Formation of the Sinus Node Head and Differentiation of Sinus Node Myocardium Are Independently Regulated by Tbx18 and Tbx3

Cornelia Wiese,* Thomas Grieskamp,* Rannar Airik, Mathilda T.M. Mommersteeg, Ajmal Gardiwal, Corrie de Gier-de Vries, Karin Schuster-Gossler, Antoon F.M. Moorman, Andreas Kispert,* Vincent M. Christoffels*

Abstract—The sinus node (or sinoatrial node [SAN]), the pacemaker of the heart, is a functionally and structurally heterogeneous structure, which consists of a large “head” within the right caval vein myocardium and a “tail” along the terminal crest. Here, we investigated its cellular origin and mechanism of formation. Using genetic lineage analysis and explant assays, we identified T-box transcription factor Tbx18-expressing mesenchymal progenitors in the inflow tract region that differentiate into pacemaker myocardium to form the SAN. We found that the head and tail represent separate regulatory domains expressing distinctive gene programs. Tbx18 is required to establish the large head structure, as seen by the existence of a very small but still functional tail piece in Tbx18-deficient fetuses. In contrast, Tbx3-deficient embryos formed a morphologically normal SAN, which, however, aberrantly expressed Cx40 and other atrial genes, demonstrating that Tbx3 controls differentiation of SAN head and tail cardiomyocytes but also demonstrating that Tbx3 is not required for the formation of the SAN structure. Our data establish a functional order for Tbx18 and Tbx3 in SAN formation, in which Tbx18 controls the formation of the SAN head from mesenchymal precursors, on which Tbx3 subsequently imposes the pacemaker gene program. (Circ Res. 2009;104:388-397.)

Key Words: heart development | progenitors | Hcn4 | Cx43 | transgenic mice

The sinoatrial node (SAN) or sinus node is the most upstream component of the cardiac conduction system. As the primary pacemaker, it serves to initiate and control the rate of electric impulses for the ordered stimulation and contraction of the cardiac chambers. The critical importance of the SAN is reflected in dysfunctions that arise on aging and disease, including sick sinus syndrome, leading to implantation in about one-half of all pacemaker recipients in the United States.1

The SAN consists of a small group of primitive variably sized myocytes with little contractile filaments and intermingled fibroblasts at the junction of the right venous entrance and the atrium.2 They form an elongated “comma-shaped” structure that is subdivided into a large “head” in the right superior caval vein bordering the atrium, and a “tail” along the terminal crest.3,4 The leading pacemaker usually originates from a small number of cells within the SAN, but its location shifts under altered physiological conditions, a phenomenon that likely contributes to both controllability and stability of pacemaker activity.2,5,6 For example, β-adrenergic stimulation in rat causes dominant pacemaker activity to shift cranially into the SAN head region,7 supporting the notion that functional specialization correlates with structural regionalization in the SAN.

The mechanisms underlying the functional regionalization, as well as the pathological mechanisms underlying SAN dysfunction, are only insufficiently understood. Histological studies initially indicated that the mouse SAN forms at approximately embryonic day (E)10 to E11 from myocardium of the sinus horns.8,9 Genetic labeling analyses confirmed the origin of the SAN from this cell population and excluded the embryonic atrial myocardium as a cellular source for this tissue.10 Yet, it remains unclear whether the SAN is formed by growth of a small prespecified population of sinus myocardium or by differentiation of mesenchymal cells into SAN cells. Analyses of the function of the Tbx3 and Shox2 genes have provided insight into the molecular program regulating formation and maintenance of the SAN.10–12 Tbx3 is expressed in the conduction system including the SAN and is required to repress atrial differentiation of the SAN. Moreover, Tbx3 was found to be sufficient for the induction of the pacemaker gene program and function in atrial myocardium.10

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From the Center for Heart Failure Research (C.W., M.T.M.M., C.d.G.-d.V., A.F.M.M., V.M.C.), Academic Medical Centre, Amsterdam, The Netherlands; and Institute for Molecular Biology (T.G., R.A., K.S.-G., A.K.) and Department of Cardiology and Angiology (A.G.), Medizinische Hochschule Hannover, Germany.
*These authors contributed equally to this work.
Correspondence to Vincent M. Christoffels, Center for Heart Failure Research, Academic Medical Centre, Meibergdreef 15, 1105AZ Amsterdam, The Netherlands. E-mail v.m.christoffels@amc.uva.nl
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Here, we explore the cellular origin and molecular mechanisms underlying SAN formation. We provide information on the morphogenesis and regionalization of this structure during normal development. We identified a progenitor source population of the SAN and show that the 2 T-box genes \( \text{Tbx3} \) and \( \text{Tbx18} \) control distinct subprograms of SAN development.

**Materials and Methods**

**Mice**

For the \( \text{Tbx18}^{\text{tm2Akis}} \) (\( \text{Tbx18}^{\text{GFP}} \)) transgenic mouse line, the \( \text{IRE} \text{S} \cdot \text{lacZ} \) knock-in construct\(^1\) was modified to harbor an enhanced green fluorescent protein (GFP) cassette in the start codon. The generation and evaluation of the \( \text{Tbx18}^{\text{tm3Akis}} \) (\( \text{Tbx18}^{\text{Cre}} \)) “lineage-tracer” transgenic line, which harbors a \( \text{Cre} \) gene at the translation start site and from which the \( \text{Pgk-neo} \) cassette was removed, will be described elsewhere. \( \text{Tbx3}^{\text{tm1Vmc}} \) (\( \text{Tbx3}^{\text{Cre}} \)),\(^10\) \( \text{R}26\text{R}^{\text{lacZ}} \),\(^14\) \( \text{Tg}^{\text{Nppa-cre}} \) (\( \text{Nppa::cre} \)), \(^15\) and \( \text{Tg}^{\text{Dll1-Tbx18}} \) (\( \text{msd::Tbx18} \))\(^13\) transgenic mouse lines have been described previously. Animal experiments were performed in agreement with national and institutional guidelines. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Results**

The SAN Head Forms From \( \text{Tbx18}^{\text{+}} \) Mesenchymal Precursors

We first analyzed the morphology, localization, and molecular subdivision of the SAN at different developmental time points in the mouse embryo. The myocardial expression pattern of \( \text{Tbx3} \) was used in all instances to accurately identify the developing and mature SAN\(^3,4\) (Figure 1A through 1D) in sections through the posterior pole of the heart. Three-dimensional reconstruction analysis revealed that at E14.5, the \( \text{Tbx3}^{\text{+}} \) SAN consists of a large domain wrapped around the superior caval vein wall like a gully and touched the right atrium and a smaller domain that extended into the atrium along the right (and left) venous valve. This structure much resembled the comma-shaped SAN described for adult mammals,\(^3,4\) with a large head (around the superior caval vein) and a smaller tail (along the venous valve, incorporated into the future terminal crest).
**Tbx18** and **Hcn4**, a marker for the developing SAN and sinus horns, \(^{11,17,18}\) but was devoid of Nkx2-5 expression. In contrast, the tail expressed **Hcn4** highly and Nkx2-5 weakly and lacked expression of **Tbx18**. Outside the **Tbx3**^+^ SAN, the common cardinal vein (future right superior caval vein) featured a **Tbx18**^+^ wall, whereas the atrium and atrial side of the venous valve expressed atrial markers connexin 40 (**Cx40**) and **Nppa** in a pattern strictly complementary to **Tbx3**. Thus, based on the expression pattern of transcription factor genes **Tbx3**, **Tbx18**, and Nkx2-5, the SAN comprises 2 distinctive regulatory domains, referred to as “head” (**Tbx18**^+^ **Tbx3**^+^ Nkx2-5^-^) and “tail” (**Tbx18**^-^ **Tbx3**^+^ Nkx2-5^-^).

At E9 to E9.5, before the sinus horn myocardium had formed, \(^{19}\) the most caudoventrally located myocardium expressed **Hcn4** and **Tbx3** (Figure 1E). The nonmyocardial mesenchyme and proepicardium that was immediately adjacent expressed **Tbx18** (Figure 1E).\(^{19,20}\) Hence, the **Tbx3**^+^ **Hcn4**^+^ myocardium (cTnI+) expanded into the **Tbx18**^+^ mesenchymal domain to form the SAN head after E9.5 (Figure 1F). This suggests that either the **Tbx3**^+^ **Hcn4**^+^ myocardium expanded and initiated **Tbx18** expression or that the **Tbx18**^+^ mesenchyme differentiated into the SAN myocardium. We next crossed a **Tbx18**^CRE^ line, in which the expression of the Cre recombinase gene mimics that of the **Tbx18** gene, with **R26RLacZ**/lacZ mice.\(^{10}\) This system irreversibly labels **Tbx18**-expressing cells and their daughters by lacZ expression (β-galactosidase activity) and allows one to follow the fate of the **Tbx18**^+^ precursors during development. Staining serial sections of an E18.5 fetus for lacZ and **Tbx3** activity revealed that indeed the **Tbx18**^+^ cells form the SAN head. In addition, the SAN tail, which does not express **Tbx18**, appeared to be derived from progenitors that once expressed **Tbx18**. In contrast, the atria remained free of lacZ expression, indicating that **Tbx18**^+^ progenitors do not give rise to atrial myocytes (Figure 2A).

To further explore the ability of **Tbx18**^+^ progenitors to form SAN, tissue fragments from the GFP-positive mesenchyme in the inflow tract region of **Tbx18**^GFP/+^ E9.5 embryos (ie, before the formation of **Tbx18**^+^ SAN) were isolated largely free of myocardium (see Figure 2B and 2D) and cultured. At day 0 of culture, 23% of the isolated explants (n=31) possessed few **MF20**^+^ cells, but 77% were completely free of contaminating **MF20**^-^ cardiomyocytes. After 4 days, however, all explants strongly expressed **Hcn4** (n=16) and 94% expressed **MF20** (n=31) (Figure 2E), indicating de novo differentiation of SAN myocardium from **Tbx18**^+^ mesenchyme. In addition, none of the **Tbx18**^+^ explants was beating at day 0, whereas all control ventricular explants were. However, at day 4 of culture, 51% of **Tbx18**^+^ explants showed synchronous contraction (n=37). Beating frequencies were higher in these explants than in control ventricular explants (n=25; Figure 2C). Because the caudal (inflow) heart regions of the early embryonic heart are known to display higher beating rates compared to ventricular myocardium,\(^{21}\) we conclude that myocardial pacemaker cells were formed de novo from **Tbx18**^+^ mesenchymal precursors in culture.

**Figure 2.** **Tbx18**^+^ progenitors form the SAN. A, Serial section in situ hybridization of a heart from a E18.5 **Tbx18**^GFP/+^-**R26RLacZ/lacZ** fetus stained as indicated. Black arrowhead indicates the **Tbx18**^+^ SAN head, the white arrowhead labels the **Tbx18**^-^ SAN tail. The entire SAN is composed of lacZ+ (=**Tbx18**^+^ derived) cells. Red arrowheads indicate atrial myocardium, free from lacZ^-^ cells. B, Whole mount in situ hybridization of an E9.5 embryo demonstrates **Tbx18** expression in caudal mesenchyme and the proepicardium. For explant cultures embryonic ventricles and **Tbx18**^+^ precursor cells from regions indicated by the squares were isolated. Both explants were used directly for immunohistochemistry (day 0) or were cultured for 4 days, followed by determination of the beating frequency and immunofluorescence analysis. C, Determination of the beating frequencies (beats per minute) of SAN progenitor clusters and embryonic ventricles after 4 days of culture. D, Immunohistochemical analysis of **Tbx18**^+^ mesenchyme using **MF20** (myocardium), **Hcn4** (SAN), and SYTOX Orange (nuclei) directly after isolation. E, Immunohistochemical analysis after 4 days of explant culture using **Hcn4**, **MF20**, and SYTOX Orange. **pe** indicates proepicardium; ev, embryonic ventricle.

**Formation of the SAN Head Requires Tbx18**

Expression of **Tbx18** in SAN precursor cells suggested a requirement for this transcription factor gene in the formation of the SAN. We therefore analyzed **Tbx18**^GFP/GFP^ mutant
embryos for defects in the SAN at different developmental stages (E10.5 to E17.5) using in situ hybridization analysis for marker genes on serial sections through the region (Figure 3 and the online data supplement, Figure I). GFP from the Tbx18GFP allele was used to monitor the Tbx18 expression domain in Tbx18-deficient embryos because it faithfully mimicked endogenous Tbx18 expression in the common cardinal vein/caval vein mesenchyme and sinus horns of Tbx18GFP/+ embryos. At E10.5 and E12.5, the GFP-negative SAN tail in the right venous valve (Tbx3+/Hcn4−) had formed, whereas the GFP-positive cTnI+/Tbx3+/Hcn4+ SAN head domain was absent in Tbx18GFP/GFP embryos (Figure 3B and supplemental Figure VI, A). Formation of atria and the atrial side of the venous valve was unaffected. Expression of atrial myocardial genes Cx40, Cx43, and Nppa was not detected in the SAN tail region in Tbx18-deficient embryos. At E14.5 and E17.5, the right superior caval vein of Tbx18-deficient embryos acquired myocardial cells (cTnI+), albeit in a delayed fashion compared to control fetuses (Figure 3B). Formation of SAN-like tissue expressing GFP, Tbx3, and Hcn4 and lacking Cx40, Cx43, and Nppa occurred but was similarly delayed (Figure 3B and 3C and not shown). Nkx2.5 was expressed in the SAN-like tissue similar to the wild-type SAN tail at these stages (supplemental Figure VI, B). Expression of Shox2, Lbh, and Odd1 in the SAN myocardium was unchanged in Tbx18-deficient embryos (supplemental Figure I, E, and data not shown). In summary, Tbx18 is required for formation of the head region of the SAN.

To further assess the defects in the formation of the SAN head in Tbx18-deficient embryos, we performed a 3D reconstruction of the SAN and the lumen of the right superior caval vein from serial section in situ hybridization analysis for Tbx3 (Figure 4A). The wild-type SAN head was found to be ~0.002 mm³ at E12.5 and rapidly increased to ~0.009 mm³ at E14.5 (~3600 cells; cell density, 0.0004 cells/μm³; supplemental Figure II, D), but thereafter the volume did not increase further (Figure 4B). In mutants of E12.5, the Tbx3+/SAN region was restricted to the venous valve part of the SAN (tail), and the head region of the SAN head was completely missing (Figure 4A and supplemental Figure V). Two days later (E14.5), after the delayed formation of myocardium of the right superior caval vein, a SAN head-like structure was formed in Tbx18GFP/GFP embryos. However, it was significantly shortened along the longitudinal axis of the right superior caval vein (supplemental Figure II, A). Quantification of the SAN volume at E12.5, E14.5, and E17.5 confirmed that the SAN, unlike in the wild-type situation, failed to enlarge after E12.5 in Tbx18-deficient embryos (Figure 4B). The proliferation rate within the Tbx18+/GFP+ SAN area was not significantly altered between the mutant and the control situation (8% to 10% 5-bromodeoxyuridine-positive; E14.5: P=0.4; E17.5: P=0.8; supplemental Figure II, B and C). Similarly, apoptosis was unaffected in Tbx18...
mutant embryos (data not shown). In addition, cell densities were not significantly different between the wild-type and $Tbx18$-deficient SAN, ruling out the notion that the reduction of the SAN volume was attributable to reduction of cell size (supplemental Figure II, D). Hence, we suggest that the smaller volume of the SAN head in $Tbx18$ mutant embryos results from the failure to expand the mesenchymal precursor population and/or to differentiate cardiomyocytes of the SAN head from precursor cells along the longitudinal axis of the right caval vein.

**$Tbx18$ Is Required to Maintain a Sharp SAN–Atrium Boundary**

The boundary between the SAN and atrial myocardium is of crucial importance, because it allows the SAN to drive the large atrium without being suppressed by its hyperpolarizing influence.

![Figure 4. Sinus node morphology in $Tbx18$-deficient embryos.](image)

$Hcn4$ and of $Cx40$ revealed a clear delineation of the 2 expression domains in the SAN region of wild-type embryos. In $Tbx18$-deficient embryos, in contrast, the boundary between SAN and atrial myocardium appeared less distinct. This may be caused by misspecification of atrial or SAN cardiomyocytes or by morphogenetic defects (Figure 5A). To investigate whether atrial cardiomyocytes redifferentiate to the SAN phenotype in the absence of $Tbx18$, we followed the fate of atrial myocardium using the Cre/loxP system and the $Nppa::cre3$ driver. We failed to detect $\beta$-galactosidase–positive cells in the $Hcn4^{-}$ SAN of both mutant ($Tbx18^{GFP/GFP}; Nppa::cre3; R26R^{loxZ}$) and control ($Tbx18^{Cre/-}; Nppa::cre3; R26R^{loxZ}$) fetuses, indicating that atrial cardiomyocytes do not redifferentiate to the SAN phenotype in the absence of $Tbx18$ (Figure 5). In addition, $Hcn4$ and $Cx40$ were never coexpressed as shown by double immunofluorescence analysis, providing evidence that SAN cardiomyocytes do not acquire...
an atrial cardiomyocyte phenotype either (Figure 5C). Taken together, disturbance of boundary formation between SAN and atrium is likely to result from morphogenetic defects rather than misspecification of atrial or SAN cells.

**Tbx3 Regulates Cytodifferentiation of the SAN Myocardium but Not SAN Formation**

We previously found that the SAN volume in Tbx3-deficient embryos is significantly smaller than of the wild-type control. To identify a possible cause, we analyzed the SAN morphology, as well as characteristics of SAN myocytes, in Tbx3Cre/Cre embryos in a similar manner to Tbx18 mutant embryos (supplemental Figure III). Three-dimensional reconstructions revealed that the morphology and length of the Tbx3-deficient SAN was not significantly different from the wild-type control (Figure 6A and supplemental Figure III, A). In addition, proliferation and apoptosis in the SAN at E12.5 were not affected, as revealed by the 5-bromodeoxyuridine incorporation assay and cleaved caspase-3 detection, respectively (supplemental Figure III, B and C, and data not shown). Finally, the measurement of the cell density did not reveal any significant differences (supplemental Figure III, D) between wild-type and Tbx3 mutant SAN. Tbx3-deficient embryos die between E11.5 and E15.5, depending on genetic background, and a fraction of mutants show developmental and growth retardation. This can be expected to reduce SAN size nonspecifically. We measured atrial wall thickness as a Tbx3-independent parameter for growth retardation and found that mutant embryos with a smaller SAN volume have thinner atrial walls (Figure 6B). These findings suggest that the reduced SAN volume in several Tbx3 mutants is caused by general growth retardation and is not caused by the absence of Tbx3 from the SAN. Molecular analysis showed ectopic expression of Cx43 and other atrial markers in the SAN of Tbx3 mutants (Figure 6C), whereas Tbx18 and Shox2 were normally expressed (data not shown). Thus, Tbx3 does not regulate basic cellular programs affecting growth and morphology of the SAN but ensures correct gene regulation in SAN cells.

**Tbx18 and Tbx3 Function Independently and Successively in SAN Development**

Our analyses indicated that Tbx18 and Tbx3 control distinct programs in SAN formation. Tbx18GFP/+;Tbx3Cre/+ double heterozygous fetuses had apparently normal SANs, indicating that Tbx18 and Tbx3 do not interact at the genetic level (data not shown). Reminiscent to the situation in Tbx18 single mutants, Tbx18/Tbx3 double mutant embryos lacked the SAN head, whereas the tail region was present (Figure 7 and supplemental Figure IV). The tail region showed ectopic expression of Cx40, in agreement with the previously described SAN-specific phenotype of Tbx3-deficient embryos. Together, our analyses of Tbx3 and Tbx18 single and compound mutant embryos indicate that both genes act heterochronically and independently. First, Tbx18 controls the formation of the head region of the developing SAN. Subsequently,
Tbx3 regulates the differentiation of myocytes in the SAN (Figure 8).

Discussion
In the present study, we have investigated the developmental origin of the SAN myocardium, its morphogenesis, its partition in distinctive regulatory domains, and the genetic requirement of Tbx18 and Tbx3 in its formation. We show that the SAN comprises at least 2 distinct domains, a large Tbx18/H11001 head in the right superior caval vein wall, which represents ~75% of the SAN volume, and a small Tbx18/H11002 tail along the venous valve (terminal crest in the mature heart). We found that the SAN originates from Tbx18/H11001 mesenchymal precursor cells that, from E9.5 onward, differentiate into SAN myocardium. Formation of the head depends on Tbx18, whereas gene regulation in cardiomyocytes of both head and tail regions depends on Tbx3. Thus, this study establishes a 2-step process of SAN formation, in which Tbx18 controls the formation of the SAN head, on which Tbx3 subsequently exerts the pacemaker gene program.

Molecular and Functional Regionalization of the SAN Into an Anterior Head and Posterior Tail Region
Based on functional, morphological, and gene expression studies, the SAN has been defined as an elongated structure with a large head, and a right-sided extension, or tail, along the terminal crest.4-7 In rodents, the head is wrapped around the superior caval vein, including the intraatrial groove region at the left side.4,7,8 The 3D reconstructions of the Tbx3 expression pattern are in full agreement with this SAN morphology (Figure 1 and elsewhere11,16), and the critical role of Tbx3 in the regulation of the SAN gene program and function10 further underscores the notion that the Tbx3 pattern accurately reflects the entire SAN domain. Shortly after E9, first the tail domain is established, and, subsequently, the SAN head domain expands rapidly along the superior caval vein to reach its definitive shape at E14.5. Our expression analysis revealed the existence of 2 domains within the SAN with distinct genetic programs: a Tbx18/H11001 Tbx3/H11001 domain corresponding to the head, and a Tbx18/H11002 Tbx3/H11001 domain corresponding to the tail. Formation of the head to a much larger extent than the tail depends on Tbx18, establishing that the SAN contains 2 distinct domains that differentially depend on the presence of this transcription factor. These data provide the first insights into the compartmentalization of the SAN, which may bear relevance to the establishment of regionalized physiological requirements within this tissue. A small region within the SAN center usually functions as dominant pacemaker during rest, although multicentric impulses may origin from both head and tail.5-7 However, during
vagal stimulation (long cycle length) or isoproterenol administration (short cycle length), the origin of the impulse was seen to shift in a species dependent manner toward the tail region along the terminal crest or toward the head.2,5–7 These studies have clearly established functional domains within the SAN, with emphasis on the head and tail regions, which underlie the regulated initiation of the impulse under different physiological (pharmacological) conditions.2,6

In 3 rare postnatal Tbx18 mutant mice, obtained by rescuing the somitic requirement of Tbx18 (Tbx18GFP/GFP, msd::Tbx1813), the SAN was severely reduced in size, as expected (data not shown). Under anesthesia, these animals showed rates of contraction and ECG parameters, including P-waves, not significantly different from littermate controls (our unpublished observations, 2008). These data suggest that under sedated conditions, a strongly reduced SAN is sufficient to function as dominant pacemaker. This observation underscores the notion that an extensive region of tissue has to be ablated to stop sinus rhythm, ie, that only a small number of cells within the SAN region is sufficient for SAN function, and that the location of this activity within the SAN is flexible.5,7,27,28

Origin of the SAN Cells and Regulation of the SAN Gene Program

There are several principle modes of SAN formation during cardiogenesis. The SAN may form (1) by proliferative growth of a small prespecified population of sinus myocardium, (2) by expansion and differentiation of mesenchymal cells directly into SAN cells, or (3) by recruiting existing adjacent myocardial cells to a small population of initially specified SAN cells. Previous analysis excluded this last mode for the development of the SAN.10 Our 3D expression analysis showed that the Tbx3+ Hcn4+ myocardium expands into the Tbx18+ mesenchymal domain along the superior caval vein to form the SAN head, suggesting that either the Tbx3+ Hcn4+ myocardium expands by proliferation and initiates Tbx18 expression or that the Tbx18+ mesenchyme is a precursor cell type that can differentiate into the SAN lineage. The Tbx18 lineage tracing experiment strongly favors the latter possibility, because cells labeled by Tbx18Cre expression and their daughters were found in the entire SAN. Moreover, they showed that the Tbx18-expressing precursors do not provide contributions to the atrial myocytes, indicating an early lineage segregation between SAN/sinus horn and atrial myocardium. Although the SAN tail is derived from the...
Tbx18-expressing precursors, Tbx18 expression is only maintained in the head, where it may have later functions in regulating head morphogenesis or gene regulation that were not addressed in this study.

Culturing of small Tbx18+ mesenchymal tissue pieces representing the putative SAN precursors revealed their propensity to differentiate into myocardium. This myocardium initiated the expression of pacemaker tissue marker Hcn4, encoding a channel important for pacemaker function.17,29 In addition, after 4 days, these differentiated explants showed significantly higher contraction rates compared to ventricular explants, consistent with an identity of caudal myocardium,21 where the dominant pacemaker resides throughout development.30 Furthermore, Tbx18-deficient embryos failed to establish a SAN head. Because proliferation, cell density, and apoptosis were not different between mutants and controls, the most likely explanation is that Tbx18 deficiency leads to a failure to form the SAN de novo from noncardiac precursors. Taken together, we conclude that myocardium forming the future SAN head is formed de novo by expansion and subsequent differentiation of Tbx18+ superior caval vein mesenchyme precursors cells to SAN myocardium. The SAN and its precursors express Islet1,11,13 a marker for the second heart field of cardiac precursors, and probably represent a subpopulation of these progenitors, which also give rise to the atrial components. However, the Tbx18+ mesenchymal population that gives rise to the sinus horns19 does not express Islet1 or Nkx2-5 and is found ventral–lateral–caudal from the tubular heart, and not dorsal, like the second heart field (our unpublished observations, 2008). These data suggest that the Tbx18+, Islet1+, Nkx2-5− SAN precursors are represented by the overlap between the Tbx18+ and Islet1+ progenitor populations found lateral from the inflow tract of the heart tube.

Investigation of Tbx3-deficient hearts did not reveal morphological changes in the SAN. Therefore, despite the important role of Tbx3 in the terminal differentiation of SAN cardiomyocytes,10 SAN morphogenesis seems to be independent of Tbx3. Furthermore, Tbx18-Tbx3 compound mutants showed an additive phenotype, whereas compound heterozygous (Tbx18+/−/GFP;Tbx3−/−/Cre) fetuses had a normal SAN. This suggests that Tbx18 acts in the mobilization or differentiation of the SAN precursor cells. Once this myocardium has been established, Shox2, important for the regulation of Nkx2.5 and Cx43,12 and Tbx3 are activated. Tbx3 subsequently ensures proper gene regulation within the SAN domain (Figure 8).

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Disclosures

None.

References


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Supplemental Methods

Mice
In order to generate double transgenic mice $Tbx3^{Cre/+}$, $R26R^{lacZ}$ and Nppa::Cre3 mice were crossed with $Tbx18^{+/GFP}$ heterozygous mice. Genomic DNA prepared from amniotic or toe biopsies was used for genotyping by PCR. Following primers were used: $Tbx18$ wild type allele (forward: GGCGAAAAGCTGGG, reverse: AAGCGTCATGATGTC), $Tbx18$ mutant allele (forward: GACACATCCTCTAC, reverse: GGTGGTTAGTATC), $Tbx3$ wild type allele (forward: AGCGGCCCAAGC, reverse: CTGCTCTTCTGTG), $Tbx3$ mutant allele (forward: see wild type allele and reverse: see $Cre$ reverse primer), $EGFP$ (forward: CGGCTCAAGAGC, reverse: CCGCGCCGAGC), $Cre$ (forward: TGGTCTCTTGTTCTGG, reverse: GCTAGGCCAATCAC), and $lacZ$ (forward: CTGCTCTTCTGTGGAA, reverse: GACACCAGCAGAC).

Collection and preparation of embryos
For timed pregnancies, vaginal plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Embryos of developmental stages between E10.5 and 17.5 were isolated for analysis. They were dissected in PBS and fixed in 4% paraformaldehyde overnight for in situ hybridization or immunohistchemistry (detection of GFP, cleaved caspase-3, BrdU and TUNEL assay), respectively. Embryos used for beta-galactosidase activity detection and immunohistchemistry ($Hcn4$, Connexin 40) were fixed in 4% paraformaldehyde for 15 min on ice and then incubated in 10% sucrose overnight. Next day, they were embedded in OCT Embedding medium and stored at -20°C.

Proliferation and apoptosis analysis
The proliferation (BrdU assay) and apoptosis (cleaved caspase-3 detection and TUNEL assay) analyses were performed as described previously.1,2

β-Galactosidase activity detection and immunohistchemistry
For detection of β-galactosidase activity 10 μm cryostat sections were fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by X-gal staining. For immunohistchemistry the following primary antibodies were used: rabbit polyclonal antibodies against $Hcn4$ (1:250, Chemicon) and GFP (1:50, Santa Cruz Biotechnology) and monoclonal antibodies against $Cx40$ (1:100, USBio) and MF20 (1:50, Hybridoma bank, Iowa City, IA, USA). Immunohistochemical analysis of $Hcn4$ and Connexin 40 was performed on 10 μm cryostat sections. In order to block endogenous mouse IgG, sections used to detect $Cx40$ were pre-incubation with an unconjugated Fab fragment goat anti-mouse IgG (H+L) (1:10, Jackson ImmunoResearch Laboratories). Secondary antibodies were Alexa 568 goat anti-rat, goat anti-rabbit (1:250, Molecular Probes) and Alexa 488 goat anti-mouse (1:400). Nuclei were counterstained with SYTOX green / orange nucleic acid stain (Molecular Probes) or DAPI (Molecular Probes), respectively. GFP expression was detected on 5 μm paraplast sections. Non-fluorescent staining was performed using kits from Vector Laboratories (ABC peroxidase kit (Rabbit IgG), DAB substrate kit).

Non-radioactive in situ hybridization
Non-radioactive in situ hybridization on sections was performed as described.3 RNA probes were kindly provided for Nkx2-5 (R. Harvey, Victor Chang Cardiac Research Institute, University of New South Wales), $Hcn4$ (B. Santoro, Center for Neurobiology and Behavior,
Columbia University, New York), Tbx3 (V. Papaioannou, Department of Biological Sciences, University of Pittsburgh), Shox2 (G. Rappold, Institute for Human Genetic, University Heidelberg, Germany) and Lbh (K. J. Briegel, Institute for Cellular and Molecular Biology, University of Texas, Austin). Other probes have been described previously.\textsuperscript{1,4-6}

**Three-dimensional reconstructions and quantification of sinus node volume**

Three-dimensional visualization and geometry reconstruction of patterns of gene and protein expression have been performed as described.\textsuperscript{7} The quantification of expression domains (e.g. sinus node volume) has been described previously.\textsuperscript{6} Files with reconstructions are available on request.

**Determination of atrial wall thickness and cell density**

The atrial wall thickness was determined using Scion Image as theoretically described previously.\textsuperscript{8} To determine the cell density in the sinus node of E14.5 old wild type and Tbx18 mutant embryos, the single nuclei within the Tbx3 labels (“masks of Tbx3” produced in Amira) were counted per each section using the program Image Pro. The area of the masks was measured using Scion Image and the cell number per area was calculated. To determine the number of cells per volume, a nucleus size of 10 $\mu$m was assumed (determined previously from embryonic chicken myocytes) and a section thickness of 10 $\mu$m was considered. The cell density is the number of cells per area divided by the nucleus diameter and the section thickness. The cell density in the sinus node of E12.5 old Tbx3 mutant and wild type embryos was estimated in a similar way but a fixed volume within the sinus node was used. The section thickness was 7 $\mu$m.

**Embryonic explant cultures**

In order to isolate Tbx18-expressing mesenchyme in the region of the developing sinus horns, both lateral parts of the EGFP-expressing area in heterozygous Tbx18 EGFP knock-in embryos at E9.5 were micro-dissected. Explants were similar cultured as previously described\textsuperscript{9}. Directly after isolation and after cell culture, respectively, samples were fixed in 4% paraformaldehyde and used for immunocytochemistry.

**Statistics**

Results are expressed as mean ± SEM. Statistical significance was tested with unpaired, two-tailed student’s t-test. (* for P < 0.05, ** for P< 0.005 and *** for P< 0.001).

**References**


Online Figure I
Gene expression patterns of the SAN and adjacent tissues in Tbx18-deficient and control (wild-type or heterozygous) embryos at different developmental stages. A, Optical section through the SAN region of the 3D reconstruction (description see figure legend 1). B, C and D, linear representations of gene expression patterns in heterozygous and homozygous Tbx18-mutant embryos at E10.5, E12.5 and E17.5, respectively. Description and color code in the model correspond to the description shown in A. Black bars indicate specific gene expression in the corresponding tissue. E, Analysis of Shox2 gene expression in Tbx18 mutant embryos (E11.5). Shown are in situ hybridizations on serial transversal sections using probes for cardiac troponin I (cTnI) to label the myocardium and Shox2. The black arrows indicate the myocardial border in wild type and Tbx18 mutant embryos and red arrows demonstrate the absence of myocardium and Shox2 expression in the left superior caval vein of Tbx18 mutant embryo. rsc, right superior caval vein; ra, right atrium.

Online Figure II
Characterization of the sinus node in Tbx18 mutant embryos. A, Determination of the length (longitudinal axis along the rscv) of the sinus node head at E14.5. B, Determination of the BrdU labeling index in the Tbx18/GFP-positive area of the sinus node at E14.5 and E17.5 in Tbx18 mutant and heterozygous embryos (n=3 and 4, respectively). C, Immunohistochemistry on E14.5 old heterozygous and Tbx18 homozygous mutant embryos using GFP (Tbx18-positive cells), BrdU (proliferating cells) and DAPI (cell nuclei). D, Determination of the cell density in the sinus node of E14.5 old wild type and Tbx18 mutant embryos (n=3).

Online Figure III
Analysis of the function of Tbx3 in sinus node development. A, Determination of the sinus node length (longitudinal axis along rscv) (E12.5, n=5 for wild type; n=3 for Tbx3^{Cre/Cre}). B and C, Proliferation analysis using BrdU incorporation (E12.5), B, Quantification of the BrdU labeling index in the sinus node head and the venous valve part of the sinus node separately (n=4). C, Immunofluorescence staining showing BrdU (red) and nuclei (sytox green, green). D, Determination of the cell density in the sinus node of E12.5 old Tbx3 mutant and wild type embryos (n=4).

Online Figure IV
Analysis of the sinus node development in Tbx18-Tbx3 double homozygous embryos (Tbx18^{+/GFP};Tbx3^{+/Cre}) at E12.5. For description of the lower panel of each genotype see Figure 7 description. Upper panel of each genotype shows the more distal part of the sinus node head indicated by the black arrow. In Tbx18 mutant and Tbx18-Tbx3 double mutant embryos the sinus node head (black arrow head in wild-type) is absent, whereas the tail (white arrow head) is present.

Online Figure V
Collection of 10 sections through the sinus node of a wild type and Tbx18 mutant embryo at E12.5 used for 3D reconstructions shown in Figure 4A. a-k, in situ hybridizations of Tbx3 in a wild type sinus node, a’-k’ in situ hybridizations of Tbx3 in a Tbx18 mutant sinus node.
Online Figure VI
Characterization of the sinus node tail. A, The sinus node tail, which expresses Tbx3, (indicated by a white arrow head) is negative for Tbx18 (wild type) and GFP (Tbx18 mutant embryo), respectively. Shown is an E12.5 old embryo.
B, In the sinus node tail, which is negative for connexin 40 (Cx40), Nkx2-5 is gradually up-regulated at E17.5 in wild type and Tbx18 mutant embryo.
Online Figure I

A

B

C

D

E

Tbx18

Tbx18

Tbx18

Tbx18

Tbx18

Nkx2-5

Nkx2-5

Nkx2-5

Nkx2-5

Nkx2-5

Hcn4

Hcn4

Hcn4

Hcn4

Hcn4

Cx40

Cx40

Cx40

Cx40

Cx40

cTnI

cTnI

cTnI

cTnI

cTnI

GFP

GFP

GFP

GFP

GFP

head

head

head

head

head

ra

ra

ra

ra

ra

tail

tail

tail

tail

tail

sh

sh

sh

sh

sh

asvv

asvv

asvv

asvv

asvv

E12.5

E12.5

E12.5

E12.5

E12.5

E10.5

E10.5

E10.5

E10.5

E10.5

E14.5-17.5

E14.5-17.5

E14.5-17.5

E14.5-17.5

E14.5-17.5

ra sh mes

ra sh mes

ra sh mes

ra sh mes

ra sh mes

Tbx18

Tbx18

Tbx18

Tbx18

Tbx18

GFP/GFP

GFP/GFP

GFP/GFP

GFP/GFP

GFP/GFP

Tbx18+/GFP

Tbx18+/GFP

Tbx18+/GFP

Tbx18+/GFP

Tbx18+/GFP

Cra

Cra

Cra

Cra

Cra

Cau

Cau

Cau

Cau

Cau

D

V

D

V

D

V

D

V

E

wt

Tbx18GFP/GFP

ra

rcv

cTnI

Shox2

ra

rcv

ra

rcv

ra

rcv

Online Figure II

A. Bar graph showing sinus node head length (µm) for Tbx18<sup>−/−</sup>GFP and Tbx18<sup>+/−</sup>GFP conditions at E14.5 and E17.5.

B. Bar graph showing BrdU labeling index (%) for Tbx18<sup>−/−</sup>GFP and Tbx18<sup>+/−</sup>GFP conditions at E14.5 and E17.5.

C. Immunostaining images of atrium and SAN regions for Tbx18<sup>−/−</sup>GFP and Tbx18<sup>+/−</sup>GFP conditions, showing BrdU and DAPI labeling.

D. Bar graph showing cell density (cells/µm<sup>3</sup>) for Tbx18<sup>−/−</sup>GFP and Tbx18<sup>+/−</sup>GFP conditions.
Online Figure III

A. Bar graph showing the sinus node length (µm) for wt and Tbx3Cre/Cre.

B. Bar graph showing the BrdU labeling index (%) for sinus node head and venous valve (tail) for wt and Tbx3Cre/Cre.

C. Images showing BrdU/Sytox staining for wt and Tbx3Cre/Cre in rscv, san, and ra.

D. Bar graph showing cell density (cells/µm²) for wt and Tbx3Cre/Cre.
Online Figure V

E12.5

wt

Tbx18\textsuperscript{GFP/GFP}
Online Figure VI

A  

E12.5

Tbx18

Tbx3

Tbx18 GFP

B  

E17.5

Nkx2-5

Cx40

rcv

san

ra

wt

Tbx18 GFP/GFP

Nkx2-5

Cx40

rcv

san

ra

wt

Tbx18 GFP/GFP