Part VIII

Conduction System and Arrhythmia

Perspective

Michael Artman

One of the fascinating and essential characteristics of the cardiovascular system is that it functions automatically without requiring conscious input. In other words, the normal heart beats regularly and spontaneously regardless of whether or not the individual is thinking about it. Electrical impulses arise in the sinoatrial node, pass through the atria, pause at the atrioventricular node, and then are distributed in a tightly orchestrated spatial and temporal manner to the ventricles for coordinated and effective pumping activity. This elegantly synchronized process repeats itself over and over approximately three billion times during the average human life. Even minor disruptions in this normal process can have devastating consequences. Considerable morbidity and mortality result from abnormalities in intrinsic cardiac pacemaker activity and conduction of impulses. Congenital and acquired arrhythmias remain a significant health problem. Consequently, a clear understanding of the molecular and cellular processes involved in the formation and maintenance of normal electrical signaling in the heart has profound implications for human cardiovascular health and disease. Furthermore, it is likely that insights into the developmental processes involved in building a cardiac conduction system will likely have implications for understanding fundamental biology applicable to other organ systems.

This part reviews and summarizes the current state of understanding of the development of spontaneous pacemaker activity and the cardiac conduction system. Christoffels' group reviews the signaling pathways and molecules involved in the

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development of the cardiac conduction system. The authors emphasize the importance of understanding the three-dimensional architecture of genomic loci to provide insight into the regulation (normal and abnormal) of gene expression. Mikawa and colleagues identify and characterize a previously unrecognized source of cells that are destined to become pacemaker cells, even before cardiac morphogenesis begins. These results will likely lead to better understanding of the mechanisms involved in pacemaker cell differentiation, which in turn may ultimately have therapeutic implications. Additional information on the mechanisms, molecular signals, and pathways involved in specification of cell fate are provided by Asai et al. and by Morikawa et al. Taken together, the papers presented in Part VIII provide an interesting, informative, and elegant overview of the current understanding of pacemaker and conduction system development. Moreover, they provide a road map for future investigations into this essential and fundamental biological process.

Regulation of Vertebrate Conduction System Development

38

Jan Hendrik van Weerd and Vincent M. Christoffels

Abstract

The cardiac conduction system (CCS) consists of distinctive components that initiate and conduct the electrical impulse required for the coordinated contraction of the cardiac chambers. The development of the CCS involves complex regulatory networks of transcription factors that act in stage, tissue and dose-dependent manners. As disrupted function or expression of these factors may lead to disorders in the development or function of components of the CCS associated with heart failure and sudden death, it is crucial to understand the molecular and cellular mechanisms underlying their complex regulation. Here, we discuss the regulation of genes driving CCS-specific gene expression and demonstrate the complexity of the mechanisms governing their regulatory networks. The three-dimensional conformation of chromatin has recently been recognized as an important regulatory layer, shaping the genome in regulatory domains and physically wiring gene promoters to their regulatory sequences. Knowledge of the mechanisms by which distal-acting regulatory sequences exert their function to drive tissue-specific gene expression and understanding how the three-dimensional chromatin landscape is involved in this regulation will increase our understanding of how disease-associated genomic variation affects the function of such sequences.

Keywords

Heart development • Conduction system • Sinus node • Atrioventricular node • Transcriptional regulation • Functional genomics • Patterning • Pacemaker

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_38

38.1 Introduction

The cardiac conduction system (CCS) initiates and propagates the electrical impulse that is required for the rhythmic and synchronized contraction of the heart. The impulse initiates in the sinoatrial node (SAN) and is rapidly propagated through the atria, thereby activating the contraction of the atrial myocardium. The impulse is then propagated through the atrioventricular node (AVN), the only electrical connection between the atria and ventricles. The AVN delays conduction of the impulse, allowing for the atrial contraction and ventricular filling to complete before the ventricles contract. Further propagation of the impulse to the fast-conducting atrioventricular bundle (AVB), bundle branches (BBs) and Purkinje network causes the depolarization of the ventricular myocardium, leading to ventricular contraction. The development of the CCS is regulated by transcription factors that act in strictly stage, tissue and dose-dependent manners [1, 2]. A disruption in the function or expression of these factors could lead to disorders in the development or function of the CCS that can lead to lethal arrhythmias and heart failure. Knowledge of the mechanisms underlying regulation of genes involved in CCS development is therefore crucial.

38.2 Genetic Pathways Controlling SAN and AVC Development

The heart is the first organ to form during embryonic development and starts as a primitive, linear tube with the inflow region at the caudal side and the outflow region at the cranial side. The slow conductive properties of the embryonic muscle cells within the primitive tube at this stage and dominant pacemaker activity at the caudal end cause a slow peristaltic pattern of contraction along the tube to propagate the blood. At this stage, the entire sinus venosus acts as pacemaker, characterized by the expression of the pacemaker channel Hcn4, a member of the family of channels responsible for the hyperpolarization-activated current *if* that is crucial for the pacemaker potential [3, 4]. The heart tube elongates by the addition of rapidly proliferating progenitor cells that differentiate to cardiac muscle. This implies that cells added to the inflow tract will acquire dominant pacemaker activity. With further development, specific regions in the heart tube start to divide rapidly and activate a working myocardial gene program, resulting in the ballooning of the primitive atrium and ventricle. Concomitant with the ballooning of the primitive atria is the formation of the sinus venosus including the SAN. Dominant pacemaker activity will gradually be confined to the SAN at the junction of the sinus venosus and atrium.

The transcriptional activator Tbx5 is required for the sinus venosus expression of Shox2 [5], a homeobox transcription factor necessary for SAN formation and function [6, 7]. Shox2 represses cardiac homeobox transcription factor Nkx2-5 [6]. In the chambers, Nkx2-5 activates chamber-specific genes including *Nppa* and high-conductance gap junction subunit-encoding genes *Gja5* (Cx40) and *Gja1* (Cx43), whereas it represses SAN/CCS-specific genes *Hcn4* and T-box transcription

factor *Tbx3* [8]. Tbx3 is required for the formation of the SAN by directly repressing atrial myocardial genes *Gja5*, *Gja1* and *Nppa* to prevent atrialization of the SAN and indirectly activating *Hcn4* and other SAN genes [9, 10].

During ballooning of the primitive cardiac chambers, the region in between the atria and ventricles does not proliferate and forms a constriction, the atrioventricular canal (AVC). Bmp2 expression in the AVC activates the expression of Tbx3 and Tbx2 [11]. Together with Msx2, these T-box factors repress the working myocardial gene program in the AVC and AVC-derived AVN [12, 13] and stimulate the pacemaker gene program and the program required for the formation of the AV cushions. Within the AVC, Tbx2 and Tbx3 interact with Nkx2-5 to repress genes that are activated by Nkx2-5 and Tbx5 in the working myocardium of the atria and ventricles [14–16]. Tbx2 and Tbx3 thus suppress working myocardial differentiation of the AVC, thereby causing the retention of the primitive phenotype of slow conduction and low rates of proliferation, providing a primitive morphological and functional constriction in between the atrial and ventricular chambers. Other factors that regulate the formation of the AVC and its border with the chamber myocardium include Wnts, acting upstream of Bmp2; Hey1 and Hey2, Notch target genes expressed in the chambers that suppress Tbx2 [17]; Tbx20, which represses BMP-mediated activation of Tbx2 in the chambers [18]; and Gata4/6, which act in complex with Smads and histone acetyltransferases (HATs) to activate AVC-specific enhancers in the AVC and with histone deacetylases (HDACs) and Hey1/2 to suppress these enhancers in the chambers [19]. The resulting pattern of conduction—fast in the atria, slow in the AVC and fast in the ventricles—results in the alternating contraction pattern of the chambers and an ECG that resembles the adult ECG (Fig. 38.1a).

38.3 Transcriptional Regulation of CCS Genes

Although the expression patterns and functions of genes involved in the development of the cardiac conduction system are relatively well studied, little is known about the molecular mechanisms underlying their regulation of expression. Tissue-specific gene expression often involves long-range regulatory elements, such as enhancers, which dictate the strictly time-, tissue- and dosage-dependent expression of their target genes. The identification and function of such enhancers is therefore highly relevant to fully understand the complex regulatory networks in CCS formation. However, to date only few studies have been carried out investigating in depth the regulation of genes driving CCS development.

The T-box transcription factor Tbx5 plays indispensable roles in the early patterning of the heart and CCS and is involved in limb development [15, 20, 21]. Mutations in *TBX5* are associated with Holt-Oram syndrome, a developmental disorder characterized by hand-heart defects [22, 23]. Using modified bacterial artificial chromosomes (BACs), the regulatory landscape of the *TBX5* locus was determined, and within this landscape, multiple cardiac-specific enhancers were identified by utilizing multiple genome-wide ChIP-seq datasets and evolutionary

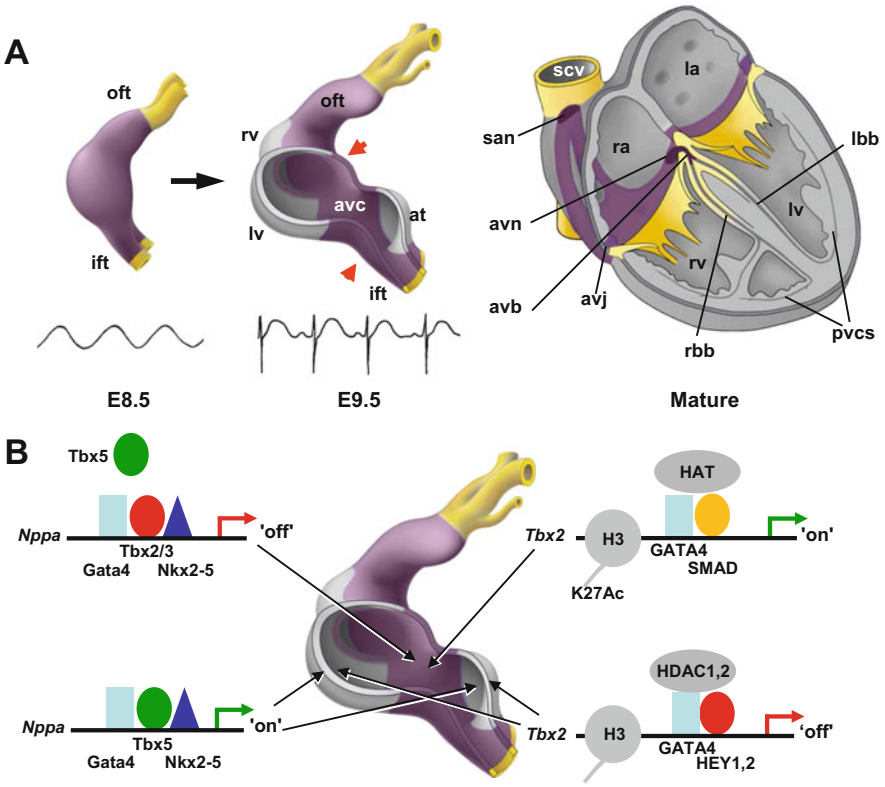

Fig. 38.1 Schematic overview of cardiac development. (**a**) The early heart tube has a primitive phenotype of slow conduction, represented by a sinusoidal ECG. With further development, regions at the outer curvatures of the primary heart tube expand and obtain a working myocardium phenotype of fast conduction (*grey*). The sinus venosus (sv), AVC, outflow tract (oft) and inner curvatures retain their primitive pacemaker-like phenotype and slow conductivity (*purple*). A more mature ECG can be derived from these hearts. Eventually, these non-chamber myocardial regions will give rise to the mature conduction system components. (**b**) The transcriptional repressors Tbx2 and Tbx3 compete with the transcriptional activator Tbx5 to regulate their target genes. In the AVC, expression of chamber myocardium genes like *Nppa* is actively repressed by Tbx2 and Tbx3, whereas in the developing chambers Tbx5 activates these genes. Transcriptional activation in the AVC is regulated by GATA binding site-dependent histone modifications which render the chromatin more (e.g. HATs) or less (e.g. HDACs) accessible for transcription factors to bind their target regulatory sequences, resulting in activation or repression of gene expression

conservation. These enhancers were shown to recapitulate part of the TBX5 expression pattern in the heart, but interestingly, none of these fragments drove reporter expression in the limbs, suggesting that the *cis*-regulation of TBX5 in the heart and limbs is compartmentalized [24]. Such knowledge can highly improve the understanding of the mechanisms underlying the development of congenital heart diseases by decoupling the heart and hand phenotypes seen with Holt-Oram syndrome, thereby presenting more compartmentalized phenotypes compared to disorders caused by protein-coding mutations.

Another example of how enhancer-mediated gene expression is involved in the tight regulation of the CCS is presented by the transcriptional repressor *Id2*. This factor was identified by serial analysis of gene expression (SAGE) as having CCS-specific expression. *Id2* is expressed throughout development in the AVB and BB and in non-CCS compartments such as the AV endocardial cushions and valves. The requirement of *Id2* for ventricular CCS structure and function was demonstrated as *Id2*-deficient mice exhibit structural and functional conduction system abnormalities, including left bundle branch block. *Id2* is cooperatively regulated by *Nkx2-5* and *Tbx5* in the developing ventricular conduction system by binding of both *Tbx5* and *Nkx2-5* to a 1052 bp fragment of the *Id2* promoter. Mutation of the *Tbx5* binding site within this promoter region completely abolished CCS expression whereas extracardiac expression was unaltered, illustrating the specificity of this transcriptional mechanism in the coordinated development of the ventricular conduction system [20].

The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. Using a transgenic BAC approach, it was shown that the regulatory regions sufficient to recapitulate the endogenous Hcn4 expression pattern in the SAN, AVN, His bundle, bundle branches and left ventricular Purkinje fibres reside within the region covered by one bacterial artificial chromosome (BAC) of 200 kbp [25]. Using transgenic mouse assays, multiple evolutionary conserved cis-acting regulatory sequences were identified to drive *Hcn4* expression in the AV conduction system. One of these regions drives reporter expression specifically in the non-chamber myocardium in a Mef2c-dependent manner. Furthermore, depletion of histone deacetylases resulted in ectopic expression of reporter activity in chamber myocardium, revealing a role for histone modifications in Mef2c-regulated enhancer-mediated expression of Hcn4 in components of the CCS [26].

More recently, Contactin-2 (*Cntn2*), a cell adhesion molecule critical for neuronal patterning and ion channel clustering, was described as a marker for the ventricular conduction system, with expression in the AVB, BBs and Purkinje fibres. Using a GFP-modified BAC, the boundaries of the regulatory domain involved in the control of *Cntn2* expression were identified, since reporter activity of the modified BAC completely recapitulates endogenous *Cntn2* expression [27]. Such knowledge facilitates in the identification of single, individual regulatory elements driving CCS development and will greatly add to our understanding of how genes involved in the complex development of CCS components are regulated.

Enhancer function is regulated by modifications of specific histone tails that mark active or poised enhancers. Active enhancers are associated with an open, accessible chromatin state, whereas poised enhancers are associated with dense, closed chromatin. Histone modifiers such as histone deactelyases (HDACs), histone methyltransferases (HMTs) and histone acetyltransferases (HATs) therefore regulate the accessibility of long-range regulatory sequences, allowing for the binding by cell type-specific transcription factors to activate transcription in a tissue-dependent manner. Specification of the AVC is regulated by Gata4, which activates AVC enhancers in synergy with Bmp2/Smad signaling to recruit HATs

such as p300 [19, 28]. This leads to H3K27 acetylation, a marker of active enhancers. In contrast, in chamber myocardium, Gata4 cooperates with HDACs and chamber-specific genes Hey1 and Hey2, leading to H3K27 deacetylation and repression (Fig. 38.1b) [19].

38.4 Common Genomic Variants Influence CCS Function

The importance of the strict regulation of the spatial and temporal expression of CCS genes is illustrated by findings from recent genome-wide association studies, which revealed common genomic variation to be associated with conduction parameters like PR interval and QRS duration. Such variation was identified in non-coding regions flanking genes encoding ion channels like *SCN5A/10A*, *KCNQ1* and *KCNH2* and cardiac transcription factors like *NKX2-5*, *MEIS1* and *TBX3/5*, indicating they might affect the function of enhancers controlling the precise regulation of these genes [29–31]. Tbx5 is broadly expressed and acts as transcriptional activator, inducing transcription of genes involved in cardiac differentiation [15, 20]. The activity domain of Tbx3 is much more restricted and confined to the developing and mature CCS, where it acts as a transcriptional repressor, thereby imposing the pacemaker phenotype on cells within its expression domain [10, 32]. Tbx3 and Tbx5 both recognize the same regulatory sequences [33], suggesting that these factors compete for binding and implicating a fine balance between activation and repression of CS genes by these factors. The precise regulation of transcription and activity of both factors is therefore crucial for proper CCS patterning, and minor changes in regulatory elements controlling the regulation of expression of these factors could thus potentially have large consequences for CCS function and development. Knowledge of the mechanisms by which such developmental genes are regulated to exert their spatio-temporal transcriptional activity is therefore crucial in the understanding of how variation identified by GWAS influences development.

38.5 3D Architecture Regulates Transcription

Physical enhancer-promoter contacts are a requirement for enhancer-mediated cell type-specific gene expression, and as such, the three-dimensional topology of chromatin plays an indispensable role in gene regulation by physically wiring long-range regulatory sequences with their target promoters [34]. Several protein complexes, including CTCF, cohesin and mediator, have been proposed to be involved in the organization of these contacts [35]. Furthermore, recent data suggest that such genomic structural organizers not only mediate single enhancer-promoter contacts but also mediate the organization of the genome in relatively cell-type invariant topologically associated domains (TADs) within which sequences particularly contact each other. Genes located within the same TAD exhibit greater expression correlation than genes located in distinct ones,

suggesting that such domains may act as a backbone for tissue-specific regulatory contacts [36]. The recent emergence of techniques aimed at capturing the 3D conformation of genomic loci [37] therefore provides valuable tools to elucidate regulatory mechanisms on the chromatin level.

38.6 Regulation of Tbx3 by a Large Regulatory Domain

The evolutionary conserved *Tbx3/5* genomic locus is one of the few genomic loci of which the 3D architecture has been studied and reveals an example of the complexity of gene regulation on the level of chromatin topology. *Tbx3* and *Tbx5* form an evolutionary conserved gene cluster derived from a primordial T-box gene [38] and, as mentioned above, play crucial roles in the formation and function of the cardiac conduction system. Using circular chromosome conformation capture sequencing (4C-seq), which captures all the genomic regions in close proximity to a chosen point of view [39], the 3D architecture of the Tbx3/5 locus was probed, and genomic regions contacting *Tbx3* or *Tbx5* were identified in different tissues (Fig. 38.2a). Interestingly, these data revealed that the regulatory landscape is in a preformed conformation that is similar in embryonic heart, brain and limb. Rather than the de novo formation of enhancer-promoter loops upon binding by cell-type relevant transcription factors to initiate transcription, the locus is in a fixed, permissive structure in which enhancer-promoter loops are pre-existing [40]. Such a permissive structure has previously been described for different loci, including the *Hox* and *Shh* gene loci. Long-range enhancer-promoter contacts in these loci were shown to be irrespective of cell type, revealing a preformed topology [36, 41]. The permissive, preformed nature of these loci was exemplified by the fact that even in the absence of a distal-acting *Shh* enhancer, contacts between the *Shh* promoter and the enhancer region still occur [41]. The benefit of such preformed regulatory landscapes is believed to lie in the ease by which tissue-specific transcription factors can utilize preformed contacts to target the gene of interest, involving only slight variations in internal contacts within an otherwise rigid and conserved structure. In agreement with this, small differences in contact profiles for the different tissue types were observed in the *Tbx3/5* locus despite the fact that the domain is largely preformed, most probably caused by cell type-specific transcription factor mediated enhancer activation. Among the multiple sites contacting *Tbx3* in the gene desert upstream of the gene, two evolutionary conserved enhancers have been identified that also contact each other. They are bound by cardiac-specific transcription factors Nkx2-5, Gata4, Tbx5 and Tbx3 and were shown to respond to a BMP-mediated signalling pathway to drive atrioventricular conduction system expression of *Tbx3* [40].

As mentioned before, Tbx3 and Tbx5 are expressed in overlapping patterns and have overlapping functions in CCS development. It could therefore be expected that both genes share common regulatory mechanisms on a genomic level. Studies on the transcriptional regulation of other clustered developmental genes, like the *Irx* and *Hox* clusters, revealed that regulatory sequences are not uniquely associated

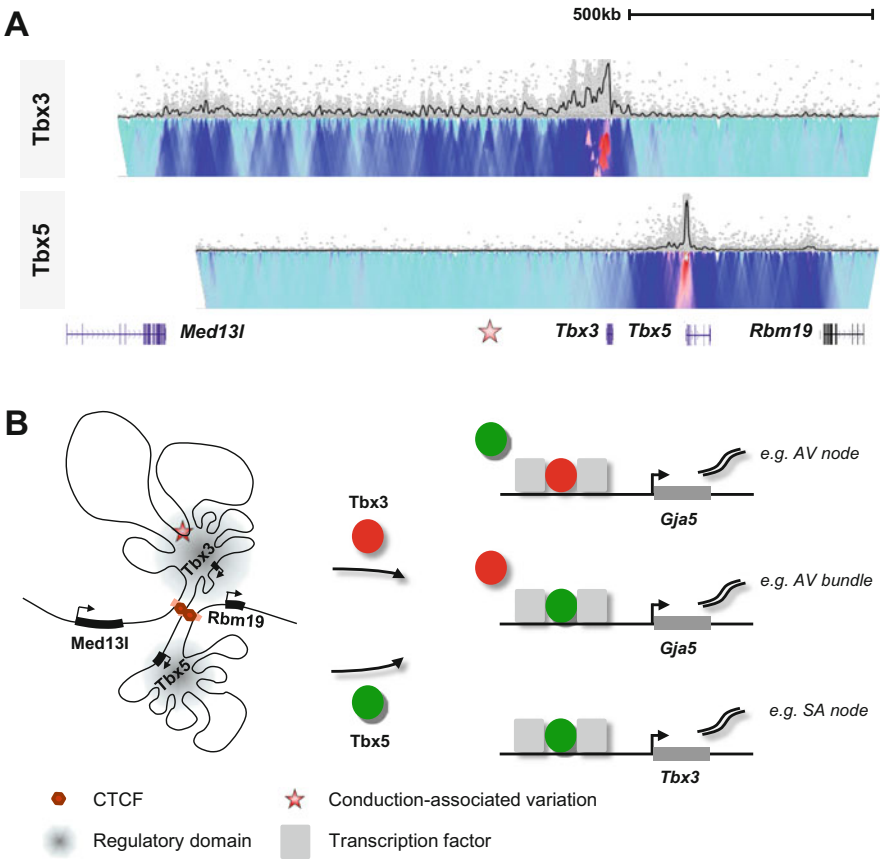

Fig. 38.2 The regulatory domains of *Tbx3* and *Tbx5* are physically separated. (**a**) Contact profiles of the *Tbx3* and *Tbx5* loci as determined by circular chromosome conformation capture (4C) reveal the genomic regions that physically contact *Tbx3* (*upper track*) or *Tbx5* (*lower track*) in mouse embryonic heart cells. *Red* and *dark blue* depict a high contact frequency, whereas *light blue* and *grey* depict a low contact frequency. The contact profiles of *Tbx3* and *Tbx5* hardly overlap, indicating that both genes do not share regulatory sequences and suggesting that common variants in humans upstream of *TBX3* as identified by GWAS (*star*) can be exclusively assigned to *TBX3* [40]. (**b**) Model of the 3D conformation of the *Tbx3/Tbx5* locus. The regulatory domains of *Tbx3* and *Tbx5* are physically separated; however, on the protein level, both genes recognize the same binding sequences and compete with each other to activate or repress their target genes, e.g. *Gja5* (Cx40). Despite the strict regulation on the chromatin level, Tbx5 also directly regulates Tbx3 by binding target sequences to activate transcription in, for example, the SAN

with single promoters, but rather are shared by multiple genes within the cluster. Such interplay between multiple enhancers coordinates the strict regulation of their expression patterns, and it has been proposed that such extensive enhancer sharing explains the conservation of the genomic organization throughout evolution [42, 43]. Interestingly, however, *Tbx3* and its flanking gene desert form a loop that is physically separated from that of the neighbouring *Tbx5* loop (Fig. 38.2b).

Genomic regions within the Tbx3 loop solely contact *Tbx3* but not *Tbx5* and vice versa, indicating enhancer sharing between these evolutionary conserved clusters is unlikely to occur [40]. The strict separation of the regulatory landscapes of *Tbx3* and *Tbx5* is not only cell type-independent, but also evolutionary conserved between mouse and human. Recent Hi-C data in human fibroblasts [36], revealing genome-wide contact profiles, reveal a similar separation of the *TBX3* and *TBX5* regulatory domains with hardly any overlap of the contact profiles. This organization of the Tbx3 locus in a ~1 Mb-scale self-regulatory domain corresponds well to the previously mentioned TADs. These domains are separated by boundary regions enriched for insulator binding protein CTCF, housekeeping genes, transfer RNA and short interspersed elements, hampering interactions of sequences within one TAD with regions exceeding the domain boundaries. Regions located within the regulatory domain of *Tbx3* are thus suggested to exclusively contact *Tbx3* and not *Tbx5* and vice versa.

38.7 Assigning Function to Genomic Variation

Understanding the 3D architecture of a genomic locus not only provides insight into the tight relationship between chromatin topology and the complex regulation of developmental gene expression, it could also provide valuable clues in the understanding of the role of functional variation as identified by genome-wide association studies on gene expression. Common genomic variation in the non-coding region upstream of *TBX3* and *TBX5* was found to influence PR interval and QRS duration in humans [29–31]. The fact that the regulatory domains of *TBX3* and *TBX5* are strictly separated indicates that the variation found in one of the domains can be exclusively assigned to its respective gene, facilitating our understanding of the functional effect of disease-associated variation.

A similar example of how knowledge on the chromatin conformation can increase our understanding of function of common variants is illustrated by studies on the *SCN5A/SCN10A* locus. *SCN5A* and *SCN10A* encode sodium channels important for conduction. GWAS implicated an intronic region in *SCN10A* as a major risk region for prolonged QRS duration [30]. The role of SCN10A in cardiac conduction however was not previously described, whereas mutations in the adjacent *SCN5A* are well established to cause several arrhythmogenic disorders, including Brugada and Long QT syndrome [44, 45]. It is therefore possible that the variation identified within the intron of *SCN10A* impacts the expression of *SCN5A*, rather than or in addition to that of *SCN10A*. Indeed, probing the 3D architecture with the promoters of both *SCN5A* and *SCN10A* and the site of the variation in the intron as point of view using 4C-seq revealed that this variant region contacts the *SCN5A* promoter and a strong enhancer downstream of SCN5A, suggesting that it might act as enhancer regulating the expression of *SCN5A* in the heart. Transgenic reporter assays revealed that indeed this enhancer is essential for cardiac *Scn5a* expression. In humans, the SNP located within the enhancer that correlates with slowed conduction is associated with lower *SCN5A* expression

[46]. Taken together, these results provide another example of how our understanding of the 3D architecture of a genomic locus harbouring functional variation can facilitate in the assignment of function to such variations, improving our understanding of the effect of disease-associated variation.

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Cardiac Pacemaker Development from a Tertiary Heart Field

39

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Abstract

Rhythmic heartbeats are paced by electrical impulses that are autonomously generated by cardiac pacemaker cells. This chapter briefly summarizes our recent findings regarding the embryonic origin of and molecular mechanism delineating cardiac pacemaker cells, showing that pacemaker cells are physically segregated and molecularly programmed, in a tertiary heart field, prior to the onset of cardiac morphogenesis.

Keywords

Differentiation • Fate determination • Fate mapping • Lateral mesoderm patterning • Pacemaker • Pacemaker cell fate specification • Tertiary heart field • Wnt

39.1 Introduction

Rhythmic heartbeat is initiated by electrical impulses evoked at the sinoatrial node (SAN) that are then conducted to atrial muscle and converge to the atrioventricular node. After a brief delay, pacemaker-initiated action potentials (APs) rapidly pass down the conduction system network and finally spread into ventricular muscle (Fig. 39.1a). The SAN was first described more than a century ago [1], and its anatomical, physiological, and molecular characteristics have been thoroughly investigated [2]. While significant progress has been made in our understanding of the mechanism responsible for the differentiation and patterning of the distal

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_39

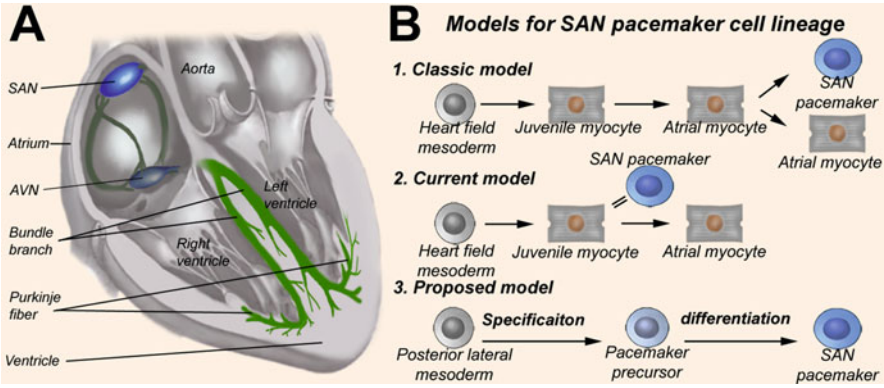

Fig. 39.1 (**a**) Diagram of the pacemaking and conduction system network consisting of distinct subcomponents. (**b**) Controversies in the developmental pathway of SAN pacemaker cells. *The classic model* assumes that this cell population differentiates from atrial myocytes. *A current dogma* suggests that SAN pacemaker cells arise from a part of the heart field mesoderm which remains as immature myocytes. These models are based on phenotypic similarities between embryonic myocytes and SAN cells in action potential shape and the expression of unique genes in common. The only way to reliably establish the origins of a cell type is to tag its antecedents. Our direct cell fate mapping studies have identified an origin and novel developmental pathway for SAN pacemaker cells (*proposed model*)

conduction components, the developmental pathway of the SAN remains controversial (Fig. 39.1b).

The identification of the definitive origin of and inductive mechanisms that define SAN pacemaker cells (PCs) will be critical for systematic investigation of developmental mechanisms, such as cell fate specification and differentiation, of this specialized cell population essential for cardiac function. We have recently identified a novel role of Wnt signaling in promoting pacemaker cell induction and differentiation, which is completely contrary to its well-documented inhibitory role in heart field induction [3].

39.2 Pacemaking Site Transitions From Left To Right During Heart Looping

Classic studies in the chick embryo have shown that as soon as the primitive heart tube forms, myocytes in the posterior inflow tract become electrically active and predominantly evoke pacemaking impulses [4]. While this population has been a priori thought as the progenitor of the SAN pacemaker, no direct cell lineage-tracing study has tested this dogma. Therefore, we have revisited this critical issue. Consistent with previous studies, our optical mapping analysis has detected that the primitive heart tube evokes APs preferentially at the left inflow. Importantly, however, our fate mapping studies have revealed that cells of the left inflow later differentiate into AV junction myocytes rather than the right side pacing cells at the

SA [5]. The above findings are consistent with a hypothesis that there are a successive series of pacemaker zones present in the early developing heart [6], indicating that the true origin of SAN pacemaker cells remains to be identified.

39.3 A Novel Cell Population That Juxtaposes the Right Atrium Takes Over Pacing Function by Mid-heart Looping Stage

While classic and current models assume that SAN PCs arise from a subpopulation of atrial precursors (Fig. 39.1b), to our surprise, our optical mapping data showed that a small region juxtaposing the right atrium preferentially evokes pacemaking action potentials [5]. This pacemaking site has often been neglected in previous studies as this region is routinely dissected away during isolation of the embryonic heart. Our optical mapping and in situ hybridization analyses [5] have identified that this pacemaker site preferentially expresses *HCN4*, a major member of the HCN gene family expressed in the heart responsible for the hyperpolarization-activated inward "funny" current, and an atrial-type myosin *AMHC1*, but is negative for a cardiac transcription factor *Nkx2-5* [9]. These are consistent with previous reports on the adult mouse SAN. Other tissues of the conduction system, such as AVN and Purkinje fibers, co-express *HCNs* and *Nkx2-5*. Thus, differentiated SAN pacemaker cells can be distinguished by these physiological, pharmacological, and molecular characteristics from other myocytes and conduction cells. Unfortunately, expression of these marker genes is dynamic and no *HCN4+/AMHC1+/Nkx2-5-* cells can be found in earlier stage embryos. It is therefore unclear when and where SAN pacemaker cell fate is induced and specified. As the heart matures through the processes of looping, heart primordium continually expands with cells being added to both the inflow and outflow segments [7, 8]. The origin of this novel pacemaking cell population needs to be determined.

39.4 The Right-Sided Pacemaking Cells Indeed Differentiate into SAN Pacemaker Cells

Our in ovo cell-tracing studies have mapped the fate of these extracardiac right-sided pacing cells to the physiologically correct SAN region of the resulting heart at stage 35 (E9) [5]. Optical mapping of these labeled hearts has shown that action potentials are predominantly evoked from these specific cells and propagate into the atrium [5]. Thus, these studies have identified a novel extracardiac cell population as SAN pacemaker precursors. However, until recently it was unknown where this cell population came from. Indeed, the fate of cells in the inflow has been controversial. No systematic fate mapping data was available about the pacemaker field posterior to the heart field at pre-heart tube formation stages. Genetic lineage tracing critically depends upon a gene that is exclusively expressed in pacemaker precursors but not in daughter cells. To our knowledge, no such gene had been identified to date. To explore the origin of SAN pacemaker cells, we performed fate

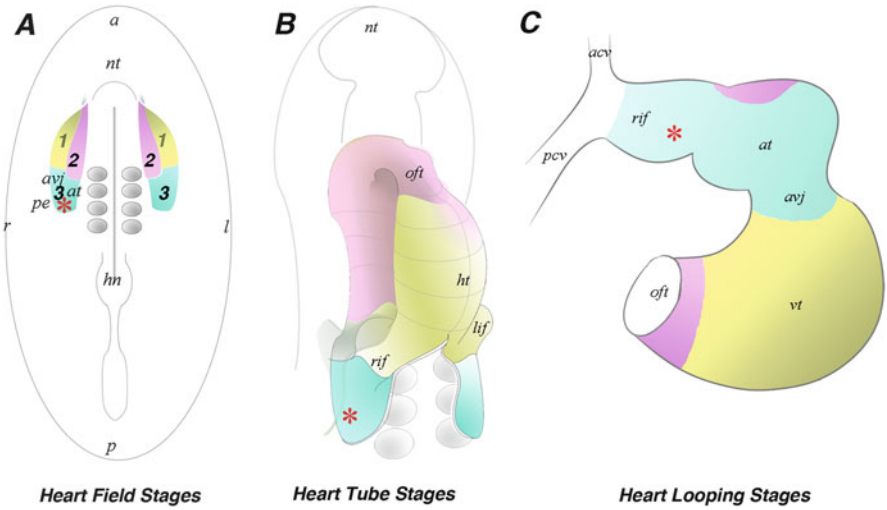

Fig. 39.2 Fate map of pacemaker field. (**a**) At early somite stages, heart precursors occupy bilateral fields within the lateral plate mesoderm. The primary heart field is indicated in *yellow*, the secondary heart field is indicated in *pink*, and the posterior tertiary heart field [5] is indicated in *blue*. Fate mapping studies indicate the progenitors of the SAN pacemaker cells reside within the tertiary heart field (*asterisk*). (**b**) At heart tube stages, the primary heart fields have fused along the midline, while the secondary and tertiary heart fields have not yet been incorporated into the heart. The pacemaker precursors maintain their position within the tertiary heart field mesoderm (*blue*). (**c**) At looping stages, the pacemaker cells have incorporated into/can be seen attaching the right inflow of the heart and begin to pace the heartbeat. *at* atria, *avj* atrioventricular junction, *ht* heart tube, *vt* ventricle, *a* anterior, *p* posterior, *r* right, *l* left

mapping for earlier embryonic stages [5; Fig. 39.2]. For example, in stage 7–8 embryos, the PCs were mapped to a small region in the right lateral plate at somite level 3. Importantly, "the pacemaker field (PF)" does not overlap with "the heart field (HF)" defined by expression of either *Nkx2-5* or *Isl1* [9, 10]. The data show for the first time that SAN precursors arise from an unpredicted small area of the lateral mesoderm immediately posterior to the known HF. It should be noted that the PF is embedded in the zone that also generates other cardiac-related tissues, such as the proepicardium, right atrium, and *vena cava* (Bressan 2013). We therefore tentatively termed this previously unrecognized mesodermal area "tertiary heart field," distinguishing it from the primary heart field and the secondary (anterior) heart field (Fig. 39.3).

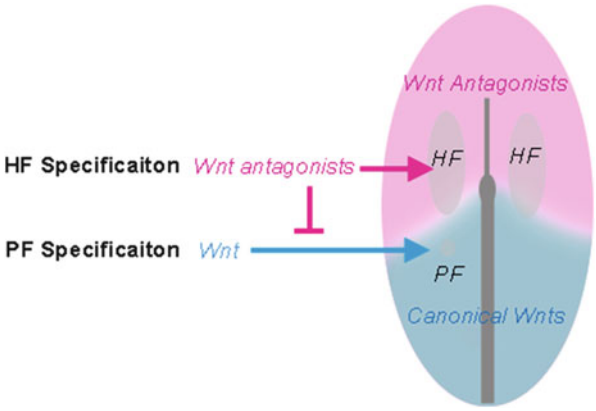

Fig. 39.3 Model for distinct roles of Wnt signaling in HF induction vs. PF induction

39.5 Pacemaker Cell Fate Specification Has Already Completed Prior to Heart Morphogenesis

The fate mapping data alone however do not provide any information as to where and when the pacemaker cell fate is specified. A protocol is needed to detect timing and location of cell fate specification apart from marker gene expression. "The cell fate specification" is defined by Harrison [11] and Slack [12] as "when a cell or a tissue becomes capable of autonomously differentiating under a neutral environment, such as in petri dish or test tube."

Using an established culture model that was previously used for studying classic HF specification, we examined autonomous pacemaker differentiation from PF, which has been mapped in stage 8 embryos (see Fig. 39.2). Under the neutral culture condition, HF explants from stage 8 embryos initiated spontaneous contractions within 24 h. Our data are consistent with previous studies on HF specification. In striking contrast, PF explants did not show any contractions during the first 24 h, but by 48 h many of them started rhythmic contractions with a higher beat rate and exhibited AP waveform characteristic of PCs. PF explants continued to rhythmically beat at a similar rate throughout the extended culture period for at least up to 120 h. The data show that cells posterior to the HF autonomously differentiate to initiate and maintain spontaneous rhythmic contractions, which are distinct from those of the HF.

39.6 PF Explants Are Sensitive to Blockers Specific for Pacemaking Ion Channel

Our pharmacological tests interrogated whether PF explants use ion channel characteristic of pacemaker cells. Our data show that a HCN channel blocker, ZD7288, induced PF explants to increase the interval between contractions by approximately

~50 % without detectable loss of rhythmicity. An L-type Ca^{2+} channel blocker, nifedipine, completely diminished spontaneous contraction of PF explants. These pharmacological responses of PF explants are characteristic of SAN pacemaker cells which evoke action potentials mainly through HCN channels and L-type Ca^{2+} channels and are distinct from cardiomyocytes which mainly use Na^{+} channels for the upstroke of action potentials.

39.7 Current Models for Molecular Regulation of SAN Pacemaker Differentiation

It was once postulated that the SAN pacemaker arises from a part of the HF mesoderm which co-expresses *Isl1* and *Tbx18*. *Pitx2c*, *Shox2*, *Nkx2-5*, *Tbx3*, and *Tbx5* are believed to play a role in SAN development. In mice, gene deletion of a laterality gene, *Pitx2c*, leads to bilateral SANs. S*hox2* deficiency results in upregulation of *Nkx2-5* and *Cx43* in the SAN domain. Both *Shox2* and *Tbx3* expression require *Tbx5*. Because *Nkx2-5* is absent in the SAN, *Nkx2-5* is suggested to suppress *HCN4* and *Tbx3*. *Tbx3* gene knockouts result in expression of atrial genes in the SAN and partial loss of SAN-specific gene expression. Ectopic expression of *Tbx3* in the atria causes arrhythmia. Taken together, *Tbx3* and *Tbx18* have been proposed as key transcription factors for SAN pacemaker differentiation. Contradictory to these models, deletion of *Tbx3*, *Tbx18*, or *Shox2* results in no or only modest pacemaker defects. Further *Tbx3* expression occurs at the AV junction and AV node where *Nkx2-5* is highly expressed. Our data have revealed, however, that *Tbx3*, *Tbx18*, and *Isl1* are absent from SAN pacemaker cells during the early stages that correspond with cell fate specification and differentiation. Thus, further elucidation was needed to better understand the molecular mechanisms that regulate the specification and formation of SAN pacemaker cells.

39.8 A Novel Role of Wnt Signaling for Pacemaker Cell Fate Specification

Heart field induction and specification are promoted by BMP signaling and are restricted to the anterior region by inhibition of Wnt signaling [3, 13]. In chick, Wnt from the neural plate ectoderm and the posterior mesoderm inhibits myocardial differentiation in paraxial and posterior mesoderm, while the secreted Wnt antagonist, crescent, produced by the anterior endoderm supports myocardial development. Noncanonical Wnts that block canonical Wnt/β-catenin signaling can act positively on HF establishment and myocardial electro gradient. Isl1-Cre/β-catenin mutants show defects in outflow tract formation with decreased expression of *Tbx2*, *Tbx3*, *Shh*, and *Wnt11*. Canonical Wnt signaling is necessary for growth of second HF-derived right ventricular myocytes during heart looping and onward but its role for early specification of the second HF remains obscure. Taken together, inhibition of canonical Wnt/β-catenin signaling is critical for restricting heart field induction

to the anterior mesoderm. In contrast to extensive studies of heart field formation, little is known about Wnt signaling in pacemaker development.

Activation of Wnt signaling in the HF diminishes the expression of several cardiac genes, including *Nkx2-5*. However, it remains largely unknown what HF fate becomes once "HF identity" is lost. Our fate mapping data indicated a surprisingly posterior origin for pacemaker cell progenitors within the *Nkx2.5* negative mesoderm, as well as an earlier timing of specification than previously believed. These results led us to reexamine the question of heart field identity following loss of *Nkx2.5*. Therefore, we investigated this untouched area of Wnt signaling on heart field cells using physiological approaches. Our data have revealed that Wnt-treated heart field cells do not lose contractility either in vivo or in vitro. Instead, they developed a rhythmic, high-rate contraction pattern similar to PF explants, including AP waveforms reminiscent of PCs [5]. A transient exposure to Wnt for only 8 h was sufficient to obtain the pacemaker-like beating pattern. Our in vivo and in vitro data have further demonstrated that inhibition of Wnt signaling for pacemaker progenitors results in a conversion of their fate to the ordinary cardiomyocyte type.

39.9 Concluding Remarks

An elucidation of the origin and molecular mechanisms that specify the pacemaker cell fate is fundamental to dissecting the earliest steps critical for SAN pacemaker development. Our data have revealed that the pacemaker cell fate is specified in a previously unconsidered embryonic region at very early embryonic stages even before heart morphogenesis begins. The work has also revealed that differential Wnt-mediated signaling cues in the lateral plate mesoderm are sufficient to induce pacemaker-like versus working myocardial fates, and that these fates are maintained throughout early cardiac morphogenesis. These results will significantly increase our understanding of the basis for the mechanisms that regulate pacemaker cell specification and differentiation.

Acknowledgments This work has been funded in part by NIH R01078921, R01HL093566, and R01HL112268.

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Endothelin Receptor Type A-Expressing Cell Population in the Inflow Tract Contributes to Chamber Formation

40

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Keywords
Endothelin • First heart field • Chamber formation

The avian and mammalian heart mainly originates from two distinct embryonic regions: an early differentiating first heart field and a dorsomedially located second heart field. It remains largely unknown when and how these subpopulations of the heart field are established as regions with different fates.

Endothelin-1 (Edn-1) acts on cardiac neural crest cells through endothelin receptor type A (Ednra) and is involved in the normal formation of pharyngeal artery-derived great vessels and ventricular septum [1]. Previously, we identified a distinct cell population defined by the expression of *Ednra* in the mouse inflow region [2]. These cells are derived from a part of the first heart field, and largely confined to the inflow region at E8.25.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_40

From the expression patterns of β-gal in the *Ednra-lacZ* knock-in mice, we thought a possibility that this *Ednra*-positive cell population might move into cardiac chambers from the inflow region. By dye injection and transplantation experiments, we showed that the *Ednra*-positive cell population moved toward not only the left and right atria but also the left ventricle.

Then, to perform lineage analysis, we have generated an *Ednra-CreERT2* mouse line (unpublished). We activated Cre recombinase by tamoxifen at E7.25 and E8.25 and analyzed the distribution of β-gal-labeled cells in the $Ednra^{CreERT2/+}$;*R26R* embryo hearts. As a result, the β-gal-labeled cells were found to contribute to the left ventricle and both atria.

To facilitate this lineage analysis, we established a mouse-chick chimera model. When we transplanted the inflow region of the $Ednra^{CreERT2/+}$;*R26R* mouse embryos (tamoxifen i.p. at E7.25) to orthotopically into chick embryos, β-gal-positive cells were detected in the right atrium and left ventricles 7 days after transplantation.

From these results, we conclude that the *Ednra*-positive cell lineage in the early inflow tract certainly contribute to chamber myocardial formation.

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Specific Isolation of HCN4-Positive Cardiac Pacemaking Cells Derived from Embryonic Stem Cells

41

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Keywords
Pacemaker • Embryonic stem cells • HCN4

Bradycardia causes slow heart beating, which has high risk for heart failure or stroke. The only available treatment for bradycardia is implantation of electronic pacemakers. However, this treatment for bradycardia has several shortcomings: requirement of operation for implantation and for exchange of battery and the lack of response to autonomic nerve regulation. The purpose of this study was to develop a biological pacemaker, which could be used for replacement of electronic pacemakers.

1. In differentiating mouse embryonic stem (mES) cells, cardiac pacemaker cells can be specifically visualized with green fluorescent protein (GFP) on the basis of their specific expression of hyperpolarization-activated cyclic nucleotide-gated (HCN) channel 4 at sinoatrial node (SAN) [1]. GFP knock-in ES cells at HCN4 locus (H7 clone) were established. The expression of GFP was specifically restricted at their contracting region in differentiating H7 ES cells.
2. Cell sorting revealed that a few cells (0.1–0.5 %) of H7 embryoid bodies (EBs) were GFP positive, of which approximately 80 % of cells showed the spontaneous beating activity with cesium-sensitive action potential. Sorted GFP+ cells expressed endogenous HCN4 and had essentially the same properties as the cardiac pacemaker cells at SAN.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_41

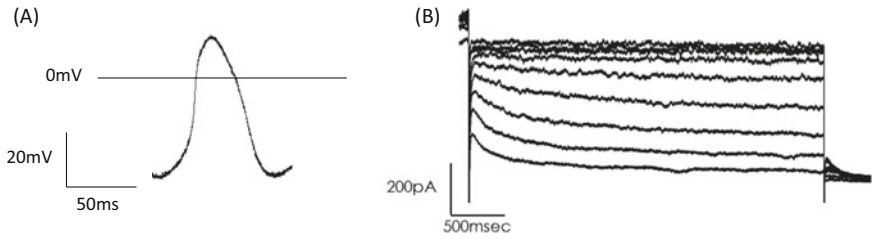

Fig. 41.1 Automaticity and I_f current from HCN4+ pacemaking cells. HCN4-GFP+ pacemaking cells derived from mESCs show typical automaticity (**a**) and I_f current (**b**)

(i) GFP+ cells expressed cardiac pacemaker specific markers such as HCN4, Cav3.1, and Connexin43 as well as cardiomyocyte-specific marker, Tropomyosin C.
(ii) Patch-clamp analysis revealed that GFP+ cells expressed pacemaker current I_f with spontaneously oscillating action potentials (automaticity) (Fig. 41.1).
(iii) GFP+ cells were capable of actively responding to adrenergic stimulation and cholinergic repression.

3. We further investigated whether mESC-derived HCN4+ cells can restore myocardial electromechanical properties. Using imaging techniques, we demonstrated that HCN4+ cells established electrical coupling with HL-1 cells, a cardiac muscle cell line derived from the mouse atrial cardiomyocytes tumor, to induce rhythmic electrical and contractile activities in vitro. Similarly, transplanted HCN4+ cells paced the hearts of rats with complete atrioventricular block, indicating that HCN4+ cells could substitute for pacemaker cells and elicit an ectopic rhythm.

These results demonstrated the potential of HCN4+ pacemaking cells derived from ES cells to act as a rate-responsive biological pacemaker and for future myocardial regenerative medicine to bradycardia.

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