Three-dimensional and molecular analysis of the developing human heart

Sizarov, A.

Publication date
2011

chapter 4

Molecular Analysis of the Patterning of the Conduction Tissues in the Developing Human Heart

Aleksandr Sizarov
Harsha D Devalla
Robert H Anderson
Robert Passier
Vincent M Christoffels
Antoon FM Moorman

published in:

Circulation: Arrhythmia and Electrophysiology. 2011
[Epub ahead of Print]

Abstract

Background – Recent studies in experimental animals have revealed some molecular mechanisms underlying the differentiation of the myocardium making up the conduction system. To date, lack of gene expression data for the developing human conduction system has precluded valid extrapolations from experimental studies to the human situation.

Methods and Results – We performed immunohistochemical analyses of the expression of key transcription factors, such as ISL1, TBX3, TBX18 and NKX2-5, ion channel HCN4 and connexins in the human embryonic heart. We supplemented our molecular analyses with three-dimensional reconstructions of myocardial TBX3 expression. TBX3 is expressed in the developing conduction system, and in the right venous valve, atrioventricular ring bundles, and retro-aortic nodal region. TBX3-positive myocardium, with exception of the top of the ventricular septum, is devoid of fast-conducting connexin40 and -43, and hence identifies slowly conducting pathways. In the early embryonic heart, we found wide expression of the pacemaker channel HCN4 at the venous pole, including the atrial chambers. HCN4 expression becomes confined during later developmental stages to the components of the conduction system. Patterns of expression of transcription factors, known from experimental studies to regulate the development of the sinus node and atrioventricular conduction system, are similar in the human and mouse developing hearts.

Conclusions – Our findings point to the comparability of mechanisms governing the development of the cardiac conduction patterning in human and mouse, which provide a molecular basis for understanding the functioning of the human developing heart prior to formation of a discrete conduction system.

Introduction

The initiation and propagation of the electrical impulse in the mammalian heart is coordinated by the conduction system. The chamber-forming heart during early development, however, does not possess a histologically distinct conduction system, nor fibrous insulation between the atrial and ventricular myocardial masses, yet is still able to generate an adult-type electrocardiogram, including atrioventricular delay.[1,2] It is the differential expression of fast-conducting gap junctional proteins in the different compartments of the embryonic heart that provides the basis for the adult pattern of conduction.[3] Recent studies in experimental animals have revealed some of the molecular mechanisms underlying the differentiation of atrial and ventricular working myocardium, along with the appearance of the different components of the conduction system.[4] To date, however, lack of gene expression data for the developing human heart has precluded valid extrapolations from experimental studies to the situation in man. Extant studies on the development of the conduction system in the human heart have been based on serial sections stained either non-specifically,[5-10] or for neural tissue antigen GlN2/Leu7/HNK-1,[11,12] the function of which in the developing heart is unknown. Furthermore, although comparable methods have been used, conclusions made from these studies have been far from consonant. To resolve these ongoing controversies, we have performed an immunohistochemical analysis, coupled with three-dimensional reconstructions, in the developing human heart of the patterns of expression of several transcription factors and proteins known to affect the myocardial conduction properties. Although we have observed important differences in gene expression, our findings point to the comparability of the mechanisms governing the patterning and development of the cardiac conduction tissues in man and mouse.

Material and Methods

Human Embryos

Collection of human embryonic material, and its preparation for histological studies, was described previously.[13] Additionally, we used three human embryos of about 8 weeks of development, one coming from the MRC–Wellcome Trust Human Developmental Biology Resource, managed by the Institute for Human Genetics of Newcastle University, United Kingdom, and the other two from the Center for anticonception, sexuality and abortion (CASA), Leiden University Medical Center, the Netherlands. Embryos were examined under a stereomicroscope for gross anomalies, and graded according to the Carnegie criteria. In total,

we have used three embryos at stage 12, five embryos at stage 13-14, six embryos at stage 15-16, four embryos at stage 18, and six embryos at stages 21-23. We included only embryos considered normal. Since the blood was not removed from the embryos, immunohistochemical stainings with some fluorochromes resulted in strong erythrocyte autofluorescence. Collection and use of the human embryonic material for research were approved by the Medical Ethical Committees of the Universities of Tartu, Estonia, Leiden and Amsterdam, the Netherlands.

Immunohistochemistry, in situ *Hybridization and 3D Reconstructions*

Immunofluorescent staining, in situ hybridization, and 3D reconstructions were performed as described.[13,14] Serial sections of the embryos collected at Leiden University Medical Center were processed by indirect immunohistochemistry. Detailed description of the used protocols, antibodies and riboprobe are given in the Data Supplement.

Limitations of Our Study

Assessment of conduction requires direct measurements in the myocardium, which cannot be performed in human embryos. The assessment of expression patterns of fast-conducting gap-junctional proteins connexin40 and -43, which play an important role in the conduction of the myocardium of the mouse heart,[15] nonetheless, may give an impression of the possible patterning of conduction. The non-standardized fixation and limited numbers of available embryos did not permit complete optimization of the staining protocol for some antibodies and probes. However, immunohistochemical and in situ hybridization stainings shown here proved to be reproducible. The signal was considered strong if intense fluorescence of the specific signal was obtained with short exposure times and weak if 2-3 times longer exposure times were required.

Results

We first assessed the patterns of expression of the hyperpolarization-activated cyclic nucleotide-gated cation channel 4 (HCN4), known to be involved in the pacemaking mechanism of the sinus node.16 Mouse embryos, in which Hcn4 was knocked-out, show extremely low heart rates and embryonic lethality.[17] In the early period of development, spanning Carnegie stages 10 through 12, and corresponding to around 22-26 days of development, HCN4 is expressed throughout the looping heart tube, including the differentiating chamber myocardium, albeit with a decreasing gradient of expression from the venous to the arterial pole (Figure 1A,B). At the venous pole, HCN4 is initially expressed very strongly not only in the myocardial, but also in the adjacent mesenchymal wall of the venous sinus (Figure 1A). At stage 16, equivalent to around 37 days of development, HCN4 is virtually absent from the ventricular chambers, but remains highly expressed in the myocardium of atrioventricular canal and atrial

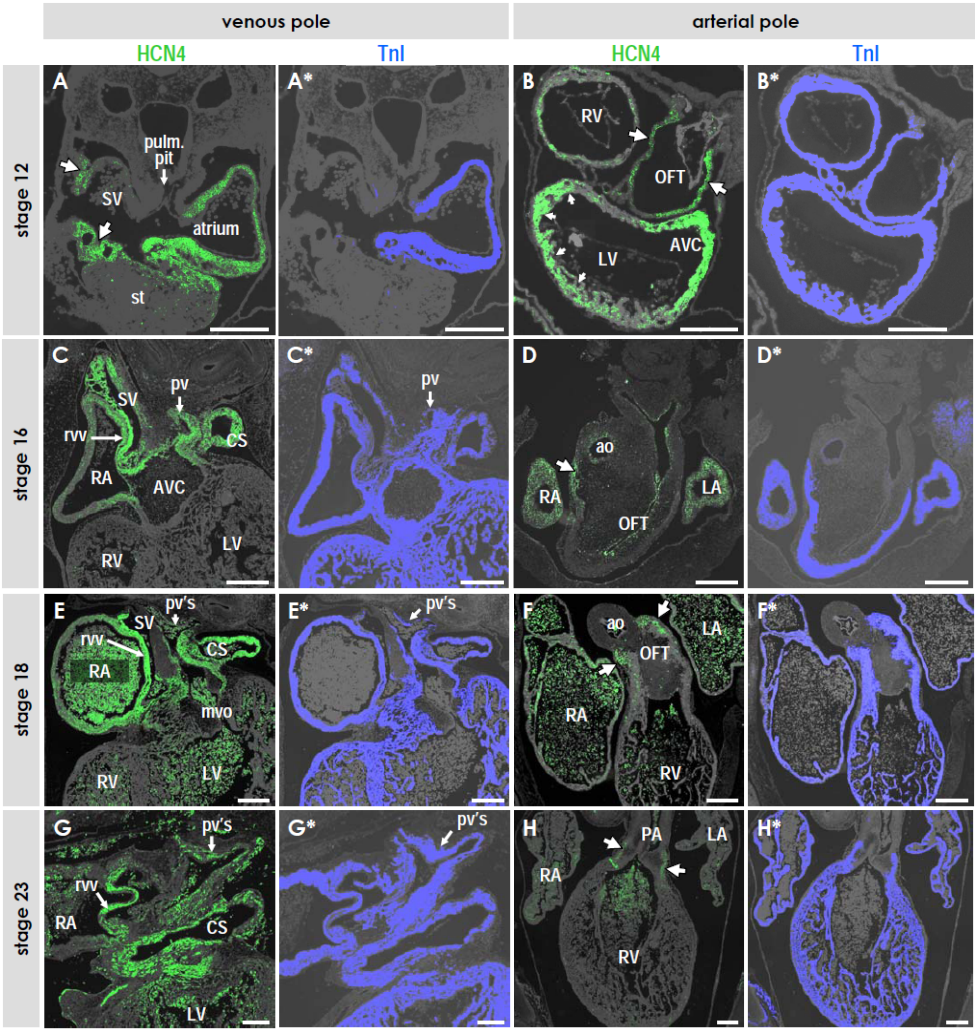


Figure 1. HCN4 expression in the developing human heart. Transverse sections were incubated with antibodies against HCN4 and troponin I (TnI) as myocardial marker. Note the strong HCN4 expression in part of the left ventricular wall (small arrows in panel B). Large arrows in panels B,D,F,H point to weak HCN4 expression in the outflow tract myocardium, while the arrows in panel A point to the mesenchymal expression of HCN4 in the young embryonic heart. See text for further description. The strong autofluorescent signal within the cavities of the atria and ventricles (panels E,F,G,H) is due to the massive presence of erythrocytes. Abbreviations: ao, ascending aorta; AVC, atrioventricular canal; CS, coronary sinus; PA, pulmonary artery; pv('s), pulmonary vein(s); OFT, outflow tract; st, septum transversum; SV, sinus venosus; RA/LA, right/left atrium; RV/LV, right/left ventricle; rvv, right venous valve. Scale bars, 200 µm.

walls, along with the myocardium at the developing veno-atrial junctions (Figure 1C-G). During late embryonic stages 18 through 23, which correspond to around 46-58 days of development, expression declines in the atrial chambers, but the strong expression seen in the sinus node primordium (see below) is also evident in the myocardium surrounding the atrioventricular junction and coronary sinus. Low expression remains in the myocardium surrounding the developing arterial valves at these late stages (Figure 1E-H).

Understanding the patterns of gene expression in the changing morphological context is facilitated by reading the results, described in the next paragraphs, along with the interactive 3D-PDF (accessible at *http://circ.ahajournals.org*).

Development of the Sinus Region

The definitive sinus node of the human heart is a comma-shaped structure located in the terminal groove at the junction of the superior caval vein and the right atrium. No such structure is seen in the early human embryos studied. The systemic venous sinus, or sinus venosus, representing the confluence of the systemic venous tributaries, is first recognisable at stage 12. Its walls are initially non-muscular,[13] but already express HCN4 (Figure 1A). As the venous sinus shifts to the right, prominent venous valves form at its junction with the right atrium. The left superior caval vein acquires its position within the left atrioventricular groove, where it is recognisable as the precursor of the coronary sinus. The walls of the venous tributaries then muscularize, with the right lateral myocardial wall of the venous sinus becoming thickened and porous,[13] remaining traceable along the right-sided sinuatrial junction in the oldest embryo analyzed (Figures 2,3). We consider this structure to be the primordium of the sinus node.

Many molecular markers have been used to delineate the precursors of the definitive conduction axis in the embryonic mouse heart,18 including the transcription factor Tbx3.[19] This factor represses the differentiation of chamber myocardium, permitting specific areas of the looping heart to develop into the conduction system.[20] Using 3D reconstructions, we assessed the myocardial expression of TBX3 relative to the primordium of the sinus node. TBX3 is expressed in the nodal primordium, in the right venous valve and the myocardium of the atrioventricular canal, and, in late embryos, the atrioventricular junctions (Figure 2). At stages 14 through 16, at the stage of formation of the primary atrial septum, TBX3 is expressed in the left venous valve, the leading edge of the atrial septal myocardium, and the floor of the right atrium, which, in turn, are contiguous with the atrioventricular canal (Figure 2A*). The expression of TBX3 outside the atrioventricular canal is weak (not shown) and disappears by the time of completion of atrial septation at stage 18 (Figure 2B**).

In the stage 12-14 embryos the entire wall of the systemic venous sinus and common atrium is positive for HCN4 (Figures 1A,3A). At later stages, HCN4 expression gradually declines in the atrial walls and becomes confined to the myocardium surrounding the venous

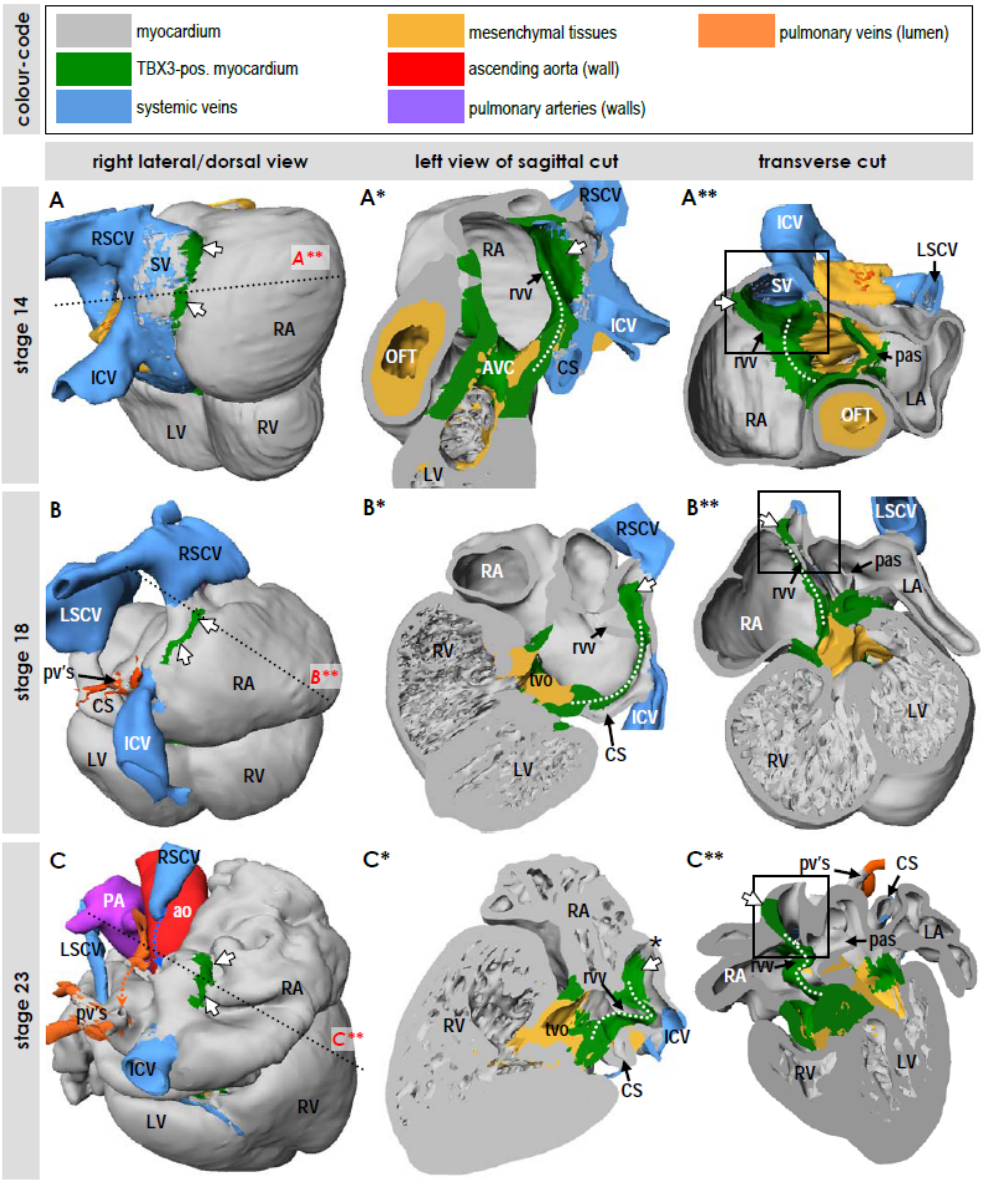


Figure 2. Three-dimensional analysis of sinus node formation based on the expression of the conduction system marker TBX3. Note, that only a small part of the myocardial wall of the venous sinus is TBX3-positive (panels A,B,C). The TBX3-positive sinus node primordium (white arrows) is in continuity with the TBX3-positive myocardium of the atrioventricular canal or, in late embryos, junction via the right venous valve (dotted white line). The lines in panels A,B,C indicate the plane of transverse cuts shown in the panels A**,B**,C**. Boxed areas are enlarged in Figures 2,3. For further description, please, see the text. Abbreviations: ICV, inferior caval vein; mvo/tvo, mitral/tricuspid valve orifice; pas, primary atrial septum; RSCV/LSCV, right/left superior caval vein; for other abbreviation see previous figure.

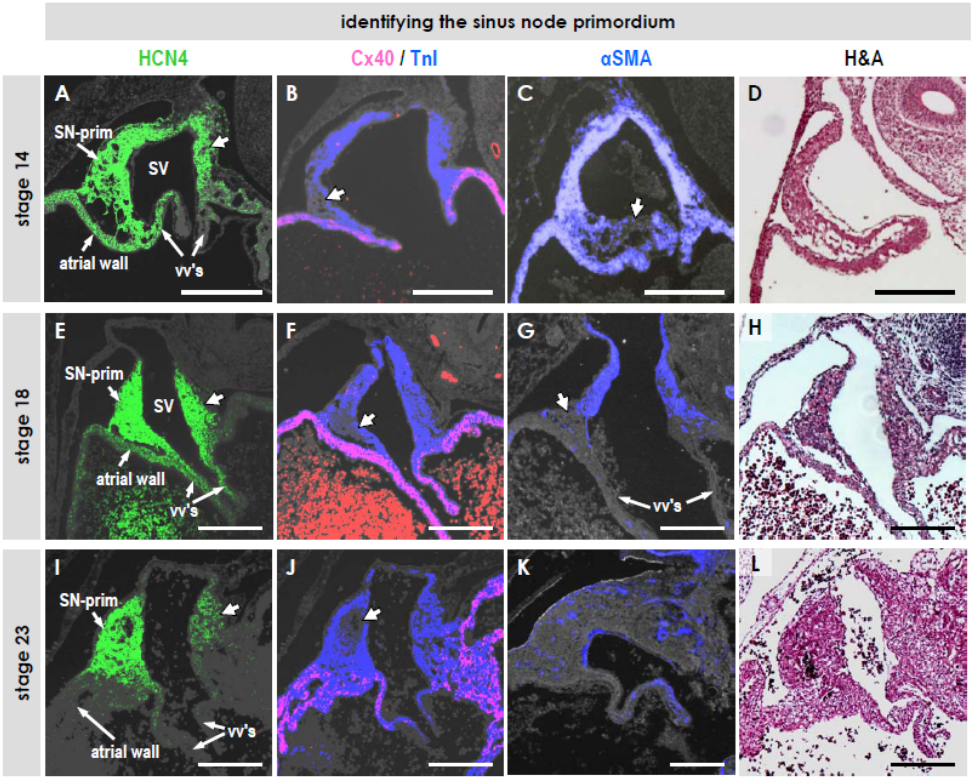
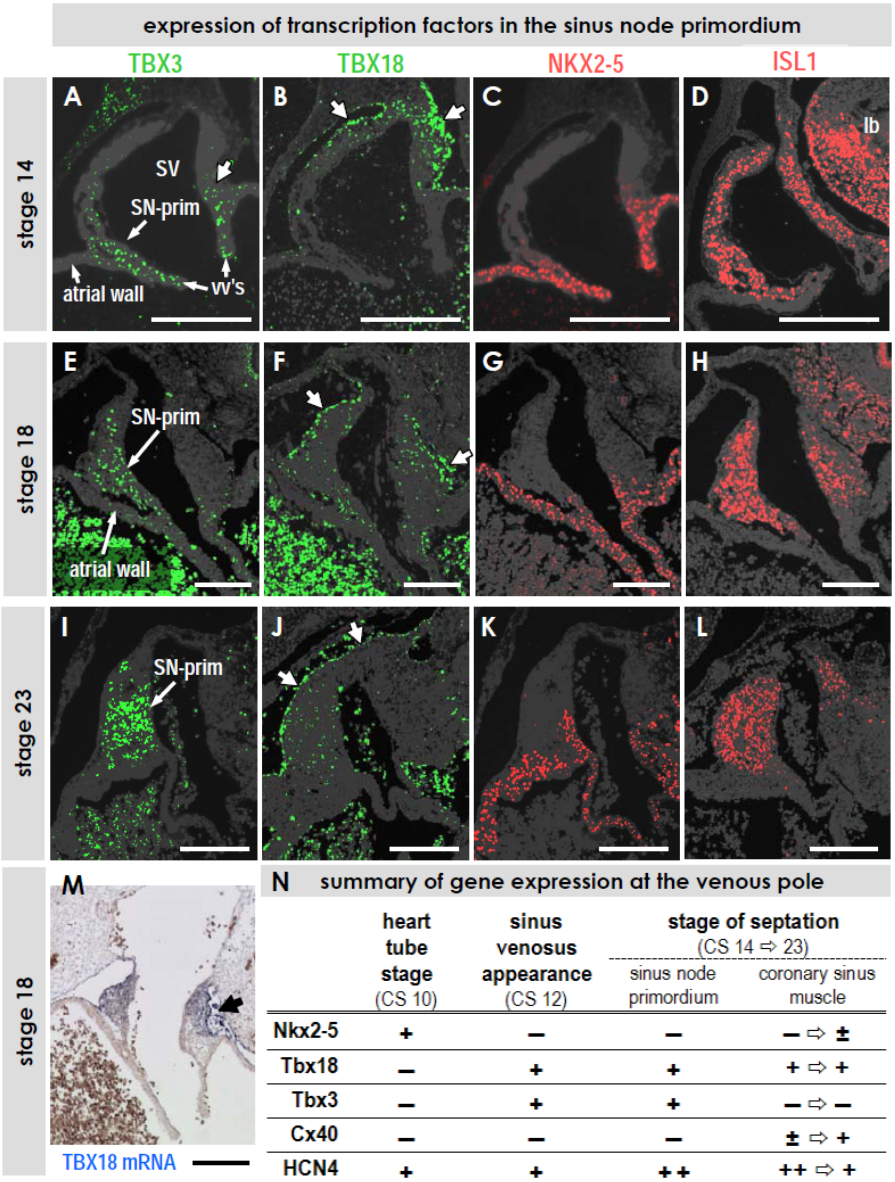


Figure 3. x Characterization of the sinus node primordium. The levels of the serial sections through the venous sinus are shown in Figure 2A**,B**,C**. The whole venous sinus is positive for HCN4, including the left-sided wall (arrows in panels A,E,I), and is negative for connexin40. Note, that a small subset of cardiomyocytes within the right lateral wall of the venous sinus is negative for TnI and αSMA already at the earliest stage (arrows in panels B,C). No histological differences were observed between the cells comprising the sinus node primordium (SN-prim) and the right atrial wall (panels D,H,L) at the stages examined. Abbreviations: SV, sinus venosus; vv's, venous valves. Scale bars, 200 μm.

tributaries (Figures 1C,E and 3E,I). In contrast to the broad expression of HCN4, only a small part of the walls of the systemic venous tributaries is positive for TBX3 (Figure 4A). This area, representing the sinus nodal primordium, increases in size at later stages (Figure 4E,I), and is from the outset virtually negative for expression of troponin I and α-smooth muscle actin (αSMA) (Figure 3B,C). As with the definitive sinus node,[21] the nodal primordium does not express connexin40 (Figure 3B,F,J). No histological differences were found between the presumed nodal primordium and the right atrial wall (Figure 3D,H,L).

In addition to TBX3, the entirety of the systemic venous sinus was positive for ISL1, expression of this gene being complementary to that of NKX2-5 (Figure 4). Unlike the strong TBX18 expression in the epicardium, we observed different levels of TBX18 protein and mRNA expression in the sinus nodal primordium. Myocardial expression of TBX18 protein was very weak (Figure 4B,F,J), but expression of mRNA was robust and broader (Figure 4M). We found fundamental molecular differences between the nodal primordium within the venous sinus wall



N summary of gene expression at the venous pole

	heart tube stage (CS 10)	sinus venosus appearance (CS 12)	stage of septation (CS 14 ⇨ 23) sinus node primordium	coronary sinus muscle
Nkx2-5	+	–	–	– ⇨ ±
Tbx18	–	+	+	+ ⇨ +
Tbx3	–	+	+	– ⇨ –
Cx40	–	–	–	± ⇨ +
HCN4	+	+	++	++ ⇨ +

Figure 4. Expression of transcription factors in the developing sinus region. Serial sections at the same level as shown in Figure 3 were incubated with antibodies (panels A-L) or riboprobe (panel M) as indicated. At early stages also the left-sided wall of the venous sinus is positive for TBX3 (arrow in panel A), expression of which gradually confines to the right side only (panels E,I). Note the very weak TBX18 expression in the venous sinus myocardium as compared to the epicardial expression (arrows in B,F,J,M). Note also the almost strict complementarity between the expression domains of NKX2-5 and ISL1 in the venous sinus. Panel N summarizes the developmental changes in gene expression occurring at the venous pole of the heart (CS indicates here Carnegie stage). Abbreviation: lb, lungbud; for other abbreviations, see previous figures. Scale bars, 200 µm.

and the myocardial sleeve of the developing coronary sinus, suggested by some investigators to form a left-sided sinus node,[22] albeit that they share the same developmental origin.[23] The myocardium around the coronary sinus, unlike the developing sinus node, does not express TBX3, initiates early expression of connexin40, and is strongly positive for αSMA. The developmental changes in gene expression at the venous pole of the heart are summarised in Figure 4N.

Development of the Atrioventricular Region at Intermediate Stages

Stages 13 through 16, equivalent to around 28-38 days of development, are marked by unmistakable growth of the chambers and formation of atrial and ventricular septal structures, along with a rightward shift of the atrioventricular canal.11 Previously we reported that in the early chamber-forming human heart myocardial expression of TBX3 is first observed in the floor of the common atrium and the atrioventricular canal.[14] By reconstructing the myocardial expression of TBX3 at later developmental stages, we have related the development of the atrioventricular conduction tissues to the changing cardiac morphology. At stage 16, TBX3 is expressed throughout the myocardium of the atrioventricular canal, contiguous with the TBX3 expression in the right venous valve, including the spurious septum, the leading edge of the atrial septum, and the right atrial wall located dorsal to the outflow tract (Figure 5A,A*). The TBX3-positive atrioventricular canal myocardium does not express connexin40 and -43. In contrast, the ventricular trabeculations are positive for both connexin40 and -43, while the atrial walls and atrial septum are strongly positive only for connexin40 (Figure 6D,E,J,K). TBX3 is not only expressed in the atrioventricular canal dorsally and ventrally, but also at the dorsal part of the base of the outflow tract, and along the crest of the ventricular septum (Figure 5A**). The weakly TBX3-positive myocardium on top of the ventricular septum, corresponding to the presumptive bundle of His dorsally, and the so-called septal branch ventrally, is negative for connexin40. Unlike the situation in mouse,[24] it does express connexin43 at this stage (Figure 6J,K).

Expression of the protein ID2, inhibitor of DNA binding, known to be important in the specification of the atrioventricular conduction axis in mouse,[25] was not limited to the developing conduction tissues in human. We observed weak expression in the atrioventricular canal musculature and ventricular trabeculations. Strong expression, however, was present in the epicardium and mesenchyme of the epicardial grooves (Figure 6C,I). As with the sinus nodal primordium, the myocardium of the atrioventricular canal and ventricular septum does not express αSMA (Figure 6F,L), although the ventricular and atrial myocardial walls largely remain positive at these stages. In contrast to the sinus nodal primordium, the compact location of the cardiomyocytes at the top of ventricular septum, where also TBX3 expression is present, allows identification of the precursors of the atrioventricular conduction axis using routine histological

staining[5-8,10] (Figure 6A,G).

Establishment of the Atrioventricular Conduction Axis in the late Embryonic heart

At the end of the embryonic period, at stages 18 through 23, the atrioventricular junction undergoes major rearrangements, with septation being completed through fusion of several mesenchymal structures, and the atrial myocardium becoming insulated from the ventricular musculature by formation of epicardially-derived fibrous tissue.[26] With these changes, the

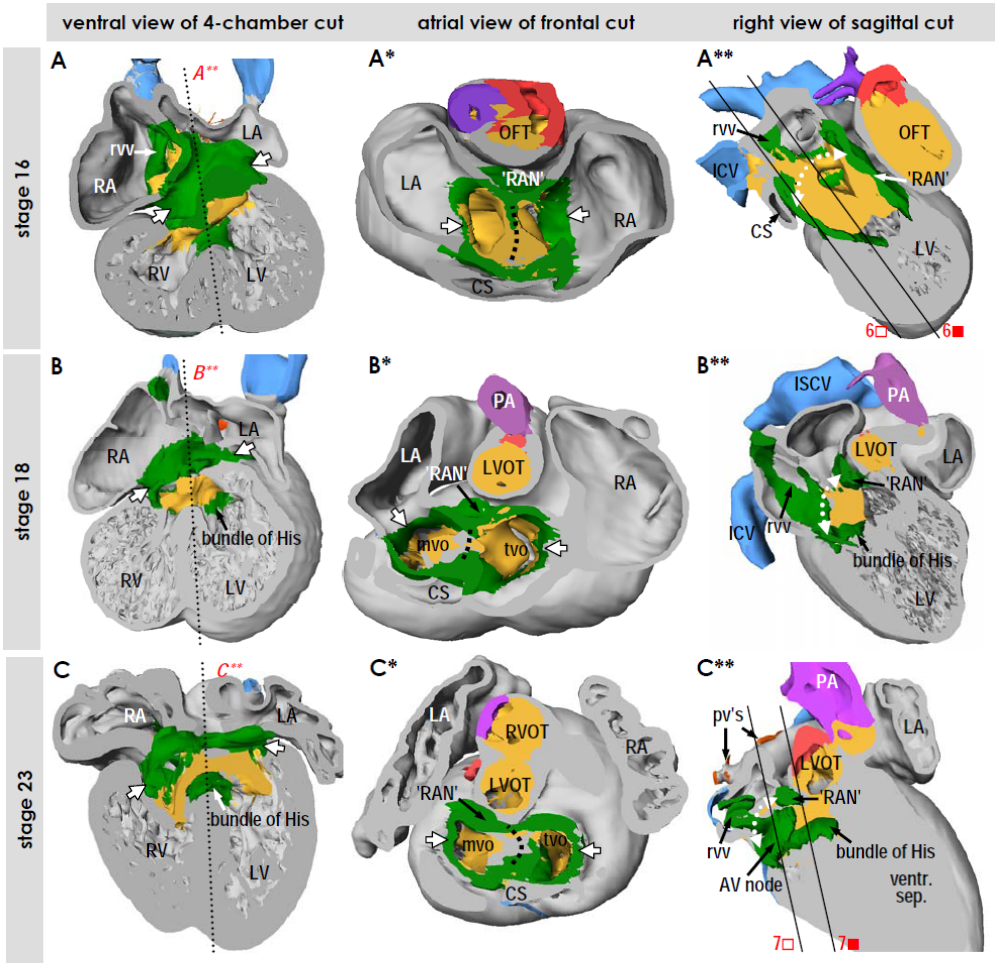


Figure 5. Three-dimensional analysis of the developing atrioventricular conduction system based on the spatial changes of myocardial TBX3 expression at three different stages. For colour-code see Figure 2. The arrows indicate the atrioventricular rings spanning the right and left sides of the atrioventricular canal. The dashed line in panels A*,B*,C* indicates the leading age, or base in the late embryos, of the primary atrial septum, which is TBX3-negative at later stages. Note the presence of the TBX3-positive myocardium also dorsal to the outflow tract, the so-called retro-aortic node region ('RAN'). The white dotted line in panels A**,B**,C** represent the continuity of the dorsal and ventral TBX3-positive myocardium, which was removed in these sagittal cuts. The lines in panels A,B,C indicate the plane of sagittal cut shown in the panels A**,B**,C**, while the lines in panels A**,C** marked by ■ and □ show the level of the sections in Figures 6 and 7. For further description, see text. Abbreviations: LVOT/RVOT, left/right ventricular outflow tract; for other abbreviations see previous figures.

expression of TBX3 in the atrioventricular canal becomes restricted to a figure-of-eight configuration in the lower rims of the atrial chambers (Figure 5B*,C*). These left and right atrioventricular ring bundles[27] converge dorsally in the developing primordium of the atrioventricular node close to the orifice of the coronary sinus, and ventrally behind the left ventricular outflow tract in the so-called retro-aortic "node" (Figure5C,C*,C**). By stage 23, the atrioventricular myocardial continuity is interrupted round the greater majority of the atrioventricular junctions, albeit in incomplete fashion (Figure 7D,F). At the dorsal aspect of the atrioventricular junctions, in contrast, the developing atrioventricular conduction axis continues to provide muscular atrioventricular continuity (Figure 5B**,C**). By this stage, the primordium of the atrioventricular node, along with the lower rims of the atrial chambers, and the muscular strands that continue to cross the forming plane of atrioventricular insulation, are negative for connexin40 and -43 (Figure 7D,E,J,K), representing slow-conducting tissues analogous to the atrioventricular canal musculature at earlier stages. The atrioventricular nodal precursors at this stage are directly contiguous with the TBX3-negative myocardium of the atrial and ventricular septa (Figure 7B). The base of the atrial septum, formed by myocardialization of the vestibular spine,[28] shows a gradient of decreasing levels of connexin40 towards the site of the nodal primordium (asterisk in Figure 7D) derived from the atrioventricular canal myocardium.[29] The ventricular septal myocytes, in contrast, express neither connexin40 nor 43 (Figure 7D,E). The TBX3-positive area of the atrioventricular nodal primordium expresses HCN4 in relatively weak heterogeneous fashion, with the part adjacent to the atrial septum being more positive than the part contiguous with the ventricular septum (Figure 7C). The atrioventricular ring bundles and retro-aortic "node" are also weakly positive for HCN4 (Figure 7C,I), as has been reported for experimental animals.[27] As with the primordium of the sinus node, the forming atrioventricular node shows a low level of troponin I (Figure 7D), and is recognisable as a lightly-stained mesh of cells by routine histology (Figure 7A), albeit that it was not yet possible at these stages to distinguish the transitional and compact portions characteristic of the definitive atrioventricular node.

When traced ventro-cranially, the developing atrioventricular conduction axis enters the forming plane of fibrous insulation as the developing bundle of His, recognizable histologically on the crest of the ventricular septum (Figures 5C,C** and 7G). The slightly darker stained cells of the developing conduction axis (Figure 7G) also express TBX3 and HCN4. After completion of ventricular septation, the developing bundle of His becomes negative for both connexin40 and -43 (Figure 7G-K). On both sides of the ventricular septum the connexin40 and -43 positive trabeculations form the myocardial strands originating from the bundle of His (Figure 7G-K), corresponding to the future bundle branches. A higher expression of NKX2-5 was observed in the developing bundle (Figure 7L), as described previously for the early human foetal heart.[30]

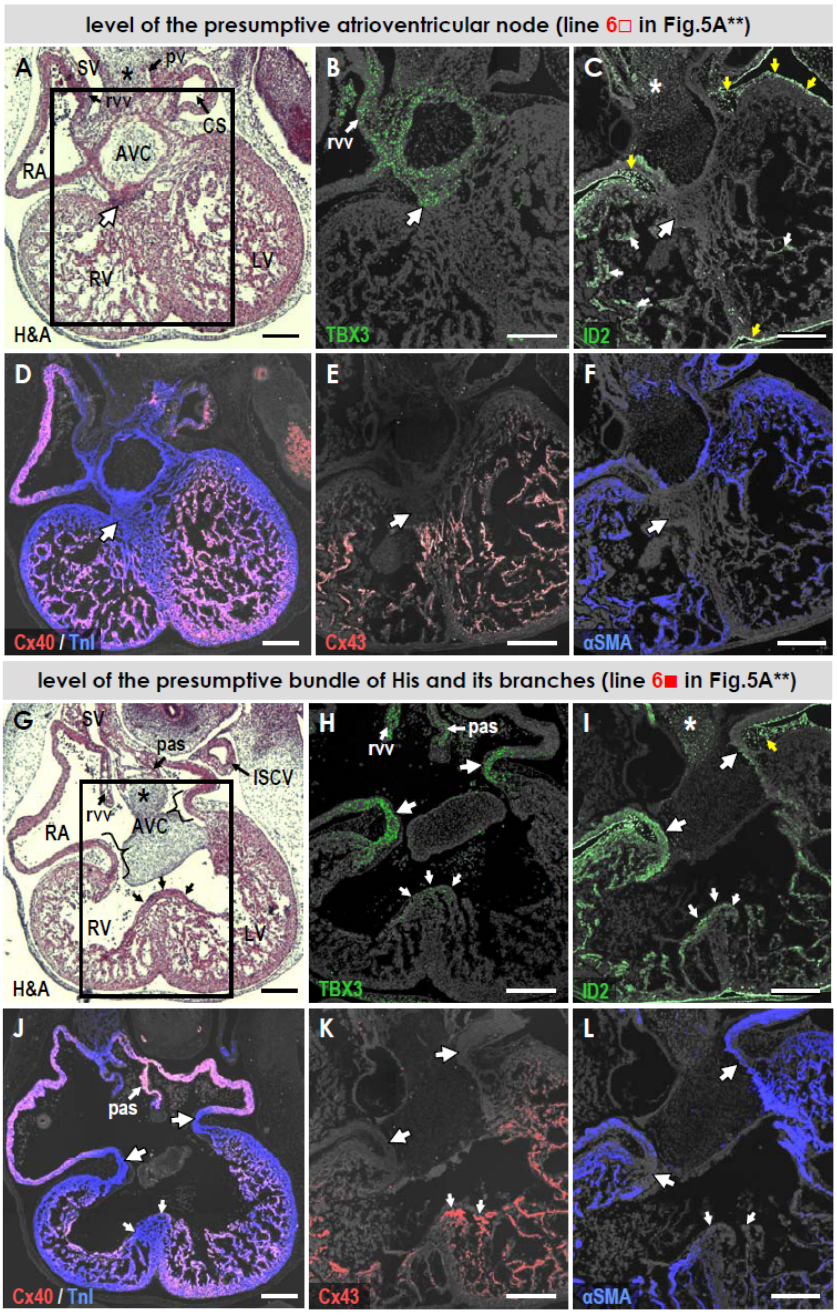


Figure 6. Morphological and molecular analysis of the developing atrioventricular conduction system in the septating heart at stage 16. Serial sections were taken through the dorsal part of the atrioventricular canal representing the primordium of the atrioventricular node (large arrows in panels A-F) and the region of the developing bundle of His and its branches (small arrows in panels G-L), corresponding to the levels shown by the lines in Figure 5A**. In panels C and I, the small yellow arrows point to strong ID2 expression in the epicardium and subepicardial mesenchyme; while the asterisk refers to the weak expression in the mesenchyme of the vestibular spine. Note, that the ventricular trabeculations are also ID2-positive. Large arrows in panels H-L indicate atrioventricular canal myocardium. For further description and abbreviations, see text and previous figures. Scale bars, 200 μm.

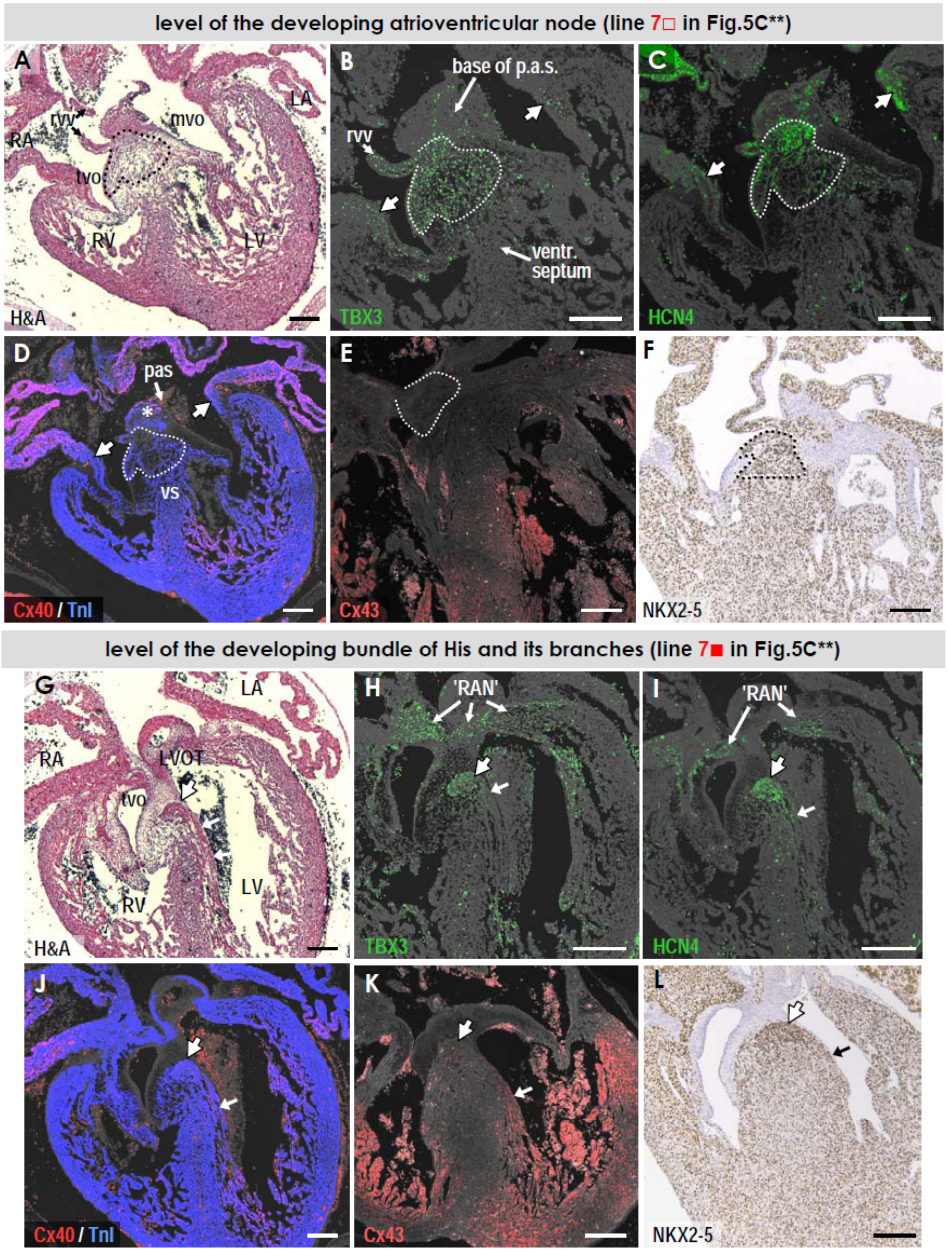


Figure 7. Morphological and molecular analysis of the atrioventricular conduction axis at the beginning of the maturation phase at stage 23. Serial sections were taken through the developing atrioventricular node (panels A-F) and bundle of His (panels G-L), corresponding to the levels shown by the lines in Figure 5C**. In panels A-F, the dotted line represents the borders of the TBX3-positive atrioventricular node primordium; while the large arrows point to the myocardium of the lower atrial rims. The asterisk in panel D refers to the base of the primary atrial septum contiguous with the atrioventricular node primordium. In the panels G-L, the large arrow points to the developing bundle of His, while the small arrows indicate the left bundle branch running along the surface of the ventricular septum. Note, that the TBX3-positive myocardium of the so-called retro-aortic node region ('RAN') expresses also HCN4 (panels H,I). At this stage the compact ventricular myocardium begins for the first time to express connexin43 (panel K), while being now completely negative for connexin40 (panels D,J). See text for further description. For abbreviations see previous figures. Scale bars, 200 µm.

In terms of expression of troponin I, the myocytes of the atrioventricular conduction axis did not differ much from the ventricular myocardium (Figure 7J), and were not yet insulated from the ventricular septal myocardium (Figure 7J).

Discussion

Although the early embryonic heart has no anatomically distinct conduction system, and demonstrates widespread muscular atrioventricular continuity, it still achieves coordinated contractions of the atrial and ventricular chambers. The spatial distribution of connexins and ion channels play a crucial role in the electrical pattering of the heart.[3] During the last decades, numerous experimental studies have revealed the molecular mechanisms governing the early specification of the conduction system.[4] The development of the conduction system in the human heart, however, has thus far been assessed only morphologically,[5-12] lack of gene expression data preventing reliable extrapolation of experimental data to the human condition. Our previous studies showed that the expression patterns of some key cardiac genes in the developing human heart are very similar to those reported for the developing mouse and chicken heart, albeit small differences in morphology were observed.[13,14] Also our current study on the molecular analysis of the patterning of the conduction tissues confirms the similarities in gene expression patterns between human and mouse.

Spatiotemporal Changes in Expression of Pacemaker Channel HCN4 Reflects Progressive Maturation of the Developing Myocardium

Working myocardium is characterized by its efficient contractile function and low automaticity, while the opposite is true for the conduction system. In the normal postnatal heart, spontaneous generation of the impulse is confined to the sinus node. In pathologic situations, arrhythmogenic activity can originate from the atrioventricular conduction axis, along with diverse myocardial structures such as the pulmonary venous sleeves, the coronary sinus, the lower atrial rims, the right ventricular outflow tract, and even the atrial appendages.[31] Despite their distinct developmental origins, all these structures share expression of the pacemaker channel HCN4 during development. At the intermediate stages, corresponding to around 6 to 7 weeks of development, we observed relatively high levels of HCN4 expression in these structures, but very low levels, if any, in the ventricular chambers. Weak expression of HCN4 is also detectable initially in the myocardium of the developing cardiac outflow tract, increasingly recognised also as a source of arrhythmogenesis in the postnatal heart.[32] During later stages, expression levels of HCN4 decline in the differentiating working myocardium at the venous pole of the heart, reflecting its ongoing maturation. In mouse, the transcription factor Nkx2-5 has been identified as an important factor in the formation of the sinuatrial boundary, suppressing Tbx3 and Hcn4 in

the atrial working myocardium.33 In contrast, we observed HCN4 expression in NKX2-5 positive areas at the venous pole of the developing human heart, suggesting a difference in the regulation of HCN4 expression between mouse and human. Maintenance of HCN4 expression in these structures during maturation, along with dysregulation of the expression of fast-conducting connexins, may provide a mechanism of the initiation of certain tachyarrhythmias.

Formation of the Sinus Node

The definitive sinus node in man has a distinct molecular architecture, which underlies its pacemaker function and differs significantly from that of adjacent atrial muscle.[34,35] Several transcription factors, including Tbx3, Tbx18 and Nkx2-5, play a crucial role in the formation of the sinus node at the right-sided sinuatrial junction in the mouse heart.[33,36] We observed similar expression patterns of these genes in the developing human heart, suggesting conservation of the molecular mechanisms governing its development. We observed almost strict complementarity between the expression domains of the transcription factors ISL1 and NKX2-5 at the venous pole (Figure 4). ISL1 is known to play a crucial role in the maintenance of the precursor state of the cardiac progenitor cells within the heart-forming regions. Its expression is switched off in the majority of the cardiomyocytes upon their differentiation.[14,37] As in the mouse,[38] the myocytes of the systemic venous tributaries, including the primordium of the sinus node, continue to express ISL1, a finding deserving further investigation.

We have endorsed previous morphological studies[5,6,9] showing that the thickening of the venous sinus wall is the first morphological evidence of the formation of the TBX3-positive nodal primordium in the embryonic human heart. However, the myocytes of the nodal primordium and the atrial wall display no obvious histological differences. Published electrophysiological data also fail to support strict localization of the pacemaker within the developing sinus node during the early stages of development,[39] which correlates well with our findings of extensive expression of HCN4 at the venous pole of the early human embryonic heart.

The specialized cardiomyocytes of the postnatal sinus node contain poorly developed myofibrils,[40] resembling the phenotype of the primary myocardium. On the other hand, the initiation of expression of fast-conducting connexins and atrial natriuretic factor, along with the gradual disappearance of smooth muscle actin in the chamber myocardium, is a sign of progressive differentiation and maturation of the working myocardium.[41] When first identified, the thickened right wall of the venous sinus in the developing human heart already contained a small subset of myocytes expressing very low levels of troponin I and αSMA, both proteins being strongly expressed in the atrial and ventricular chamber myocardium at these stages (Figures 3,6). This troponin I- and αSMA-negative area within the wall of the venous sinus does express TBX3, known to control the genetic programming of the sinus node development in

mouse.[36] This suggests that TBX3 also controls very early specification of the myocytes of the systemic venous sinus into the pacemaker lineage in human.

Human hearts with right atrial isomerism,[42] as well as mouse hearts with complex malformations resembling right atrial isomerism due to knock-out of the left-right axis regulator Pitx2c,[33] display bilateral sinus nodes. In normal human embryonic hearts we did not observe in the myocardial wall of the coronary sinus the molecular phenotype characteristic of the developing sinus nodal primordium. Our observations, along with the absence of reports about bilateral sinus nodes in the setting of the normally arranged laterality, lend no support to the hypothesis of the presence of bilateral sinus nodal primordia[22] in the normal developing heart.

Formation of the Atrioventricular Conduction Axis

To date, morphological studies on serial sections of human embryos have resulted in contradictory conclusions concerning the development of the atrioventricular conduction axis in man.[5-8,10-12] Thus, it has been suggested that the atrioventricular node develops through fusion of the ventral and dorsal parts of the atrioventricular ring of “histologically specialized” cardiomyocytes.[8,12] We have found no evidence to support this hypothesis. At the intermediate stages of development, the entire atrioventricular canal is positive for the conduction system marker TBX3,[19] with somewhat thicker portions dorsally and ventrally. The lumen of the canal, along with the atrioventricular endocardial cushions, separate these parts of the TBX3-positive atrioventricular canal myocardium. Subsequently, the mesenchymal tissues of the forming central fibrous body remain interposed between the developing atrioventricular node and the so-called retro-aortic “node”, the latter structure regressing in normal hearts, albeit persisting in certain complex cardiac malformations.[43]

Recent lineage studies in mouse have shown that, albeit atrioventricular canal musculature and the myocardializing vestibular spine both originate from a Tbx2-positive lineage, the compact part of the atrioventricular node develops in its entirety from the atrioventricular canal musculature,[29] without contributions from the coronary sinus musculature, specifically expressing transcription factor Tbx18,[24] or vestibular spine-derived myocardium, which is, unlike the atrioventricular node, marked by the MEF2c-AHF-Cre transgene.[29] In the human embryonic heart, we failed to observe TBX18 expression in the TBX3-positive atrioventricular nodal primordium (data not shown).

In the developing mouse heart, the expression of the connexins, which ensure rapid myocardial conduction is suppressed in the atrioventricular canal by the transcription factors Tbx3 and Tbx2.[20] The cardiomyocytes on the crest of the ventricular septum, which form the atrioventricular conduction axis in mouse, also express Tbx3 at very early stages.[19,24] The developing and maturing bundle of His in the mouse heart remains negative for connexin40 and 43.[24] In the late embryonic human heart, we found a similar arrangement, where the cells of the

developing axis, after completion of ventricular septation, remained negative for both connexin40 and -43 (Figure 7J,K), corroborating that at these stages the developing bundle of His is a slow-conducting entity as in early fetal hearts.[44] In contrast to the situation in mouse, we found connexin43 expression on top of the ventricular septum (Figure 6E,K), the functional significance of which is unclear. Despite such differences in the timing of expression of connexin43, and in the intensity of ID2 expression, the similar patterns of expression of key regulatory and functional proteins in the developing human atrioventricular conduction system suggest evolutionary conservation of the general embryonic blueprint for the formation of atrioventricular conduction axis in man and mouse.

In the normal human postnatal heart, the atrioventricular conduction axis is the only muscular connection between the atrial and ventricular muscle masses. Defective formation of the plane of atrioventricular insulation in the mouse, particularly in the absence of transcription factor Tbx2,[45] and Bmp-receptor 1a/Alk3,[46] has been shown to be associated with the persistence of accessory muscular bundles, which are capable of producing ventricular pre-excitation. In man, the connections, which are responsible for the Wolff-Parkinson-White syndrome, possess fast conducting properties and express connexin43.[47] Even up to the neonatal period, when formation of the atrioventricular insulation plane is relatively complete, multiple accessory myocardial strands are still to be found crossing the fibrous plane of insulation.48 These remnants of the atrioventricular canal myocardium, however, are negative for connexins40 and -43, and would thus constitute slowly conducting pathways. The failure of disappearance of these strands, therefore, cannot be the sole mechanism for ventricular pre-excitation. An additional requirement is the further differentiation of this primary myocardium into fast-conducting working myocardium.[45]

In contrast to the postnatal situation, where the atrioventricular node and the bundle of His are isolated from the myocardium of the ventricular septal crest by fibrous tissue, the TBX3-positive precursors of these structures seen at late embryonic stages are not yet isolated from the ventricular myocardium by fibrous tissue (Figure 7). In these stages the cardiomyocytes making up the atrioventricular node and the penetrating atrioventricular bundle do not express fast-conducting connexins, thus producing an effective functional isolation in the setting of absent fibrous insulation. It is persistence of the original muscular continuity between the myocytes of the atrioventricular conduction axis and the crest of the ventricular septum that accounts for the so-called Mahaim connections, characterized by slow conduction.

Acknowledgments

We are indebted to the personnel of the Gynaecology Department of the Tartu University Hospital, and to Dr. M.Aunapuu and Prof. A.Arend from the Anatomy Institute of the University of Tartu, Estonia, for their help with the collection of the human embryos. We thank J.Hagoort for his continuous support and invaluable help with the preparation of the interactive 3D-PDF file, Chantal Schreurs for laboratory assistance, and Prof. W.H.Lamers for critically reading the manuscript.

Sources of Funding

This work was supported by the European Community's Framework Programmes' contracts LSHM-CT-2005-018630 ("HeartRepair") and Health-F2-2008-223040 ("CHeartED"). Collection of human embryonic material was supported by grant 7301 from the Estonian Science Foundation.

Disclosures

None

Reference List

1. Paff GH, Boucek RJ, Harrell TC. Observations on the development of the electrocardiogram. *Anat Rec.* 1968;160:575-582.
2. de Jong F, Opthof T, Wilde AA, Janse MJ, Charles R, Lamers WH, Moorman AF. Persisting zones of slow impulse conduction in developing chicken hearts. *Circ Res.* 1992;71:240-250.
3. Schram G, Pourrier M, Malnyk P, Nattel S. Differential distribution of cardiac ion channel expression as a basis for regional specialization in electrical function. *Circ Res.* 2002;90:939-950.
4. Christoffels VM, Smits GJ, Kispert A, Moorman AF. Development of the pacemaker tissues of the heart. *Circ Res.* 2010;106:240-254.
5. Walls EW. The development of the specialized conducting tissue of the human heart. *J Anat.* 1947;81:93-110.
6. Navaratnam V. The development of the specialized musculature of the human heart. *Ceylon J Med Sci.* 1965;14:68-80.
7. Anderson RH, Taylor IM. Development of atrioventricular specialized tissue in human heart. *Br Heart J.* 1972;34:1205-1214.
8. Wenink ACG. Development of the human cardiac conduction system. *J Anat.* 1976;121:617–631.
9. Anderson RH, Ho SY, Becker AE, Gosling JA. The development of the sinoatrial node. In: Bonke FIM (ed). The Sinus Node: Structure, Function and Clinical Relevance. *Martinus Nijhoff Medical Division*, 1978;166-182.
10. Truex RC, Marino TA, Marino DR. Observations on the development of the human atrioventricular node and bundle. *Anat Rec.* 1978;192:337-350.
11. Wessels A, Vermeulen JL, Verbeek FJ, Virágh S, Kálmán F, Lamers WH, Moorman AF. An immunohistochemical analysis of the distribution of the neural tissue antigen G1N2 in the embryonic human heart. *Anat Rec.* 1992;232:97-111.
12. Blom NA, Gittenberger-de Groot AC, DeRuiter MC, Poelmann RE, Mentink MMT, Ottenkamp J. Development of the cardiac conduction tissue in human embryos using HNK-1 antigen expression: possible relevance for understanding of abnormal atrial automaticity. *Circulation.* 1999;99:800-806.
13. Sizarov A, Anderson RH, Christoffels VM, Moorman AF. Three-dimensional and molecular analysis of the venous pole of the developing human heart. *Circulation.* 2010;122:798-807.
14. Sizarov A, Ya J, de Boer BA, Christoffels VM, Lamers WH, Moorman AF. Development of the building plan of the human heart: morphogenesis, growth and differentiation. *Circulation.* 2011;123:1125-1135.
15. Jansen JA, van Veen TA, de Bakker JM, van Rijen HV. Cardiac connexins and impulse propagation. *J Mol Cell Cardiol.* 2010;48:76-82.
16. Mangoni ME, Nargeot J. Genesis and regulation of the heart automaticity. *Physiol Rev.* 2008;88:919-982.
17. Stieber J, Herrmann S, Feil S, Löster J, Feil R, Biel M, Hofmann F, Ludwig A. The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. *Proc Natl Acad Sci USA.* 2003;100:15235-15240.
18. Gourdie RG, Harris BS, Bond J, Justus C, Hewett KW, O'Brien TX, Thompson RP, Sedmera D. Development of the cardiac pacemaking and conduction system. *Birth Defects Res.* 2003;69:46-57.

19. Hoogaars WM, Tessari A, Moorman AF, de Boer PA, Hagoort J, Soufan AT, Campione M, Christoffels VM. The transcriptional repressor Tbx3 delineates the developing central conduction system of the heart. *Cardiovasc Res.* 2004;62:489-499.
20. Christoffels VM, Burch JBE, Moorman AFM. Architectural plan for the heart: early patterning and delineation of the chambers and nodes. *Trends Cardiovasc Med.* 2004;14:301-307.
21. Davis LM, Rodefeld ME, Green K, Beyer EC, Saffitz JE. Gap junction protein phenotypes of the human heart and conduction system. *J Cardiovasc Electrophysiol.* 1995;6:813-822.
22. Jongbloed MR, Mahtab EA, Blom NA, Schalij MJ, Gittenberger-de Groot AC. Development of the cardiac conduction system. *ScientificWorldJournal.* 2008;8:239-269.
23. Christoffels VM, Mommersteeg MT, Trowe MO, Prall OW, de Gier-de Vries C, Soufan AT, Bussen M, Schuster-Gossler K, Harvey RP, Moorman AF, Kispert A. Formation of the venous pole of the heart from an Nkx2-5-negative precursor population requires Tbx18. *Circ Res.* 2006;98:1555-1563.
24. Bakker ML, Boukens BJ, Mommersteeg MT, Brons JF, Wakker V, Moorman AF, Christoffels VM. Transcription factor Tbx3 is required for the specification of the atrioventricular conduction system. *Circ Res.* 2008;102:1340-1349.
25. Moskowitz IP, Kim JB, Moore ML, Wolf CM, Peterson MA, Shendure J, Nobrega MA, Yokota Y, Berul C, Izumo S, Seidman JG, Seidman CE. A molecular pathway including Id2, Tbx5, and Nkx2-5 required for cardiac conduction system development. *Cell.* 2007;129:1365-1376.
26. Kolditz DP, Wijffels MC, Blom NA, van der Laarse A, Hahurij ND, Lie-Venema H, Markwald RR, Poelmann RE, Schalij MJ, Gittenberger-de Groot AC. Epicardium-derived cells in development of annulus fibrosis and persistence of accessory pathways. *Circulation.* 2008;117:1508-1517.
27. Yanni J, Boyett MR, Anderson RH, Dobrzynski H. The extent of the specialized atrioventricular ring tissues. *Heart Rhythm.* 2009;6:672-680.
28. Snarr BS, Wirrig EE, Phelps AL, Trusk TC, Wessels A. A spatiotemporal evaluation of the contribution of the dorsal mesenchymal protrusion to cardiac development. *Dev Dyn.* 2007;236:1287-1294.
29. Aanhaanen WT, Mommersteeg MT, Norden J, Wakker V, de Gier-de Vries C, Anderson RH, Kispert A, Moorman AF, Christoffels VM. Developmental origin, growth, and three-dimensional architecture of the atrioventricular conduction axis of the mouse heart. *Circ Res.* 2010;107:728-736.
30. Thomas PS, Kasahara H, Edmonson AM, Izumo S, Yacoub MH, Barton PJR, Gourdie RG. Elevated expression of Nkx-2.5 in developing myocardial conduction cells. *Anat Rec.* 2001;263:307-313.
31. Issa ZF, Miller JM, Zipes DP (eds). Clinical Arrhythmology and Electrophysiology: Companion to Braunwald's Heart Disease. *Saunders:* 2009.
32. Boukens BJ, Christoffels VM, Coronel R, Moorman AF. Developmental basis for electrophysiological heterogeneity in the ventricular and outflow tract myocardium as a substrate for life-threatening ventricular arrhythmias. *Circ Res.* 2009;104:19-31.
33. Mommersteeg MTM, Hoogaars WMH, Prall OWJ, de Gier-de Vries C, Wiese C, Clout DEW, Papaioannou VE, Brown NA, Harvey RP, Moorman AFM, Christoffels VM. Molecular pathway for the localized formation of the sinoatrial node. *Circ Res.* 2007;100:354-362.
34. Chandler NJ, Greener ID, Tellez JO, Inada S, Musa H, Molenaar P, DiFrancesco D, Baruscotti M, Longhi R, Anderson RH, Billeter R, Sharma V, Sigg DC, Boyett MR, Dobrzynski H. Molecular architecture of the human sinus node: insights into the function of the cardiac pacemaker. *Circulation.* 2009;119:1562-1575.
35. Gaborit N, le Bouter S, Szuts V, Varro A, Escande D, Nattel S, Demolombe S. Regional and tissue specific transcript signatures of ion channel genes in the non-diseased human heart. *J Physiol.* 2007;582:675-693.
36. Hoogaars WMH, Engel A, Brons JF, Verkerk AO, de Lange FJ, Wong LY, Bakker ML, Clout DE, Wakker V, Barnett P, Ravesloot JH, Moorman AF, Verheijck EE, Christoffels VM. et al. Tbx3 controls the sinoatrial node gene program and imposes pacemaker function on the atria. *Genes Dev.* 2007;21:1098-1112.
37. Ma Q, Zhou B, Pu WT. Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of *Cre* activity. *Dev Biol.* 2008;323:98-104.
38. Sun Y, Liang X, Najafi N, Cass M, Lin L, Cai CL, Chen J, Evans SM. Islet1 is expressed in distinct cardiovascular lineages, including pacemaker and coronary vascular cells. *Dev Biol.* 2007;304:286-296.
39. Sedmera D,Wessels A, Trusk TC, Thompson RP, Hewett KW, Gourdie RG. Changes in activation sequence of embryonic chick atria correlate with developing myocardial architecture. *Am J Physiol.* 2006;291:H1646-H1652.
40. Op't Hof T. The mammalian sinoatrial node. *Cardiovasc Drugs Ther.* 1988;1:572-597.
41. Moorman AF, Christoffels VM. Cardiac chamber formation: development, genes, and evolution. *Physiol Rev.* 2003;83:1223-1267.
42. Ho SY, Seo JW, Brown NA, Cook AC, Fagg NL, Anderson RH. Morphology of the sinus node in human and mouse hearts with isomerism of the atrial appendages. *Br Heart J.* 1995; 74: 437–442.
43. Kurosawa H, Becker AE. Atrioventricular Conduction in Congenital Heart Disease: Surgical Anatomy. *Springer-Verlag Berlin:* 1987.
44. Janse MK, Anderson RH, van Capelle FJ, Durrer D. A combined electrophysiological and anatomical study of the human fetal heart. *Am Heart J.* 1976;91:556-562.

45. Aanhaanen WT, Boukens BJ, Sizarov A, Wakker V, de Gier-de Vries C, van Ginneken AC, Moorman AF, Coronel R, Christoffels VM. Defective Tbx2-dependent patterning of the atrioventricular canal myocardium causes accessory pathway formation in mice. *J Clin Invest.* 2011; 121:534-544.
46. Gaussin V, Morley GE, Cox L, et al. Alk3/Bmpr1a receptor is required for development of the atrioventricular canal into valves and annulus fibrosus. *Circ Res.* 2005;97:219-226.
47. Peters NS, Rowland E, Bennett JG, Green CR, Anderson RH, Severs NJ. The Wolff-Parkinson-White syndrome: the cellular substrate for conduction in the accessory atrioventricular pathway. *Eur Heart J.* 1994;15:981-987.
48. Hahurij ND, Gittenberger-De Groot AC, Kolditz DP, Bökenkamp R, Schalij MJ, Poelmann RE, Blom NA. Accessory atrioventricular myocardial connections in the developing human heart. *Circulation.* 2008;117:2850-2858.

Supplementary Methods

Immunofluorescence and Immunohistochemical Staining

Paraffin-embedded embryos were sectioned at 5 to 7 µm. Sections were mounted onto silane-coated slides, deparaffinized in xylene, rehydrated in graded ethanol series and washed in phosphate-buffered saline (PBS, pH 7.4).

Triple immunofluorescent staining was performed as described previously.[1] Here we report the results of staining with the following primary antibodies: goat polyclonal for connexin40, rabbit polyclonal for NKX2-5, goat polyclonal for TBX3, goat polyclonal for TBX18, rabbit polyclonal for ID2 (all from Santa Cruz Biotechnology; diluted 1:250), mouse monoclonal for connexin43 (diluted 1:100), goat polyclonal for Islet-1 (1:250; Neuromics), rabbit polyclonal for HCN4 (1:250; Chemicon) and mouse monoclonal for α-smooth muscle actin (Sigma-Aldrich, 1:500). Every combination contained a mouse monoclonal antibody for troponin I (1:250; Chemicon) as myocardial marker. After washing in TNT (100 mM Tris, pH 7.4; 150 mM NaCl; 0.05% Tween-20, Sigma-Aldrich) sections were incubated at room temperature for 1.5-2 hr in the dark with a mix of fluorochrome-coupled secondary antibodies containing donkey-anti-goat Alexa568, chicken-anti-rabbit Alexa488 and donkey-anti-mouse Alexa680 (all diluted 1:250; Molecular Probes, Invitrogen). For TBX3 and TBX18 biotinylated anti-goat antibody (1:250; Jackson ImmunoResearch) and a Tyramide Signal Amplification kit (Perkins & Palmer) were used according to the manufacturer's instructions.

For indirect immunohistochemical staining sections were pre-treated with PBS supplemented with 0.05% Tween-20 to reduce non-specific antibody binding. Then consecutive sections were incubated overnight at room temperature with different primary antibodies in repetition through the whole series. Here we report the results of staining with the goat polyclonal antibody for NKX2-5 (Santa Cruz Biotechnology; diluted 1:4000 in PBS with 1% bovine serum albumin). The next morning, after washing the sections for 3 times in PBS, they were incubated with appropriate biotin-coupled secondary antibodies for 1 hr at room temperature and washed again 3 times in PBS. For NKX2-5 binding visualization a horse-anti-goat antibody (Vectors Laboratories) was used at a dilution of 1:200 in PBS supplemented with 1.5% horse normal serum. Then the slides were incubated with the ABC reagent (Vector Laboratories) for 45 min, and washed again in PBS. Then 3-3'di-aminobenzidin tetrahydrochloride (Sigma-Aldrich) diluted 400 µg/mL in Tris-maleate buffer (58 mM Tris; 50 mM maleic acid, pH 7.6; 50 mM NaOH) and supplemented with 5% H_2O_2 was added to the sections to develop the colour at the binding site of the primary antibody. Reaction was stopped by rinsing the sections in distilled water, after which they were counterstained in 0.1% hemotoxylin (Merck) for 10 seconds, followed by rinsing in running tap water for 10 minutes. The slides were then dehydrated in graded ethanol series, washed in xylene and mounted with Entellan (Merck).

Staining for General Histology

Subsequent to immunofluorescent staining and photography, sections were washed in PBS and stained with hematoxylin and azophloxin according to a standard protocol. Then sections were dehydrated in a graded ethanol series, washed in xylene and covered with Entellan (Merck).

In situ *Hybridization on Sections*

In situ hybridization was performed as described previously.2 Here we report the results of staining with a TBX18 probe, transcribed from nucleotides 1039-1790 of the human TBX18 gene (GenBank reference nr NM_001080508). The template for the TBX18 probe was amplified using polymerase chain reaction from total cDNA synthesized using reverse transcription from RNA extracted from adult atrial myocardium and subcloned into pBluescript SK-II plasmid according to the standard protocols.

Three-Dimensional Reconstructions

Three-dimensional reconstructions from the fluorescently stained serial sections were performed essentially as described previously.[1,2] Some structures (e.g, dorsal mesenchyme, veins and arteries) were not labelled in their entirety for the sake of clarity. The myocardial expression domain of TBX3 from every third section of the series was projected upon the myocardium label without quantification. For technical reasons the areas containing scattered or graded patterns of expression were similarly labelled as the areas containing strong gene expression. The extent of the labels for other structures, which have not been specifically stained on the sections, was defined according to general embryology knowledge.[3,4] The 3D data from the Amira viewer were exported into Adobe Acrobat 9 Pro Extended (Adobe Systems Inc., www.adobe.com) to generate the file in interactive 3D portable document format.[5]

Supplemental References

1. Sizarov A, Anderson RH, Christoffels VM, Moorman AFM. Three-dimensional and molecular analysys of the venous pole of the developing human heart. *Circulation*. 2010;122:798-807.
2. Sizarov A, Ya J, de Boer BA, Christoffels VM, Lamers WH, Moorman AF. Development of the building plan of the human heart: morphogenesis, growth and differentiation. *Circulation*. 2011;123:1125-1135.
3. O'Rahilly R, Müller F. Developental Stages in Human Embryos. Including a revision of Streeter's "Horizons" and a survey of the Carnegie Collection. *Carnegie Institution Washington*: 1987, publication 647.
4. Gasser RF. Atlas of Human Embryos. *Harper and Row*: 1975.
5. de Boer BA, Soufan AT, Hagoort J, Mohun TJ, van den Hoff MJB, Hasman A, Voorbraak FJM, Moorman AFM, Ruijter JM. The interactive presentation of 3D information obtained from reconstructed datasets and 3D placement of single histological sections with the 3D portable document format. *Development.* 2011; 138:159-167.