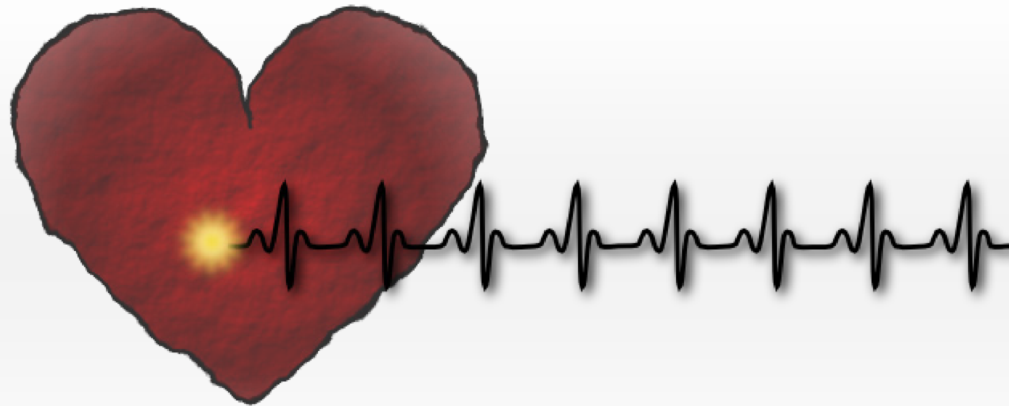
ORIGIN AND FUNCTION OF THE CARDIAC PACEMAKER



Master thesis

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Abstract

The rhythmic beating of the heart is initiated and controlled by an intrinsic pacemaker system. Cardiac contractions commence at very early embryonic stages and coordination is crucial for survival. Abnormalities account for a number of congenital and acquired heart defects in humans. In the mammalian heart, the dominant pacemaker is situated in the sinoatrial node in the upper wall of the right atrium. Specialised myocardial cells develop the potential to generate spontaneous electrical activity. Discrete conduction pathways transmit the electrical signal to the working cardiomyocytes, stimulating muscle contractions. A network of transcription factors controls patterning of the sinoatrial node and surrounding working myocardium. The underlying mechanisms of pacemaker cell development and function are still not fully understood. The heart shows high evolutionary conservation. Intrinsic pacemaker systems are already present in invertebrates and are believed to have evolved in an ancestral bilaterian. On the molecular level, pacemaker mechanisms are less well understood in lower vertebrates. The zebrafish *Danio rerio* has emerged as a promising model to study pacemaker development and function. Detailed characterisation of its pacemaker system is required to tap the full potential of the extensive experimental techniques available in zebrafish.

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Structure and function of the cardiac pacemaker

The hearts striking ability to continuously and reliably drive blood circulation has fascinated generations of researchers. It was in the beginning of the 20th century that the discussion about the hearts intrinsic initiation of contraction and regulation of the heartbeat started to unravel [37]. Arthur Keith was the first to identify a structure in the human heart, nowadays known as the sinoatrial node [39]. His anatomical description shifted the focus to this "*remarkable remnant of primitive fibres persisting at the sino-auricular junction*" [39], which was demonstrated to correspond to the location of initial cardiac excitation [47].

Box 1

Abbreviations

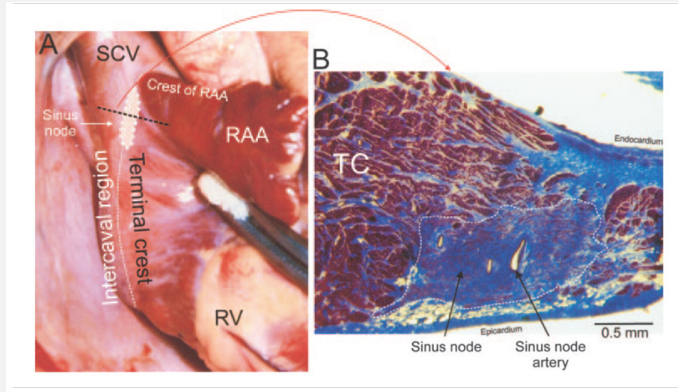
A	(common) atrium
AP	Action potential
AV	atrioventricular
AVB	atrioventricular bundle
AVC	atrioventricular canal
AVN	atrioventricular node
BA	bulbus arteriosus
CA	conus arteriosus
CCS	cardiac conduction system
ECG	echocardiogram
ICV	inferior caval vein
IFT	inflow tract
LA	left atrium
LV	left ventricle
Mya	Million years ago
OFT	outflow tract
RA	right atrium
RV	right ventricle
SAN	sinoatrial node
SCV	superior caval vein
SV	sinus venosus
V	(common) ventricle

An intrinsic pacemaker and conduction system, comprised of several distinct domains, control the basal rate of the cardiac rhythm. It includes specialised pacemaker cells, which are able to spontaneously generate an electrical pulse and a well coordinated network of cells transmitting the electrical signal to the contractile working myocardium. This review focuses on the anatomic and (electro-) physiological properties of the subpopulation of cardiac pacemaker cells in the sinoatrial node, the primary site of electrical impulse generation in the healthy mammalian heart. The main emphasis is on the embryonic development of the primary pacemaker as well as its evolutionary origin. The following chapter will mainly focus on the situation in human hearts. Important differences in other mammalian systems such as mouse are mentioned. The secondary pacemaker site, the atrioventricular node and the peripheral conduction system are discussed briefly. For further information on these aspects, recent reviews are pointed out as references.

The sinoatrial node (SAN or sinus node) holds the primary pacemaker of the mammalian heart. It has been described as a subepicardial, crescent shaped structure with a roundish head and elongated tail [55].The SAN is located in the upper wall of the right atrium, near the entry of the superior vena cava in human (Fig. 1A and 2). Localisation in the right atrium is conserved in all mammals, but the location/position within the atrium can differ, such as described for rabbit [56], rat [57] and mouse [58]. The SAN is a myocardial structure

Figure 1

Anatomic and histological description of the human sinoatrial node



The sinus node in an adult human heart situated at the junction of the superior caval vein and the right atrium., descending towards the terminal crest (A). The sinus node is histologically distinct from surrounding atrial myocardium (B) Masson's trichrome stained section, taken approximately at the dashed line in A. White dashed lines mark the sinus node.

SCV = superior caval vein; RAA = right atrial appendage; RV = right ventricle; TC = terminal crest (Crista terminalis) (figure adapted from Dobrzynski et al., 2007 [2])

and the nodal cells histologically resemble cardiac myocytes. However they are smaller and have only poorly developed muscle cell properties, such as sarcomeres and sarcoplasmic reticulum, resulting in a merely rudimental contractile system [55]. This is typical for cells specialised in conduction, since they function electrically, not mechanically. Apart from the node-specific cellular phenotype, the SAN area can also be delineated by the presence of a connective tissue matrix discriminating it from non-nodal cardiomyocytes [55] (Fig. 1B). This non-excitable, fibrous layer plays an important role in the function of the sinus node. It protects nodal cells from hyperpolarisation by the surrounding cardiomyocytes [59]. Despite the distinct sinus node structure, nodal cells have been found in extranodal areas as well. Chandler et al. reported the observation of nodal cells intermingled with working myocardial cells in the Crista terminalis (muscular junction between the SCV and the right atrium ,Fig. 1) separated from, but immediately adjacent to the SAN [60, 61]. This paranodal area is described as having an intermediate phenotype, but a distinct function could not be verified. However, it remains debatable whether this paranodal structure in fact harbours pacemaker cells or merely constitutes of poorly developed cardiomyocytes intermingled with the working myocardium.

The SAN is densely innervated by vagal and sympathetic nerve endings, which enables modulation of the cardiac rhythm by nervous system stimulation [62]. It has been shown, that systemic influences constantly modulate the heart rate. In addition, perfusion with messenger factors (e.g. hormonal control) and the chemical environment can influence heart rate (extensive review [63]). Nevertheless, generation of spontaneous action potentials is clearly an intrinsic property and there is no need for extrinsic stimulation to maintain the basic cardiac rhythm [64].

The electrical impulse from the pacemaker cells initiates depolarisation and action potential generation in the excitable cardiomyocytes located in immediate vicinity to the SAN. The leading pacemaker site is believed to be located in the centre of the

node. It has been shown that various modulating factors, such as systemic influences, medication and temperature or tension can lead to a shift on the leading pacemaker site within the SAN [63, 65]. Fedorov et al. argued for the existence of discrete bundles of excitable cells (exit pathways) originating from the SAN passing through the insulating fibrous layer into the surrounding atrial myocytes in dog and human [59, 66]. Thus allowing the electric signal to pass through the insulating cell layer. Alternatively, it has recently emerged that myocytes can form direct electrical coupling connection with fibroblast [67, 68]. How this interaction might affect electrical signal propagation, has not been established yet. Currently, mainly in vitro and theoretical studies have been done to address coupling between cardiomyocytes and non-cardiomyocytes [69, 70]. Depolarisation is quickly transmitted through a preferentially ordered myofibril pathway in the fast-conducting atrial working myocardium. Each heartbeat is an electrical-mechanical coupling. Excitation of the cardiomyocytes is immediately followed by tissue contraction expelling blood from the atrium into the ventricle. Despite having two ventricles and two atria, there is only one primary pacemaker site in the four-chambered heart. The left atrium is stimulated via a conduction pathway linking it to the SAN. Thus, coordinated simultaneous contraction of both atria is achieved (see Fig. 2). After depolarisation, the working myocytes are quickly repolarised in order to be excitable for the following electrical signal from the pacemaker. At the end of atrial depolarisation, the excitation signal reaches the atrioventricular node (AVN).

The Atrioventricular junction, including the AVN is the second major site in the heart with potential pacemaker autorhythmicity, albeit at a lower rate than SAN pacemaker cells [8, 12, 13, 51, 52]. The faster rate of conduction in the SAN overrules the pacemaker potential of the AVN and His-Purkinje system [63]. If the SAN pacemaker fails to generate automatic excitation, such as in SAN failure of block, the AVN automaticity can become dominant and facilitate ventricular contraction (backup system) [63]. The AVN is situated in the floor of the right atrium in close vicinity to the tricuspid valve and atrioventricular canal (Fig. 2).

The main function of the AVN is its pacemaking and conduction slowing properties. For recent reviews on structure and function of the AVN and ventricular conduction system see [8-13, 71]. The AVN-AVB (atrioventricular bundle) connection significantly slows down the electric signal to allow the atria to fully contract and fill the ventricles before ventricular contraction. Therefore, it controls timing of transfer of the electrical signal from the atrium to the ventricle, allowing coordinated and optimal hemodynamic performance. Atrium and ventricle are physically separated by a fibrous tissue layer. Insulation inhibits premature “leakage” of the electric signal to the fast conducting ventricular myocytes, preventing pre-excitation arrhythmias [72].

The AVN transfers the electrical signal via the atrioventricular bundle to the bundles of His in the ventricular septum (Fig. 2). The bundle of His is a myocardial muscle string, which crosses the fibrous tissue insulation of the AVN and reaches into the ventricular septum. Thus it forms the only connection between the AVN and the ventricular part of the conduction system [12]. It is a rapidly

conducting pathway. Even though cells resemble the primitive phenotype of nodal cells, they show fast conducting properties [73]. Ectopic connection pathways, for instance remnants of embryonic connection structures in the adult heart are implicated in cardiac arrhythmias such as re-entrant tachycardia [9]. Through the right and left bundle branches the stimulation signal is conducted to the Purkinje fibre network, which innervates the myocardium of both ventricles and transmits the electrical signal to the cardiomyocytes (Fig. 2) [74]. In contrast to the nodal cells, working cardiomyocytes cannot autonomously trigger an action potential. They are force-generating cells with mechanical activity that require external stimulation.

Cellular specification – at the heart of intrinsic pacemaking capacity

Pacemaker cells differ from the surrounding cardiomyocytes. This is mainly manifested by intracellular characteristics such as poor contractile properties, slow intercellular communication and signal conduction and expression of specific ion-channels allowing an inherent pacemaker potential [65]. Underlying cardiac electrical activity are membrane currents, fluxes of positive ions (sodium, calcium and potassium) and negative ions (chloride) across the cell membrane [55, 60].

The ability to initiate spontaneous depolarisation of cardiac myocytes requires a highly specialised cellular architecture, as it has been described for the pacemaker cells of the SAN and AVN. The core feature of nodal cells is their distinct electrophysiology, the pacemaker ppotential [60] (extensive review [63]). A slow, steady depolarisation across the cell membrane (raising the membrane potential) occurs at the end of one action potential and upon reaching the threshold, the next action potential is triggered (Fig. 3). Pacemaker cells possess a number of specialised ion-channels that allow the automaticity of membrane de- and repolarisation [75]. The cell type-specific electrophysiological properties are manifested in the nature of the action potential in working cardiomyocytes of the chambers and pacemaker cells in the node (Fig. 3). A characteristic property of the working myocardium action potential is the plateau phase, which is not seen in other action potential (e.g. neuronal). The plateau phase is a delay in repolarisation after the peak depolarisation and mainly attributed to the transient calcium current $I_{Ca,L}$. The action potential in pacemaker cells lacks a stable resting potential, resulting in onset of slow depolarisation right after repolarisation ceases. Though still debated, this is assumed to be a result of the lacking the potassium current I_{KI} and presence of the funny current I_f and special Ca^{2+}-channels characteristically found in cells with pacemaker potential. Characteristic action potentials for ventricular myocytes and SAN pacemaker cells with an indicative graph of the corresponding ion currents are depicted (Fig. 3). Atrial action potentials are not shown. They have been shown to differ from ventricular action potentials, especially in the shorter repolarisation phase [76, 77]. The rhythmic contractions of the heart can be measured as an electrocardiogram (ECG, see Box 2), which is an important diagnostic procedure to assess heart function.

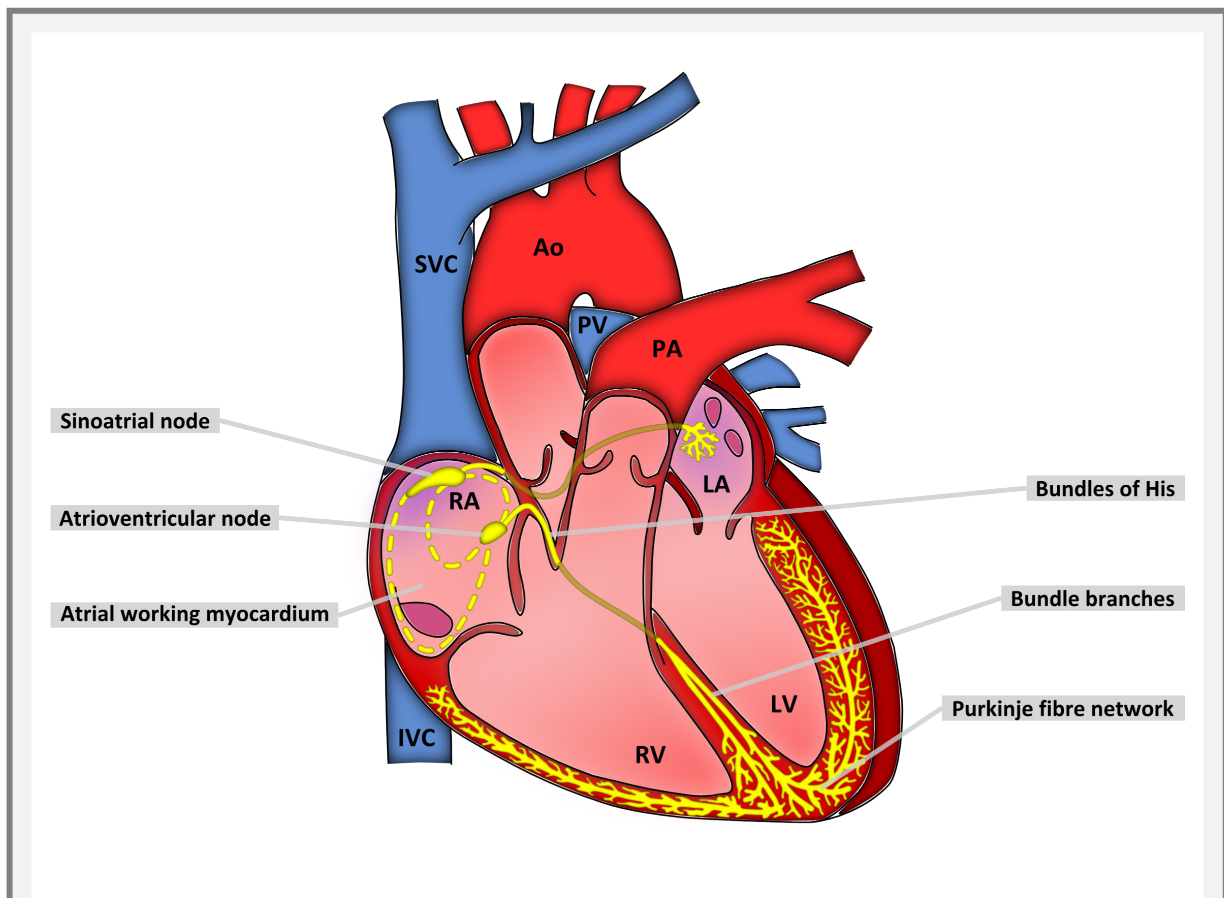


Figure 2

Major components of the cardiac conduction system in the human heart

The first impulse of a heartbeat is generated by the primary pacemaker cells within the centre of the sinoatrial node at the roof of the right atrium. The depolarisation signal is transmitted by the slow conducting peripheral nodal cells and passed on to the surrounding atrial cardiomyocytes. Through a direct conduction pathway (Bachmann's bundle or anterior interatrial band [1]) depolarisation is transported to the left atrium simultaneously. The electric signal rapidly travels through the fast-conducting working myocardium, causing the atria to contraction. When reaching the atrioventricular node at the floor of the right atrium, adjacent to the septum, the electrical impulse slows down. As the only connection to the ventricular part of the conduction axis, the depolarisation signal has to pass through the slow conducting AVN cells. Conduction delay allows the atria to contract fully and fill the ventricles with blood. The distal part of the AVN passes into the bundle of His. The fast conducting bundle descends into the interventricular septum and branches out into the bundle branches, which ultimately connect to the Purkinje fibre network. A mesh-like network of fibres innervating the ventricular myocardium and allowing rapid induction of the cardiomyocytes. Ventricular contraction pumps the blood from the ventricles into the pulmonary artery and aorta respectively.

Anterior view of the adult human heart. RA = right atrium; RV = right ventricle; LA = left atrium; LV = left ventricle; IVC = inferior vena cava; SVC = superior vena cava; PA = pulmonary artery; PV = pulmonary vein; Ao = Aorta

Reviews for further reading: [8-14]

Potassium channels

The largest group of cardiac ion-channels are the potassium (K^+) channels. They are very diverse in myocytes and vary regionally and between species. Normally most channels are closed, with some exceptions. In working myocardium cells, the inward rectifier current I_{KI} is responsible for maintaining the stable diastolic resting potential, which is estimated to be around -85 to -90 mV. I_{KI} is absent from pacemaker cells, partly responsible for their lack of a stable resting membrane potential in favour of constant slow depolarisation. I_{to} is a transient K^+ outward current that is important for initial repolarisation [78]. It is rapidly activated and immediately deactivated again in phase 1. Though also found in ventricular cardiomyocytes, it is most abundant in the atria [77]. It has been suggested, that I_{to} influences the balance between inward and outward currents during the plateau phase controlling duration and refractoriness of the action potential. By shortening the action potential in atrial myocytes, I_{to} could ensure swift repolarisation in preparation for the next action potential. Reduction in I_{to} has been reported to prolong action potentials, potentially contributing to cardiac arrhythmias. Two isoforms of I_{to} have been identified $I_{to,1}$ (constituted by K(v4.2) and K(v4.3)) and $I_{to,2}$ (formed by K(v1.4) and K(v1.7)) [2]. The delayed outward rectifier currents I_{Kr} , I_{Ks} and I_{Kur} all contribute to repolarisation [79]. Alterations in these potassium currents can modulate QT-phase leading to potentially dangerous arrhythmia [5]. I_{Ks} is of particular interest for heart rate control [80]. Because of its slow kinetics, channels cannot sufficiently close during shortened diastolic intervals, leading to an accumulation of opened K^+ channels, faster depolarisation phase ultimately further enhancing the heart rate. Furthermore, its activation is influenced by elevated Ca^{2+} levels and β-adrenergic stimulation. The recently identified I_{Kur} is an ultra-rapid outward current only found in atrial cardiomyocytes and important for fast repolarisation [81]. There are also ligand-dependent potassium channels, namely $I_{K,Ach}$, which is activated by elevated levels of acetylcholine and $I_{K,ATP}$ activated in response to reduced intracellular ATP concentration such as observed during acute ischemia [82].

Sodium channels

The cardiac Na^+-channel $Na_V1.5$ is the major voltage-gated ion channel responsible for depolarisation and present in working cardiomyocyte and node periphery, but absent from the node centre [19, 60, 71, 83]. Na^+ inward current is responsible for fast depolarisation in phase 0, inactivated at peak depolarisation and virtually absent in plateau phase. The α subunit is encoded by SCN5A. Mutations in SCN5A and other subunits are associated with several arrhythmia syndromes, such as long-QT and Brugada syndrome, showing a wide variety of disease phenotypes [21, 84]. It is not entirely clear how deregulation of one cardiac channel can be the underlying cause of such a diverse range of phenotypes. It highlights the importance of the cardiac sodium channel for normal pacemaking and conduction.

Figure 3

The cardiac action potential

Action potential of a ventricular working myocardium cells and the corresponding ion currents (A). Showing a rapid depolarisation, plateau of the deferred repolarisation phase and final return to the stable resting potential. The plateau phase is due to a cardiomyocytes-specific Ca^{2+} current and prolongs the cardiac action potential duration to about 200-300ms in human. In comparison, the basic neuronal action potential lasts for 1-5ms.

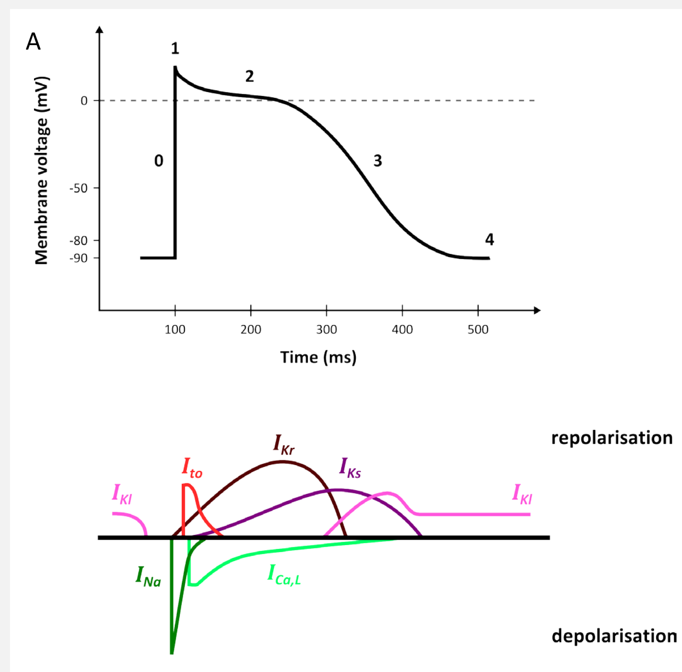
In contrast, the action potential of a nodal pacemaker cell and the corresponding ion currents (B). There is no stable resting potential. The continuous slow depolarisation eventually reaches the threshold, initiating an action potential. There is no plateau phase due to lack of the specific Ca^{2+} current. Upon cessation of repolarisation, the membrane slowly depolarises again autonomously.

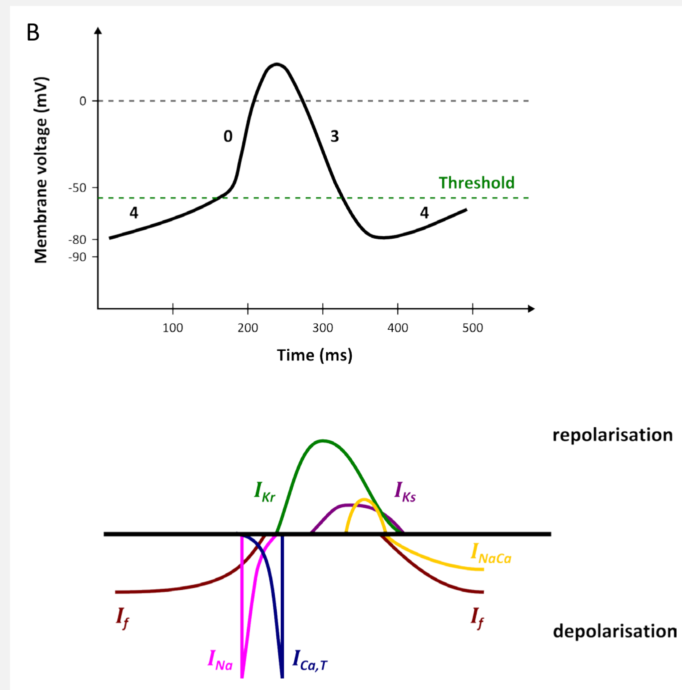
Phase 0: Depolarisation, either triggered by external stimulus (working myocardium) or intrinsic pacemaker potential (nodal cell). Facilitated by fast activation of I_{Na} and $I_{Ca,T}$ (pacemaker).

Phase 1: Peak of depolarisation and fast repolarisation. I_{Na} and $I_{Ca,T}$ (pacemaker) are deactivated. I_{to} activation causes initial repolarisation but is not maintained.

Phase2: Plateau phase. Activation of $I_{Ca,L}$ (working myocardium) is activated upon depolarisation and directly inactivated again. Due to slow kinetics $I_{Ca,L}$ prevails and prolongs the repolarisation phase. Balance between inward and outward currents.

Phase3: Terminal repolarisation I_{Kr} , I_{Ks} and I_{Kur} (atrial cardiomyocytes) are activated leading to an outward potassium current. I_{Cl} has been reported to be activated by high Ca^{2+} concentrations and enhance repolarisation. Activation of the Na^{+}/Ca^{2+} exchanger I_{NaCa} enhances repolarisation in pacemaker cells.





Phase4: Stable resting phase, maintained by I_{Kl} (working myocardium). Activation of I_f and I_{NaCa} in the pacemaker cells leads to automatic, slow depolarisation.

Note: Inward currents, depolarising the cell membrane are pointing downwards. Outward currents, repolarising the membrane are pointing upwards.

[2, 14, 18-21]

Calcium channels

There are two major types of voltage-dependent cardiac Ca^{2+} channels, L-type (long lasting) and T-type (transient) [2, 60]. L-type channels, responsible for $I_{Ca,L}$, activated upon depolarisation and inactivated at onset of the plateau phase have very slow kinetics. $I_{Ca,L}$ therefore lasts very long and, in combination with high channel conductance, facilitates maintenance of the plateau phase and delay in repolarisation, characteristic for working cardiomyocytes. However, $I_{Ca,L}$ has also been reported in nodal cells, where it is assumed to contribute to action potential upstroke [60]. They are also located at the sarcolemma, releasing Ca^{2+} from the sarcoplasmic reticulum upon depolarisation [85]. Ca^{2+} inflow is also important for the cardiomyocytes mechanical properties, as Ca^{2+} inflow triggers Ca^{2+} release from sarcoplasmic reticulum leading to sarcomere activation and ultimately cell contraction [86]. Sarcoplasmic Ca^{2+} release in reaction to electric stimulation plays a central role in excitation-contraction coupling. T-Type channels, responsible for $I_{Ca,T}$ open rapidly upon onset of depolarisation, but close again quickly. They are important for the acceleration of the action potential. Even though, $I_{Ca,T}$ is found in working myocardium, its contribution to upstroke of the action potential has not been proven, possibly due to it being overrun by the much stronger sodium current. Because of its rapid kinetics, $I_{Ca,T}$ is found in cells undergoing rhythmic electrical behaviour such as SAN and AVN pacemaker cells. $I_{Ca,T}$ has not yet been verified in human SAN but in various other mammalian species [56, 87].

The funny current

A unique current that has emerged as the major contributor to the generation of spontaneous pacemaker activity and rate control is the funny current I_f. It is found specifically in cardiac cells with pacemaker potential in the SAN, AVN and Purkinje fibres in the ventricular conduction system [2, 18, 19]. I_f is a mixed nonselective Na^+/K^+ inward current facilitated by hyperpolarisation-activated HCN channels (hyperpolarisation-activated, cyclic-nucleotide gated). HCN4 is the main constitutive subunit of funny channels in pacemaker cells. Upon membrane repolarisation after an action potential (phase 4), I_f-channels open when the membrane potential reaches the I_f threshold of approximately -40/45mV [64]. As a result, pacemaker cells slowly depolarise during diastole and automatically initiate the next action potential. HCN channels are controlled by the intracellular concentration of cAMP. It binds directly to the channel and alters the activation of diastolic depolarisation. cAMP is a second messenger in response to β-adrenergic (e.g. adrenaline, causing sympathetic acceleration) and muscarinic M2 (e.g. acetylcholine, causing parasympathetic deceleration) receptor stimulation [88]. Therefore I_f is suggested to be responsible for heart rate modulation by the autonomic nervous system [89]. The funny current is not completely continuous, it stops when the threshold for L-type Ca^{2+} current is reached, initiating the next action potential. At positive voltages after depolarisation, it is completely turned off and even carries a slight outward current for a short period (Fig. 3) [64]. The degree of activation of I_f determines the

frequency of the action potential firing. Further mechanisms of funny channel control have been described as well [90]. If I_f is blocked, heart rate slows down [91]. Since funny channels are highly specific to cardiomyocytes, they are a potential target for drugs aimed at controling the heart rate, such as the blocking agent ivabradine [92]. In contrast, perfusion with adrenaline leads to an acceleration of the heart rate [93]. A newly emerged interest in funny channels aims at delivering them into non-pacemaker cardiomyocytes to initiate pacemaking capacity as a novel approach to develop a biological pacemaker [94].

Calcium-clock versus voltage clock

While action potential generation and the interplay between the various ion currents have been thoroughly investigated, there is still a debate about the exact driving force behind the pacemaker automaticity and initiation of the electric signal. Two theories have been established to explain the underlying mechanism. Initially, rhythmic changes in the membrane voltage, induced mainly by the funny current and specific activity of T- and L-type Ca^{2+}-channels, have been proposed (the "membrane voltage clock") [64]. Recently, a second mechanism, the "calcium clock" has been implicated to contribute to the generation of pacemaker automaticity. Underlying is an increased intracellular Ca^{2+} concentration caused by spontaneous rhythmic release of Ca^{2+} from the sarcoplasmic reticulum [95]. Elevated levels of Ca^{2+} also activate a sodium-calcium exchanger, known to be expressed in nodal cells and initiating spontaneous depolarisation. At present, it is assumed, that both mechanisms together create the sinus rhythm [96]. Interplay between the membrane voltage clock and the calcium clock has also been suggested to link sympathetic stimulation to an increase in the heart rate. There is an ongoing debate about the significance of the different components on dynamic pacemaking regulation. Sympathetic stimulation leads to an increase in intracellular cAMP. It has been shown that cAMP can modulate HCN channels conductivity and influence the heart rate either by direct binding to HCN channels [88], or through the cAMP activated protein kinase PKA (in mouse) [97]. Furthermore, cAMP concentrations have been shown to modulate cycling Ca^{2+} levels, alternating the mechanisms of the calcium clock [96]. Abnormalities in both pathways can cause arrhythmia, highlighting the joined role in generating the basal pacemaker rhythm and modulating heart rate in response to nervous system stimulation [98-100]. Future research can hopefully narrow down the definite role of the different components and mechanisms to further understanding of cardiac rhythm control.

Chloride channels

Cardiomyocytes have been shown to express several chloride channels, such as the voltage-gated and volume-dependent CIC-3 and CIC-2 channels. It remains controversial to what extend chloride channels participate in cardiac function. Repolarisation-promoting effects by CIC-2-dependent Cl^--influx have been proposed [101]. Huang et al. implicated a function of an endogenous Cl^- inward rectifier channel in pacemaker function under stress conditions in small rodents [101]. Furthermore, autonomic regulation of action potential rate

by chloride currents has been found in several mouse model studies. In addition, Ca^{2+}-dependent Cl^{-}-channels have been reported to participate in the onset of repolarisation in depolarised cardiomyocytes. Little evidence of chloride channel activity has been obtained for human heart. Nevertheless, several cardiac pathologies have been linked to abnormal chloride currents [102, 103].

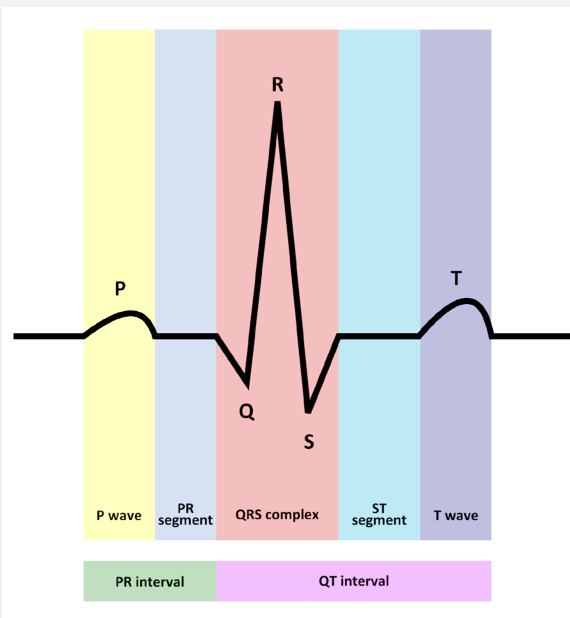
Stretch-activated ion channels

A further class of cardiac ion channel are the stretch-activated or mechano-sensitive channels. Such channels are assumed to act in response to mechanic tension especially in the ventricular myocytes during contraction. A non-specific cation (potassium) channel has been described [104]. Stretch-activated channels have been linked to a mechanoelectric feedback mechanism, which could contribute to the control of action potential duration [105]. Ward et al. could show elevated levels of Ca^{2+} and Na^{+} during diastole in a mouse model due to stretch activated channels [106]. These might contribute to development of dilated cardiomyopathy. However, the field of mechano-sensitive cardiac ion-channels remains controversial. More insight into the relation between electric and mechanical activity in the heart will be needed.

Box 2

Interpretation of cardiac electrical activity by electrocardiography

An electrocardiogram (ECG) essentially follows the electrical signal through the cardiac cycle or heartbeat. The start is shown by the **P wave**, which represents atrial muscle depolarisation and contraction following initiation by the SAN. It spreads through the atria towards the AVN. The **PR segment** represents the conduction delay at the AVN and transmission of the signal through the bundles, bundle branches and Purkinje fibres to the ventricles. P wave and PR segment form the **PR interval**, which corresponds to the time needed by the electrical signal to travel from the SAN through the AVN to the ventricular apex. The PR interval is a clinical estimate of AVN function. Repolarisation of the atrial myocytes is masked by the much stronger signal of ventricular depolarisation and not visible. The rapid depolarisation of the ventricular cardiomyocytes is illustrated in the **QRS complex**. The following **ST segment** is an isoelectric phase during which the ventricular cardiomyocytes are depolarised.



Repolarisation of the ventricles is represented by the **T wave**, the last step of the cardiac cycle. QRS complex, ST segment and T wave are combined in the **QT interval**, which thus represents the time it takes for the ventricular cardiomyocytes to recover. Alterations, especially a prolonged QT interval are of clinical significance and pose a risk for ventricular tachyarrhythmias [4-6].

Gap junctions

Cardiomyocytes of the working myocardium cannot trigger action potentials autonomously. They need external stimulation. In order to conduct the initial depolarisation signal from the pacemaker cells throughout the working myocardium, the cells are tightly coupled. Gap junctions composed of connexins allow intercellular communication. Connexins are found in various cell types. They constitute a family of transmembrane proteins, which assemble in the plasma membrane as hexamers, forming a hemichannel (or connexon). Neighbouring cells can communicate via gap junctions consisting of two adjacent hemichannels [107]. This allows exchange of nutrients, metabolites and other small peptides. Most importantly, they allow exchange of ions, facilitating propagation of the local membrane depolarisation. The resting potential in a cardiomyocyte is elevated by positive ion influx from the adjacent cell, lifting its membrane potential above the threshold to trigger an action potential, which is passed on to the next cell. Thereby rapid conduction of the electrical signal is possible. In the heart, predominantly Cx40, Cx43, Cx45 and Cx31.9 (ortholog of mouse Cx30.2, expression in human still subject of discussion) are expressed [60, 108] (Fig. 4). These cardiac connexins differ in conductance, Cx40 and Cx43 are fast conducting, Cx45 and Cx31.9 are slow conducting [109]. Therefore, a specific connexin expression pattern results in a characteristic cellular conduction phenotype [107]. Slow conduction has been described in the SAN and AVN. Chamber myocytes are fast conducting. The pattern of connexins at the junction of nodal to working myocardium cells has not yet been fully determined [107]. Furthermore, differential expression of cardiac connexins is also important in the developing heart and conduction system [110].

Within the SAN and AVN, cells are poorly coupled and show a reduced density in connexin expression. Cx45 is the predominant gap junction protein in nodal cells and Cx40 was found expressed in the peripheral part of the node [15, 57, 65, 111]. Electric intercellular coupling increases from centre to vicinity of the SAN and border with atrial muscle cells. This favours the exit of the electrical signal from the node centre and prevents retrograde hyperpolarisation [65]. Furthermore, Cx30.2 has been identified in the AVN and conduction system of the mouse, where it is involved in the delay of the electrical signal at the AVN due to its very low conductance [112]. The human ortholog Cx31.9 does not show node- or conduction system specific expression and is hardly expressed in the heart at all [113]. Cx40 is also expressed in atrial myocytes and parts of the conduction system, but absent from the ventricular working myocardium [60, 71, 107, 109]. In a recent publication, Greener et al. [71] studied expression levels of connexins in the human AV conduction axis. They could show that Cx40 and Cx45 were most abundant in the AVN and peripheral bundle system, whereas Cx43 showed a reverse expression profile. Cx43 is highly abundant in the ventricular working myocardium . It is less expressed in the atrial myocardium, co-localising with Cx40 and in parts of the conduction system excluding SAN and AVN [60, 73, 108, 109]. Connexins are highly redundant and can also form heteromeric channels, but reduced expression eventually leads to a reduction in cellular conductance [109, 114]. In adult cardiomyocytes, gap junctions are mainly

found at the long end of the cell, connecting to an adjacent cell and enhancing unidirectional fast conduction. Remodelling of connexins has been described in adult heart disease such as ischemia and heart failure [15]. It enlarges gap junction heterogeneity, resulting in impaired conduction. For instance, in a patient study, increased heterogeneity in ventricular Cx43 distribution correlated with impaired ventricular conduction and elevated risk parameters for ventricular arrhythmias and sudden cardiac death [115]. A cardiac-restricted knockout mouse for Cx43 was described by Gutstein et al. as having a similar phenotype. Mice suffered from slow ventricular conduction, spontaneous ventricular arrhythmias eventually leading to sudden cardiac death [116]. This highlights the importance of the expression and distribution of gap-junction proteins for cardiac function.

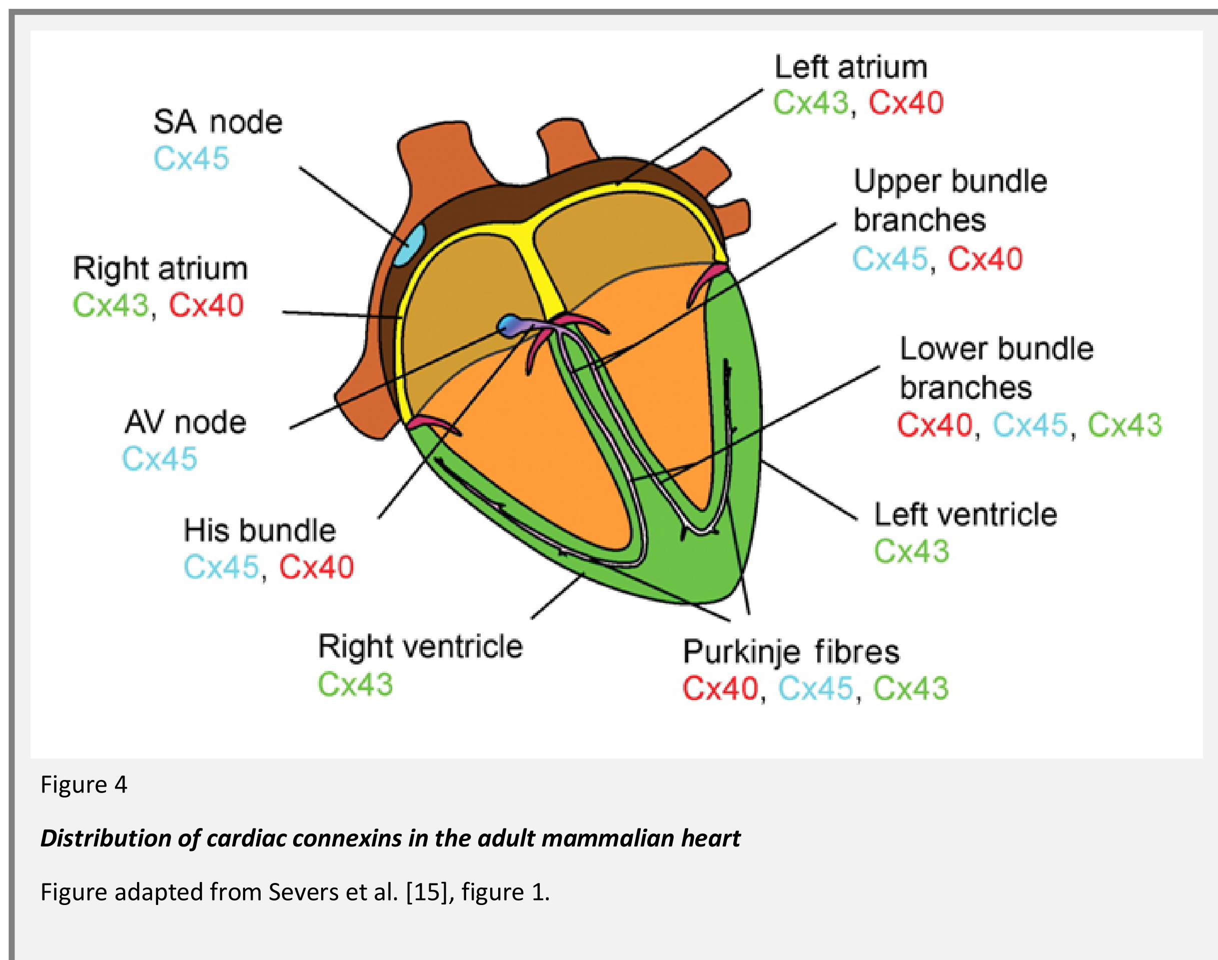


Figure 4

Distribution of cardiac connexins in the adult mammalian heart

Figure adapted from Severs et al. [15], figure 1.

Mechanisms during embryonic development

During vertebrate development, the heart is the first functional organ to form. Profound defects in early heart development often terminate embryonic development at early stages. Survival to later embryonic stages and adulthood is impossible without heart function and blood circulation. Biomechanical forces created by the hemodynamic blood flow contribute to numerous developmental processes in the embryonic circulation system [9, 117]. Therefore, the heart has to fulfil its function well before completing morphogenesis. Even though, the onset of cardiac contraction precedes the formation of a morphologically distinct pacemaker domain, correct development and function of the primary pacemaker is crucial for proper heart function in late embryonic and adult stages.

Development of the SAN and pacemaker primordium occurs during early heart development concurrent with the formation of the earliest heart structure. Therefore, it is crucial to examine pacemaker development in the context of general heart morphogenesis. The morphological development of the heart has been discussed in detail in numerous publications (see Box 3).

Special consideration should be given to the developing sinus venosus. It contains the sinus node primordium and is the source of dominant pacemaker activity in the early embryonic heart. Before establishing a distinct SAN, the cardiomyocytes at the inflow pole of the heart tube dictate the heart beat as a dominant pacemaker.

Onset of pacemaker automaticity in the embryonic heart

Nishii et al. [118] reported the first sign of contractile activity in small groups of cardiomyocytes in the early mouse heart tube (E8.25). Furthermore, they reinforced earlier observations stating that electric activity even precedes the first contractions, suggesting that the release of spontaneous depolarisation is independent of the maturation of surrounding contractile cardiomyocytes [9, 118]. The early onset of rhythmic cardiac contractions and their propagation has been attributed to regional specialisation facilitated by the differential expression of cardiac ion channels and their distribution across the heart tube [119]. No distinct pacemaker domain can be distinguished at these early stages. Nevertheless, the observation of independent rhythmic electrical impulses indicates the presence of cells with pacemaker potential. The contractions originate from the developing sinus venosus at the inflow tract and slowly travel towards the arterial pole of the heart tube.

Functionally, the vertebrate embryonic heart has recently been described as a dynamic suction pump able to create unidirectional blood flow before the development of distinct chambers and valves [120]. There is still an ongoing debate about the exact mode of embryonic heart function, with data supporting a peristaltic pumping mechanism or a suction pump mechanism respectively [120, 121].

With progression of development, transition from an early peristaltic to the pulsatile contraction pattern seen in adults, correlates with the development of complex heart structures such as valves, chambers and the conduction system. Mainly the establishment of a leading pacemaker at the SAN, the delay at the AVN and the distinct transmission properties of the conduction system facilitate the adult contraction pattern [9]. After cardiac looping, chamber formation and septation are completed, the SAN comes to lie in the upper right atrial wall adjacent to the systemic inflow region in mammalian hearts [8]. This illustrates the close structure-function relationship in the developing heart.

During elongation, when cells are added to both poles of the heart tube, the leading pacemaker site continuously shifts to the newly added cells [122]. This indicates that the primitive cells added to the heart tube either already possess an intrinsic pacemaker potential or acquire it simultaneously. It has been hypothesised that this shift is due to progressing maturation of cells into chamber cardiomyocytes accompanied by loss of pacemaker potential, possibly due to downregulation of Tbx18 and Hcn4 expression and initiation of Nkx2.5 expression in maturing cardiomyocytes [9, 123].

The phenotype of myocardium comprising the early embryonic heart tube resembles the nodal cell phenotype. Although it shows automaticity, the poor contraction and slow transmission of the electric signal result in a slow, peristaltic contraction pattern [16, 124]. This can be measured as a sinusoidal ECG [9].

It raises the question whether all primitive myocytes have the ability to elicit spontaneous action potentials and whether the pacemaker potential is an acquired phenotype in nodal cells or repressed in cardiomyocyte differentiation. The early contractions of the heart tube already show a simple coordination system, with the leading site of contraction initiation at the posterior venous pole, before a distinct node structure has developed. The embryonic heart generates coordinated atrial and ventricular contraction as early as at the onset of their formation, despite not having developed a discrete pacemaker and conduction system or atrioventricular insulation [12]. During cardiomyocyte differentiation, the chamber working myocardium acquires fast-conducting and contractile properties to accommodate the increasing need for fast and powerful contractions to ensure sufficient heart function in the growing organism. Contraction initiation and coordinated transmission becomes progressively restricted to a specialised pacemaker and conduction system.

The myocytes in the embryonic heart tube express Nkx2.5. Adjacent to the inflow tract, a population of Nkx2.5-negative myocytes are prevented to mature into working myocardium and instead give rise to the SAN [123]. It has been shown, that the sinus venosus myocardium develops from Nkx2.5 negative, Tbx18 positive non-cardiac mesenchymal progenitors, which are added to the posterior pole during elongation, instead of arising from specialised myocardial cells [125]. After the initial differentiation, sinus venosus and SAN grow by continuous slow proliferation [126]. The sinus venosus later on acquires atrial properties, restricting pacemaker function to the distinct SAN [123]. A cascade of transcription factors has been established as causative to this spatial divergence in myocytes development (Fig. 3).

Regulatory transcription factor network patterns the SAN

Patterning of the distinct regions of the heart and development of the SAN is facilitated by a regulatory network of transcription factors (see schematic Fig. 5). Cells in the mature SAN are specialised myocardial cells, but show a primitive phenotype. During differentiation, development into working myocardium is actively inhibited in pacemaker cell, retaining the primitive myocyte phenotype. SAN cells express pacemaker-specific ion channels (HCN4) and gap junction proteins (CX45, CX30.2), allowing generation of spontaneous action potentials. Differential gene expression determines development of a nodal versus a working chamber myocardium phenotype. With respect to SAN development, the most important factors are the T-box transcription factors TBX18, TBX5 and TBX3, as well as the homeobox protein NKX2.5 and short stature homeobox2 (SHOX2). Especially TBX3 and NKX2.5 have a central and antagonistic function in pacemaker differentiation.

The family of T-box transcription factors controls various developmental processes, including heart development [127-129]. *Tbx20* is expressed in the first and second heart field and required for heart tube elongation and chamber formation [127, 130]. It activates several cardiac chamber differentiation factors including *Nkx2*.5 [131]. *Tbx18* is expressed in presomitic mesoderm from E7.75 onwards in mouse [132]. It induces myocardial differentiation in the mesenchymal precursor population and is essential for sinus horn formation [125]. Due to their distinct developmental potential and genetic makeup, these *Tbx18*-positive precursors have been considered either a distinct sublineage of the second heart field or a third heart field [125, 127]. The expression pattern of *Tbx18* is complementary to *Nkx2*.5, confining sinus horns from the atria and is presumably due to upstream regulatory processes [125, 126]. *Tbx18*$^{-/-}$ mice fail to form sinus horns leading to a deformed SAN lacking the head, but still expressing *Hcn4* indicating functional differentiation [125, 126]. Indeed, it has been shown that *Tbx3* is responsible for SAN cell differentiation independent of *Tbx18* [126]. *Tbx18* actively represses atrial natriuretic factor (*Anf*) and atrial differentiation in the developing SAN by competing with *Tbx5* and repressing *Gata*-4 and *Nkx2*.5 [133]. TBX5 is an early patterning factor and is expressed in all cells derived from the first heart field. Potentially restricted by retinoic acid signalling, TBX5 implies posterior identity to the heart tube [127]. It interacts with NKX2.5 and GATA-4 to drive atrial working myocardium differentiation by recruiting ANF [127, 134, 135]. Mori et al. used an allelic series of *Tbx5* mouse mutants to show pronounced dosage sensitivity during heart development [136]. TBX5 negatively regulates the SAN promoting factor SHOX2, which is specifically expressed in venous pole myocardium in mouse, zebrafish and xenopus [137-141]. SHOX2 is crucial for SAN development, deficiency leads to atrialisation of the SAN, secondary to ectopic *Nkx2*.5 expression and impaired pacemaker function [137-139]. Through repression of *Nkx2*.5, SHOX2 enables expression of a genetic cascade including *Tbx3* in the SAN primordium, driving pacemaker differentiation [138]. Inhibition of *Shox2* by the asymmetry factor PITX2 inhibits ectopic left-sided pacemaker formation [123, 142]. SHOX2 activates BMP4 at

the inflow pole in concert with SAN promotion as shown in xenopus, mouse and human (in vitro) [137]. Differentiation of the SAN cells to acquire a pacemaker phenotype is driven by TBX3. It is expressed in the nodes and conduction system components, but not in the working myocardium [143]. TBX3 inhibits expression of chamber myocardium differentiation factors such as *Anf*, *Cx40* and *Cx43* [143-145]. *Tbx3*$^{-/-}$ mice are embryonic lethal between E11-E13, but form a SAN primordium [123]. The expression boundary to *Nkx2.5* expressing chamber myocardium is still set up, confining TBX3 function to prevention of a chamber-specific expression programme in an already spatially restricted node [123]. If ectopically expressed in the heart tube prior to myocardial differentiation, TBX3 inhibits expression of *Anf* and *Cx40* and abrogates chamber formation [123]. A similar molecular role has been described for TBX2 [146]. However, it is exclusively expressed in non-chamber myocardium of the AVC and OFT, but absent from the SAN in mouse [127] and human [146]. *Tbx2*$^{-/-}$ mice develop a normal SAN [127].

The homeobox protein NKX2.5 shows a radically opposite expression pattern to TBX3. *Nkx2.5* is expressed in the early mouse embryo from E7.5, first in the cardiac progenitor cell pool subsequently forming the cardiac crescent [147]. Until E9-E9.5 in mouse, *Nkx2.5* is expressed in all myocardial cells from both the first and second heart field. *Nkx2.5* null mice form a heart tube containing cells originating from both heart fields, but fail chamber formation, indicating that *Nkx2.5* is required for differentiation rather than migration of cells [148, 149]. *Nkx2.5* expression seems to be the default status in cardiomyocytes, as not much is known about specific activators and patterning in the developing heart depends on negative regulation of *Nkx2.5*. STAT3 has been implicated as a mediator of cardiomyocyte differentiation in vitro by promoting, amongst others, *Nkx2.5* expression [150]. Differentiating sinus horn myocardium cells from E9.5 onwards fail to express *Nkx2.5* and do not initiate a chamber myocardium expression profile [125]. The mesenchymal progenitor pool of the sinus venosus needs to be devoid of *Nkx2.5* expression to initiate SAN differentiation. NKX2.5 represses *Tbx3* and *Hcn4* expression in the heart tube, thus inhibiting SAN development and driving maturation into chamber working myocardium. Furthermore, it shows laterality by preferential expression on the left side of the heart at later stages (from E10.5-E11.5) [147]. Expression of *Nkx2.5* is controlled by SHOX2, strictly excluding it from the venous pole and developing SAN [151]. Ectopic expression of *Nkx2.5* in the SAN primordium inhibits pacemaker cell development, leading to hypoplastic sinus venosus and arterialisation [151]. In contrast, knockout mouse studies showed that NKX2.5 is crucial for initiation of an atrial expression pattern in primitive cardiomyocytes and directly regulates expression of *Anf* and cardiac connexins [152-155]. *Nkx2.5*$^{-/-}$ mice are embryonic lethal around the time of sinus venosus development and chamber differentiation [152]. The significance of *Nkx2.5* expression is not limited to embryonic development.

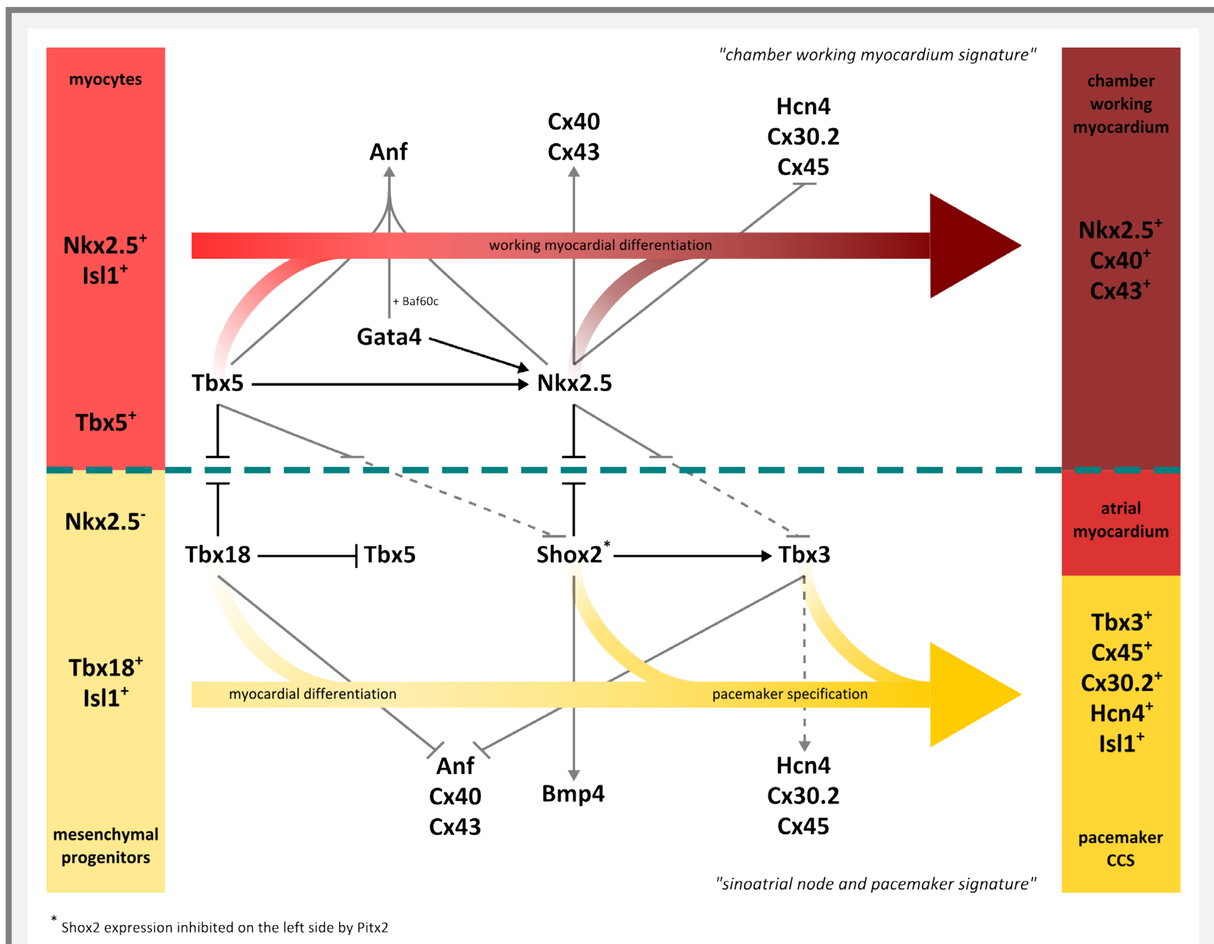


Figure 5

Important factors in the specification of pacemaker cells in the SAN and atrial working myocardium

SAN cells arise from a *Tbx18*$^{+}$ *Nkx2.5*$^{-}$ mesenchymal progenitor population located adjacent to the *Nkx2.5*$^{+}$ posterior heart tube myocytes. TBX18 is the main driving factors of myocardial differentiation in the mesenchymal progenitors. It delineates the SAN primordium by competing with TBX5 and functionally repressing atrial differentiation factors such as *Gata4*, *Nkx2.5* and *Anf*. SHOX2 inhibits *Nkx2.5* expression and activates *Tbx3*. It is a direct target of laterality factor PITX2 and inhibited in the left compartments of the developing heart. TBX3 is the main factor to directly or indirectly activate pacemaker-specific factors. TBX5 interacts with GATA4 and NKX2.5 to initiate atrial working myocardium differentiation. TBX5 can repress *Shox2*, inhibiting the SAN promoting factor in the working cardiomyocyte lineage. NKX2.5 is the main determining factor for chamber myocardial cells and activates working myocardium-specific factors.

The transcription factor network leads to the establishment of specific gene expression signatures. The SAN is characterised by high expression of *Hcn4*, *Cx30.2* (mouse) and *Cx45*, corresponding with low expression or absence of *Cx40*, *Cx43* and *Scn5a* in embryo and adult. Atrial working myocardium shows a contrary expression pattern with high expression of *Cx40*, *Cx43*, *Scn5a* and *Anf* corresponding to low or absent expression of *Cx30.2* (mouse), *Cx45* and *Hcn4*.

Note: With developmental progression, pacemaker activity becomes restricted to cells within the SAN. The remaining cells of the sinus horns gradually activate an atrial myocardium expression pattern (express *Cx40*; repress *Hcn4*).

Conduction defects in inducible mouse models indicated the necessity of *Nkx2.5* expression in perinatal [156] and, to a progressively lesser extent, in postnatal hearts, when cardiomyocytes have ceased differentiation [157].

Additional factors involved in SAN development are ISL1, GATA4, COUP-TFII and HCN4. Islet1 is a LIM/homeodomain transcription factor expressed in cardiogenic precursor cell populations in the lateral plate mesoderm [22, 158]. It is important for the maintenance of the precursor state in cardiac progenitor cells and required for cellular migration into the heart tube, contributing to the OFT, right ventricle and atria [22]. SAN cells originate from multipotent *Isl1*-positive progenitors and while *Isl1* expression is downregulated upon working myocardial differentiation, it remains expressed in the nodes in mouse [159] and human [36, 160]. In human embryonic hearts, the entire sinus venosus is positive for ISL1, overlapping expression with TBX3, but strictly complementary to NKX2.5 [12]. Laugwitz et al. showed that ISL1 positive, self-renewing cardiac progenitors could be found in the postnatal heart of human and mouse, implicating a possible cardiac repair capacity [161]. It has been proposed that during cardiogenesis, cells arise from a common ISL1 positive multipotent cardiac progenitor cell pool [158, 162]. Therefore, it has been speculated that ISL1 has a role in maintaining the primitive expression phenotype in pacemaker cells, but further investigation on the role of ISL1 in pacemaker development is necessary to pinpoint its function. TBX20 and Forkhead transcription factors have been implicated in the regulation of *Isl1* expression in the cardiac progenitors [131, 163, 164]. BMP and FGF-signalling pathway components as well as MEF2C are potential downstream targets of ISL1 [22, 165]. In zebrafish, Isl1 has been shown to be important for differentiation and lengthening of the inflow pole from progenitor cells. In *isl1* mutants, Bmp4 expression is reduced at the venous pole, whereas early cardiomyocyte markers (Nkx2.5) remained unaffected [38]. A regulatory feedback loop between ISL1 and BMP4 has been observed in mouse dental development [166].

GATA4 is a zinc-finger transcription factor. In differentiating cardiomyocytes, it interacts with TBX5 and NKX2.5 to activate *Anf* expression [127, 134, 135]. In vitro studies could show that GATA4, TBX5, NKX2.5 and BAF60C are sufficient to induce mouse non-cardiac mesoderm or human embryonic stem cells to differentiate into contractile cardiomyocytes [127, 134, 167]. It was also shown that GATA4 is the key factor to initiate cardiomyocyte differentiation, whereas TBX5 is crucial to establish the contractile phenotype [134]. GATA4 interacts with the orphan nuclear receptor COUP-TFII to synergistically activate *Anf* and promote atrial cardiomyocyte differentiation [168]. Early development of the posterior heart tube depends on expression of COUP-TFII and mutant mice fail to progress atrium and sinus venosus development past the initial specification [169]. BAF60C is part of a heart specific chromatin-remodelling complex that allows transcription factors to access the DNA and in differentiating atrial cardiomyocytes, it is required for GATA4 interaction with TBX5 and NKX2.5 [170]. The exact mechanism of the BAF60C-GATA4 interaction remains to be determined.

In addition to the role of PITX2C in inhibiting ectopic pacemaker development on the left

side of the heart, in vitro studies have indicated that it can actively induce chamber myocardial differentiation [171]. It remains to be determined whether the observed activating effect is directly caused by PITX2C or a secondary effect due to inhibition of repressors such as SHOX2.

HCN4 is a SAN specific marker in developing and adult mouse heart [172]. It constitutes the main potassium channel responsible for pacemaker automaticity and is highly restricted to pacemaker cells in the adult mouse heart and crucial for mature pacemaker function [64, 173]. *Hcn4* null mutants are embryonic lethal due to the failure to establish a pacemaker structure, resulting in extremely low heart rates [173, 174]. It is expressed by various structures during development, but expression levels decline in working myocardium upon maturation. *Hcn4* expression in working myocardium is restricted by NKX2.5 [139].

In summary, TBX3 is the main driving factor for SAN development, while NKX2.5 determines the working myocardial fate. NKX2.5 is the upstream regulator of cardiomyocyte differentiation and has to be strictly controlled in the developing SAN [143]. However, the complex and multifactorial regulation of the transcription factor network is demonstrated by the observation that single manipulation of either factor is not sufficient to transform myocytes into the corresponding phenotype [123, 151, 152]. It is assumed that working cardiomyocyte differentiation is the default pathway in myocytes development [34]. Therefore, establishment of a functional SAN depends on two mechanisms, active inhibition of cardiomyocyte differentiation and activation of the SAN specific gene expression pattern. Understanding of the complex network will be necessary to succeed in experimentally generating a biological pacemaker or enhance understanding of congenital heart disease and arrhythmias. Recently, Wiese et al. reported that addition of suramin, (aka germanin) an ion channel regulatory compound, could induce differentiation of mouse ES cells into sinus node-like cells as shown by the characteristic gene expression and electrophysiology patterns. They hypothesised that suramin simultaneously reduces *Nkx2.5* and enhances *Tbx3*, *Tbx2* and *Hcn4* expression [175].

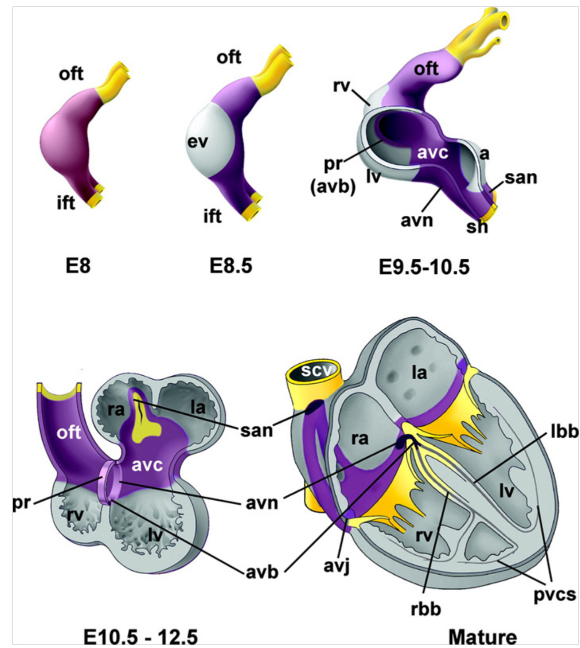
Comprehensive studies on the developing human heart are sparse, mainly due to limitations in the acquisition and experimental manipulation of human embryonic material. The question is to what extend data obtained from other mammals (mouse, rabbit etc.) is conferrable to humans? A few studies have elucidated the anatomic structures of human embryonic hearts at different stages [12, 35, 36, 176, 177]. Early human embryonic heart lacks distinct conduction system and displays widespread atrioventricular continuity (no delay), still achieves coordinated contractions of atria and ventricles [12]. Overall, they documented a great overlap in structural and molecular properties of the human heart with more commonly studied mammalian species (e.g. mouse). Regarding the important transcription- and patterning factors, TBX18, TBX3, NKX2–5, ISL1, HCN4, and the common connexins were found in the human embryonic heart with similar expression patterns as in mouse [12, 35].

Furthermore, a vast number of human pathologies have been attributed to these factors and resemble phenotypes observed in animal studies (Box 5). For example, PITX2 is also an important laterality factor in human. Right atrial isomerism due to lack of PITX2

leads to bilateral sinus node development with ectopic pacemaker activity [178]. Deregulation of TBX5 is known to cause Holt-Oram Syndrome, a congenital disease presenting a wide range of heart defects, including cardiac conduction abnormalities [179]. Congenital heart defects in human often present a high variability in severity and distinct disease characteristics. Considering the complex and highly interrelated network of factors involved in heart and specifically pacemaker and conduction system development, it is not surprising.

Box 3

Basic overview of vertebrate heart development.



Heart development starts soon after gastrulation in the early embryo and can be subdivided broadly into five successive but overlapping phases. Initial specification of cardiac precursors in bilateral regions of the anterior lateral plate mesoderm. They fuse at the midline and form the cardiac crescent [16]. The cells of the cardiac crescent fold ventrally towards the midline to give rise to the linear heart tube. Rhythmic contractions of the linear tube commence shortly after its establishment. The heart tube then undergoes rightward looping acquiring left-right polarity. The cardiac chambers form from distinct areas within the heart tube subsequently separated by valves and septa (in mammals). The cells of the early heart tube contribute to the future left ventricle and atrioventricular canal (AVC) [22]. The outflow tract, right ventricle, atria and inflow tract (including the sinus venosus) form during looping of the heart tube by addition of mesenchymal precursor cells from the mesoderm of the secondary heart field, which includes the anterior heart field that gives rise to the OFT and right ventricle [24-27].

Though still a matter of debate, it is assumed, that cells contributing to heart formation arise from two distinct pools of progenitors, the first and second heart field. The first heart field comprises the bilateral groups of progenitors in the anterior lateral plate mesoderm that form the cardiac crescent and subsequently give rise to the early linear heart tube. In the mature heart, cells originating from the first heart field only contribute to the left ventricle. The second heart field is an additional pool of progenitor cells located adjacent to the cardiac crescent. When the heart tube starts elongating, cells from the second heart field migrate and attach to both poles. They give rise to the left atrium and components of the CCS including the nodes.

Although species-specific differences exist, the basic patterning of the heart is conserved in mammals and most vertebrates. Even invertebrates show a striking degree of conservation in heart morphogenesis and molecular development.

Chamber myocardium (gray), nonchamber myocardium (purple), inflow tract (ift), sinus venosus (sv), outflow tract (oft), embryonic ventricle (ev), right ventricle (rv), left ventricle (lv), primary ring (pr), atrioventricular canal (avc), atrioventricular node (avn), sinus node (san), sinus horns (sh),atrioventricular junction (avj), atrioventricular canal (avc), atrioventricular bundle (avb), right atrium (ra), left atrium (la), right bundle branch (rbb), left bundle branch (lbb), superior caval vein (scv), peripheral ventricular conduction system (pvcs)

Reviews for further reading: [16, 25, 32-36] (in mammals); [25] (in chicken); [3, 17] (in zebrafish); [42] (in drosophila); [25, 43-45] (on the second heart field); [49] (transcription factor network); [9, 10, 50-52] (AVN and cardiac conduction system development)

Figure adapted from Christoffels et al., 2009 [9]

On the evolution of the cardiac pacemaker

The heart is an evolutionary success story. During the course of heart evolution, novel structures and functions have been added to the primitive ancient pump. The network of transcription factors described during mammalian embryonic heart development shows a high degree of conservation. Similar signalling pathways controlling muscle growth, patterning, and contractility have been found in animals as distantly related as humans and drosophila [16, 42, 180-190]. The phenotypic consequences of human congenital heart defects, caused by mutations in this network oftentimes highlight the significance of evolutionary achievements during the adaption of the heart to human physiology. For example, the need for separation of the systemic and pulmonary circuit by septation is obvious in profound septal defects.

Even the most basic heart-like structure shares the common crucial feature of all hearts, the ability to rhythmically contract and pump fluid through the body. The heart is the motor of a fluid-based transport system for nutrients, metabolites and oxygen throughout the body. Even animals with radically different lifestyles, such as insects, fish, birds and terrestrial animals show a striking conservation in cardiac development and function [180, 185, 188-190]. The cardiac pacemaker and conduction system in the mammalian heart can be considered the latest important advancement in increasing cardiac efficiency. Mammals possess a sophisticated network of pacemaker nodes, preferentially coupled cardiomyocytes and a fast conduction system enabling coordinated, sequential contraction of the chambered heart. In comparison, the primitive tubular pumps in invertebrates resemble early mammalian embryonic hearts both in structure (slow-conducting, poorly coupled myocytes, lack of valves and a conduction system) and function (peristaltic contraction pattern) [36, 71, 121, 181, 184, 189, 191]. It is appealing to hypothesise, that these analogies reflect the ancestral background of the mammalian heart. Thus, analysis of heart morphogenesis from an evolutionary perspective might help understanding mechanisms observed during embryonic development. Many of the morphological changes of the heart have been attributed to physiological adaption of an ancestral cardiac network to an increase in body size and complexity. With regard to the pacemaker and conduction system, it is unclear when the distinct structures evolved.

Concerning the evolutionary development of the heart, several hypotheses have been established. It has been well documented, that body size (and increasing complexity) and heart size of an organism are closely correlated, a phenomenon known as scaling [185, 192]. Furthermore, heart rate and cardiac output correlate with body size, with a fast-beating heart in small animal and a slower heart beat in bigger animals [185, 192, 193]. The larger an organism grows, the bigger the stroke volume of the heart has to be to insure sufficient circulation [182, 185, 192]. This led to the assumption, that the heart capacity poses a main growth restriction on the organism. In order to overcome this restriction, heart structures became increasingly more complex

during evolution, while the underlying principle of a muscular pump remained highly conserved.

The rhythmical heartbeat is the central functional characteristic of the cardiac pumping organ. The ability to contract and transform an electrical signal into physical energy to actively transport fluid is the identification feature of hearts. When discussing the evolutionary development of the heart, it is necessary to define basic characteristics, a structure ought to have in order to be classified as a heart. The basic definition of a heart might be an arrangement of cells forming a hollow contractile structure able to actively transport fluid (haemolymph or blood). Thus, this definition includes the very basic peristaltic pumping organs found in invertebrates. The cardiac pacemaker might be understood as the specialised, intrinsic structure initiating the cellular contractions.

In 1968 M. Anderson gave a definition for the cardiac pacemaker in her work on *Ciona intestinalis*:

> *"The heart of the tunicate Ciona intestinalis is a simple tube composed of epithelial muscle cells which - though isolated from any neural connexions - is capable of producing repeated contractions at reasonably constant frequency. It is thus a myogenic pacemaker system in which spontaneous rhythmic activity is derived from some intrinsic source." [194]*

Regarding the cardiac pacemaking system, several questions should be addressed. Did the distinct pacemaker evolve out of necessity to accommodate the growing heart in higher organisms to ensure a controlled contraction pattern, increase in blood pressure and hemodynamic force? Did it evolve to ensure coordinated, unidirectional blood flow in a separated systemic-pulmonary circuit? Was it crucial as a mediator to allow heart regulation by the nervous system? Is the human cardiac conduction system the latest and most sophisticated addition to the cardiac network?

The first heart-like organ is believed to have evolved in an ancestral bilaterians about 500 mya [16, 180, 188]. This ancestral "heart" was likely a simple tubular structure, consisting of a single layer of pulsatile cells to force fluid through pericellular interstices without an enclosed vascular system [180]. The initial appearance of muscle-like cells is not entirely clear, but is said to have emerged from the gastrodermis prior to the divergence of Cnideria and Ctenophora from bilaterians [186]. Muscle cells are of mesodermal origin and present in all triploblastic animals. Mesodermal cells specifying into early primitive myocytes arose first in bilaterians [180]. It remains to be determined at what stage a subset of cells became functionally dominant to coordinate the cellular contractions. Morphologically it might have resembled the simple tubular heart found in *Amphioxus* [195].

Diversity of heart pumps in protostomia

In protostomia, several approaches to maintain circulation have emerged. Unlike

their fellow protostomian relatives, nematodes (roundworms) such as *C. elegans* do not have a heart. However, they have a contractile pharynx (foregut), which has cardiomyocyte-like electrical properties [186]. It is still subjected to debate, to what extend the nematode pharynx can be considered homologous to the vertebrate heart or as a related muscular pump that has arisen simultaneously during evolution [196]. Gut and circulation system structures are evolutionary related and during embryogenesis in insects, molluscs and annelids, heart tube formation begins with an invagination from the gut and continuity persists through to adulthood [180]. It is assumed that all heart pumps in bilaterians arose from a common coelomic progenitor [190]. Nervous innervation in the pharynx is believed to underlie the initiation and control of contraction. Similarities to vertebrate cardiomyocytes have been found in the ionic channels responsible for changes in membrane polarisation [197]. Nevertheless, a distinct and independent pacemaker system has not been identified [196, 197]. The nematode pharynx is a myoepithelial structure and not of mesodermal, but ectodermal origin [186]. Hence, the intrinsic pacemaker potential of higher organism seems to be a property of mesodermally derived cells.

The cephalopods are an exception in the invertebrate group, as they have a closed circulatory system, multi-chambered muscular hearts, valves and are commonly considered to have the most complex invertebrate heart [198]. Circulation is facilitated by three heart pumps, two pumps at the gills and one systemic heart which appear to have a independent pacemaker [199].

Bilateral pacemaker system in Drosophila melanogaster

Heart formation in arthropods has been widely studied and the most prominent model organism is the fruit fly *Drosophila melanogaster.* The drosophila heart (also referred to as the dorsal vessel) is a tubular organ consisting of a single layer of contractile mesodermal cardioblasts and an overlying pericardial cell layer [180, 191]. As generally found in arthropods, drosophila has an open circulatory system with a dorsally positioned heart able to pump haemolymph through the body [200]. The heart functions as a linear peristaltic pump. There are no distinct chambers, but an aortic valve structure at the anterior opening supports fluid flow direction [180, 186, 200]. On the posterior side, four pairs of ostia with a cellular flap functioning as a valve open into the heart lumen [191, 200, 201]. Ostia are believed to be the drosophila analogue to vertebrate inflow tract structures. It has been shown that ostia formation depends on a distinct gene expression programme, similar to the differential development of sinus venosus structures versus chamber myocardium in vertebrates [202]. The primary pacemaker is situated at the caudal end of the heart and peristaltic contractions move anteriorly to expel haemolymph into the aorta [203].

The heartbeat in drosophila is of myogenic origin, since larvae lack nervous innervation of the heart, only an intrinsic pacemaker potential can initiate the peristaltic contractions [204-206]. After metamorphosis, the heart receives neuronal input [206, 207]. Molecularly, the cardiac muscle cells in drosophila and

mammalian species show a striking degree of similarity. *Tinman*, the drosophila homologue of NKX2.5 is the determining factor underlying cardiomyocyte differentiation [200]. *Seven-up*, the homologue of vertebrate COUP-TF 1 and 2 is specifically expressed in the posterior part of the dorsal vessel in myocardial cells giving rise to the ostia [200]. Furthermore, homologues of important mammalian cardiac factors also partaking in drosophila cardiogenesis are *tailup* (ISL1), *pannier* (GATA4), *dorsocross* (TBX) [208-210].

Pharmacological studies could show, that the important ion-channels found in mammalian myocytes are also present in drosophila [211]. The only major difference was the substitution of the inward sodium current with an inward calcium current as the main depolarization current [200, 211]. The mechanism underlying the pacemaker potential in drosophila has not been identified. DMIH, a homolog of the pacemaker-specific HCN4 is present. It similarly encodes for a subunit of the slow inward hyperpolarisation-activated potassium channel (I_h-channels) [212]. However, whether I_h- is present in the drosophila pacemaker remains to be determined. The *Ork1* gene, encoding a two-pore domain potassium channel facilitating an open rectifier K^+-current, has been demonstrated as a critical component of the drosophila pacemaker system [213]. However, it seems to rather have a heart rate modulating effect by regulating the duration of the slow diastolic depolarisation without influencing the basal cardiac automaticity [213]. Whether pacemaker depolarization in drosophila relies on a mechanism similar to the funny current I_f or a calcium clock mechanism as described in mammals remains to be determined.

A feature not present in vertebrates is the ability to reverse the direction of fluid flow through the adult heart [200, 201]. The reason for cardiac reversal is not entirely clear, but it is unique to open circulatory systems and seemingly supports maintenance of fluid circulation. Apart from insects, an alternating pacemaker mechanism is also found in tunicates [184]. It shows periodic reversal of the pumping direction to an anterior-to-posterior pattern, generating two alternating pacemaker phases [200, 201, 206, 214]. Therefore, drosophila is believed to have two independent pacemakers at the post-metamorphosis stage (Fig. 6A) [191]. The primary pacemaker is situated at the caudal tip of the dorsal vessel, extending to the central pair of ostia. A second pacemaker is localised at the anterior exit of the heart around the aortic valve [191]. Imaging studies could show that cardiac reversal shows a periodic pattern, suggesting that it is part of the normal circulation as opposed to a specialised feature or backup mechanism. Retrograde haemolymph flow as a result of reversed pacemaker dominance has been attributed to neuronal stimulation of the anterior pacemaker [201]. In the currently proposed model, neuronal stimulation, likely by glutamatergic neurons, activates or enhances the anterior pacemaker, enables it to overrule the dominant posterior pacemaker and reverse the contraction direction from posterior-to-anterior to an anterior-to-posterior pattern [206]. The hypothesis of nervous stimulation as the underlying cause of cardiac reversal is supported by the observation, that drosophila larvae lacking nervous innervation of the heart, do not show reversal of the direction of blood circulation. At larval stages the caudal pacemaker is the dominant pacemaker [191].

In summary, the pacemaker system in the drosophila dorsal vessel is divided into an autonomous caudal pacemaker maintaining continuous fluid flow and an additional inducible secondary pacemaker at the anterior outflow tract with the potential to reverse the contraction direction. It therefore combines two reasonable mechanisms by which repeated rhythmic contraction can be achieved, nervous system control with an external pacemaker and intrinsic control by an independent myogenic pacemaker. In contrast, the mammalian SAN is always the dominant pacemaker (under healthy conditions) and responsible for both the spontaneous rhythmic contractions and heart rate modulation in response to nervous system stimulation.

Despite the large evolutionary distance between arthropods and mammals, there is compelling evidence supporting a close relationship between their heart structures. This indicates that a basic tubular heart had been present in the common bilaterians ancestor and although gene regulatory modifications to accommodate the growing organism lead to morphologically distinct structures, common basic mechanisms are still conserved [180].

Basic circulation system in early deuterostomia

A basic, but well-studied organism is the tunicate *Ciona intestinalis* (ascidia, urochordata). It has an open circulatory system with a curved, V-shaped heart (Fig. 6B) [215]. The tube consists of cardiac myoepithelium containing striated myofilaments and an outer pericardial lining, but no endocardium [216]. Deuterostome evolution coincided with a multiplication and functional divergence of contractile proteins [180]. There are no chambers or valves discernible and the tube itself does not show morphological polarity [188]. It is situated ventrally, close to the stomach and opens into single vessels at the posterior end connecting to the endostyle, at the anterior end to the dorsal part of the pharynx [194, 216]. A series of studies by Kriebel et al. in the 1960s morphologically and physiologically characterised the pacemaker system in tunicates [217-221]. Early electrophysiology and microscopy studies could localise two independent myogenic pacemakers, one at the posterior and one at the anterior opening of the Ciona heart tube generating rhythmic peristaltic contractions [180, 194]. It is unclear, whether one of the pacemakers has a dominant function. The contractions show an alternating pattern and velocity is similar and independent of direction [198, 217, 219]. The reversal of the pumping direction has been speculated to be a compensating mechanism for inefficiency of the unidirectional fluid flow or a reaction to external stimuli [188, 190, 194, 219, 222]. Nervous system innervation of the heart appears to be absent [219, 223]. Humoral modulation and pharmacological alterations of pacemaker frequencies have been reported, but whether a similar mechanism of control as in higher vertebrates exists is unclear [221, 224].

Several factors important during mammalian heart development are also conserved in Ciona, which possesses orthologues of NKX, GATA and HAND factors [222]. Recently, Stolfi et al. identified *Islet*-expressing migratory cells in developing Ciona

embryos, which follow mechanisms homologous to the SHF in vertebrates [225]. However, these cells did not contribute to the heart, but to adjacent pharyngeal structure. It can be speculated, that further evolutionary progress in cardiac differentiation factors lead to a reallocation of *Isl*^{+} cells [225]. This indicates an *Islet*-expressing precursor population is highly conserved and might have already been present in the early bilaterians ancestor. Evidence for a distinct cardiac conduction system has not been found in the Ciona heart. Electrical coupling is believed to be facilitated by tight junctions between adjacent myocardial cells without a preferred conduction pathway or direction [217-219, 222].

Transition to vertebrate hearts is accompanied by chamber development

The evolutionary step from invertebrates to vertebrates included significant remodelling of the cardiac and circulatory system. The transition from a linear tube to a chambered heart is still not fully understood. Therefore, it is also not clear, whether the positioning and function of the primary pacemaker in the complex vertebrate heart is a vertebrate-specific evolutionary novelty or secondary to the major morphological remodelling of the heart tube.

Today, the closest living invertebrate relative of vertebrates is Amphioxus (also known as lancelet, *Branchiostoma lanceolatum*) [180, 195]. This aquatic species constitutes the modern representative of the subphylum Cephalochordate. Amphioxus has a closed circulation system with multiple contractile vessels functioning as rudimentary pumps (Fig. 6C) [180, 195]. There is no consensus on whether amphioxus pumps should be considered as hearts or as a rudimentary, heartless contraction system. The pumps lack continuous endothelium and striated muscle, but they are initially expressing a NKX2.5 homologue [188]. It remains unclear, whether the pumps have a common embryonic origin or whether there is a dominant pump. Furthermore, nothing is known about the origin of the peristaltic contractions and whether there is any kind of pacemaker-like structure. It is generally assumed, that the pumps originate from the subintestinal vessel, supporting the hypothesis of a common ancestral intestinal structure of the urochordate heart [188, 195]. Anatomical studies of the vasculature in Amphioxus revealed a high degree of similarity to vertebrates [226]. Interestingly, amphioxus has a ventrally located blood reservoir situated just upstream of the contractile pumps at the junction of the major veins [188]. Due to its function in storing blood, it has been referred to as the amphioxus sinus venosus [226]. However, it does not show contractile capacity itself and the location just adjacent to the hepatic vein challenges the consideration as an ancestral sinus venosus structure [188]. Despite its simple circulation structure, amphioxus bears a characteristic universally found in all chordates. It places its pumping organs upstream of the gills or pharyngeal vessels. The blood is pumped through the gills and on to the body, through a dorsal vessel the it is transported posteriorly and returning towards the heart/pump structure via a main ventral vessel [188].

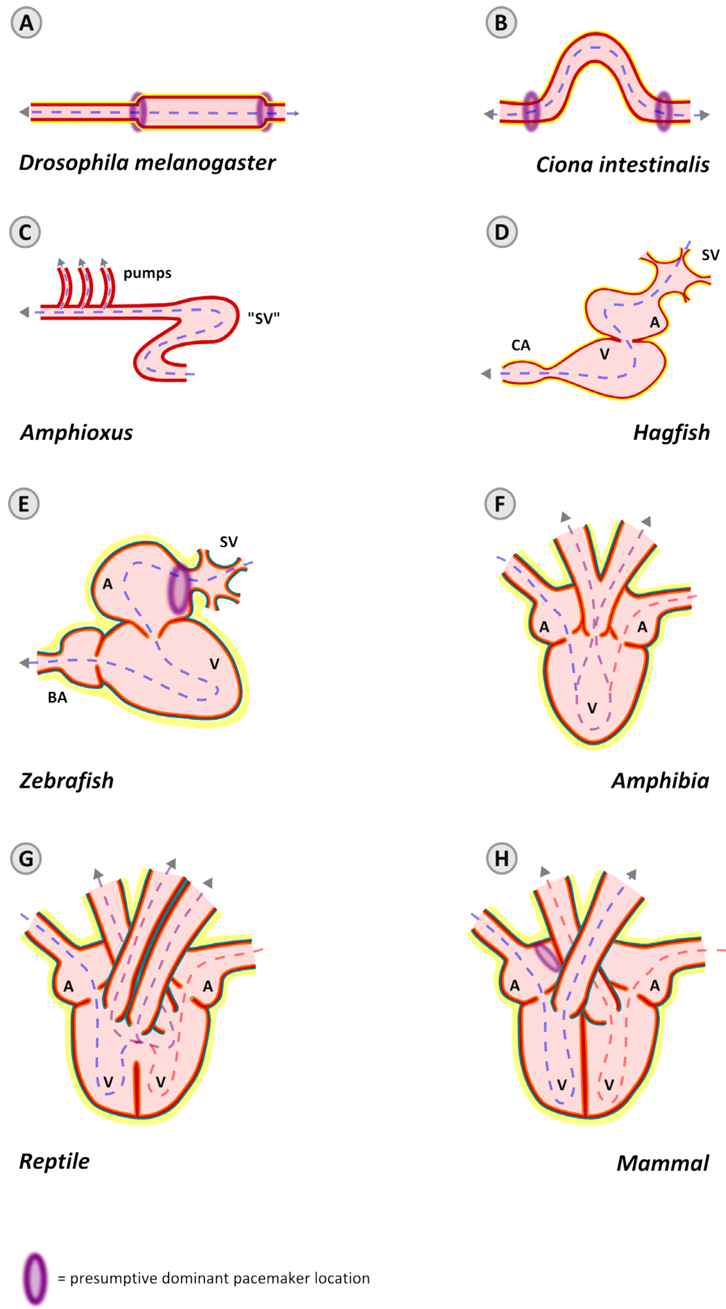


Figure 6

Illustration of heart evolution

(A) Drosophila dorsal vessel and (B) Ciona heart with bilateral pacemaker structures. (C) Rudimentary pumps in Amphioxus. (D) Two-chambered hearts in hagfish and (E) zebrafish (hypothesised pacemaker location). (F) Three-chambered amphibian heart with truncus arteriosus. (G) Reptilian heart with three major arteries. (H) Four-chambered mammalian heart, primary pacemaker in the SAN. (A-E anterior on the left, posterior on the right), Arrows indicate direction of blood flow.

red= myocardial/muscle layer; orange= endocardium; green= epicardial layer; yellow= pericardial sac; A= atrium; V= ventricle; SV= sinus venosus; CA= conus arteriosus; BA= bulbus arteriosus

Evolutionary closely related to the cephalochordates are the craniates, considered at the bottom of the vertebrate phylum. Hagfish (*Myxine glutinosa*) are craniates, as they have a complete bony skull, but they are not considered true vertebrates due to the lack of vertebrae [227, 228]. Lampreys (*Petromyzontidae*) have rudimentary vertebrae and can therefore be considered vertebrates [229] (more specifically ancestral fish). Together they constitute the modern representative of the *Agnatha* class of vertebrates. However, no universal consensus has been reached about their position in the phylogenetic tree and whether they should be considered true vertebrates [228]. However, concerning their cardiac morphology with a chambered, looped heart, they can be placed alongside the remaining classes of fish (*Chondrichthyes* and *Osteichthyes*) [227-229]. Putting these two species at the junction of vertebrate development, it is worthwhile to investigate their cardiac and circulatory system.

The linear, peristaltic pumps of invertebrates are considered too inefficient for the needs of more complex animals. Fluid energy might be misdirected by uncoordinated contractions and strictly linear tubes bear the risk of stream reflections and retrograde flow [188]. Hagfish and lampreys have an S-shaped heart, comparable to the embryonic heart of teleost fish. It is tilted anterior-ventrally to position the inflow chamber dorsally to the outflow chamber (Fig. 6D) [229]. When compared to linear hearts, this is considered to improve mechanical efficiency in pushing blood from the inflow- to the outflow-chamber [188]. Despite the main systemic heart, hagfish also have a secondary pumping organ, the portal vein heart located caudal to the systemic heart at the junction of gut and liver [227].

Both hagfish and lampreys have four cardiac compartments, a sinus venosus, an atrium, a ventricle and a conus arteriosus, a structural blueprint resembling teleost fish [188]. Early electrophysiological recordings failed to localise the onset of contractions to a specific structure, but could show characteristic pacemaker action potentials and slow spontaneous diastolic depolarisation in cardiac cells spread over all compartments in several species of hagfish [227]. Even though hagfish show a mature ECG comparable to vertebrates, electrical conduction throughout the heart was described as slower than in vertebrates, probably indicating the lack of a coordinated conduction system and poor intercellular coupling [230].

Towards the complex vertebrate heart

With the early chordates, a rapid structural and functional diversification of the cardiac system commenced. A looped, chambered, trabeculated heart with functional valves, facilitating unidirectional blood flow through an enclosed circulation system and in higher vertebrates eventually controlled by a coordinated pacemaker and conduction system [16, 180, 185, 186, 188, 198, 231].

All vertebrates have a closed circulatory system with an endocardial layer lining the heart [188]. This also abolishes the possibility to supply the myocardium by direct perfusion. Instead, an epicardial layer and coronary artery system is established to serve the thickening

myocardial layer [188]. A basic configuration of alternating slow-conducting and poorly contracting pacemaker components with fast-conducting myocardium appears to be conserved in all higher vertebrates with multi-chambered hearts [124].

Two-chambered heart in fish

Fish have a single circuit circulation system, with the heart positioned upstream of the gills. Therefore, a single pumping system is sufficient in fish. The best-studied representative is the teleostei zebrafish (*Danio rerio*) [17, 232]. The adult zebrafish heart consists of two contractile chambers, a single atrium and a single ventricle delineated by valves at the atrioventricular junction (Fig. 6E). Furthermore, it has an enlarged outflow tract, the bulbus arteriosus. The sinus venosus is present during embryonic development, but partly regresses to the venous pole upon maturation. The heart consists of three layers, endocardium, myocardium and epicardium [17, 232]. The ventricle of mature zebrafish hearts is a thick muscular pump and highly trabeculated [9, 233].

During embryonic development the onset of myocardial contractions is observed shortly after formation of the linear heart tube and originates from the venous pole [17]. It initially has a peristaltic contraction pattern comparable to invertebrate hearts, though it has also been described as a dynamic suction pump mechanism [120]. Upon maturation, it shifts to a sequential atrial-ventricular contraction pattern similar to the mammalian pattern [3]. A mature ECG pattern can be measured in zebrafish [9]. A recent optogenetic study could localise the functional pacemaker of embryonic zebrafish hearts (3 dpf) at the inner curvature of the atrium immediately adjacent to the venous pole and restricted to a small number of cells [29]. Voltage dynamics visualisation could show that depolarisation origins from the sino-atrial region [3, 234]. This location is thought to be conferrable to the SAN in mammalian hearts. Upon further maturation, the pacemaker site became restricted to the right site of the sino-atrial junction, in agreement with the right-sided localisation of the SAN in the septated mammalian heart [29]. A secondary pacemaker region was found at the atrioventricular canal, but was unable to maintain contractions after ablation of the primary pacemaker, a redundancy readily observed in mature mammalian hearts. However, detailed pacemaker studies in the adult zebrafish heart have not been done so far. Moreover, it remains unclear whether zebrafish hearts develop a mature cardiac conduction system [3, 124, 233]. The presence of a secondary pacemaker node at the AV-junction in embryonic hearts and the observed sequential contraction patterns in advanced stage embryos and mature zebrafish indicate the presence of a coordinated atrial conduction pathway with delay at the AV junction, but so far, no sufficient evidence for a ventricular conduction system could be presented [3, 235]. Some studies favoured a ventricular conduction system in zebrafish facilitating a mammalian-like apex-to-base ventricular contraction pattern [233]. However, the conduction system in zebrafish is still subjected to debate. The overall electrophysiological properties of isolated atrial and ventricular cardiomyocytes are similar to mammals [23]. The important ion currents for depolarisation, plateau phase and repolarisation (I_{Na}, $I_{Ca,L}$ and

I_{Kr}) are similarly distributed as in mammalian hearts. $I_{Ca,T}$, which has a prominent role in mammalian pacemaker cell depolarisation was expressed in all cardiomyocytes of the zebrafish heart and unlike the mammalian situation, expression persists in mature cardiomyocytes [23]. The adult zebrafish ECG is comparable to the mammalian ECG and has even be proposed as a possible model for the assessment of pharmacological agents for treatment of human arrhythmias [236-238].

Interestingly, temperature acclimation studies in another teleostei, the rainbow trout, could also locate the primary pacemaker at the sino-atrial junction [239]. It furthermore highlights an extrinsic regulation mechanism found in many ectothermic animals, temperature-dependent heart rate modulation by directly interfering with cardiac ion channels [239-244].

In mammals, pacemaker cells have been intensively characterised concerning their molecular and electrophysiological properties. The expression of a hyperpolarisation-activated slow rectifier potassium channel is considered the inherent property of mammalian pacemaker cells. Similar studies in zebrafish have not been conducted so far, mainly due to the lack of a pacemaker cell-specific marker and the presumably small number of pacemaker cells. Characterisation studies of the cardiac mutant *slow-mo* argued for the existence of a hyperpolarisation-activated inward potassium current I_h [245, 246]. The mutant presents with bradycardia persisting into adulthood. However, unlike the funny current (I_f) in mammalian pacemakers, I_h was found in all cardiomyocytes and the underlying genetic components have not been identified, rendering it unsuitable as a pacemaker cell marker [245, 246]. There is a zebrafish homologue of the pacemaker-specific potassium channel subunit HCN4, and expression analysis might shed light on the presumptive pacemaker potential of the cells localised in the sino-atrial junction. Expression data for homologues of the mammalian cardiac connexins (CX30.2, CX40, CX43, CX45) is sparse in zebrafish [247]. *cx43* (homologue of CX43) expression has been observed in the embryonic heart [3, 248]. *cx45.6* (homologue of CX40) is expressed in the chamber myocardium of ventricle and atrium, similar to the mammalian expression pattern [3, 249]. Both pacemaker-specific mammalian connexins (CX30.2, CX45) have not been described in zebrafish so far. Given the importance of TBX3 in mammalian pacemaker specification, the expression pattern of *tbx3* in the developing zebrafish heart could clarify a possible evolutionary conservation.

Interestingly, the transcription factor *Isl1* has been identified as an important factor in cardiomyocyte differentiation at the venous pole [38]. *Isl1* expression persists into adulthood and is highly restricted to a small number of cells at the venous pole [38]. Mutant embryos display arrhythmia and bradycardia [38]. Taken together, this indicates a role for *Isl1* in zebrafish pacemaker development and if so, it might constitute a useful pacemaker-specific marker. If speculating, that the pacemaker system in zebrafish is closely related to the mammalian pacemaker system except for lacking modifications required in the septated heart (such as an inter-atrial conduction pathway) and seemingly the ventricular conduction system, zebrafish could prove to be an excellent model to study pacemaker dynamics during development and disease (Box 4). In summary, zebrafish could be a promising

model to address pacemaker development and function. Considerable homology between zebrafish and mammalian heart development and physiology has been shown [17, 23, 232]. However, substantial gaps in the understanding of the zebrafish cardiac pacemaker system will need to be addressed, which could eventually help to clarify general questions concerning vertebrate pacemaker physiology.

Separation of the double circulation circuit in tetrapods

The terrestrialisation of vertebrates and emergence of tetrapods during the late Devonian period (around 370 mya [250]) brought a major lifestyle shift for the early vertebrates, the transition from water- to air-breathing [251]. With it came the division of the circulation system into a systemic and pulmonary circuit. It is assumed to have evolved sometime during the separation of teleost fish and amphibians [252, 253]. Therefore, amphibians are the earliest class of terrestrial vertebrates to have a double circulatory system [180, 252]. They have two separate atria and a single anatomically unseptated ventricle giving rise to the truncus arteriosus, leaving them with a three-chambered heart (Fig. 6F) [252, 254]. Functionally, trabeculation of the ventricle provides a varying degree of separation of the oxygenated and deoxygenated blood [186]. Phenotypically, amphibian hearts resemble the human congenital disease “Persistent truncus arteriosus”.

Information on the evolutionary step from gills to lungs might be acquired from the lungfish, a fish that can manage gas exchange either through its tetrapod-like lungs or remaining gills [255-257]. Lungfish are considered to be animals that are at the beginning of a evolutionary transition from water- to air-breathing [257]. Their heart retains a transitory state between fish and tetrapods. There is a distinct sinus venosus, partially separate atria and ventricle, conus arteriosus and persisting bulbus cordis, resulting in sufficient separation of deoxygenated blood (right) and oxygenated blood (left) [255, 256]. Arbel et al. conducted electrophysiological studies on the heart of the African lungfish (*Protopterus ethiopicus*) and described the leading pacemaker site to be in the sinus venosus, close to the left cardinal vein, but not in the sinoatrial junction [257]. The precise location was found to shift considerably between different parts of the sinus venosus and the atria [257].

In the chambered heart of higher vertebrates, the dominant pacemaker shows a strict laterality and is situated in the upper wall of the right atrium. There is no obvious functional reason for the right-sided dominance of the pacemaker. It could have been purely due to the location of the interatrial septum during development. Localisation of the pacemaker in the amphibian heart could clarify, whether the right sided location is an ancestrally established feature. Furthermore, the presence of two atria requires coordinated contraction, a mechanism ensured in mammalian hearts by the Bachmann’s bundle or anterior interatrial band (Box 1) [1].

Atrial septation in amphibians

A well studied amphibian model organism is the clawed frog *Xenopus laevi* [252]. Heart rate measurements in Xenopus larvae [258] and adults [254] showed that the ECG pattern is comparable to mammals and contractions showed sequential atrial-ventricular contraction with a delay at the AV junction. Furthermore, drug sensitivity studies showed that the pacemaking system in Xenopus interacts with sympathetic and parasympathetic nervous system input [258]. The location of the primary pacemaker was not addressed in this study. It would be interesting to check whether the amphibian pacemaker shows a similar right-sided laterality as the mammalian SAN.

As described for zebrafish, the presence of an organised ventricular conduction system in Xenopus is unclear. In higher vertebrates and mammals the ventricular conduction pathway is situated in the septum, which is absent in fish and amphibians. It has been hypothesised, that the ventricular trabeculae or discrete conduction pathways in the ventricular wall might constitute a fast pathway to the apex of the ventricle [233, 257]. Neither histological, nor microscopy studies have been successful in sufficiently outlining a potential ventricular conduction network in fish or amphibians.

Beginning ventricular septation in reptiles

Reptile hearts are similar to amphibian hearts, but show a varied degree of ventricular septation [259, 260]. The reptile heart is commonly described as a three-chambered heart similar to the amphibian heart (Fig. 6G). The sinus venosus is well developed and the first reservoir to receive blood from the venous system, sometimes it is considered a fourth chamber [260-262].

A notable exceptions are crocodilians, which have fully septated ventricles resulting in a four-chambered heart [263]. Pulmonary and systemic circulation are almost completely separated and it has thick muscular ventricular walls, allowing the generation of high systemic blood pressure, presumably solving the growth limitation problem in bigger animals [189, 264].

Other reptilian species, such as sea turtles have developed a muscular ridge, the sphincter muscle at the pulmonary artery, allowing controlled blood flow from the ventricle [260]. If the sphincter contracts, blood is diverted back into the ventricle, across the incomplete septum into the aorta [265]. This allows a controlled bypassing of the pulmonary circuit. It has been speculated, that the common ventricle allows to shuttle deoxygenated blood to the body in order to regulate the body temperature [266]. Furthermore, reptilians have two aortas in addition to the pulmonary artery. The right part of the ventricle is connected to the pulmonary vein and one aorta, which are interconnected via an actively regulated shunt [265, 267]. This ability to bypass the pulmonary system is lost in birds and mammals. It remains unclear whether cardiac shunting is a favourable process in lower vertebrates or mainly represents a transitory step from a two to a four-chambered heart [263, 265]. High pumping pressure as generated by the mammalian heart is only feasible in securely separated circulation circuit in order to avoid pressure overload of the pulmonary system [189].

Data on the embryonic development of reptilian hearts is sparse. Early morphological

development is similar to that of higher vertebrates [231, 268, 269]. An initially peristaltic contraction pattern of the early heart tube has been observed in several reptilian species [268]. The mature contractions in the reptile heart originate from the sinus venosus, resulting in a characteristic SV wave in ECG measurements that precedes the P wave (atrial depolarisation) [262]. The sinus venosus of lower vertebrates has even been considered "the pacemaker chamber" [193]. The pacemaker might be less compact than in mammals, leading to a widespread pacemaker area across the sinus venosus.

Reptiles, as well as amphibians, are poikilothermic animals and use the circulatory system to regulate their body temperature [266]. This gives additional significance to heart rate control and regulation. Nervous system innervation of the heart has been described for various reptiles, amphibians and fish [266, 270-272]. Whether these directly interact with the pacemaker structure could not be clarified yet, due to a lack of understanding of the pacemaker system in these animals. When comparing the mammalian heart to that of lower vertebrates, especially the development of the distinct atrioventricular conduction system constitutes a noticeable difference. The network of SAN, AVN, and ventricular His-Purkinje system described for mammals can also be found in avian species, but not in lower vertebrates. The intriguing question is what the evolutionary pressure or driving force for conduction system development might have been.

Given the close evolutionary relationship between birds and reptiles, determining the status of a cardiac conduction system in reptiles might shed light on its evolutionary origin and whether it is in fact the latest functional addition to the vertebrate heart. Especially the crocodilian heart might be of interest. Its fully septated heart morphologically closely resembles the avian and mammalian heart. A SAN with spontaneously depolarising pacemaker cells has been morphologically outlined in the crocodile heart [189, 273]. Histological studies on *Crocodylus johnstoni* ventricles could not outline a distinct conduction system, specialised myocardial tissue or purkinje cells as seen in mammalian hearts [274]. However, it could be shown that ventricular excitation travels along distinct pathways in the ventricular septum towards the apex of the heart in order to activate an apex-to-base contraction pattern simultaneously in both ventricles [274] (the same observation in alligators [267]). A functional AVN delay has been observed in alligators [267].

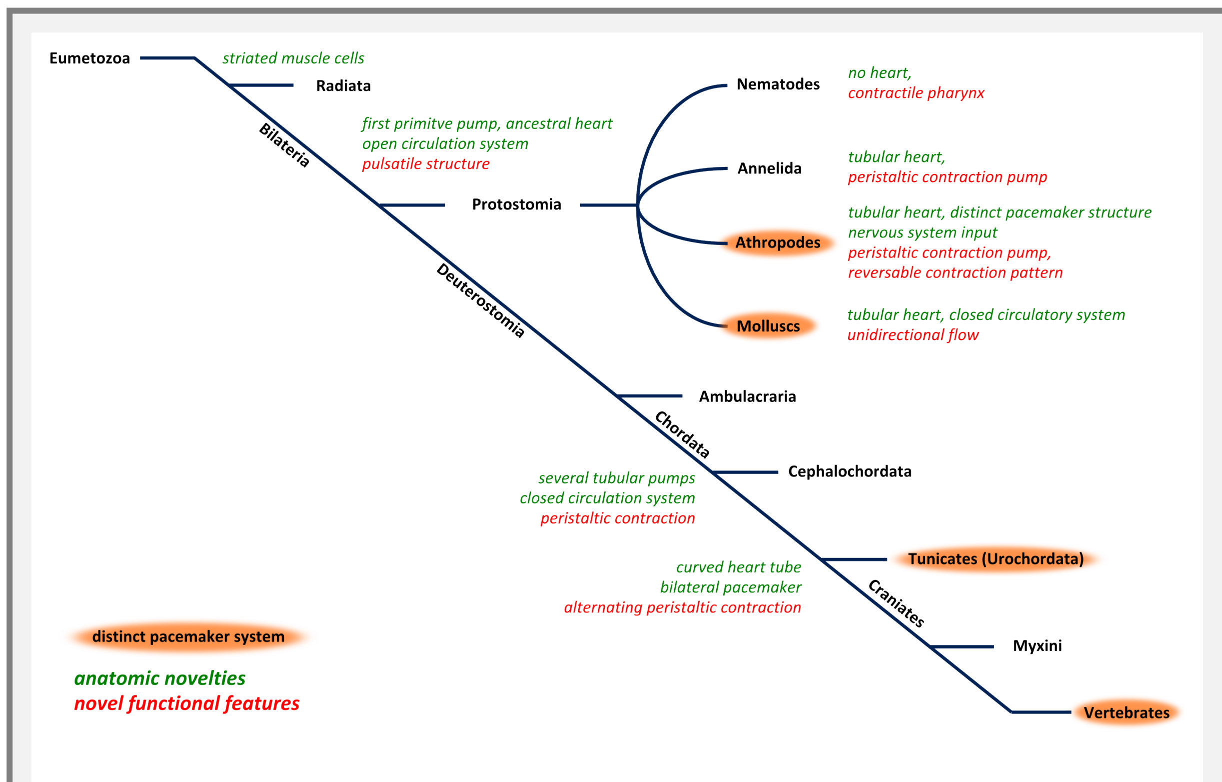
It is still subject to debate, whether reptilian hearts are to be considered ancestral to avian and mammalian hearts, or whether they have evolved independently from a common ancestor. To elucidate this, more research in the lower vertebrates should be done. The distinct nodal pacemaker in the atrium seems to be conserved in fish and frog. The distinct conduction system appears to be bird/mammal-specific. One of the few molecular studies in reptilian species development uncovered that TBX5, critical during mammalian heart patterning, is also essential during patterning of the reptilian ventricular septum [259]. This indicates, that the partly septated reptilian ventricle is a transitory step towards the fully septated mammalian ventricles. Since the ventricular conduction system components are situated within the ventricular septum, it might be assumed that the intraseptal conduction

pathways evolved during the transition from reptilians to bird.

Complex pacemaker and cardiac conduction system in birds and mammals

All endothermic (warm-blooded) animals, birds and mammals, have an intrinsic, myogenic pacemaker system coupled to a coordinated cardiac conduction system to allow highly controlled cardiac contraction (Fig. 6H). Heart development and function is highly similar, even though histological differences exist (for review [12, 16, 34-36, 49, 124, 185, 189, 275, 276]).

A drawback when focusing on non-model organisms, especially lower vertebrates is the lack of recent and genetic studies. Identification of components of the pacemaker and conduction system was mostly done by histological, morphological or functional (electrophysiological) studies. To further characterise the presumptive nodes and pacemaker structures, it would be beneficial to assess molecular markers used in mammalian systems to delineate the pacemaker cells. For example, the described transcription factor network controlling pacemaker development in mammalian embryos (Fig. 5). It has been shown, that core transcription factors, such as Nkx2.5 or Mef2 are conserved in all modern animals with hearts [186]. It is a common evolutionary mechanism, that gene duplication and acquisition of new gene functions promote progression and diversity [186, 277]. Reflecting this, in the heart of higher vertebrates, many genes have partially redundant functions and mutations often affect a very specific part of the heart, whereas defects are much more profound in simple hearts [186].



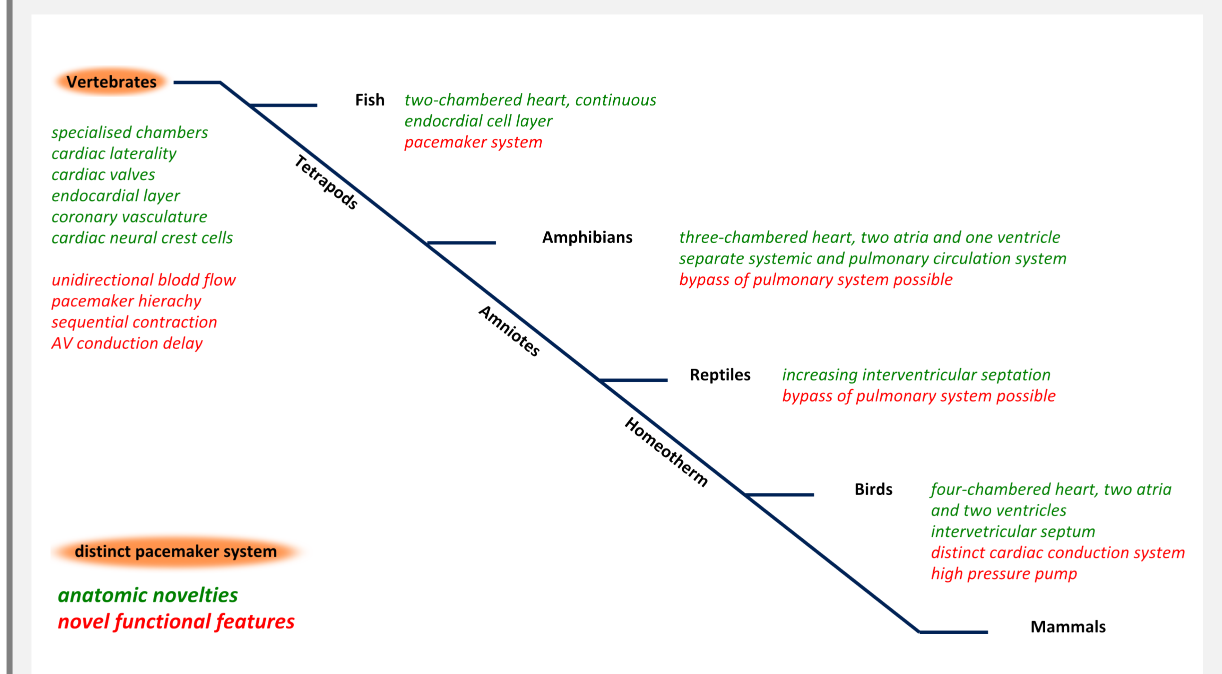


Figure 7

Evolutionary adaption of the cardiac circulation system

With regard to important morphological (green) and functional (red) novelties. (Top) Heart and pacemaker evolution of existent Eumetazoans, from the presumptive common bilaterian ancestor to vertebrates and (bottom) within the vertebrate subphylum.

(Orange) All groups with an intrinsic pacemaker system, includes all vertebrates

Concluding remarks

After summarising the evolutionary journey of the cardiac pacemaker, the questions proposed in the beginning can be reconsidered. Did the distinct pacemaker evolve out of necessity to accommodate the growing heart in higher organism to ensure a controlled contraction pattern, increase in blood pressure and hemodynamic force?

The concept of a myogenic pacemaker seems to have evolved in a very early, common bilaterian ancestor. Therefore, it is unlikely to be a sole adaption to a growing heart size. The coordination of atrial contraction, AVN delay and ventricular contraction is highly conserved in chambered hearts and necessary to ensure a functional, sequential contraction pattern. Even though, the ventricular conduction system has likely evolved at a much later stage, there are relatively large and physically active animals (e.g. crocodiles) that do not seem to have a distinct conduction system.

Did it evolve to ensure coordinated, unidirectional blood flow in a separated systemic-pulmonary circuit?

It is assumed, that the distinct pacemaker system evolved as a mechanism to regulate contraction and fluid flow direction. The development of the conduction system appears to coincide with the definite separation of systemic and pulmonary circulation in endothermic animals. Nevertheless, it has been indicated that it might already be present in lower vertebrates.

Was it crucial as a mediator to allow heart regulation by the nervous system?

Nervous system innervation as a control of cardiac function is highly conserved. Drosophila seems to have an additional pacemaker linked to the nervous system, whereas the SAN pacemaker cells in mammals combine intrinsic pacemaker potential and susceptibility to nervous system and humoral modulation. It cannot be excluded, that the nervous system can exert influence directly onto non-pacemaker cardiomyocytes. In some species, the pacemaker cells seem to be less concentrated and rather diffusely spread over the sinus venosus.

Is the mammalian cardiac conduction system the latest and most sophisticated addition to the cardiac network?

The coordinated SAN-AVN-His/Purkinje system appears to be the major cardiac advancement in the highest classes of vertebrates [189].

It could be shown, that a unique, evolutionary conserved network of factors control embryonic development of the cardiac pacemaker system. It leads to the establishment of a coordinated system outermost crucial for survival. Whereas many aspects of the cardiac pacemaking have been addressed and unravelled, especially the molecular details of pacemaker development in part remain elusive.

Recent development and emerging questions

Severe cardiac arrhythmias and other abnormalities in heart function requiring medical intervention have been treated with implantable electronic devices for the last 50

years [278, 279]. Sophisticated modern pacemaker devices are specifically designed for the treatment of various heart rhythm problems. These artificial pacemakers can monitor, support or even substitute for the hearts intrinsic pacemaking system. Formerly deleterious heart defects can be treated effectively, significantly increasing overall survival [278].

However, some concern regarding artificial pacemakers remains. Especially in the paediatric patient population, lifelong pacemaker treatment is difficult [280, 281]. Problems, such as dependence on batteries, damage to the heart tissue and restrains on the individuals lifestyle remain [94]. With the advancement of (stem-) cell culturing and manipulation, the idea of creating biological pacemakers emerged. Several recent publications discussed the problems with artificial pacemakers and the current advancements in the field of biological pacing [94, 282]. Ultimately, a biological pacemaker aims at (re-) establishing a functional, independent intrinsic pacemaker. In vitro studies could show that manipulation of ion channels in cardiomyocytes can induce rhythmic, autonomous depolarisation. Such engineered cells could provide improvement of arrhythmias in animal model, but were not sufficient to ensure heart function [282]. Biological pacemakers are still far from being applicable in medical treatment. It remains debatable, whether biological pacemaking will be a sufficient alternative to the artificial pacemaker. Technical advancements of the electronic devices might eventually solve many current complications. It even bares potential, a biological pacemaker cannot provide, such as constant remote monitoring of the heart function [283]. Significant improvement of biological pacemaker strategies might enable their use at least as a supplementary system, as it has been shown in animal studies [282]. There are numerous challenges concerning the stable induction of pacemaker automaticity, the cellular transfer and adaption of the graft cells to the heart, the formation of functional intercellular communication with the working myocardium and possible long-term effects. A detailed understanding of the intrinsic pacemaker system is essential. Furthermore, it is crucial to examine the structure of the pacemaker node, in order to reveal, how pacemaker cells are embedded in their surrounding, how they are connected to each other and to the working myocardium. With the aim of rebuilding a pacemaker node from engineered cells, detailed knowledge of the natural architecture, development and function is of great importance. Insight into the development of pacemaker cells will enhance our understanding of their molecular setup and possibly point out crucial factors in maintaining the pacemaker potential. Embryological studies are difficult or impossible to do in human and mouse. The zebrafish could prove to be a valuable model organism (Box 4). Efficient labelling of the pacemaker cells would allow monitoring their embryonic development and elucidating the genetic network governing pacemaker development in fish.

Box 4

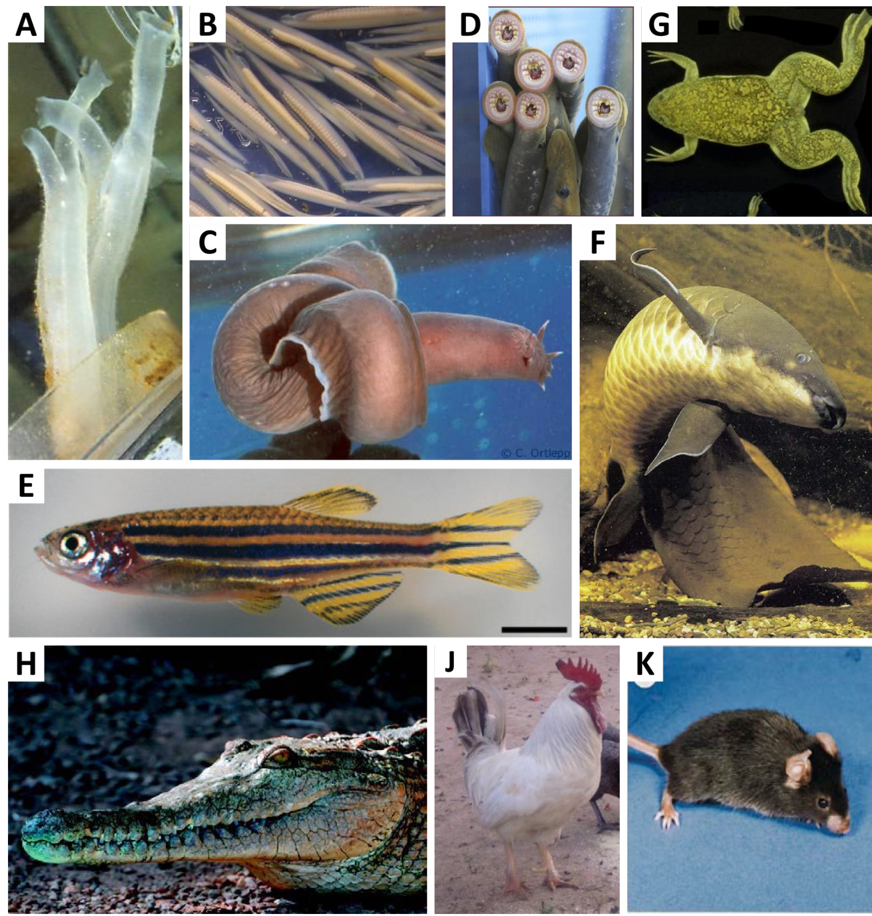
Zebrafish – an emerging model to study cardiac pacemaking.

Despite its extensive use as a model organisms in a wide field of scientific research, the cardiac pacemaker in zebrafish has not been sufficiently described. Given the evolutionary position of bony fish near the bottom of the vertebrate subphylum, it might constitute a useful link between simple tubular hearts and the complex structures of higher vertebrates. Zebrafish is a well established model organism in cardiac research and especially useful to conduct embryological studies [17]. Characterising the cardiac pacemaker and conduction system in zebrafish could enable experimental studies not possible or considerably more difficult in more complex model organisms. The ability of zebrafish embryos to initially survive without circulation, the use of forward genetics and other molecular techniques, as well as easy handling and transparent embryos favour zebrafish as a model to study the embryonic development of the pacemaker and conduction system (if it proves to be present in zebrafish). Furthermore, it has been suggested that cardiac ion-channels in resemble the human physiology. Zebrafish could provide a suitable model to test pharmacological agents targeting Channelopathies or correcting acquired deficiencies [23]. A number of zebrafish mutants mimic human conduction system defects [3]. Identification of the underlying genetic defects in zebrafish might shed light on the human condition. Concerning the SAN pacemaker, especially mutants displaying an altered beating pattern (e.g. bradycardia, tachycardia or arrhythmia) could be of interest (see table below).

The recently described bradycardia phenotype in the *isl1* mutant embryos and the observation, that Isl1 is expressed in the presumptive pacemaker location at the inflow tract indicates that it might have a role in pacemaker function [29] [38]. In case the *isl1*-expressing cells are pacemaker cells, it could be a useful marker to delineate the pacemaker system in zebrafish. The functional relationship between the Isl1 deficiency and the bradycardia phenotype remains to be determined. The pacemaker function could be impaired by lack or misexpression of cardiac ion channels, impairment of the pacemaker potential (e.g. by interfering with I_f) or a result of disturbed transmission of the electrical signal. As mentioned, Isl1 is considered to be a patterning factor in cardiac progenitor cell populations and assumed to be involved in the maintenance of a primitive progenitor phenotype. Expression in the node is also conserved in mouse and human. The intriguing question is, why it remains expressed specifically in these cells. Since it is a transcription factor, it might regulate genes that are pacemaker-specific or important to maintain the pacemaker potential. Therefore, it would be useful to identify interaction partners of Isl1 in pacemaker cells.

Name	***Conduction phenotype***
silent ventricle (siv)	noncontractile ventricle
tremblor (tre)	atrial and ventricular fibrillation
hiphop (hip)	variable AV block
bullseye (bue)	no heartbeat at 24 hpf, AV block at 36-48 hpf
kingpin (kgp)	atrial and ventricular fibrillation
brady (bra)	slow heart rate
dococ (dcc)	uncoordinated ventricular contraction
hobglobin (hob)	AV block at 48 hpf, silent ventricle at 96 hpf
slipjig (sli)	peristaltic contraction without AV delay
daredevil (ddl)	AV block, silent ventricle at 120 hpf
mobitz (mbz)	AV block, sinus pause at 120 hpf
elektra (elk)	AV block

adapted from Chi et al., 2008; Table 1 [3]



Box 5

Representative images of discussed organisms

A: Tunicate *Ciona intestinalis* [7]; **B**: Amphioxus (Lancelet) *Branchiostoma lanceolatum* [28]; **C**: Hagfish *Myxine glutinosa* [30]; **D**: Sea lamprey *Petromyzon marinus* [31]; **E**: Zebrafish *Danio rerio* [40]; **F**: Lungfish *Neoceratodus forsteri* [41]; **G**: Clawed frog *Xenopus laevi* [46]; H: Freshwater crocodile *Crocodylus johnstoni* [48]; **J**: Chicken *Gallus domesticus* [53]; **K**: Mouse *Mus musculus* [54]

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