Embryological Origins: How Does the Right Ventricle Form

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Abstract

The heart originates from a group of cardiac progenitor cells that form the cardiac tube, which develops into a complex four-chambered beating organ. Several tissues signal to stimulate cardiac progenitors to acquire cell fate and differentiate. The timing of differentiation of cardiac progenitors defines the first and second heart fields. The first heart field gives rise to the left ventricle. The second heart field, located anterior to the first heart field, is added to the cardiac tube to give rise mainly to the outflow tract and the right ventricle. Several epigenetic mechanisms including histone and DNA methylation stabilize the transcriptional programs controlling cardiac development.

Keywords

Cardiac development • Second heart field • Cardiac progenitors • Right ventricle • Cardiomyocyte differentiation • Transcriptional regulation

Introduction

The heart develops from cardiac progenitor cells that originate during gastrulation and which move through the primitive streak and emerge as a bilateral cardiac field that fuses in the embryonic midline to form the cardiac crescent and the heart tube later on. The cardiac tube, in which cardiac progenitors start differentiating into cardiomyocytes, serves as a scaffold for addition of cardiac progenitors through the arterial and venous poles. The cardiac tube undergoes complex morphogenetic movements including looping and septation that result in the division of the heart into chambers [1, 2]. Understanding how these processes occur has been the goal of very active research. Early experiments aimed at identifying the cells acting as the building blocks that form the heart identified the population of progenitors cells that are added to the cardiac tube

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[3]. These observations fueled further research that confirmed the existence of two groups of cardiac progenitors, known as the first and second heart fields. The first heart field forms the cardiac crescent and the cardiac tube, and gives rise mainly to the left ventricle. The second heart field, added to the cardiac tube, contributes the totality of outflow tract and the right ventricle [4]. A recent finding of a group of progenitors regulated by canonical Wnt signaling that gives rise to pacemaker cells in chicken led to proposing the existence of a "tertiary" heart field [5]. The exact nature of the molecular cues that induce segregation of these progenitors is not clear, however extensive research using mainly animal and cellular model systems has made significant progress in uncovering the transcriptional pathways that regulate differentiation of cardiac progenitors and cardiogenesis.

This chapter provides an overview of the process of cardiac development, starting with a concise explanation of the morphogenetic events that transform the cardiac crescent into the four-chambered heart. Then, the events that led to the identification of the cardiac fields are discussed, emphasizing the discovery of the second heart field and its contribution to cardiogenesis. The transition from proliferating to differentiating cardiac progenitors and its relevance for development of the second heart field and right ventricle is discussed. Finally, recent findings on the molecular mechanisms controlling differentiation and maturation of cardiac myocytes are summarized.

Overview of Cardiac Development

Early Cardiac Development

The heart is the first organ to function during embryogenesis. The formation of the heart is a complex process that starts very early during development. The earliest cardiac progenitors arise during gastrulation [6, 7], during which the three embryonic layers ectoderm, mesoderm and endoderm are patterned. Multipotent cardiac progenitors arise from a cell population expressing mesoderm posterior 1 homolog, or Mesp1. Mesp1-expressing progenitors give rise first to the first heart field, and then to the second heart field at E6.75 in the gastrulating mouse embryo. Progenitors of the first heart field give rise to either cardiomyocytes or endothelial cells. Progenitors of the second heart field give rise to cardiomyocytes, endothelial cells, and smooth muscle cells [7]. Thus, cardiac progenitors are hierarchically segregated early during gastrulation. Studies using stem cell models of cardiac differentiation, as well as cell lineage tracing in animal models have shed light on some of the intermediate stages in the progression from stem cells to specialized differentiated cardiac lineages (Fig. 1.1) [8].

The cardiac progenitors emerge through the primitive streak and they take position cranially to the forming neural tube and surrounding the neural plate at approximately 18 days of human development. The cardiac progenitors aggregate to form two bilateral primitive heart fields, which fuse in the midline to form a horse shoe-shaped tube from splanchnic mesoderm known as the cardiac crescent. The cardiac crescent harbors a population of cardiac progenitors known as the first heart field, which contributes to the formation of the left ventricle and portions of the atria. As a result of ventral folding of the embryo in a cranial to caudal direction, the limbs of the cardiac crescent coalesce and fuse in the midline to form the linear heart tube (Fig. 1.2). Co-migration of cardiac and vascular progenitors allows for the formation of the endothelial heart tube, which is separated from the primitive myocardium by cardiac jelly, and which will form the endocardium. At this stage, day 22 of development, dilations and constrictions of the heart tube define the *truncus arteriosus*, *bulbus cordis*, primitive ventricle and atrium, and the *sinus venosus* (Fig. 1.3). The transition from cardiac progenitors into differentiating cardiomyocytes results in coordinated contraction of the linear heart tube and blood flow from the *sinus venosus* towards the cranial portion of the embryo. The linear heart tube is then extended by proliferation of resident differentiating cardiac progenitors and by contribution of additional ones

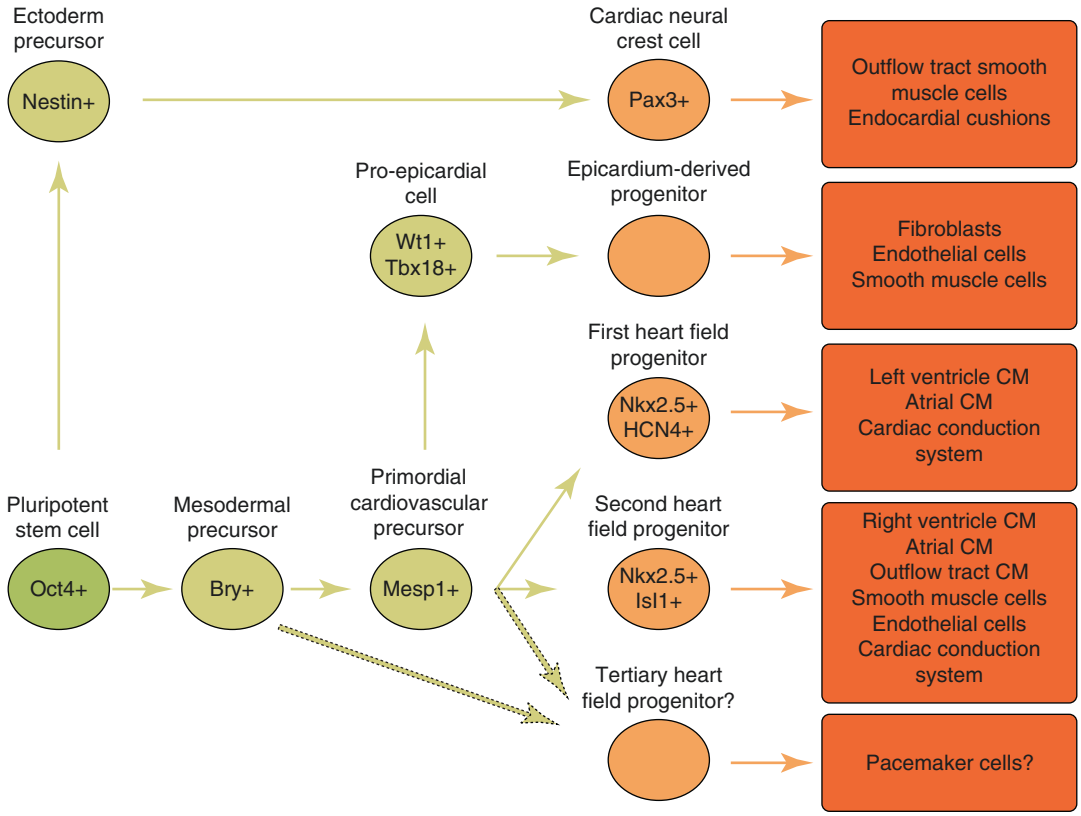

Fig. 1.1 Cardiac cell lineage progression. Intermediate stages generated from pluripotent stem cells towards differentiated cardiac lineages. Markers expressed in the intermediate progenitors are indicated. *CM* cardiomyocytes. Progression towards tertiary heart progenitors is speculative and is indicated with *dotted arrows*. Modified from [8]

originated from splanchnic and pharyngeal mesoderm, which migrate into the linear heart through the arterial and venous poles. This population of cardiac progenitors added to the linear heart is known as the second heart field. Cardiac progenitors of the second heart field give rise to the totality of the right ventricular myocardium, the outflow tract and an important proportion of the atria (Fig. 1.2) [4, 9, 10].

Cardiac Looping

Looping of the heart tube is the first visual evidence of embryonic asymmetry. Progenitors of the second heart field continue to be added during the process of looping, in which torsion forces cause the elongating heart tube to simultaneously twist and rotate rightwards, resulting in the formation of a C-shaped cardiac tube at embryonic day 23 (Fig. 1.3). These morphogenetic movements push the ventral portion of the linear tube towards the right outer curvature of the C-shaped tube, while the dorsal portion of the linear heart separates from the dorsal pericardial wall and forms the inner left curvature. Elongation continues at the arterial and venous poles, and the cardiac tube arranges into and S-shape structure, in which the outflow and inflow tracts come closer together cranially. Displacement of the *bulbus cordis* caudally, ventrally and rightwards, leftwards displacement of the primitive ventricle, and dorsal and cranial displacement of the primitive atrium by embryonic day 28, results in the proper spatial arrangement of the future cardiac chambers (Fig. 1.3). The rightwards movement

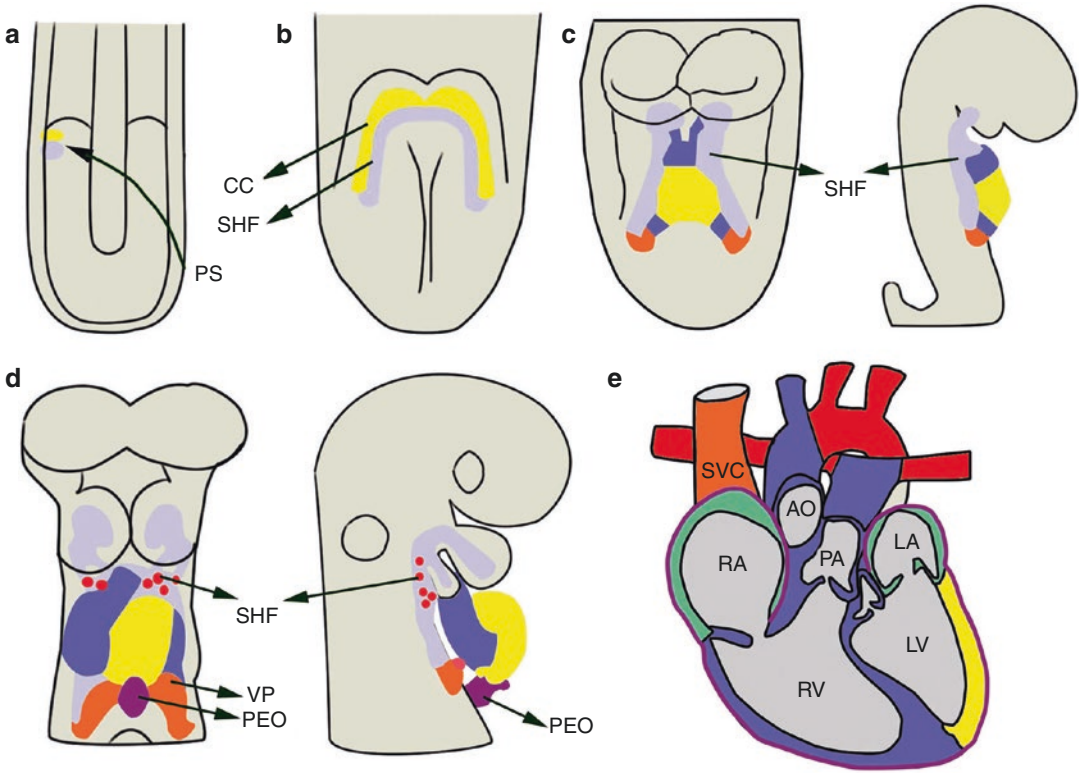

Fig. 1.2 Cardiac development. (**a**) Cardiac progenitors migrate anteriorly from the primitive streak (PS) to form two bilateral cardiac fields that (**b**) fuse in the midline to form the cardiac crescent (*yellow*) with the second heart field (*purple*) located medially. (**c**, **d**) Front (*left*) and lateral (*right*) views of the (**c**) linear, and (**d**) looping heart tube. Progenitors of the second heart field and the neural crest (*red*) migrate into the looping heart. (**e**) Fully developed heart. *Green colored atria* represent contribution of first and second heart field progenitors. The proepicardial organ gives rise to the epicardium. *AO* aorta, *CC* cardiac crescent, *LA* left atrium, *LV* left ventricle, *PEO* proepicardial organ, *PA* pulmonary artery, *SHF* second heart field, *RA* right atrium, *RV* right ventricle, *SVC* superior vena cava, *VP* venous pole. (**a**–**d**) modified from [10]. (**e**) Modified from [126]

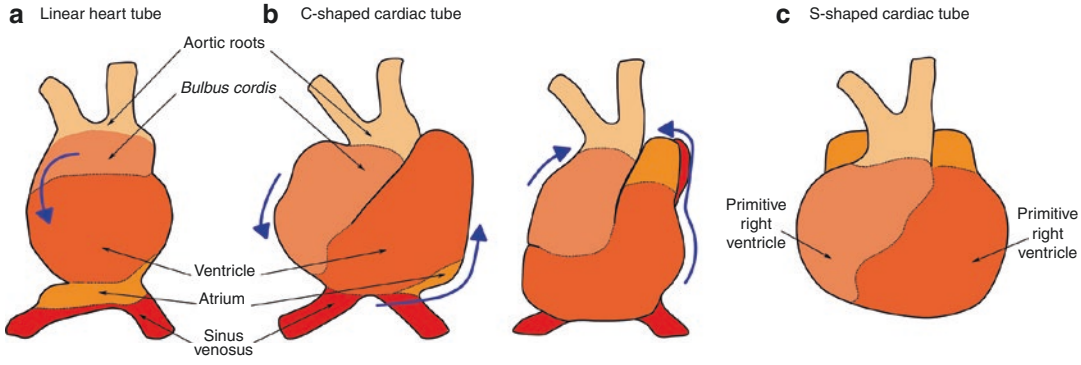

Fig. 1.3 Cardiac looping. The linear heart tube (**a**) twists and rotates rightwards resulting in the formation of the C-shaped tube (**b**). Elongation at the arterial and venous poles force arrangement into the S-shape tube (**c**), in which the outflow and inflow tracts come closer together cranially. Displacement of the *bulbus cordis* caudally, ventrally and rightwards, leftwards displacement of the primitive ventricle, and dorsal and cranial displacement of the primitive atrium, results in the proper spatial arrangement of the future cardiac chambers. Modified from [127]

of the heart tube and the establishment of asymmetry is regulated by leftwards flow in the node, which is a structure formed at the distal tip of the primitive streak containing motile cilia. The leftwards flow in the node elicits asymmetrical activation of diverse signaling pathways controlling left-right asymmetry, and include Ssh, Notch, Wnt, and FGF signaling. Nodal signaling activates expression of *FoxH1*, resulting in downstream asymmetric activation of left-right determinants *Nodal*, *Lefty* and *Pitx2c* [11].

After looping is complete, cells from the pro-epicardial organ (Fig. 1.2), which is a transient group of cells originated from lateral plate mesoderm and located ventro-caudally to the base of the heart [12], migrate towards the surface of the heart to form the epicardium and later contribute to formation of the coronary vasculature, early *sinus venosus*, and endocardium [13].

Cardiac Septation

The process of cardiac septation has been extensively reviewed [1, 14, 15]. Cardiac septation occurs during the fourth to the seventh week of embryogenesis and completely defines the cardiac chambers, separating the left from the right side and establishing the pulmonary and systemic circulatory systems. Studies on animal models have identified numerous molecular pathways that regulate cardiac septation and valve development, highlighting the complexity of the process. TFG, BMP, SMAD, Notch, Wnt, EGF, calcineurin/NFAT and VEGF signaling pathways are important regulators of cardiac septation. Transcription factors also required for the process are Pax3, Pbx and Meis, GATA, and T-box factors. In addition, epigenetic mechanisms controlling the expression of genes, including microRNAs and chromatin regulators also play central roles [16].

Ventricular Septation

Septation of the common ventricle occurs during the fifth week of development, and is completed by the ninth week. The muscular projection that will separate the ventricle starts forming during the looping process, when the walls of the future right and left ventricles grow concomitantly and coalesce resulting in the formation of the primitive interventricular septum, or interventricular ridge, at the base of the common ventricle [17]. The interventricular septum grows and extends posteriorly towards the endocardial atrioventricular cushions, leaving a space known as interventricular foramen. The interventricular foramen is closed when the interventricular septum fuses with the conotruncal septum, which forms from the fusion of conotruncal ridges derived from the endocardial cushions [18, 19].

The molecular processes patterning the ventricles are poorly understood. Comparative studies of the expression of the developmental regulator *Tbx5*, which is a key transcription factor regulating cardiac differentiation, have provided interesting insight. While *Tbx5* is homogenously expressed in the early developing single ventricle of turtle and lizard, it is restricted to precursors of the left ventricle in chicken and mouse. In later stages of development, *Tbx5* is preferentially expressed in a left to right gradient in the turtle ventricle, which by then develops an interventricular primary septum-like structure. Consistent with a key function for *Tbx5* in cardiac septation, genetically modified mice that mimic the reptilian *Tbx5* expression pattern develop a single ventricle. Thus, expression of *Tbx5* may constitute a patterning cue for ventricular septation [20].

Atrial Septation

The process of atrial septation has also been extensively reviewed [1, 14, 21]. Cardiac septation starts in the atrioventricular (AV) canal, a constriction of the looped cardiac tube that defines the primitive ventricle and atria. A subset of endothelial cells on the ventral and dorsal surface of the AV canal undergo endothelial to mesenchymal transition and migrate into the underlying cardiac jelly to form the endocardial cushions, which grow and fuse separating the right and left sides of the AV canal and partially separating the primitive atria from the ventricle. Then, the primitive atrium starts septation through the growth of the *septum primum*, which is a muscular appendage arising from the roof of

the left side of the chamber that grows towards, but does not reach the endocardial cushion [21], leaving an orifice known as the *ostium primum* or atrial foramen (Fig. 1.4). Apoptosis in the elongating *septum primum* creates a perforation known as the *ostium secundum*. The mesenchymal cap at the end of the growing *septum primum* fuses with the endocardial cushion in the AV canal. Before fusion of the *septum primum* with the endocardial cushion, another muscular appendage grows from the roof of the atrial chamber in the right side of the *septum primum* [21], known as *septum secundum*, which grows downwards overlapping the *ostium secundum* but does not reach the endocardial cushion. The partial overlap of the *septum secundum* with the *septum primum* and *ostium secundum* results in incomplete septation of the embryonic atria, which communicate through the *foramen ovale* (Fig. 1.4). This communication allows oxygenated blood coming from the placenta to reach the fetal circulation. Atrial septation is completed after birth, when increased pressure in the left atrium pushes the *septum primum* laterally towards the *septum secundum* closing the *foramen ovale*.

Outflow Tract Septation

Given the relevant function of the outflow tract in development of the right ventricle, its early stages of development will be discussed in more detail later in the "Development of the second heart field" section.

The process of septation of the outflow tract has been extensively reviewed [15, 16]. Septation of the outflow tract starts during the fifth week of development, and consists in the division of the common outflow chamber and *truncus arteriosus* to form the outlets and valves of the right and left ventricles, the pulmonary trunk and the aorta, respectively (Fig. 1.4). At the fifth week of human development, the outflow tract myocardium extends from the aortic sac [22]. At this stage, the proximal (conal) and distal (truncal) portions of the outflow tract can be distinguished by the presence of the conotruncal curvature, also known as the "dog-leg bend" [23]. Two pairs of endocardial ridges, the conotruncal and intercalated ridges, follow a spiral path and give rise to cushions that develop along the proximal and distal outflow tract. The spiral arrangement of the endocardial ridges positions the pulmonary trunk around the aorta. These cushions are formed in part with migrating mesenchyme derived from the neural crest. Septation starts with the fusion of the distal cushions, located towards the aortic sac, followed by fusion of the proximal cushions. Fusion of the distal portion of the proximal cushions with distal intercalated cushions will generate the aortic and pulmonary valves [15]. Around the 50th day of gestation, fusion of the proximal part of the outflow tract cushions results in formation of an embryonic outlet septum within the right ventricle. This septum is muscularized by invasion of parietal cardiac myocytes [24]. The septum then fuses with the muscular ventricular septum, confining the outlet of the aorta to the left ventricle and separating the ventricles. Further muscularization of the endocardial cushions [25], contribution of progenitor cells originated in the neural crest, and apoptosis [26] are required for complete separation of the outflow tract into the pulmonary artery and aorta.

Trabeculation

Trabeculae are projections of cardiac myoctes covered by endocardium that extend into the ventricle. Trabeculae are very important in the developing heart, as they extend the surface area favoring cardiac oxygenation and nutrient uptake before development of the coronary vasculature [27]. Trabeculae begin to form after looping of the cardiac tube by delamination of myocytes from the ventricular wall into the cardiac jelly [28]. Coordinated myocardial proliferation and differentiation results in the definition of two myocardial layers, the compact and trabecular myocardium. This process requires reciprocal communication between myocardium and endocardium via NOTCH and bone morphogenetic protein (BMP) signaling. NOTCH is activated exclusively in endocardial

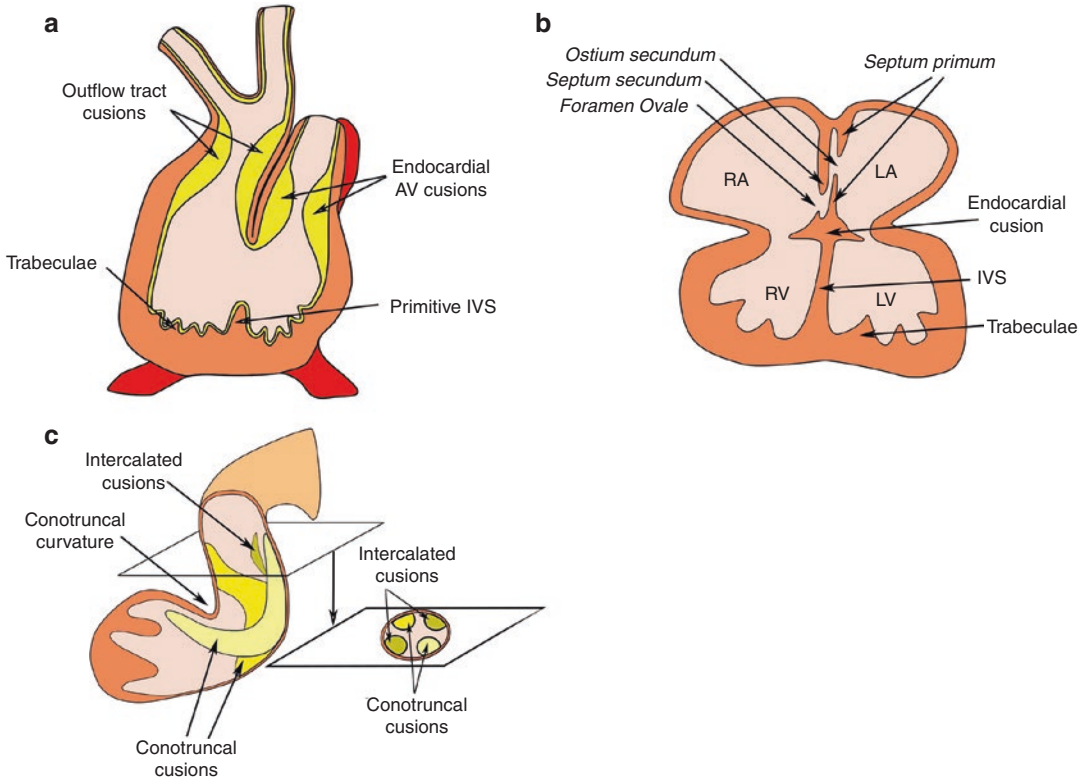

Fig. 1.4 Cardiac septation. (**a**) The primitive interventricular septum (IVS) is formed as a result of concomitant growth of the walls of the future ventricles colliding at the base of the common ventricle. The primitive IVS grows towards and fuses with the endocardial atrio ventricular cushions. (**b**) Septation of the atria results from the growth of the *septum primum* and *septum secundum* from the roof the common ventricle towards the endocardial cushion. Apoptosis in the elongating *septum primum* creates the *ostium secundum.* The septum secundum grows downwards overlapping the *ostium secundum* but does not reach the endocardial cushion. The partial overlap of the *septum secundum* with the *septum primum* and *ostium secundum* results in incomplete septation of the embryonic atria, which communicate through the *foramen ovale.* Modified from [127] (**c**) The proximal (conal) and distal (truncal) portions of the outflow tract can be distinguished by the presence of the conotruncal curvature. Two pairs of endocardial ridges, the conotruncal and intercalated ridges, follow a spiral path and give rise to cushions that develop along the proximal and distal outflow tract. Septation starts with the fusion of the distal cushions, located towards the aortic sac, followed by fusion of the proximal cushions. Fusion of the distal portion of the proximal cushions with distal intercalated cushions will generate the aortic and pulmonary valves. Modified from [16]

cells at the base of the trabeculae, resulting in expression of ephrin B2 (EPHB2) and subsequent expression of the secreted ligand neuregulin (NRG1) which stimulates differentiation of adjacent trabecular myocytes. Simultaneously, endocardial NOTCH stimulates proliferation of adjacent cardiomyocytes by activating BMP10 [29]. Trabeculae then undergo extensive maturation. Trabeculae stop growing towards the ventricles and thicken radially; causing them to compact to the point that trabecular myocardium is indistinguishable from compact myocardium and forms the complex trabecular network observed in the mature heart [30].

The Cardiac Fields

The previous sections highlight the fact that development of the heart entails complex morphogenetic processes involving multiple cell types, but how the cellular complexity of the

heart is generated during embryogenesis has not been discussed. As mentioned at the beginning of this chapter, cardiac progenitor cells developing during gastrulation give rise to the multiple cardiac cell types (Fig. 1.1), however the precise mechanisms that specify the fate of cardiac progenitors or the molecular cues that restrict their anatomical identity during development are not completely understood.

The first evidence for the existence of a group of cells giving rise to cardiac structures, or a cardiac field, was provided by the demonstration that lateral plate mesoderm located bilaterally to the primitive node of head-process stage chick embryos can generate myocardium and beating tissue in transplantation experiments [31]. Cells in this primitive bilateral cardiac field express the transcription factors Nk2 homebox 2, or *Nkx2-5*, and *Isl* Lim homeobox 1, or *Isl1*, contain progenitors of the first and second heart fields [32, 33] and migrate as a cohesive group and fuse at the midline. However, the location and timing for specification of such progenitors is poorly understood. A study shed light on this issue. Analysis of the expression of markers of early cardiac differentiation and the VEGF-1 receptor *Flk1* [6], which marks a multipotent progenitor population that differentiates into hematopoietic, endothelial, smooth muscle and cardiac myocytes [34, 35], demonstrated that cardiac progenitors are specified from mesoderm very early during gastrulation before formation of the bilateral cardiac field [6]. This study identified a molecular signature for the progression of mesoderm towards commitment to the cardiac lineage. A subpopulation of mesoderm cells expressing *Mesp1* during early gastrulation activate expression of *Smarcd3*, *Flk1*, and a marker of the anterior portion of the second heart field (AHF): the *Mef2cAHF* enhancer [36], followed by expression of specific markers of the cardiac lineage *Tbx5* and *Isl1* [6]. Therefore, expression of *Smarcd3* could be a very early marker of specification of the cardiac field from mesoderm during gastrulation. The endoderm adjacent to the lateral splanchnic mesoderm, the overlying endoderm, and the neural tube and notochord, signal positively and negatively to regulate cardiac differentiation through bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and canonical and noncanonical Wnt pathways [37]. This signaling induces mesodermal progenitors expressing Mesp1 to activate transcription factors including *Isl1*, *Tbx5*, *Nkx2-5* and *Gata4*, and the chromatin remodeler *Baf60c*, which induce downstream expression of cardiac differentiation markers [38–41].

Once the cardiac progenitors in the primitive heart field converge in the midline, they form the cardiac crescent, which corresponds to the first heart field (Fig. 1.2). Cells in the cardiac crescent are labeled by expression of the ion channel *Hcn4*, which is also a marker of the conduction system [42], myosin light chain 2a or *Mlc2a* [43], *Tbx5* [44], and myosin light chain 1 [45], encoded by *MLC3F* [46]. The limbs of the cardiac crescent fuse in the midline to form the linear heart tube, in which cardiac myocytes start differentiating, as evidenced by expression of muscle-specific proteins and the presence of beating [47–49]. Lineage tracing experiments for *Hcn4*-expressing cells have shown that the first heart field contributes myocardium to portions of the left ventricle and atria [43].

Isl1 expression is turned off as progenitors start differentiating [47] and is absent in the heart tube, but remains present in undifferentiated cardiac progenitors located in the splanchnic mesoderm posterior to the first heart field [4], therefore *Isl1* is considered as a marker of cardiac progenitors [4, 50]. This population of *Isl1* positive cells includes the second heart field. Cells in the second heart field are more proliferative, and their differentiation is delayed compared to first heart field progenitors [51, 52]. Progenitors of the second heart field are added to the linear and looping heart tube through the arterial pole anteriorly, and through the venous pole posteriorly to form the outflow tract, the right ventricle and a proportion of the atria [4].

A recent study traced the origin of chicken pacemaker cells to a field outside the first and second heart fields, a so-called tertiary heart field [5]. However, the origin of pacemaker cells remains controversial.

The Second Heart Field

Progenitors of the second heart field contribute the totality of the right ventricular myocardium, the outflow tract and the majority of the atria [9]. In addition, defects during its development cause several congenital cardiac diseases. The origin and development of the second heart field will be discussed in more detail in the following sections.

Discovery of the Second Heart Field and Its Contribution to Cardiogenesis

Cell labeling experiments using iron oxide performed by Victoria de la Cruz and colleagues in 1977 showed that the outflow tract was added after the cardiac tube was formed, leading to the hypothesis that this region originated from a secondary source of myocardium [3]. Later studies defined the identity of this cardiac progenitor population. Lineage tracing analysis for cells expressing a transgene driven by *FGF10* revealed contribution to the right ventricle, *conus* and *truncus* [53]. Other studies in which cells residing in the pharyngeal arches cranial to the cardiac tube were chemically labeled also revealed contribution to the *conus* and *truncus* [54]. The observation that cardiac field markers *Nkx2-5* and *Gata4* are expressed in the pharyngeal mesoderm caudal to the outflow tract, which also express a marker of migratory cells, suggested that this discrete cell population could migrate towards the cardiac tube and contribute to the formation of the heart. Indeed, cell-labeling experiments revealed contribution to the *conus* and *truncus*. This cell population was named the "second heart field" [55], which refers to the progenitors in the splanchnic mesoderm that migrate into to the linear heart through the arterial and venous poles [4] and that contribute myocardium to the distal outflow tract and smooth muscle of the great arteries in the arterial pole [55, 56]. The later discovery of *Isl1*, unified these findings, as it is expressed in the cell population that contributes to the right ventricle, *conus* and *truncus* (Figs. 1.2 and 1.3) [4]. Based on the results from cell labeling and lineage tracing experiments mentioned, the second heart field, labeled by *Isl1* expression, has been divided in "subfields": the anterior heart field, and the second heart field, which refer to the distinct cardiac progenitor populations added to the heart tube at different times [51]. Progenitors in the anterior heart field are labeled by expression of the *FGF10* transgene and migrate into the cardiac tube to at the arterial pole to form the right ventricle and the outflow tract [53]. A transgene driven by the promoter and enhancer of *Mef2c* is also expressed in the anterior heart field, but it is not restricted, as it also labels atrial progenitors and the muscular atrial septa [36, 57], which are contributed posteriorly to the cardiac tube through the venous pole. The differential contribution, and gene expression patterns of second heart field progenitors at the arterial and venous poles of the heart tube suggest that these sub-regions are pre-patterned [10, 58–60]. Retinoic acid signaling might be involved in pre-patterning the second heart field [61], as *Raldh2* encoding retinaldehyde dehydrogenase, an enzyme required for retinoic acid synthesis, functions in establishing the posterior boundary of the second heart field [62, 63] and patterning the embryonic heart [64]. Accordingly, mutation of *Raldh2* results in posterior expansion of second heart field marker genes [62, 63], and abrogation of retinoic acid signaling in pharyngeal mesoderm disrupts alignment and septation of the outflow tract [65].

The relevance of the second heart field in cardiogenesis was demonstrated by studies of the function of *Isl1* in the mouse and subsequently confirmed in *Xenopus* and zebrafish. The heart in homozygous null mice for *Isl1* does not loop and does not develop an outflow tract, right ventricle or an important proportion of the atria. Accordingly, *Isl1* is required for cell proliferation and survival in the pharyngeal endoderm and splanchnic mesoderm, and for migration of cardiac progenitors [4]. Further studies confirmed that impairment of second heart field development impairs cardiac elongation and looping [66, 67]. Similarly, knockdown of *isl1* in *Xenopus* results in abnormal cardiac development and reduced expression of cardiac differentiation

marker genes [68]. Studies in zebrafish revealed a that *isl1* is required for complete cardiomyocyte differentiation [69], and further studies have strengthened the existence of a second heart field in zebrafish [70, 71]. These findings indicate that an important function of *Isl1* in cardiac differentiation and patterning has been conserved during evolution, which is in line with requirement of the second heart field for cardiac morphogenesis and human health. Perturbation of second heart field development resulst in defective alignment of the ascending aorta with the left ventricle [66, 72], which occurs in human conotruncal defects like tetralogy of Fallot, overriding aorta and double outlet right ventricle [73]. Abnormal second heart field development can also cause defective atrial and atrioventricular septation in the venous pole of the heart [57, 74].

Development of the Second Heart Field

Cardiac progenitors segregate from mesodermal cells during gastrulation [6, 75]. After migration of the primitive cardiac field towards the midline, the anterior lateral splanchnic mesoderm forms the cardiac crescent, in which cardiac progenitors start differentiating. Concomitantly, splanchnic mesoderm containing the second heart field locates medially to the cardiac crescent [32, 53, 61]. Separation of the heart tube from the dorsal wall of the pericardial cavity separates the second heart field, leaving the heart tube connected to the pharyngeal mesoderm through the arterial and venous poles [76]. While differentiation starts in the first heart field, the second heart field remains in a proliferative and undifferentiated state in the caudal pharyngeal region [52], however, the mechanisms promoting cell renewal are not understood. A recent finding indicates that the second branchial arch serves as a microenvironment for cardiac progenitor cell expansion, and that a cell autonomous function of *Numb* and *Numb* like (*Numbl*) is required [77]. Active research has uncovered complex interactions between the microenvironment of the second heart field in regulating the balance between proliferation and differentiation of the second heart field, as well as the pathways promoting cell fate acquisition and segregation of progenitors. The microenvironment surrounding second heart field progenitors, include the lateral plate and paraxial mesoderm, adjacent endoderm, overlying ectoderm, dorsal aortas, notochord, and neural tube; which signal through retinoic acid, fibroblast growth factor (FGF), Wnt, bone morphogenetic protein (BMP), and sonic hedgehog (Shh) to regulate the fate, differentiation, and behavior of cardiac progenitors of the second heart field. These proteins activate a hierarchical signaling cascade that ultimately affects the activity of key transcription factors and their downstream targets to establish gene expression patterns [10, 78].

Proliferation and Survival of Second Heart Field Progenitors

Stimulatory and repressive activities of BMP, FGF and canonical Wnt signaling turn on the cardiac gene expression program in anterior lateral splanchnic mesoderm [37] by activating expression of transcriptional regulators including *Isl1*, *Nkx2-5*, *Mef2c* and *Gata4*, and the chromatin remodeler *Baf60c* [1, 38]. During formation of the linear heart tube, proliferation, survival and delayed differentiation of second heart field progenitors is controlled by Notch, Wnt/b-catenin, FGF and Hedgehog (Hh) signaling from the pharyngeal endoderm [78, 79]. Notch signals repress cardiac differentiation by preventing activation of *Mef2c* expression [80], and by activating *Hes1*, which is required for cardiac progenitor cell proliferation, and prevents precocious differentiation [81], upstream Wnt/b-catenin [82]. Wnt/b-catenin signaling activates *Isl1* gene expression by binding to its regulatory region [83], activates *Gata6* expression [84], and promotes expansion of *Isl1*-expressing progenitors [85, 86], and its loss results in defects in outflow tract and right ventricle development [83, 86].

Inactivation of Wnt/b-catenin is accompanied by loss of FGF signaling [86, 87], which regulates second heart field progenitor proliferation in the dorsal pericardial wall mainly through the

ligand Fgf8, with contribution from Fgf3, and Fgf10 [79, 88–91]. Inactivation of *Fgf10* exacerbates cardiac defects in *Fgf8* mutants, and compound inactivation of *Fgf10* and *Fgf3* results in decreased expression of *Isl1*, and unstable *Nkx2-5* expression, leading to shortening of the outflow tract [91]. *Isl1* acts upstream the Wnt and FGF signaling pathways by regulating the expression of *Tbx1*, which regulates FGF ligand expression [4, 92], is required for proliferation of the pharyngeal mesoderm [93], and is mutated in human DiGeorge syndrome, associated with conotruncal congenital cardiac abnormalities [94]. *Tbx1* is an important promoter of progenitor proliferation and suppressor of differentiation. Tbx1 inhibits activation of BMP target genes, interacts with Sp1, which activates cardiac gene expression, targeting it for degradation, and represses the expression of *Mef2c*, which is required for differentiation [95–97]. The homeodomain transcription factor *Six1* and its coactivator *Eya1* act upstream *Fgf8* and downstream *Tbx1* in the regulation of second heart field proliferation and survival [61, 98].

Shh from the pharyngeal mesoderm is also required for proliferation of the second heart field, and for neural crest development [99]. The forkhead box transcription factors *Foxf1a* and *Foxf2* regulate atrioventricular septation, and integrate Tbx5 and Hedgehog pathways by the synergistic activity of the hedgehog transcriptional regulators Tbx5 and Gli1 in activation of *Foxf* gene expression [100].

Differentiation of Cardiac Progenitors and Cardiogenesis

T-box transcription factors regulate the transition from proliferating to differentiating cardiac progenitors by controlling BMP signaling [101]. BMP signaling from the distal outflow tract induces this transition at the arterial pole by inducing neural crest derivatives to express *Msx1*, which represses *FGF* gene expression, thus opposing FGF signaling [102]. Cardiac progenitors start differentiating as they enter the cardiac tube and express cardiogenic transcription factors [38]. It is not clear if progenitors of the second heart field follow a specific differentiation pathway different from that followed by first heart field progenitors. Identifying the transcriptional programs controlling first and second heart field differentiation is subject of active research. In the first heart field, transcription factors including Nkx2-5, Gata4, Tbx5, the basic helix-loop-helix transcription factors Hand1 and 2, and Mef2c regulate cardiac cell differentiation in the cardiac crescent and the developing heart. Gata4 associates with Baf60c/Smarcd3, acting as a link with the BAF chromatin remodeling complex, to activate expression of Nkx2-5, which physically interacts with Gata4 to activate the cardiac gene expression program [40]. Indeed, forced expression of *Gata4*, *Tbx5* and *Baf60c*/*Smarcd3* induces ectopic myocardial differentiation and beating in mesoderm, suggesting these factors might act as master regulators of the cardiac differentiation program [41]. These findings also suggest that chromatin structure dynamics is important for the establishment of the transcriptional program regulating cardiogenesis. Indeed histone acetylation and methylation are key for cardiomyocyte differentiation and cardiogenesis [103].

The histone acetyltransferase P300 regulates the expression of cardiac genes *Nkx2-5*, *Mef2c*, *Hand1/2*, *ANP*, *BNP*, *a-MHC*, and *b-MHC*, at least in part by interacting with Gata4, Nkx2-5 and Mef2c [103]. Accordingly, deficiency of P300 results in abnormal cardiogenesis with decreased trabeculation and defective differentiation [104]. In addition, P300 directly acetylates GATA4 and Mef2c, increasing their transcriptional activity [105, 106]. Removal of acetyl marks also alters cardiac development. The histone deacetylases HDAC5 and 9 interact with Mef2c, suppressing its transcriptional activity, and their inactivation results in lethality with thinning of the myocardial wall [107, 108].

Histone methylation has a key function in the orchestration of the cardiac transcriptional program. Analysis of the global distribution of histone methylation marks in stem cell-derived cardiomyocytes revealed that cardiac differentiation is regulated by coordinated epigenetic

transitions [109]. Furthermore, a chromatin signature distinguishes cardiac genes encoding transcription factors and soluble proteins regulating cardiac differentiation, from lineage-specific genes regulating cardiac function and maintenance. This chromatin signature consists in enrichment of tri-methylation of lysine 4 of histone H3 (H3K4me3) and H3K36me3, which are histone marks associated with gene activation, and lack of H3K27me3, which is a mark of inactive genes [110].

The fundamental role of histone methylation in cardiac differentiation is highlighted by the discovery that de novo mutations in genes involved in H2K4 and H3K27 methylation are enriched in the transcriptome of patients with congenital heart disease [111]. Accordingly, deficiency of histone methyltransferases affects cardiac development. Deficiency of the H3K4 methyltransferase *Smyd1* results in embryonic lethality with defective cardiomyocyte maturation and right ventricular development [112].

Deletion of the H4K36 methyltransferase Wolf-WHSC1 has been found in patients with Wolf-Hirschhorn Syndrome, associated with congenital heart defects [113]. WHSC1 interacts with Nkx2-5 and represses transcription. Accordingly, deficiency of WHSC1 causes lethality with ventricular septal defects [114].

H3K27me3 stabilizes the transcriptional program of differentiating cardiac progenitors by suppressing the expression of the transcription factor *Six1* [115], which is required for development of second heart field progenitors, but is suppressed during differentiation [98]. Inactivation of the H3K27me3 methyltransferase *Ezh2* in cardiac progenitors results in embryonic lethality with decreased cardiomyocyte proliferation and septal defects [116, 117]. Inactivation of *Ezh2* in progenitors of the second heart field does not cause developmental cardiac defects, but results in adult cardiac disease with right ventricular hypertrophy and increased fibrosis due to derepression of a transcriptional program characteristic of skeletal muscle downstream of *Six1* [115]. Therefore, it is possible that altering the epigenetic environment early during early differentiation of cardiac progenitors results in long-term gene expression misregulation that may predispose to postnatal cardiac disease.

The H3K9 mono and di-methyltransferases G9a and GLP act as transcriptional repressors and their combined deficiency causes atrioventricular septal defects [118], and silencing of the H3K9me3 Suv39h1 promotes expression of genes controlling cell cycle progression, resulting in increased proliferation [119].

Thus, repressive histone methylation stabilizes the cardiac gene expression program by repressing non-cardiac gene expression [103, 115].

Given that dynamic histone modification transitions regulate cardiac differentiation [109], it is not unexpected that removal of histone methylations is also important for cardiogenesis. Proteins harboring the Jumonji (Jmj) domain are histone demethylases that regulate gene expression [120], and deficiency of several of them affects cardiac development. Deficiency of the H3 and H4 arginine demethylase Jmjd6 causes perinatal death with ventricular septal defects and double outlet right ventricle. UTX is a H3K27 demethylase encoded in the X chromosome expressed in the developing heart, and its deficiency results in embryonic lethality with reduced expression of *Nkx2-5* and *Tbx5*, defective cardiac tube looping, and lack of chamber development [121]. UTX is recruited to cardiac genes through interaction with Nkx2-5, Tbx5, Gata4 and Baf60c, and influences cardiac enhancer activity by it interaction with the MLL3/4 complex, which mediates H3K4 methylation [121].

Dynamic DNA methylation has recently been shown to regulate the maturation of cardiac myocytes. DNA methylation represses gene expression, and loss of DNA methylation accompanies activation of genes expressed in adult mature cardiomyocytes, like the adult troponin *Tnni3*. In contrast, de novo methylation induced by the DNA methyltransferases DNMT3A/B occurs in genes expressed in immature newborn cardiomyocytes but that are silenced in mature hearts, like the fetal troponin *Tnni1* [122]. DNA methylation acts in concert with histone methylation to regulate the transition from newborn to adult

gene expression program. For instance, *Isl1*, which is expressed embryonically, and silenced upon differentiation, remains demethylated in newborn and adult cardiomyocytes, however it is occupied by the repressive histone mark H3K27me3 [122].

Thus, multiple epigenetic processes coordinate the gene expression programs controlling cardiac differentiation and maturation. However, we still do not completely understand the epigenetic control of cardiovascular development. Furthering this field will be required to uncover the roots of cardiovascular disease and will put us in a better position to design future preventive and therapeutic strategies.

Conclusions and Future Directions

Development of the right ventricle entails complex morphogenetic processes that transform a group of progenitor cells in the cardiac fields into a four-chambered heart. Coordinated signaling pathways instruct key transcription factors to tightly regulate gene expression and orchestrate cardiac development. The study of transcriptional pathways involved in the establishment and differentiation of the cardiac fields has led to important discoveries that could revolutionize the approaches to treat diseases caused by loss of myocardium. Recent studies found a combination of transcription factors that induce the cardiac phenotype in non-cardiac myocytes. These pioneering experiments showed that forced expression of *Gata4*, *Tbx5* and *Baf60c*/*Smarcd3* induces ectopic myocardial differentiation and beating in mesoderm in vivo [41]. Subsequent experiments showed that induction of *Gata4*, *Mef2c* and *Tbx5* can induce a cardiomyocyte-like phenotype in fibroblasts [123, 124]. Furthermore this combination of transcription factors can induce trans-differentiation of cardiac fibroblasts in post-infarction scars into beating cardiomyocytes [125]. These findings suggest that forced expression of key cardiogenic transcription factors holds a potential therapeutic use, however, the efficiency of the induction of the cardiac phenotype is low in the reported protocols. Uncovering the so far unknown molecular cues that induce the cardiac progenitor fate early during gastrulation and that control cardiac development could provide the missing elements required for controlled and efficient induction of cardiomyogenesis, and thus revolutionize the approaches to treat cardiac disease.

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