Development of the Pacemaker Tissues of the Heart

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Pacemaker and conduction system myocytes play crucial roles in initiating and regulating the contraction of the cardiac chambers. Genetic defects, acquired diseases, and aging cause dysfunction of the pacemaker and conduction tissues, emphasizing the clinical necessity to understand the molecular and cellular mechanisms of their development and homeostasis. Although all cardiac myocytes of the developing heart initially possess pacemaker properties, the majority differentiates into working myocardium. Only small populations of embryonic myocytes will form the sinus node and the atrioventricular node and bundle. Recent efforts have revealed that the development of these nodal regions is achieved by highly localized suppression of working muscle differentiation, and have identified transcriptional repressors that mediate this process. This review will summarize and reflect new experimental findings on the cellular origin and the molecular control of differentiation and morphogenesis of the pacemaker tissues of the heart. It will also shed light on the etiology of inborn and acquired errors of nodal tissues. (Circ Res. 2010;106:240-254.)

Key Words: conduction system | pacemaker | sinus venosus | atrioventricular canal | development

The contractions of the heart are initiated and coordinated by electric signals from pacemaker tissues. At the entrance of the right atrium, sinus node (sinoatrial node [SAN]) myocytes generate the impulse to activate the atrial myocardium. After rapid propagation through the atria, the impulse is delayed in the atrioventricular node (AVN) and further propagated to the fast-conducting atrioventricular bundle (AVB), bundle branches (BB), and Purkinje fiber network, from which the mass of the ventricular working myocardium is activated. The components of the conduction system contain cardiomyocytes with pacemaker activity and other specific nodal properties that discriminate them from atrial and ventricular working myocardium (Figure 1). The SAN serves as the primary pacemaker, whereas the AVN and ventricular conduction system act as secondary (accessory) pacemakers to secure...
repetitive ventricular contraction in conditions of SAN failure or AV block.\(^1\) In addition, the lower rim of the atrial myocardium, just above the AV junction, and the myocardium surrounding the systemic venous return contain nodal-like cells, which, under pathological conditions, can acquire ectopic pacemaker activity.\(^2\)–\(^5\)

Disease, congenital malformations, aging, or somatic gene defects may cause dysfunction of the pacemaker tissues resulting in severe arrhythmias.\(^6\)–\(^7\) Although the electrophysiological properties, structure and cellular composition of the nodes have been extensively studied,\(^1\)\(^,\)\(^6\)–\(^8\)\(^,\)\(^10\) pathologies associated with the nodes are still far from understood. SAN and AVN development is cellularly and molecularly intertwined with that of the chambers. Therefore, the elucidation of the mechanisms underlying heart development in general will make a crucial contribution to our understanding of the functional regulation of the nodes. These insights may provide leads for the therapeutic intervention of pacemaker and conduction system diseases.

The mode of initiating and propagating the depolarizing impulse through the simple hearts of lower vertebrates, hearts of vertebrate embryos, and multichambered hearts of adult higher vertebrates (including human) is essentially identical (Figure 1). In fact, even in the absence of discernible nodal and conduction system components in simple hearts, a mature ECG can be derived. Thus, the coordinated generation and propagation of the depolarizing impulse is deeply rooted in the basic design of the vertebrate heart, indicating that the cellular and molecular mechanisms that drive the formation of pacemaker tissues are evolutionary conserved. This review discusses current

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Figure 1. Schematic overview of heart development in higher vertebrates. A, The early heart tube has a primitive phenotype (gray) and sinusoidal ECG. Chamber myocardium (blue) expands from the outer curvatures of the primary heart tube, whereas nonchamber myocardium (gray) of the sinus venosus (sv), AVC (avc), outflow tract (oft), and inner curvatures does not expand. The chamber heart has a mature type of ECG. B, The basic configuration of slow conducting pacemaker and fast conducting chamber components is found in the adult fish and human heart. C, The sinus node (san) forms in the sinus venosus, the AVN (avn), and atrioventricular junction (avj) in the atrioventricular canal. The ventricular septum crest part of the primary ring (pr) will form the AVB. a indicates atrium; ev, embryonic ventricle; ift, inflow tract; la, left atrium; lbb, left bundle branch; lv, left ventricle; pvcs, peripheral ventricular conduction system; ra, right atrium; rv, right ventricle; scv, superior caval vein; rbb, right bundle branch.
insights into the cellular origin of the nodal pacemaker tissues, their formation and function in relation to other cardiac components, and the molecular mechanisms controlling nodal tissue development. The formation of the AV bundle, BBs, and Purkinje network has been reviewed elsewhere.11–13

Development of the Heart and Its Basic Electric Configuration

A simple heart tube is formed subsequent to the formation of a cardiac crescent by fusion of the 2 heart-forming regions of the lateral plate mesoderm in gastrulation stage mammalian embryos. Not all cells of the (pre)cardiac mesoderm differentiate immediately. Cells located medially in the cardiac mesoderm are kept behind and become positioned dorsally and caudally to the arising heart tube. These cells, referred to as second heart field,14 proliferate rapidly and serve as a progenitor pool that continuously provides myocardium to both poles of the heart tube.15 Before their differentiation to myocardium and addition to the heart tube, these cells drastically decrease their proliferation rate.14,15 Labeling studies have indicated that the cardiac crescent and early heart tube only represent the outer curvature (apex) of the left ventricle and parts of the AVC and atria, whereas the remainder of the heart derives from cells added later.14,16–18

The initial embryonic heart tube myocardium possesses a phenotype that resembles that of the nodal tissues in displaying automaticity, poor contraction, and slow transmission of the depolarizing impulse.19–21 Sarcomeres and sarcoplasmic reticulum are not well-developed in this primary myocardium.19,20,22 Caudal pacemaker activity in this slow-conducting heart tube results in sluggish, unidirectional peristaltic contractions that are reflected in a sinusoidal ECG. During further elongation of the heart tube, the developing ventricular and atrial chambers acquire a working myocardial phenotype and rapidly expand by highly increased levels of proliferation (Figure 1A).20,23 The underlying molecular programs upregulate genes for high conductance gap junctions that contribute to the rapid transmission of the electric impulse, of mitochondrial genes associated with the increase in mitochondrial number and activity, and of genes for sarcomere components.19,20,24

Cardiac regions including the sinus venosus, the AVC, inner curvatures, and the outflow tract do not differentiate into chamber myocardium, retain low proliferation rates and will consequently form constrictions.19,20 Dominant pacemaker activity remains localized at the intake, the sinus venosus.25 The AVC retains its embryonic mode of conduction, which is much slower that that of the newly formed chambers. Thus, the impulse between the rapidly propagating atrial and ventricular chambers is effectively delayed.19,21 Concomitant with chamber formation, the initially peristaltic contraction mode of the embryonic heart tube is replaced by a pattern of serial and rapid contractions of the atrial and ventricular compartment(s), and the derived ECG starts to resemble that of a mature heart (Figure 1A).26 This basic configuration of alternating slow-conducting and poorly contracting pacemaker components (sinus venosus, AVC, outflow tract) and fast-conducting myocardial components (atria and ventricles) can be found in embryos and adults of all vertebrates with multi-chambered hearts, including human (Figure 1B).20 Higher vertebrates, such as mammals, will develop a morphologically distinctive SAN in the sinus venosus, an AVN within the AVC, and an AV bundle and ventricular conduction network to efficiently and coordinately activate the ventricles. The structural differentiation of these components varies widely between species. They are hardly present or not discernable in fish, they are very well-developed in large mammals, and in the mouse they are in-between. Thus, species-specific morphological adaptations are likely to have arisen during evolution.

The Sinus Node and Its Markers

The mature SAN is located in the intercaval region at the entrance of the right atrium.6 The SAN has an elongated structure described as “comma-shaped” with a “head” at the superior caval vein-atrial border and a “tail” along the terminal crest.6,22 However, the shape, position and composition of the SAN varies considerably between species.6,9 The SAN core is surrounded by an area that displays a myocardial phenotype and expression profile approximately in-between that of the working atria and the SAN.6,28 and an outer ring of connective tissue and arteries.9,22 This SAN periphery plays a role in the functional protection of the SAN from the resistive load of the atrium, allowing the SAN to activate the atrium through narrow superior and inferior SAN exit pathways.7,9

Classically, the SAN has been delineated using histological properties associated with nodal myocytes, including pale glycogen rich cells, poorly developed myofibrils, connective tissue, acetylcholine esterase activity, and HNK-1 expression.1,9 In addition, biosynthetic enzymes for acetylcholine and catecholamines are transiently expressed in the embryonic and fetal SAN and other parts of the conduction system,30 providing useful immunohistochemical markers for this tissue.

More recently, a panel of SAN-specific molecular markers has been established that relate to the distinct electrophysiological properties of this tissue (Figure 2).1,6,10,27,28,31–35 Pacemaker activity requires a relatively high intercellular resistance (low conductivity) to protect it from the suppressing hyperpolarizing influence of the atrium.1 Because gap junctions play an important role in the intercellular conduction velocities, it is not surprising that the SAN expresses a set of Cx45, Cx30.2, and Cx30 subunits32,36 that form gap junction channels with very low conductance. In contrast, the adjacent fast-conducting atrial working myocardium mainly expresses Cx40 and Cx43 subunits that assemble high-conductance gap junction channels.6,28,32,35 Furthermore, Scn5a, encoding Nav1.5, the main sodium channel responsible for rapid depolarization and conduction, is low in the SAN compared to the adjacent atrial myocytes.10,34 Thus, the Cx30.2/Cx45-high, Cx40/Cx43/Scn5a-low gene expression profile provides a useful set of markers for both fetal and adult SAN tissue (Figure 2A). Hcn4 is a member of the family of channels responsible for the hyperpolarization-activated current, I_h, that plays a key role in the pacemaker potential of the SAN.1,37 Hcn4 is enriched in pacemaker tissues, including the
SAN,6,37–39 and serves as an excellent positive nodal marker in the mature heart. The gene for atrial natriuretic peptide, Nppa, is expressed in atrial myocytes, not in the SAN or other nodal cells, providing a complementary negative marker. In rabbit, neurofilament is frequently used to mark the nodal tissues including SAN.10 Finally, the T-box transcription factor Tbx3 is specifically expressed in the developing and the mature conduction system, including the SAN, of mammals and chicken.39,40 Further information on structure, function, composition and “molecular architecture” of the mature SAN can be found in some excellent reviews.1,6,9

Formation of the Sinus Venosus and SAN
The sinus venosus comprises the SAN, the venous side of the bilayered venous valves and the right and left sinus horns (Figures 2B and 3). Based on histological sections, a SAN primordium was observed at embryonic day (E)10.5 in mouse.41 Virágh and Challice described that sinus muscle cells, including the SAN primordium, form from loose mesenchymal cells of the pericardial wall from E11.5.42 From that stage onward, a nodal structure can be easily observed in the right sinus horn at the junction with the atrium. In the left sinus horn of the mouse41,42 and human heart, the transient development of a small SAN was noted (ASizarov, AFM Moorman, unpublished observations, 2009). This structure probably degenerates, but may develop to a full SAN in case of atrial right isomerism,43 a process that is repressed by the transcription factor Pitx2c (see below).39

Recent molecular analyses revealed that a Tbx3+ SAN primordium develops within the right side of the Hcn4+/Cx40-negative sinus venous myocardium as early as E9.5 to E10 (Figures 2B and 3A).39 This initial Tbx3+ thickening in the sinus venosus most likely represents the primordium of the SAN extension along the right venous valve, the “tail.”27,39 This SAN primordium continues to grow by proliferation and myocardial differentiation of mesenchymal progenitors (see below) to form a “head” component that will extend into the right sinus horn. The SAN and the venous side of the bilayered venous valves express Tbx3 and Hcn4, whereas the atrium and the atrial aspect of the venous valves express Cx40 and Nppa in a complementary manner (Figure 4A).27,39

Cellular Origin of the Sinus Venosus and SAN
Until E9 to E9.5, the homeobox transcription factor Nkx2.5 is expressed in all myocardium derived from the first and second heart field but not in mesenchymal cells at the caudal-ventral-lateral side of the inflow tract.44 These cells express the T-box transcription factor Tbx18 in a strictly complementary pattern.27,44 Intriguingly, the myocardium of the sinus venosus and SAN, which differentiates after E9 to E9.5, expresses Tbx18 but not Nkx2.5, suggesting that the sinus venosus and SAN myocardium differentiates de novo from Tbx18+/Nkx2.5-negative mesenchymal progenitors (Figures 2B and 3A). Indeed, genetic lineage analyses (Nkx2.5−/Cre;R26RlacZ and Tbx18−/Cre;R26RlacZ) provided strong evidence for the origin of the entire sinus venosus from these noncardiac progenitors (Figure 4A and 4B).27,44 When isolated and cultured, the Tbx18− noncardiac precursors differentiated into Hcn4+/Nkx2.5-negative pacemaker myocardium that was beating much faster than cultured age-matched embryonic ventricular myocardium.27 Taking into account that these cells are precursors of the sinus venosus, this finding is consistent with
the much older observation that the elongating heart tube shows an increase in beat rate.\textsuperscript{45,46} Furthermore, \textit{Tbx18}-deficient mice fail to form the large head component of the SAN (Figure 4C), demonstrating that \textit{Tbx18} is required for the recruitment of these mesenchymal precursors to the cardiac lineage.\textsuperscript{27,44} Myocardial differentiation of the sinus venosus and SAN occurs between E9.5 and approximately E11 or later in mouse. After this period, the sinus venosus and SAN continuously gain size by slow proliferation.\textsuperscript{27,47} The \textit{Tbx18}\textsuperscript{+} sinus horn precursors do not express the transcription factor Is11, an operational marker of second heart field cells, before their differentiation.\textsuperscript{44} However, both the precursors of the SAN and the SAN itself coexpress Is11 and \textit{Tbx18}, indicating that the SAN progenitors have features of both second heart field (Is11\textsuperscript{+}) and sinus venosus progenitors (Tbx18\textsuperscript{+}).\textsuperscript{39,44,48}

SAN growth may be mediated by recruitment of surrounding myocardial cells that acquire the SAN phenotype (Figure 4D). However, when emerging atrial cells (Npp\textsubscript{a}\textsuperscript{−}) directly adjacent to the developing SAN (Tbx3\textsuperscript{+}) were labeled, the SAN remained unlabeled (Figure 4E). This observation suggests rather that SAN growth is achieved by proliferation of a subpopulation of specified SAN primordial cells. In turn, daughters of Tbx18\textsuperscript{+} sinus horn and SAN cells, which can be traced from approximately E8.5 to E9 onward in a Tbx18\textsuperscript{−/−};\textit{R26\textsubscript{R} lacZ} mouse, did not contribute to the atria (Figure 4B).\textsuperscript{27} Therefore, atrial and SAN lineages segregate at least as soon as they initiate the expression of these atrial and SAN markers.

**Tbx3 Transcriptional Repression and SAN Development**

Mature SAN cells resemble embryonic myocytes,\textsuperscript{9,19,20,22,42} contrasting the phenotype of atrial working myocytes with their well-developed contractile apparatus, as recognized as early as in 1907 by Keith and Flack,\textsuperscript{49} who described the SAN cells as “palely stained undifferentiated fibers.” It is thus conceivable that prospective SAN cells are actively inhibited from differentiation into working myocardium. Indeed, the transcriptional repressor Tbx3 has recently emerged as the molecular executor of this developmental restriction. Tbx3 is expressed selectively in the SAN primordium from E9.5 to E10 onward in the mouse.\textsuperscript{39,40} The SAN expression of Tbx3 is conserved in human and chicken. Tbx3 deficiency results in loss of \textit{Lhb} expression and expansion of working myocardial gene expression (\textit{Cx40, Cx43, Nppa, Snc5a}) into the SAN domain (Figure 2A).\textsuperscript{28} In contrast, conditional atrial misexpression of Tbx3 in mice led to repression of working myocardium genes, and activation of SAN genes (eg, \textit{Hcn4, Cx30.2, Cav3.1}) but did not change the expression of pan-specific myocardial genes.\textsuperscript{34} Gene expression profiling identified another 1500 transcripts representing direct or indirect targets of Tbx3 in the atria (WM Hoogaars, VM Christoffels, unpublished observations, 2009). Intriguingly, forced atrial expression of Tbx3 led to the ectopic development of functional pacemaker tissue identifying Tbx3 as a key regulator of the SAN phenotype (Figure 2C).\textsuperscript{34}

**Molecular Mechanisms of SAN Development**

Because of the potential of Tbx3 to confer the pacemaker gene program, restriction of \textit{Tbx3} expression to the developing SAN is mandatory. The expression of \textit{Hcn4} becomes restricted to the sinus venosus as well. A Shox2-Nnk2.5 repressor pathway was shown to regulate this process (Figure 2B and 2C). \textit{Hcn4} is initially expressed in the caudal end of the Nnk2.5\textsuperscript{+} heart tube (Figure 3A).\textsuperscript{37,38} It is subsequently downregulated in this domain, and activated in newly differentiated Tbx18\textsuperscript{−}/Nnk2.5\textsuperscript{−}negative myocardial cells of the sinus horns, effectively shifting its expression domain into the
Because Tbx3 is activated in the Nkx2.5-negative SAN primordium within the sinus venosus as well,39 Nkx2.5 may repress these genes, whereas it is required to activate Cx40, Nppa, and many other genes associated with atrial myocardial differentiation.39 Indeed, Nkx2.5-deficient embryos show ectopic expression of Hcn4 and Tbx3 in the heart tube,39 and fail to activate Cx40.3935 suggesting that Nkx2.5 represses Hcn4 and Tbx3, thereby confining their expression to the forming Nkx2.5-negative sinus venosus (Figure 3). Intriguingly, in Nkx2.5-deficient embryos, pacemaker activity originated from the early ventricular region, indicating derepression of a functional pacemaker program in the heart tube.39

Analyses of the Shox2 gene in mouse and frog have pointed to a role for the paired-related homeodomain transcription factor as a negative upstream regulator of Nkx2.5 expression in the sinus venosus (Figure 2B).52 Shox2-deficient mouse and zebrafish embryos display bradycardia, consistent with the ectopic expression of Nkx2.5 in the sinus venosus, subsequent loss of the pacemaker program, and ectopic activation of the chamber myocardium program.5455

The T-box transcription factor gene Tbx5 is expressed in a caudal-high gradient in the developing heart and is crucial for the formation of the early inflow/atrial region54 and for the expression of Shox2 and Tbx3 (Figure 2C).53 Tbx5 expression in all atrial and venous myocardium (or the formation of the Tbx5-positive cell population therein) is controlled by retinoic acid signaling,54 indicating that a retinoic acid-Tbx5 axis activates, rather than spatially restricts, Shox2 and Tbx3 expression in the SAN region. The transcription factor Pitx2 is known to control asymmetrical morphogenesis of the heart.57 In Pitx2c-deficient fetuses, SANs form at both the right and left sinus atrial junction. These SANs have indistinguishable molecular signatures, including Tbx3 expression,
sustained that Pttx2c functions within the left/right pathway to suppress a default program for SAN formation in the left sinus venosus (Figure 2B).39

**Evolutionary Conservation of Sinus Venosus Development**

Given the functional importance of pacemaker activity, it is not surprising that the molecular programs underlying sinus venosus and SAN formation appear highly conserved. In *Drosophila*, cardioblasts fated to differentiate into working myocytes express tinman, the *Drosophila* homolog of Nkx2.5, whereas the 6 pairs of cardioblasts that surround the inflow ostia, resembling the vertebrate sinus venosus, do not express tinman but the dorsocross T-box factor homologs instead.58 Inactivation of tinman results in ectopic expression of dorsocross, and loss of expression of *Sur*, encoding a K+ channel subunit, in tinman-positive cardioblasts. Tinman itself is repressed in ostia precursors by seven-up (*Scr*), the homolog of the orphan nuclear receptor Nrl2 (Coup-TF2), which in mouse is selectivity expressed in the early inflow tract.59

Similar to the mouse, the caudal (inflow) pole of the zebrafish heart tube is formed by differentiation of isl1+ cells.60 Loss of isl1 results in failure to differentiate and lengthen the inflow pole from progenitors and to increase beat rates during development (relative bradycardia). The formation of the definitive sinus venosus was not addressed in this study. However, expression of *Tbx18* in the sinus venosus is conserved in zebrafish.61* Xenopus*,62 chicken,63 mouse,64 and human (A Sizarov, VM Christoffels, AFM Moorman, unpublished observations, 2009), suggesting the existence of a *Tbx18*-controlled module for sinus venosus development in vertebrates.

**Confinement of Pacemaker Activity to the Sinus Venosus and SAN**

Whereas pacemaker activity is tightly associated with the SAN in the adult heart, pacemaker activity initiates more broadly and is only gradually confined to the developing SAN. First pacemaker activity can be recorded before the onset of contractions of the just-differentiated myocardium in the embryo.25,65 In the initial heart tube the myocytes at the caudal end, the intake, possess the fastest cycle, dictating the beat rate.25,46 Pacemaker activity then shifts to the left side of the inflow tract,25,65 for reasons yet unknown. With myocytes added to the inflow tract, the sinus venosus will form (Figure 3).44 Notably, during the lengthening of the heart tube, dominant pacemaker activity remains associated with the caudal end,25 implying that pacemaker activity continuously shifts to the cardiac cells most recently differentiated from the progenitor pool at the systemic venous side (Figure 3B). Indeed, once formed, the sinus venosus (sinus horns and SAN primordium) acts as pacemaker in the embryo and early fetus,25 consistent with the expression of Hcn4 and repression of working myocardial “high conductance” genes Cx40, Cx43, and *Scr*5a at this site. The mechanism underlying the shift of dominant pacemaker activity to the newly added sinus venous cells may involve the repressive activity of Nkx2.5 as discussed above.

At some as yet undefined prenatal stage, pacemaker activity becomes confined to the actual SAN structure (Figure 3B). The reason for this is unclear, but it is conceivable that the sinus horn myocardium, with the exception of the SAN itself, matures to obtain a phenotype comparable to the atrial working myocardium.39,42 This “atrialization” of sinus horns is confirmed by characteristic shifts of marker gene expression. The sinus horns activate Cx43 and *Scr*5a and, after approximately E12, also Cx40.39 Expression of these genes continues to be low or absent in the SAN, which maintains the expression of Cx45 and Cx30.2. Moreover, the expression of *Hcn4* in the entire sinus venosus becomes restricted to the SAN during fetal stages.38,39

**Sites of Ectopic Pacemaker Foci and Arrhythmias: Remnants of Pacemaker Development?**

Although the sinus venosus acquires atrial properties and loses pacemaker activity, the extent to which this occurs is unknown. It is possible that atrialization of sinus venosus-derived structures (superior caval vein [right sinus horn], crista terminalis [right venous valve], coronary sinus ostium [atrial entrance of the left sinus horn], and ligament of Marshall [remnant of the left sinus horn]) does not occur completely in some individuals. As a result, pacemaker or focal automatic activity may persist and give rise to nonpulmonary vein paroxysmal atrial fibrillation.86,87 HNK-1/Leu-7 immunohistochemistry revealed several strands of internodal tracts between the SAN and AVN in the dorsal atrial wall,68,69 the function of which have not been defined. These tracts express Tbx3, CCS-lacZ and other conduction system markers (Figure 5A),40,70 suggesting they may have a nodal phenotype. These tracts may be remnants of the original Tbx3+/Cx40-negative nodal cell at the connection of the sinus venosus and atrium that connects the SAN and AVN. When SAN and AVN separate during expansion of the venous region, the intervening tissue forms strands between the venous entrance and the dorsal atrial septum (Figure 5A).

Pulmonary vein myocardium represents another major site of ectopic foci that initiate paroxysmal atrial fibrillation, and its formation has been discussed in the context of conduction system development. However, the pulmonary myocardium forms independently from the sinus venosus, and displays an atrial working myocardial phenotype and gene program from the outset (eg, Nkx2.5’/Cx40+ and Tbx18/Hcn4-negative). Hence, the mechanisms underlying arrhythmias from the pulmonary myocardium are, in all likelihood, different from those underlying arrhythmias from the residual nodal tissues of the sinus venosus and AV junction.

**The AVN and Its Markers**

The second major site in the heart with potential pacemaker activity is the AV junction, which includes the AVN. The adult AVN is a complex and heterogeneous structure, consisting of multiple components and different cell types with
distinctive gene expression profiles. The region of AV nodal–like myocytes extends from the AVN to encircle the vestibule of the tricuspid valve (right AV ring bundle) and the mitral valve (left AV ring) to contact anteriorly and form what has been called a “retrograde node” (Figure 5B).5,40

Similar to SAN cells, AVN and AV junction cells are smaller and more primitive (poorly developed myofibrils and sarcoplasmic reticulum, lack of complex intercalated discs, high glycogen content, lower mitochondrial content, etc) than working myocytes.19 Their expression profile is in agreement with their pacemaking and conduction-slowing properties, Cx40/Cx43/Scn5a-negative and Cx45/Cx30.2(mouse)/Hcn4/Tbx3-positive.8,71,78 In addition, several transgenes have been identified that mark the AVN and junction, including minK-lacZ, cGata6-lacZ, CCS-lacZ, cTnI-promoter-lacZ, and BAC(Tbx3-GFP) (Figure 5).17,24,75-77 Collectively, these genetic markers can be used to distinguish the AV nodal myocardium from the surrounding working myocardium. The AVN/junction has distinctive subdomains, which may express the markers to a different extent.8,71,78 Furthermore, the frequently used marker Cx45 seems to be AVN restricted only at the protein level, as Cx45 mRNA has been found at similar levels in nodal and working myocardium.30 The AVN is connected to the fast-conducting (Cx40/Scn5a-positive) proximal AVB.32,74,79

Formation of the AVN and AV Junction Myocardium From the Embryonic AVC

Functional and morphological studies initially suggested de novo formation of the AVN and proximal AVB in the lower part of the atrial septum, but derivation from the left sinus horn (coronary sinus), from the “AV ring,” AVC, or from multiple tissues has also been discussed (reviewed elsewhere). Current studies, however, support the view that the embryonic AVC contains the precursors of the AVN, of the AV ring bundle(s), of the support of the AV valves, and of the lower rim of the atrium (Figure 5).17,24,75,81,82,83 In general, markers robustly expressed in the embryonic AVC become restricted to the AVN, AV valves-encircling myocardium of the AV junction and anterior node. After birth, the expression domains of minK-lacZ, Tbx3 and BAC(Tbx3-GFP) become further restricted to the AVN and small parts of the right AV junction only (Figure 5B).5,40,83 Together, these molecular findings argue that the mature AVN and nodal AV junction myocardium are derivatives, or remnants, of the prenatal AVC myocardium.19,82

The initial heart tube consists of precursors of the left ventricle and most of the AVC.14,17,18 LacZ expression driven by an AVC-specific chicken Gata6-enhancer at early stages marks the limbs of the cardiac crescent.17 Similarly, Tbx2 is expressed in the limbs of the cardiac crescent (E7.5), inflow tract (E8), and, subsequently, in the early AVC (E9).18 Classic studies and Tbx2Cre-mediated fate mapping indicated a lineage relation between these embryonic tissues,16,18 suggesting that the early AVC itself is largely derived from the cardiac crescent, which is derived from the first heart field. In the embryo, the electric impulse traverses the myocardium of the AVC on its way from the atria to the ventricles,19,21,84,85 with the dorsal AVC region being the preferential site of conduction.84,85 In agreement, AVN primordial cells (high glycogen content) are observed in the dorsal AVC as early as E9.5, shortly after chamber differentiation (Figure 6A).78,81,86 They form a (Tbx3+) inner layer that faces the dorsal AV cushion and extends deeply into the left ventricle, where it interconnects with both the (Cx40+) trabeculae and the emerging interventricular septum (Figure 6A through 6C). The dorsal outer wall myocardium disappears, probably by apoptosis. The ventral and lateral walls of the AVC harbor loose spongy myocardium that becomes penetrated by connective tissue to form the annulus fibrosus. From E11 onward until completion of septation, the AVN primordial cells at the distal end proliferate and expand into the dorsal AV cushion and form the definitive AVN node. The cells of the ridge of the interventricular septum form the AV bundle and remain in contact with the AVN (Figure 6D an 6E).86

Figure 5. Tbx3 expression delineates the conduction system. A, Reconstruction of the Tbx3 expression domain (red) delineates the san, AVC, AVN, internodal tracts, AVB, and BBs. The myocardium has been removed revealing the lumen of the chambers. The san and AVC/AVN express Tbx3 throughout development. B, Tbx3BAC-Egfp expression in the E9.5 AVC is progressively restricted to the AVN, right atrioventricular ring bundle (ravrb), and retroaortic AVN (ran) derived from the AVC. Stages shown are E11.5, E17.5, and 3 weeks after birth.

Lineage Relations Between the AVC and the Definitive AV Conduction System

Based on morphological studies, the AVN has been proposed to forms in its entirety from a small subpopulation of proliferating AVC cells. However, cellular contributions from outside the AVC could not be excluded. Retrospective clonal analyses in chicken represented the first lineage analyses of the AV conduction system.11,87 These studies settled the debate regarding a neurogenic versus cardiac
origin of the conduction system components by showing that conduction system myocytes share a common origin with other myocytes. In these studies, rare single cell–derived clones in the AV conduction system, including the right AV ring bundle, were found to always extend into the adjacent working myocardium. The myocardium of the forming AVB, were found to always extend into the adjacent working myocardium. Because AV myocardial cells do proliferate and provide an extensive contribution to the adjacent working myocardium, an extensive contribution to the adjacent working myocardium, an extensive contribution to the adjacent working myocardium, and a small portion of the AV junction (Figure 5B). First, the AV conduction system components grow by ongoing accretional recruitment of developing working myocytes to an initial framework represented by the early AVC, a process that continues until the end of septation. Alternatively, the early AVC may not only form the AV conduction system components, but may additionally provide cells to the adjacent chambers. This model has recently gained support by a genetic lineage tracing experiment: mouse AVC cells labeled between E8 and E9.5 by Tbx2-driven Cre, corresponding to the early AVC, give rise to the definitive AVN and AV ring bundles, but they also provide an extensive contribution to the adjacent working myocardium. Because AV myocardial cells do proliferate significantly, their increase in number is likely to be sufficient to generate cells of both the AV conduction system components and the developing working myocardium.

The AVB is formed from the crest of the developing ventricular septum, the left and right BBs develop from the subendocardial myocytes along the ventricular septum. In contrast to the AVN, the AVB and BB are not derived from the Tbx2+ AVC, indicating that the precursors of these AV conduction system components segregate very early in development (mouse E8) and have been exposed to distinctive regulatory signals.

**Differentiation and Maturation of the AVN and Junction**

Although gene expression and cell lineage studies have uncovered that the AVN develops from embryonic myocardium within the AVC, the molecular programs that drive the localized formation of a structurally complex AVN have remained elusive. Because the developing AVN and AV ring bundles always face the cushion mesenchyme, signals from the cushion mesenchyme may mediate or participate in the maintenance and further specialization of the pacemaker phenotype. Support for this hypothesis comes from zebrafish studies that showed simultaneous ectopic induction of AVC myocardium and cushion formation in Apc mutants and loss of AVC in cloche mutants that do not develop endocardium. Furthermore, immigrating epicardial and neural crest cells may influence the maturation of these components. The expression of minK-lacZ, Tbx3, Tbx3-GFP and other markers in the embryonic AVC becomes restricted to the AVN and small portion of the AV junction (Figure 5B). This finding indicates disappearance of AVC myocardium by apoptosis or by differentiation into working myocardium. Ectopic beats may originate from the remaining AV junction myocardium, an Hcn4+/Tbx3+ Cx43-negative nodal tissue, around the tricuspid and mitral valve. Other than that, not much is known regarding regulatory mechanisms of postnatal maintenance or degree of interindividual variations in the extent of these tissues. During development, the conduction velocity rapidly increases in the chambers, but also changes in the AVC, implying further specialization of the AVC phenotype. We recently performed genome-wide expression analyses of the developing AV nodal and working myocardium. The profiles were compatible with a more embryonic, less working muscle–typical phenotype of the
AVC and AVN. Moreover, AV nodal cells express an extensive neurogenic gene program. Cross-comparison of the different data sets revealed that the majority of differentially expressed transcripts in the E10.5 embryonic AVC maintained differential expression in the E17.5 late fetal AVN. These data indicate maintenance of properties during AVN development. However, the profile of E17.5 AVN substantially differs from that of the E10.5 AVC, revealing that the AVN substantially specializes during development.

Atrioventricular Insulation and Accessory Pathways

Soon after the establishment of the AVC, connective tissue from the AV cushion and subepicardial mesenchyme starts to invade the AV myocardium to form the annulus fibrosus, which physically separates and insulates atria from ventricles. The AVB remains as only connection between the AVN at the atrial side of the fibrous body with the ventricles. However, this process of insulation is gradual and often not completed before birth. AV reentrant tachycardias are caused by the presence of abnormal accessory myocardial bundles connecting the atrial and ventricular myocardium. Usually, these bundles conduct rapidly, such as the Kent bundles in Wolff–Parkinson–White syndrome, suggesting they are of a working myocardial phenotype. Accessory bundles with AV-nodal properties (slow), as found in Mahaim tachycardia, are rare and usually occur at the tricuspid side. These bundles probably derive from the remnants of prenatal AVC myocardium, which is more extensive around the tricuspid valve (Figure 5B).

The formation of accessory bundles probably involves abnormalities in both the formation of the fibrous insulation and the differentiation of the original slow-conducting AVC myocardium to fast-conducting working myocardium. Mutations in PRKAG2 have been associated with ventricular preexcitation (Wolff–Parkinson–White syndrome). Expression of a mutant isoform leads to disruption of the fibrous insulation and direct contact between the atrial and ventricular myocardium, allowing the electric signal to by-pass the AVN-AVB connection. Furthermore, inhibition of epicardium formation results in disturbed formation of the fibrous insulation and prenatal ventricular preexcitation. These data provide evidence for the importance of the fibrous insulation to prevent peri- and postnatal ventricular preexcitation arrhythmias.

Regulatory DNA Sequences Reveal AVC-Specific Transcriptional Modules

Analysis of the molecular pathways involved in AVC specification and formation has greatly benefited from the dissection of regulatory DNA elements conferring AVC-restricted patterns of activity, including the chicken Gata6-enhancer, a cardiac troponin I–promoter, an Nppa regulatory module, a Cx30.2-enhancer, and a large genomic fragment of Tbx3.

Cardiac troponin I and Gata6 are not specifically expressed in the AVC, indicating that these genes have multiple regulatory modules that define their complete pattern. In contrast to Tbx3 itself, the Tbx3 genomic fragment was not active in the AVB (Figure 5B), showing that gene expression in the AVC and the AVB depends on distinct regulatory sequences and pathways.

The activity of the chicken Gata6-enhancer and Cx30.2-enhancer depends on GATA elements to which Gata4 (and other Gata family members) binds. The Cx30.2-enhancer in addition requires binding sites for T-box genes. The Cx30.2-enhancer and Cx30.2 itself are less active in Gata4+/− and Tbx5+/− embryos. Moreover, Gata4+/− mice display shortened P-R intervals, consistent with Cx30.2 downregulation. Together, these data indicate Tbx5 and Gata4 are responsible for Cx30.2 activity in the AVC. Tbx5 and Gata4 are expressed in both the AVC and working myocardium, suggesting the existence of additional cofactors for AVC-restricted activity of Cx30.2 and the chicken Gata6-enhancer.

Nppa expression marks the differentiating chambers, and has been a very useful negative marker for the AVC. The proximal Nppa promoter contains a small regulatory module that is activated cooperatively by Tbx5 and Nkx2.5, and that is active in the chambers but inactive in the AVC in vivo. Unexpectedly, mutation of either the T-box element or Nkx2.5-binding site in the Nppa promoter resulted in loss of repression in the AVC, whereas chamber activity was maintained. This study suggested that chamber-specific activity is mediated by active transcriptional repression by a T-box factor in the AVC.

T-Box Factors and Nkx2.5 in AVC Development

Tbx2, a T-box factor that acts as a repressor, is selectively expressed in all primary (nonchamber) myocardium, including the AVC. Tbx2 competes with Tbx5 for binding of T-box elements in Nppa and Cx40 promoters and for interaction with Nkx2.5, thus repressing Nppa in the AVC. Subsequent gain- and loss-of-function studies showed that Tbx2 is a general repressor of the chamber-specific gene program in the AVC (Figure 7). In zebrafish, tbx2b expression is restricted to the AVC. Morpholino knockdown of this gene resulted in loss of the AVC and of AV conduction delay.

The AVC also expresses Tbx3 (Figures 5A and 6B), which is equivalent to Tbx2 in its ability to inhibit chamber differentiation and gene expression. Tbx3-deficient embryos develop a limited AVC defect, whereas Tbx3-deficient embryos have a normal AVC and display cardiac defects only at sites where Tbx2 is not expressed (SAN, AVB). Simultaneous inactivation of both Tbx2 and Tbx3 results in loss of the AVC phenotype (VM Christoffels, A Kispert, unpublished observations, 2009), confirming functional redundancy of the 2 T-box genes in AVC development.

Mxs2 (Drosophila muscle segment homeobox related) expression marks the AVC in chicken and mouse. Its function and ability to form a complex with Tbx2 and Tbx3 that represses Cx43 expression render Mxs2 a possible component of the regulatory pathway controlling AVC for-
mation. Nevertheless, mice that are deficient in Mx2 do not display conduction defects.

Nkx2.5 and Tbx5 are broadly expressed in the embryonic heart, with Tbx5 displaying a more restricted caudocranial graded pattern of expression that is maintained in the atria and AV conduction system. Both factors are crucial activators of chamber-specific genes and essential for chamber differentiation. Nkx2.5 and Tbx5 play important activators of chamber-specific genes and essential for chamber differentiation. Nkx2.5 and Tbx5 have been identified as activators of AV conduction system (AVC) genes.

Dominant mutations in Nkx2.5 and Tbx5 (Holt–Oram syndrome) cause congenital heart defects and AV conduction defects, even though their development appeared unaffected. Nkx2.5 and Tbx5 play important roles in AV conduction system development.

Figure 7. Molecular pathways regulating the development and boundary establishment of the AVC and chamber myocardium.

Bone Morphogenetic Protein Signaling Controls Tbx2 Expression and AVC Development: Implications for Ventricular Preexcitation

AVC-restricted expression of Tbx2 (and possibly Tbx3) is mediated by bone morphogenetic protein (Bmp) signaling (Figure 7). Bmp2 that is expressed remarkably early and specifically in the embryonic AVC, is required for AVC specification and Tbx2 expression, and is sufficient to activate Tbx2 and Tbx3. Tbx2 expression in the AVC in vivo relies on a small Smad-dependent enhancer upstream of the transcription start site. In the zebrafish heart, the transcription factor slf/foxn4 is required for AVC formation. Foxn4 and Tbx5 have been identified as activators of Tbx2b expression that act through conserved T-box element and Foxn4 sites. In mouse, these sites are not required for Tbx2 enhancer activity in the AVC, suggesting that AVC restriction of Tbx2 has been achieved by different molecular pathways in vertebrate evolution.

Tbx20 is required for heart tube formation and chamber development. Importantly, Tbx20 deficiency leads to widespread ectopic expression of Tbx2 in the entire heart tube. The phenotype of Tbx20 mutants is reminiscent of that of embryos in which Tbx2 is ectopically expressed in the entire primitive heart tube, indicating that Tbx20 represses Tbx2. In addition to this function, Tbx20 was found to be required for chamber formation independently of Tbx2. Although Tbx20 may repress Tbx2 directly, putative T-box elements conferring repression were not required for Tbx20-mediated repression in vivo. Instead, Tbx20 was found to interact with Smad1/5, thereby interfering with Bmp-Smad activation of this enhancer, thus providing an indirect mechanism to deactivate Tbx2 in the early heart tube and confine its expression to the AVC.

Notch-Hey signaling in the chambers delimits the Bmp2-Tbx2 pathway, hence AVC formation. In forming chamber myocardium of chicken, Serrate-Notch2 signaling activates the expression of Hey1. Hey1 together with Hey2 then repress Bmp2. Bmp2-activated Tbx2 represses Hey1 and Hey2 in the AVC, in turn, generating a feedback inhibition loop that sharpens the chamber–AVC border. Similarly, in mouse, ventricular Hey2 represses Bmp2 and Tbx2, whereas atrial Hey1 represses Tbx2, thereby confining the expression of Bmp2 and Tbx2 to the AVC.

Abrogation of BMP signaling in the AVC by cGata6-Cre-mediated inactivation of Bmpr1a/Alk3, the type IA receptor for BMP, resulted in the disruption of the fibrous insulation. Myocardial AV connections expressing Cx43 developed that were sufficient to cause preexcitation, and AVN morphology was changed. BMP signaling in the AVC is essential for the formation of AV cushions and the expression of Tbx2 (Figure 7). Because inactivation of Tbx2 causes the formation of abnormal accessory myocardial bundles and preexcitation (W Aanhaanen, B Boukens, VM Christoffels, unpublished observations, 2009), the BMP-Tbx2-pathway in the AVC may play an important role in coordinating AV insula-
tion. Intriguingly, a 20p12.3 microdeletion that includes BMP2 was found to predispose to Wolff–Parkinson–White syndrome.121

Conclusions

Until very recently, our understanding of the etiology of cardiac arrhythmias has been compromised by the lack of insight into the molecular and cellular mechanisms underlying development and function of the conduction system. Insight into ion channel gene regulation was scarce, the development of the tissue architecture, cellular composition, and innervation of the nodes and bundle not well understood, and the spatial and temporal integration of conduction system development with that of the heart in toto is insufficiently explained. Fortunately, this unsatisfying situation has started to change by the identification of progenitor cell sources of the SAN and AVN, the definition of their lineage contributions, and the revelation of key transcriptional circuits that control specification and differentiation of the conduction system components. We can now explain the “primitive” phenotype of conduction system myocytes as a consequence of local transcriptional repression of working myocardial differentiation. These transcriptional pathways provide a link between cellular differentiation, morphogenesis, and the regulation of expression of ion channels and gap junctions that define the electrophysiological function of the mature tissues. Finally, the important interaction between heart function and cardiac developmental gene regulation has been uncovered. Genome-wide analyses of tissue-specific transcription factor–DNA interactions and their dependence of coregulatory factors within the heart will hopefully bring us closer to understanding the complexity of the regulatory mechanisms underlying normal and aberrant development and function of the cardiac conduction system.

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Disclosures

None.

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