1 Normal and Abnormal Cardiac Development

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Introduction

In this chapter, the main events of cardiac morphogenesis are discussed. We focus on morphologic descriptions and insights based on the molecular biologic approaches in animal models that have enhanced and modified our understanding of normal and abnormal cardiac development, including relevance for adult disease with a developmental background.

Advances and limitations in studying human development

The normal cardiovascular development of the human embryo in its crucial stages from 2 to 8 weeks' gestation has to be deduced from postmortem morphologic studies of abortion material [1]. In this category we are mainly dealing with spontaneous abortions and do not know whether the material reflects normal morphogenesis. Descriptions in the literature referring to normal and abnormal human development do not emphasize this aspect. An addition to early detection of human embryonic malformations, mainly providing information on disturbed genes and chromosomes, is provided by amniocentesis, chorionic villus biopsies, and subsequent FISH (fluorescent *in situ* hybridization) analysis with genetic markers. However, these are not examined within the first crucial 8 weeks of development. Fetal diagnosis is a rapidly expanding area with increasing technical possibilities of ultrasound and echo-Doppler investigations *in utero*. The earliest observations indicating normal or abnormal heart development refer to 11–12 weeks' gestation [2]. Consequently, our knowledge of detailed cardiac morphogenesis relies on describing processes in animal species, the main embryonic models being avians (chick and quail) and rodents (mouse and rat) and more recently the zebrafish. With the development of transgenic techniques, the mouse embryo has become important, and we will regularly refer to mouse embryo models when discussing certain abnormalities of cardiac development.

Knowledge about an embryonic lethal phenotype after a gene knockout and the absence of a phenotype might contribute little to the understanding of human congenital cardiac malformations [3]; 85% of the diagnosed human cardiac malformations are described as having a multifactorial origin. Epigenetic, environmental, biomechanical, and hemodynamic factors have been underestimated in research on cardiogenic programming. Their role in the development of cardiac malformations has previously been acknowledged, however, and has led to the so-called mechanistic classification [4]. There are a few recent publications linking hemodynamics to cardiovascular developmental abnormalities [5–8], but their relation to gene expression and cardiogenic patterning is unclear. A multidisciplinary approach combining clinical knowledge with basic science will lead to new insights into developmental processes.

Formation of the cardiogenic plates and the cardiac tube

The cardiac developmental program starts with the formation within the splanchnic mesoderm of the bilateral cardiogenic plates, which give rise to the myocardium and probably to parts of the endocardium (Figure 1.1). The splanchnic mesoderm at the endoderm/mesoderm interface differentiates into the vascular endothelium [9] and part of the endocardium [10,11]. The evidence for a cardiogenic plate origin of the endocardium supports a dual origin for this layer of the heart [12].

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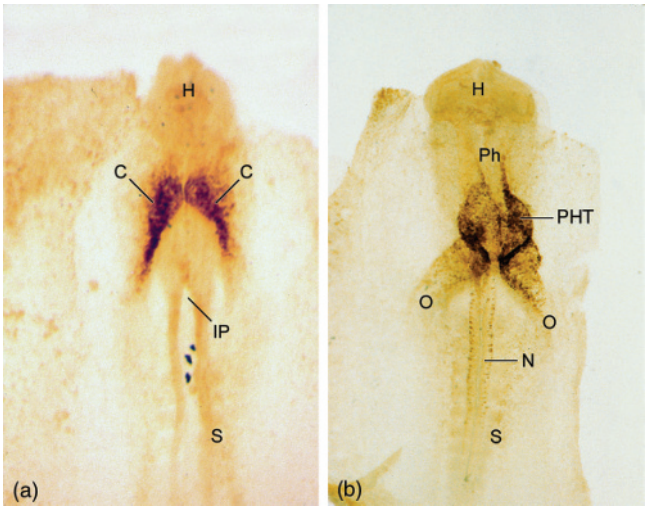

Figure 1.1 (a) Whole mount of a quail embryo (stage HH 8) viewed from the ventral aspect, showing the bilateral cardiogenic plates (C) that have not yet fused across the midline. At this stage, the staining is done by a nonspecific neurofilament antibody. (b) Whole mount of the fused primary heart tube (PHT) of a quail embryo (stage HH 10) viewed from the ventral aspect. The staining is by an anti-smooth muscle actin antibody, showing the myocardial lining of the tube. H, head region; IP, intestinal portal; N, neural tube; O, omphalomesenteric vein; Ph, pharyngeal region; S, somite. (Copyright Leiden University Medical Center.)

The bilateral asymmetric cardiogenic plates can be delineated early in embryonic life because several transcription factors and proteins are expressed. These expression patterns distinguish a first or primary heart field (PHF) laterally flanking the second heart field (SHF) component of the cardiogenic plate (Figure 1.2a). Whereas the first heart field differentiates, the secondary component remains part of the body wall mesoderm before its cells are recruited and incorporated into the poles of the cardiac tube. With formation of the cardiac tube, the pericardial coelomic cavity becomes continuous across the midline and the ventral mesocardium disappears. The cardiac tube is thereafter solely connected to the dorsal body wall or splanchnic mesoderm by the dorsal mesocardium that runs from the developing pharyngeal arches (arterial pole) to the sinus venosus (venous pole) (Figure 1.3). At this stage, the tube consists of an inner endocardial and an outer myocardial layer separated by cardiac jelly (Figures 1.2b and 1.3a).

Initially, the primitive cardiac endothelial network is remodeled into a single endocardial tube that connects the omphalomesenteric veins to the pharyngeal arch vasculature (Figure 1.1). The asymmetric cardiac jelly surrounding the endocardial tube suggests bilateral endocardial tubes, giving the wrong impression that two endocardial tubes have to fuse. From the onset, however, the endocardial tubes are connected by endocardial cells that cross the midline [13]. Real cardia bifida can occur spontaneously and can also be produced experimentally by retinoic acid overdose in the chicken embryo [14] or in a zebrafish mutational screen [15]. Therefore, each cardiogenic plate can potentially give rise to an independent cardiac tube, implying that fusion of the cardiogenic plates is unnecessary for the onset of cardiac formation. Nevertheless, cardia bifida is lethal to the embryo as further cardiac development is hampered and no connection with the endothelium of the pharyngeal vascular system is established.

Looping of the cardiac tube

The single cardiac tube is never completely straight as both cardiogenic plates have different dimensions [12]. Normally the cardiac tube loops to the right (D-loop) (Figure 1.2). Abnormalities in looping such as L-loop or anterior-loop formation are related to ventricular inversion, which differs from laterality problems as seen in abnormalities of the atrial situs.

The mechanisms underlying the looping direction are poorly understood, but several regulating genes have been described, such as sonic hedgehog, nodal and activin receptor IIa [16]. In mouse mutants iv/iv and inv, the laterality of the heart is also affected. The iv gene has been mapped to chromosome 12 in the mouse and is syntenic to chromosome 14q in the human. In the human, this abnormality is reflected in the heart by atrial isomerism and is discussed below when considering atrial development and septation.

During looping, the outflow tract becomes more ventrally positioned, moving in front of the atrioventricular (AV) canal. The arterial and venous poles remain fixed to the dorsal body wall (Figure 1.4 and Videoclip 1.1). Both remodeling of the inner curvature (site of the disruption of the dorsal mesocardium) and asymmetric addition of SHF-derived myocardium to the primary heart tube are essential for proper looping.

Contribution of first and second heart fields

Recent mouse studies, based on various transgenic mouse models with cell tracing [17–19], have shown that the primary heart tube does not contain all components necessary for the future mature heart [20]. The first heart field provides only for the AV canal and the future left ventricle (LV), implying that the primary heart tube already has additions of the second heart field (SHF) at both poles. The primary heart tube connects the omphalomesenteric veins at the venous pole via a small atrial component, the AV canal, and a primitive LV and small outflow tract component to the aortic sac and the first pair of pharyngeal arch arteries at the arterial pole (Figures 1.2 and 1.3).

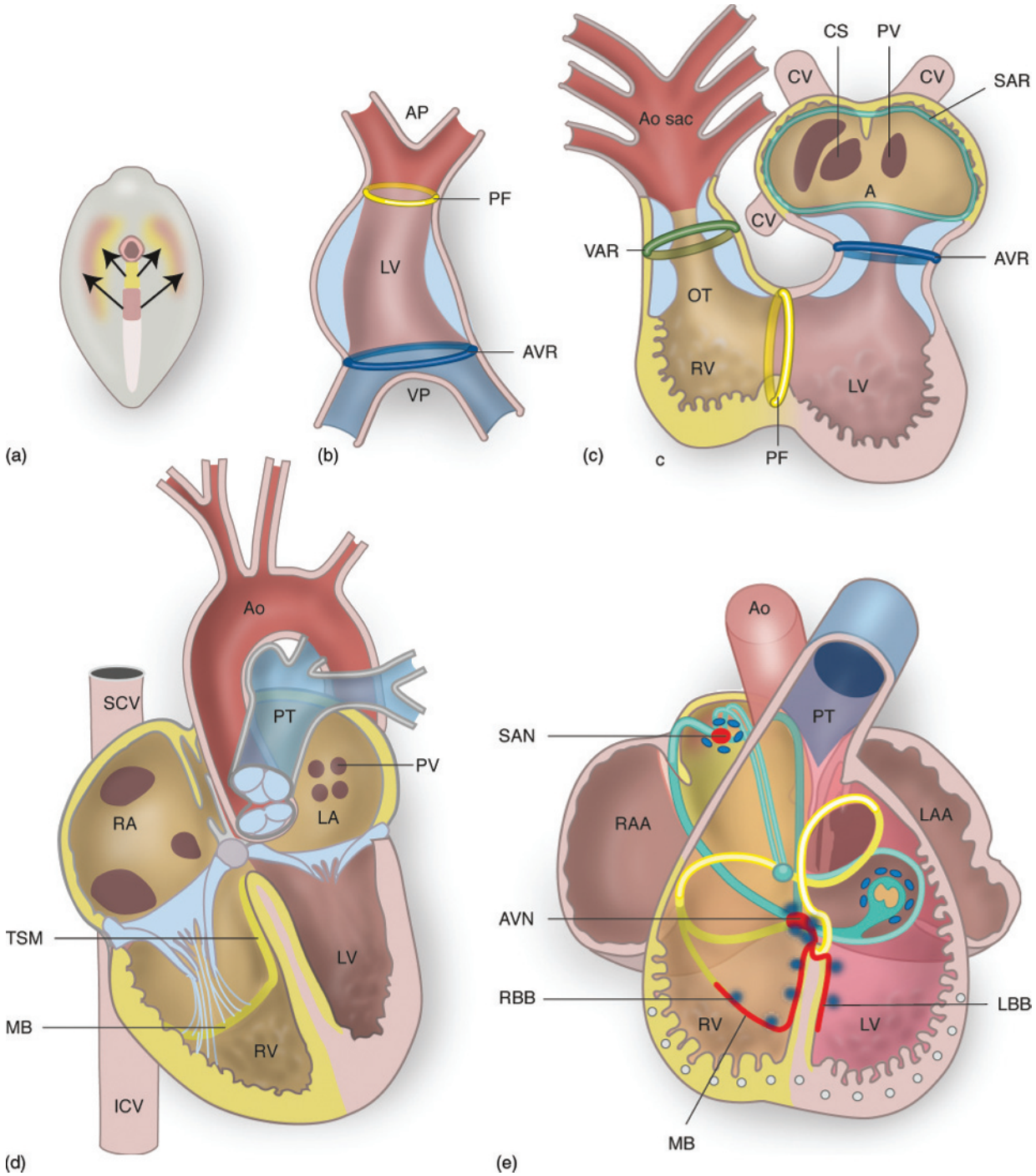

Figure 1.2 Development of the heart from the first and second heart fields. (a) In the primitive plate, bilateral fields of cardiac mesoderm are present. Progenitor cells migrate from the primitive streak to the bilateral mesoderm (arrows). Cells depicted in yellow will contribute to the second heart field-derived parts of the heart, whereas cells depicted in brown depict the primary heart fields that will contribute the primary myocardial heart tube. (b) Schematic representation of the primary heart tube, consisting of endocardium and myocardium, with myocardial jelly between the two layers. Initially the primitive heart tube consists mainly of the AV canal and the LV. (c) After looping, several transitional zones can be distinguished in the tube, namely the sinoatrial transition (light blue, SAR) in between the sinus venosus and common atrium, the AV transition (dark blue, AVR) in between the common atrium and common ventricle, the primary fold (yellow, PF) in between the primitive right ventricle (RV) and LV, and a ventriculoarterial transitional (green, VAR) zone at the outflow tract (OT) of the heart. Second heart field-derived parts of the heart are depicted in yellow. (d) The heart after completion of atrial and ventricular septation. Due to outgrowth of the RV, a remodeling of the PF has occurred, and it has divided into a lateral septal part, the trabecula septomarginalis (TSM), that contains the right bundle branch [RBB, see (e)] and continues into the moderator band (MB). (e) Part of the transitional zones will contribute to definitive elements of the cardiac conduction system, depicted in red. Bright blue dots depict neural crest cells that contribute to the network of autonomic nerve fibers surrounding the sinoatrial node (SAN) and atrioventricular node (AVN). Shaded blue dots surrounding elements of the cardiac conduction system indicate neural crest cells with an inductive role in conduction system development. A, common atrium; AP, arterial pole; Ao, aorta; Ao sac, aortic sac; CV, cardinal vein; CS, coronary sinus; ICV, inferior caval vein; LA, left atrium; LAA, left atrial appendage; LBB, left bundle branch; LV, left ventricle; PT, pulmonary trunk; PV, pulmonary veins; RA, right atrium; RAA, right atrial appendage; SCV, superior caval vein; VP, venous pole. (Copyright Leiden University Medical Center.)

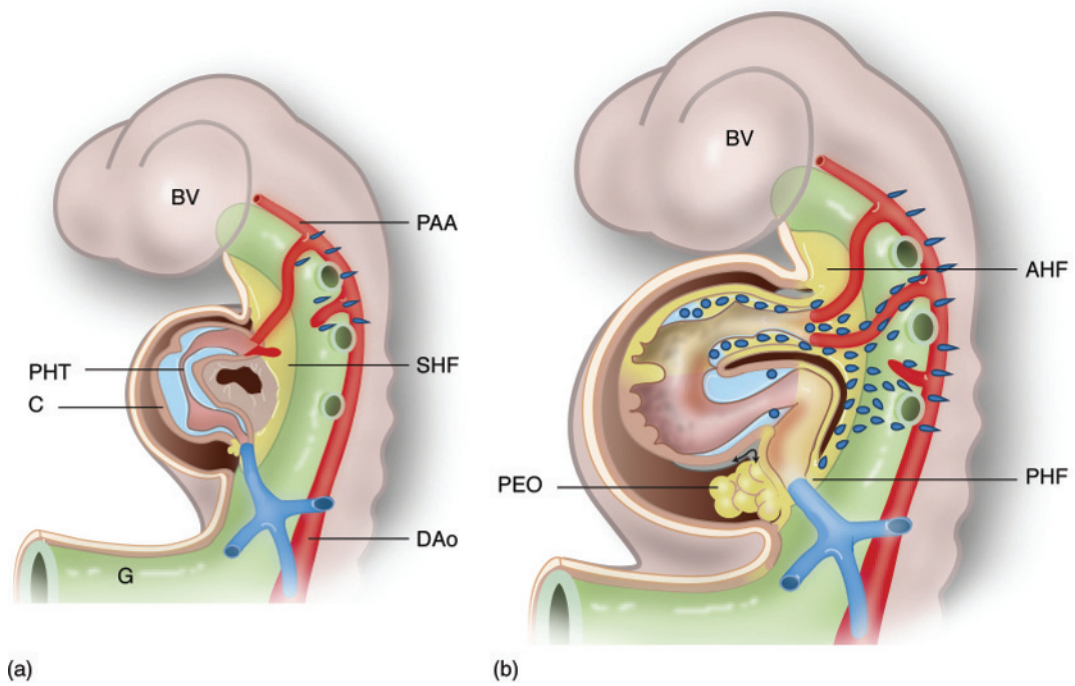

Figure 1.3 (a) Schematic representation of the primary heart tube (PHT, brown) after fusion of the bilateral plates of mesoderm. The tube is lined on the inside by cardiac jelly (blue). The mesoderm of the second heart field (SHF) is depicted by the yellow area behind the primary heart tube, and will during development contribute myocardium to both the arterial and venous poles of the heart [depicted by the yellow myocardium in (b)]. (b) The heart tube after contribution from the first and second heart fields have been made. The second heart field can be divided into the anterior heart field (AHF) and posterior heart field (PHF). The yellow lobulated structure that protrudes into the pericardial cavity at the venous pole of the heart is the pro-epicardial organ (PEO). Neural crest cells (depicted by blue dots) migrate from the neural crest along the arterial and venous pole into the heart. BV, brain ventricles; C, coelomic cavity; DAo, dorsal aorta; G, gut; PAA, pharyngeal arch arteries. (Copyright Leiden University Medical Center.)

The cardiac splanchnic mesoderm consists of so-called SHF. This precardiac mesoderm is added at both the arterial and venous poles of the heart, mainly contributing myocardium but also smooth muscle cells of connecting vessels.

The mesodermal cell population grows in a caudocranial direction [21]. Recruitment starts at the arterial pole and almost the complete myocardium of the right ventricle (RV) including the outflow tract and the larger part of the ventricular septum is derived from the SHF. The smooth muscle cells of the aortic sac are derived from this source, although probably asymmetric with respect of contribution to the pulmonary and aortic aspects. More restricted studies of the outflow tract have led to a confusing nomenclature with respect to anterior heart field [22] and secondary heart field [23], the latter often being confused with SHF that contributes to both arterial and venous poles.

At the venous pole, the myocardium lining the sinus venosus derives from SHF mesoderm referred to as posterior heart field (PHF) [24]. Incorporation of the sinus venosus implies that the myocardium of the sinoatrial node, the venous valves, the atrial septum, and the cardinal and pulmonary veins also come from this source. A further mesenchymal derivative of the SHF is the proepicardial organ (PEO), which is crucial for many aspects of differentiation of the heart (see below).

Several transcription factors and morphogenetic genes and cascades are important in the precardiac mesoderm of both first heart field and SHF [25]. Specification of the precardiac cells is accompanied by early expression of TGFβ family members, including BMP4 (bone morphogenetic protein), followed by the earliest known marker for the cardiogenic lineage – the homeobox (Hox)-containing gene Nkx2.5 (homolog to tinman in *Drosophila*) [26] and the zinc finger-containing GATA 4/5/6 cluster of transcription factors [27]. Mesp1 [28] and Mef2c [29] are also early cardiac mesoderm markers. Recently, the platelet-derived growth factor receptor (PDGFRα) was added to this list [30]. Patterning of the heart field from arterial to venous pole is accompanied by the expression of T-box gene family members Tbx1, 5 and 20, Fgf 8 and 10, and Isl1. Finally, differentiation during heart tube formation involves, for example, MLC and MHC, alpha cardiac actin and troponin I, and RhoA [31]. Mouse models in which these genes are used for cell tracing and complete or conditional knockout provide essential data on their relevance for normal and abnormal cardiac development. In some instances, such as Nkx2.5, [32] human mutations are known.

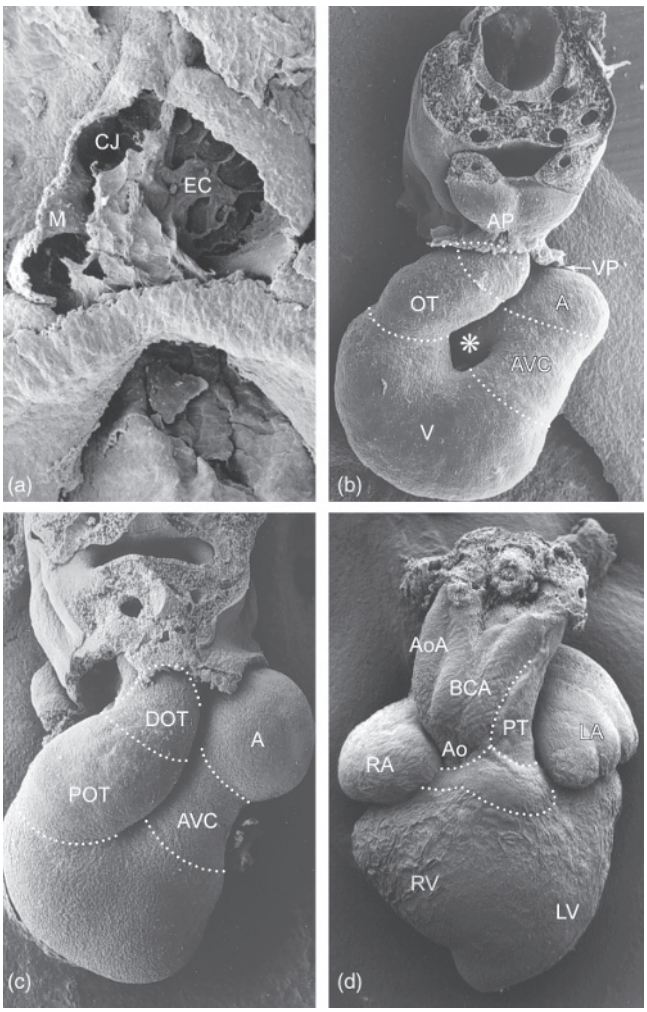

Figure 1.4 Scanning electron micrographs of the developing heart. (a) The fused heart tube (also see Figure 1.1) has been opened to show the endocardial cells (EC) inside the myocardium (M) and the cardiac jelly (CJ). (b) The heart tube normally loops to the right showing a venous pole (VP) and an arterial pole (AP) and the inner curvature (asterisk). (c) As the inner curvature tightens, the outflow tract becomes positioned in front of the venous pole. (d) With completion of the looping process, the AP is wedged in between the atrioventricular orifices that connect the right atrium (RA) to the right ventricle (RV) and the left atrium (LA) to the left ventricle (LV). A, atrium; AoA, aortic arch (right sided in birds); Ar, aortic root; AVC, atrioventricular canal. BCA, brachiocephalic arteries; DOT, distal outflow tract; OT, outflow tract; POT, proximal outflow tract; PT, pulmonary trunk; V, ventricular loop. (Copyright Leiden University Medical Center.)

Segmentation of the heart tube

The primary heart tube consists of myocardium lined on the inside by cardiac jelly and endocardium. A number of genes are expressed along the anterior/posterior axis and there is from the onset a right–left designation. Chamber outgrowth or ballooning, intricately regulated by a balance of Tbx2 and Tbx3 transcription factor expression [33], brings out more clearly the segments (atrial and ventricular chambers) and the transitional zones. These areas stand out against the myocardial trabeculated atrial and ventricular walls. Figure 1.2b–e depicts the cardiac segments and transitional zones. Starting at the inflow at the venous pole, we can distinguish the sinus venosus, the atrium, the atrioventricular canal, the primitive LV, the primary fold, and the primitive RV that develops into a trabeculated part and a part lined by endocardial outflow tract cushions. In general, the endocardial cushion-lined transitional zones form the atrioventricular and semilunar valves and function initially as temporary valves accompanying peristaltic contractions of the cardiac tube. The myocardium of the sinus venosus (considered as a transitional zone), the AV canal, the primary fold, and the endocardial cushion-lined outflow tract are important for the formation of the future cardiac conduction system. Furthermore, these transitional zones are involved in septation.

Neural crest and epicardium contributions

For many years, the neural crest and epicardial cells were described as extracardiac contributors essential for proper differentiation of the developing heart. With new insights into the contribution of the SHF, we need to adjust their relevance.

Neural crest cells are an extracardiac source of cells that migrate from the neural crest through the mesoderm of the SHF to the cardiac tube. The main entrance site into the heart is at the arterial pole, but they also reach the venous pole of the heart [34,35] (Figures 1.3b and 1.5). These neural crest cells differentiate into smooth muscle cells of the great arteries and into the cells of the autonomic nervous system that are needed to innervate the great arteries and the coronary arteries, and for the nodes of the cardiac conduction system (Figures 1.2e and 1.5). The neural crest cells that migrate into the heart do not differentiate into a particular cardiac cell but go into apoptosis. Through release or activation of growth factors such as TGFβ they may induce myocardialization of the outflow tract septum and, at the venous pole, differentiation of the cardiac conduction system [36,37]. They are also important in the interaction with the SHF cells, mainly in the pharyngeal region, so that genetic mutations of both cell types can lead to congenital heart disease. This is best exemplified in the Tbx1-related 22q11 deletion syndrome [38].

The epicardium develops from the proepicardial organ, an epithelial derivative of the PHF at the venous pole (depicted in Figure 1.3b). These cells differentiate into smooth muscle cells and cardiac fibroblasts and migrate to many cardiac structures where their function is less known [39]. Suggestions, based on cell tracing in transgenic mouse

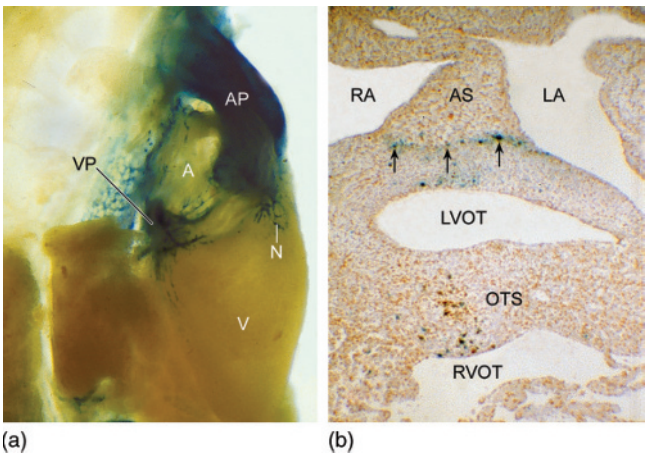

Figure 1.5 (a) Whole mount staining of a chicken heart (stage HH 35) that shows the neural crest-derived cells after a retroviral transporter gene marker containing lac-Z. The neural crest cells are present at the arterial pole (AP) as smooth muscle cells in the vessel wall and over the heart as fine nerve fibers (N). The neural crest cells also reach the venous pole (VP) of the heart, where they enter the atrioventricular region through the dorsal mesocardium. (b) A section through the inflow and outflow tract of a chicken heart in which the neural crest cells are seen in the outflow tract septum (OTS) and also at the base of the atrial septum (AS) (arrows), where they have arrived through the dorsal mesocardium. The brown staining of the outflow tract septum (OTS) neural crest cells by the TUNEL [TdT-mediated dUTP (deoxyuridine triphosphate) nick end labeling] technique detected apoptosis of these cells. A, atrium; LA, left atrium; LVOT, left ventricular outflow tract; RA, right atrium; RVOT, right ventricular outflow tract; V, ventricle. (Copyright Leiden University Medical Center.)

models, that epicardial cells can differentiate into myocardial [40] and endothelial cells [41] have been refuted.

Cardiac differentiation and development of cardiac malformations

Sinus venosus incorporation and atrial septation

The sinus venosus in the developing heart forms an intermediate transitional zone between the systemic cardiac veins and the developing atrium proper, and now receives much attention as the myocardium of the sinus venosus is derived from the PHF mesoderm, showing specific gene expression patterns. On the basis of endothelial vascular patterns, scanning electron microscopy data, and immunohistochemistry, we demonstrated that the sinus venosus is incorporated not only into the dorsal wall of the right atrium but also into the dorsal wall of the left atrium [42]. Here, it encircles the entrance of the future pulmonary veins. The sinus venosus also contributes to the posterior wall of the left atrium and pulmonary veins, as suggested for both the mouse and the human embryo [43,44], and earlier postulated by Van Praagh and Corsini [45]. Other groups, focusing on gene expression patterns, regard the pulmonary veins (pulmonary pit lined by pulmonary ridges) as having their own origin independent of the sinus venosus [46]. All explanations have in common that the veins are connected to the cardiac tube by way of the dorsal mesocardium to the PHF mesoderm in the dorsal body wall. In the fully developed human heart, this area is demarcated by the epicardial/pericardial fold. The above morphogenesis of the sinus venosus also provides new data on the septation of the atria (Figure 1.6). The primary atrial septum is a structure that initially consists of atrial myocardium, but later becomes fibrous, and is derived from the PHF-derived myocardium. It forms an arch that runs from posterior to anterior and is lined on the inside by cushion-like tissue, called the mesenchymal cap. At this site, also PHF mesoderm, formerly referred to as spina vestibuli but now named the dorsal mesenchymal protrusion (DMP) [47], contributes to atrial and ventricular septation. The DMP provides cells to the inferior atrial septum and borders the mesenchymal cap on the right side. Fusion of the mesenchymal cap with the AV cushions is essential to close the primary atrial foramen.

The PHF mesoderm and also the derived myocardium have characteristic gene patterns that partly differ from the outflow tract. This refers to the transcription factors Tbx18, 20 [48], Shox2 [49], the functional marker HCN4 [50], and the growth factors RhoA [31] and PDGFRα [30,51]. The sinus venosus myocardium is Nkx2.5 negative before incorporation into the dorsal atrial wall and remains as such in the sinoatrial node. Transgenic mouse studies of these genes and some human mutations correlate with abnormalities in PHF-derived structures, including conduction system disturbances.

The primary atrial septum becomes perforated to form the ostium secundum that is never completely closed off by the septum secundum. The complex of the lower rim of the septum secundum and the ostium secundum is called the foramen ovale (Figure 1.6, arrow). The muscular secondary atrial septum is in its basal and dorsal part fused with the DMP. The major anterior and superior parts of the secondary atrial septum are merely a folding of the atrial wall forming the limbus fossa ovalis on the right side of the atrial septum.

Consequences for abnormal development

The above data provide new insights into abnormal pulmonary venous connections and also atrial septal defects (ASDs) and atrioventricular septal defects (AVSDs).

Abnormal pulmonary venous connection

As the plexus for forming the pulmonary veins has extensive connections to the cranial and caudal parts of the cardinal veins [52], persistent connections can lead to supracardiac and infracardiac pulmonary venous connection patterns. For cardiac abnormal pulmonary venous connection, the pulmonary veins do not grow out of the left atrial dorsal wall but

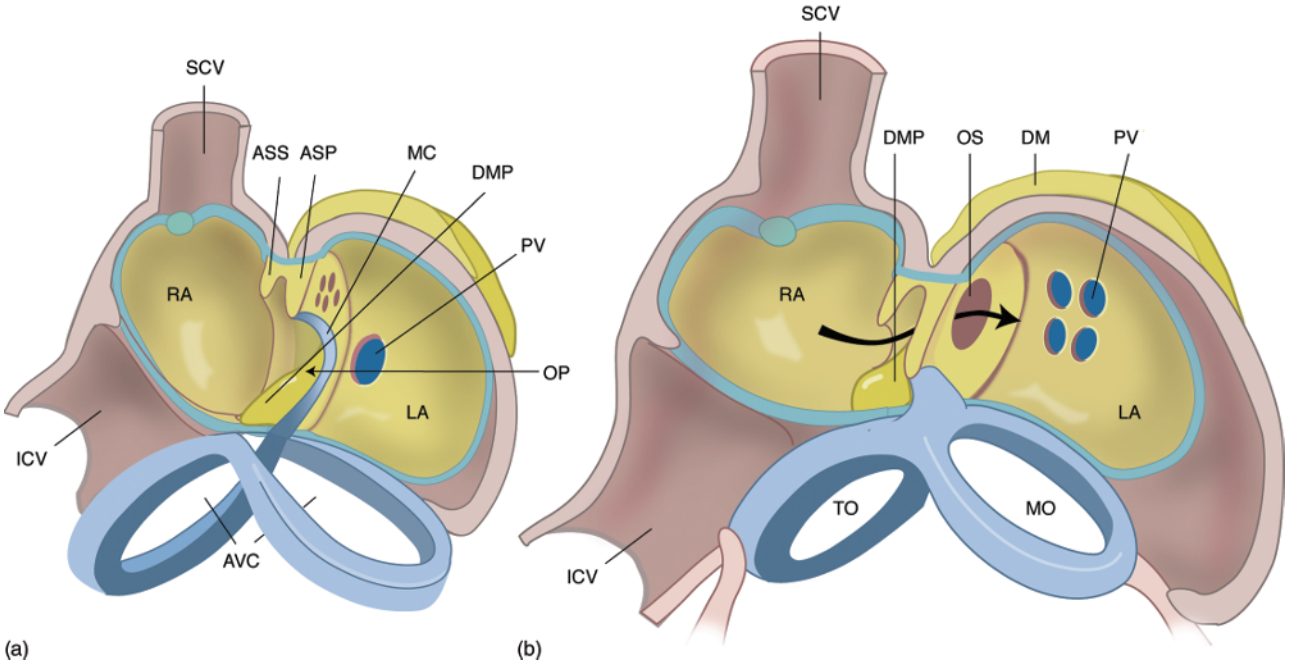

Figure 1.6 (a) Atrial septation starts out with formation of a septum primum (ASP) that grows out from the roof of the common atrium towards the AV canal (AVC). The AV cushions continue over the basal part of the primary atrial septum as the mesenchymal cap (MC). Initially, there is an opening at the basal part of the primary atrium septum, called the ostium primum (OP). Subsequently, probably by a process of apoptosis, several holes will form in the septum primum, that will eventually coalesce to form the ostium secundum [OS, see (b)]. The septum secundum (ASS) will grow out later in development from the roof of the common atrium. In between these structures, at the base, a protrusion of second heart field mesoderm called the dorsal mesenchymal protrusion (DMP) is present. (b) During further development, the ostium primum is closed by fusing the endocardial cushions with the dorsal mesenchymal protrusion. The septum secundum has grown out to form a wedge-shaped septum that during the embryologic and fetal phase will (owing to a higher pressure on the right side) allow the passage of blood towards the left side via the ostium secundum (arrow). The complex of the lower rim of the septum secundum and the ostium secundum is called the foramen ovale (FO) (arrow). After birth, the left atrial pressure rises and the FO will be functionally closed by the primary atrial septum that is being pressed to the septum secundum. The right atrium (RA) receives systemic blood via the superior caval vein (SCV), inferior caval vein (ICV), and coronary sinus (not shown). The left atrium (LA) receives pulmonary venous blood via the pulmonary veins (PV). DM, dorsal mesocardium; TO, tricuspid ostium; MO, mitral ostium. Second heart field-derived myocardium is depicted in yellow. (Copyright Leiden University Medical Center.)

are connected to the left atrial wall through incorporation of the sinus venosus. Disturbance of genes in the PHF can lead to abnormal formation of the wall of the pulmonary veins and the left atrium [53]. Familial total anomalous pulmonary venous connection (TAPVC) has been mapped to chromosome 4p13-q12 in the region near the PDGFRα gene. A knockout mouse of this gene shows TAPVC [51]. Interestingly, the DMP and mesenchymal cap are very hypoplastic in this model, leading to AVSD (see below). A recent review described the current clinical, genetic, and developmental data on pulmonary venous development and abnormalities [54]. Only pulmonary veins connected to the left atrium acquire a myocardial cuff [44]. This cuff is lacking in veins that connect to the right atrium or a spatium pulmonale.

Atrial septal defects

The most common defect is the septum secundum defect (ASD II), in which there is a discrepancy between the septum secundum (demarcated on the right side by the limbus) and the free edge of the fenestrated septum primum. In normal circumstances they overlap as two crescents (Figure 1.6) that fuse after birth. Defective development, including perforations, of the valve of the septum primum, the so-called valve of the foramen ovale, can also lead to an ASD. It is necessary to distinguish between retarded closure of the foramen ovale and a real secundum ASD.

Abnormalities in formation of the base of the atrial septum secundum can lead to so-called sinus venosus ASD, where both the inferior and superior caval veins are closely related to the defect and the pulmonary veins are often abnormally positioned [43].

Based on our new knowledge of addition of the PHF to both the atrial septal components and also the pulmonary veins, some genes are good candidates for study. We already know human mutations in Tbx5 (Holt–Oram syndrome) [55], Nkx2.5 [56], and the PDGFRα region [51] that explain the separate or combined abnormalities in atrial septation, pulmonary venous connection, and in

some patients conduction system problems particularly related to pace-making.

Atrioventricular septal defects

AVSDs are intriguing malformations with many postulated causes, including deficient differentiation of the AV valves and the endocardium lining these valves. This has been extensively studied [57] for the trisomy 21 (Down syndrome) and the syntenic trisomy 16 mouse model without resolution. In the human embryo with AVSD, however, studies of the disposition of the conduction system demonstrated a deficiency of the spina vestibuli (now DMP) and the mesenchymal cap [58], now confirmed in mouse models [47,59]. The primary ASD resulting from non-fusion with the AV cushions can now be explained by the hypoplasia of the mesenchymal cap lining the lower rim of the primary atrial septum. The deficiency of the ventricular inlet septum in humans still needs clarification [60]. The fact that the AV valve tissue in AVSD seems structurally normal confirms that abnormal AV endocardial cushion differentiation is not the primary problem.

Although the heart has two left- and two right-sided chambers, asymmetry is a dominant feature in both form and function. The ventricular asymmetry is determined during looping, whereas the atrial differences are determined by genetic regulation involving, for example, Pitx2 [61,62]. Pitx2 acts in breaking symmetry in early development, is present in the left-sided plate mesoderm only, and has subsequent roles in differentiation of the inflow and outflow segment of the heart. Pitx2 mutant mice present with right atrial isomerism, suggesting inhibition of the left program. Pitx2 mutants may present syndrome-like malformations also involving other organs, for instance the spleen, showing polysplenia in left isomerism and asplenia in right isomerism. Furthermore, DNA sequence variations close to Pitx2 have been described in patients with atrial fibrillation and atrial flutter [63]. Morphologists and clinicians are aware of the differences in the right and left atria, the most obvious being the appendage. Furthermore, the right posterior wall is trabeculated whereas the left is smooth. Usually, atrial situs correlates with bronchial anatomy (see Chapter 50). Lung lobulation, difficult to assess for the clinician, is less reliable.

Ventricular inflow tract septation and the formation of the RV inlet

The RV myocardium with all its components, including at least the right part of the ventricular septum, is derived from the anterior SHF [18]. At the border between the primitive LV and the developing RV, a myocardial ring called the primary ring or fold, previously referred to as bulboventricular fold, can be distinguished [64]. The primary fold is considered a transitional zone and attracts a great deal of attention because it forms the major part of the ventricular inlet and trabecular septum and contains precursors of the AV conduction system.

The primary fold borders on the inner curvature of the heart where it coalesces with the right side of the AV canal (Figure 1.2c). The lower part of the primary fold becomes a real septum by local condensation of the ventricular trabeculae combined with ballooning of the apices of both the LV and RV. Closure of the primary interventricular foramen between the RV and LV takes place by fusion of the inferior and superior atrioventricular cushions in combination with one of the outflow tract endocardial ridges that is connected to this superior cushion.

The role of the primary fold as progenitor of the main body of the ventricular septum deserves special attention. A proper septum is only established when a RV with the tricuspid valve and its orifice is formed. This has to be achieved during development and is important for forming the right ventricular inlet compartment. The right part of the AV canal with the adjoining part of the primary fold has to be transferred to the right side (for remodeling of the primary fold, see Figure 1.2c–e and Videoclip 1.2). Our opinion is that this is achieved by a widening in the dorsal wall of the ventricle adjacent to the primary fold. We have been able to support this developmental concept in a model for Mahaim conduction [65]. With growth of the initial minute inflow part of the RV, a new posterior wall of the right ventricle is formed. In this way, the RV consists eventually of three parts: the RV inlet, bordered by the remnants of the primary fold (trabecula septomarginalis and moderator band), the RV trabecular part (embryonic proximal ventricular outlet segment), and part of the distal ventricular outlet segment underneath the pulmonary orifice.

From an RV view, the ventricular septum is made up of three parts (Figure 1.7):

1 The inlet septum that is formed concurrently with expansion of the RV inflow.

2 This is separated from the trabecular part of the septum by the crista supraventricularis (composed of the continuum of the ventriculo-infundibular fold and the trabecula septomarginalis and also contains the outlet septum; see Figures 1.7 and 1.8), and the moderator band.

3 The muscular outflow tract "septum" or infundibulum derives its myocardium from the distal endocardial cushion-lined outflow tract or conotruncal region (see below).

Consequences for abnormal development

Isolated or multiple muscular VSDs can result from noncompaction of the myocardial trabeculae. Several mouse models present an extensive spongy myocardium that show both myocardial and epicardial differentiation problems as a basis (see below).

Tricuspid valve and orifice abnormalities

Abnormal looping of the heart tube can lead to the tricuspid valve not being optimally brought above the RV, causing a spectrum of tricuspid atresia and hypoplasia, to straddling

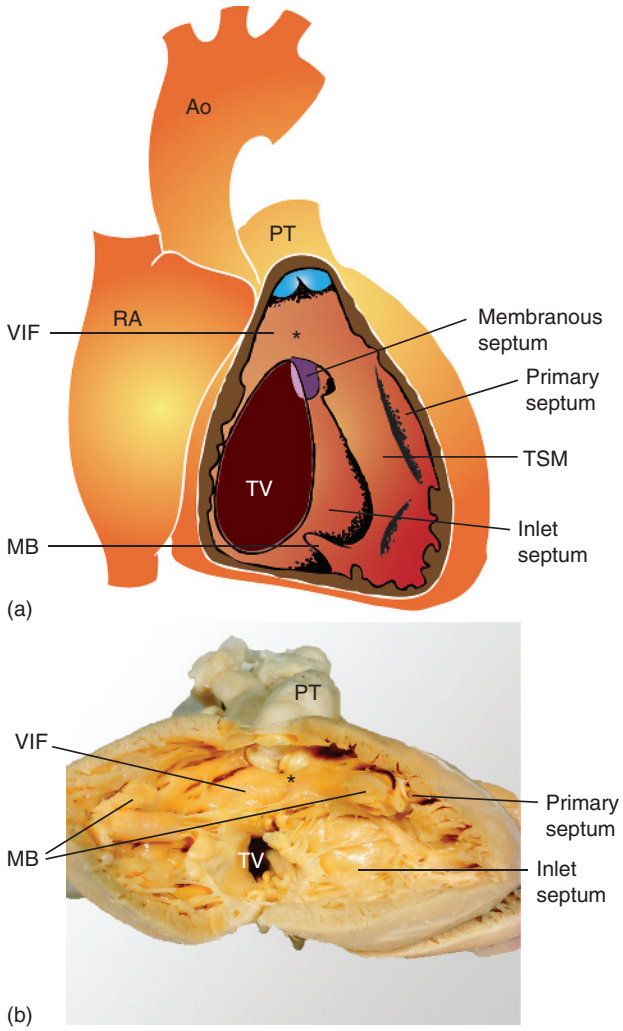

Figure 1.7 Schematic representation (a) and human specimen (b) demonstrating the elements of the ventricular septum after septation has been completed, as viewed from the right side. The ventricular inlet septum is below the tricuspid valve (TV) ostium, and separated from the primitive septum by the myocardial crista supraventricularis. The crista supraventricularis consists of the ventriculo-infundibular fold (VIF), the trabecula septomarginalis (TSM), and the outlet septum (asterisks) that in the normal heart cannot be distinguished as a separate structure. The TSM (that contains the right bundle branch) becomes continuous with the moderator band (MB). The membranous septum is part of the fibrous heart skeleton. Ao, aorta; PT, pulmonary trunk; RA, right atrium. (Copyright Leiden University Medical Center.)

tricuspid valve, and complete double inlet left ventricle. Severe deficient looping can also lead to double outlet right ventricle (DORV). In knockout mouse models with disturbed epicardial differentiation, the abnormalities result in abnormal looping, for example, the mutant RxRα [66], Sp3 [67], Ets1/2 [68], and TGFβ2 [69] mice. It is also possible that primary myocardial problems can cause these abnormalities.

Most perimembranous VSDs and also the outflow tract malalignment defects are the result of abnormal outflow tract septation (see below).

We consider the AVSD anomaly to result from abnormal fusion of the DMP, the mesenchymal cap, and the AV cushion mass [47,58].

Ventricular outflow tract septation

The myocardial contribution of the SHF, referred to as anterior [22] or secondary heart field [70], forms almost the complete RV. The relevance of neural crest cells [35] for outflow tract septation is still important but no longer unique, following studies of the 22q11 deletion syndrome in both patients and mouse models [38]. This complicated syndrome has a high incidence of outflow tract malformations, including aortic arch anomalies, persistent truncus arteriosus, and tetralogy of Fallot. Eventually, the transcription factor Tbx1 was found to be the crucial gene. This gene was not expressed in neural crest cells but in the mesoderm of the SHF. An intricate interaction between neural crest and SHF cells takes place and disturbed genes that are essential for either cell group can lead to outflow tract malformations.

We refer to the septation of the ventricular outflow tract as "separation" as in the normal heart the subpulmonary infundibular or muscular septum is mainly a free-standing sleeve of muscle in front of the vessel wall of the ascending aorta (Figure 1.8). Outflow tract separation has been described for the human embryo [71] and proved similar in animal species such as chick and mouse [64].

Outflow tract separation starts in the embryonic distal outflow tract that is lined by endocardial cushion tissue. This tissue consists of two opposing spiraling ridges. One ridge runs in a laterodorsal direction where it borders the myocardium of the primary fold at the future site of the ventriculo-infundibular fold in the full-grown heart. The other ridge runs ventroanterior to the myocardium of the primary fold as well as the superior atrioventricular cushion. This merging takes place in the bend of the inner curvature of the embryonic heart tube. The endocardial outflow tract ridges are the source of extensive nomenclature confusion. Some authors consider these ridges to consist of proximal or conal ridges (leading after septation to the conal septum) and distal or truncal ridges (leading to a truncal septum). Pexieder [72] clarified this nomenclature confusion. We indicated in a scanning electron micrograph both boundaries and ridges in their full length (Figure 1.8). It is practical to distinguish proximal and distal ridges, which are clearly visible as separate structures in the chicken embryo but are more continuous in humans and rodents (mouse and rat). The proximal ridges mainly form the muscular outflow tract septum whereas the distal ridge area is important for semilunar valve formation and the septation of the arterial orifice level.

Understanding outflow tract separation starts with acknowledging that the arterial orifice level indicated by the mesenchymal (vessel wall) joining the myocardial (outflow tract heart) boundary is not an oval or a circle in one plane but has a three-dimensional saddle shape (Figure 1.9). This

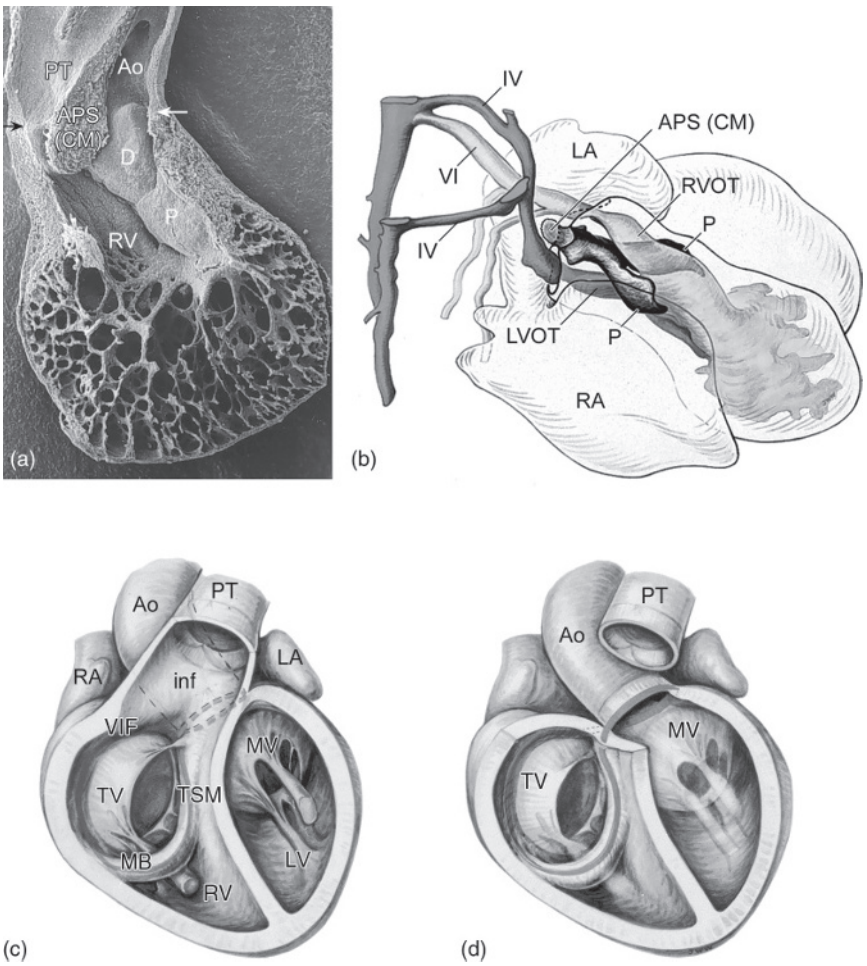

Figure 1.8 (a) Scanning electron micrograph of a preseptation chicken heart showing the proximal (P) and distal (D) outflow tract ridges. The borderline between myocardium (lined on the inside by endocardial cushions) and arterial wall is indicated by arrows. The distal cushions remodel into semilunar valves. (b) Septation of the outflow tract is achieved by fusion of the outflow tract ridges and an ingrowth of condensed mesenchyme (CM), also called the aortopulmonary septum (APS). The APS extends two prongs into the ridges. (c) After septation of the outflow tract, a muscular subpulmonary infundibulum (inf) is formed, which separates the right ventricular outflow tract (RVOT) from the outside world and the aorta (Ao). In a normal heart, the actual outflow tract septum separating the left ventricular outflow tract (LVOT) and RVOT is minimal. (d) Depiction of the difference in length of the RVOT and the relative tilted position of the aortic and pulmonary orifice. LA, left atrium; LV, left ventricle; MB, moderator band; MV, mitral valve; PT, pulmonary trunk; P, prong of CM; RA, right atrium; RV, right ventricle; TSM, trabecula septomarginalis; TV, tricuspid valve; VIF, ventriculoinfundibular fold; IV, VI, pharyngeal arch arteries. (Copyright Leiden University Medical Center.)

brings future aortic orifice more lateral and lower compared with the future pulmonary orifice. During normal looping, this orifice is brought even deeper into the heart, referred to as wedging of the aorta. We recently found (unpublished data) an asymmetric contribution of the SHF to the myocardium of the outflow tract. This Nkx2.5-expressing mesoderm differentiates into myocardium mainly confined to the subpulmonary region, explaining the relative growth of the subpulmonary outflow tract and the rotation to an anterior position with regard to the aortic orifice (see Videoclips 1.3 and 1.4). Molecular biologic experiments using retrospective clonal analysis [73] also showed a difference in subpulmonary and subaortic myocardium but did not link it to the asymmetric addition of SHF. This explains the known asymmetry in the outflow tract [74] for the human embryo. The final result is the well-known difference in position and plane of both arterial orifices.

The highest (most distal part) of the myocardium, always lined on the inside by endocardial cushion tissue, is positioned in the intersection between the sixth and fourth pharyngeal arch arteries. It is exactly at this site that the condensation of extracardiac mesenchyme takes place. The condensed mesenchyme extends two prongs that enter the endocardial cushions together differentiating into the aortopulmonary septum [64] that undergoes myocardialization. Our own chicken chimera studies and also the neural crest indicator

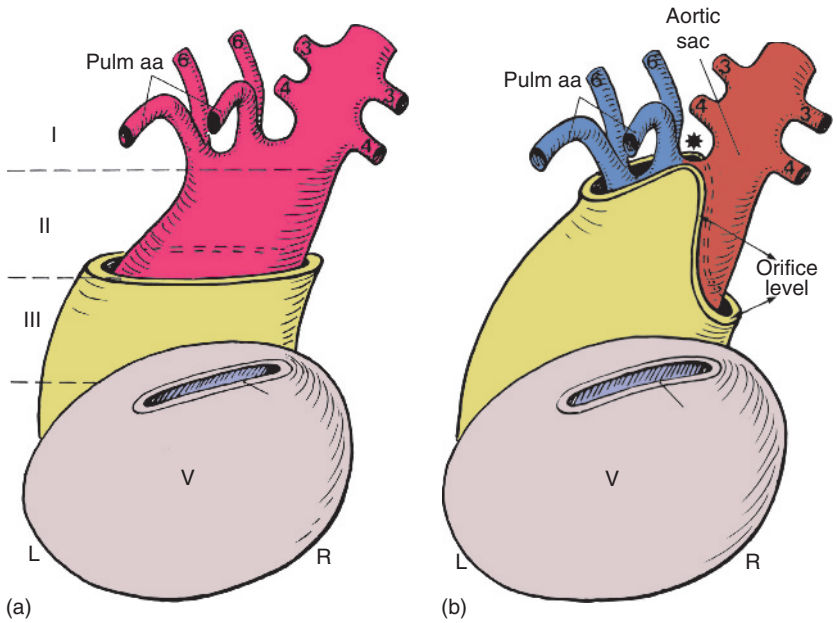

Figure 1.9 Concepts of outflow tract formation. (a) Early concept that assumed that the myocardium surrounding the aorta and pulmonary trunk was distributed symmetrically. Levels I–III indicate the supposed levels of separation, I being the great arteries, II the arterial orifices, and III the outflow tract. (b) In contrast, later work demonstrated that the myocardium in this area actually has a saddle shape, demonstrating the increase in length of the pulmonary trunk in respect to the aorta. The asterisk indicates the level of initiation of separation of the arterial pole. pulm aa, pulmonary arteries; V, ventricle. (Copyright Leiden University Medical Center.)

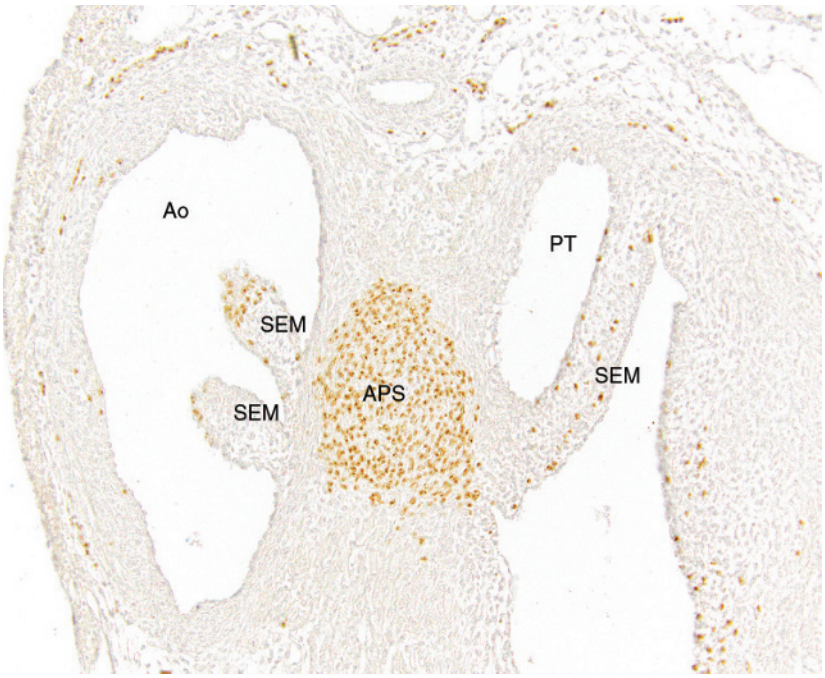

Figure 1.10 Section of the outflow tract of a chicken–quail cardiac neural crest chimera (stage HH 34). The quail cardiac neural crest cells (dark nuclei) fill almost completely the condensed mesenchyme of the aortopulmonary septum (APS). The semilunar valve leaflets (SEM) also show neural crest-derived cells. Ao, aorta; PT, pulmonary trunk. (Copyright Leiden University Medical Center.)

mouse show this condensed mesenchyme to be composed mainly of neural crest cells extending into the distal ridges and the semilunar valves and also the surrounding myocardium [75,76] (Figures 1.5 and 1.10). Staining for apoptosis reveals that most of the neural crest cells at this level have gone into apoptosis, which is particularly prominent during the stage of myocardialization of fused endocardial outflow tract ridges (Figure 1.5b). We postulate that this neural crest cell death program plays an active role in stimulating outflow tract myocardialization. This process

is much less obvious at the distal valve level, where a lump of condensed mesenchyme persists and turns into a fibrous structure (conus tendon).

In completing normal outflow tract separation, the myocardial outflow tract "septum" has merged seamlessly with the ventriculo-infundibular fold and the trabecula septomarginalis. The lower rim borders upon the anterior tricuspid orifice and is called in normal hearts the crista supraventricularis. The final result is an RV outflow tract that is long and surrounded by myocardium whereas the LV outflow tract is short and only partly surrounded by myocardium. In the normal heart, only a small stretch of musculature can really be called a septum between both outflow tracts (Figure 1.8). This situation differs markedly from specimens with disturbed outflow tract septation.

Consequences for malformations

Tetralogy of Fallot, double outlet right ventricle, malalignment defects

The main events in cardiac outflow tract septation are linked to addition of SHF and neural crest cells and their interaction. As a consequence, malformations in outflow tract septation, a major cause for CHD, are linked to disturbances of genes that are crucial for the development of both cell populations. Tbx1 is primarily expressed in the SHF mesenchyme and not in neural crest cells. Hence the phenotype of this malformation is based on a misdirection of neural crest cell contribution based on an abnormal interaction with SHF cells. Patients with solely a Tbx1 mutation have been reported to develop a 22q11 deletion phenotype [38].

van Mierop and Kutsche [77] suggested the possible impact of neural crest in outflow tract septation. Kirby *et al*. [78] were the first to show experimentally the link between outflow tract malformations and the neural crest. They ablated the neural crest in chicken embryos, ending up with a spectrum of outflow tract malformations ranging from a simple VSD to DORV and, in the extreme, a persistent truncus arteriosus (common arterial trunk).

Recent studies have refined this concept, adding specific SHF-expressed genes to the causes. Examples are semaphorin [79], TGFβ [69], and Pax3 [80] mutant mice. We recently showed a role for VEGF isoforms in development of tetralogy of Fallot in a VEGF120/120 mutant mouse [81]. There was selective apoptosis in the subpulmonary myocardium, which we know to have a distinct SHF-derived contribution that differs from the subaortic region.

Epigenetic or environmental factors also play a role, as we showed in embryos from maternal diabetic rats that presented with DORV and a too short outflow tract [82]. For the development of bicuspid aortic valve, some genes in patients have recently been described such as Notch1 [83] and Axin1 linked to endoglin [84]. This draws attention to a possible role of hemodynamics and blood flow that has been neglected. Our studies of the chicken venous clip model showed the importance of alteration of blood flow for the development of a spectrum of outflow tract malformations, including semilunar valve abnormalities [85]. The eNOS (a shear stress responsive gene) mutant mouse showed development of a bicuspid aortic valve [86] after fusion of specific outflow tract endocardial cushions.

Transposition of the great arteries, including DORV with subpulmonary VSD (also termed the Taussig–Bing malformation), remains an enigma. Nakazawa and co-workers [87] showed that retinoic acid treatment of mouse embryos could lead to transposition of the great arteries. There seems to be a link to laterality problems during addition of SHF mesoderm at the outflow tract. In animal models with transposition, the proximal outflow tract ridges had a straighter position. This fits nicely with the morphology of the heart in human infants with transposition in which there is not simply a reverse of the great arteries but also a straighter outflow tract septum [88].

Outflow tract septation is a very vulnerable process. Abnormalities can be evoked by all key players in this area – myocardium, endocardial cushion tissue, and neural crest.

Atrioventricular and semilunar valve formation

Both valve types differentiate from the endocardial cushion tissue lining the AV canal for AV valve formation and the distal outflow tract for semilunar valve formation. The main morphologic difference is that the AV valves are connected to the ventricular wall by chordae and papillary muscles, whereas the semilunar valves do not have a tension apparatus.

The endocardial cushion tissue itself is remodeled into valve leaflets [89,90]. The chordae are also derived from the endocardial cushion tissue and are not differentiated from the papillary muscles (Figure 1.11). The endocardial cushions are formed by contributions of several cell types derived from the endocardium and myocardium in which the process of endothelial to mesenchymal transformation is very important [91]. More recent data show the addition of epicardium-derived cells (EPDCs) to the AV cushion tissue [92] (Figure 1.11). Periostin has been described as an important regulator in AV valve development [93]. Differentiation of the AV valve leaflets has not attracted much attention. Oosthoek *et al*. [89] showed for the chicken, mouse, and the human embryo expression of a number of layered differentiation markers that differ on the atrial and the ventricular side.

Semilunar valve development [94] takes place at the distal part of the outflow tract ridges on the borderline of mesenchyme and myocardium. Reconstructions of the human embryo [64] showed that the orifice level does not lie in one plane but has a saddle shape with the future aortic orifice already more dorsolateral and caudal than the

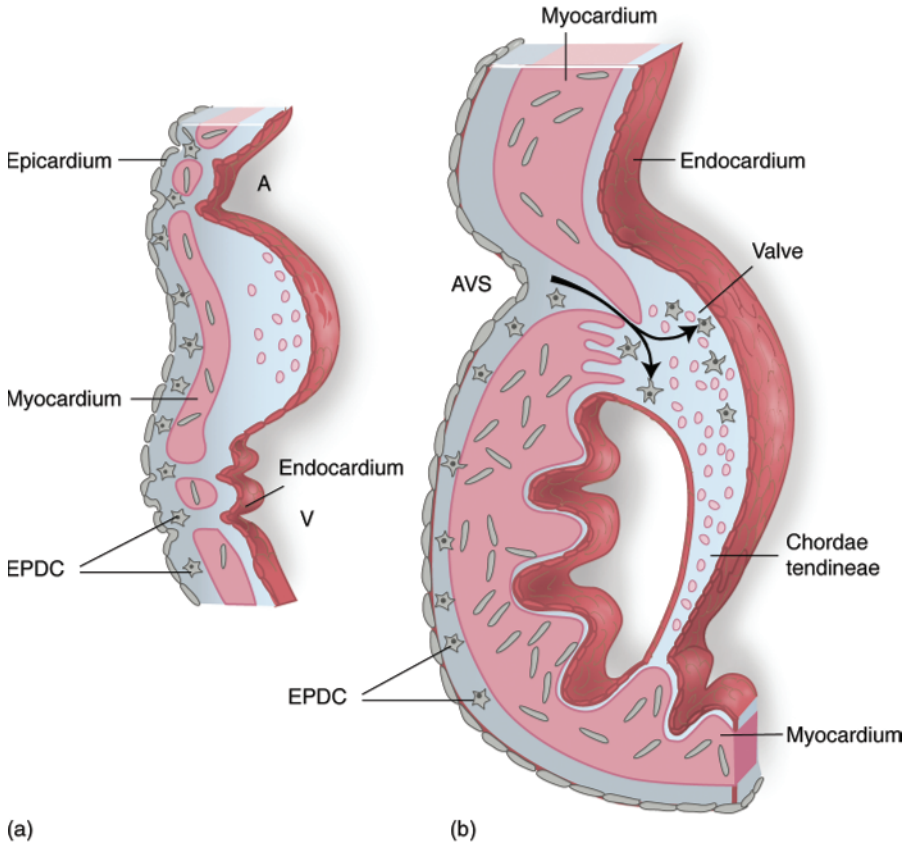

Figure 1.11 (a) The cardiac valves differentiate from the endocardial cushion tissue that is lining the AV canal for AV valve formation. AV valves are connected to the ventricular wall by chordae and papillary muscles. Both valve leaflets and the chordae are derived from endocardial cushion tissues that are remodeled from thick spongy structures into thin valve leaflets. The endocardial cushions are formed by contributions of several cell types derived from the endocardium (depicted as small circles) and myocardium. Epicardium-derived cells (EPDCs), depicted as star-shaped cells) also contribute to AV cushion formation. (b) These are derived from the epicardium and migrate towards the endocardium [arrows] once the myocardial continuity between atrium and ventricle has been disrupted by formation of the annulus fibrosis. Delamination of the valve occurs under the influence of the EPDCs, resulting in detachment of the endocardial cushion from the myocardial wall. A, atrium; AVS, AV sulcus; V, ventricle. (Copyright Leiden University Medical Center.)

pulmonary side. With septation of the outflow tract, the two main endocardial ridges are fused by the condensed mesenchyme of the aortopulmonary septum (Figure 1.8), so that four cushion masses can be distinguished, two in each orifice. To achieve three valve leaflets in each orifice, two intercalated cushion swellings are seen positioned on the far side of the facing valve cushions. In the aorta, the intercalated valve swelling develops into the noncoronary cusp. The two facing semilunar valve sinuses receive the main stems of the right and left coronary artery. For a proper attachment of the semilunar valve leaflets to the underlying myocardium and the above vessel wall, a collagen "ring" is formed [95].

Consequences for abnormal development

Atrioventricular valve abnormalities

Both in human CHD and in experimental animals, information is lacking on abnormal differentiation of the AV valve leaflets. There are reports of tricuspid valve insufficiency during fetal life which can lead to intrauterine death. Furthermore, polyvalvar disease seems to have a genetic background [96]. As the valves are delaminated from the underlying myocardium, ventricular septation abnormalities can also lead to abnormal attachments and formation of the tricuspid and mitral valve; examples are AVSDs, straddling tricuspid and mitral valves. Studies on AV valve differentiation [97,98] provided new information on normal and abnormal papillary formation, especially with regard to the subject of parachute mitral valve in which there is usually asymmetric formation of the mitral valve rather than a real parachute. Abnormal atrioventricular valve leaflets such as seen in Ebstein's malformation are not easily explained from embryology but point towards incorrect undermining from the ventricular myocardium. The animal model using lithium treatment does not resolve the mechanism. Recently, the roles of EPDCs [99] and periostin [93] have been described. Transgenic mice (N-FATc [100] and Sox4 [101]) show specific expression in the endocardial cushion tissue. Knockouts show a spectrum ranging from absence to underdevelopment

of the mitral and tricuspid valves and also of the semilunar valve leaflets. These models are embryolethal and have not been evaluated. We postulate that absence of AV valve leaflets in the human embryo leads to early intrauterine death, as we have not seen that abnormality in humans.

Semilunar valve abnormalities

Several processes described for the AV cushions are also pertinent for the endocardial outflow tract cushions that develop into semilunar valve leaflets, with deficiencies leading to absent or very hypoplastic aortic and pulmonary valves [102]. Absence of aortic leaflets is lethal for the human fetus whereas absence of pulmonary valve leaflets is compatible with term delivery.

Extracardiac cells migrate into the developing valves, as proven for the neural crest cell population [75]. The mechanism underlying the formation of commissures and the sites of formation still need elucidation. A recent publication on the development of the bicuspid aortic valve shows beautifully the difference between a genetic Syrian hamster model and the eNos knockout [103]. Finally, hemodynamic factors [85] may play a role.

Cardiac conduction system (CCS) development

The impulse from the sinoatrial node (SAN) is propagated via Bachman's bundle to the left atrium, and via the internodal myocardium to the atrioventricular node (AVN). Although internodal tracts with specific histologic, immunohistochemical, and molecular characteristics [104–108] can be distinguished in the atria, their functionality is yet to be determined. The AVN is a compact cellular node, covered by transitional cells [109,110]. From this node, the common bundle or bundle of His supplies the ventricles (see Chapter 55).

During development of CCS, the sequence of activation changes from an immature base-to-apex activation pattern of the primary heart tube to a mature apex-to-base activation pattern, in accordance with the development of the His–Purkinje system. Pre-excitation of the ventricles can still occur after septation due to persisting myocardial AV bridges across the AV junction [111]. Insulation of the atrial myocardium from the ventricular myocardium occurs by development of the annulus fibrosis, which begins by fusion of epicardial sulcus tissue with endocardial cushion tissue at the ventricular site of the AV junctional myocardium and moves the original AV myocardium to an atrial position [90]. For this process, correct ingrowth of EPDCs is mandatory [112]. EPDCs produce in the AV annulus the extracellular matrix molecule periostin that is involved in fibrosis of the myocardium of the AV canal [112]. Fibrosis of the annulus however remains incomplete until late fetal stages [111].

In the developing and the adult heart, myocardium of the (putative) CCS can be distinguished from the surrounding working myocardium, based on histologic characteristics, and also by the expression patterns of several immunohistologic and molecular markers [113]. Multiple genes, cells, and their interactions are involved. Furthermore, the developing CCS seems to be much more extensive than in the adult CCS. After looping of the heart has started, several *transitional zones* (Figure 1.2b–e) can be distinguished from the surrounding working myocardium [104,106,107,114]. These transitional zones are the sinoatrial transition comprising the sinus venosus segment connecting to the primitive atrium, the atrioventricular transition or primitive AV canal between the primitive atrium and primitive left ventricle, the primary fold that separates the primitive left ventricle from the primitive right ventricle, and the ventriculoarterial transition at the junction of the primitive right ventricle with the truncus arteriosus or putative outflow tract of the heart (Figure 1.2). The so-called "ring theory" hypothesizes that these transitional zones contribute to elements of the CCS [114].

Due to further looping of the primitive heart tube, the zones meet in the inner curvature of the heart where they contribute to AVN formation. During development, part of this embryonic myocardium differentiates into working myocardium. The transcription factors Tbx2/3 function as transcriptional repressors of this chamber formation process [108,115], whereas Tbx5 is required for maturation and differentiation of the CCS [116]. The latter transcription factor is also involved in molecular pathways for CCS specification, which includes the inhibitor of DNA-binding Id2 [117]. Parts of the zones that do not differentiate into working myocardium form elements of the mature cardiac conduction system (Figure 1.2).

As explained above, the first heart field contributes to the primary heart tube that includes the primitive AV canal. The myocardium at the venous pole of the heart, the sinus venosus, that includes the SAN, is incorporated later to the heart from the PHF [17,24]. This venous myocardium is characterized by the expression of several markers, including podoplanin [24] linked to RhoA [31], Shox2 [49,118], Tbx18 [119], and the functional marker HCN4 [50], and by a lack of expression of the transcription factor Nkx2.5 [24,48].

A transient left-sided SAN precedes the formation of the definitive right-sided SAN [31], making the complete embryonic sinus venosus myocardium a potential pacemaking area.

Whether the SHF also contributes to the elements of the AVN is debatable. Although a contribution from the SHF has been suggested [120], the compact part of the AVN appears to come from the primitive AV canal (first heart field derived), except from the lower part that is derived from the primary fold [121]. However, the origin of the atrial septal component of the AV conduction axis and of the transitional cells still needs to be elucidated. In addition to a direct contribution, an interaction between the tissues from the different transitional zones may also contribute to CCS formation as a

result of induction. Generally, based on histologic studies, the transitional cells are regarded as an atrial or sinus venosus contribution to the AVN [109,110]. The His bundle and bundle branches are most likely derived from the myocardium of the primary fold that undergoes extensive remodeling during development (see Videoclip 1.2). The newly incorporated SHF-derived myocardium forms the right ventricular inflow tract with expansion of the primary fold tissue, after which the medial part of the ventricular septum consists of the trabecula marginalis, containing the right bundle branch, which remains continuous with the moderator band that runs to the lateral right ventricular wall. The peripheral Purkinje fibers develop from differentiating ventricular cardiomyocytes [122] in close association with both the coronary arteries and EPDCs [92,122,123]. In the chick, EPDCs are important for inducing Purkinje fiber formation [124].

Neural crest cells also reach the venous pole of the heart, where they probably play an inducing role in CCS formation and maturation [34,37].

Consequences of abnormal development in relation to arrhythmias

Clinical arrhythmias are related to anatomic predilection sites and many of these ectopic pacemaker foci are preferentially encountered in specific parts of the right and left atrium that are related to the sinus venosus. These include the crista terminalis [125,126], the adult counterpart of the embryonic right venous valve, being related to initiating/perpetuating atrial flutter. The myocardium of the caval veins [127] and coronary sinus [128] are clinically known to initiate arrhythmias. In the left atrium, atrial fibrillation has been attributed to arrhythmogenic foci that originate from the pulmonary veins [129]. The sinus venosus myocardium, which includes the myocardium surrounding the caval and pulmonary veins, is derived from the SHF, and is characterized by specific gene expression patterns including absence of Nkx2.5 expression [24,48]. During development, the sinus venosus myocardium largely differentiates towards a chamber phenotype and acquires expression of Nkx2.5, but loses some of the characteristic early conduction system markers such as Tbx18, CCSLacZ, and Shox2 [113]. Failure of this chamber differentiation, and also re-expression of the embryonic phenotype, may explain the occurrence of clinical arrhythmias originating specifically in these areas.

AV re-entrant tachycardias are based on accessory myocardial bundles connecting atrial and ventricular tissue, thus bypassing the insulating function of the AV groove. The best known is the bundle of Kent, present in Wolff–Parkinson–White (WPW) syndrome [130]. As described above, accessory AV myocardial continuities may persist in the embryo until late stages [111]. Insulation of the annulus fibrosis requires EPDCs. In animal models in which the epicardial outgrowth is inhibited, accessory connections causing ventricular pre-excitation, as in WPW syndrome, are observed. Valvar anomalies such as Ebstein-like malformations due to non-delamination of valves are also part of the phenotype of epicardial deficiency in avian models [99], perhaps explaining the frequent association of Ebstein's anomaly of the tricuspid valve and WPW syndrome [131]. A special form of re-entrant tachycardia is Mahaim tachycardia, during which antidromic re-entrant tachycardia occurs over an accessory bundle with AV node-like conduction properties [65]. Arrhythmias originating in the right ventricular outflow tract can be related to the ventriculoarterial transition and studies on the role of Tbx2 mutations are ongoing [115].

Whether disturbed migration and subsequent apoptosis of neural crest can be linked to human rhythm problems is uncertain. After neural crest ablation in chick embryos, there is a lack of differentiation of a compact lamellar organization by the His bundle and of (electrical) isolation from the working myocardium, and also failure of the conduction system to convert to a mature apex-to-base activation pattern [36]. Genes with differentiation abnormalities of the SHF-derived cardiac conduction system have been described but a link to human malformations has not been made with the exception of Nkx2.5 [56] and Tbx5 [117] mutations. Mutations in both genes cause ASDs and conduction disorders, explained by deficient contributions from the SHF, since both the atrial septum and elements of the CCS are SHF derived.

Development of the epicardium and the coronary vasculature

The epicardial epithelium growing out from the proepicardial organ serves as a covering layer of the myocardium. This epithelium differentiates into epicardium-derived cells (EPDCs) entering the subepicardial space and migrating into the myocardial wall where they differentiate further into the cardiac fibroblasts (also forming the annulus fibrosis) and the smooth muscle cells of the coronary vessels [39,132].

The coronary vasculature is the last part in the developing embryonic heart that is essential for its survival as a beating pump. It provides nutrients to the cardiac wall which cannot survive solely on diffusion from the cardiac lumen. In a normal human embryo at about 6 weeks of development, the coronary arteries contact the two facing semilunar sinuses of the aorta (the right and left sinus of Valsalva) [133,134].

The development of the coronary endothelial network takes place within the confinement of the subepicardial covering of the heart (see Figure 1.12) adjacent to the liver primordium. The microvasculature originates from the sinus venosus endothelial lining where it runs through the liver and enters the subepicardial space [135,136]. Patterning of the main branches of the coronary arteries is largely guided by the underlying AV sulcus (derived from EPDCs) and the position of the interventricular septum, even when abnormally positioned. Development of a peri-truncal microvascular network that remodels into arteries and veins

precedes the differentiation of these main branches [137]. The variation in the main branching pattern at the orifice level seems related to the shortest distance to the area that has to be perfused and might, therefore, depend on hypoxia-dependent gene expression.

The coronary venous drainage is through the large veins accompanying the major coronary arteries and ending in the coronary sinus. A number of small anterior veins enter the anterior part of the right atrium. The development of this system has been followed in both chicken–quail chimeras and transgenic mice. In studying the human embryo, we have been unable to find an extensive Thebesian network connecting the ventricular lumen to the coronary veins. There is evidence, however, of an extensive arteriovenous collateral network. The coronary veins have a myocardial media at their connection to the atria and only more distally is this replaced by a vascular wall containing smooth muscle cells [138].

Consequences for malformations

Cardiomyopathies

The EPDCs that enter the myocardium are essential for the development of the compact myocardial layer. Inhibition of epicardial outgrowth [139] or disturbance of the differentiation of the PHF and epicardium results in a very thin myocardium. This lack of myocardial differentiation could be the origin of cardiomyopathies such as noncompaction cardiomyopathy [39]. The important role of the epicardium for myocardial differentiation has also resulted in the use of EPDCs as cell therapy to ameliorate cardiac function after myocardial infarction [140].

Coronary vascular abnormalities

The coronary arteries grow into the aortic wall, and do not sprout from it (Figure 1.12). Why normally the two facing sinuses of the aorta harbor a coronary artery, leaving the nonfacing (noncoronary cusp) and the pulmonary sinuses empty, remains elusive. We postulate that the unequal contributions of the SHF to the aortic and pulmonary segments of the early aortic sac designates this pattern and depends in part on Tbx1. The interaction with the neural crest is shown after its ablation in the chick embryo. In persistent truncus arteriosus, the coronary arteries enter only the aortic side of the orifice [141]. For humans, this was confirmed in a pathomorphologic study [142].

An explanation for coronary fistulas, more common in the right ventricular wall, combined with diseased coronary arteries is incompletely understood. EPDCs are necessary for correct ingrowth of the coronary arteries into the aorta [68,143] and studies demonstrate transient connections between endocardium and coronary microvasculature through lacunas in the ventricular myocardium. If these connections persist, fistulas could develop. In fetal life, large fistulas are a primary abnormality, leading secondarily to atresia of the pulmonary orifice [144,145]. Hypoplasia of the coronary artery wall and the plasticity of coronary vessels is influenced by growth factors such as VEGF [146] and PDGF [147].

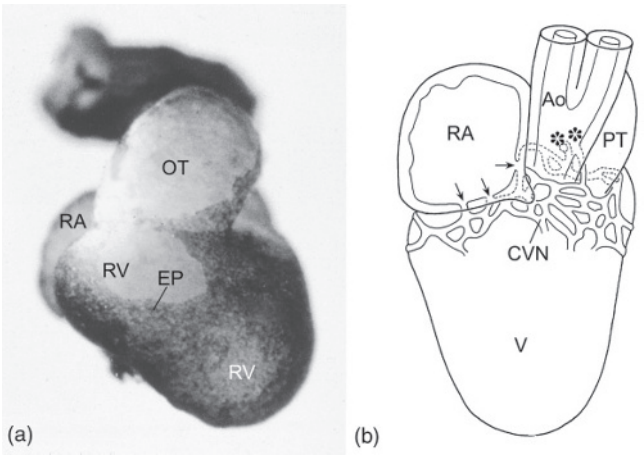

Figure 1.12 (a) Cytokeratin whole mount staining of the epicardium (EP) of a quail heart (stage HH 24). The epicardium grows out from the epicardial organ at the sinus venosus and covers the outer part of the outflow tract (OT) and the right ventricle (RV). (b) Schematic view of the coronary vascular network (CVN) at the peritruncal area underlying the epicardium. This network grows at a quail stage HH 32 into the aorta (two lumenized sprouts, asterisks) and into the anterior part (arrows) of the right atrium (RA). Ao, aorta; PT, pulmonary trunk; V, ventricle. (Copyright Leiden University Medical Center.)

Development of the aortic arch system and pulmonary arteries

The aortic arch and its branches develop from an essentially bilaterally symmetric pharyngeal arch artery system. In the human embryo this is remodeled into a left aortic arch and prenatally a left ductus arteriosus (Figure 1.13). The first and second pairs of arch arteries are transformed into craniofacial arteries whereas the third pair provides the common stem of the carotid arteries. The fourth and sixth pairs (the fifth pair does not appear in most mammals) develop asymmetrically. The left fourth pharyngeal arch artery persists as the aortic arch, whereas the right fourth forms a small segment of the subclavian artery. The sixth pair forms a transient connection on both sides between the pulmonary trunk and the descending aorta. On the right side, the distal part of the sixth pair disappears early, leaving the left one to persist as the ductus arteriosus until birth. In human embryos, the pulmonary arteries might contact the aortic sac directly and are never inserted into the sixth arch artery. The right and left subclavian arteries have to move cranially from the seventh intersegmental level to the aortic arch. During normal development, the early left

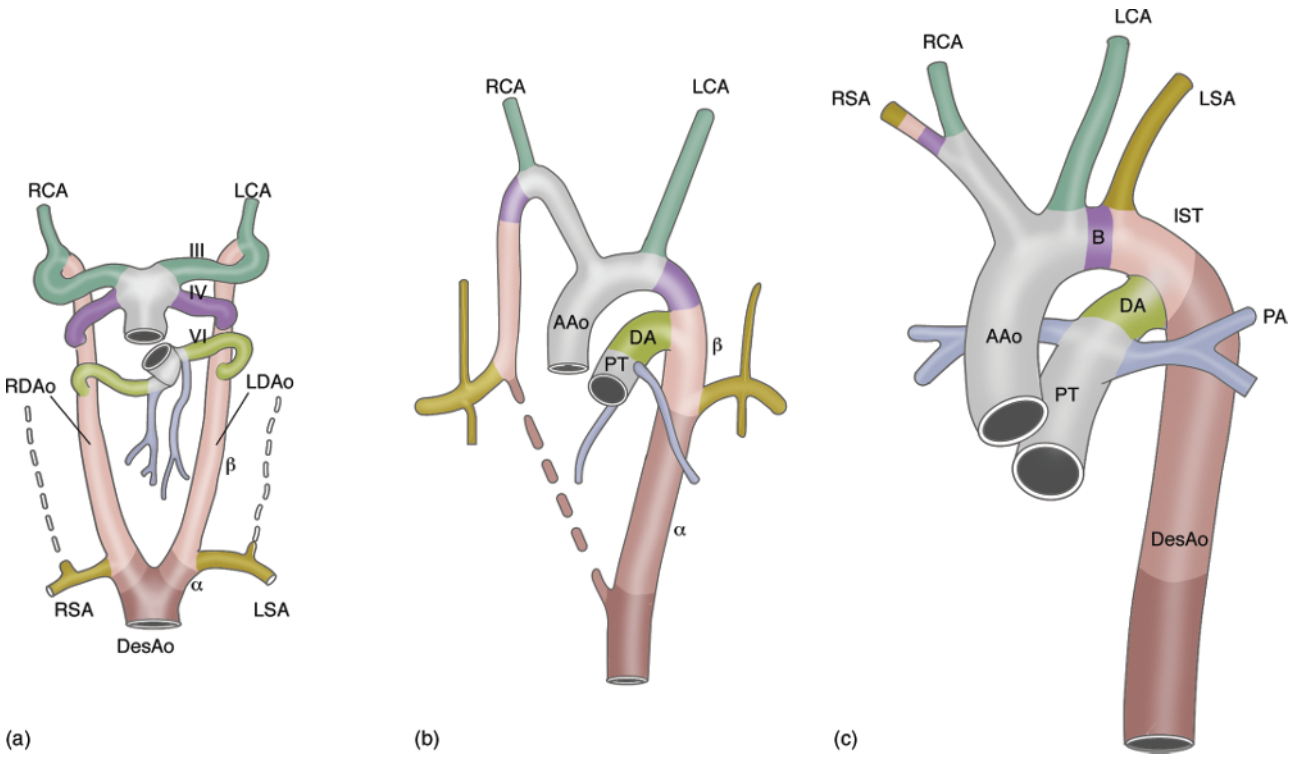

Figure 1.13 (a) Schematic view of the remodeling thoracic arterial vasculature, from an almost symmetric system with a number of pharyngeal arch arteries (depicted are III, IV, and VI) to a left aortic arch. Dashed lines represent regressing vessels. α and β indicate the aortic segments proximal and distal to the left subclavian artery. (b and c) After the left subclavian artery (LSA) has migrated into its proper position the aortic arch has an isthmus (IST) and a segment B (vulnerable in the 22q11 deletion syndrome). AAo, ascending aorta; DA, ductus arteriosus; DESAo, descending aorta; LCA, left carotid artery; LDAo, left dorsal aorta; PA pulmonary artery; PT pulmonary trunk; RCA, right carotid artery; RDAo, right dorsal aorta; RSA, right subclavian artery. (Copyright Leiden University Medical Center).

subclavian artery crosses over the ductus arteriosus entrance, thereby creating the isthmus.

The study of the pharyngeal system in the chicken and mouse shows the significance of several tissues in this region, including the neural crest, the anterior heart field, and even the foregut endoderm. The latter is involved in molecular signaling but not in cellular contributions. The significance of the cardiac neural crest, positioned between the otic placode and the third somite level, is evident as ablations of this rhombencephalic crest in chicken embryos result in abnormalities of the third, fourth, and sixth pharyngeal arch arteries and also the outflow tract of the heart. The neural crest migrates through the circumpharyngeal region and is influenced by signals from the foregut, including the sonic hedgehog and Tbx1 pathways. Next, the neural crest joins the anterior SHF area ventrally to the foregut and makes differentiation of the pharyngeal arches and the enclosed arteries even more complicated. The smooth muscle cells of the root of the ascending aorta and the pulmonary trunk derive from the anterior SHF [23], whereas the medial smooth muscle cells of the arteries come from the cardiac neural crest [148], as is true for the fibroblasts of the adventitia and for the surrounding ganglia. Vessels not populated by neural crest cells include the subclavian arteries, the distal and intrapulmonary arteries, and the coronary arteries. The data from chicken and mouse studies cannot be simply transferred to the human embryo as reliable markers for the various vessels and the outflow tract are lacking. Note that the mouse embryo lacks the human isthmus section located between the left subclavian artery and the entrance of the ductus arteriosus (in later life a ligament).

Consequences for abnormalities

Aortic arch abnormalities

Abnormalities of the aortic arch system, including those encompassed in the 22q11 deletion syndrome (DiGeorge syndrome), show a phenotype that is paramount in certain parts of the fourth arch derived segment and also the ductus arteriosus/left pulmonary artery connection, which is sixth arch derived. The problem is mainly right sided and is probably related to the right-sided dominance of the anterior heart field-related expansion of the pulmonary trunk compared with the aortic root. The disappearance of vessel segments is accompanied by increased local apoptosis programs [149] that are at least partly governed by sonic hedgehog signaling. The postulation that hemodynamics [5,7,8] influence normal arch artery development is gaining

importance, particularly for hypoplasia and coarctation [150]. Shear stress-induced genes include Kruppel-like factor (KLF2), TGFβ and endothelin-1 [151]. The downstream mechanism is complicated, as shown by malformations including DORV, VSD, aortic arch malformations, and abnormal semilunar valves caused by rerouting the venous return in chicken embryos.

The relatively common coarctation of the aorta, localized on the border of the ductus arteriosus and the aortic arch, cannot be explained from a mouse model as this segment is missing. Interruption, hypoplasia, and local coarctation of the isthmus seem best explained by hemodynamic factors related to left ventricular outflow tract obstruction.

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