The role of Tbx2 in the development of the atrioventriculair canal and conduction system of the heart. "Making the beat go on and on"

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Chapter 1

Origin and development of the atrioventricular myocardial lineage: insight into the development of accessory pathways

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Abstract

Defects originating from the atrioventricular canal region are part of a wide spectrum of congenital cardiovascular malformations that frequently affect newborns. These defects include partial or complete atrioventricular septal defects, atrioventricular valve defects and arrhythmias, such as atrioventricular re-entry tachycardia, atrioventricular nodal block and ventricular preexcitation. Insight into the cellular origin of the atrioventricular canal myocardium and the molecular mechanisms that control its development will aid in the understanding of the etiology of the atrioventricular defects. This review discusses current knowledge concerning the origin and fate of the atrioventricular canal myocardium, the molecular mechanisms that determine its specification and differentiation, and its role in the development of certain malformations such as those that underlie ventricular preexcitation.

Keywords: Atrioventricular conduction system, Atrioventricular canal, Preexcitation, Lineage analysis, WPW, T-box transcription factors
Introduction

Organ development is a beautiful but very complex process that is difficult to understand. Heart development is no exception, especially because the function of the heart is constantly required for development of the organ itself and the embryo as a whole. Experimental manipulations of the heart cause malfunction, which, in turn, feed back to affect heart development. This complexity and sensitivity to intrinsic and extrinsic factors is reflected in the frequency of the occurrence of congenital heart disease, which affects almost 1% of newborns. In the western world it is a major cause of intrauterine and early postnatal death.1, 2

The four-chambered heart develops from a peristaltically contracting embryonic heart tube to which different types of progenitor cells are added. One particular structure in the developing heart, the atrioventricular canal, stands out as it is involved in many aspects of heart development. It is involved in alignment of the atria and ventricles and in the formation of septae and valves.3, 4 Furthermore, the atrioventricular canal provides the delay in impulse propagation required for coordinated contraction of the atria and ventricles, and is involved in the formation of the atrioventricular conduction axis including the atrioventricular node.5-9 As a consequence, partial or complete atrioventricular septal defects, atrioventricular valve defects and arrhythmias such as atrioventricular re-entry tachycardia, atrioventricular nodal block and ventricular pre-excitation, can have their origin in misregulated development of the atrioventricular canal region.

Insight into the developmental and molecular mechanisms, including the cellular origin and fate, that underlie the formation of the atrioventricular canal myocardium, will aid in the understanding of atrioventricular canal related defects. We will discuss current knowledge concerning the origin and fate of the atrioventricular canal myocardium, the molecular mechanisms that determine its specification and differentiation, and its role in the development of certain malformations such as those that underlie ventricular pre-excitation.
Chapter 1

The origin and fate of the atrioventricular canal myocardium

Morphological criteria and expression patterns to define the origin and fate of the embryonic atrioventricular canal

While the myocardium of the developing atria and ventricles differentiates and expands, the atrioventricular canal myocardium retains the tubular heart phenotype, which includes slow rates of proliferation, and does not expand. As a consequence, it appears as a constriction (Figure 1). The atrioventricular canal myocardium provides the signals to the endocardium to initiate the formation of the cushions, which participate in atrioventricular septation and valve formation. The endocardium, in turn, provides signals to maintain the atrioventricular myocardial phenotype. The epicardium in the atrioventricular sulcus forms the subepicardial mesenchyme that will later contribute to the formation of the annulus fibrosus. In the adult heart, the atrioventricular myocardial phenotype can still be found in rings around the orifices of the atrioventricular valves.

Figure 1. The heart tube differentiates and expands to form the ventricular and atrial myocardium at the ventral and dorsal side, respectively. The atrioventricular canal does not differentiate and expand, and appears as a constriction between the chambers. (A) Marker Nppa is expressed in the developing chambers, whereas Tbx2 expression demarcates the atrioventricular canal myocardium in a pattern complementary to that of Nppa. (B) The atrioventricular canal consists of three different lineages. The middle layer consists of myocardium, at the luminal side the endocardium is found, and the outer layer is formed by the epicardium. The atrioventricular cushions initially develop by extracellular matrix formed by the atrioventricular myocardium. Later, endocardial cells will give rise to the cellular content of the cushions. ev, embryonic ventricle; avc, atrioventricular canal; la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle; avcs, atrioventricular cushion; end, endocardium; epi, epicardium; myo, myocardium.
The morphogenesis of the atrioventricular canal has been described in detail on the basis of its morphology and the evolving spatio-temporal patterns of expression of marker genes. The different cell population in the heart could be distinguished on the basis of their ultra-structure using electron microscopy, glycogen content, and difference in enzyme activity. Using these differences, the atrioventricular canal region could be identified in consecutive stages of development and its changing aspects monitored. The similarities between the early atrioventricular canal myocardium in the embryo and the atrioventricular node and atrioventricular ring bundle in the formed heart were noticed: compared to adjacent atrial cells, atrioventricular cells have a high glycogen content, a poorly developed sarcoplasmatic reticulum and myofilaments, and few junctional complexes. Consistently, several important functional properties of the embryonic atrioventricular myocardium, including automaticity and slow conduction, were also found to be maintained in the corresponding region of the formed heart. Indeed, careful examination of serial sections of consecutive stages suggested that the atrioventricular node was derived from the atrioventricular canal.

Following these studies, expression patterns were used to distinguish the atrioventricular canal from adjacent atrial or ventricular myocardial cells in order to define the morphogenetic mechanisms of its formation. One of those studies used the distribution of GlN2 staining, an epitope raised against the ganglion nodosum of chicken. In the heart, GlN2 is selectively detected in the atrioventricular junction, except for the left side, and in the embryonic interventricular ring. The expression pattern at consecutive stages was reconstructed to understand the morphological changes of the atrioventricular canal and interventricular ring during development. These studies indicated that the embryonic atrioventricular canal forms the lower rim of the atria (vestibules), the muscular support of the atrioventricular valves and the atrioventricular node and the right atrioventricular ring bundle of the formed heart. The interventricular ring myocardium was suggested to form the atrioventricular bundle and septal branch.

Several transgenic markers (regulatory DNA sequences coupled to a reporter gene) have been reported that identify the atrioventricular canal myocardium. The activity patterns of the transgene again point to the relation between the embryonic atrioventricular canal and the mature atrioventricular node and atrioventricular ring bundle. During development, the Gata6-LacZ construct, which contains a regulatory fragment of chicken Gata6, is expressed in the caudal limbs of the embryonic day (E)7.5 cardiac crescent, the inflow tract of the E8.5 heart tube, and atrioventricular canal of the E9.5 tubular heart. In the adult heart, its activity is limited to the atrioventricular node and right atrioventricular ring bundle. These observations suggest that the underlying molecular mechanisms that drive expression in these areas are similar. Because of the half-life of β-Galactosidase it also indicated a possible cellular relation between the caudal limbs of the cardiac crescent, the inflow tract of the early heart tube and the atrioventricular canal of the chamber forming heart, in agreement with the conclusions from physical cell labeling studies.
As stated before, it is impossible to settle issues of cellular origin on the basis of phenotypic characteristics or spatiotemporally expression patterns. Resolution of these type of questions requires tracers of lineages that are able to target to defined embryonic loci and then passed with stability from one generation of cells to the next along further development.38

Physical cell labeling to assess the origin and fate of the embryonic atrioventricular canal
A classical method used to trace the fate of cells in the embryo is to physically label a particular region in the embryo with iron particles, and assess the position of these labels at later stages of development.37, 39-44 Iron-particles do not incorporate into the cell membrane and might travel independently from the cells, which complicates the interpretation of the results. The use of cell-membrane bound dyes effectively addressed this issue. However, in highly proliferative cell populations the dye dilutes rapidly and becomes undetectable, allowing short-term fate maps only. Furthermore, these experiments were mainly performed in chicken embryos and extrapolated to mammalian embryos, which are difficult to label and culture up to stages relevant for cardiac development.

The first fate map studies of the developing heart mainly focused on the location of the cardiac precursors in the mesoderm and their relation with the components of the early embryonic heart. They identified a bilateral (crescent shaped) cardiac field just after gastrulation that provides the cells for the myocardium of the heart tube.39-44 The caudal limbs of the heart field at stage 8 were suggested to contribute to the inflow tract of the stage 9 embryo.45 The inflow tract of the heart formed the primitive inlet of the left ventricle at stage 12, which in all likelihood is equivalent to what we now would call the atrioventricular canal.37 Subsequently, the primitive inlet contributed to the left ventricular free wall.37 This last finding was hardly given attention, but was recently supported by three independent studies (Figure 2A).8, 46, 47 Although these fate map studies have provided insight into the dynamics of cellular contributions to the definitive heart, the spatial resolution is limited and not suitable to trace the origin of small sub-components such as the atrioventricular node. Furthermore, they are not suitable to address the origin and fate of specific cellular lineages.

Genetic lineage analysis to trace the origin and fate of the atrioventricular myocardial lineage
The Cre-LoxP system allows the genetic labeling of specific cell lineages in which a particular gene or regulatory sequence is active during development. The system uses the property of Cre to recombine DNA fragments that are flanked by two recognition sites called LoxP sites. The orientation of the LoxP sites determines the outcome of the recombination event.48 Typically, in genetic lineage tracing studies, a reporter gene such as Egfp or LacZ is placed downstream of a ubiquitous promoter followed by a transcriptional stop signal flanked by LoxP sites.49, 50 Upon Cre-mediated recombination, the transcriptional stop signal is deleted and the reporter gene is irreversibly activated in the cell and its daughter cells, irrespective of whether or not
Origin and Development of the Atrioventricular Myocardial Lineage

Figure 2. During cardiac development, the caudal limbs of the cardiac crescent will form the inflow tract and subsequently the atrioventricular canal myocardium, and ultimately will contribute to the left ventricular free wall. The embryonic ventricle will form only the apex and ventricular septum. (A) Physical cell labeling of a Hamburger Hamilton stage 9 stage chicken embryonic heart (comparable to mouse E8.0) using a fluorescent dye. Cells in the caudal-most part of the inflow tract have been labeled, and are incorporated in the atrioventricular canal and subsequently in the left ventricle (modified from van den Berg et al. 2009). (B) Schematic representation of the Tbx2\(^{+/}\)-lineage analysis. In Tbx2\(^{+/}\);Z/EG embryos, the caudal limbs of the cardiac crescent robustly express Tbx2/Cre but no reporter gene expression (= recombination) is seen yet. At E8.5, Tbx2/Cre is expressed in the inflow tract and reporter gene expression is found in a similar pattern. At E9.25, Tbx2/Cre is expressed in the atrioventricular canal myocardium and the reporter is similarly expressed. No expression of the reporter is seen in the embryonic ventricle. At E10.5, Tbx2/Cre expression remains in the atrioventricular canal myocardium but the reporter is now found additionally in the left ventricular wall, indicating a contribution of the Tbx2/Cre \(^{+/}\) atrioventricular canal to the left ventricle. (C) At E17.5 the entire left ventricular free wall is found to be derived from the Tbx2\(^{+/}\) embryonic atrioventricular canal myocardium. ev, embryonic ventricle; ift, inflow tract; avc, atrioventricular canal; oft, outflow tract; la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle; pv, pulmonary vein; lsh, left sinus horn; dm, dorsal mesocardium; av, atrioventricular.
Cre remains active. This system thus allows mapping the fate of a genetically defined lineage throughout development and in the adult. One of the disadvantages of the Cre-LoxP system is that activation of the reporter by recombination can occur at low, sometimes undetectable, levels of Cre. Cells in which the reporter has been activated by undetectable levels of Cre are prone to be unjustly related to cell population in which Cre was expressed at sufficient levels to be detected. Careful examination of the spatio-temporal pattern of Cre and the reporter gene is therefore essential for the proper interpretation of genetic lineage analyses.

The first genetic lineage tracing that aimed to monitor the origin and fate of atrioventricular canal myocardium, used the chicken Gata6-enhancer to drive Cre expression. First recombination was found in the inflow tract of the E8.5 mouse heart. At E9.5, activated reporter activity was confined mainly to the atrioventricular canal. In the adult, the atrioventricular node and right atrioventricular ring bundle were completely recombined, indicating a cellular relation with the embryonic atrioventricular canal. Cells with an activated reporter were also found in the wall of the left ventricle, but dismissed as ectopic expression of the enhancer construct driving Cre. Unfortunately, in this study the expression of Cre itself during development was not documented and appeared mosaic. Therefore, lineage relations and aberrant expression of Cre could not be distinguished, which complicated the interpretation of the data.

Recently, a Tbx2Cre allele was made in which Cre was placed in the endogenous locus of Tbx2. Tbx2 is expressed in those regions that retain the primary phenotype of the primitive heart tube, including the atrioventricular canal myocardium, and is not expressed in the working myocardium of the ventricle. Tbx2 is required and sufficient for atrioventricular canal development and for the repression of working myocardial differentiation. The expression pattern and functional aspects of Tbx2 made its endogenous locus an excellent location to target Cre and trace the fate of the atrioventricular canal cells and their contribution to the adult heart.

The earliest robust recombination was found in the inflow tract of the heart at E8.5. At E7.5-8.0 Cre was expressed in the limbs of the cardiac crescent, suggesting a lineage relation with the inflow tract. At E9.25 recombination was found in the atrioventricular canal and not in the embryonic ventricle or atria, confirming the relation between the E8.5 inflow tract and the E9.25 atrioventricular canal (Figure 2B). The fate of the atrioventricular canal in the formed heart was found to be more extensive than previously anticipated. The Tbx2-Cre lineage confirmed the relation between the atrioventricular canal and the lower rim of the atria, the muscular support of the valves and the atrioventricular node. More surprisingly, the free wall of the definitive left ventricle was formed by cells that were part of the atrioventricular canal at embryonic stages, whereas the unrecombined embryonic ventricle only formed the apex and left side of the ventricular septum of the heart (Figure 2B). The large contribution of atrioventricular canal myocardium to the left ventricular wall implies that misregulation of early atrioventricular canal development can affect a larger area than previously anticipated, including the left ventricular free wall.
The atrioventricular canal regulatory network

The Bmp2-Tbx2/3 regulatory axis is required for development and homeostasis of the atrioventricular canal

*Bmp2* is expressed in the early cardiac progenitor cells and promotes differentiation of mesodermal cells into cardiomyocytes. Bmp-signaling has been implicated in the induction of Nkx2.5, Tbx20, Mef2c and Gata factors that drive cardiac differentiation. These broadly expressed transcription factors are equally important for working myocardial differentiation of the atria and ventricles, and the development and maturation of the conduction system.

After its initial broad expression in the cardiac crescent, Bmp2 expression becomes restricted to the atrioventricular canal myocardium. Here, Bmp2 is essential for the activation of *Tbx2* (and possibly *Tbx3*) via a Smad-dependent enhancer in mice. In zebrafish, the *Alk2* receptor, encoding a Bmp-receptor, was found to be required for activation of *Tbx2* in the atrioventricular canal. In zebrafish, *Foxn4* was found to regulate *Tbx2* via a *Tbx2*-enhancer domain that contains a Foxn4 site. The role of Foxn4 in mammalian atrioventricular canal development has not been studied. Moreover, the putative Foxn4 binding site was not required for correct *Tbx2* expression.

*Tbx2* and *Tbx3* are two highly homologous transcription factors with comparable functional properties. In the developing heart, *Tbx2* is expressed in the inflow tract, the atrioventricular canal and outflow tract. *Tbx3* is expressed in a similar pattern but includes expression in the crest of the ventricular septum that forms the atrioventricular bundle and in the developing sinus node. *Tbx2* is more robustly expressed in the myocardium of the left side of the atrioventricular canal. *Tbx2* expression diminishes during development and is not observed in the adult heart, whereas *Tbx3* is expressed in the developing and adult conduction system, including the remaining atrioventricular canal myocardium and node. Both *Tbx2* and *Tbx3* are able to suppress the expression of working myocardial genes including *Cx40*, *Nppa*, *Cx43* and *Chisel*. Furthermore, *Tbx2* and *Tbx3* have been implicated in the regulation of senescence and proliferation via a p21 dependent pathway and direct binding to *Nmyc*. Overexpression of either gene in the early heart tube results in the repression of working myocardial differentiation in the ventricle. In mice, loss of *Tbx2* leads to ectopic expression of working myocardial genes in the left side of the atrioventricular canal myocardium. Furthermore, in the same region, the proliferation rate increased to become similar to that of the working myocardium of the left ventricle. In mutants, the left side of the atrioventricular canal is broader, shorter and appears trabecularized. This indicates a functional requirement of *Tbx2* for the repression of working myocardial differentiation in the atrioventricular canal myocardium. Interestingly, in *Tbx2/Tbx3* double knockout mice the expression of *Bmp2* is lost, indicating a positive feed-back mechanism for *Bmp2* expression in the atrioventricular canal (VMC and A. Kispert, unpublished observations).
The Bmp2-Tbx2/3 regulatory axis is limited by Tbx20 and Notch-Hey signaling

Tbx20 suppresses Tbx2 expression in the early developing heart. Tbx20 was reported to directly bind to a T-box binding element (TBE) in the promoter of Tbx2. However, the putative TBE’s were found to be dispensable for Tbx2 expression in the atrioventricular canal in vivo. Instead, its expression depends on an upstream Smad-dependent enhancer, suggesting that an indirect mechanism underlies Tbx20-dependent regulation of Tbx2. Upon BMP-mediated activation of the receptors, Smad4 forms a complex with Smad1 or Smad5 in the nucleus to activate genes. Tbx20 can bind to Smad4, thereby competing with Smad1 or 5 for binding to Smad4, thus interfering with the activation complex. Because Tbx20 is expressed in the entire heart tube, this mechanism could contribute to the suppression of Tbx2 activation by low levels of BMP-signaling in the heart tube, allowing specific regions to differentiate into chamber myocardium. In this hypothesis, the high levels of Bmp2-mediated signaling in the atrioventricular canal are sufficient to allow the formation of Smad1/5-Smad4 complex that activates Tbx2 expression.

The Notch-Hey pathway has been implicated in limiting the expansion of the atrioventricular canal phenotype. Activation of the Notch receptor by its ligands, e.g. Delta-like and Jagged, leads to expression of Hey. Hey1 and Hey2 are two homologous factors that are expressed in the atrial and ventricular myocardium, respectively, and are not expressed in the myocardium of the atrioventricular canal. Ectopic Notch activation in the cardiogenic mesoderm resulted in ectopic activation of Hey1 in the myocardium of the atrioventricular canal. This ectopic activation led to shortening of the atrioventricular canal, in which the expression of Bmp2 and Tbx2 was reduced. Additional knockout of Hey1 rescued the atrioventricular phenotype. Similarly, in chick and mouse, overexpression of Hey resulted in loss of Bmp2 and Tbx2 expression in the atrioventricular canal, whereas loss of Notch-Hey led to expansion of the Bmp2-Tbx2 regulatory network. In addition, in chick, the Bmp2-Tbx2 axis was found to repress Hey1 and Hey2, but this repression was not found in mouse. The early zebrafish heart tube expresses Bmp4, which later becomes restricted to the atrioventricular canal. After mutation of Hey2 homolog Gridlock, expression of Bmp4 remained expressed in the heart tube.

Importantly, the role of Notch signaling in the myocardium is different from its role in the endocardium. In contrast to the repression of the atrioventricular canal phenotype by myocardial activation, endocardial activation of Notch-Hey seems to promote the atrioventricular canal phenotype. In zebrafish, Notch1b activation in endocardium is required for the patterning of the atrioventricular canal myocardium. In mice, constitutive Notch activation in the endocardium resulted in an expansion of the atrioventricular canal endocardial phenotype to the ventricular compartment, but did not affect the myocardial expression of atrioventricular canal markers.

These data indicate an alluring concept of the development and homeostasis of the atrioventricular canal. Broadly expressed transcription factors drive working myocardial differentiation (Nkx2.5, Tbx5/20, Gata4/6). Robust expression of Bmp2 in the atrioventricular canal precursors drives the expression of Tbx2 (and possibly Tbx3), which repress working
myocardial differentiation, allowing these cells to obtain a specialized nodal phenotype. Bmp2 expression is limited by the Notch-Hey signaling pathway in the ventricular compartment. At the border of the atrioventricular canal the expression of Tbx2 and Tbx3 tapers off and may not be sufficient to repress working myocardial differentiation, allowing cells to be added to the ventricular compartment. Two potential mechanisms drive this transition into working myocardium and make it robust: Firstly, the Notch-Hey axis represses the expression of Bmp2 and Tbx2, and secondly, Tbx20 abolishes Bmp-driven Tbx2 expression in the prospective chamber myocardium.

Figure 3. In the adult heart two non-working myocardial rings are found around the orifices of the atrioventricular valves representing maintained embryonic atrioventricular canal myocardium. (A) Images obtained from a 3-dimensional reconstruction based on the expression patterns of proteins that are involved in the initiation and propagation of the electrical impulse. In red myocardium is indicated that has an expression profile in-between that of the atrial working myocardium and nodal myocardium, called transitional atrioventricular ring myocardium. Underneath, a second ring is present, depicted in yellow, representing myocardium with a nodal-like expression pattern and called the nodal atrioventricular ring myocardium. The caudal part of the nodal atrioventricular ring forms the atrioventricular node, indicated in green, which connects to the atrioventricular bundle, indicated in blue. The non-myocardial tissue is indicated in grey. (B) Schematic representation of the different expression domains and lineage relations. Lineage analyses indicated that the atrioventricular node is derived from the atrioventricular myocardium and does not receive contributions from the sinus horn myocardium, the atrioventricular bundle or dorsal mesenchymal protrusion. The atrioventricular bundle and lower nodal cells are derived from the embryonic interventricular ring myocardium. Note that the cellular relation and expression profile of the atrioventricular bundle and lower nodal cells are similar. ravr, right atrioventricular ring; lavr, left atrioventricular ring; tavr, transitional atrioventricular ring; navr, nodal atrioventricular ring; ao, aorta; astc, atrial septum transitional cells; asnc, atrial septum nodal cells; rab, retro-aortic root branch; ine, inferior nodal extension; cavn, compact atrioventricular node; inc, lower nodal cells; avb, atrioventricular bundle; sb, septal branch; av, atrioventricular.
Structure and growth of the atrioventricular conduction axis

The atrioventricular conduction axis and its components

In the adult heart, the electrical impulse originating in the sinus node rapidly propagates over the atria and then reaches the atrioventricular node. The atrioventricular node and bundle are the only muscular connection between the atria and the ventricles. The atrioventricular node delays the electrical impulse, which allows the atria to fully contract before the ventricles are activated. When the impulse reaches the atrioventricular bundle, it is rapidly propagated to both bundle branches and the downstream Purkinje fiber network that efficiently activates both ventricles. The atrioventricular node, along with the atrioventricular bundle and Purkinje system, can function as subsidiary pacemaker in case of sinus nodal failure. Furthermore, the relatively long refractory period of the atrioventricular node allows it to serve as safeguard in case of fast atrial arrhythmias, protecting the ventricle from detrimental fast or irregular rhythms. Several arrhythmias, such as atrioventricular block and reentrant tachycardia, have their anatomic substrates within the axis.

The atrioventricular conduction axis has been defined by histological appearances and anatomic criteria described in two papers that were the outcome of a meeting of the German Pathological Society in 1910. Macroscopically, the tendon of Todaro, the septal leaflet of the tricuspid valve and the orifice of the coronary sinus form an imaginary triangle (of Koch) and demarcate the atrioventricular nodal area. The beginning of the atrioventricular bundle, where it borders the atrioventricular node, is anatomically defined by the point of penetration into the central fibrous body, noting a coinciding change of the cellular and histological appearances in the sheep heart as described by Tawara. In other mammals such as rabbit, rat and mouse, this coinciding change is less strict. In these species, atrioventricular bundle-like cells were found at the atrial side and were later called the lower nodal cells.

Atrioventricular myocardium is still present in the adult heart.

In the adult heart the entire atrioventricular conduction system, including the atrioventricular node, atrioventricular bundle and bundle branches is delineated by Tbx3 expression. The different functional domains within this Tbx3+ population can be identified by the expression patterns of the different proteins that are associated with the initiation and propagation of the impulse, including Hcn4, Cx40, Nav1.5, Cx45, Cx30.2 and Cx43. The expression patterns of different combinations of proteins were used to make 3-dimensional reconstructions of the atrioventricular junction, including the atrioventricular conduction axis, of several species. The atrioventricular node is located at the caudal side of the atrioventricular junction and connects to the atrioventricular bundle. At the atrial side of the point of penetration of the membranous septum, cells with an expression pattern similar to the atrioventricular bundle were found at the same location of the previously identified lower nodal cells (Figure 3A and B).
In mice, the expression pattern in the myocardium lining the orifices of the mitral and tricuspid valves, also referred to as the atrial vestibules, indicated the presence of a transitional and a nodal ring of myocytes with a phenotype comparable to that of the embryonic atrioventricular myocardium (Figure 3A).9 In dogs, a layer of cells with a nodal-like electrical phenotype was found in the atrioventricular junction around the valves. In-between these nodal-like cells and the atrial working myocardium, cells with an intermediate phenotype were found, which were called the transitional cells.108 Together with the genetic lineage analysis of the atrioventricular canal myocardium, these data suggest that the embryonic atrioventricular canal myocardium is maintained, and represented by the nodal and transitional myocardial rings in the adult heart (Figure 3B).

**Development and growth of the atrioventricular conduction axis components**

The atrioventricular conduction system has been suggested to form by slow proliferation of the primary myocardium of the embryonic atrioventricular canal and embryonic interventricular ring, which form the atrioventricular node and ring bundles and the atrioventricular bundle, respectively.6-8, 14, 31 Others have suggested that the limited proliferation rate could not explain the growth of the atrioventricular conduction axis,109, 110 implying that contributions from other lineages to the atrioventricular conduction axis are required. For this and other reasons, several lineages have been suggested to contribute to the atrioventricular conduction system, including the atrial septum, the dorsal mesenchymal protrusion, the sinus horn myocardium and multipotent myocardial cells adjacent to a conduction system framework present in the embryonic heart.109, 111-114

To address this issue, low-titers of retrovirus were used to label single cells in the chicken heart of Hamburger Hamilton stage 13-17, corresponding to 2-3 days of incubation (E2-3). The virus was modified such that it was able to integrate in the genome and subsequently transcribe the LacZ reporter gene, but not able to replicate and produce new virus particles. These properties allowed the analysis of the clonal outgrowth of single infected cells. Hearts were analyzed at Hamburger Hamilton stage 39-43 (E14 to E18) to address the development of the conduction system as identified by EAP-300 expression.109, 115 These retrospective clonal analyses revealed that single cell-derived clones in the atrioventricular ring bundle and atrioventricular bundle always extended into the adjacent working myocardium.109, 115 Radioactive thymidine dilution assays suggested that the core of the atrioventricular bundle was retracted from the cell cycle earlier than the peripheral cells.107, 109 These data led to the formulation of the recruitment hypothesis in which a conduction system framework is formed early in development, which grows by recruitment of adjacent multipotent myocardial cells. The presence of a signal from the framework cells inducing recruitment was proposed.116 How the framework or the multipotent cells could be identified molecularly was not indicated in this model.

Importantly, at the stage of labeling by viral infection, the atrioventricular canal myocardium, representing the atrioventricular conduction system precursors or early
framework has already been formed, whereas the adjacent myocytes have already initiated the chamber (working) differentiation program. Therefore, the clones that were found to extend into adjacent working myocardium could be derived from atrioventricular myocardium, i.e. from the early framework itself rather than from adjacent cells (Figure 4A). The non-dividing core of the framework indicated by the thymidine dilution assays might then reflect merely a difference in timing of maturation within the myocardium of the atrioventricular junction and bundle myocardium. Tbx3 expression marks the conduction system components as soon as they emerge in the developing heart, including the atrioventricular myocardium, and is maintained in the components of the adult heart.25, 76, 82, 98 As such, the Tbx3+ population can be seen as the conduction system framework in the early developing heart. Recently, we defined the number of Tbx3+ cells and proliferation rates in the atrioventricular myocardium at consecutive gestational stages. These data indicated that the embryonic Tbx3+ cells proliferate sufficiently to form the adult components of the atrioventricular conduction axis.9 Furthermore, genetic lineage tracing of the Tbx2+ primary myocardium of the atrioventricular canal and physical cell-labeling studies indicated that this myocardium contributed to the working myocardium (Figure 2B and Figure 4B).8, 37, 46, 47 Subsequent additional lineage analyses showed that the atrioventricular node does not receive contributions from the atrioventricular bundle, sinus horn myocardium or the dorsal mesenchymal protrusion.9 From these data it can be concluded that the growth of the Tbx3+ myocardium is not dependent on, and does not require, recruitment of adjacent Tbx3-negative (chamber) myocardium.

Does this mean that the recruitment theory can be dismissed? If multipotent cardiac progenitors that will form both conduction system and working myocytes are present in the heart, as suggested by the recruitment model, these cells should lie within the Tbx3+ primary myocardium. Possibly, all Tbx3+ primary myocardial cells have the potential to become working myocardium and can be seen as a multipotent progenitor pool, whereas myocardium that has already differentiated into working myocardium of the atria or ventricles very likely does not contribute to the atrioventricular conduction axis anymore. It is also possible that the Tbx3+ population can be divided in a core central conduction system and adjacent multipotent cells. However, markers for such subpopulations are lacking. Moreover, to date a signal inducing recruitment has not been identified.
Atrioventricular canal development and the formation of accessory bundles

The role of the annulus fibrosus in the formation of accessory pathways

In the embryonic heart, the entire ring of the atrioventricular myocardium electrically connects the atria with the ventricles. In contrast, in the adult heart, the atrioventricular conduction axis provides the only myocardial and electrical connection between the atria and ventricles. The remainder of the atrioventricular junction is separated by a collagenous sheet known as the annulus fibrosus, which is formed by epicardium-derived cells that ingress the atrioventricular junction. 1-3 in 1000 individuals have accessory myocardial pathways that cross the annulus fibrosus and bypass the atrioventricular conduction axis. These accessory pathways are known as bundles of Kent and Mahaim. Bundles of Kent are associated with fast conduction and express Cx43. Mahaim bundles are predominantly located at the right atrioventricular border and are associated with slow conduction. Accessory connections may cause preexcitation of the ventricle, circus movement tachycardia, and in the presence of atrial fibrillation, even life-threatening ventricular tachycardia, as seen in Wolff-Parkinson-White syndrome patients. The actual number of individuals that have an accessory connection might be underestimated, because accessory connections may be present but not be functional.

It is commonly thought that the annulus fibrosus is required for an appropriate atrioventricular delay by blocking conduction over the atrioventricular junction, and that malformation of the annulus fibrosus, during development or later in life, causes preexcitation. Several observations challenge this assumption. In the embryo an appropriate atrioventricular delay is present without the presence of an annulus fibrosus. Remnant strands of this myocardium between the atrium and ventricle have still been observed in normal hearts around and after birth. Furthermore, in the adult heart, after formation of the annulus fibrosus has been completed, slow-conducting atrioventricular canal-type myocardium remains present around the orifices of the mitral and tricuspid valve (Figure 3A). In adult lower vertebrates, including reptiles, fish and amphibians a functional atrioventricular delay is found without the presence of an annulus fibrosus. These data suggest that malformation of the annulus fibrosus might theoretically provide a functional pathway with atrioventricular nodal-like properties (Mahaim bundles), but does not explain the existence of fast conducting bundles (Kent bundles), as present in Wolff-Parkinson-White syndrome patients. Taken together, it is very likely that phenotype of the atrioventricular myocardium plays a central role in the atrioventricular delay, and that defects in the development of atrioventricular myocardium could underlie formation of functional accessory pathways.

Disruption of the regulatory network for atrioventricular development underlies accessory pathway formation

Recently, myocardium-specific ablation of Tbx2 was found to cause erroneous gene expression and dysmorphogenesis of part of the embryonic atrioventricular canal. The atrioventricular
myocardium at the left side was observed to differentiate into working myocardium and the annulus fibrosus was found to be malformed (Figure 5A). Together, this leads to persistent atrioventricular myocardial connections that have acquired fast-conducting properties and to preexcitation of the ventricles.\textsuperscript{135} The myocardial pathways were seen to develop mainly at the epicardial side, probably due to the redundant function of $Tbx3$, which continued to be expressed at the endocardial side.\textsuperscript{135} In these $Tbx2$ mutants the malformation of the annulus fibrosus can be caused by disturbed epicardial ingression\textsuperscript{6} at the level of the atrioventricular sulcus due to dysmorphogenesis of the atrioventricular myocardium in $Tbx2$ mutants. However, the epicardium-derived mesenchymal material, which is formed before ingression, was not formed at the left side in myocardium-specific $Tbx2$ mutants (Figure 5A). Furthermore, the patterning of the epicardium was altered as revealed by changed expression of marker genes. These findings indicate the existence of a $Tbx2$-dependent signal in the atrioventricular myocardium that is involved in the formation of the annulus fibrosus. Consistently, deletion of the Bmpr1a receptor (Alk3) (activated by, among others, Bmp2) in the atrioventricular myocardium in mice resulted in accessory pathways and atrioventricular nodal defects.\textsuperscript{125,136} In addition, ectopic induction of activated Notch in the myocardium affected normal formation of the atrioventricular myocardium and led to the formation of functional accessory pathways.\textsuperscript{137} In agreement with these observations, in human, micro-deletions of $BMP2$ and Notch ligand $JAGGED1$ have been associated with ventricular preexcitation as part of a syndrome, including congenital defects.\textsuperscript{138,139}

The significance of these findings is that erroneous patterning of the embryonic atrioventricular myocardium can be the primary event in the formation of fast conducting accessory pathways. Therefore, genetic or epigenetic mechanisms and environmental factors that affect the $Bmp2-Alk3-Tbx2-Notch-Hey$ regulatory network and other components important for atrioventricular canal development may cause dysmorphogenesis of the atrioventricular canal, the acquisition of fast-conducting properties and malformation of the annulus fibrosus. Together, these changes lead to the formation of functional accessory pathways and preexcitation (Figure 5B).

### Linking familial ventricular preexcitation and atrioventricular canal development

Several genes have been associated with the formation of accessory pathways in familial preexcitation, including $LAMP2$ (encoding lysosome-associated membrane glycoprotein 2) and $PRKAG2$ (encoding regulatory gamma(2)-subunit of the AMP-activated protein kinase).\textsuperscript{140,141} Mutations in $LAMP2$ or $PRKAG2$ cause a hypertrophic cardiomyopathy-like phenotype due to defective glycogen storage, which is distinguished by electrophysiological abnormalities, particularly ventricular preexcitation. It is thought that the annulus fibrosus thins and becomes disrupted by toxicity of excess glycogen.\textsuperscript{128,129} The loss of the annulus fibrosus is thought to reestablish atrioventricular connections. However, in a mouse model of cardiomyocyte-specific overexpression of mutated PRKAG, accessory pathways only developed when the
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Mutated PRKAG was overexpressed during development. When overexpression was initiated in adulthood, glycogen storage disease and conduction system degeneration occurred, but accessory pathways did not develop. Whether PRKAG plays a role in atrioventricular canal development has yet to be elucidated.\textsuperscript{142, 143}

Ebstein’s anomaly is a rare syndrome that affects mostly the right atrioventricular junction, but coinciding left sided malformations have been described post-mortem.\textsuperscript{144} It is characterized by incomplete delamination of the mural and septal leaflets of the tricuspid valve.

\textbf{Figure 4.} (A) Schematic to depict the recruitment model (red dots) and the early specification-growth model (green dots) for conduction system development. According to the recruitment model, a labeled cell in the working myocardium and adjacent to the atrioventricular canal myocardium will contribute to the central conduction system. According to the early specification-growth model, the cells of the atrioventricular canal myocardium provide the cells of the future atrioventricular canal and to the adjacent working myocardium. (B) Schematic representation of the combined results from genetic lineage analysis and physical cell labeling of the atrioventricular canal myocardium. Labeled cells of the embryonic atrioventricular canal myocardium (dark green dot) can contribute to both the adult atrioventricular canal and left ventricle. Labeled cells of the embryonic left ventricle (orange dot) will only contribute to the ventricle and not to the atrioventricular canal. The proliferation rate and cell number of the Tbx3\textsuperscript{+} atrioventricular canal myocardium at consecutive stages indicated that the atrioventricular myocardium does not require recruitment to explain its growth. These combined data are in favor of a model in which the precursors of the conduction system are specified early and grow to form the definitive components of the conduction system. la, left atrium; lv, left ventricle; avc, atrioventricular canal.
Figure 5. (A) At E10.5, myocardium-specific Tbx2-mutants have ectopic expression of working myocardial genes in the left atrioventricular canal. Tbx3 expression is maintained at the luminal side of the atrioventricular canal myocardium. At E12.5, myocardium-specific Tbx2 mutants lack the accumulation of the subepicardial mesenchyme. Adult myocardium-specific Tbx2 mutants develop functional accessory bundles and preexcitation of the ventricles. The atrioventricular conduction system, including the atrioventricular node and bundle, remained intact. (B) Model of the transcriptional regulatory network in the atrioventricular canal myocardium that depends on a Bmp2-Tbx2-Notch axis for its correct patterning, formation of the annulus fibrosus, and generation of atrioventricular delay. avcm, atrioventricular canal myocardium; epi, epicardium; lv, left ventricle; avcs, atrioventricular cushion; sm, subepicardialmesenchyme; mv, mitral valve; af, annulus fibrosus; ab, accessory bundle.
and the right ventricular inlet becomes functionally part of the right atrium.\textsuperscript{145, 146} Defects in atrioventricular canal development are likely to underlie the anomaly. Interestingly, ventricular preexcitation is highly associated with this syndrome, suggesting that development of the atrioventricular canal plays a role in preexcitation in these patients.\textsuperscript{147, 148} Nevertheless, this role remains to be elucidated.

**Conclusion**

The atrioventricular canal is an embryonic structure playing important roles in the delay of impulse from the atrium to the ventricle, in the development of the atrioventricular conduction system and the valves, and in the alignment of the chambers. The atrioventricular canal is a transient embryonic structure, and recent lineage analyses have revealed it will ultimately give rise to the atrioventricular node and the atrioventricular junction myocardium (ring bundles), slow-conducting pacemaker-like tissue that remains present after birth. Moreover, it provides a much larger contribution to the ventricles and atria than previously appreciated. As a consequence, a large component of the left ventricular free wall for example, has been subject to the specific signaling environment found in the embryonic atrioventricular canal, suggesting that errors in this embryonic structure may affect the left ventricle.

Genetic manipulation of zebrafish, chick and mouse has revealed an evolutionary conserved molecular mechanism by which the atrioventricular canal phenotype is enforced and maintained. Broadly expressed transcription factors drive myocardial gene expression and differentiation, while localized Bmp-signaling in the presumptive atrioventricular canal represses working myocardial differentiation through Tbx2 and Tbx3. This repressive pathway is limited to the atrioventricular canal by at least two mechanisms, the myocardial Notch-Hey axis and Tbx20, respectively, which abolishes Bmp-driven Tbx2 expression in the prospective chamber myocardium. Manipulation of the atrioventricular canal regulatory network revealed that genetic or epigenetic mechanisms and environmental factors that affect the Bmp2-Alk3-Tbx2-Notch regulatory network may lead to the formation of accessory atrioventricular bundles. These accessory bundles can cause ventricular preexcitation, a feature of the Wolff-Parkinson-White syndrome. Importantly, these observations revealed that erroneous patterning of the early embryonic atrioventricular canal can be the primary event in the formation accessory pathways, and underlines the close relationship between the embryonic atrioventricular canal and the development of the atrioventricular conduction system.
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