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Chapter 1

A molecular and genetic outline of cardiac morphogenesis

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Abstract

Perturbations in cardiac development result in congenital heart disease, the leading cause of birth defect-related infant morbidity and mortality. Advances in cardiac developmental biology have significantly augmented our understanding of signalling pathways and transcriptional networks underlying heart formation. Cardiogenesis is initiated with the formation of mesodermal multipotent cardiac progenitor cells, and is governed by cross-talk between developmental cues emanating from endodermal, mesodermal and ectodermal cells. The molecular and transcriptional machineries that direct the specification and differentiation of these cardiac precursors are part of an evolutionarily conserved programme that includes the Nkx-, Gata-, Hand-, T-box- and Mef2-family of transcription factors. Unravelling the hierarchical networks governing the fate and differentiation of cardiac precursors is crucial for our understanding of congenital heart disease and future stem cell-based and gene therapies. Recent molecular and genetic lineage analyses have revealed that subpopulations of cardiac progenitor cells follow distinctive specification and differentiation paths, which determine their final contribution to the heart. In the last decade, progenitor cells that contribute to the arterial pole and right ventricle have received much attention, as abnormal development of these cells frequently results in congenital defects of the aortic and pulmonary outlets, representing the most commonly occurring congenital cardiac defects. In this review, we provide an overview of the building plan of the vertebrate four-chambered heart, with a special focus on cardiac progenitor cell specification, differentiation and deployment during arterial pole development.
The transcriptional and molecular machineries that control heart development are tremendously complex and sensitive to genetic and environmental disturbances. This is reflected by the high incidence of congenital cardiac anomalies in the human population, affecting approximately 1% of all live births. Consequently, our insight into the etiology of heart malformations is directly linked to our understanding of cardiac development.

The heart is the first functional organ in the developing embryo and, initially, is present as a tube-shaped muscular pump. Blood is propelled uni-directionally from the caudal or venous pole to the cranial or arterial pole. The addition of cells to both poles will cause the tube to elongate. The primary myocardial cells that make up the heart tube at this stage will subsequently start to proliferate and differentiate at localized regions to give rise to the cardiac chambers, whereas subregions of non-chamber myocardium will be specified into the cardiac conduction system. Complex morphogenetic processes underlie the transformation of the tubular heart into a heart that is composed of four cardiac chambers with corresponding cardiac valves, venous inlets and arterial outlets.

Aberrant development of any of the different cardiac components can potentially result in congenital cardiac defects. Malformations of the great arteries and right ventricle are among the most commonly occurring congenital heart defects, yet the molecular mechanisms that underlie the development of these cardiac regions are still not fully understood. In this review, our current knowledge of the underlying molecular pathways and transcriptional machineries that direct proliferation, differentiation and specification of cardiac progenitor cells will be discussed. In Part 1 of this review, we provide a general overview of cardiac morphogenesis. In Part 2 we emphasize the development of the cardiac progenitor cells that contribute to the heart after the initial formation of the embryonic heart tube, in particular, the arterial pole of the heart.

Anatomy of the adult four-chambered heart

The heart is a rhythmically contracting muscular pump that propels blood throughout the body, thereby establishing a circulatory system, in which oxygen and nutrients are delivered to and waste products are disposed from all the organs. The adult heart is composed of two atrial and two ventricular chambers, separated from each other by septa to prevent intermingling of oxygen-rich and -poor blood and by cardiac valves to guarantee unidirectional blood flow and prevent regurgitation of blood (Figure 1a). De-oxygenated blood fills the right atrium via the superior and inferior caval veins, and passes the tricuspid valve to enter the right ventricle, before it is forwarded to the lungs through the pulmonary trunk and its branches. This part of the circulatory system is known as the pulmonary circulation (Figure 1b). Oxygenated blood from the lungs is forwarded to the pulmonary veins to enter the left atrium. After passing the mitral valve, blood will enter the left ventricle to exit via the aorta to nourish the body through the arterial vascular system of the body, as such making up the systemic circulation (Figure 1b).

The atria and the ventricles contract sequentially and force blood toward either the lungs or the body. This propagation is highly dependent on the functional properties of the
cardiac pacemaker and conduction system. The cardiac beat is initiated in the sinus node, which is located at the junction of the superior caval vein and the right atrium (Figure 1c). From the sinus node, the electrical impulse spreads over the atria and toward the atrioventricular node and to the atrioventricular junction. The impulse is delayed when travelling from the atrioventricular node to the atrioventricular bundle and bundle branches in the ventricular septum and walls; this delay ensures ventricular filling. Impulse propagation via the bundle branches subsequently contributes to an apex-to-base contraction of the ventricles, by which blood from the right ventricle enters the pulmonary circulation and blood from the left ventricle enters the systemic circulation.

![Diagram of the heart and circulation system](image)

**Figure 1.** The adult human heart and circulatory system. (a) The human heart is composed of two atria and two ventricles, separated by septa and valves. De-oxygenated blood (present in blue components) enters the right atrium (ra) from the inferior (icv) and superior caval vein (scv), and is forwarded to the lungs. Oxygenated blood (present in red components) is forwarded from the lungs to the pulmonary veins (pv), and subsequently to the body via the aorta (Ao). (b) The circulatory system can be divided into a systemic and pulmonary circulation with oxygen-rich (red) and oxygen-poor blood (blue). (c) Schematic overview of the conduction system of the heart, with myocardial (blue) and non-myocardial (yellow) parts. The electrical impulse is initiated in the sinus node (sn) and travels to the atrioventricular node (avn), where it is delayed as the impulse is propagated to the atrioventricular bundle (avb) and bundle branches (bb), activating the ventricles from apex-to-base. Abbreviations: aa, aortic arch; avj, atrioventricular junction; DaO, descending aorta; epi, epicardium; la, left atrium; lv, left ventricle; mv, mitral valve; pa, pulmonary artery, pt, pulmonary trunk; rv, right ventricle; tv, tricuspid valve; vs, ventricular septum.

The cardiac chambers as found in the adult heart have long been suggested to be present in the embryonic heart, and subsequent growth of these pre-specified embryonic segments was thought to give rise to their adult counterparts. Recent molecular and genetic lineage analyses have challenged this developmental view by demonstrating that the heart acquires its final form upon addition of cells outside the embryonic heart, including cardiac progenitors and neural crest-derived cells. Moreover, the cardiac conduction system originates from embryonic primary myocardial cells that have retained aspects of their primitive phenotype, rather than being specified from a different precursor pool. Our view of the developing heart has changed remarkably over the years and current progress has led to significant novelties in cardiac embryology and progenitor cell biology. In the following sections, we will outline the currently accepted paradigm of the developing four-chambered heart.
Part 1: Contemporary concepts of heart development

Formation of cardiogenic mesoderm

During the process of gastrulation, three germ layers emerge in the developing embryo; a dorsal ectodermal layer, a ventral endodermal layer, and a mesodermal layer in between these layers. Subsequent to the formation of these layers, the intra-embryonic coelom develops within the mesoderm, dividing it into a somatic and splanchnic mesodermal layer flanking the ectoderm and endoderm, respectively. Precursors of the heart are induced from the splanchnic mesodermal cells that make up the anterior part of the primitive streak, also known as lateral plate mesoderm.\textsuperscript{10,11} In the third week of human embryonic development, or around 6 days of mouse development, these induced cardiogenic mesodermal cells migrate craniolaterally to form two bilateral heart-forming fields.\textsuperscript{12,13}

In vertebrates, the transcriptional programmes that guide the specification of splanchnic mesoderm into cardiogenic mesoderm require the T-box transcription factor Eomesoderm (Eomes).\textsuperscript{14} In the primitive streak stage embryo, Eomes directly activates the basic helix looped helix transcription factor Mesoderm Posterior 1 (Mesp1)\textsuperscript{15}, which has been identified as an important regulator of cardiac specification and migration prior to the formation of the early heart.\textsuperscript{16-19} Mesp1-induced specification of cardiogenic mesoderm is achieved by down-regulation of pluripotency genes and up-regulation of cardiac transcription factors such as the Zinc-finger factor Gata4, the homeobox protein Nkx2-5, the MADS-box containing enhancer factor Mef2c, but also Smarcd3, a transcriptional cofactor in the SWI/SNF chromatin remodeling complex.\textsuperscript{16} In agreement with the role of Mesp1, ectopic hearts were observed when Mesp1 mRNA was injected into Xenopus embryos\textsuperscript{20}, and it was found to induce multipotent cardiovascular progenitors from embryonic stem cells \textit{in vitro}.\textsuperscript{21} Similar to Mesp1, family member Mesp2 is expressed in early mesodermal cells that are destined to give rise to cranial and cardiac mesoderm.\textsuperscript{19} In Mesp1 null embryos, Mesp2 is highly up-regulated and may be compensating for the loss of Mesp1\textsuperscript{18}, albeit Mesp1 has been shown to be more important in promoting cardiac differentiation.\textsuperscript{17,20,21} Importantly, in mouse embryos lacking both Mesp1 and Mesp2, cardiac mesoderm was found to be absent.\textsuperscript{18,19}

Although the myocardial potential of induced cardiogenic mesodermal cells has been appreciated, these cells only acquire a myocardial phenotype when imposed accordingly by stimuli emanating from neighboring cells, which include the anterior visceral and definitive endoderm, ectoderm and precursors of the future notochord (Figure 2).\textsuperscript{22,23} Signalling from anterior endodermal cells positively regulates cardiac specification, whereas signalling from the neural plate, somatic and axial mesoderm represses heart formation.\textsuperscript{24} The relevant stimulatory signalling pathway components include Hedgehog- (Hh) and Wnt ligands, fibroblast growth factor (FGF) signals, and the members of the Transforming growth factor (Tgf) superfamily Nodal and bone morphogenetic proteins (BMPs).\textsuperscript{25-29} Inhibitory regulation is mostly achieved through the Wnt signalling pathway, which itself is inhibited by the Wnt antagonists Dickkopf1 (Dkk1) and Crescent.\textsuperscript{30} The importance of endodermal Wnt signalling was demonstrated by conditionally ablating endodermal β-catenin, the intracellular mediator of canonical Wnt signalling, which resulted in the induction of multiple cardiac foci in adjacent mesodermal cells.\textsuperscript{31} Repressing endodermal Wnt/β-catenin furthermore impacts on the endodermal expression of the homeodomain transcription factor Hex\textsuperscript{32}, which, in...
turn, is required for commitment of adjacent mesoderm into the cardiogenic direction. Wnt ligands Wnt11 and Wnt3a in somatic mesoderm as well as BMP-inhibitors Noggin and Chordin present in the dorsal neural tube and notochord combinatorially restrict cardiac commitment (Figure 2). In addition, transcription factor Gata6 was found to regulate the levels of Wnt2, which is required in embryonic stem cells to promote cardiac differentiation of precardiac mesoderm via the non-canonical Wnt pathway. The formation of cardiomyogenic precursors requires stringent regulation, as emphasized by a recent report, in which the transcription factor Scl was shown to suppress cardiac differentiation in prospective endothelial and endocardial cells.

**Figure 2.** Induction of cardiogenic mesoderm. Formation of cardiogenic cells from splanchnic mesoderm occurs through cross-talk between multiple tissue types as depicted in an embryonic day (E) 7.5 mouse embryo. Wnt signaling from the neuro-ectoderm (ne) inhibits cardiogenic mesoderm formation. BMP-inhibitors Noggin and Chordin from the notochord (ntc) inhibit Wnt signaling (Wnt8 and Wnt3a), allowing cardiac commitment in subregions of somatic mesoderm. Endodermal BMP-, FGF- and Hh-signaling underlies cardiac differentiation and the activation of genes encoding cardiac transcription factors, including Nkx2-5, Gata4/5/6, Tbx5, Myocardin and Mef2c. Abbreviations: end, endoderm; hm, head mesenchyme. See text for details.
Formation of the cardiac crescent and tubular heart

Between 17 and 19 days of human development, and on embryonic day 7.5 in mouse, the mesodermal cardiac precursors in the heart-forming fields migrate to the midline and join, giving rise to a cardiac crescent of differentiating cardiomyocytes.\textsuperscript{38} The evolutionarily conserved signalling pathways that guide cardiac differentiation\textsuperscript{39} in turn activate the expression of cardiac transcription factors, of which Nkx2-5, Gata4, T-box transcription factor Tbx5, Hand2 and Mef2c are deemed crucial.\textsuperscript{40-43} In agreement with this, mutations in genes encoding transcription factors have been reported in patients with congenitally malformed hearts.\textsuperscript{1} Cardiac transcription factors often act as co-factors in both differentiated and undifferentiated cardiac precursors to regulate a number of target genes, which control the onset of myocardial differentiation as well as progenitor cell fate, demonstrating the complexity of the developmental genetics of congenital heart defects.

Around 19-21 days of human embryonic development, differentiation of cranial splanchnic mesoderm and folding of the embryonic disc leads to the transformation of the cardiac crescent into a bowl-shaped heart\textsuperscript{44} (Figure 3a, b). The fusion of the bilateral heart-forming regions and subsequent formation of the embryonic bowl-shaped heart has been suggested to depend on the contractile properties within the underlying endoderm.\textsuperscript{45} The embryonic heart at these early stages is composed of an inner endocardial layer and is ventrally enveloped by acellular cardiac jelly and an outer myocardial layer (Figure 3a).\textsuperscript{44,46} The cardiac myocytes border the coelomic splanchnic mesodermal wall, with the cardiac jelly lying directly against the ventral wall of the foregut (Figure 3a). At these stages, the heart is bilaterally symmetrical and the caudal side is connected to the venous tributaries, yolk sac and placenta. The heart subsequently detaches from the ventral pharynx when the cardiac precursors fuse to form a cardiac tube (Figure 3c, c', d, d'), albeit a dorsal continuity transiently remains present as the dorsal mesocardium (Figure 3c', e, f, arrow). The newly formed tubular heart only remains attached to the coelomic wall at the arterial and venous poles (Figure 3c', d').

Because the cells that contribute to the bowl-shaped heart are the first cardiac precursors to differentiate, they are collectively referred to as the primary or first heart field (FHF), a population of cells pivotal for the development of the embryonic ventricle. Most of the cardiogenic mesoderm, however, remains present as an undifferentiated subpopulation medially and posteriorly to the cardiac crescent.\textsuperscript{4} Along the course of development, these undifferentiated cells maintain their high proliferative level and are gradually added to the heart, by which the primitive heart tube elongates (Figure 3b-d). Because of the developmental characteristics and delayed differentiation, these precursors are commonly referred to as the second heart field (SHF).\textsuperscript{38} It should be emphasized, however, that this terminology is largely based on the temporal aspect of the onset of differentiation and addition to the heart. With ongoing development, the lateral margins of the forming heart tube migrate to the midline, fuse, and detach from the dorsal coelomic wall. By this process, a closed heart tube is formed and at the same time the left and right halves of the SHF cells fuse in the midline to form the dorsal pericardial wall (Figure 3). Consequently, further elongation of the heart tube can only be achieved by addition of cells from SHF precursors at the arterial and venous poles of the heart.
Elongation of the tubular heart

The initial description of the SHF population dates back several decades ago, when studies performed in chicken embryos elegantly demonstrated the dynamic contribution of cells to the heart, mainly after the formation of the cardiac crescent and heart tube.\textsuperscript{47-49} More recently, retrospective clonal analysis in the early developing mouse heart revealed that labelled cells could be traced back in either the left or right ventricle, outflow tract and atria, pointing toward a growth process in which the onset of differentiation correlates with the addition of cells to the heart.\textsuperscript{50} Using a different genetic strategy, the expression of an Mlc1v-nlacZ transgenic construct that had integrated upstream of the Fgf10 gene was first found to be restricted to the pharyngeal mesoderm and at later stages was also expressed in right ventricular and outflow tract myocardium.\textsuperscript{5} Lineage tracing experiments showed that these pharyngeal mesodermal cells contribute to the elongating outflow tract, and that outflow tract myocardium becomes incorporated into the right ventricle in both mouse\textsuperscript{5} and chicken.\textsuperscript{7} In addition, Cai and co-workers (2009) demonstrated that the cardiac precursors of the heart are located contiguous with and medial to the cardiac crescent, which provides cells to both poles of the heart. These findings have led to the current paradigm that the heart tube elongates by recruitment of SHF cells that give rise to the outflow tract, right ventricle and ventricular septum and to the remaining part of the left ventricle and the atria.\textsuperscript{4,24,51} Taken together, the FHF represents the cardiac precursors of the primitive myocardial crescent or bowl, and is important for the developing primitive ventricle of the tubular heart, whereas the remainder of the heart requires the ongoing contribution of SHF cells.

Early growth and looping of the embryonic heart

As soon as differentiation of cardiac progenitor cells into the myocardial lineage has initiated, proliferation ceases.\textsuperscript{52} This was didactically shown by three-dimensional proliferation mapping, which revealed that the proliferation rate of differentiated cardiomyocytes in the chicken, mouse and human embryonic heart tube is relatively low, compared to the rapidly dividing cardiac precursors of the SHF in the dorsal pericardial wall (Figure 3e’, 3f’, arrow\textsuperscript{44, 53-55}), which enter the cell cycle every \textasciitilde 5.2 hours.\textsuperscript{55} Differentiation of cardiac precursors initially occurs all the way through the tissue connecting the forming heart tube to the surrounding splanchnic mesoderm.

The addition of differentiating cardiac progenitor cells to the heart is paralleled by asymmetric growth of the elongating heart tube, establishing a dextral movement of the heart known as cardiac looping (Figure 3c, d). The underlying mechanism of the formation of this dextral loop remains unclear. It is intimately associated with the development of laterality in the embryo, which is established during gastrulation and depends on a monocilia-rich structure called the Hensen’s node.\textsuperscript{56} These monocilia are composed of nine pairs of doublet microtubules and one central pair of microtubules, of which adjacent pairs of doublet microtubules are connected by dynein motors to generate rotational movements of nodal monocilia.\textsuperscript{57} The direction of these movements is essentially determined by the central pair of microtubules, which, as a result, establishes an asymmetric
leftward extracellular fluid flow of secreted growth factors and signalling molecules. These signalling events activate a bilateral asymmetric pattern of gene expression and determine the left-right (L-R) axis of the embryo and developing organs. In agreement with this, presomite stage mouse embryos cultured under artificial rightward nodal flow conditions exhibited inverted (leftward) cardiac looping and right-sided expression of Pitx2 and Nodal, which normally are expressed in left lateral plate mesoderm. These findings suggest that abnormal nodal fluid flow disrupts the L-R gene programme and thereby impacts on the direction of cardiac looping. Subsequent to the rupture of the connection of the heart to the dorsal pericardial wall, or dorsal mesocardium, differentiating progenitor cells can only be added to the heart via its arterial and venous poles.

Figure 3. Formation and elongation of the human embryonic heart tube. (a) At Carnegie stage (CS) 9, the arterial lumen is surrounded by cardiac jelly, enveloped by myocardium and ventrally borders the foregut endoderm. (b) The embryonic bowl-shaped heart (ht, ventral view), directly connected to its precursors in coelomic wall, are migrating toward each other (b', arrows). (c) A detached CS10 heart, attached to what we know as the dorsal pericardial wall. The heart precursors in this wall are now connected (compare c' and d' with b', ventral view). The heart now is tube-shaped, with an arterial outflow tract (oft), embryonic ventricle (ev) and venous inflow tract (ift) and remains attached to the dorsal pericardial wall at the arterial and venous poles. (d) At CS 11, the heart tube has elongated by the ongoing addition of cardiac precursors to both poles, and the cardiac chambers are beginning to emerge. (e, f) The dorsal connection of a stage 9-10 heart with the coelomic or dorsal pericardial wall (white arrow in e and black arrow in f) is known as the dorsal mesocardium (dm). The cardiac precursors in the dorsal wall have a much higher proliferation rate than the differentiated myocardium (note decreased Ki67-positive nuclei; compare white arrow with white arrowhead in e' and black arrow with black arrowhead in f'). Abbreviations: ca, caudal; cr, cranial; d, dorsal; l, left; r, right; rv, right ventricle; lv, left ventricle; v, ventral. Ki67 is a cell division marker (Adapted from 44).
**Lineage analysis of the first and second heart field**

The extent to which cardiac progenitor cells constitute the various regions of the definitive heart has been vigorously explored, but has also remained a subject of ongoing debate. Differences in gene expression profiles have been appreciated between FHF and SHF cells, which could point toward two distinct pools, but could also indicate that these cells segregate early from a common precursor pool. Moreover, the precursors of the myocardial sinus horns develop from an Nkx2-5-negative precursor pool distinct from the SHF, substantiating the complexity of the concept of multiple heart fields.

The fate of cardiac precursors and their contribution to the heart has been studied by labelling groups of cells mapping within the cardiogenic region of the primitive streak in the chicken embryo. Compared to mammals, avian embryos offer the advantage that cardiac precursors and subdomains are easier to manipulate. In a recent study, the fate of avian pre-cardiac mesoderm was determined by means of plasmid electroporations and computational time-lapse microscopy, revealing that the cardiac precursors in the bilateral primary heart-forming region change their relative position as gastrulation ensues. These findings suggest that cardiac precursor migration results from convectional displacements of groups of cells rather than cell-autonomous migratory processes.

Although certain labelling studies in avian embryos are of significance for our insight into early heart formation, they fall short if we want to verify the exact contributions of cardiac precursors to the adult heart. For this, a genetic approach has been developed to lineage-trace precursors during embryonic development. This procedure requires transgenic mice, in which the expression of a gene called Cre (Causes recombination event) is under the transcriptional control of a cell type-specific gene or derived regulatory sequence (Figure 4). These mice can be crossed with a different transgenic mouse harbouring a stop codon flanked by Cre-recombinogenic loxP sites under the control of a ubiquitous promoter and upstream of a reporter gene (e.g. lacZ or GFP). When both genetic elements are expressed in the offspring, cell type-specific Cre recombination results in the excision of the stop codon and expression of the reporter gene in these cells. Upon excision, the cell and its daughters will irreversibly express the reporter gene, irrespective of future Cre expression (Figure 4b). Cre-mediated recombination can also be deployed at later time-points, for example at fetal stages, by linking Cre to the estrogen receptor ligand binding domain and using tamoxifen to induce nuclear translocation of Cre. Using Cre-loxP approaches, it was confirmed that Mesp1 represents one of the earliest markers of cardiac progenitor cells as almost all cardiac lineages were labeled, except for some cells in the conduction system and cardiac cushions. It has also been demonstrated that most of the right ventricle and arterial trunks are added after the formation of the primitive heart tube. (Figure 4c). Tbx2, encoding a transcriptional repressor, is initially expressed in the caudal limbs of the cardiac crescent and inflow tract but never in the embryonic ventricle and was found to be activated in the anterior cardiac precursors and the outflow tract. Interestingly, Tbx2 progeny was found to encompass a large portion of the left ventricular wall and base, in addition to the right ventricular wall and septum.
Figure 4. Genetic lineage tracing using Cre-mediated recombination. (a) Transgenic mice carrying Cre under the transcriptional control of a cell type-specific gene, can be crossed with transgenic mice harbouring a STOP codon flanked by Cre-recombinogenic loxP sites under the control of a ubiquitous promoter and upstream of a reporter gene (e.g. GFP). When both genetic elements are expressed in the offspring, cell type-specific Cre-recombination will remove the STOP codon, allowing the reporter gene to be expressed in the cell and its daughters, irrespective of future Cre expression. (b) Schematic representation of Cre-mediated reporter gene expression. When neither Cre nor GFP is or has been expressed, the cell is depicted as yellow with a grey nucleus. Upon expression of Cre (purple nucleus), Cre-mediated excision of the STOP codon will result in GFP expression and protein accumulation (green cell with purple nucleus). When Cre expression subsides, GFP continues to be expressed because the STOP codon has irreversibly been removed by Cre (green cell with grey nucleus). (c) Representative example showing recombination of a GFP-reporter line when crossed to Mef2-AHF-Cre mice (see68). GFP is present in all the progeny of cells that once expressed Mef2c-AHF, including the right ventricle (rv), outflow tract (oft) and second heart field (shf).

These findings imply that, as opposed to previous concepts, the cardiac progenitor cells that make up the cardiac crescent only contribute a part of the future left ventricle rather than the entire left ventricle. As such, the progressive addition of cells to the heart is required at very early stages of cardiac development. The extending embryonic heart tube is therefore more responsive to disturbances that impact on cellular contributions than previously anticipated, which consequently result in severe cardiac abnormalities.

Although the progressive addition of precursors to the heart has been demonstrated, the concept of multiple heart fields may potentially be confusing. It still remains plausible that genetically and developmentally similar heart precursors segregate early after their formation24, by which some precursors differentiate into the myocardial cells of the embryonic bowl-shaped heart, while others remain undifferentiated for an extended period of time. In agreement with this view is the observation that Nkx2-5Cre and Isl1Cre are
expressed in overlapping domains of the lateral plate mesoderm\textsuperscript{70}, and that all cells in the myocardial heart tube are \textit{Nkx2-5}-positive\textsuperscript{71} but also are derived from an \textit{Isil}-positive precursor pool.\textsuperscript{71, 72} It should be noted, though, that a recent study demonstrated that a subpopulation of the \textit{Isil}-positive cells found in the heart is derived from neural crest cells.\textsuperscript{73}

The undifferentiated SHF population is positioned dorsally and posteriorly to the tubular heart. Pro-differentiation signals at the two ends of the heart tube will guide the acquisition of these precursors toward myocardial cells. The SHF population that primarily contributes cells to the arterial pole has been termed the anterior SHF, and the cells that are predominantly added to the venous pole are referred to as the posterior SHF cells. The initiation of differentiation of both anterior and posterior SHF cells is similarly delayed, but these SHF subpopulations display distinct molecular marks, suggesting that different genetic programmes are required for the onset of differentiation of these subpopulations, and possibly even for the trajectory of migration, being anterior or posterior.

\textbf{Figure 5}. The ballooning model of cardiac chamber formation\textsuperscript{7}. The embryonic day (E) 8 mouse heart tube has an anteriorly located outflow tract (oft) and a caudal inflow tract (ift) and consists of primary myocardial cells with a primitive phenotype (grey). At E8.5, localized proliferation and differentiation will cause myocardium to expand or balloon out from the outer curvature, revealing the first signs of chamber myocardium of the embryonic ventricle (ev). Between stages E9.5-12.5, the common atrium (a), right and left atria (ra, la) and ventricular chambers will continue to develop (right and left ventricle; rv, lv). Blood travels from the venous pole (vp) to the developing atria. The myocardial cells of the outflow tract (oft), inner curvature, atrioventricular canal (avc) and sinus horns (sh) retain their primitive primary myocardial phenotype. Subregions of non-chamber myocardium will be specified into components of the cardiac conduction system. During subsequent development, the chamber-forming heart remodels into a four-chambered heart with atrioventricular valves, an atrial and ventricular septum (as, vs), and venous inlets and arterial outlets (modified from\textsuperscript{7}).

\textit{Development of the cardiac chambers}

The primitive heart tube is composed of slowly proliferating myocardium\textsuperscript{44, 53-55}, and is characterized by poor automaticity and contractility and a low conduction velocity.\textsuperscript{9} Based on these features, these cardiomyocytes are known as the primary myocardium (Figure 5). As the tubular heart continues to develop, its outer curvature initiates a distinct gene programme by which localized differentiation and proliferation causes expansion of atrial and ventricular chambers. The atrial and ventricular chambers are physically separated by
the atrioventricular canal, which retains primary myocardial properties (Figure 5). At early stages, blood drains from the venous pole into the common atrium and travels via the atrioventricular canal and embryonic ventricle to the outflow tract. Interestingly, this configuration resembles that of the hearts of lower vertebrates.\(^2\) \(^74\) During subsequent development, the differentiating myocardial cells of the future atrial and ventricular chambers acquire fast conduction, high contractility, high automaticity, and well-developed sarcomeric structures, which will ensure rapid contractions and efficient blood pumping\(^9\). The cardiomyocytes of the developing cardiac chambers thus acquire a so-called working myocardial phenotype. The process by which localized regions of the tubular heart expand to form the atrial and ventricular chambers resembles the inflation process of a balloon, and, hence, has been dubbed the ballooning model of chamber formation.\(^2\)

**Transcriptional control of chamber-specific gene expression**

Studies have revealed that *Nppa*, the gene encoding atrial natriuretic factor, is one of the earliest markers of the highly proliferative and rapidly expanding cardiac chambers.\(^75\) The transcription factors Hand1, Irx4 and Irx5, Nkx2-5, Gata4, Tbx2, Tbx3, Tbx5 and Tbx20 are all known to be required during chamber morphogenesis and regulate a number of genes, such as those encoding the gap-junction proteins Connexin40 (Cx40) and Connexin43 (Cx43).\(^2\) \(^9\) Some of these transcription factors, including Nkx2-5 and Gata4, are known to mark the entire cardiac crescent.\(^71\) Even though *Nkx2-5* is expressed in myocardial cells and in cardiac progenitors\(^41,\) \(^71,\) \(^76,\) \(^77\), *Nkx2-5*-deficient mouse embryos still develop a tubular heart, but do not initiate cardiac looping and chamber formation\(^78,\) \(^79\). Therefore, Nkx2-5 is crucial for the developing heart tube and chambers, but redundant during the early phases of cardiogenesis.

Haploinsufficiency of the genes encoding the cardiac transcription factors Nkx2-5, Gata4 and Tbx5 can cause congenital cardiac malformations, including atrial and ventricular septal defects, whereas homozygous loss of any of these genes causes hypoplasia of the cardiac chambers at the tubular heart stage.\(^1\) Importantly, these factors are expressed in both primary and working myocardium (Figure 6a). For example, Tbx5 is predominantly expressed in the precardiac venous mesoderm, inflow tract, atrioventricular canal, and in the embryonic ventricle, and therefore is not fully restricted to working myocardium (Figure 6a).\(^80,\) \(^81\) Family member *Tbx20* is expressed throughout the heart tube, and *Tbx20* knockout mouse embryos display signs of arrested chamber development.\(^82-85\) Hence, the regulation of chamber-specific gene expression seems to require an additional set of transcriptional regulators that are more specifically expressed to confine chamber morphogenesis to localized domains.

Cardiac transcription factors function in parallel as co-factors to form multi-protein transcriptional complexes that regulate localized differentiation and specification of primary myocardial cells.\(^86,\) \(^87\) *Nppa* is never expressed in the primary heart tube, or in the primary myocardial cells of the chamber-forming heart, which could be the result of activation of the chamber-specific gene programme or repression of chamber formation in primary myocardium. Interestingly, two other members of the T-box family, Tbx2 and Tbx3, are predominantly expressed in primary myocardial cells (Figure 6a).\(^88-90\) The repressive role of
Tbx2 during chamber development was demonstrated through transgenic analyses, in which a 0.7 kb regulatory sequence of Nppa was cloned upstream of the β-galactosidase encoding LacZ reporter construct, revealing a chamber-specific expression pattern similar to the endogenous pattern of Nppa expression. This genomic element was found to repress lacZ expression when cloned upstream of a regulatory sequence that was abundantly expressed in the primary myocardium of the atrioventricular canal. The transcriptional repression observed in this study was demonstrated to depend on the cooperative action of Nkx2-5 and Tbx2. Additional studies have indicated that Tbx2 and Tbx3 individually repress the differentiation of primary myocardium into working myocardium (Figure 6b), but also function redundantly to repress chamber morphogenesis and induce endocardial cushion formation.

Whether Tbx2 and Tbx3 are repressed in the working myocardial cells, or activated in primary myocardium was addressed in a recent study, in which multiple Smad binding sites were identified within an atrioventricular canal- and outflow tract-specific enhancer upstream of the transcription start site of Tbx2, allowing BMP-mediated activation of the gene in primary myocardial cells. The expression of Tbx2 in the primitive heart tube is suppressed by Tbx20, which binds Smad1 and Smad5 and thus prevents the formation of Smad-protein complexes that activate Tbx2 (Figure 6c). Although the transcriptional repression of chamber-specific gene expression by Tbx2 and Tbx3 has been demonstrated to rely on their DNA binding capacity, these repressors have also been reported to interact with histone deacetylases, and might regulate chamber repression through histone modification as well. Nevertheless, the abovementioned findings have provided novel insights but also multiple levels of complexity into cardiac chamber morphogenesis and the numerous angles by which myocardial cells are specified.

**Development of the pacemaker and cardiac conduction system**

The adult mammalian heart propagates blood like a rhythmically contracting pump, in which atrial contraction is followed by the delayed contraction of the ventricles. The principal components that initiate and propagate the action potential necessary for these synchronized contractions are the pacemaker cells and the conduction system. Except for the Purkinje fibres, the primary characteristics of these components are similar to those found in primitive embryonic myocardium, being glycogen rich, poorly developed sarcomeric structures and fewer mitochondria. Indeed, in the chamber-forming heart, a subset of the primary myocardial cells will retain their embryonic phenotype to form the future sinus node, atrioventricular node, atrioventricular bundle and its branches. Along the years, the molecular underpinnings that promote the diversification of cardiac precursors into primary and working myocardium have received much attention and have indicated that a network of regulatory proteins is required during pacemaker and conduction tissue specification, of which some will be discussed here.

The sinus node is the dominant pacemaker in the heart and originates from Nkx2-5-negative and Tbx18-positive precursors added to the heart tube, forming the sinus venosus. The adult sinus node strongly expresses Hcn4, a hyperpolarization-activated cyclic nucleotide-gated potassium channel, contributing to the currents essential for
Pacemaker function. In the embryonic heart, the whole sinus venosus expresses Hcn4 and can initiate the electrical impulse in its entirety. Shox2, a homeodomain transcription factor, confines the region where the sinus node primordium can develop by repressing Nkx2-5, which prevents the activation of Hcn4 and Tbx3. Tbx3 is expressed in the developing sinus node and not in the surrounding atrial myocardium or sinus venosus myocardium. In sinus node precursors, Tbx3 initially represses the expression of working myocardial genes and subsequently initiates the pacemaker gene programme. The remainder of the Tbx3-negative sinus venosus will initiate the working myocardial gene programme. The potent role of Tbx3 in controlling the sinus node gene programme was demonstrated by ectopically expressing Tbx3 in developing atrial and adult working cardiomyocytes in mouse, which induced pacemaker genes and functionality, as such reprogramming their original working myocardial phenotype into cells with sinus node-like characteristics. At early stages of sinus node development, Tbx18 specifies both the head and tail structures of the sinus node from mesenchymal cells, but is not required in the sinus node tail-precursors.

Pacemaker activity is subsequently propagated to the atroioventricular node and atrioventricular bundle. In the embryonic heart, blood is forwarded from the atrial chambers to the slowly conducting atroioventricular canal, before filling the fast-conducting developing ventricles. Thus, during this process, the Tbx2/Tbx3-positive but Cx40- and Nppa-negative atroioventricular canal delays impulse propagation analogous to the adult atroioventricular node. In agreement with this early role, comparative gene expression profiling of the embryonic atroioventricular canal and the fetal atroioventricular node revealed similarities in gene programmes, indicating that the atroioventricular canal-specific gene programme is largely maintained in the atroioventricular node.

In the embryonic heart, ventricular activation follows a trajectory that starts from the dorsal Cx40-negative atroioventricular canal and runs to the developing Cx40-positive trabecular myocardium. At later stages, the atroioventricular bundle, bundle branches and Purkinje fibres start to express Cx40. The specification of the atroioventricular bundle occurs through a network of transcription factors, including Tbx5, Nkx2-5, Id2, Irx3 and Tbx3. Tbx3 represses the working myocardial gene programme in the developing atroioventricular bundle, but some genes required for fast conduction, including Cx40, will gradually be activated in these cells at later fetal stages, despite the presence of Tbx3.
These findings indicate that conduction tissue development depends on temporally repressed and subsequently activated gene programmes. Moreover, both Tbx3 and Tbx5 regulate functional aspects of the conduction system by binding to conserved enhancers of cardiac sodium channel genes Scn5a and Scn10a, known to be vital for cardiac excitability.87,116 Recently, Notch signalling was shown to promote the expression of conduction system-specific genes in neonatal cardiomyocytes, reprogramming them into cells with the electrophysiological characteristics of conduction system cells.117 All these intricate molecular processes are crucial in determining form and function of the conduction system of the four-chambered heart.
Part 2: Development of the arterial pole of the heart

Evidence is accumulating that impaired development of the SHF results in a wide spectrum of cardiac defects, including conotruncal, ventricular, atrial septal and atrioventricular septal defects,118-120 subsequently affecting systemic and pulmonary blood flow. The arterial pole is the primary affected component in approximately one third of all congenital heart disease cases.121, 122 In the developing heart, the arterial or cranial pole comprises the outflow tract, aortic sac and the pharyngeal arch arteries. In the adult heart, the arterial pole starts from where the myocardial base and the smooth muscle cells of the aorta and pulmonary trunk join. Thus, the embryonic arterial pole remodels in such a way that the smooth muscular walled ascending aorta and the pulmonary trunk directly attach to the myocardial investments of the great vessels.123 During this transition, the mesenchymal cardiac cushions in the lumen of the outflow tract will develop into the semilunar valves to prevent regurgitation of blood. Interestingly, during development, the primary myocardial cells and cushion mesenchyme that make up the cardiac outflow tract already function as a sphincter with valve-like characteristics (Figure 7), based on the poor conducting and primitive properties of outflow tract cardiomyocytes compared to working myocardium.2 Furthermore, a separation of the trajectory that blood follows when traveling from both atra to the outflow tract is already apparent in the chamber-forming heart (Figure 7). Developmental aberrations of the SHF and myocardial outflow tract have been reported to affect cardiac valve morphogenesis.124 As such, several cell populations are crucial for the developing arterial pole, emphasizing the importance of unravelling the molecular mechanisms that orchestrate their contribution to the heart. In Part 2 of this review, we will focus on the development of the arterial pole of the heart and how recent insights will aid in understanding the underlying disturbances that cause common congenital conotruncal defects.

![Figure 7. The cardiac cushions function as sphincters and separate right and left blood flow. A schematic representation of the developing embryonic four-chambered heart. Primary myocardial (grey) and endocardial cells making up the walls of the outflow tract (oft) and atrioventricular canal (avc) are involved in cushion mesenchyme formation. The spatial orientation of cushion mesenchyme (as illustrated in dark and light yellow and through cross-sections) establishes that blood flow from left and right cardiac compartments are already separated at these early stages. For abbreviations see Figure 5.](image-url)
Signalling pathways during second heart field development

Heart tube elongation requires that SHF precursors persist as undifferentiated cells, but also continue to be present in significant numbers to replenish the SHF cells that have initiated a myocardial gene programme and have been added to the arterial pole. These developmental characteristics are maintained by the expression of genes that suppress differentiation and promote proliferation\textsuperscript{59}, which have received considerable attention in the past 10 years. The signalling pathways required to induce cardiac differentiation and maintain cell populations in the developing embryo include intercellular ligands and receptors of the FGF, BMP, Hh, Notch, retinoic acid (RA) and Wnt signalling pathways (also reviewed elsewhere by\textsuperscript{39, 52}).

The Wnt signalling pathway

The canonical and non-canonical Wnt signalling pathways play important roles during early cardiogenesis, which include the induction and inhibition of cardiomyogenic differentiation\textsuperscript{125-129}. The canonical Wnt pathway is mediated through binding of extracellular Wnt ligands to the Frizzled-LRP5/6 receptor complex. This results in the activation of intracellular Dishevelled, which stabilizes cytoplasmic β-catenin that now translocates to the nucleus, binds TCF/LEF and induces transcription of target genes\textsuperscript{130}. The non-canonical Wnt signalling pathway functions downstream of activated Dishevelled, which promotes the activation of G proteins Rac and Rho, as such the activation of c-Jun N-terminal kinase (JNK) and Rho-associated kinase (ROCK), and will activate gene transcription via the ATF/CREB complex\textsuperscript{131}. Over-expressing Wnt3α and Wnt8 results in cardiomyogenic inhibition\textsuperscript{132}, indicating that canonical Wnt signalling needs to be antagonized. This occurs through Dkk1 and Crescent\textsuperscript{133} which allow the transcriptional activation of Nkx2-5 and Tbx5 and of genes encoding structural muscle proteins. In addition, decreased Wnt signalling is crucial for BMP-mediated cardiogenic induction\textsuperscript{53}. Following cardiac induction and heart tube formation, it has been demonstrated that ablation of β-catenin in the SHF population\textsuperscript{125} or in Mesp1 descendants\textsuperscript{134} reduces the number of Isl1-positive cardiac precursors. When β-catenin is constitutively activated in the SHF population, the Isl1-positive expression domain expands, stressing out the importance of balanced Wnt/β-catenin dosage during cardiac progenitor cell development\textsuperscript{125, 134-137}. The Wnt proteins Wnt5α and Wnt11 are individually known to act through the non-canonical Wnt pathway during early cardiogenesis\textsuperscript{28, 131, 132, 138-141}. Recent findings demonstrated that they are co-required to confine the repressive action of prolonged canonical Wnt signalling in SHF progenitors\textsuperscript{142}. These combined data suggest that cardiac induction is inhibited and proliferation and cellular multipotency is maintained by canonical Wnt, and that non-canonical Wnt signalling promotes cardiac differentiation by inhibiting canonical Wnt signalling, which is crucial later in development.

The FGF signalling pathway

The FGF signalling pathway is a target of Wnt signalling in the SHF and, therefore, has important implications for progenitor cell expansion. Fgf8 expression in the anterior endoderm plays a vital role during the early specification of cardiogenic mesoderm\textsuperscript{25} and
loss of Fgf8 at later stages abrogates SHF proliferation, survival and heart tube elongation.143, 144 Fgf8 and Fgf10, as well as the Fgf receptor 1 (Fgfr1) are furthermore required during cardiac looping by positively regulating differentiation.145-148 Decreasing Fgf8 dosage in pharyngeal endoderm and SHF cells results in aortic arch artery anomalies, outflow tract shortening and ventriculo-arterial alignment defects.149,150 FGF ligand expression during SHF deployment is also affected in the absence of Tbx1145, 148, 151 and Isl1.67 Further evidence for the role of FGF signalling pathway components during SHF expansion and outflow tract elongation was demonstrated in mice lacking Fgfr1 and Fgfr2, in a gain-of-function mouse model with an excessive inhibitory effect on Fgf signalling through Sprouty2 and in mice lacking Frs2α, a mediator of Fgf receptor function147,152, all of which displayed a shortened outflow tract and myocardial differentiation defects. These findings indicate that multiple FGF ligands exert their role during outflow tract development, supported by the observation that mice mutant for the Fgf receptor Fgfr2-IIib display more severe phenotypes than Fgf10 null mutants.153 The mechanism by which FGF ligands cause SHF cells to be deployed to the outflow tract is not yet fully clear, but an important role has been proposed for additional growth factor signalling modules governed by the Tgfβ superfamily154, by which SHF migration, proliferation and differentiation are carefully balanced.

Tgfβ signalling

The Tgfβ superfamily is responsible for signalling in cardiac progenitors through ligands like BMP, which are also downstream effectors of the Wnt/β-catenin pathway. BMPs are known to drive myocardial specification and differentiation and negatively regulate FGF signalling.155-157 Excessive BMPs have been shown to impose a myocardial phenotype on non-cardiogenic mesoderm.158 To exert their developmental roles, BMPs bind and activate type 1 and type 2 BMP receptors, which will result in phosphorylation of Smad1/5/8 proteins, association with co-activator Smad4, and subsequent activation of transcription.157 Bmp signalling is inhibited by Smad6 and 7, which disrupt cardiac progenitor cell development.159,160 An important BMP family member, Bmp2, directs early specification of cardiogenic mesoderm, but is also required for differentiation events in the distal outflow tract, possibly by recruiting SHF cells8, 29, 161, 162, as such revealing critical roles during outflow tract elongation. Reports on other BMP ligands during outflow tract development emphasize the molecular intricacy of these developmental processes. For example, loss of Bmp4 in mice affects outflow tract septation, smooth muscle development and endocardial cushion formation and compound homozygosity for Bmp4 and Bmp7 null alleles severely impairs outflow tract elongation.163 Bmp4 expression is furthermore decreased in mice lacking important transcription factors such as Isl1, Tbx2, Tbx3 or the chromatin-complex protein Smarcd3.67,90,164,165 Absence of the BMP receptor Bmpr1 in cardiac progenitors demonstrated inducing roles for BMPs during cardiac specification134, but in the SHF population or in neural crest cells primarily affects outflow tract septation166 or elongation.167 BMP signalling also regulates differentiation by inducing the expression of the microRNA 17-92 cluster, which suppresses Isl1 and Tbx1.168 However, Isl1 deficiency decreases the expression of BMP ligands as well82. The importance of BMPs in cardiac precursors has furthermore been demonstrated in Nkx2-5 null mutants, in which Bmp2 is
upregulated, leading to overspecification and proliferation abnormalities in cardiac progenitors.\textsuperscript{71}

**Hh signalling**

The binding of a Hedgehog (Hh) ligand to the trans-membrane receptor Patched (Ptc) is followed by internalization of this Hh/Ptc-protein complex, upon which its inhibitory function on another membrane-bound protein, Smoothened (Smo), will attenuate. This will subsequently lead to the conversion of Gli transcription factors into transcriptional activators.\textsuperscript{169} In 2001, Garg and colleagues demonstrated that Sonic hedgehog (Shh), a key player in the Hh-signalling pathway, regulates transcription factor Tbx1 through the activation of Forkhead (Fox) transcription factors. Further studies have indicated that Shh is involved in maintaining the size of the SHF population and plays important roles during outflow tract septation\textsuperscript{170} and posterior SHF development through Tbx5.\textsuperscript{171} Loss of Shh causes a phenotype that remarkably resembles that of Tbx1-deficient embryos.\textsuperscript{170} Disrupting the Hh signalling pathway via the Hh receptor Smoothened (Smo) resulted in severe heart tube elongation defects, reminiscent of mouse embryos null for both Shh and Indian Hedgehog (Ihh).\textsuperscript{172} Smo is also expressed in the SHF, and conditional deletion of Smo in these cells also results in outflow tract elongation defects and a common arterial trunk, indicating that SHF cells require Hh signalling for proper deployment and development.\textsuperscript{173, 174}

**Notch signalling**

When the membrane-bound ligands Serrate, Delta and Jagged bind to an adjacent trans-membrane Notch receptor, proteolytic cleavage of this receptor will trigger the release of Notch intracellular domain (NICD) into the cytoplasm, which will translocate to the nucleus to activate target genes.\textsuperscript{175} In the absence of Notch1 in mice, a heart tube is formed, but defective differentiation of cardiac progenitors results in embryonic lethality between embryonic day 9.5 and 10.5.\textsuperscript{176} The expansion of cardiac precursors depends on Notch-mediated phosphorylation of β-catenin, which, together with Isl1 expression, is negatively regulated by Notch1.\textsuperscript{177} On further notice, conditional deletion of Notch signalling in Isl1- or Mef2c-positive cells and progeny results in severe outflow tract malformations, but also affects aortic arch system remodelling.\textsuperscript{178} The crucial role of the Notch pathway in cardiac progenitor cells was furthermore emphasized in a recent study, demonstrating that the Wnt/β-catenin pathway regulates Nkx2-5, Isl1 and Smarcd3 and BMP signalling is required for Gata4 and Mef2c activation downstream of Notch signaling events.\textsuperscript{179}

**Retinoic acid signalling**

SHF development requires input from a multitude of interacting signalling pathway components and the notion that the retinoic acid pathway is of great importance herein is steadily increasing.\textsuperscript{39} Retinoic acid (RA) is the active derivative of vitamin A and regulates a number of processes during cardiogenesis.\textsuperscript{180} RA dosage is maintained by the enzyme
retinaldehyde dehydrogenase 2 (Raldh2), which is expressed in pharyngeal mesoderm.\textsuperscript{181} Earlier studies have already recognized the role of retinoic acid dosage during cardiac patterning.\textsuperscript{182-184} Loss of Raldh2 disturbs cardiac looping and atrial development as it expands SHF genes like Tbx1, Fgf8, and Isl1\textsuperscript{181,185,186}, suggesting that the boundary of anterior and posterior SHF is severely affected. However, the cells that misexpressed these SHF genes did not have the potential to differentiate into functional cardiomyocytes.\textsuperscript{185} Increased levels of retinoic acid were also found to downregulate Tbx1 expression in avian embryos\textsuperscript{187}, whereas Raldh2 expression expanded anteriorly in the absence of Tbx1 in mouse embryos and RA metabolizing enzymes were downregulated.\textsuperscript{146,187-190} The observation that Raldh2-deficient mouse embryos display venous pole abnormalities\textsuperscript{185,187} suggests that retinoic acid signalling is required during posterior SHF development. The role of RA in the arterial pole was demonstrated in transgenic mice harbouring a RA reporter gene, which was expressed in the inferior wall of the outflow tract and in the SHF.\textsuperscript{154} These cells were additionally affected in the absence of Raldh2.\textsuperscript{191} Endogenous RA is thought to be required to replenish SHF cells of the distal outflow tract, and loss of RA function in these cells affected both SHF development and septation due to epithelial-to-mesenchymal transition defects mediated by Tgfβ signalling.\textsuperscript{154}

**Characteristics and development of the second heart field**

It has been firmly established that the SHF is a cardiac precursor population that extends the heart tube and properly shapes the arterial and venous poles of the heart.\textsuperscript{1} The right ventricle, (part of) the ventricular septum, the myocardial and smooth muscle cells of the arterial outlets and the atria are all dependent on the ongoing accumulation of differentiating SHF cells.\textsuperscript{38,52,192} In a previous study, the LIM homeodomain transcription factor Isl1 was found to mark the majority of the SHF\textsuperscript{67}, although, importantly, much of the embryonic heart was positive for β-galactosidase in their lineage analysis. Unfortunately, demarcating the derivatives of heart progenitors has resulted in inconsistent results.\textsuperscript{24,51} These differences could potentially result from variable sensitivities of mouse reporter lines to Cre-mediated recombination, which could also vary between different genetic backgrounds. In addition, in chicken embryos, highly proliferative undifferentiated Isl1-positive cardiac precursors were found to contribute to both poles\textsuperscript{3}, in agreement with the observation that most, if not all, cardiac progenitors once expressed Isl1.\textsuperscript{71}

The induction of cardiomyogenesis in SHF cells following the formation of the cardiac crescent is the prolongation of the heart tube. Analogous to the first heart field, this induction is mediated by Fgf8, expressed in the SHF, outflow tract and lateral endoderm and by Bmp2, among others (Figure 8b, c).\textsuperscript{6,149,193} The cells in the SHF divide at a rapid pace and are maintained as undifferentiated cells unless instructed otherwise. Key molecules detected in the SHF are the transcription factors Tbx1 and Isl1 and the growth factor Fgf10 (Figure 8b, c).\textsuperscript{38} Proper deployment of SHF cells and concomitant development of the outflow tract has been attributed to Isl1\textsuperscript{67}, whereas Tbx1 is involved in positively regulating proliferation and negatively impacting on differentiation in the SHF.\textsuperscript{146,194,195} Interestingly, both Isl1 and Tbx1 have been identified in multipotent progenitors\textsuperscript{194, 196}, reflecting their roles in maintaining the multipotent state of progenitor cells.\textsuperscript{197,198}
It is pivotal that SHF cells are deployed correctly and at a controlled pace. For this, SHF cells need to remain undifferentiated, which greatly depends on BMP signalling, whereas FGF-signalling directs the rate of proliferation to preserve the SHF population size. Fgf8 plays a significant role herein, and is supported by Fgf3 and Fgf10. These FGFs are expressed in pharyngeal mesoderm and epithelia and depend on Wnt/β-catenin and Jagged1/Notch signalling for cross-talk with neighbouring cells. Loss of any of these signalling pathways in either the SHF, endoderm or neural-crest cells affects heart tube elongation, including Hh-signalling events from a ventral expression site of the pharyngeal endoderm adjacent to the SHF population, which additionally aids in directing cardiac progenitor cell differentiation. These signalling processes and components depend on a variety of upstream regulatory proteins, including T-box transcription factors.

**T-box transcription factors and cardiac progenitor cell development**

Members of the T-box family of transcription factors are crucial developmental regulators, and six of the 17 different vertebrate T-box factors are expressed in the heart, where they are required for myocardial growth, patterning and cellular identity. Transcriptional activation or repression is mediated by a highly conserved T-box element, which binds DNA, but also interacts with transcription factors and proteins involved in chromatin remodelling and histone modifications.

In the elongating heart tube, the continuous addition of Tbx5-positive cells to the venous pole supports the shaping process of the left ventricular wall, the atrial chambers and atrial septum. These cardiac components are therefore collectively denoted to be posterior SHF derivatives. Tbx5 expression gradually declines when traced from the inflow tract to the right ventricle and the outflow tract is negative for Tbx5 expression, but whether it is derived from a Tbx5-negative precursor pool remains to be assessed. Ectopic expression of Tbx5 in all cardiomyocytes results in looping defects and the embryonic heart largely retains a left ventricular identity. Furthermore, loss of Tbx5 affects posterior SHF contribution, growth and the expression of cardiac genes in the tubular heart, indicating that the tubular heart and posterior SHF depend on Tbx5 for proper growth.

Similar to Tbx5, Tbx20 transcripts can be found in both the first and second heart field, but in contrast to Tbx5, Tbx20 is expressed in all myocardial and endocardial cells. Aberrant levels of Tbx20 have been associated with tetralogy of Fallot, with valvular disease and cardiomyopathy and with disturbed cardiac homeostasis. Although a tubular heart develops normally in Tbx20-deficient embryos, cardiac elongation is halted, suggesting that loss of Tbx20 in cardiac precursors and cardiomyocytes disrupts cardiac looping and chamber morphogenesis, evidenced by down-regulation of chamber-specific genes such as Nppa, Chisel and Irx4. In addition, knockdown of Tbx20 results in right ventricular and outflow tract hypoplasia, and Tbx20 has been demonstrated to activate the Mef2c anterior SHF enhancer and to repress myocardial Isl1, demonstrating that Tbx20 plays an important role in SHF deployment and development.
Figure 8. Contribution of cardiac progenitor cells and cardiac neural crest cells to the developing heart. (a) Ventral view of an E9.0 mouse embryonic heart and dorsal pericardial wall (grey). The dorsal pericardial wall can be divided into the anterior second heart field (b), posterior second heart field (c) and caudal cardiac progenitor cells (d). (b-d) Examples of some of the major developmental regulators (e.g. Nkx2-5, Tbx1 and Isl1) in the different subdomains of the dorsal pericardial wall. Cardiac neural crest cells (blue cells, e) migrate to the dorsal pericardial wall, where second heart field cells reside (green cells), and subsequently to the arterial pole. (g) Second heart field development is known to depend on intercellular signalling events between pharyngeal endoderm, mesoderm and cardiac neural crest cells. The migration of cardiac neural crest cells into the heart subsequently contributes to the formation of the aortico-pulmonary septum and the cardiac valves.

The transcriptional repressor Tbx2 is expressed in the SHF and adjacent neural crest cells, in primary myocardial cells of the outflow tract, inner curvature, atrioventricular canal and, at early stages, part of the inflow tract. Ectopic expression of Tbx2 in the entire heart results in elongation defects of the outflow tract, which is suggested to be caused by down-regulation of cell adhesion molecules Alcam and N-cadherin in the outflow region of the heart, disrupting SHF migration and deployment. Interestingly, in these dysmorphic Tbx2-misexpressing hearts, Tbx20 was found to be decreased specifically in the outflow tract, whereas in Tbx20 mutant mice Tbx2 was ectopically expressed in the entire cardiac crescent and tubular heart, potentially maintaining the primary myocardial phenotype. Additional analyses revealed that the Tbx20-dependent regulation
of Tbx2 is mediated by Bmp/Smad signalling, where Tbx20 binds activated Smad1 and Smad5, preventing them to form protein complexes with Smad4, which is required to activate target genes like Tbx2 (Figure 6c).

The transcriptional repressor Tbx2 shares significant structural and functional homology with its closely related paralog Tbx3 and both factors overlap in multiple regions of the developing embryo.88, 90, 93, 213-215 The cardiac expression domain of Tbx3 is largely restricted to the non-chamber myocardial cells that make up the atrioventricular canal, in the sinus node primordium, and at later stages in the central components of the cardiac conduction system.89, 90, 93, 112, 165 Expression of this factor was also observed in pharyngeal epithelia, neural crest cells and a subdomain of the SHF.90,165 Loss of Tbx3 in mice affects SHF and outflow tract signalling pathways resulting in failed outflow tract elongation and a double outlet right ventricle at later stages.112,165 However, as Tbx3 is predominantly expressed in neural crest-derived cells, these results indicated an important role for Tbx3 in this cell population during heart tube extension. This finding is in agreement with the observation that neural crest ablation affects cardiac looping and outflow tract elongation in chicken embryos216, pointing toward vital intercellular signalling events between neural crest-derived cells and the SHF.

As both Tbx2 and Tbx3 share common target genes in overlapping cardiac and pharyngeal regions, these transcription factors can potentially act in a functionally redundant manner. In support of this suggestion, recent findings have demonstrated that loss of three of the four Tbx2 and Tbx3 alleles (Tbx2+/−;Tbx3+/− and Tbx2/−;Tbx3/− mouse embryos) causes severe outflow tract and right ventricular hypoplasia and aberrant endocardial cushion formation and atrioventricular patterning.90,94 Mesbah and colleagues (2012) also demonstrated that these genes are required upstream of BMP-, FGF- and Hh signalling pathways in the SHF and pharyngeal endoderm, disrupting crucial intercellular signalling during outflow tract development.

In addition to the combinatorial function of Tbx2 and Tbx3 during cardiac morphogenesis, they also control outflow tract development with another member of the T-box family, the transcriptional activator Tbx1, during earlier stages of heart tube extension90. Tbx1 has been identified as a major candidate in the etiology of most of the phenotypes found in 22q11.2 deletion syndrome, the most common deletion syndrome in humans, characterized by a spectrum of defects including craniofacial and aortic arch defects.217,218 The expression of Tbx1 is mostly restricted to the SHF, pharyngeal endoderm and head mesenchyme.90 and genetic lineage analysis in mouse revealed that Tbx1-positive cells contribute to the outflow tract myocardium, right ventricle, endocardium, and mesenchymal cushions from embryonic day E8.25 onward.148,219,220

Tbx1 is known to play a prominent role in controlling the balance between proliferation and differentiation in the SHF194, as loss of Tbx1 affects SHF development leading to hypoplasia of the distal outflow tract at midgestation and, at fetal stages, to a common arterial trunk.148,195 It has become clear that the number of neural crest cells in the pharyngeal region is affected by loss of Tbx1, conceivably impacting on the number of SHF cells and possibly on outflow tract septation.90,221 Cardiac progenitor cell development furthermore relies on Tbx1, which regulates Wnt5a via Baf60a and suppresses Mef2c, thereby affecting differentiation and target gene activation.197,222 Although little is known about the upstream regulators of Tbx1, studies have indicated that Shh plays a role in activating Tbx1223, by inducing the expression the Fox transcription factors Foxa2, Foxc1 and
Foxc2, which subsequently act on the Tbx1 promoter. However, Tbx1 seems to maintain its own expression by acting on Foxa2 through an autoregulatory feedback mechanism.\textsuperscript{145} Importantly, transgenic analyses in mouse performed by Zhang and Baldini (2010) demonstrated that removing or mutating the Shh-responsive Fox-binding site in an Tbx1 enhancer did not compromise embryonic development \textit{in vivo}. In addition to Shh and Fox proteins, Tbx1 activity in pharyngeal epithelia was demonstrated to depend on Ripply3.\textsuperscript{224}

In Tbx2;Tbx3 compound null mutant mouse embryos, Tbx1 was ectopically expressed in the ventral endodermal cells directly adjacent to the SHF.\textsuperscript{90} In turn, Tbx1 was found to be required for the expression of Tbx2 and Tbx3 in pharyngeal and neural crest-derived mesenchyme, although this effect is likely to be mediated by altered intercellular signalling between pharyngeal mesoderm and epithelia and neural crest cells as Tbx1 is not expressed in the neural crest. In addition, the expression of Tbx2, Tbx3, Isl1, Fgf8 and Fgf10 was decreased in the dorsal pericardial wall where SHF cells reside, suggesting that Tbx1 also functions upstream of these key proteins in the developing SHF.\textsuperscript{90,225} Importantly, embryos lacking Tbx1 and either Tbx2 or Tbx3 display severe pharyngeal and heart tube elongation phenotypes, illustrating overlapping roles for Tbx2 and Tbx3 and providing evidence for a crucial cross-regulating Tbx1/Tbx2/Tbx3 network during outflow tract and SHF development.\textsuperscript{90} In these compound embryos, combinatorial defects are observed such that proximal to the elongating heart tube FGF signalling is elevated and BMP signaling is down-regulated. Thus, Tbx2/Tbx3 function redundantly to assist Tbx1 in coordinating proliferation as well as repression of differentiation of cardiac progenitor cells located in the SHF. Important genetic and molecular interactions involving Tbx1 have also been identified for other genes, including Crkl, Fgf8, Pitx2, Gbx2, Chd7, Six1/Eya1\textsuperscript{186,226-231}, all potentially contributing as candidate modifier genes to the phenotypic severity of cardiopharyngeal abnormalities in TBX1 haplo-insufficient 22q11.2 deletion syndrome patients.

\textbf{Role of cardiac neural crest cells in second heart field development and deployment}

The transformation of the myocardial outflow tract into the separated pulmonary trunk and aorta requires the cellular contribution of a distinct precursor pool called the neural crest cells, in addition to SHF cells.\textsuperscript{232} Neural crest cells are multipotent migratory cells that delaminate from the lateral margin of the neural plate in a rostro-caudal wave during early embryogenesis, and provide a substantial contribution to a variety of different cell types, including nervous system cells, smooth muscle cells and melanocytes.\textsuperscript{233,234} Neural crest cells give rise to cranial and trunk neural crest, and a subregion of cranial neural crest cells migrates toward the outflow tract. In the developing mouse embryo, genetic lineage analysis using Wnt1-Cre mice and the \textit{Rosa26} transgenic reporter line demonstrated that neural crest cells invade the proximal and distal outflow tract cushions and contribute to the formation of the semilunar valves, the tunica media layer of the smooth muscular wall of the great vessels, aortic arch and the proximal carotids.\textsuperscript{235-237} Neural crest cells were also found to moderately contribute to the smooth muscle cells of the proximal coronary arteries and intermediate branches.\textsuperscript{236} The subpopulation of cranial neural crest cells that is added to the heart is thus known as the cardiac neural crest and is crucial for the development and septation of the outflow tract into separate pulmonary and systemic vessels.\textsuperscript{238} In addition to the requirement of cardiac neural crest cells during cardiac morphogenesis, they also
contribute to the formation of the thymus, parathyroid and thyroid glands, which develop from pharyngeal endodermal pouches. In this section, we will focus on cross-talk between cardiac neural crest cells and pharyngeal mesoderm during early heart development.

Cardiac neural crest cells originate in the neural folds, running from the otic placode to the third somite and migrate into the third, fourth and sixth pharyngeal arches. They subsequently migrate toward the dorsal pericardial wall, from where they enter the outflow tract (Figure 8e-g). The requirement of cardiac neural crest cells during outflow tract septation is supported by the finding that when ablating these cells in chicken embryos, the aortico-pulmonary septum does not form, resulting in persistent truncus arteriosus. Interestingly, SHF cells failed to move to the myocardial outflow tract in neural crest-ablated embryos, which consequently displayed abnormal cardiac looping and outflow tract shortening, whereas SHF-derived vascular smooth muscle cells developed normally. These findings illustrate the obligatory role of cardiac neural crest cells and their migration to the heart, where they explicitly control the migration of the myocardial precursors of the outflow tract.

When neural crest cells migrate to the pharyngeal arches, they largely surround and somewhat intermingle with the mesodermal cores within the arches that largely contain craniofacial muscle precursors. These pharyngeal arches are bordered by pharyngeal epithelia. Therefore, neural crest cells could also be expected to interact with cells in these layers other than pharyngeal mesoderm. Indeed, elevated levels of Fgf8 were observed in the caudal pharyngeal endoderm upon neural crest ablation, additionally affecting SHF migration to the heart, which in turn could be rescued by diminishing excess FGF ligands. Mouse embryos lacking Tbx1 display interrupted neural crest migration, resulting in a common arterial trunk, only form the first pharyngeal arch, and neural crest cells that would normally contribute to more caudally positioned arches are redirected toward the first arch. Importantly, Tbx1 is predominantly expressed in pharyngeal endoderm and mesoderm, including the SHF, but not in neural crest cells, suggesting cell-non-autonomous roles of Tbx1 during cardiac neural crest migration.

The Hh ligand Shh has also been demonstrated to affect the SHF and cardiac neural crest population from a ventral endodermal expression site, evidenced by a decrease in Nkx2-5 expression. Disrupting endodermal Shh-signalling in mouse embryos results in increased apoptosis of pharyngeal mesoderm and cardiac neural crest cells, causing outflow tract septation defects, similar to the full Shh mutant. Moreover, conditional ablation of the Hh receptor Smoothened (Smo) in neural crest cells and SHF cells revealed a critical role for Smo in both cell populations, in which neural crest migration and SHF development are intricately linked. Thus, multiple signalling pathways regulate the ongoing movement of myocardial precursors and neural crest cells into the heart. Hence, understanding the interactions between neural crest cells and the various cell- and tissue types present in the cardiopharyngeal region will likely increase our insight into outflow tract morphogenesis and remodeling.
Future perspectives

The past fifteen years have been very exciting for cardiac developmental and molecular biologists and have led to significant insights into the developmental origin of the various tissue types that make up the heart. Current progress has furthermore shed light on the molecular and transcriptional machineries that determine cardiac form and function. This knowledge is essential if we want to understand the developmental origin of congenital cardiac defects, and to devise future molecular, genetic and cell-based therapies. The signalling pathways that underlie cardiac induction, differentiation, proliferation and cell fate determination of cardiac progenitor cells are vigorously being addressed, but have proven to be more complex than originally anticipated. In addition to the well-known transcription factors and growth factors, it has become clear that epigenetic marks and chromatin-remodeling subunits such as Smarcd3 (Baf60c), Arid1a (Baf250a) and Brg1 are pivotal in cardiomyogenesis. In line with this, the combined function of Gata4, Tbx5 and Smarcd3 has been reported to be sufficient to direct non-cardiogenic posterior mesoderm and extra-embryonic amniotic mesoderm into beating cardiomyocytes. More recently, postnatal cardiac or dermal fibroblasts were demonstrated to have the potential to differentiate into myocardium-like cells by adding only Gata4, Tbx5 and Mef2c. This combination also induced functional and beating cardiomyocytes from resident non-myocytes in vivo. Adding the basic helix-loop-helix transcription factor Hand1 to the Gata4/Mef2c/Tbx5-mix has been reported to have a positive impact on cardiac function following myocardial infarction. These cardiogenic transcription factors seem to be crucial and sufficient during cardiac muscle cell formation, in vitro and in vivo, but their usage as a therapeutic strategy warrants further investigation. The analysis of the transcriptional control of signalling pathways required to allow differentiation and survival have revealed hurdles that we first need to overcome. Nevertheless, our ability to control the development of cardiac progenitor cells or induce cardiac cells from non-myocardial tissue types will set the pace to explore the possibility to mend the malformed and damaged postnatal heart.

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