The development of the venous pole of the heart
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Chapter 4

Formation of the venous pole of the heart from an Nkx2-5-negative precursor population requires Tbx18

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Abstract

The venous pole of the mammalian heart is a structurally and electrically complex region, yet the lineage and molecular mechanisms underlying its formation have remained largely unexplored. In contrast to classical studies that attribute the origin of the myocardial sinus horns to the embryonic venous pole, we find that the sinus horns form only after heart looping by differentiation of mesenchymal cells of the septum transversum region into myocardium. The myocardial sinus horns and their mesenchymal precursor cells never express \textit{Nkx2-5}, a transcription factor critical for heart development. In addition, lineage studies show that the sinus horns do not derive from cells previously positive for \textit{Nkx2-5}. In contrast, the sinus horns express the T-box transcription factor gene \textit{Tbx18}. Mice deficient for \textit{Tbx18} fail to form sinus horns from the pericardial mesenchyme and have defective caval veins, whereas the pulmonary vein and atrial structures are unaffected. Our studies define a novel heart precursor population that contributes exclusively to the myocardium surrounding the sinus horns or systemic venous tributaries of the developing heart, which are a source of congenital malformation and cardiac arrhythmias.
Introduction

The systemic venous return of the heart consists of multiple anatomical components including the proximal myocardial part of the right superior and inferior caval veins, the coronary sinus (the persisting left caval vein in the mouse), and the sinus venarum. These structures are thought to be the mature counterparts of the right and left sinus horns in the embryo, which are the myocardial parts of the common cardinal veins upstream of the venous valves that bulge into the pericardial cavity. These, in turn, are presumed to derive from the embryonic venous pole or inflow tract of the forming heart and the common cardinal veins. Developmental disorders of the heart, which include malformations of the pulmonary and systemic venous returns, represent the most common human birth defects. In addition, several specific components of the venous returns are found to be the origin of arrhythmias. Recent 3D reconstruction and genetic analyses have greatly improved our insight into the morphogenesis of the systemic and pulmonary venous returns. Nevertheless, the cellular origin of the components of the systemic and pulmonary venous return and the genetic mechanisms underlying their formation are not known.

The embryonic heart of amniotes initially represents a tube consisting of precursor cells for most of the left ventricle and a small portion of the atria. Outflow tract, right ventricle, and large portions of the atria are only subsequently recruited from a second lineage of mesenchymal cells. Nkx2-5, that encodes a homeobox transcription factor, is expressed in both the first and second lineages of the heart, and plays pivotal roles in early and late steps of cardiogenesis. Using genetic lineage analysis, we show that the myocardial sinus horns and their precursor cells do not express Nkx2-5. The sinus horns form only after heart looping in the presence of Tbx18. We demonstrate that Tbx18 is essential for the formation of the sinus horns from the mesenchyme of the pericardial wall and for their myocardial differentiation but not for formation of the atria or pulmonary veins.

Materials and Methods

Mice

The Nppa-Cre, Nkx2-5-IRES-Cre, R26R, Nkx2-5GFP and Tbx18lacZ transgenic mouse lines have been described previously. Construction of the GFP null allele of Tbx18 (Tbx18GFP) will be described elsewhere. Tbx18GFPGFP and Tbx18lacZlacZ mice are phenotypically indistinguishable. For timed pregnancies, vaginal plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Em...
Bryos and fetuses were dissected in PBS and fixed in 4% paraformaldehyde overnight. Genomic DNA prepared from amnion or tail biopsies was used for genotyping by PCR. H. Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

**β-galactosidase activity detection and immunohistochemical analyses**

Detection of β-galactosidase activity on 20 µm cryostat sections was performed as described. For immunohistochemistry on 5 to 10 µm embryo sections, the following primary antibodies were used: monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:2000) (Santa Cruz Biotechnology); monoclonal antibody against Desmin (1:50; Monosan Clone D33); rabbit polyclonal antibody against GFP (1:150; Santa Cruz Biotechnology). Proliferating cells were marked with an antibody against phosphorylated histone H3 (ser10; 1:50; Cell Signalling). Adjacent sections were stained for GFP to define the Tbx18-positive region. Mitotic indices were calculated as fraction of labeled cells to the total number of DAPI positive nuclei within a GFP-positive region. Quantification of proliferation by detection of BrdU incorporation was performed as described. TUNEL staining for apoptotic cells was done on 10 µm sections of embryos according to the protocol supplied with the in situ cell death detection kit, POD (Roche).

**Non-radioactive in situ hybridization and three-dimensional reconstruction**

Non-radioactive in situ hybridization analysis of 10-12 µm embryo sections was performed as described. The following probes were kindly provided: Isl1 and Hcn4 by S. Evans (Skaggs School of Pharmacy, University of California, San Diego) and B. Santoro (Center for Neurobiology and Behavior, Colombia University, New York). Probes for chicken Tbx18 and Isl1 were derived from ChEST 519c15 and 861p11 (BBSRC ChickEst databank, MRC geneservice). Three-dimensional visualization and geometry reconstruction of patterns of gene expression determined by in situ hybridization was carried out as described previously. Files with reconstructions are available upon request.
Results

Definitions

Systemic venous tributaries/return
The whole of veins that return the systemic blood to the developing or mature heart.

Inflow tract/venous pole
Generic terms for most upstream (caudal) component of the heart, regardless of developmental stage.

Venous valves
Valvular folds guarding the sinoatrial orifice (entrance to the right atrium). They form after E9.5 from Nkx2-5+ caudal inflow tract myocardium (this study).

Sinus horns
The myocardial parts of the common cardinal veins upstream of the venous valves that bulge into the pericardial cavity. They form after mouse E9.5 and express Tbx18 (this study).

Figure 1. Dorsal views of 3D reconstructions of hearts of consecutive stages of development. The Cx40-positive atrial myocardium is shown in green, the Cx40-negative myocardium of the sinus horns in gray, the lumen of the caval veins in orange-brown, the lumen of the forming pulmonary vein in red, and the atrioventricular cushion mesenchyme in yellow. The mesenchyme to which the sinus horns are attached has been removed to expose the embedded veins. The volume of the reconstructed Cx40-negative myocardium of the sinus horns is given underneath the stage. The size bar represents 300 μm. icv indicates inferior caval vein; l/ra, left/right atrium; l/rcv, left/right cardinal vein; l/rsh, left/right sinus horn; mm, mediastinal myocardium.
Expression and 3D reconstruction analysis of sinus horn development

In a previous study, 3D reconstruction analyses were performed to gain insight into the morphologically complex area of the developing atria and venous pole. To get a clearer picture of the formation of the sinus horns we here extended this analysis. At E9.5, when the inflow tract is a bilaterally symmetrical structure, a very small Cx40-myocardial component has formed (Figure 1). At subsequent stages, the walls of the left and right common cardinal vein bulge into the pericardial cavity, forming reflections at the pericardial lining. By E10.5, the Cx40-myocardial volume of the sinus horns has gained five fold in size. The common entrance has shifted to the right due to the stronger increase of Cx40-myocardium at the left compared to the right sinus horn. The sinus horns continue to expand until the intrapericardial component has become almost completely enveloped by myocardium at E12.5. The lumen of the pulmonary vein in the dorsal mesenchyme becomes visible from E11.5 onward. It is surrounded by Cx40+ mediastinal myocardium at the border of the Cx40- sinus horn component (Figure 1). The mesenchyme lining the pulmonary vein lumen will become Cx40+ myocardium from E12.5-13.5 (not shown).

At E9.5, all myocardium (cTnI+) expressed Nkx2-5 (Figure 2A). In addition, Nkx2-5 was expressed in the Isl1+ cTnI- second field of cardiac precursors (Figure 2A and 2B). At this stage, Tbx18 was expressed in mesenchyme abutting the Nkx2-5+ myocardium at the venous pole of the heart (Figure 2A, left panels). Thus, in the region of the developing sinus horns 2 distinct cell populations were present, the Nkx2-5+ caudal myocardium and the adjacent Tbx18+ mesenchyme. Surprisingly, the sinus horn myocardium forming from E9.5 on failed to express Nkx2-5, but was positive for Tbx18 (Figure 2A and 2D). The expression domains of Nkx2-5 and Tbx18 continued to be adjacent and mutually exclusive. At E9.5 and E10.5 the transcription factor gene Islet1 (Isl1) was expressed in the dorsal mesenchyme that includes the second lineage but also in the myocardial component of the inflow tract and outflow tract (Figure 2B). However, the Isl1+ and Tbx18+ domains were largely exclusive, with a marginal overlap in the myocardial-mesenchymal border zone (Figure 2B). To assess whether the genetic mechanism of sinus horn formation was evolutionary conserved, the expression patterns of Tbx18, Nkx2-5 and Isl1 were analyzed in chicken hearts. The patterns of these genes were conserved between mouse and chicken (Figure 2C), indicating that the formation of the sinus horns may be governed by a general and conserved mechanism. Since only 2 sinus horn precursor populations are present in the inflow tract region, the Nkx2-5+ myocardial precursors, on one hand and the adjacent Tbx18+ mesenchymal precursors on the other hand, formation of the sinus horns is likely to follow 1 of 2 possible mechanisms. The sinus horns may form from the inflow tract myocardium by proliferation, losing Nkx2-5 expression but gaining Tbx18 expression. Alternatively, the sinus horns may expand by
Figure 2. Marker analysis of the venous pole of the heart. Adjacent serial sections of the sinus venosus region of mouse (A and B) or chicken (C) embryos in columns. Stages and probes are indicated. Black arrows in A and C indicate the border between Nkx2-5 and Tbx18 expression domains; in B, absence of Tbx18 from Isl1+ myocardial domains. Red arrows in A indicate Nkx2-5 expression in the second lineage/second heart field; in B, Isl1 expression in the myocardium; and in C, absence of Isl1 expression from the forming sinus horn. At E11.5 the myocardial sinus horns express Tbx18 in their entirety (D). β-Galactosidase activity of the lacZ gene in the Tbx18 locus mimics the Tbx18 mRNA pattern, indicating a lack of contribution of Tbx18+ cells to the atria or venous valves (E). Sinus horn derived caval veins express Tbx18, pulmonary veins do not (F). a indicates atrium; am, atrial myocardium; Cra, cranial; Cau, caudal; D, dorsal; dm, dorsal mesocardium; ep, epicardium; L, left; lscv, left superior caval vein; oft, outflow tract; pe, proepicardium; pv, pulmonary vein; R, right; sh(m), sinus horn (myocardium); V, ventral. For other abbreviations, see the legend of Figure 1.
Figure 3. Genetic lineage analysis of the sinus horns. *Nppa-Cre / R26R* double transgenic hearts stained for β-galactosidase activity (A and B). Recombination occurred in the atrial myocardium (black arrows), but not in the adjacent superior caval vein myocardium (red arrows). Some recombination was observed in the caval veins (blue arrows). A whole mount X-gal stained E9.5 *Nkx2-5-IRES-Cre / R26R* embryo (C) and transverse (D) and sagittal sections (E, F) of an E10.5 embryo. Sister sections of an E11.5 wild-type (G) and *Tbx18* mutant littermate (I). Sister sections of an E9 wild-type (K) and *Nkx2-5* mutant littermate (M). A model of the formation of the sinus horn myocardium (O). Arrows with crosses indicate that one type of cell did not contribute to the other type. Dashed lines show lineage relations. (P-U) PCNA and nuclear staining showing labeling in the myocardium of the atrium and mesenchyme, but not sinus horn, at E11.5 (P-S), and in the sinus horn at E14.5 (T and U). li indicates liver; pa, pharyngeal arch. Other abbreviations as in the legends of Figure 1 and 2.
recruitment and myocardial differentiation of the adjacent Tbx18+ mesenchyme, maintaining Tbx18, and not initiating Nkx2-5 expression.

**Lineage analysis of sinus horn development**

We addressed the origin of the sinus horn cells by using the two-component Cre-loxP system. The first component is a transgene expressing Cre in a cell-type specific manner. The second component is a conditional lacZ reporter, R26R, which is irreversibly activated by Cre. \(^1\) Progeny of labeled cells continue to express β-galactosidase even after Cre expression has been terminated, permitting to map the fate of these cells. We first analyzed the contribution of atrial (Cx40+, Nppa+) myocardium to the Cx40- Nppa- sinus horns during development. The Nppa-Cre line drives Cre expression specifically in Cx40+ Nppa+ atrial myocardium from E9.5 onward, but not in the inflow tract myocardium, resulting in detectable β-galactosidase labeling at E10.5 \(^1\) and robust labeling at E11.5 (Figure 3A). At E17.5, we observed almost complete recombination within the atria of Nppa-Cre / R26R double transgenic animals, whereas the myocardial cells of the caval veins bordering the atria remained almost free of recombined cells (Figure 3B). The few recombination events detected in the caval veins were probably due to sporadic activity of the Nppa promoter. In line with the expression analysis, this experiment shows that the sinus horns are not derived from atrial myocardium.

We next deployed the Nkx2-5-IRES-Cre knock-in line, which drives expression of Cre in a pattern identical to that of Nkx2-5\(^1\) (Figure 3C). Because all myocardial cells express Nkx2-5 at the start of sinus horn formation at E9.5 (Figure 2A), we could test whether the Nkx2-5- sinus horn myocardium is derived from the Nkx2-5+ caudal myocardium. In Nkx2-5-IRES-Cre / R26R double knock-in embryos of E10.5, all myocardium except that of the sinus horns was labeled by β-galactosidase (Figure 3D and 3E). These results show that the sinus horns are not derived from Nkx2-5+ cells, suggesting that they are derived from the adjacent Tbx18+ posterior pericardial mesenchyme instead. This is supported by the pattern of β-galactosidase activity from the lacZ reporter gene in the Tbx18 locus that recapitulated Tbx18 expression in the myocardial sinus horns at E12.5 (Figure 2E). In contrast, atrial cells, venous valve cells and atrial septal cells failed to express β-galactosidase, a lineage marker due to its longevity, indicating that they are not derived from Tbx18+ precursors.

The complementary expression of Nkx2-5 and Tbx18 may suggest mutual transcriptional repression. However, neither Tbx18 nor Nkx2.5 were derepressed in Nkx2.5 and Tbx18 mutants, respectively, indicating that upstream factors and/or patterning processes are responsible for the formation of the border between the sinus horns and atria (Figure 3G-N).
We analyzed the presence of PCNA, a marker for proliferating cells, in the sinus horns at a stage of recruitment, E11.5, and after recruitment, E14.5 (Figure 3P through 3U). At E11.5, almost all atrial cells proliferated, but only a few of the cells of the myocardial sinus horns revealed PCNA expression. In contrast, a large fraction of the non-myocardial cells of the mesenchyme bordering the sinus horns was PCNA-positive (Figure 3S). At E14.5, PCNA staining was detected in a large fraction of the sinus horn cells (Figure 3T and 3U).

Collectively, these results exclude that the sinus horns are formed by prolif-

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Table 1. Genotype and sinus horn morphology of embryos from matings of Tbx18-/- mice. Assessment of the formation of a left sinus horn structure, defined as a venous structure bulging into the intrapericardial cavity. Each embryo in the mutant group (-/-) has 1 or more littermates in the control (+/+, +/-) group. Only embryos were included, of which the entire region of interest (sinus horns or common cardinal veins, venous valves, dorsal atrial wall, pulmonary vein) was analyzed on serial sections. NA indicates not applicable. *No. of embryos with myocardium in the wall of the sinus horns (+/+, +/-) or embedded common cardinal vein (-/-) per no. of total embryos analyzed. †Limited myocardium was observed in the walls of the systemic veins in these embryos. One of these E12.5 embryos has been 3D reconstructed (Figure 4). ‡The systemic veins were embedded in the pleuro-pericardial membrane. These veins had a myocardial wall. §The pulmonary vein was normal and the dorsal atrial wall intact in these fetuses. ¶One representative fetus was 3D reconstructed (see Figure 4).
Figure 4. Sinus horn defects in Tbx18 mutant hearts. Section in situ hybridization analysis of heterozygous (Tbx18lacZ/+ or Tbx18GFP/+) embryos (left column) and of mutant (Tbx18lacZ/lacZ or Tbx18GFP/GFP) embryos (right column). Probes and stages are as indicated. G and I show sister sections, as do H and J. Black arrows indicate sinus horns and the equivalent structure in the mutant, respectively. Dashed lines indicate the pericardial wall. Red arrows in panel L point to notches in the lung that accommodate the mis-positioned caval veins. Panels E and F show dorsal views of reconstructions of hearts. Color codes are as in Figure 1. Black arrows highlight absence of Cx40-negative sinus horn myocardium in the mutant. Panels M and N show a reconstruction of an E17.5 Tbx18GFP/GFP heart. In M, gray shows all myocardium, orange the lumen. The pulmonary vein has been removed. The dorsal atrial wall is intact. N shows a lumen cast, all myocardium has been removed. Before entering the right atrium, the lumen of the small and malformed systemic venous return does not make contact (black arrow) with the well-developed pulmonary vein. For abbreviations, see legends of Figures 1 and 3. The bar represents 300 µm.
Figure 5. Proliferation and apoptosis in Tbx18−/− sinus horns. (A-I) Immunohistochemically stained serial sections of E10.5 Tbx18GFP/+ embryos are shown at the left, of Tbx18GFP/GFP embryos at the right. (A, B, D-I) Black arrows indicate BrdU and phosphorylated histone H3 (pH3), respectively, positive cells in the GFP-expressing area. (C) Labeling index is defined as the ratio of BrdU-positive cells to total cell number in the GFP-positive area. No significant difference in cell proliferation was detected between the two genotypes. (J-M) TUNEL assay on sections of E10.5 embryos. Black arrows indicate apoptosis in foregut (fg) endoderm and in the mesenchyme between dorsal aorta and cardinal vein. For abbreviations, see the legend of Figure 1.
eration of existing Nkx2-5+ caudal myocardium. They rather suggest that the sinus horns are formed by myocardial differentiation (recruitment) and subsequent proliferation of Tbx18+ mesenchyme (Figure 3O).

**Systemic venous defects in Tbx18-deficient mice**
The specific expression of Tbx18 in the systemic venous return suggested a functional involvement of the gene in the formation of this heart region. To investigate the role of Tbx18 in this process, we analyzed Tbx18-deficient mice for phenotypic alterations in this region. Heterozygous Tbx18lacZ/+ and Tbx18GFP/+ fetuses were unaffected. Hearts of Tbx18+/- embryos appeared grossly normal. Similarly, the morphology and gene expression profiles of the pulmonary veins appeared unchanged in Tbx18-deficient mice, consistent with the absence of Tbx18 expression in the mesenchyme and the myocardium of the developing pulmonary veins (Figure 2F). We detected GFP expression around the common cardinal veins both in Tbx18GFP/+ and Tbx18GFP/GFP embryos (the GFP signal being stronger in the Tbx18GFP/GFP mutants), indicating that Tbx18 is not required for the formation of the Tbx18+ precursor population (Figure 4A and 4B). At E9.5, the inflow tracts of mutant and control embryos appeared similar on sections, but at E10.5 all mutants had failed to form a left sinus horn in the pericardial cavity (Figure 4A and 4B and Table 1). Instead, the left and right common cardinal veins remained embedded in the latero-dorsal mesenchyme, and until E12.5 their walls did not express myocardial markers (Figure 4C and 4D and Table 1). The malformation was restricted to the venous GFP-expressing domain.

In some E12.5 embryos primary myocardial cells protruded into the walls of the common cardinal veins, indicating they had initiated differentiation into the myocardial phenotype (Table 1), as is illustrated by the 3D reconstructions of a Tbx18lacZ/+ and a Tbx18lacZ/lacZ heart (Figure 4E and 4F). The caudal myocardium, including the sinus horn myocardium of mutants and littermate controls expressed Bmp4, Tbx5, Serca2a, cTnl, aMhc, Mic2a, Cx43, aSMA and Desmin at comparable levels (not shown).

From E14.5 on, Tbx18 mutant hearts featured thinner and smaller veins that were abnormally positioned within the membranous wall that separates the pericardial and pleural cavities (Figure 4G through 4L). This membrane derives from the mesenchyme that holds the common cardinal veins prior to sinus horns formation. At this stage, the venous walls had differentiated into myocardium like in the wild-type and acquired a small and deformed Hcn4+ sinoatrial nodal region (Figure 4I and 4J). At a more cranial level the veins were shifted laterally and were surrounded by lung tissue (Figure 4K and 4L). The defect was restricted to the sinus horns, as the pulmonary vein and dorsal atrial wall (mediastinal wall) were normal (Figure 4M, N). Taken together, these results indicate that in Tbx18 mutants the sinus horns fail to
develop from the pericardial mesenchyme. Myocardial differentiation of the walls of
the systemic veins is delayed, but not completely blocked in Tbx18⁻/⁻ embryos.

Because all Tbx18 mutant embryos failed to form sinus horns, we investigated whether proliferation of the Tbx18-expressing sinus horn precursor cells was decreased at E10.5, when the defect became first visible. Serial sections of E10.5 Tbx18<sup>GFP/+</sup> and Tbx18<sup>GFP/GFP</sup> embryos were alternately stained for GFP to identify the Tbx18-positive area, and for BrdU reflecting proliferation rates. No differences in proliferation rates were noticed (Tbx18<sup>GFP/+</sup>: 0.147± 0.022 vs. Tbx18<sup>GFP/GFP</sup>: 0.157± 0.022; P = 0.11, Figure 5A through 5E). Both heterozygous and Tbx18-deficient embryos displayed comparable fractions of cells in M-phase of the cell cycle within the GFP-positive mesenchyme (Figure 5F through 5I; Tbx18<sup>GFP/+</sup>: 2.2% vs. Tbx18<sup>GFP/GFP</sup>: 2.6%; P = 0.396), confirming the BrdU analysis. These results indicate that sinus horn defects are not caused by decreased proliferation in mutants. To address whether Tbx18-deficiency affects programmed cell death, apoptosis was examined by TUNEL analysis and cleaved-caspase 3 staining on serial sections of E9.5 to11.5 Tbx18<sup>GFP/+</sup> and Tbx18<sup>GFP/GFP</sup> embryos. Apoptosis was not detected in the GFP-positive region of either genotype (Figure 5J through 5M).

**Discussion**

*Sinus horns form after E9.5 from a novel cardiac precursor population*

Previous studies have suggested that all components of the multi-chambered mammalian heart derive from 2 fields of cardiac precursor cells. The left ventricle that is already represented in the early linear heart tube forms from a first field, whereas all other cardiac components are subsequently added from a second field that lies anterior and dorsal to the linear heart tube. These fields, or lineages, are marked by expression of the homeobox transcription factor genes Nkx2-5 and Isl1. Thus far the cell lineages of the systemic venous tributaries have only been investigated at stages earlier than E9.5. Our present study indicates that Nkx2-5⁺ caudal cardiac cells constitute the venous pole myocardium up to E9.5, but are then taken up into the forming atria and venous valves (Figure 6). Thus, the “sinus horns” of the early heart tube consist of atrial chamber and venous valve precursors, whereas the “real” sinus horns of the systemic venous return only form after E9.5. These sinus horns will subsequently form the sinus venarum and myocardial components of the right superior and inferior caval vein and persistent left caval vein (coronary sinus in human).

The mesenchymal precursor population of the sinus horns not only fails to express Nkx2-5, the majority of the precursors also lack Isl1 expression prior to their
differentiation. Instead, they uniquely express Tbx18, and will give rise to myocardium that is distinguished from the atrial myocardium by its Tbx18+ Nkx2-5- Cx40- expression profile (Figure 6). In addition, the Tbx18+ population is positioned ventral-caudal-lateral to the second heart field, meeting the Isl1+ precursors at the site where the venous pole myocardium is recruited. Therefore, we conclude that the sinus horns are formed from a precursor population that is genetically distinct from the first and second heart fields. Alternatively, the sinus horn precursor population may be considered as a molecular distinct sub lineage of the second field.

Figure 6. Model of sinus horn formation. (A) A simplified scheme of the venous return region. Colors represent mesenchyme and myocardium, respectively, with phenotypes as indicated. Arrows show direction of processes. The pericardial cavity (pc) enlarges by proliferation of its wall. The proliferating Tbx18-expressing wall mesenchyme becomes positioned into the pericardial cavity and differentiates to myocardium, thus forming the sinus horns. (B) A scheme depicting roles and genetic interactions for Nkx2-5 and Tbx18 in the differentiation and morphogenesis of the atrial myocardium and sinus horn myocardium. Factor X and Y generate two compartments and activate Nkx2-5 and Tbx18, respectively. Nkx2-5 is required for further differentiation into atrial cells, and for the activation of atrial genes. Tbx18 is required for differentiation and formation of sinus horns from the pericardial mesenchyme compartment. A number of genes / pathways were not affected by the absence of Tbx18. Gray arrows depict processes, black arrows (genetic) interactions or lack thereof (red crosses). For abbreviations, see the legends of Figures 1 and 3.
**Tbx18 regulates morphogenesis and myocardial differentiation of the sinus horns**

Recruitment and myocardial differentiation of pericardial mesenchymal precursor cells are processes common to the formation of sinus horns and the development of cardiac components like the outflow tract from the anterior heart field. The genetic control of the latter relies on the function of *Nkx2-5*, *Isl1* and of other factors including *Mef2C*, *Foxh1*, *Fgf8* and *Tbx1*, whose mutation primarily result in defects in the anterior portion of the tubular heart. In contrast, defects in *Tbx18* mutants are restricted to the sinus horns and derived caval veins, indicating that the molecular program underlying the development of the sinus horns is profoundly distinct from that of other components of the heart.

*Gata4*, *Gata6*, *Raldh2* and *Coup-TFII* and *Tbx5*-mutants show defects of the atrial and venous pole region, suggesting involvement of these factors in embryonic inflow tract development. However, these defects are manifested at E9.5 or earlier, when the progenitors of the atria still reside in the inflow tract. Although these factors certainly play a prominent role in the formation of the caudal aspect of the heart tube, one cannot infer a function in regulating the formation or differentiation of the sinus horns from the *Nkx2-5- Tbx18*+ precursor population at this moment. Moreover, the expression of these genes is not affected in *Tbx18* mutants (data not shown), suggesting that *Tbx18* acts downstream or in an independent molecular pathway.

Although the *Cx40- Nppa*-expression profile of the sinus horn myocardium resembles that of the primary myocardium of the atrioventricular canal and the outflow tract, several findings argue against a common regulatory program. In the primary myocardium of the atrioventricular canal, *Tbx2* and *Tbx3*, two closely related T-box transcription factors, counteract *Nkx2-5/Tbx5* mediated activation of *Cx40* and *Nppa* expression, thus, preventing expansion of the chamber phenotype into this region. In contrast, neither *Tbx2/Tbx3* nor *Nkx2-5* is expressed in the sinus horn myocardium. Loss of *Tbx18* caused reduction and delay in myocardial differentiation, but did not result in ectopic expression of *Nppa* or *Cx40* in the sinus horn myocardium. This suggests that the absence of *Nkx2-5* from the sinus horns is sufficient to prevent activation of atrial chamber-specific genes in the sinus horns (Figure 6B). This is in line with the observation that both expression of chamber-specific genes and chamber formation are severely affected in *Nkx2-5* mutant mice.

**Congenital malformations and arrhythmias of the venous pole**

Recent studies indicated that the development of the systemic and pulmonary venous returns involves physically separated and phenotypically distinct types of myocardium. However, not all studies fully agree on this issue because the systemic and pulmonary venous returns also share phenotypic properties. To address
these issues, however, insight into the position and nature of the mesenchymal pre-
cursors of the venous returns is required. Our study now provides some of these in-
sights. We found that Tbx18 is critical for the formation of the sinus horns but not for
the pulmonary venous return. Moreover, the systemic venous returns and their pre-
cursors express Tbx18 and not Nkx2-5, whereas the pulmonary venous return and
its precursors show the opposite profile. These observations show that distinct reg-
ulatory mechanisms are required for each structure, and indicate that the precursors
have a distinct phenotype. Nevertheless, because systemic and pulmonary vein pre-
cursors at the caudal pole of the heart are in close proximity, it is conceivable that er-
rors in patterning and misdirection of fate of either population may result in
malformations such as communications between the sinus venosus and pulmonary
vein, as seen in abnormal venous return.4 It will be interesting to identify additional
genes involved in the specification of the precursor sub-populations to test this hy-
pothesis.

Foci of ectopic beats that initiate atrial arrhythmias are typically found in my-
ocardium surrounding the pulmonary vein. However, a fraction of these ectopic beats
arises from the sinus horn-derived structures.7-9 Although the mechanism behind the
formation of these ectopic foci has not been solved, the molecular pathways may be
distinct for the pulmonary vein and systemic venous return structures, the latter in-
volving small regions with nodal, spontaneously depolarizing activity. We observed
that at late fetal stages the primary myocardial sinus horns (caval veins), initiate Cx40
expression and lose expression of Hcn4, which is essential for embryonic and adult
cardiac pace-making.32 Therefore, the sinus horns loose their nodal phenotype. It is
conceivable that some of the primary myocardium of the sinus horns does not suffi-
ciently mature and persists. This would provide a molecular explanation for the for-
modation and the non-random distribution of these foci.

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References

1. Sadler TW. Langman’s medical embryology, ed8th, USA, Lippincott Williams & Wilkins, 2000, pp 3-504


11. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell. 2003;5:877-889


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<tr>
<td>30</td>
<td>Franco D, Campione M, Kelly R, Zammit PS, Buckingham M, Lamers WH, Moorman AFM. Multiple transcriptional domains, with distinct left and right components, in the atrial chambers of the developing heart. <em>Circ Res.</em> 2000;87:984-991</td>
</tr>
<tr>
<td>31</td>
<td>deRuiter MC, Gittenberger-de Groot AC, Wenink ACG, Poelmann RE, Mentink MMT. In normal development pulmonary veins are connected to the sinus venosus segment in the left atrium. <em>Anat Rec.</em> 1995;243:84-92</td>
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