chamber differentiation in the embryonic heart tube.

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