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Developmental Aspects of the Electrophysiology of the Heart: Function Follows Form

Alex V. Postma, Vincent M. Christoffels, and Antoon F. M. Moorman

Abstract

The cardiovascular system is the first organ system to form and function in the developing embryo. In a typical lifetime the heart performs roughly 2,000 million contraction-relaxation cycles (2.3×10^9), to supply the whole body and all of its organs with oxygen and nutrients. To this end, an intricate and complex organ developed, encompassing multiple chambers containing electrical and force producing components, with nodes to activate the chambers and valves to prevent regurgitation. The cardiomyocytes of a primitive heart can be considered as a nodal cell because they display automaticity and are poorly coupled, which, together with slow propagation, gives rise to peristaltic contraction. The introduction of dominant pacemaker activity at the intake of the heart perfected such a heart into a one-way pump. Subsequently, highly localized, fast-conducting cardiac chambers were added to this nodal tube, resulting in the four-chambered hearts. Concomitant with the formation of such chambers, an adult type of electrocardiogram (ECG) can already be monitored in the embryo. Thus, cardiac design, i.e. the positioning of the atrial and ventricular chambers within the nodal tube, principally explains the coordinated activation of the heart reflected in the ECG. A crucial question is why some areas of the embryonic heart tube do not participate in the formation of atrial or ventricular working myocardium and mature in a nodal direction. As a generalized hypothesis we propose that the chamber-specific program of gene expression is specifically repressed by T-box factors and by other transcriptional repressors. Consequently, aberrant expression of these factors might be at the basis of ectopic automaticity, malformations of the conduction system and congenital heart disease in general.

Keywords

Heart development • Conduction system • Transcription factor • Congenital heart disease • Nodal cell • Cardiomyocyte • Pacemaker • Cardiac design • Electrocardiogram • Electrophysiology

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Introduction

The cardiovascular system is the first organ system to form and function in the developing embryo. In a typical lifetime the heart performs roughly 2,000 million contraction-relaxation cycles (2.3×10^9), to supply the whole body

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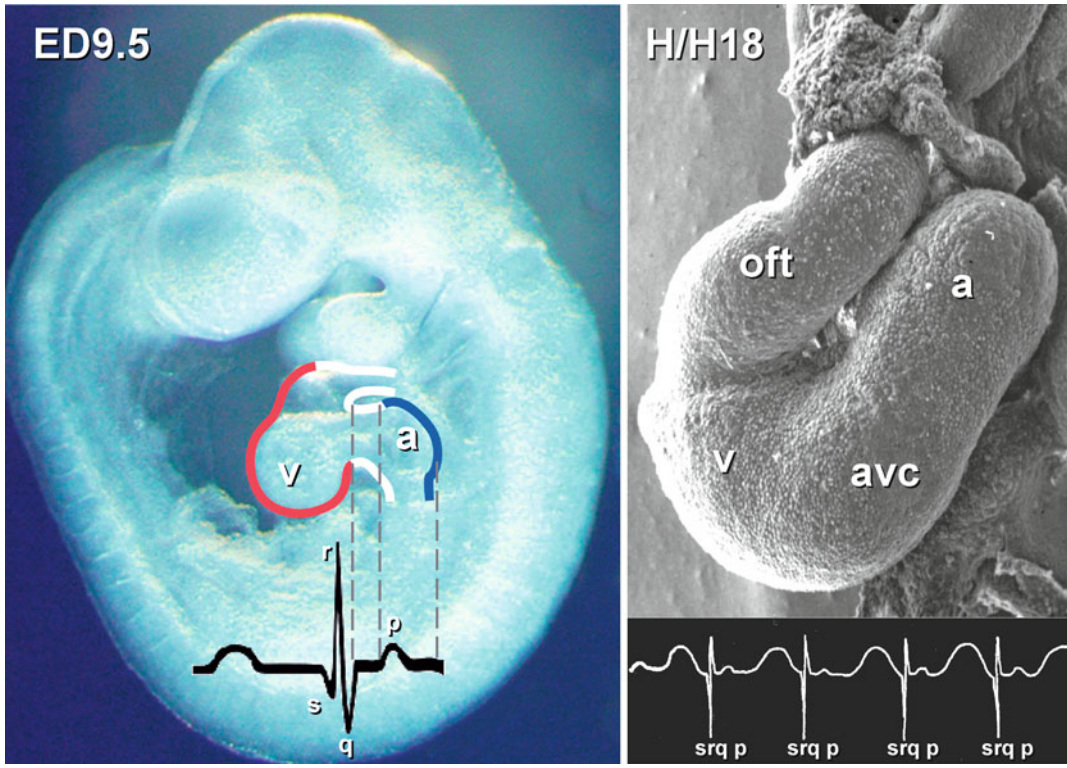

Figure 3–1. Concomitant with the formation of chambers (atria, ventricles), an adult type of electrocardiogram (ECG) can already be monitored in the embryo [2]. Scanning electron microscopic photographs of the developing chicken heart with matching electrocardiograms. At H/H 18, locally fast-conducting chamber myocardium has differentiated as reflected in the electrocardiogram. *A* atrium, *avc* atrioventricular canal, *oft* outflow tract, *V* ventricle. Note that the ECGs are displayed mirrored to match with the position of the chambers in the embryonic heart. The apparent T-wave has not been labeled since it reflects the depolarization of the muscularized outflow tract at this stage rather than the repolarization of the ventricle

and all of its organs with oxygen and nutrients. To this end, an intricate and complex organ developed, encompassing multiple chambers containing electrical and force producing components, with nodes to activate the chambers and valves to prevent regurgitation. In contrast, in early vertebrate embryos and primitive chordates, the heart merely constitutes a myocardial mantle enfolding a ventral aorta, in which the blood is propelled by peristaltic contractions. Like the nodal cells in the formed heart, the cardiomyocytes of such a primitive heart display high automaticity and are poorly coupled. This results in slow propagation of the depolarizing impulse and a matching peristaltic contraction. Due to the development of polarity along the heart tube, a dominant pacemaker activity develops at the intake of the heart, leading to the evolution of a one-way pump. Although dominant pacemaker activity implies development of sinus node function, only in mammals a morphologically distinct node actually develops [1]. The addition of highly localized, fast conducting cardiac chambers to the straight heart tube is an evolutionary novel event, and resulted in the four-chambered hearts of birds and mammals with a synchronous contraction and a dual circulation. Already with the onset of the formation of chambers, an adult type of electrocardiogram (ECG) can be monitored in the embryo (Fig. 3.1) [2]. Thus, cardiac design, i.e. the positioning of the atrial and ventricular chambers within the straight heart tube, rather than the invention of nodes, principally explains the coordinated activation of the heart reflected in the ECG. An important question to address is

why some areas of the embryonic heart tube do not participate in the formation of the atrial or ventricular working myocardium and mature in a nodal direction. In short, the chamber-specific program of gene expression is repressed by T-box factors and by other transcriptional repressors [3, 4]. Consequently, aberrant expression of these factors might be at the basis of ectopic automaticity and congenital malformations of the cardiac conduction system in the formed human heart.

Early Peristaltic Hearts

A typical circulatory system is made of pumps and transporting vessels. Nature uses two different schemes to make muscle-pumping devices. In one version, also utilized by the intestine, peristalsis is the driving force. In contrast, in adult vertebrates an alternative version involving chambers and valves is used (see next section). In the peristaltic version, a wave of contractions runs along the muscle mantel enfolding the main blood vessel, and this action pushes the encompassed fluid ahead in either direction. Such a system is not particularly efficient, but it allows the steady movement of fluids and slurries. During evolution, polarity evolved in the primitive peristaltic chordate hearts and this resulted in dominant pacemaker activity at one end of the cardiac tube, transforming such a heart into a one-way pump. All regions of peristaltic hearts possess poorly coupled cells and intrinsic automaticity, by which depolarizing impulses propagate slowly along the tube, resulting in matching peristaltic waves of contraction [5–9]. The advantage is that hearts using these slow contractions do not require well-developed contractile structures like those present in the chamber myocardium of higher vertebrates.

Development of Chambered Hearts

It is important to appreciate that the basic characteristics of muscle cells comprising a peristaltic heart are similar to those comprising the nodes of a chambered heart [4], as this facilitates the understanding that the design of chambered hearts is derived from the peristaltic heart. Though they share design characteristics, the chambered heart and its associated functional requirements are obviously far more complex than a peristaltic heart. Chambered hearts are the more powerful hearts that can cope with the increasing demands imposed by a growing microcirculatory resistance due to the evolutionary development of liver and kidneys. To achieve this, the atria became the drainage pool of the body to allow efficient filling of the ventricles, while the ventricles themselves became the power pumps. Like peristaltic hearts, chambered hearts are directional because dominant pace making activity remains localized at the intake of the heart. A logical further addition to these chambered hearts was the development of one-way valves at both the inflow and the outflow of a chamber. This permitted that, with relaxation, a chamber could be prevented from refilling from the downstream compartment, and prevented, with contraction, backflow into the preceding compartment. Remarkably, the areas in which these valves evolve display many nodal characteristics, and are the same areas that in the vertebrate embryonic heart will not, or will only later develop into chamber myocardium [10]. Thus, cardiac valves are always found in regions of nodal-like myocardium. This holds true for the sinuatrial region, atrioventricular junctional region and also for the myocardial outflow region of the embryonic heart. Interestingly, the outflow tract myocardium in human can extend as far downstream as the semi-lunar valves. Spontaneous activity and even tachycardias originating from this area have been reported [11] underscoring the notion that this myocardium is persisting embryonic nodal-like tissue.

Development of the Cardiac Chambers and Conduction System

The four-chambered heart of mammals develops from a single tube, which initially contains the precursors for the left ventricle only, or even less. With development, the precursors for the

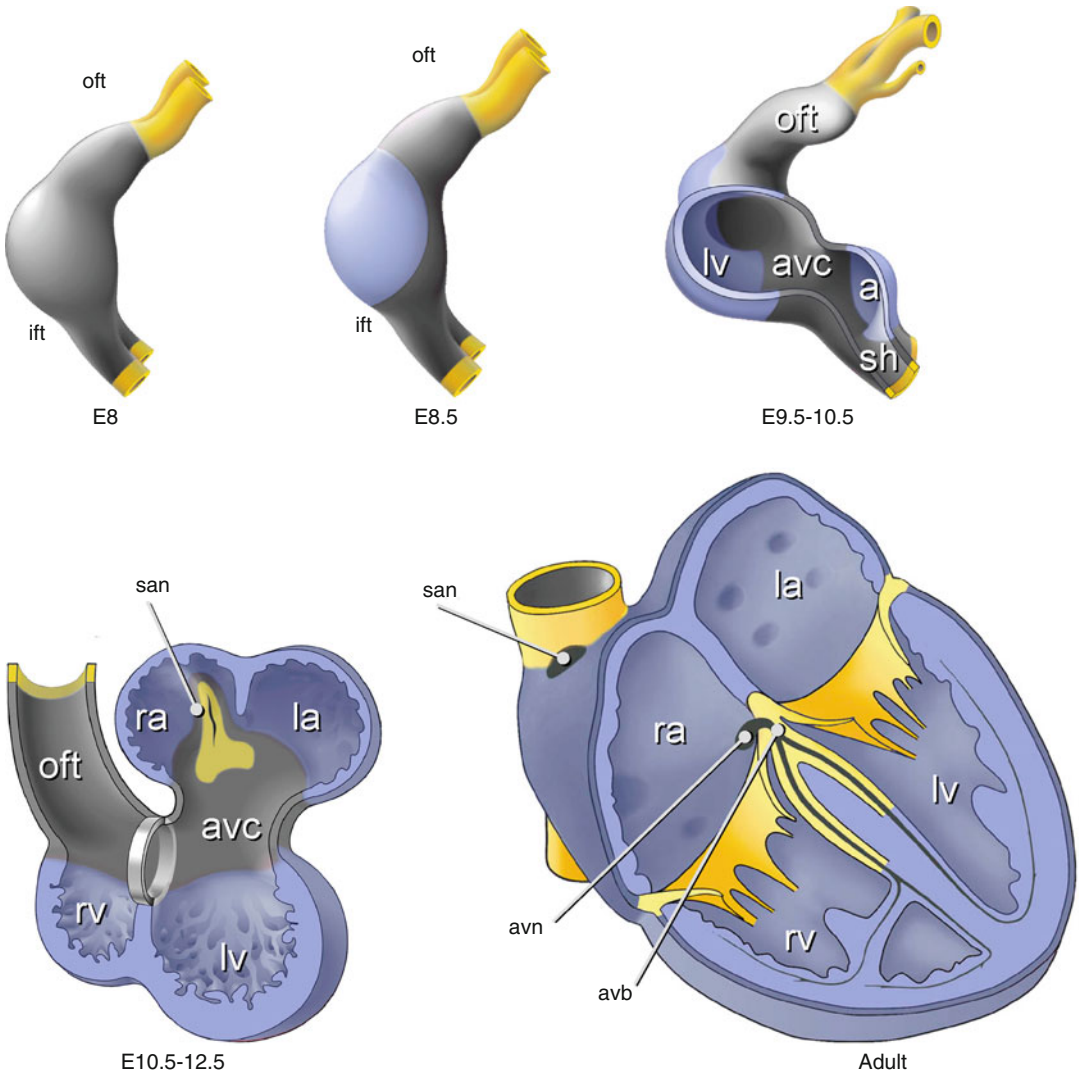

Figure 3–2. Schematic overview of heart development in higher vertebrates. Chamber myocardium (*blue*) expands from the outer curvatures of the primary heart tube, whereas non-chamber myocardium (*grey*) of the inflow tract (*ift*), sinus horns (*sh*), atrioventricular canal (*avc*), outflow tract (*oft*), and inner curvatures does not expand. Sinus horn myocardium gives rise to the sinoatrial node (*san*), atrioventricular canal myocardium to the atrioventricular node (*avn*) and atrioventricular junction. First three panels show a left-lateral view of the heart

remainder of the heart are added at the arterial and venous poles (Fig. 3.2) [12, 13]. The heart tube continues to elongate as a result of the recruitment of myocytes and bends ventrally and rightwards in a process called looping, which occurs around mouse embryonic day 8.5–9 (E8.5–9), comparable to 23 days in human development [14–16]. At this time, the heart tube has started to form a distinct ventricular chamber at its original ventral side, while the future atrial appendages differentiate slightly later at the dorsal and caudal end [17–19].

The myocardium of the original heart tube and the myocardium subsequently added to its poles, displays slow conduction and contraction, along with the property to spontaneously depolarize (automaticity), which is mainly Cx45 dependent [20], resulting in peristaltic-like, rhythmic contractions. Dominant pacemaker activity is always located in the newly formed

myocardium at the venous pole [21, 22]. This generates a unidirectional blood flow from the venous to the arterial pole. While the ventricular and atrial chamber myocardium differentiates and rapidly proliferates at specific sites within the heart tube, the remainder of the myocardium maintains the primary phenotype. Once chambers have developed, this primary myocardium can be recognized as the sinus venosus, the atrioventricular canal (AVC) and the outflow tract (OFT). The sinus node develops from a small component of the sinus venosus [22, 23] and the AVN and AV junction myocardium develop from the AVC [24, 25]. Both nodal components still display phenotypic features of the primary myocardium they develop from, such as automaticity, slow conduction and poorly developed sarcomeres and sarcoplasmatic reticuli [26]. The maintenance of the primary phenotype of the myocardium is fundamental and regulated by the transcriptional repressor genes *Tbx2* and *Tbx3* [3, 27], which are selectively expressed in the primary myocardium and developing and mature conduction system [16]. A deficiency of Tbx3 in the myocardium results in expansion of the expression of working myocardial gens *Cx40*, *Cx43*, *Nppa* and *Scn5a* into the sinus node domain. In contrast, forced expression of Tbx3 leads to development of ectopic functional pacemaker tissue [27]. Both Tbx2 and Tbx3 are required to pattern and form the AVC in a redundant fashion [28]. Thus, Tbx2 and Tbx3 inhibit differentiation into working myocardium, which allows for the development of components of the cardiac conduction system. In conclusion, the primary myocardium lies at the basis of the pacemaker tissues of the cardiac conduction system.

In contrast, formation of the chambers is marked by the differentiation of localized regions of primary myocardium of the embryonic heart tube into the fast-conducting working myocardium of the chambers. A hallmark feature of this working myocardium is the expression of a chamber-specific gene program [4]. This includes genes for rapid propagation of the action potential such as *Cx40* and *Cx43* and the secreted factor Nppa (Natriuretic precursor peptide type A, also known as Anf). Moreover, working myocardium expresses the α-subunit of the sodium channel Nav1.5, encoded by *SCN5a* and develops a functional sarcoplasmatic reticulum [29]. At the cellular level, a local increase in cell size followed by local re-initiation of proliferation marks this area [30]. At the morphological level, initiation of chamber formation is marked by the appearance of trabecules, sponge-like myocardial structures developing at the luminal side of the outer curvatures of the developing heart, particularly in the future ventricular chambers. This series of events has been dubbed the ballooning model of chamber formation [4]. Both the chamber-specific gene program and gene expression in the maintained primary myocardium are regulated amongst others by cardiac transcription factors such as Nkx2-5, Gata4 and a number of T-box transcription factors (Tbx), such as Tbx5 and Tbx20 [15]. Disruption of any of these crucial factors leads to misspecification of working myocardium or primary myocardium which, in turn, eventually can result in local heterogeneities and arrhythmias.

T-box Transcription Factors Regulate Compartmentalization of the Heart

An important question is why some areas of the (embryonic) heart do not participate in the formation of atrial and ventricular working myocardium and mature in a nodal direction, such as the sinus venosus and the atrioventricular canal. To gain insight into this process we studied the regulation of the *Nppa* gene in more detail. *Nppa* is never expressed in nodal tissues from fish to human, and in the embryonic heart it marks the developing atrial and ventricular working myocardium [31]. While investigating the mechanism behind the chamber-specific expression of *Nppa*, we established that both a single TBE site (DNA binding/recognition site for T-box transcription factors) and adjacent NKE site (Nkx2-5 binding element), are present in the *Nppa* promoter and are required for repression of Nppa in the atrioventricular canal [3] and outflow tract [17]. T-box factors are evolutionary highly conserved transcription factors, which are important regulators of (cardiac) development. Presently at least 17

different T-box genes with diverse functions in development and disease are known [32]. In a search for the T-box factors that could act as a repressor for the *Nppa* gene, we observed that *Tbx2* is expressed in inflow, atrioventricular canal, inner curvature and outflow myocardium. Moreover, expression of *Tbx2* and *Tbx3*, a transcriptional repressor with a similar role, is confined to primary (non-chamber) myocardium, remarkably mutually exclusive to *Nppa*, *Cx40*, *Cx43*, and other chamber-specific genes [3, 33, 34]. These findings point to a model in which chamber formation (e.g. atria, left and right ventricle) and differentiation is driven by broadly expressed factors, in addition to which a supplementary layer of localized repressors inhibits this process in regions where chambers do not develop [35]. *Tbx2* gain and loss of function experiments have demonstrated that *Tbx2* is indeed able and required to inhibit chamber formation and expression of chamber marker genes [3, 36]. *Tbx3* is expressed in a sub-domain of the *Tbx2* domain, and whereas it is able to block chamber formation when expressed ectopically, its deficiency does not lead to obvious defects in atrioventricular canal patterning, indicating functional redundancy with *Tbx2*.

But how do *Tbx2* and *Tbx3* exert their functions? Both factors act as repressors of transcription and share DNA binding properties and target genes [37–41]. They effectively compete with *Tbx5*, a transcriptional activator, for TBE-binding, and for *Nkx2-5* on NKE binding, thereby repressing chamber-specific genes and chamber differentiation [3, 34, 35]. Interestingly, lineage studies indicate that the sinus node develops from *Tbx18*-expressing precursors at the junction between the right sinus horn and the right atrium, whereas the atrioventricular node develops from the atrioventricular canal [23, 28]. The sinus node precursors induce expression of *Tbx3*, whereas the atrioventricular node precursors express both *Tbx2* and *Tbx3*. *Tbx2* is absent from the sinus node and sinus venosus and during development, *Tbx2* becomes down-regulated. *Tbx3* expression is maintained specifically in the nodes, thereby providing the only transcription factor found to date to be expressed specifically in the nodes (Fig. 3.3) [34, 36]. As mature nodes display many features that resemble primary myocardium in the embryo, it is attractive to hypothesize that their formation is the result of repression by *Tbx2* and *Tbx3* maintaining the primary phenotype.

Concluding, in a generalizing view one may envision that *Tbx2*, *Tbx3* and/or other transcriptional repressors suppress the chamber-specific program of gene expression, allowing the regions where these factors are expressed to further mature into the nodal direction. Aberrant expression of such factors might thus be at the basis of ectopic automaticity and congenital malformations of the cardiac conduction system in the formed human heart.

Mutations in Transcription Factors Can Cause Congenital Heart Defects and Arrhythmias

As the above mentioned transcription factors are essential for the proper development of the cardiac (conduction) system, it is hardly surprising that mutations in these key genes lead to congenital heart defects. Mutations in *Tbx5* in humans lead to the Holt-Oram syndrome (HOS), which is characterized by anterior pre-axial limb and cardiac malformations [42] (see below). Mutations in *Tbx3* cause ulnar-mammary syndrome characterized by defects in breast development, apocrine gland, limb and genital formation [43], one study reporting ventricular septal defects and pulmonary stenosis [44]. Moreover, mutations in *Tbx5* interacting partners such as *Nkx2-5* and *Gata4*, like *Tbx5* itself, are known to cause septum defects [45] and, in the case of *Nkx2-5*, atrioventricular conduction defects [46] (see below). Presently no diseases are known to be caused by *Tbx2* mutations. A more common congenital disorder called DiGeorge syndrome, is caused by a 1.5–3 megabase genomic deletions of the chromosome 22q11 region which includes the *Tbx1* gene [47]. Patients with DiGeorge syndrome are characterized by a variety of abnormalities including absence/hypoplasia of the thymus, cleft palate, facial dysmorphism and

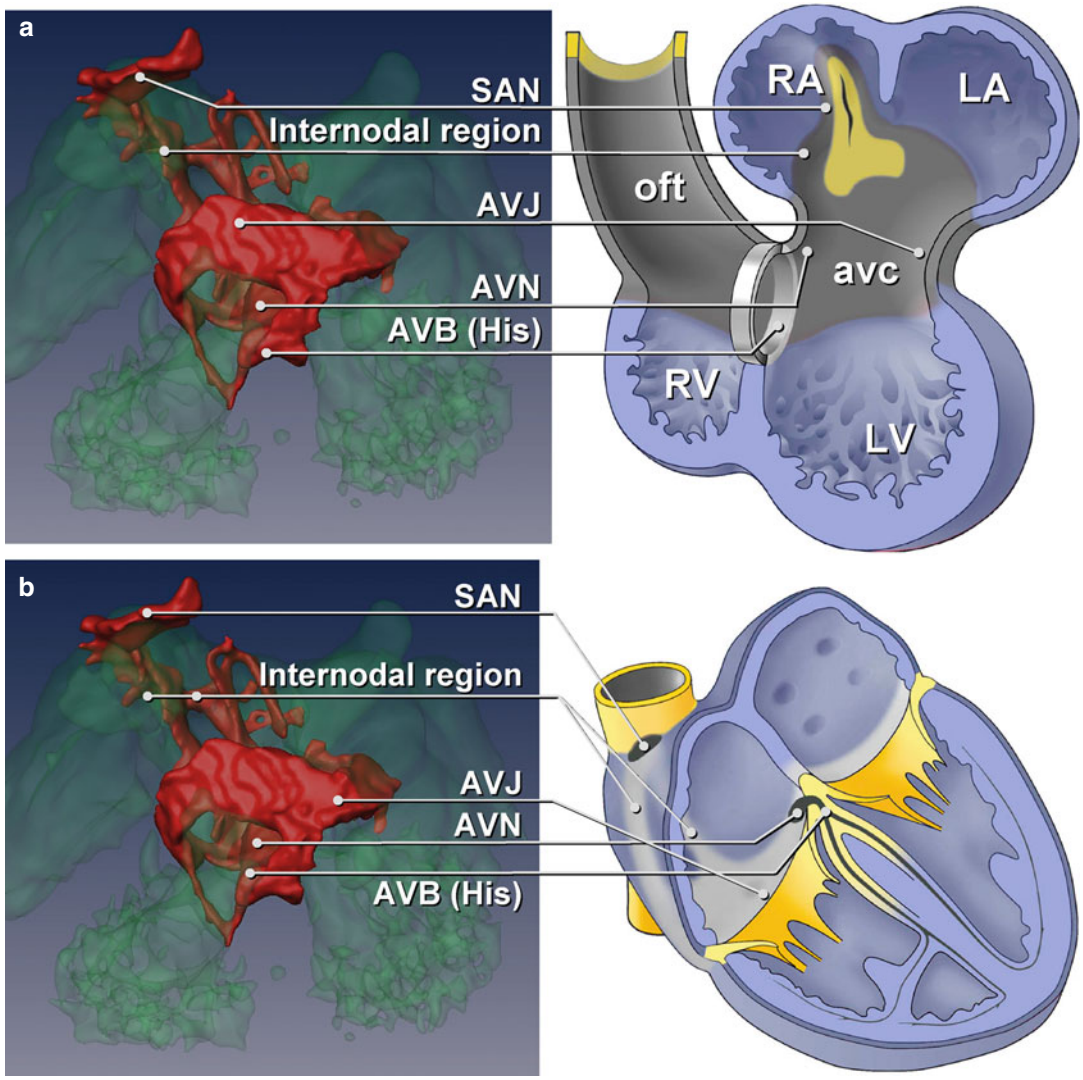

Figure 3–3. Tbx3 (*red*) expression visualized in the heart at ED10.5 (**a**). Tbx3 marks the sinoatrial node, internodal region, AV junction, AV node and the AV bundles with respect to the location of the atria, the atrioventricular canal, the ventricles and the outflow tract (**b**) and with respect to the adult heart

cardiovascular anomalies such as aortic arch malformation, outflow tract defects and VSDs. Recently it was postulated that a synergistic interaction between *Tbx1* and *Nkx2-5* might be responsible for the varying heart malformations of DiGeorge syndrome [48]. As, *Tbx1*-deficient mice phenocopy important aspects of DiGeorge including outflow tract abnormalities [49], it is believed that *Tbx1* might modulate, in part, the outflow tract defects seen in DiGeorge patients.

Nkx2-5, AV Conduction and Atrial Fibrillation

One of the most intensely studied transcription factors involved in cardiac development is Nkx2-5. This homeobox transcription factor is expressed in the heart and other tissues and is part of the core cardiac transcriptional network essential for cardiac development. Targeted disruption of *Nkx2-5* in mice causes

early embryonic lethality, with cardiac development arrested at the linear heart tube stage, prior to looping [50]. Cardiac expression of Nkx2-5 continues throughout development and into adult life. In humans dominant mutations in *NKX2-5* cause a variety of cardiac anomalies as well as atrioventricular abnormalities. Atrioventricular conduction abnormalities and atrial septal defects (ASD) are, in fact, the most common clinical presentations. However, other abnormalities such as ventricular septal defects, double-outlet right ventricle, tetralogy of Fallot and tricuspid valve abnormalities have also been noted [46, 51]. Cardiac dysfunction and sudden death have also been reported in mutation carriers. Atrioventricular conduction disease can occur in the absence of associated congenital heart defects [46, 51]. It is progressive in nature and electrophysiological studies have indicated that the conduction abnormality affects specifically the AVN. The fact that conduction defects can occur in the absence of cardiac structural malformations suggests that NKX2-5 has a function in conduction system development that is independent of its role in cardiac morphogenesis, and the fact that conduction disease is progressive with increasing age underscores the importance of normal *NKX2-5* function in maintenance of AV conduction in adult life. Recent findings that genetic variation in the region of the *NKX2.5* gene modulates the PR-interval in the general population seem to support such a role. The association signal detected at this locus however is located at some distance from the *NKX2-5* gene and further research will be required to establish an unequivocal link between the genetic variation underlying this signal, NKX2-5 expression levels and the link to the PR-interval [52]. This is further underlined by studies using transgenic mice that carry a loss of function allele (DNA-non binding mutant) for *NKX2-5*. These transgenic mice were born with structurally normal hearts but displayed progressive atrioventricular conduction defects and heart failure. PR-prolongation was observed at 2 weeks of age, rapidly progressing into complete AV block by 4 weeks of age. A dramatic decrease in expression of gap junctional channels, Cx50 and 43, probably contributed to the conduction phenotype [53]. Geno-phenotype correlations on the various Nkx2-5 mutants *in vitro* suggested that the principle determinant of the two most common phenotypes (AV block, atrial septal defect) was the total dose of Nkx2-5 capable of binding to DNA [54]. It turned out that *Nkx2-5* haploinsufficiency in the conduction system results in a significantly reduced number of normal cells, and that the specific functional defects observed in the knockout mice could be attributed to hypoplastic development of the conduction system. Thus, the postnatal conduction defects arising from *Nkx2-5* mutations may results from a defect in the development of the embryonic conduction system. This conclusion was in line with experiments on heterozygous *Nkx2-5* knockout mice with eGFP expression, knocked in the *Cx40* locus, permitting the visualization of the conduction system. Indeed hypoplasia and disorganization of the Purkinje fibers was observed in the heterozygous *Nkx2-5* mice and this was associated with abnormal ventricular electrical activation [55]. Analysis showed that maximal Nkx2-5 levels are required cell-autonomously and that a reduction in Nkx2-5 levels is associated with a delay in cell cycle withdrawal from surrounding Cx40 negative myocytes. This suggests that the formation of the peripheral conduction system is time- and dose-dependent on the transcription factor Nkx2-5, which is cell-autonomously required for the postnatal differentiation of Purkinje fibres. Experiments on Id2, a member of the Id gene family of transcriptional repressors, showing AVB-specific expression, proved that a critical transcriptional network, including Tbx5, Nkx2-5, and Id2, is required for the differentiation of ventricular myocytes into specialized cells of the conduction system [56]. Taken together, Nkx2-5 is required for development and homeostasis of atrio-ventricular conduction system (AVCS) and Purkinje fibers. In the case of the AVB some details of the molecular mechanism have been elucidated (Id2, Tbx5). It is noteworthy that Tbx3 also cooperates with Nkx2-5 [27] providing a link between this factor and Tbx3 in the AVB and AVN.

Some mutations in *Nkx2-5* are not linked to AV block, but to atrial fibrillation [57], an arrhythmia long thought to have a possible

origin in development [58]. In the majority of cases of paroxysmal atrial fibrillation, the myocardial sleeves surrounding the pulmonary veins at the orifice to the left atrium carry the triggers and possibly the substrate for the arrhythmia. Cells with presumed pacemaker activity have been found in the pulmonary veins of rat hearts [59] and in the pulmonary veins of patients with atrial fibrillation [60]. However, these observations are controversial, since lineage studies have demonstrated that the pulmonary myocardium is formed from a distinct lineage of precursor cells, different from the lineage that forms the muscle encompassing the sinus venosus (myocardium around caval veins, including the sinus node and around coronary sinus) [23, 61–63]. In contrast to the sinus muscle, pulmonary myocardium expresses from the outset transcription factor Nkx2-5 and its target gap-junction gene *Cx40*, suggesting it has an atrial working phenotype from the outset. Intriguingly, when Nkx2-5 protein levels are lowered experimentally, the pulmonary myocardium switches to a Cx40-negative, Hcn4-positive phenotype, similar to sinus nodal–like cells, resembling the sinus muscle. Thus, a reduction in the expression level of a single transcription factor, Nkx2-5, activates a gene program potentially sufficient to provide automaticity in the pulmonary myocardium. This observation suggests that inter-individual variation in Nkx2-5 dosage could be an important contributing trigger to the development of atrial fibrillation. Also, intriguingly, as mentioned above, genetic variation in the region of *NKX2.5* has been associated with the PR-interval in the general population, which when prolonged, is in turn associated with risk of AF [64]. Taken together, variations in the (regulatory) sequence of the *Nkx2-5* gene (and/or its interacting partners) are prime candidates for further research into the AV conduction and atrial fibrillation.

Tbx5, Atrial Fibrillation and Left-Right Chamber Differences

Another crucial factor in cardiac development is TBX5, which belongs to the evolutionarily conserved T-box family of transcription factors that bind DNA through a highly conserved DNA binding domain called the T-box. The consensus DNA sequences of the target genes where T-box factors bind are called T-box binding elements (TBEs) [65]. Tbx5 expression displays a posterior-anterior gradient along the heart tube with the most intense expression at the inflow tract of the heart. After chamber development has been initiated, Tbx5 expression is found in the sinus venosus, atria, AVC, left ventricle, including the left aspect of the ventricular septum, and to a lesser extent in the right ventricular trabecules [31, 66]. In humans, *TBX5* haploinsufficiency has been shown to cause Holt-Oram syndrome (HOS), which includes congenital heart defects, conduction system abnormalities and upper limb deformities [42, 67]. Heterozygous *Tbx5* knockout mice recapitulate many of the phenotypic abnormalities observed in Holt-Oram syndrome patients [68]. Expression of both *Nppa* and *Cx40*, targets of Tbx5, was also reduced in these mice during development. Complete deletion of *Tbx5* in mice leads to embryonic death (around E9.5) with failure of heart tube looping and an underdeveloped caudal part [68]. Furthermore, Tbx5 overexpression in an embryonic cell line results in significant upregulation of *Nppa* and *Cx40*, and, counter intuitively, in cells displaying a spontaneous beating phenotype [69, 70]. Moreover, as mentioned earlier, Tbx5 is required for the differentiation of the AVB in the crest of the ventricular septum [71].

Recent work further links TBX5 to arrhythmogenic mechanisms and atrial fibrillation in particular. Atrial fibrillation has occasionally been described in sporadic patients with Holt-Oram [72], though principally in the setting of congenital heart disease and the resultant hemodynamic effects (atrial enlargement). We recently reported on a family in which affected patients have mild skeletal deformations (a hallmark feature of HOS) and very few have congenital heart disease. However, the great majority presented only with paroxysmal atrial fibrillation at an unusually young age [73]. Sequencing of *TBX5* revealed a novel mutation, p.G125R, co-segregating with the disease. The mutant protein displayed significantly enhanced DNA-binding properties, which resulted in

augmented expression of *Nppa*, *Cx40*, *Kcnj2* and *Tbx3* genes in comparison to wild-type TBX5, i.e. functioned as a gain-of-function factor. This is in contrast to all known Holt-Oram mutations to date, which result in a loss-of-function phenotype. This TBX5 gain-of-function mechanism probably underlies the paroxysmal atrial fibrillation and mild HOS phenotype. The mechanism underlying atrial fibrillation in TBX5 gain-of-function mutants may involve direct stimulation of TBX5 target genes involved in familial atrial fibrillation (*NPPA*, *KCNJ2*, *CX40*). Alternatively, it may involve TBX5-stimulated *TBX3*, as it was recently shown that *TBX3* is highly sensitive to *TBX5* dosage [74], and TBX3 controls the sinus node gene program and induces pacemaker activity in atrial muscle [27]. There is thus evidence in support of a role of *TBX5* in the development of (paroxysmal) atrial fibrillation, a proposition that was recently further substantiated by the fact that genetic variation within the *TBX5* gene was found to be associated with AF [75].

The left and right ventricle have a different morphology, tissue architecture, geometry, and function, and myocytes of the right and left ventricle differentiate to similar, but not identical phenotypes [76]. Differences in the regulatory and gene programs of progenitors of the left and right ventricle probably underlie the differences in these phenotypes. Tbx5 likely contributes to this, as it is expressed in an antero-posterior gradient in the heart tube regulated by retinoic acid [77]. The left and right ventricles are specified along the antero-posterior axis, and, as a consequence, the developing left ventricle robustly expresses *Tbx5*, in contrast to the right ventricle [66]. This suggests that *Tbx5* is necessary for left ventricular identity, and provides, in part, the boundary between the left and right ventricle [78]. Consequently, target genes of *Tbx5* are likely differentially regulated between the left and right ventricles. In fact, target gene *Cx40*, mimics the pattern of Tbx5 [68] and is differentially expressed between the left and right ventricle in the developing and adult heart. Seeing that the right ventricle especially is prone to the development of various arrhythmias, such as those seen in Brugada Syndrome [79] and Arrhythmogenic Right Ventricular Dysplasia (ARVD) [80], and given the fact that *Tbx5* is actively involved in segregating between the left and right ventricle [78], it is tempting to speculate that target genes of *Tbx5* might contribute to or oppose arrhythmias. Recent work has shown that numerous genes are influenced by differences in *Tbx5* dosage, including genes expressed during heart development such as transcription factors (*Tbx3*, *Irx2*), cell-cell signalling molecules, and ion channels (*Cx40*, *KCNA5*) [74]. This, together with the link between *TBX5* and atrial fibrillation, warrants further investigation into *TBX5* and its downstream genes in relation to arrhythmias and ion channel genes.

Tbx2, Accessory Conduction Pathways and Defective Patterning and Gene Regulation Within the Atrioventricular Canal Myocardium

Wolff–Parkinson–White (WPW) syndrome is an arrhythmogenic defect characterized by a normal conduction system and one or more accessory AV connections, bypassing the AV node and His bundle, which cause ventricular preexcitation and predispose to re-entrant supraventricular tachycardia [81]. These abnormal accessory pathways may conduct faster than the AV node and this leads to ventricular preexcitation, evidenced by a short PR interval and a slurred upstroke of the QRS complex on the ECG. The majority of WPW occurs isolated and sporadic, affecting 1–3 persons per 1,000 [81], most patients with WPW syndrome have structurally normal hearts, though in a small portion of WPW patients, accessory pathways occur in association with congenital heart defects, such as Ebstein anomaly [82]. Nonetheless, autosomal dominant WPW families have been described [83, 84]. *PRKAG2* and *LAMP2* gene mutations were found in familial left ventricular hypertrophy and preexcitation, while a *PRKAG2* mutation was detected in a single family with isolated WPW [85, 86]. Patients with mutations in the *PRKAG2* gene have a variable combination of glycogen storage cardiomyopathy, progressive conduction system disease including sinus

bradycardia and atrioventricular block, ventricular preexcitation, arrhythmias, and sudden death [85]. Ventricular preexcitation is presumably caused by a disruption of the annulus fibrosus, which electrically insulates the atrial and ventricular muscle masses, distinct from the muscular-appearing bypass tracts observed in typical WPW syndrome [87]. However, in a cardiomyocyte-specific overexpression model of mutated PRKAG, accessory pathways only developed when the mutated PRKAG was overexpressed during development. When overexpression started in adulthood, glycogen storage disease and conduction system degeneration occurred but accessory pathways did not develop, suggesting that a developmental defect may underlie pre-excitation in PRKAG mutants [88]. In addition, various *NKX2-5* gene mutations in patients with CHDs are also associated with WPW, and point to a role of this critical cardiac transcription factor gene in WPW pathogenesis [51, 56].

A milder form of arrhythmias involving accessory pathways is the atrioventricular reentrant tachycardia (AVRT). This is the most common type of supraventricular tachycardia in both the fetus and the newborn [89]. Although AVRT can be potentially life-threatening and are sometimes difficult to control with antiarrhythmic drug therapy, in general these tachycardias resolve spontaneously within the first months of life, and >60 % of patients require no antiarrhythmic drug therapy and remain free of symptoms after the age of 1 year [90]. This self-resolving character of most perinatal AVRTs suggests that the majority of the accessory pathways eventually disappear after birth. Studies in animal models have shown that accessory AV myocardial connections are present until the late stages of cardiac development [91]. Moreover, a recent study on human hearts from neonates without episodes of supraventricular tachycardia found that isolation of the AV junction is a gradual and ongoing process [92]. The right lateral accessory myocardial AV connections in particular are commonly found at later stages of normal human cardiac development. These transitory accessory connections may therefore act as a substrate for AV reentrant tachycardias in fetuses or neonates.

The molecular mechanisms by which these accessory bypasses are formed are, however, poorly understood. The atrioventricular (AV) node and AV bundle are the only muscular connections that cross the annulus fibrosus. However, during heart development, in the absence of an annulus fibrosus, the AV canal myocardium still maintains adequate AV delay, allowing for the synchronized alternating contraction of the atria and ventricles [93, 94]. Remnant strands of this AV myocardium can however be observed in normal hearts around and after birth (see above). They disappear when the annulus fibrosus is fully formed [92, 95]. These strands are likely to maintain the slow conduction properties of the AV myocardium. Moreover, slow-conducting AV canal–type myocardium remains present around the orifices of the mitral and tricuspid valve in the adult heart [25, 96]. Together, this suggests that AV canal myocardium plays a central role in the AV delay and that defects in AV canal myocardium could underlie formation of functional accessory pathways. The AV canal myocardium is specified early in cardiac development by the expression of bone morphogenetic protein 2 (Bmp2) in the AV canal myocardium progenitors in the early heart tube, where it stimulates the expression of Tbx2 [97], a transcription factor required for the development of the AV canal [25]. Indeed in a recent study by Aanhaanen et al. [98], it was shown that myocardium-specific inactivation of *Tbx2* leads to the formation of fast-conducting accessory pathways, malformation of the annulus fibrosus, and ventricular preexcitation in mice. The AV accessory pathways ectopically express proteins required for fast conduction (Cx40, Cx43, and sodium channel, SCN5A). Several other genes and pathways have been implicated in AV canal development such as the Bmp and Notch pathways. Interestingly, microdeletions of BMP2 and JAGGED1, a Notch ligand, have been associated with ventricular preexcitation in human [99, 100]. Moreover, deletion of the *Alk3* receptor (activated by Bmp2) in the AV canal myocardium also results in accessory pathways and AV nodal defects in mice [101]. The fact that Notch signalling is involved in maturation of the AV canal-derived myocardium was underscored by another recent paper in which it was shown that activation of Notch

signalling in the developing myocardium of mice produces fully penetrant accessory pathways and ventricular preexcitation [102]. Conversely, inhibition of Notch signalling in the developing myocardium resulted in a hypoplastic AV node, with specific loss of slow-conducting cells expressing Cx30.2 and a loss of physiologic AV conduction delay. In conclusion, these results indicate that defective patterning and gene regulation within the AV canal myocardium, via disruption of the AV canal regulatory network, leads to malformation of the annulus fibrosus, formation of accessory AV connections, and ventricular preexcitation.

Sarcoplasmic Reticulum and Heart Development

Having discussed the building plan of vertebrate hearts and their conduction system, it is relevant to look at the developmental aspects of their most prominent features, namely contraction and electrical activity. Regular beating is already observed in the very early period of heart development from embryonic day E9 onwards [103], as mentioned above. This implies that an intracellular system of contraction and cardiac automaticity is already established. Interestingly, the atrioventricular canal and the outflow tract are characterized by slow conduction velocity [10], a low level of gap junction and connexin expression [104] and low SR activity [29]. This supports the idea that these flanking segments can function as one-way valves, because they fulfill all the requirements needed for a long contraction duration resulting in a peristaltic contraction. However, the radical change from a peristaltic slowly-contracting heart tube to one with fast-contracting chambers necessitates proper control on free intracellular calcium ions. This, in turn, requires the regulated movement of calcium ions across both the sarcolemmal and sarcoplasmic membranes. Clearance of intracellular calcium can be obtained by two ways, either by extrusion of the calcium into the extra cellular space by the Na^{+}-Ca^{2+} exchanger (NCX) or by the sarcoplasmic-endoplasmatic Ca^{2+}-ATPase (SERCA2) into the sarcoplasmic reticulum (SR). SERCA2 itself is regulated by the non phosphorylated form of another protein, phospholamban [105].

The calcium handling system was the first ionic system to be studied extensively during heart development, however mostly in the late fetal heart stage (embryonic day 16.5 and later) [105]. At this stage most calcium required for contraction is derived from the calcium influx through the voltage dependent calcium channels (L type and T type). Although a distinct SR is not morphologically observed in fetal myocytes, calcium influxes nevertheless are capable of triggering calcium releases through the calcium release channels (so-called ryanodine receptors, RYRs) [29]. It is thought that, in contrast to the mature situation, the calcium stores for the RYRs in the fetal myocytes are very small organelles, which are located far from the L and T type channels on the surface membrane. In this way, calcium flowing through the calcium membrane channels diffuses into the intracellular space, where it stimulates the immature SR. The whole chain of calcium induced calcium release is thus slowed down, and produces the slow kinetics of calcium signals in fetal myocytes.

In contrast, the expression pattern of the various genes involved in the calcium handling has been studied more thoroughly throughout embryonic heart development. SERCA2 and PLB can already be observed as early as the cardiac crescent stage (embryonic day 7.5 in the mouse), even before myocardial contraction has begun. In the forming heart tube, however, there is already polarity in expression, as SERCA2 is more abundant in the posterior region, or venous pole, of the heart and decreases towards the anterior regions, or arterial pole, whereas PLB, in contrast, shows a complementary distribution [106]. At the stage of the primitive cardiac tube (E8), several additional calcium-related genes start to be expressed. Whereas PLB and SERCA2 are expressed in opposite gradients, RYR, NCX and NaK-ATPase are distributed homogeneously over the cardiac tube. They remain homogeneously distributed throughout further embryonic development of the heart [106]. It thus seems that the control of calcium homeostasis is determined solely by the SERCA/PLB system. At the stage of cardiac looping (E8.5), when the first

signs of left/right asymmetry and identity are manifested in the embryo, the calcium related genes maintain their previous patterns of expression. In the final stage (E16.5), termed fetal heart, SERCA2 is more expressed in atrial myocardium than in the ventricular myocardium, whereas PLB is once again expressed in the opposite pattern. Additionally, SERCA2 has low expression in the AVC and OFT. Interestingly, SERCA2 and PLB display a differential expression between the trabeculated and compact layers of the ventricular myocardium. Contrarily, the expression of both genes is very weak in different components of the cardiac conduction system, atrioventricular node, and the bundle of His, which is in line with the nodal-like morphological origin of these components, as discussed earlier. The expression of the other components of the calcium metabolism, RYR, NCX and NaK-ATPase is homogenously in the different regions of the fetal heart.

Connexins and Heart Development

The propagation of cardiac impulses is mainly determined by the capacity to rapidly carry changes in the membrane potential of cardiomyocytes. This propagation is mediated by gap junctions, which are aggregates of hydrophobic cell-cell channels that allow the intercellular exchange of ions, metabolites and second messengers of up to 1 kDa in size [107]. The aqueous pores are formed by serially linked hemi-channels (connexons) provided by apposing cell membranes. One connexon hemi-channel is composed of six transmembrane proteins called connexins (Cx), which are encoded by 21 connexin genes [108]. Although the main purpose of gap junctions in the heart is the conduction of the depolarizing impulse across the myocardium, evidence exists that connexins also play a role in cardiac morphogenesis, as homozygotic Cx43-knockout mice die shortly after birth from a pulmonary outflow tract stenosis due to cardiac malformation [109]. Analogous, in humans, visceral heterotaxia and hypoplastic left heart syndrome have been found to be associated with mutations of the Cx43 gene [110, 111].

Differences in the expression of the connexins are consistent with a functional myocardium (atria and ventricles) model in which the working myocardium has the capacity to transmit the cardiac impulse more quickly than the adjacent myocardium of the inflow tract, atrioventricular canal and outflow tract. This guarantees synchronized contraction without the need for a specialized system (as discussed earlier). The expression of the main connexin, Cx43, is detected for the first time in the embryonic heart stage (E10.5). Cx40 has a similar pattern of expression although more reduced [106]. Cx40 (from ED9.5) and Cx43 mRNA (from 10.5) are detectable in atria and ventricles, but not in their flanking myocardium (inflow tract, atrioventricular canal and outflow tract) [112]. Even though Cx40 and Cx43 mRNA eventually become expressed in the inflow tract, they remain undetectable in the sinus node, the atrioventricular canal (including atrioventricular node) and outflow tract [107]. Expression of Cx40 is maximal in the fetal period and declines towards birth. At the stage of the fetal heart (E16.5), Cx43 is restricted to the ventricular myocardium and it is barely detectable in the atrial myocardium in rat, while the mouse atrium expresses a lot of Cx43 [106, 107]. Analogous to SERCA2 and PLB, Cx43 expression is low in the trabeculated layer and higher in the compact layer [106]. In contrast, Cx40 expression complements Cx43 expression, as Cx40 is expressed mainly in the atrial chambers and shows a transitory differential expression between the right and left ventricles under the influence of Tbx5, as discussed earlier.

It is interesting to note that based on the expression of Cx40 and Cx43 during development, two populations of myocytes can be distinguished, viz. cardiomyocytes that do not express Cx40 and Cx43, and cardiomyocytes that do express both. The first group includes the myocardium of the sinus node, the atrioventricular canal (including the atrioventricular node), bundle of His and the outflow tract. The second population includes the working myocardium of the atria and the ventricles, and, later in development, the myocardium of the inflow tract (excluding the sinus node). These patterns further that the structures that do not express Cx40/43 are derived from the embryonic myocardium of the sinus venosus and atrioventricular canal, respectively.

Ion Channels and Heart Development

The regulation of the action potential (AP) is determined by a large variety of ion currents. During the depolarization of the myocytes massive amounts of sodium ions are pumped into the cell, whereas the subsequent repolarizations are characterized by a balance of different potassium currents flowing into and out the myocyte. Many genes are involved in maintaining this dynamic balance, the most prominent ones being SCN5A, carrying the initial sodium current, and KCNQ1 and KCNH2, carrying the subsequent potassium currents. Several modulator genes, such as KCNE1,2 and SCN1b also play a role. As much as is known about their function and expression in the adult heart, virtually nothing is known about the different components of the action potential during the various embryonic stages. Attempts to characterize the various individual currents electrophysiologically during development have been hampered by technical difficulties though some studies were published on this topic [113–115].

In general, the action potentials and resting potentials in cardiomyocytes are altered greatly during development, e.g. both the rate of rise and the overshoot increases along with the duration of the AP. These electrophysiological changes are mainly produced by developmental changes in ion channels, e.g. changes in amount, the type and the kinetic properties. The fast sodium current (mainly encoded by SCN5a and SCN1b), which is responsible for the upstroke of the AP, is markedly increased during development. There are few functional sodium channels present at the earliest stage but the density increases progressively during development. Though the current has a significant sustained component in the earlier stages, this decreases during development, thereby contributing in part to the abbreviation of the AP [115]. The main potassium current in fetal ventricular myocytes is I_{Kr} (mainly encoded by KCNH2 and KCNE2), whereas I_{Ks} (encoded by KCNQ1 and KCNE1) is lacking or very small, though in the early neonate I_{Ks} becomes the dominant repolarizing current. I_f, or the funny current (encoded by the HCN gene family), is the pacemaker current and as such contributes prominently to cardiac rhythm [116]. In the early embryonic human heart, wide expression of the pacemaker channel HCN4 is seen at the venous pole, including the atrial chambers. The HCN4 expression becomes confined during later developmental stages to the components of the conduction system [16]. From mouse studies it is known that mice lacking HCN4 globally, as well as selectively from cardiomyocytes, die between ED9.5 and 11.5, displaying a strong reduction in I_f and bradycardia [117]. The few studies that investigated I_f electrophysiologically during development did so only in ventricular cells. They show that I_f is prominent at E9.5 in ventricular myocytes and decreases together with loss of regular spontaneous activity of ventricular cells towards the neonatal stage, which is accompanied by a subtype switch from HCN4 to HCN2 [118, 119]. So while the I_f current of the sinus node type is present in early embryonic mouse ventricular cells, the ventricle tends to lose pacemaker potency during the second half of embryonic development. Moreover, kinetics were found to be changed, as the threshold voltage to evoke I_f significantly lowers from neonatal myocytes to adult ones [120]. The developmental expression pattern of the HCN4 gene shows that it can already be detected in the cardiac crescent at ED7.5, while at ED8 it is symmetrically located in the caudal portion of the heart tube, the sinus venosus, where pacemaker activity has previously been reported [21]. Further in development, HCN4 becomes asymmetrically expressed, occupying the dorsal wall of the right atrium and eventually becomes restricted to the junction of the right atrial appendage and the superior vena cava in concordance with the site at which the sinus node is located in the postnatal and adult heart [121]. In the adult heart, the sinus node, atrioventricular node and ventricular conduction system mainly expresses HCN4, whereas expression of HCN2 is homogenously low in the heart [119]. The molecular pathways underlying the developmental regulation of cardiac HCN channels are not yet known. Recently the myocyte enhancer factor 2 (MEF2) was shown to interact with an enhancer of HCN4 and regulate its transcription [122]. As MEF2 expression is increased in the atrium, it could possibly account in part for the spatial distribution of HCN4 [123].

Likewise, NKX2-5 is a strong candidate for the developmental regulation of HCN4, as it has been implicated in HCN4 repression and its confinement to the sinus venosus in the developing heart [22].

Ion Channels Have a Possible Non-electrogenic Role in Heart Development

Recent work has demonstrated that heart development does not only rely on interactions of transcription factors and target genes, but, surprisingly, that ion channels themselves play a novel and possibly non-electrogenic role in heart development. The first evidence for this emerged from a study in which a strain of zebrafish called island beat was identified. These fish exhibited ventricular growth failure and non-contraction, while the atrium exhibited rapid, isolated, and uncoordinated contractions. Positional cloning led to the identification of mutations in the pore-forming α(alpha)1C subunit of the L-type calcium current [124]. Thus, abolishment of this current caused the ventricle to fail to grow and remain electrically silent, which demonstrated that the L-type calcium current is essential for normal cardiac form and function during embryonic development. This was followed by the discovery that knockouts for *Scn5a*, the cardiac sodium channel, led to early embryonic lethality. Hearts of knockout mice showed uncoordinated contractions, and developmental defects were seen, such as a common ventricular chamber, with reduced chamber size, reduced trabeculation of the ventricular wall, and a reduced number of thin, spindle-like cardiomyocytes [125]. This ventricular defect in *Scn5a* knockout embryos is unlikely to reflect a generalized failure of cardiac development, as the endocardial cushions of the atrioventricular canal, the common atrial chamber, and the truncus arteriosus appeared normal. This suggested the possibility that ion channels have an important influence on ventricular development. Indeed, antisense knockdown of zebrafish homologue *Scn5a* results in marked cardiac chamber dysmorphogenesis and perturbed looping [126]. Moreover, these abnormalities were associated with decreased expression of the myocardial precursor genes *Nkx2.5*, *Gata4*, and *Hand2* and significant deficits in the production of cardiomyocyte progenitors. Interestingly, these early defects did not appear to result from altered membrane electrophysiology, as prolonged pharmacological blockade of sodium current failed to phenocopy the ion channel knockdown. Moreover, embryos grown in the calcium channel blocker-containing medium (similar to the island beat experiments described earlier) had hearts that did not beat but developed normally. This demonstrated that voltage-gated ion channels have important roles in vertebrate heart development and supports the hypothesis that such roles might be mediated by nonelectrogenic functions of the channel complex. Additional evidence for the role of ion channels in heart development comes from studies on mice lacking Hcn4 (carrier of the I_f pacemaker current), either globally or specifically in the heart. Such mice die during embryonic development between E9.5 and E11.5, as mentioned earlier [117]. In summary, recent results argue that ion channels are required for heart development in addition to their canonical role as regulators of heart rhythm. Moreover, the data suggest that this developmental role is mediated by a nonelectrogenic function of the ion channel.

Conclusion

The heart evolved from a myocardial tube in primitive chordates to a four-chambered heart with synchronous contraction and dual circulation in higher vertebrates. Each cardiomyocyte of a primitive heart can be considered as a nodal cell because it displays automaticity and is poorly coupled, which, together with slow propagation, gives rise to peristaltic contraction. The introduction of dominant pacemaker activity at the intake of the heart perfected such a heart into a one-way pump. Subsequently, highly localized, fast-conducting cardiac chambers were added to this nodal tube, resulting in the four-chambered hearts. Interestingly, concomitant with the formation of such chambers, an adult type of electrocardiogram (ECG) can already be

monitored in the embryo. Thus, cardiac design, e.g. the positioning of the atrial and ventricular chambers within the nodal tube, principally explains the coordinated activation of the heart reflected in the ECG. A crucial question is why some areas of the embryonic heart tube do not participate in the formation of atrial or ventricular working myocardium and mature in a nodal direction. As a generalized hypothesis we propose that the chamber-specific program of gene expression is specifically repressed by T-box factors and by the other transcriptional repressors. Consequently, aberrant expression of these factors might be at the basis of ectopic automaticity, malformations of the conduction system and congenital heart disease in general.

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