On the connective tissue regulator Follistatin-like 1
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Development of the Human Heart

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Abstract

Molecular and genetic studies around the turn of this century have revolutionized the field of cardiac development. We now know that the primary heart tube, as seen in the early embryo contains little more than the precursors for the left ventricle, whereas the precursor cells for the remainder of the cardiac components are continuously added, to both the venous and arterial pole of the heart tube, from a single center of growth outside the heart. While the primary heart tube is growing by addition of cells, it does not show significant cell proliferation, until chamber differentiation and expansion starts locally in the tube, by which the chambers balloon from the primary heart tube. The transcriptional repressors TBX2 and TBX3 locally repress the chamber-specific program of gene expression, by which these regions are allowed to differentiate into the distinct components of the conduction system. Molecular genetic lineage analyses have been extremely valuable to assess the distinct developmental origin of the various component parts of the heart, which currently can be unambiguously identified by their unique molecular phenotype. Despite the enormous advances in our knowledge on cardiac development, even the most common congenital cardiac malformations are only poorly understood. The challenge of the newly developed molecular genetic techniques is to unveil the basic gene regulatory networks underlying cardiac morphogenesis.
Introduction

In the last decades the field of cardiac development has been revolutionized, rendering many views on cardiac development from the nineties and further back obsolete. The idea of a heart tube, containing all the future components, that just needed to grow, has been shattered by molecular lineage tracing and cell division studies. A pool of rapidly proliferating precursor cells outside the heart will give rise to most of the adult heart. Three dimensional reconstructions of human and mouse embryos, stained for key molecules in heart development, provided extra insight into this process. While textbooks are currently beginning to incorporate the new findings in cardiac development, an overview on cardiac development discussing the major novelties of the last 15 years is highly needed for anyone studying inborn defects. We will discuss the major steps of cardiac development focusing on growth, formation of primary and chamber myocardium and the development of the cardiac electrical design.

In the full grown adult, a system of parallel circulations exists. Oxygen-deprived blood from the body enters the heart at the right atrium and is propelled by the right ventricle towards the lungs. The oxygenated blood, in turn, enters the heart at the left atrium and is pumped by the left ventricle via the aorta into the systemic circulation. The different components of the heart ensure an efficient contraction-relaxation cycle of the atria and the ventricles (Figure 1). The sinus node situated at the roof of the right atrium generates the electrical impulse that travels through the atria. When the activation front reaches the atrioventricular node, conduction is delayed. Then the front travels from the node via the fast-conducting bundles and bundle branches to reach the peripheral conduction system of the ventricles, the Purkinje fibers. The rapid propagation of the electrical current in the ventricles ensures that the entire ventricular mass contracts simultaneously, allowing efficient propulsion of the blood.

In small invertebrates, the distances between the environment and their cells are small enough to cope without a circulatory system. With the increase in size and activity of an organism, a circulatory system is required for the delivery of nutrients, and removal of wastes. It is, therefore, of no surprise that the heart, an organ shared in species varying from worms to man, is the first organ to function during embryonic development. The rhythmically contracting pharynx of nematodes, such as Caenorhabditis elegans, might be interpreted as the most primitive form of a heart. Cardiac transcription factors, like NK2 homeobox 5 (NKX2.5) or myocyte enhancer factor 2 (MEF2) homologues, are expressed in the pharynx muscles of these nematodes. Transcription factors of the NKX2, MEF2, GATA binding protein (GATA), T-box and heart and neural crest derivatives expressed transcript (HAND) family proved to be involved in cardiac development, ranging from the tubular hearts observed in the fruit fly Drosophila melanogaster to the four-chambered human heart [7 Olson, EN (2006)]. Often these factors were first identified in Drosophila before
their role in vertebrate heart development was demonstrated [8 Bodmer, R (1993)].

In most fish, the phylogenetically oldest vertebrates, the heart is composed of two chambers, a collecting atrium and an ejection ventricle (Figure 1). During evolution, from lungfish onward, a lung formed by means of a sac bulging out of the foregut and the circulatory system acquired an extra component, the pulmonary circulation. The capillary network surrounding the lung is supplied with blood from the gill arteries. The lungfish’s atrium is divided into two parts, in the right component the systemic blood flow empties, the left component is connected to the pulmonary veins. Although the heart is anatomically not completely septated, the blood flow is functionally divided into a systemic and pulmonary circulation. The hearts of amphibians and reptiles have a similar anatomical configuration, allowing these animals to change blood volumes, but not pressures, between the pulmonary and systemic circulation. In birds and mammals the heart is anatomically fully separated into four-chambers, which permits these species to vary pressure, but not volume [9 Randall, DJ (1970)].

Figure 1. The vertebrate circulatory system.
Panels A-A’ depict two types of circulation, panel A shows the serial circulation, as seen in fish and mammalian embryos, which, however, receive oxygen through the umbilical vein from the placenta rather than through the gills. Panel A’ depicts the parallel circulation as found in mammals. Panel B represents a schematic drawing of a human heart with the non-myocardial tissues in yellow, the working myocardium in blue and in black the myocardium of the conduction system. Abbreviations: SCV: superior caval vein; ICV: inferior caval vein; CS: coronary sinus; PV: pulmonary vein; SN: sinus node; AVN: ativoventricular node; BB: bundle branches; MB: moderator band; RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle.
Formation and growth of the linear heart tube

Origin of the cardiac mesoderm

Like all other cardiovascular components, the heart largely is a mesodermal derivative, albeit that some parts of the heart, such as the cushions of the outflow tract, have a contribution of the ectoderm-derived cardiac neural crest. Early in human development, at Carnegie Stage (CS) 8, equivalent to 3 weeks of human development (Table 1), when the embryo is a flat tri-laminar disc, the cardiac precursors reside in two symmetrical parts of the mesoderm, lateral to the stomatopharyngeal membrane, the future mouth (Figure 2A-B). The mesoderm is divided by the intra-embryonic coelom into a somatopleuric layer facing the ectoderm, and a splanchnopleuric layer facing the endoderm. The latter portion of the mesoderm gives rise to the heart. The bilateral precursor pools unite in the midline, cranial to the stomatopharyngeal membrane, forming the cardiac crescent [10 Moorman, AFM (2007)], [11 Sizarov, A (2011)] (Figure 2B).

The differentiation of the cardiac crescent into cardiomyocytes is dependent on signals derived from the adjacent endoderm. Agonists and antagonists of the Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF) and Wingless Type (WNT) families of growth factors are expressed by the endoderm and ectoderm in a complementary fashion resulting in a unique signaling environment at the endodermal side, which drives cardiac differentiation [12 Harvey, RP (2002)]. At this stage already, the cardiac crescent can be identified by the presence of many cardiogenic transcription factors, and even some sarcomeric genes [13 Somi, S (2004)].
Chapter 1

Figure 2. Folding of the embryo and formation of the heart tube.

The embryo starts as a flat disc (Panels A,B), containing the three germ layers, the ectoderm (Ecto), mesoderm (Meso) and endoderm (Endo). The mesoderm adjacent to the endoderm, between the future transverse septum (TS) and the future pharyngeal mesoderm (PM), will give rise to the heart. The outer lining of the flat disc is termed the navel ring and forms the outer lining of the future umbilicus. Panel A-A'''. With ongoing folding of the embryo the embryonic gut that runs from the stomatopharyngeal membrane (SM) to the cloacal membrane (CM) is formed. The heart (HT) becomes positioned ventrally to the foregut (FG) caudally to the head and cranially to the umbilical cord and transverse septum. Panel B displays the division of the heart-forming field into two (sub)fields, the first heart field (1), which will give rise to the linear heart tube and the second heart field (2), which will remain in continuity with the first heart field during subsequent development, and adds cardiomyocytes to the developing heart. Note that the strict borders drawn here in reality are gradual. Panel C through G display the formation of the heart tube from a flat horseshoe-shaped cardiac crescent, to a gully of cardiac mesoderm eventually forming a tube by merging at the back side of the heart. The connection of the heart to the dorsal body wall is termed the dorsal mesocardium (DM). The heart tube (grey) forms by addition of cells from the flanking splanchnic mesoderm (yellow). Red line: peripheral border; blue line: central border. After closure of the dorsal mesocardium, cells of the second heart field can only be added to the heart via the arterial and venous poles (AP and VP). Panels H and I show a Tbx2 lineage tracing demonstrating that the ventricular (V) cells at E9.5, that do not express Tbx2, eventually contribute to the ventricular septum, which has not recombined (Panel I). The left ventricular (LV) free wall and the entire right ventricle (RV) are derived from cells that once expressed Tbx2 and thus were primary myocardium as in the atrial floor (AF), atrioventricular canal (AVC) or outflow tract (blue color). Panel J displays the quantification of cell proliferation in the CS10 human embryo, displayed in panels G' and G'', based on staining with the proliferation marker Ki67. High proliferation is seen in the extracardiac splanchnic mesodermal pool of precursor cells. The colors depict the percentage of Ki67-positive cells; the dotted line depicts the border between myocardium and the splanchnic mesoderm. Abbreviations: RA: right atrium LA: left atrium. Modified from [11 Sizarov, A (2011)], [18 Aanhaanen, WT (2009)], [10 Moorman, AFM (2007)]
Table I. Stages of human development with corresponding events in cardiac development.

A time line of events taking place in human cardiac development is summarized. Data was obtained from [104 Arraez-Aybar, LA (2008)], [97 Oostra, RJ (2007)], [105 O’Rahilly, R (1987)]

<table>
<thead>
<tr>
<th>Carnegie stage</th>
<th>Human DPC</th>
<th>Mouse DPC</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS8</td>
<td>17-19</td>
<td>7</td>
<td>The cardiac crescent forms</td>
</tr>
<tr>
<td>CS9</td>
<td>19-21</td>
<td>7.5</td>
<td>The embryo folds, the pericardiac cavity is placed in its final position, gully of myocardium forms, the endocardial plexus forms, cardiac jelly forms</td>
</tr>
<tr>
<td>CS10</td>
<td>22-23</td>
<td>8</td>
<td>The heart beats, the endocardial tubes fuse, the mesocardium perforates, looping starts, the ventricle starts ballooning</td>
</tr>
<tr>
<td>CS11</td>
<td>23-26</td>
<td>8.5</td>
<td>The atria balloon, the pro-epicardium forms</td>
</tr>
<tr>
<td>CS12</td>
<td>26-30</td>
<td>9.5</td>
<td>The septum primum appears, the right venous valve appears, the muscular part of the ventricular septum forms, cells appear in the cardiac jelly, the epicardial growth starts.</td>
</tr>
<tr>
<td>CS13</td>
<td>28-32</td>
<td>10.5</td>
<td>The atrioventricular-cushions form, the pulmonary vein attaches to the atrium, the left venous valve appears, epicardial mesenchyme appears first in the atrioventricular sulcus.</td>
</tr>
<tr>
<td>CS14</td>
<td>31-35</td>
<td>11.5</td>
<td>The atrioventricular-cushions approach one another, the outflow ridges become apparent, capillaries form in the epicardial mesenchyme</td>
</tr>
<tr>
<td>CS15</td>
<td>35-38</td>
<td>12</td>
<td>The atrioventricular cushions oppose one another, the secondary foramen forms, the distal outflow tract septates the outflow tract ridges reach the primary foramen.</td>
</tr>
<tr>
<td>CS16</td>
<td>37-42</td>
<td>12.5</td>
<td>The primary atrial septum closes, the outflow tract ridges approach the interventricular septum. The entire heart is covered in epicardium</td>
</tr>
<tr>
<td>CS17</td>
<td>42-44</td>
<td>13.5</td>
<td>Secondary atrial septum appears, the sinus node becomes discernable, the left and right atrioventricular connection becomes separate, the proximal outflow tract becomes septated, the semilunar valves develop.</td>
</tr>
<tr>
<td>CS18</td>
<td>44-48</td>
<td>14.5</td>
<td>Pappilary muscles appear, the atrioventricular valves start to form</td>
</tr>
<tr>
<td>CS19</td>
<td>48-51</td>
<td>15</td>
<td>The left venous valve fuses with the secondary septum, the mural leaflets of the mitral and tricuspid valve are released.</td>
</tr>
<tr>
<td>CS21</td>
<td>53-54</td>
<td>16</td>
<td>The main branches of the coronary artery become apparent</td>
</tr>
<tr>
<td>CS22</td>
<td>54-56</td>
<td>16.5</td>
<td>The chorda tendinae form</td>
</tr>
<tr>
<td>CS23</td>
<td>56-60</td>
<td>17.5</td>
<td>The septal leaflet of the tricuspid valve delaminates</td>
</tr>
</tbody>
</table>

**Folding of the embryo and formation of the heart tube**

As development progresses, during CS9, the flat embryo starts to fold. Folding of the cranial part of the embryo into ventro-caudal direction brings the heart-forming region in its final position (Figure 2A-A”). The cardiac precursors are initially situated anterior (cranial) and lateral to the stomatopharyngeal membrane (the future mouth), but posterior (caudal) from the mesoderm that forms the transverse septum, which contributes to the formation of the diaphragm. During the process of folding the cardiac precursors end up between the mouth at the cranial side, the diaphragm at the caudal side, and ventral to the foregut, as in the adult situation.

By folding of the embryo, the lateral parts of the cardiac mesoderm are brought together, forming the ventral part of the heart tube. The inner curvature of the cardiac crescent forms the dorsal side of the tube, and is contiguous with the dorsal mesocardium, the attachment of the heart to the body wall. The horseshoe-shaped cardiac crescent forms
a tube with two caudo-lateral inlets, or venous pole, and one cranio-medial outlet, or arterial pole (Figure 2 C-G).

The peripheral part of the cardiac crescent will eventually face the transverse septum and forms the venous pole of the heart, whereas the central part of the crescent, which forms the outflow tract, is contiguous with the pharyngeal mesenchyme [14 Lescroart, F (2010)] (Figure 2B). This intimate association of the cardiac and facial region during development might explain the high incidence of combined cardiac and facial malformations.

**Growth of the heart via addition of cells**

The linear heart tube manages to grow, despite the lack of proliferation in the cardiomyocytes, but does so by the addition of newly differentiated cardiomyocytes, derived from the surrounding mesoderm. Proliferation studies in chicken revealed that a pool of rapidly proliferating cells is present at the venous pole of the heart [15 van den Berg, G (2009)]. Labeling of these cells with fluorescent dyes demonstrated that cells from this proliferating pool migrate into the heart tube and differentiate into cardiomyocytes at all places where the myocardium is attached to the body wall, i.e. at the venous and arterial poles, as well as at the dorsal mesocardium. After rupture of the dorsal mesocardium, at CS10, cells can only be added to the heart at the venous and arterial poles (Figure 2F-G).

Mouse and human cardiac development display a similar course, with a highly proliferative precursor pool in the dorsal pericardial wall, or splanchnic mesoderm, and a significantly lower proliferative cardiac tube [11 Sizarov, A (2011)], [16 de Boer, BA (2012)] (Figure 2J).

In experimental studies using mice and chicken embryos it was shown that cells that initially reside in the outflow tract eventually end up in the right ventricle, whereas newly added cells, form the outflow tract [17 Kelly, RG (2001)]. Cells, initially forming the embryonic left ventricle, end up in the ventricular septum of the formed heart [18 Aanhaanen, WT (2009)] (Figure 2H,I). Cells that are added later at the venous pole of the heart form the left ventricle and atria. Therefore, the identity of a cardiomyocyte is not fixed and depends on its location in the developing heart. The stop of cell division of the initially formed cardiomyocytes is a reversible event. With the development of the chambers, a subset of cardiomyocytes re-enters the cell cycle and starts to proliferate again.

In many textbooks the linear heart tube is described as a segmented structure, already containing all the compartments of the adult heart, which just needed to grow. At the turn of this century, several studies demonstrated that the heart grows by gradual addition of cells [19 Mjaatvedt, CH (2001)], [17 Kelly, RG (2001)], [20 Waldo, KL (2001)], [21 Cai, CL (2003)]. This notion revolutionized the field, albeit, in retrospect, similar results were already obtained from chicken lineage tracing experiments in the 1970’s [22 De la Cruz, MV (1977)].

It has been proposed to divide the cardiac precursor pool into two groups of cells, cells that form the initial heart tube and cells that are added to this linear heart tube at a later
developmental stage [23 Buckingham, M (2005)], [24 Dyer, LA (2009)], [25 Rochais, F (2009)]. These two populations are called the first and second heart field, respectively (Figure 2B). The cells of the second heart field are found medially to the first heart field in the cardiac crescent stage. Subsequent to folding of the embryo and the formation of the heart tube, the second heart field is located in the dorsal pericardial wall, producing the rapidly proliferating population of cells that are added to the heart tube. So far, there are no unambiguous molecular markers to distinguish both fields, albeit gradually differences develop at the cranial as opposed to the caudal site of the field. The new concept is of great value as this emphasizes growth of the heart by addition of cells from an extra-cardiac precursor pool localized in the splanchnic mesoderm of the dorsal pericardial wall.

**Endocardium**

The inner lining of the heart, the endocardium, develops concomitantly with the cardiomyocytes within the heart field, during CS9, simultaneously with the formation of the linear heart tube. First a vascular plexus forms and unites into two hollow endocardial tubes, which, in turn, fuse to form a single tube (Figure 2E). Although the myocardial and endocardial progenitors develop in the same region, it has been demonstrated that individual cells differentiate either into endocardium or into myocardium, but not into both [26 Cohen-Gould, L (1996)]. Whether the cells that form the endocardium are intrinsically different from the future myocardial cells, or whether their location dictates their future phenotype, is unknown.

The endocardial cells lining the heart resemble the other endothelial cells that line all other blood vessels in the developing embryo. It is of no surprise that the molecular programs involved in the differentiation into endothelial or endocardial cells display many similarities [27 Harris, IS (2010)]. However, the initial steps involved in the differentiation seem to be different, as mutant mice and zebrafish exist that have no endocardium, but show normal endothelial development. Moreover, in the developing endocardium, the cardiac transcription factor Nkx2.5 is shown to drive endothelial gene programs [27 Harris, IS (2010)].

Taken together, studies on the growth of the linear heart tube revealed several major characteristics of cardiac growth. The linear heart tube grows not via division of myocytes, but by addition of cells from a proliferating pool of precursors. Secondly, when being added to the heart their fate is not fixed and their identity depends on their eventual location. The endocardium develops simultaneously with the myocardium and is a specialized endothelial cell type derived from the splanchnic mesoderm that via an unknown mechanism differs from the myocardial precursors.
From one tube to four chambers

Building plan of the heart

All cardiomyocytes 1) share the capability to contract, mediated by their sarcomeres and calcium supply from the sarcoplasmatic reticulum, 2) are capable of spontaneous depolarization, regulated by ion pumps and channels within their membrane, and 3) are electrically coupled to their neighboring cells, via gap junctions. Based on their strength of contractility, firing frequency, and conduction velocity, the cardiomyocytes can be subdivided into different groups (Figure 3).

The myocytes of the atrial and ventricular chambers are fast-conducting and have well-developed sarcomeres, by which they can generate higher contraction forces compared to the primary myocardium of the linear heart tube. Nodal cardiomyocytes, in contrast, have a high frequency of automaticity and have poorly developed sarcomeres. They are poorly electrically coupled to one another and to the surrounding atrial cells, which allow them to build up an electrical charge to drive the depolarization of the chambers. Because the nodal cardiomyocytes are poorly coupled they are slow-conducting cells. The cells populating the bundle branches of the ventricular conduction system have an intermediate phenotype being equipped for fast conduction, but otherwise have a nodal phenotype [28 Moorman, AFM (2003)].

Figure 3. Differentiation of the primary myocardium.

Panel A shows a flow chart of the differentiation of primary and secondary myocardium into the different kinds of adult type myocardium. The upper table in panel B shows the characteristics of different kinds of myocardium, in terms of their contraction and electrophysiological behavior. The second table summarizes the expression pattern of different T-box transcription factors in different parts of the heart. Note that the primary myocardium always expresses either Tbx2, Tbx3, or both. Panel C shows
Development of the human heart

A schematic of the chamber-forming, or ballooning heart. The primary myocardium is indicated in grey and the secondary myocardium in blue, note the grey area at the top of the ventricular septum (white dotted line) that retains characteristics of the primary myocardium and will become the atrioventricular bundle. Abbreviations: IFT: inflow tract; AR: atrial roof; AF: atrial floor; AVC: atrioventricular canal; IC: inner curvature; LV: left ventricle; RV: right ventricle; OFT: outflow tract.

**T-box transcription factors are key regulators of the fate of cardiomyocytes**

A family of transcription factors called T-box transcription factors plays a key role in the regulation of cardiomyocytes identity (Figure 3). They are expressed in different parts of the developing heart, thereby determining the electrical patterning of the heart. TBX5 and TBX20 are expressed in most parts of the heart. TBX5 is expressed in a gradient from the venous pole to the right ventricle and is absent in the outflow tract. Both factors are important activators of the chamber program of gene expression. TBX2 and TBX3 function as repressors of the working myocardium gene program keeping the inflow tract, or venous pole, atrioventricular canal and outflow tract myocardium “primary”, allowing these regions to develop another fate. TBX1 is expressed in the outflow tract precursors of the heart and TBX18 is expressed at the venous pole.

Both mouse models and human diseases in which these genes are impaired reveal cardiac defects associated with the area in which they are expressed, showing their importance in cardiac development [29 Plageman, TF, Jr. (2005)], [30 Boogerd, CJ (2009)].

**The ballooning model of chamber formation**

The myocardium of the tubular heart is called primary myocardium, and is composed of “primitive” or nodal-like cells. With the process of looping an S-shaped heart develops with inner and outer curvatures, during CS9-10 (see left right axis in heart development) (Figure 4). At the outer curvature of the heart differentiation along with re-initiation of cell division of the cardiomyocytes occurs, giving rise to the future ventricles (Figure 4). The same process of differentiation and proliferation occurs at the venous pole of the heart tube, by which the atrial appendices grow bilaterally (Figure 4). Although the atria are dorso-laterally formed, they eventually extend cranially at the left and right side of the outflow tract as in the formed heart (Figure 4 K). The differentiating and proliferating myocardium can be termed secondary or chamber myocardium. Via this process of differentiation and proliferation, the future chambers of the heart balloon from the primary heart tube (Figure 4 D,H,L). Histologically chamber formation becomes evident when the extensive extracellular matrix between endocardium and myocardium, known as cardiac jelly (see development of the valves) disappears and trabeculations become evident. The chamber myocardial cells start to express different genes like atrial natriuretic factor (ANF/NPPA), (Figure 4 A-D) and the gap-junction protein connexin40 (CX40/GJA5), allowing the formation of fast-conducting channels.

Whereas the ventricles and atria expand and differentiate, cells in the floor of the atrium, the atrioventricular canal, the inner curvature and the outflow tract maintain the features of the original primary heart tube (Figure 4 L). Parts of this primary myocardium will
differentiate into the nodal cells as present in the formed heart. As stated earlier it is important to appreciate that at these stages the heart still grows via addition of cells and that the identity of the cardiomyocytes changes with their position in the developing heart.

Figure 4. Formation of the cardiac chambers.
Panel A-D display a developmental series of mouse embryos; whole mount RNA in situ hybridization for the embryonic chamber marker atrial natriuretic factor (Anf) is used as a marker for differentiation into chamber myocardium. Schematic drawings of these chamber-forming hearts are shown in panels E-G and I-L. Grey: primary myocardium; blue: chamber-forming myocardium. The arrows in panel K indicates the expansion of the chambers eventually leading to the adult configuration, with the ventricles positioned ventro-caudally to the atria. Panel H shows an electron microscopic photograph of a CS14 human heart, demonstrating the similarity with the mouse E11.5 heart and the schematic shown in panel L. For didactic purposes, in the schematic in panel L, the outflow tract is hinged toward the right side, in vivo it is positioned ventrally to the heart, as depicted in panel D and H. Panels A,E,I. The heart tube (HT) consists from venous to arterial pole (VP, AP) solely of primary myocardium. Panels B,F,J. The first chamber to start ballooning is the embryonic ventricle (V) at the outer curvature of the heart. Panels C,G,K. The heart tube now has started to loop, and acquired an S shape. An embryonic left and right ventricle are now visible; also the atria (A) start to balloon, towards the left and right side; the myocardium of the outflow tract (OFT), inner curvature (IC), as well as the atrioventricular canal (AVC) remains primary myocardium. Abbreviations: RA: right atrium; LV: left ventricle; OFT: out flow tract. Modified from [31 Christoffels, VM (2000)], [97 Oostra, RJ (2007)]

Atrial chamber formation
At the dorso-lateral sides of the primary heart tube the atrial chambers form in symmetrical fashion. The myocardium differentiates into working myocardium and starts to proliferate more rapidly than the surrounding primary myocardium, which results in the formation of two pouches: the future atrial appendices. The eventual morphology of
these two appendages is under control of left-right signaling (see left right axis in heart
development). The developing atrial chamber myocardium, marked by the expression
of working myocardium genes like ANF, is flanked by primary myocardium of the sinus
venosus and atroventricular canal [31 Christoffels, VM (2000)] (Figure 4L). Downstream
with respect to the blood flow, the atroventricular canal myocardium separates the atria
from the ventricles. Upstream, the primary myocardium of the sinus venosus separates
the systemic veins from the atria. The initially formed atrial chamber myocardium only
gives rise to the trabeculated, atrial appendices in the formed heart. All smooth-walled
myocardium found in the full grown heart is added later during development.
Initially the veins draining to the heart are embedded in the mesenchyme at the venous
pole of the heart. With ongoing development the connecting veins become “excavated”
from this mesenchyme, by expansion of the pericardial cavity. In this way the common
cardinal veins, which are the confluence of the left and right superior and inferior cardinal
veins, become incorporated within the pericardial cavity. They become ensleeved by
myocardium and this confluence of the systemic veins is then called sinus venosus, or left
and right sinus horns. Eventually both sinus horns connect to the right atrium (Figure 5).
The right sinus horn becomes incorporated into the dorsal part of the full grown right
atrium, and is termed sinus venarum. In human the left sinus horn becomes the coronary
sinus, upon which the coronary venous circulation drains. The border between the atrial
chamber myocardium and the sinus myocardium can be observed in the adult heart as the
terminal crest. The valves flanking the orifices of the coronary sinus and inferior caval vein
are termed Thebesian and Eustachian valves, respectively (Figure 5).
The formation of the myocardium surrounding the systemic and pulmonary venous return,
are independent processes. Unlike the systemic venous connections the pulmonary vein
develops and connects to the heart after the formation of the initial heart tube and start
of chamber formation (Figure 6). Mesenchyme dorsal to the heart differentiates into a
vascular plexus surrounding the embryonic foregut. At CS 13, the cranial component of this
plexus connects as a solitary pulmonary vein to the heart through the dorsal mesocardium
in the midline and cranial to the atroventricular node (Figure 6E,F). Albeit the pulmonary
venous return is a midline structure eventually the pulmonary vein will drain into the left
atrium, because the primary atrial septum develops at the right side (Figure 6E, asterix),
by which the midline structures, including the pulmonary vein, become incorporated into
the morphologically left atrium. Subsequent to its connection to the forming left atrium
the pulmonary vein and its bifurcations become ensleeved by myocardium (Figure 6A-
D), which differentiate de novo. The transcription factor paired-like homeodomain 2
transcript variant c (PITX2c) plays a crucial role in the differentiation of the pulmonary
vein myocardium. Interestingly, it does so at the left and the right side. Therefore, this
transcription factor seems not to function as a mere laterality marker [32 Mommersteeg,
MTM (2007)]. During development, the muscularised pulmonary veins incorporate into
the left atrium, up to their second bifurcation, resulting into four pulmonary orifices in the left human atrium. The pulmonary myocardial sleeves do not extend to a great extent upstream of the pulmonary orifices and intermingle with fibrous tissue, which may be part of the cause of the frequent development of arrhythmias originating at the pulmonary orifices [32 Mommersteeg, MTM (2007)]. In contrast, in adult mice, as in human embryos, there is only one pulmonary vein orifice, but three in sheep and camels [33 Nathan, H (1970)]. In mouse the pulmonary myocardial sleeves extend up to the 5th bifurcation, the function of which has remained unknown as yet.

Figure 5. Formation of the venous pole.
Panel A-F show cross-sections of 3D reconstructions of human hearts, myocardium is in grey, systemic veins in blue, pulmonary veins in orange and cardiac mesenchyme in yellow. Panel A, B depict the developing right and left atrium (RA, LA), the right atrium is connected to both the right superior (RSCV) and inferior caval vein (ICV) as well as the left superior caval vein (LSCV), via the coronary sinus (CS). The entire systemic venous pole is connected to the right atrium through one orifice, flanked by the left and right venous valves (LVV and RVV), at this stage. The primary septum (PS) is growing right from the opening of the pulmonary vein (PV). In panel C, D the right superior caval vein and coronary sinus are still connected to the atrium via a single orifice, which has now been muscularised, the secondary foramen (SF), appears in the primary septum. Panel E, F show that all veins connect separately to the atrium, via myocardialized orifices. The primary atrial foramen (PAF) is closed and the secondary septum (SS) is growing between the left venous valve and the primary septum, being already partially fused to the secondary septum. The right venous valves can now be separated in a part that flanks the coronary sinus and a part that flanks the right superior caval vein, the future Thebesian and Eustachian valves respectively. Abbreviations: OF: oval foramen. Modified from [98 Sizarov, A (2010)]
**Figure 6. Development of the pulmonary vein from a Nkx2.5 positive precursor pool.**
Panels A-D show 3D reconstructions of the dorsal side of the right and left atrium (RA, LA). In blue the systemic venous return is depicted, composed of the right and left sinus horn (RSH, LSH). The developing pulmonary vein (PV) (in orange), becomes myocardialised (in grey) after connecting to the left atrium (LA). In panels E, F the pulmonary vein is about to connect to the atria, right to this connection the primary septum will develop (asterix). In panels G, H the Nkx2.5 lineage is shown in blue and the myocardium in brown. Note that the left and right sinus horns are composed of myocardium, yet are not derived from the Nkx2.5 lineage. The pulmonary vein and its surroundings are derived from the Nkx2.5 lineage. Abbreviations: AVC: atrioventricular canal; RV: right ventricle; FG: fore gut Modified from [32 Mommersteeg, MTM (2007)], [99 Soufan, AT (2004)] EM photos were a generous gift of Prof. N. Brown

**Formation of the ventricles**
At the outer curvature of the looped heart tube of CS10 the ventricles form by reinitiating proliferation, along with the start of a “chamber program of gene expression”, encompassing the expression of the hallmark genes *CX40* and *ANF* (Figure 2 and 4) [28 Moorman, AFM (2003)]. Initially a spongy, or trabecular type of myocardium develops. The trabecules grow by addition of cells at their base, by which the forming ventricle expands, or balloons exteriorly [16 de Boer, BA (2012)]. It is important to appreciate that the trabecules do not grow towards the lumen. If this were the case, the ventricle would not grow and would become filled with a mass of trabecules.

How the left and right ventricles obtain their different morphology is not understood, as yet, albeit some differences between the left and the right ventricle in terms of gene expression are known. For instance the cardiac transcription factor *TBX5* is expressed in a
gradient tapering off toward the right ventricle [34 Takeuchi, JK (2003)]. Two reporter mice harboring a LacZ gene regulated by elements surrounding either Myosin light chain 1 or Myosin light chain 3 gene show expression in either the left or right ventricle, respectively [35 Franco, D (2006)]. As will be discussed later, left-right signaling seems not to play a major role in the development of the distinct morphology of the left and right ventricles, which is to be expected because the right and left ventricle initially develop along the cranio-caudal axis of the embryo.

The initially formed ventricular chamber myocardium has, in its entirety, a trabecular phenotype. The compact layer only develops later, at CS14, under influence of the epicardial-derived fibroblasts, the interaction of which with the trabecular myocardium induces a cascade of proliferation and differentiation resulting in the formation of the compact ventricular layer [36 Ieda, M (2009)], [37 Lavine, KJ (2005)]. Embryos in which the pro-epicardium is removed, or in which the formation of epicardium is impaired, do not form a compact myocardial layer, leading to early embryonic death ( [38 Perez-Pomares, JM (2002)], [39 Yang, JT (1995)], [40 Kwee, L (1995)])). Compaction is thus not a process by which trabeculated myocardium becomes compact, but primarily a process by which the myocardium at the epicardial side of the ventricular wall proliferates to form the compact layer. When the compact outer layer starts to form, proliferation in the ventricular trabeculations ceases. This is at a stage that the heart is hardly more than a few millimeters, which makes it clear why the trabeculated myocardium, albeit retaining its original thickness, becomes inconspicuous in the adult heart [41 Christoffels, VM (2009)] (Figure 7). Interestingly, the early markers of chamber formation ANF and CX40 remain restricted to the original trabeculated myocardium, whereas, the compact ventricular layer does not express these markers [11 Sizarov, A (2011)].

Taken together, the heart is composed of cardiomyocytes that differ in three basic characteristics, being conduction, contraction and automaticity. Tbox transcription factors play a crucial role in the regulation of these differences. Cardiac chambers develop by proliferation and differentiation in localized areas of the primary heart tube, a process called ballooning. The trabeculated myocardium of the atrial appendages in the formed heart is derived from the initially ballooned atrial chambers, whereas the smooth walled part of the atria develops from the myocardium formed along the connecting veins. The ventricles develop at the outer curvature of the heart tube, initially forming trabeculated myocardium. Proliferation ceases in the trabeculated myocardium at the luminal side, along with an increase of proliferation at the pericardial side, by which the compact myocardium forms.
Figure 7. Development of the compact myocardium.
This figure shows a developmental series of mouse hearts stained for the expression of Cx40 mRNA and photographed at equal magnifications. Subsequent to an initial growth the trabecular myocardium no longer expands, whereas the compact myocardium continues to grow and will increase in size relative to the inner trabecular layer. Modified from [100 Christoffels, VM (2009)]
Building the ECG

**The earliest ECG recordings**

For efficient propulsion of blood, a coordinated contraction of the heart is required. In the early primary heart tube, at CS9-10, the depolarizing impulse travels slowly from the venous to the arterial pole of the heart, resulting in a matching peristaltic wave of contraction, which ensures a unidirectional flow of blood. At this stage the ECG has a sinusoidal morphology [42 Hoff, EC (1939)], [43 Christoffels, VM (2010)] (Figure 8).

When chamber formation starts around CS10, or about 3 weeks of human development, the myocardium of the newly formed chambers conducts the depolarizing wave faster than the primary myocardium [44 de Jong F. (1992)]. The myocardium flanking the chambers, at the venous pole, atrioventricular canal and outflow tract, retains its primary phenotype, preventing backflow of blood (see development of the valves). The alternating fast and slow conduction in the chamber-forming heart, can be registered in an ECG, which resembles that of the formed heart [42 Hoff, EC (1939)] (Figure 8H-J). Of note, solely by virtue of the arrangement of different types of cardiomyocytes, an adult-like ECG with atrioventricular delay can be obtained, whereas neither, electrical insulation by connective tissue, nor differentiated nodes and conduction system, are present at this stage.

![Figure 8. Development of the conduction system.](image)

Panels A-E show 3D reconstructions of the adult and E12.5 mouse conduction system. The molecular phenotype of the primary myocardium remains to be expressed in the atrioventricular region of the adult heart, termed the atrioventricular ring (AVR); the
The sinus node

From the first beat of the heart tube onward to the last beat of the adult heart, the electrical impulse travels from the venous to the arterial pole. Thus, dominant pacemaker activity always is at the venous pole of the heart. Since the heart grows by addition of cells during development, the most recently differentiated cells, added at the venous pole always possess dominant pacemaker activity.

The sinus node develops in the myocardium added at the venous pole of the heart (Figure 8F,G, Figure 3A). A conspicuous feature of this sinus myocardium is the absence of the key cardiac transcription factor NKX2.5, which, in turn, is under control of the transcription factor short stature homeobox 2 (SHOX2). Loss of SHOX2 results in ectopic NKX2.5 expression, hypoplasia of the sinus myocardium and bradycardia in mice [45 Blaschke, RJ (2007)]. In agreement with these observations ectopic expression of SHOX2 results in a decrease of NKX2.5 expression, whereas overruled SHOX2 inhibition, by over-expression of NKX2.5, results in hypoplasia of the sinus node [46 Espinoza-Lewis, RA (2009)]. Thus, the absence of NKX2.5 is a prerequisite for normal sinus node development.

The sinus myocardium is further characterized by the expression of TBX18. Lineage tracing experiments of TBX18-expressing cells have revealed that the entire sinus myocardium is derived from TBX18 positive cells [47 Mommersteeg, MT (2010)]. In the absence of TBX18 the sinus node develops hypoplastically [48 Wiese, C (2009)] (Figure 8 F,G).

The transcriptional repressor TBX3 also is expressed in the developing sinus myocardium, albeit only at the right side (Figure 8D,E). TBX3 prevents chamber formation by repressing the chamber-specific gene program. In the adult stage the sinus node still expresses TBX3, and, when overexpressed in adult atrial working myocardium, ectopic pacemaker function can be observed [49 Hoogaars, WM (2007)].

Thus, a gene regulatory network involving TBX18, SHOX2-mediated repression of NKX2.5 expression, and TBX3 inhibition of chamber formation, underlies the development of the sinus node.
sinus myocardium, in which eventually the sinus node will develop. Initially the entire sinus myocardium expresses this program, which, among others results in the expression of the ion-channel hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4). HCN4 is responsible for the spontaneous depolarizing “funny” current, a major component of pacemaker activity. With subsequent development this gene program becomes restricted to the sinus node proper via an as yet unknown mechanism and the remainder of the sinus venosus will differentiate into smooth-walled atrial myocardium upon activation of Nkx2.5 expression.

**Pulmonary venous myocardium differs from systemic venous myocardium.**
The pulmonary veins are often the source of ectopic depolarization in atrial fibrillation [50 Haïssaguerre, M (1998)], [51 Postma, AV (2009)]. A direct developmental link between the pace-making precursors of the sinus node and the pulmonary venous myocardium, however, cannot be made. The pulmonary vein myocardium differs fundamentally from the sinus venosus myocardium in both origin and transcriptional control. Genetic lineage tracings have demonstrated that pulmonary vein myocardium has a different origin than the sinus myocardium, and differentiates, de novo, after connecting to the developing atria [32 Mommersteeg, MTM (2007)] (Figure 6 G,H). In addition, the genetic programming of the pulmonary myocardium differs from the one in the sinus venosus myocardium. NKK2.5 is expressed in the pulmonary vein mesenchyme prior to myocardial differentiation, and sinus venosus markers such as TBX18 are never expressed in this myocardium. So, although in disease the pulmonary vein myocardium displays high automaticity, like the sinus venosus myocardium during development, the two structures differ fundamentally based on lineage and genetic programming.

**Atrioventricular node**
As described above the myocardium of the atrioventricular canal, maintains its primary phenotype, owing to the repressive action of the transcription factors TBX2 and TBX3 [43 Christoffels, VM (2010)] (Figure 3). These, in turn, are induced by the BMP-signaling pathway, via a complex mechanism involving another T-box transcription factor, TBX20 [52 Singh, R (2009)]. In contrast to the sinus node, NKK2.5 is expressed in the atrioventricular canal myocardium. Also the ion channel HCN4 is expressed in the atrioventricular node, but not the fast-conducting connexin, CX40, explaining its high automaticity but slow conduction.

Also the atrioventricular bundle and bundle branches express the transcriptional repressor TBX3, but nonetheless express CX40, allowing the bundles to conduct fast. A lineage analysis of this region has shown that the atrio-ventricular node, is derived from atrio-ventricular canal myocardium, whereas the atrio-ventricular bundle, as well as the bundle branches are derived from ventricular myocardium [53 Aanhaanen, WT (2010)] (Figure
In principle the electrical properties of the atrioventricular canal myocardium suffice to guarantee proper atrioventricular delay. This is indeed the case in hearts of lower vertebrates and in the embryonic hearts of birds and mammals [54 Jensen, B (2012)]. In the formed avian and mammalian hearts, however, the atria and ventricles are isolated by an insulating plane of fibrous tissue, with the atrioventricular node and bundle being the only connection between the atria and the ventricles. This might be interpreted as adding another layer of safety to the electrical design of the mammalian heart, preventing ventricular tachycardias.

**Peripheral ventricular conduction system**

Subsequent to propagation of the electrical impulse through the bundle branches the Purkinje fibers of the formed heart transmit the depolarizing wave toward the ventricular working myocardium via an endo-to epicardial front of activation. The ventricular conduction system is able to conduct fast by virtue of the fast-conducting gap junctions, composed of CX40 and gap junction protein, alpha 1 (GJA1/CX43).

Early in development, when no Purkinje fibers are visible, the trabeculated myocardium expresses fast-conducting connexins (Figure 7). This trabeculated myocardium can be considered as both the “working myocardium” and the “conducting myocardium”, activating the ventricular chamber from base to apex. During subsequent development the growth of these trabeculations ceases significantly when compared to the growth of the compact layer of myocardium [16 de Boer, BA (2012)]. This leaves the initial trabeculations as small structures on the endocardial side of the heart expressing fast-conducting connexins. It is, therefore, logical that these trabeculations are the eventual Purkinje fibers of the heart, as recent lineage studies have confirmed [55 Miquerol, L (2010)].
Septation

Septation of the heart can be subdivided into the septation of four different compartments, 1) the atria, 2) the ventricles, 3) the primary myocardium of the atrioventricular canal and primary (or interventricular) foramen, and 4) the outflow tract.

Different components contribute to the septation of the heart. Apart from the muscular septa that form in the atrial and ventricular chambers, also the non-muscular endocardial or atrioventricular cushions, outflow tract ridges and the dorsal mesenchymal protrusion (DMP), also called vestibular spine, participate in the septation of the heart (Figure 9).

The endocardial cushions initially are a-cellular structures filled with cardiac jelly produced by the myocardium. Prior to septation these structures are populated by endocardially-derived cells in case of the atrioventricular canal and in the case of the outflow tract ridges, also by neural crest-derived cells (see development of the valves).

The DMP develops differently at the dorsal side of the heart and is contiguous with the dorsal atrioventricular cushion and the mesenchymal cap at the leading edge of the primary atrial septum (Figure 9B). This group of cells has an extra-cardiac origin, the distinction between endocardium- and DMP-derived cells can be observed on the basis of genetic lineage tracings and their anatomical position in the heart [56 Snarr, BS (2007)], [57 Goddeeris, MM (2008)]. In humans the DMP is anatomically more pronounced than in the frequently studied mice. Mal-development of the DMP results in atrioventricular septal defects and absence of the DMP is observed in fetuses with Down syndrome [58 Webb, S (1999)], [59 Blom, NA (2003)].

Figure 9. Septation of the atria and primary foramen.

Panel A shows a schematic drawing of the chamber-forming heart, with the atrioventricular canal (AVC) and outflow tract (OFT) cushions and ridges. The two arrows going down, through the atrioventricular canal, in the ventricle, represent the blood flow during diastole. The two arrows pointing toward the direction of the outflow tract, represent the blood flow during systole. Note that the primary foramen (PF) is the cross road of the blood running from the right atrium (RA) to the right ventricle (RV), and the blood running from the left ventricle (LV) to the outflow tract. Panels B-F depict sagittal sections at the level of the dotted line in panel A. In panel B the ventral and dorsal endocardial cushions (nr1-2) are growing towards each other. In C the primary atrial foramen (PAF) is closing due to the in growing of the primary atrial septum (PS), with its mesenchymal cap (MC), the dorsal...
mesenchymal protrusion (DMP) and the endocardial cushions. From C-D In the primary septum small holes appear merging to form the secondary foramen (SF). From D-F the secondary septum (SS) grows to the right side of the primary septum covering the secondary foramen and the rest of the PAS, leaving at the right surface of the atrial septum only the oval fossa (OF) uncovered. Abbreviations: LA: left atrium; A: atrium; V: Ventricle; 3,4:septal and parietal outflow tract ridges

**Atrial septation**
During uterine life, the atria are not entirely separated from one another. This is so because in this period of life little blood flows via the lungs to the left atrium. To guarantee sufficient flow of blood to the left side of the heart, atrial septation occurs in phases, permitting right-left atrial shunting.

Atrial septation starts with the formation of a crescent-shaped structure, the primary septum (Figure 9 B,C), at CS12. The septum grows towards the atrioventricular canal, by proliferation from the cranio-dorsal wall of the atrium at the right side of the midline. The mesenchymal cap on the edge of the primary septum is ventrally contiguous with the ventral atrioventricular cushion, and dorsally with the DMP and the dorsal atrioventricular cushion. The primary atrial foramen or ostium primum is lined by this mesenchymal complex and permits communication between the two atria. With ongoing growth of the atrial septum this ostium primum closes (Figure 9D), at CS16. However, already at CS15, in the septum primum small holes develop, mediated by apoptosis, which merge forming the secondary foramen, or ostium secundum (Figure 9 C,D). After closure of the primary foramen the secondary foramen supports the right-left shunting of blood.

Subsequent to the formation of the secondary foramen, from CS17 onwards, the muscular wall of the right atrium folds down to form a secondary septum at the right side of the primary septum, covering the secondary foramen (Figure 9D-F Figure 5). As blood pressure in the fetus is higher in the right atrium than in the left, blood can flow from the right to the left side. After birth when the pressure gradient inverts, the primary and secondary septum are squeezed together, by which the secondary foramen, closes. The oval fossa is the part of the primitive septum that remains uncovered by the secondary septum (Figure 9F).

**Ventricular septation**
The interventricular septum in the formed heart is composed of both a myocardial and a membranous part. The development of the muscular part is described below; the membranous part will be discussed with the septation of the primary heart tube.

When the right and left ventricles form by expansion from the primary heart tube the cells in between them do not follow the chamber myocardial gene program, and do not balloon, but form the top of the ventricular septum. In fact the top of the septum is the most primitive part as was already recognized by Keith and Flack in the beginning of the previous century [60 Keith, A (1906)]: “The evidence is now accumulated which shows that the interventricular septum is not developed by a process of up-growth as His proposed;
its development is the result of an opposite process; the ventricles are outgrowths; or bulgings from the primitive cardiac tube; the septum is that part of the tube which remains between the outgrowth; hence the upper border of the septum represents the least changed part of the lumen of the embryonic heart and it is there that the atrioventricular bundle is found”. Indeed the top of the ventricular septum expresses the transcriptional repressor Tbx3 (Figure 8D). The crest of the ventricular septum connects with the caudal, or dorsal, atrioventricular cushion. The septum grows by a process called apposition, meaning that cells are added to the septum largely from the adjacent left ventricular free wall, and it becomes apparent at CS12.

**Septation of the atrioventricular canal and primary foramen**

Prior to septation, the blood flow is already separated into a left- and right-sided circulation, with limited mixing of the two, due to the fact that the bloodstream is laminar [61 Hogers, B (1995)]. The primary foramen is the region of the primary heart tube in between the parts of the heart tube from which the ventricles expand (Figure 9A). This region often is called the interventricular foramen, albeit this term formally is a misnomer, since it is never situated between the two ventricles, but on top of them, at the inner curvature. Through this foramen the right atrium connects with the right ventricle at diastole, and the left ventricle connects to the aorta at systole. This situation, obviously, is maintained in adult life, so the primary foramen never closes but becomes separated, by the membranous septum into a left and right part, at the end of CS18.

The atrioventricular canal is divided by two cushions, the ventral and dorsal endocardial cushion, into a left and a right half. Also the outflow tract is divided by two cushions, known as the septal and parietal outflow tract ridges, forming a pulmonary and an aortic channel (Figure 9A). The terms septal and parietal are based on the proximal attachment of these ridges to the primary ring region, being the circumference of the primary foramen. The left flow is guided by the atrioventricular cushions to the left ventricle and then by the outflow tract ridges to the outflow tract through the primary ventricular foramen. The right flow is directly guided to the right ventricle also via the interventricular foramen and then, with systole into the outflow tract (Figure 9A). Proper physical separation of the primary foramen is achieved by the fusion of the atrioventricular cushions and outflow tract ridges, resulting into the left ventricular outlet and the right ventricular inlet. In the adult heart the membranous part of the ventricular septum is the remnant of the fused atrioventricular and outflow tract cushions.
Separation of the outflow tract

At CS 12, the outflow tract is a myocardial tube that runs from the developing ventricles to the aortic sac. The aortic sac is connected to the, initially symmetrical, pharyngeal arch arteries. During subsequent development the cushions in the outflow tract separate the outflow tract, resulting into a fully separated pulmonary and aortic channel at CS18 [62 Sizarov, A (2012)]. The separation occurs in a distal to proximal order by which the outflow tract becomes gradually divided, starting at CS14. The cushions lay in a spiral fashion in the outflow tract, which gives rise to an 180° twist of the eventual pulmonary and aortic arteries. In case of a transposition of the great arteries this spiraling appears not to have taken place, and the aorta and pulmonary trunk are situated next to each other in the frontal plane, instead of a dorso-ventral positioning. During the process of septation, the outflow tract myocardium becomes largely incorporated into the right ventricle; the distal myocardial border retracts halfway to the level of the semilunar valves. This ensures that the coronary orifices are normally embedded in non-contractile vascular tissue, ensuring unhindered nourishment of the coronary vasculature during contraction.

Along with the separation of the outflow tract, a complex system of 3 pairs of pharyngeal arch arteries remolds into the aortic arch and pulmonary trunk (Figure 10). In short, the pharyngeal arch artery system is traditionally composed of 6 paired arteries although the 5th pair never develops in human. The 1st and 2nd pairs regress early in development before the remodeling into the aortic arch system takes place. Therefore, the outflow tract is, via the aortic sac, connected with three pairs of pharyngeal arch arteries, the 3rd, 4th and 6th (Figure 10A-D).

The pulmonary trunk, will be separated from the ascending aorta, by a protrusion of the pharyngeal mesenchyme, termed the aorto-pulmonary septum, that grows into the aortic sac and connects distally to the spiraling OFT ridges. This separates the 6th from the 4th pharyngeal arch artery (Figure 10E-H). Defects in this septation rarely occur and give rise to a distal connection between the aorta and the pulmonary trunk, termed an aorto-pulmonary window.

At the junction of the aortic sac to the 6th pharyngeal arch artery, the pulmonary arteries develop. At the right side, the distal part of the 6th pharyngeal arch artery is underdeveloped, compared to the left side, and will eventually obliterate, at the left side this forms the ductus arteriosus, which closes shortly after birth.

The 3rd and 4th pairs of pharyngeal arch arteries together with the left dorsal aorta will form the eventual aortic arch system (Figure 10I-L), the dorsal aorta between the 3rd and 4th pharyngeal arch arteries will obliterate, separating the future carotic artery from the descending aorta. The 7th intersegmental artery, which sprouts from the aorta at the level of the developing limbs, will give rise to the subclavian arteries. The right-sided dorsal aorta distal to the subclavian artery connection will obliterate, resulting in the final aortic arch with a left sided descending aorta (Figure 10M).
Figure 10. Development of the OFT and arterial pole.

In panels A, E, I cranial views of 3D reconstructed human hearts are shown; the systemic arteries are in red, systemic veins in blue, pulmonary arteries in purple, and pulmonary veins in orange, the myocardium is grey and the foregut is green. Panels B, C, F, G, J, K show the aorta and pharyngeal arch arteries (numbered 3rd, 4th, 6th) surrounding the embryonic foregut. The cartoon presented in panels D, H, I, M summarize the remodeling of the pharyngeal arch arteries in which different colors depict the different vessels. Abbreviations: R/LSA: right/left subclavian artery; R/LCCA: right/left common carotid artery BT: brachiocephalic trunk AAo: ascending aorta PT: pulmonary trunk LA: ligamentum arteriosum PA: pulmonary artery. Modified after [62 Sizarov, A (2012)] and cartoons were drawn from [102 Rana, MS (2013)].
The cardiac connective tissues

Most of the adult heart volume is made up by cardiomyocytes; however, a significant 20% of the cells in the heart is made up by the fibroblasts, smooth muscle and endothelial cells [63 Banerjee, I (2007)]. During development there are four sources of connective tissue cells in the heart: the endocardium, the DMP, the cardiac neural crest and the epicardium. In addition also bone-marrow derived cells populate the heart after development [64 Zeisberg, EM (2010)]. The first three populations are discussed in the sections on the development of the valves and septation, the development of the epicardium will be discussed below.

Figure 11. Development of the AV and OFT valves and their contributing tissues.
Panels A-H show sections through mouse hearts in a plane comparable to the schematic heart in panel S, boxed is enlarged in panels I,K,M,O. In panels B,D,F,H the lineage contributions of the epicardium is displayed at different developmental stages. The epicardial lineage marker WT1 was used, epicardial lineage is depicted in red, myocardium in green. Panels I-P show schematic drawings of valve development in both the atrioventricular canal as well as the developing outflow tract. Red is epicardium (Ep).
grey is primary myocardium and yellow is endocardial cushion tissue (EC). Note that in the outflow tract the cells are primarily neural crest derived and not endocardial derived as in the atrioventricular canal. In panel P the contribution of the different cushions and ridges to the eventual valves (panel T) is depicted. Panels Q,R depict the pro-epicardium (PE) in a 3 day old chicken embryo in panel R, the pro-epicardium is attached to the heart tube (HT) spreading out forming the epicardium. Abbreviations: RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle; MC: mesenchymal cap; LC: lateral cushion; VAVC: ventral atrioventricular cushion; DAVC: dorsal atrioventricular cushion; SR: septal ridge; PR: parietal ridge; IR: intercalated ridge; S: Septum; PT: pulmonary trunk; Ao: Aorta. Modified from [79 Wessels, A (2012)]

**Epicardium**

Originally the epicardium was thought to be derived from the outer layer of the myocardial heart tube, which was called the myo-epicardium, although the famous anatomist Wilhelm His the elder, already in 1885 considered this theory to be unlikely. Moreover, in 1909 Kurkiewicz identified the pro-epicardium as the source of the epicardium, yet it was until the late sixties of the previous century before these studies to be revealed from oblivion and the findings to be confirmed by electron microscopy. [65 Manasek, FJ (1969)], [66 Manner, J (2001)]

The pro-epicardium is a medusa-like structure, which develops at the venous pole of the linear heart tube at CS10 (Figure 11Q,R). While the heart is looping, at CS11, the villi of the pro-epicardium make contact with the outer surface of the heart, first at the dorsal side of the atrioventricular canal and then grow to cover the entire heart tube with epicardium, at CS16. The epicardium then undergoes epithelial to mesenchymal transformation giving rise to a mesenchymal layer, called the sub-epicardial mesenchyme, between the myocardium and the mesothelial epicardial outer layer, (CS15). In this sub-epicardial mesenchyme the coronary arteries develop. Some cells of the sub-epicardial mesenchyme invade into the heart, forming the cardiac fibroblasts that will populate part of the atrioventricular valves and isolating plane. Apart from their contribution to the fibrous skeleton of the heart, these fibroblasts also supply signals necessary for normal cardiomyocyte development, such as the growth of the compact myocardium (see formation of the ventricles).

**Development of Cardiac vasculature**

The coronary veins are formed by sprouting from the sinus venosus, and cover the heart by expanding the already connected vessels. This process is called angiogenesis, or sprouting, and the endothelium of the coronary veins is derived from the sinus venosus. The differentiation of the endothelial cells of the coronary arteries seems more complex. The coronary arteries develop in the sub-epicardial mesenchyme between CS16-18. A coronary plexus forms in the mesenchyme, which later remodels into the coronary artery system. Lineage tracings have shown that the smooth muscle cells lining the coronary arteries are derived from the epicardial cells. Coronary artery formation involves vasculogenesis, a process by which vessels are newly made and connect later to the circulation. Whether the endothelial cells of the coronary arteries are derived from the sprouting veins, or from a
different precursor pool present in the sub-epicardial mesenchyme, is unsettled as yet [67 Red-Horse, K (2010)] [68 Katz, TC (2012)].

After the coronary tree has developed the connection with the aorta is established. The signals that promote the coronary arteries to grow towards the aorta and perforate the aortic wall connecting with the systemic circulatory system, are unknown as yet. When the development of the outflow tract is altered, for instance in the absence of Tbx1, the coronary arteries fail to connect to the cardiac outflow channels in a proper fashion, demonstrating the intimate association between the development of the outflow tract and of the coronary arteries [69 Theveniau-Ruissy, M (2008)].

**The insulating plane**

The myocardium of the atria and ventricles becomes separated by an insulating plane. This insulation comprises both the annulus fibrosus in the strict sense, being the fibrous tissue surrounding the atrioventricular junctions, and the atrioventricular sulcus mesenchyme, which resides at the outer surface of the heart [70 Becker, AE (1978)].

At the endocardial side the annulus fibrosus is in continuity with the fibrous tissues of the valves and is implicated for the support and proper function of the valvular apparatus [71 Angelini, A (1988)]. Both endocardially as well as epicardially derived cells contribute to the insulating plane, as demonstrated by recent lineage studies [72 de Lange, FJ (2004)], [73 Zhou, B (2010)], [53 Aanhaanen, WT (2010)].

At the epicardial side in the atrioventricular sulcus, mesenchyme, derived from the epicardium [73 Zhou, B (2010)] is present. Interestingly, in patients with pre-excitation due to an atrioventricular accessory pathway (Wolf-Parkinson-White syndrome), often the fibrous part of the plane of insulation is found to be intact and the accessory pathway is present within the sulcus mesenchyme [70 Becker, AE (1978)]. Inhibited outgrowth of the epicardium, results in accessory connections between the atria and the ventricles as seen in Wolf-Parkinson-White syndrome [74 Kolditz, DP (2008)]. Deletion of either the bone morphogenetic protein receptor 1a (BMPR1a), or the transcription factor TBX2, or impairment of Notch signaling in the atrioventricular canal, gives rise to additional myocardial connections, demonstrating that the Notch/BMP/TBX2 signaling axis is involved in the development of the atrioventricular insulating plane [75 Gaussin, V (2005)], [76 Aanhaanen, WT (2011)], [77 Rentschler, S (2011)].

Taken together the fibroblasts populating the heart are derived from four different precursor pools, the epicardium, endocardium, DMP and neural crest. These cells will populate the future valves and parts of the septae, as well as the coronary vasculature. Apart from their roles as producers of the fibrous skeleton of the heart, the fibroblasts also produce signaling molecules, which are indispensable for the normal growth of the myocardium.
Development of the valves

Unidirectional flow in the primitive heart
In the formed heart, valves ensure a unidirectional flow of blood; in diastole the arterial valves prevent regurgitation of blood into the ventricles and in systole the atrioventricular valves prevent regurgitation into the atria. However, in the primary heart tube, which is devoid of valves, the blood flow is unidirectional; here regurgitation is prevented by the complete closure of the cardiac lumen during the peristaltic waves of contraction. If the heart tube would consist of just myocardium, its lumen could only be partly reduced. The full closure of the heart tube is achieved by the presence of cardiac jelly, extracellular matrix depositions in between the endocardium and the myocardium, which functions as a stuffer substance, already present at CS9.

With ongoing development chambers are formed, which no longer produce cardiac jelly. Also the contractile properties of the chamber myocardium changes allowing for fast synchronous contractions. The venous pole, atrioventricular canal and outflow tract myocardium, retains its primary phenotype. The cardiac jelly in these regions remodels into cushions and ridges, which will eventually differentiate into the valves. The slow conducting and long-lasting contractile properties of the primary myocardium, together with cardiac cushions, enable these regions to function as sphincters, preventing backflow of blood [10 Moorman, AFM (2007)].

Cushion formation
Whereas in the regions of the forming atria and ventricles, the cardiac jelly disappears, the cardiac jelly is sculptured into two major cardiac cushions in both the atrioventricular canal and outflow tract (Figure 9A, Figure 11). During subsequent development in both the atrioventricular canal and outflow tract minor cushions are formed, the so-called lateral cushions and intercalated ridges, respectively. The valves develop from the minor cushions and from the major cushions (Figure 11), albeit that the major cushions in the atrioventricular canal primarily contribute to the septation of the primary heart tube. Though the cushions are initially a-cellular, they become populated with cells from different sources.

Both the atrioventricular cushions as well as the outflow tract ridges are first populated by endocardially-derived cells, starting at CS12. Part of the endocardium overlaying the cushions undergoes a process called epithelial to mesenchymal transformation. During this transformation a subset of the cells loses their epithelial characteristics and turn into mesenchymal cells, which migrate into the adjacent cardiac jelly. The interaction between the myocardium and the endocardium is crucial for this process. BMPs produced by the outflow tract and atrioventricular canal myocardium, signal through the cardiac jelly to the underlying endocardium. Subsequently, transforming growth factor beta (TGFβ) and Notch
signals regulate the EMT, resulting in the population of the cushions with mesenchyme. [78 Luna-Zurita, L (2010)].

Although the endocardially-derived cells obviously populate the atrioventricular cushions, it has been a controversial topic whether epicardially-derived cells contribute to the atrioventricular-valves. In quail-to-chicken chimeric embryos, pro-epicardially-derived cells were observed in the atrioventricular-, but not outflow tract, valves. However, genetic lineage tracings in mice showed that the majority of cells within the valves are endocardially-derived. Genetic lineage tracing of epicardially-derived cells within the cardiac valves has until recently been lacking. It has become clear that the mesenchyme populating the atrioventricular cushions initially is endocardially derived, but with ongoing development, the lateral atrioventricular cushions become increasingly populated with epicardially derived cells [79 Wessels, A (2012)] (Figure 11).

Although the heart primarily is a mesodermal organ, a subset of ectodermal cells, the neural crest cells, migrates through the developing embryo, and plays an important role in the development of the arterial pole of the heart. The neural crest cells are set apart as the neural tube closes and contribute to the development of many different organs, including the heart, in which case the cells are termed cardiac neural crest cells. Experimental ablation of the cardiac neural crest in chicken embryos results in many different congenital heart diseases, among most frequently common trunk, pulmonary atresia and double outlet right ventricle. The cardiac neural crest functions both by supplying cells necessary for normal development of the outflow tract, and by secreting growth factors essential for normal development of the surrounding tissue [80 Hutson, MR (2003)], [81 Stoller, JZ (2005)].

The atrioventricular valves

The dorsal and ventral atrioventricular cushions are important for septation, as described above. Within the atrioventricular canal the valves are formed from the dorsal and ventral atrioventricular cushions as well as from the lateral atrioventricular cushions. To form valves, the cushions need to detach from the underlying myocardium and transform into the thin valve leaflets attached with chordae tendineae to the papillary muscles. The different valve leaflets detach from the myocardium via different mechanisms (Figure 11). The septal leaflet of the tricuspid valve and the aortic leaflet of the mitral valve, arise from the fused dorsal and ventral atrioventricular cushions. The part of the cushions forming the aortic leaflet of the mitral valve is never supported by myocardium, but protrudes from the beginning into the left ventricular lumen (Figure 11C,I,K). The septal leaflet of the tricuspid valve detaches by apoptosis from the underlying myocardium, being the last leaflet formed at CS23 (Figure 11 M-O). The mural leaflets of the atrioventricular valves, i.e. the anterior and posterior leaflet of the tricuspid valve and the parietal leaflet of the mitral valve are derived from the lateral atrioventricular cushions. These valves do not detach
from the underlying myocardium by apoptosis, but by excavation or ventricularization; the ventricles grow behind the cushions creating a space, liberating the valve leaflets, starting at CS19 (Figure 11 K-O). During this process a small amount of cardiomyocytes remain in the forming valve, which are removed by apoptosis during subsequent development.

**The semilunar valves**

In addition to the two outflow tract cushions (or ridges) involved in septation, two intercalated ridges form in the proximal part of the outflow tract. The parietal outflow tract ridge gives rise to the right aortic and pulmonary valve, the septal outflow tract ridge to the left aortic and pulmonary valve and the left and right intercalated ridges to the posterior aortic and anterior pulmonary valve leaflets, respectively (Figure 11 J,L,N,P). In contrast to the atrioventricular valves the arterial valves are not anchored via chorda tendineae, but their semilunar shape prevents them from prolapsing. The semilunar shape is achieved by apoptosis of the ridges at the distal side of the future valve leaflet, and its characteristic morphology is discernible at CS18. Subsequently, the initially shaped valves elongate by proliferation of the remaining mesenchyme and eventually thin to obtain the adult semilunar valve leaflets. The coronary orifices are found in the aorta just downstream of the myocardium in the cusps that face the pulmonary trunk.

**Remodeling of the embryonic valves**

The last stage of the atrioventricular and outflow tract valve development, involves their maturation in which the valve leaflets become slender by the remodeling of the extracellular matrix, becoming organized in three layers. (1) The atrialis of the atrioventricular valve leaflets and the ventricularis of the outflow tract valves, i.e. the side of the valve facing the inflow, which is primarily composed of elastic fibers and provides motility of the leaflets. (2) The fibrosa, on the opposite side is providing stiffness to the valve leaflets. (3) The spongiosa, in between the previous two layers, is composed of proteoglycans for compressibility of the valve leaflets [Hinton, RB (2011)].

In patients suffering from valve disease the organization of the different layers is disrupted. A large set of extracellular matrix genes such as elastins and collagens is involved in congenital valve disease, however not only mutations in extracellular matrix molecules result in valve defects, also dysregulation of the connective tissue transcription factor sex determining region Y-box 9 (SOX9), or disrupted signaling, like Notch and BMP, results in remodeling of the extracellular matrix and calcification as observed frequently in human valve disease [Hinton, RB (2011)].

Taken together the atrioventricular and semilunar valves develop from the atrioventricular cushions and outflow tract ridges that separate the atrioventricular canal and outflow tract. During their development, the lineages populating the valves change. Whereas
the atrioventricular valves are composed of endocardium- and epicardium-derived cells, the semilunar valves are primarily derived from the endocardial lineage, albeit a large contribution of the neural crest occurs. The final stage in valve development involves a maturation process in which different layers of different types of extracellular matrix are formed.
Left right axis in heart development

Establishment of the left-right axis
In human, as in all other vertebrates, many organs show differences between the left and right side of the body. One of the first signs of these morphological differences becomes apparent, when the linear heart tube starts to bend towards the right side, a process termed looping, which commences at CS9. The molecular mechanisms facilitating this breach of symmetry, however, are initiated earlier during development. Albeit much is known about the basic principles of the establishment of left-right asymmetry [83 Logan, M (1998)], [84 Nonaka, S (2002)], [85 Nonaka, S (1998)], much has to be learned about this process in human development.

In mammals the nodal cilia located at the anterior border of the primitive streak, sweep from right to left, resulting in a leftward flow of extra-cellular fluid, which, in turn, activates at the left side the expression of the TGFβ super-family member Nodal. Via an auto-regulatory feedback loop, Nodal concentrations rise temporarily on the left side. A spread of this signal to the right side is inhibited by midline expression of the Nodal inhibitor Lefty1 [86 Chen, CM (2010)] (Figure 12 A).

There are two theories for the transduction of the cilia-mediated flow into a left sided expression of Nodal. Either the flow is mechanically sensed by sensory cilia on the left side of the node or, small excreted vesicles containing retinoic acid and the growth factor sonic hedgehog are moved by this flow to the left side [86 Chen, CM (2010)]. Be this as it may, the left-sided expression of Nodal is crucial in establishing the left-right axis. The Nodal signal is transduced, upon its binding, together with the cofactor Cripto (CFC1), to the activin receptor complex. The transduction of this signal results in the expression of the transcription factor PITX2c, which is an important regulator for conferring a sense of leftness to the body. PITX2c remains expressed on the left side long after the expression of Nodal ended. (Figure 12B)

Establishment of the left-right axis is thus a matter of defining the left side in molecular terms. Impairment of the right-to-left flow by impaired cilia function results in randomization of the left-right patterning. Disruption of Nodal signaling gives rise to loss of left-sided signals and thus to right isomerism. Insufficient inhibition of Nodal at the midline results in an expansion of left-sided signals and thus causes left isomerism. Interestingly, cardiac looping seems to be independent of PITX2c. PITX2c mutants display right isomerism, thus two morphologically right atria, but cardiac looping is unaffected. This indicates that in addition to the PITX2c pathway, another pathway plays a role in the looping of the heart [12 Harvey, RP (2002)] (Figure 12 A).
**Development of the human heart**

Figure 12. Left right signaling during development of the heart. Panel A displays a schematic representation of the signaling cascades involved in heart development. The initial event translating the leftward flow of the cilia in Hensen’s node is the left sided expression of nodal, that via the activine receptor 1b and 2b (\textit{Acvr1b} \textit{Acvr2b}) and co-receptor cripto (\textit{Cfc1}) regulates the expression of FoxH1 and Pitx2c. Note that there are two separate pathways one involving Pitx2c, determining atrial identity, and the other determining looping direction, or ventricular topology. In panel B a RNA-ISH for Pitx2c on chicken hearts is shown, where only the left half of the heart tube (arrows) is positive. Panels C,D show scanning electron microscopic photographs of the venous pole of a E10.5 mouse heart. Panel C represents the WT situation and D the Pitx2c KO situation showing an almost perfectly symmetrical set of atria, both marked as right atrium (RA), no pulmonary vein (PV) orifice and no atrial septum (AS).

**Processes differentially affected by left-right signaling**

While the linear heart tube grows a looping occurs. Looping can be subdivided into two different stages. During a process termed C-looping, the heart loops to the ventral side, owing to the continuous addition of cardiac cells to the heart tube, whereas the arterio-venous distance remains constant. The heart turns then to the right, a process termed D-looping, at CS10. This places the left side of the heart towards the ventral side of the embryo. Interestingly, explants of linear heart tubes show upon culture spontaneous looping suggesting that laterality information has already been imposed on the heart at an earlier stage [87 Manning, A (1990)]. During the looping process the dorsal mesocardium, breaks through, at CS10. Eventually the venous pole of the heart is situated cranially to the
rest of the heart. The ventral side of the primary heart tube becomes the outer curvature of the looped heart and the dorsal side the inner curvature.

The left and right ventricles differentiate and expand at the ventral side of the heart tube. In contrast to the atria, which develop in bilateral fashion, the right ventricle develops downstream from the left one. Consequently, isomerism of the ventricles does not exist. Although the identity of the ventricles itself is determined by their cranio-caudal position, rather than by left-right signaling pathways, their mutual positioning is. If the heart loops to the left instead of the right side, the right ventricle will be positioned on the left side of the body. Assuming that the atria and outflow tract receive correct left-right signals the left atrium will be connected to the right ventricle and the morphological right ventricle will be connected to the aorta. This situation is known as a congenitally corrected transposition of the great arteries, also termed double discordance. How left-right signaling affects cardiac looping is not clear, but it is known to be independent from the Pitx2c pathway and the transcription co-factor Cited 2 is involved [88 Bamforth, SD (2004)].

Unlike the ventricles, the atria are formed bilaterally in a symmetric fashion. The developing atria receive left-right signals and develop differently according to these signals. The identity of the atria is assessed by the morphology of the cardiac auricles, or atrial appendices, albeit formally this is the mere identity of the atrial appendages. Upon disruption of PITX2c-mediated laterality, both atria develop with right atrial appendages, demonstrating the role of PITX2c in the establishment of atrial sidedness [89 Franco, D (2003)] (Figure 12 C,D).

Both the arterial and venous poles are subject to left-right signaling. The connection of the outflow tract, linking the right ventricle to the pulmonary artery and the left ventricle to the aorta, is influenced by left-right patterning. Also the development of the pharyngeal arch arteries, which determine the morphology of the aortic arch and pulmonary trunk, are under control of left-right patterning.

It is equally important to appreciate that both the connections of the different veins to the atria as well as the development of the veins themselves, for instance the left superior caval vein or azygos system, are influenced by the left-right signaling pathway.

**Pathogenesis of discordant connections**

As described above, virtually all components of the heart are subject to left-right signaling. An intriguing aspect however, is that the molecular pathways involved in the asymmetric development differ between the distinct components of the heart. Perturbations high in the hierarchy of the left-right signaling cascade, like in Kartagener syndrome, result typically in randomization of the situs, either resulting in situs solitus or situs inversus. In the latter case, the anatomy is mirrored, but all cardiac connections are concordant [90 Kennedy, MP (2007)]. Discordant connections arise when tissue-specific left-right signaling is effected and thus some parts develop normally and others mirrored. For example if only
cardiac looping is altered both atrio-ventricular as well as ventriculo-atrial connections are discordant. Several studies suggest a role for the TGFβ super family signaling in cardiac heterotaxias [91 Fakhro, KA (2011)], [92 Karkera, JD (2007)], [93 Monteiro, R (2008)].

Taken together, the establishment of a left-right axis is partly understood. Nodal cilia mediating a right to left flow in the gastrulating embryo are crucial for the establishment of the normal left-right asymmetry of the body. Although the ventricles develop according to a cranio-caudal patterning, their left–right location in the body depends on the direction of looping. In contrast to the ventricles, the atria develop in a bilateral fashion and are under control of the left-right pathway, and therefore can be inverted or isomeric. The molecular pathways regulating asymmetric development differ between the distinct parts of the heart, allowing discordant connections to develop.
Future perspective.

In the last two decades the field of heart development has been revolutionized. The fact that the heart grows by addition of cells and the heart tube thus, does not contain all the precursors for the full grown heart has been well-accepted. In addition, genetic lineage tracings have demonstrated that, both the cardiomyocytes as well as the fibroblast in the heart are comprised of different populations. The working model of a tube of primary myocardium from which, by differentiation and proliferation, chambers balloon out, has been proven by lineage, gene expression and electrophysiological studies. In spite of this enormous knowledge, only in a discouraging low percentage of patients with cardiac malformations, a genetic or environmental cause can be found. The hypothesis that several (genetic) events have to take place in one patient before a cardiac malformation takes place becomes more and more likely. It is therefore that the interactions of the known players in cardiac development, needs to be studied. Novel techniques identifying regions in the genome on which transcription-factors act, driving their target genes, have already provided us with new disease-causing loci [94 van den Boogaard, M (2012)], [95 Arnolds, DE (2012)], [96 Smemo, S (2012)]). To further unravel how the cacophony of individual factors is orchestrated into the, rhythmically beating, full-grown heart, will be our next challenge.