REVIEW

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Animal models of congenital defects in the ventriculoarterial connection of the heart

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Abstract The embryonic heart functions as a pump without one-way valves. To accomplish this, a long, slowly conducting myocardial structure, the outflow tract, functions as a sphincter at the arterial pole of the heart. During subsequent development tissue remodeling in the outflow tract and immigrating cells of the neural crest are responsible for connecting the right ventricle

with the pulmonary trunk and the left ventricle with the aorta, that is, for the developmental formation of the ventriculoarterial junction. Most congenital malformations of the ventriculoarterial junction stem from disturbances that result in developmental arrest or in abnormal pattern formation (“real” teratology). Abnormal pattern formation can in turn originate from problems with laterality or from aberrant or incomplete formation of structural elements. Genetically modified animals with well-defined gene deficiencies are beginning to provide insight in the signal-transduction pathways and structural elements that are responsible for normal development.

Key words Heart · Embryology · Malformations · Animal models · Genetic manipulation

Abbreviations ASD Atrial septal defect · BAV Bicuspid aortic valve · DORV Double-outlet right ventricle · PDGF Platelet-derived growth factor · TGA Transposition of the great arteries · VSD Ventricular septal defect

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Introduction

Congenital malformations of the heart are the most common birth defects in man, accounting for 0.5–1% of live births, 10% of stillbirths, and possibly up to 20% of spontaneous abortions [1–3]. The majority of congenital heart defects are due to abnormal development of the valves and membranous septa [4]. Thus far, the systematic analysis of these defects has almost entirely rested on findings in human neonates with these structural anomalies. As a consequence little direct information is available about the etiology and pathogenesis of the malformations. In particular it is usually not known to what extent a particular malformation can be traced to a single morphogenetic event or process. Furthermore, since congenital heart disease in man may often have a multifactorial basis, the classical genetic approach, i.e., the identification of mutations that link the primary defect to the

phenotype, will probably be inefficient. However, recent progress in molecular and developmental biology has changed the general approach for the study of cardiovascular diseases profoundly. In particular the technique to genetically modify the expression of genes *in vivo* has already proven to be a powerful approach to identify genes that are involved in the development of specific cardiac malformations. The increasing availability of such genetically modified mouse mutants (for recent reviews see [5–7]) will therefore most likely prove to be a major source of new, molecular insight into the developmental mechanisms underlying normal cardiogenesis (for recent reviews see [8, 9]) and into the etiology of cardiovascular anomalies.

The present review highlights a much overlooked part of the embryonic heart, the outflow tract (Fig. 1). Prior to the development of the semilunar valves, the embryonic outflow tract functions as a sphincter to prevent regurgitation of blood between heart beats. During cardiac septation the outflow tract provides the structural components to form the definitive architecture of the ventriculoarterial connection, that is, the connection of the left ventricle to the aorta and of the right ventricle to the pulmonary trunk. In the formed heart the remaining tissues of the outflow tract are found in the infundibular portion of the right ventricle. We first discuss the normal development of the outflow tract. Next, as a first step toward a molecular understanding of the development of the outflow tract and its congenital pathology, we describe the genetic (animal) models that are presently available. Finally, we discuss the major congenital malformations of the outflow tract and current hypotheses concerning their pathogenesis.

The normal development of the outflow tract

We will follow the development of the outflow tract as it unfolds in mammalian embryos, in particular the human embryo, as we are most familiar with it. However, no difference is made in the description between the findings in mammalian and avian species when, in the view of the authors, development in the two classes of vertebrates is comparable. To compare developmental stages of human embryos with those of frequently used animal models, the atlas of Butler and Juurlink [10] is useful.

Definition

The outflow tract (bulbus, conotruncus; for a review of the nomenclature see [11–13]) is defined as that part of the embryonic heart that connects the embryonic right ventricle with the branchial arch arteries (Fig. 1). Our demarcation of its boundaries is based on external inspection, histochemical staining, and functional analysis of embryonic hearts (e.g., [14–19]) but closely follows that provided by Pexieder [12]. Externally its proximal, ventricular boundary can easily be identified as a furrow

Fig. 1 *Left*, schematic diagram of the embryonic heart illustrating the topographic relations of the inflow tract (1), the atrial segment (2), the atrioventricular canal (3), the left (4a) and right (4b) ventricular segments, the outflow tract (5), and the aortic sac (6), from which the branchial arteries arise. The proximal portion of the outflow tract (connected to the embryonic right ventricle) is also known as conus, while the distal portion (connected to the aortic sac) is also known as truncus. The interventricular foramen is located between the left and right ventricular segments. *Right*, Development of segmental differences in conduction velocity in the embryonic heart (modified from [16]). Rapidly conducting myocardium (white) is found in the developing atria and ventricles only, whereas the slowly conducting myocardium (gray) of the outflow tract, the atrioventricular canal, and, temporarily, the inflow tract flank these segments

on the myocardial surface, whereas its distal, arterial boundary is taken to coincide with the distal boundary of the myocardium (but see “The formation of the semilunar valves”). In human embryos the outflow tract can be recognized by these criteria up to at least the end of the first trimester. Internally the distal boundary is also taken to coincide with the distal boundary of the myocardium. Proximally its boundary can be seen to correspond with those of the endocardial ridges of the outflow tract in young embryos and with the boundary between the developing trabeculae of the embryonic right ventricle and the nontrabeculated, bilayered wall of the outflow tract. In the early fetal period this boundary can be traced to the supraventricular crest, the medial (conal) papillary muscle, and the lower boundary of the smooth-walled infundibulum of the right ventricle.

Early development

The origin of the outflow tract can be traced to the distal portion of the “primary” myocardial heart tube, that is, to that portion of the heart tube that lies downstream the developing ventricular segments [20]. In this portion of the heart tube, cardiac jelly, an initially acellular stuffer substance that is produced by the myocardium, and that is composed of glucosaminoglycans [21, 22], separates the myocardial sheath from the endocardial lining of the lumen. While this description is systematically correct, it must be kept in mind that myocardium continues to be

formed at both the arterial and venous poles of the heart tube for some time after the heart has become recognizable as such [15, 23, 24]. The development of the ascending limb of the heart loop is a prominent feature of Carnegie stage 11 human embryos (approx. 24 days of development), followed in the next few days by its subdivision into the embryonic right ventricle proximally and the outflow tract distally [25, 26]. In the mean time the middle, ventricular segment of the loop "rotates" in such a way that the embryonic right and left ventricles acquire their right-lateral and left-lateral positions, respectively. The resulting configuration of the heart loop is known as the D- or dextroloop of the heart tube. The junction between the preexisting sagittal and the newly formed transverse components of the loop is temporarily identifiable as a characteristic bayonet-shaped bend [26] or "knee" [27] midway in the outflow tract segment at stage 14 (3.5 weeks). This bend is usually taken as the boundary between the proximal (so-called "conus") and the distal (so-called "truncus") portions of the outflow tract. During the second half of the 5th week of development (Carnegie stage 15), differential growth of the right ventricle causes it to attain a more ventral position. Possibly as a result, the outflow tract straightens [23, 25, 26].

The cell origins of the myocardial and endocardial components of the developing outflow tract have recently been reviewed [28] and are not be addressed extensively here. It therefore suffices to mention that the endocardium of the outflow tract derives both from progeny of precursor cells that are shared with the myocardium [24, 29–31] and from the progeny of neural crest derived cells in the pharyngeal arch region [32].

Function and fate of the outflow tract segment

In early embryos the outflow tract is a relatively long structure that comprises most of the ascending limb of the heart tube (see e.g., [25]). Because of the low mitotic activity, particularly in its myocardial portion ([33, 34]; and our own unpublished observations in the rat embryo), the relative size of the outflow tract continually declines during development. As a result its description in a segmental analysis of the (mal)formed neonatal heart is reduced to that of the ventriculoarterial connection [35]. In fact this marked change in size and shape underlies many of the uncertainties that remain with respect to the developmental fate of the structures of the outflow tract. In our view, the following structural and functional aspects of the outflow tract must be taken into account to understand its morphogenetic role in normal and abnormal heart development:

- Prevention of regurgitation of blood in the preseptation heart
- Separation of the left-ventricular (subaortic) and right-ventricular (subpulmonary) arterial outlets
- Formation of the semilunar valves

- Formation of the outlet portion of the muscular ventricular septum

The outflow tract functions as a sphincter in the preseptation heart

As long as the embryonic heart has no properly functioning one-way valves, another mechanism to guard efficient propulsion of the blood is necessary. This is provided by the slow conduction of the depolarizing impulse [16, 18, 19], a functional property that the outflow tract shares with the other persisting portions of the "primary" myocardium, i.e., the atrioventricular canal between the atrial and ventricular segments, and the inflow tract upstream of the atrial segment (Fig. 1). This highly characteristic property, in conjunction with a long-lasting contraction [16] and the abundant presence of endocardial jelly as stuffer material, allows the atrioventricular canal and outflow tract to function as sphincters to prevent regurgitation of the blood prior to the development of the one-way atrioventricular and semilunar valves, respectively [16, 20, 36]. The structural features that confer the ability to function as a sphincter upon the outflow tract, persist as long as the outflow tract remains externally recognizable. This implies that the transition of a sphincteric valve mechanism to a one-way valve mechanism is gradual and prolonged, and that the initially voluminous valve leaflets do not yet have to be very pliable because they simultaneously function as stuffer material.

The formation of the endocardial ridges of the outflow tract

The beginning of the formation of endocardial jelly by the myocardium [22] closely follows the formation of the cardiac tube itself [25, 37]. This temporal linkage makes sense because the peristaltoid contraction pattern of the tubular heart requires the presence of stuffer material to produce occlusion during contraction and hence one-way propulsion of the blood [38]. As long as the embryonic heart is characterized by a peristaltoid contraction pattern (up to Carnegie stage 14, or 4.5 weeks after conception), the jelly forms a complete cuff around the lumen. In such hearts a ventral bloodstream along the greater curvature and a dorsal bloodstream along the lesser curvature of the heart tube can be distinguished in the ventricular and outflow segments [39–41]. The physical basis for the presence of separate blood streams in these as yet unseptated hearts is the low Reynolds number (the ratio of inertial and friction forces in the flow), which has been estimated to be less than 0.2% of that in the adult [42].

Up to stage 15 (5 weeks after conception) the endocardial jelly of the outflow tract encloses an oblong, transversely oriented lumen. The remodeling of the cardiac jelly of the outflow tract into a dextrodorsal [43, 44] or parietal [45], and a sinistroventral or septal ridge be-

Fig. 2 **A** Schematic drawing of the topography of the endocardial ridges in the outflow tract (1) and their connection with the aorticopulmonary septum (3; lower cut surface of 3 is continuous with the upper cut surface of 1). Arrowhead, "bayonet" bend; the portion of the outflow tract that is proximal to the bayonet bend is known as the conus, and that distal to the bend is known as the truncus. *aps*, Aorticopulmonary septum; *iac*, inferior atrioventricular endocardial cushion; *ir*, "intercalated" endocardial ridge; *ivs*, interventricular septum; *la*, left atrium; *lv*, left ventricle; *pr*, parietal endocardial ridge; *ra*, right atrium; *rv*, right ventricle; *sac*, superior atrioventricular endocardial cushion; *sr*, septal endocardial ridge; *III*, third branchial artery; *IV*, fourth branchial artery; *VI*, sixth branchial artery; ***, condensed mesenchyma in endocardial ridge. (Modified from [51]) **B** Schematic drawing of the spiraling course of the endocardial ridges of the outflow tract (light gray, septal ridge; intermediate gray, parietal ridge; dark grey, intercalated ridges). 2, 3, Cross sections are shown just above and below the bayonet bend. Due to the disappearance of the myocardium from the distal portion of the outflow tract the ventriculoarterial junction appears to rotate (compare top of upper segment with top of lower segment in 3). Abbreviations as in A. **C** Schematic diagram depicting the course of the ventrocranial and dorsocaudal channel in the outflow tract of hearts with a D-loop (left) or a L-loop configuration (right). *D/L-loop*, position of embryonic right ventricle relative to embryonic left ventricle in D- and L-loop configuration, respectively; *A*, outlet channel connected to 3rd and 4th arch arteries; *LV*, embryonic left ventricle; *PT*, outlet channel connected to 6th arch arteries; *RV*, embryonic right ventricle

gins at Carnegie stage 14 in human embryos [25, 44] and proceeds from proximal to distal to reach the arterial pole at stage 15 (5 weeks; Fig. 2). However, beyond the "bayonet" bend the lumen remains completely surrounded by endocardial jelly/ridge tissue so that, in addition, two "intercalated" endocardial ridges can be identified in this portion of the outflow tract. At its ventricular end the septal ridge attaches to the right side of the interventricular septum, whereas the parietal ridge continues into the lateral wall of the atrioventricular canal as its right-lateral endocardial cushion. The plane through both endocardial ridges therefore differs from that through the simultaneously developing muscular interventricular septum, thus creating the outline of a conduit which passes from the left ventricle *over* the interventricular septum to the *dorsal* segment of the outflow tract (the future sub-aortic outlet), and one that passes directly from the right ventricle to the *ventral* segment of the outflow tract (the future subpulmonary outlet).

The course of the endocardial ridges is usually described as spiral, in such a way that the parietal (dextro-dorsal) ridge turns ventrally and to the left, whereas the septal (sinistroventral) ridge turns dorsally and to the right. A still unsettled question is whether this spiral course is imposed by the blood stream or develops autonomously (see [39, 40]). Thus it has been suggested [46] that the spiral course results from the fusion at the "bayonet" bend of a proximal and a distal set of endocardial ridges with a mutually perpendicular orientation. However, separate proximal and distal portions of the endocardial ridges have been delineated only in human and canine embryos [46, 47], so that continuous endocardial ridges with a more abrupt change in orientation at the position of the "bayonet" bend is the more plausible explanation. In support of the latter notion it should be mentioned that the spiral course reflects the "rotation" of the outflow tract that results from the formation of the D-loop of the heart tube remarkably well [25], albeit that the D-loop of the heart tube already forms at Carnegie stages 11–12 (3.5 weeks of development), that is, 1 week earlier.

In any case the growth and rightward expansion of the right atrioventricular junction and the right ventricle [46, 48–50], and the development of the septal and parietal endocardial ridges in the outflow tract are thought to be responsible for directing the originally ventral bloodstream from the left atrium via left ventricle to the dorsal channel in the outflow tract and the originally dorsal bloodstream from the right atrium via the right ventricle to the ventral channel in the outflow tract [40, 51]. The establishment of this definitive streaming pattern coincides with the transition from a peristaltoid to a rapid, concentric contraction pattern of the ventricles [16, 18]. This transition in turn is caused by the rapid accumulation of gap junctions in the ventricles and atria [19]. Gap junctions do not accumulate in the outflow tract [19] so that its contraction pattern remains peristaltoid.

The aorticopulmonary septum

The aorticopulmonary septum is a neural crest derived mesenchymal structure that is responsible for establishing separate connections between the subaortic channel of the outflow tract with the 3rd and 4th branchial arch arteries, on the one hand, and the subpulmonary channel of the outflow tract with the 6th branchial arch arteries, on the other (Fig. 2). The aorticopulmonary septum is continuous on the left side with the parietal endocardial ridge and on the right side with the septal ridge. Due to the spiraling course of the endocardial ridges the subaortic channel occupies a dorsal position in the proximal part of the outflow tract and a dextroventral position in its distal part, whereas the subpulmonary channel occupies ventral and sinistrodorsal positions.

Considerable controversy continues to exist on the mechanism by which the outflow tract is septated. The classical concept is that apposition and fusion of the spiraling endocardial ridges from distal to proximal leads to the formation of the aorticopulmonary septum [25, 43, 44], much as the fusion of the endocardial cushions in the atrioventricular canal. However, the endocardial ridges of the outflow tract differ from the endocardial cushions in the atrioventricular canal in that the former become extensively populated by extracardiac mesenchymal cells that can be traced to the branchial arches, and that originate largely from the neural crest [52–54]. In addition, the mesenchyma between the 4th and 6th arches becomes an identifiable structure of condensed mesenchyma that can already be visualized convincingly with antibodies to smooth-muscle actin as the future aorticopulmonary septum ([55, 56]; Ya et al., submitted).

The neural crest derived cells in both endocardial ridges form two pillars of condensed mesenchyma, which become continuous with the mesenchyma between the 4th and 6th arches distally and which are attached to the myocardial wall proximally. During fusion of the ridges both mesenchymal pillars also fuse to form an arch in what can now be recognized as the aorticopulmonary septum (Fig. 2). In the concept of Thompson and

coworkers [50, 55–58], the condensed mesenchyma of the aorticopulmonary septum and pillars form, together with the distal myocardial boundary of the outflow tract, a “septation-complex” that translocates towards the ventricle. Although we subscribe to the general description of the architecture of the septation complex, a major problem of the concept is that it implies that the septation complex, that is, the junction of the outflow tract with great arteries, rotates over 180° [59–61] (see next section).

The formation of the semilunar valves

The semilunar valves are the most distal structures of the formed heart. They become morphologically recognizable as soon as the partitioning of the distal outflow tract is completed (Carnegie stage 16, 5.5 weeks of gestation [43, 44, 60]). The most characteristic feature of the valves is the three-foliate appearance of their cusps. The left and right cusps of both the aortic and pulmonary semilunar valves develop from the fused septal and parietal endocardial ridges, the incisure between them still identifying the outline of each of the ridges. A third cusp develops from each of the intercalated ridges, which are present only beyond the “bayonet” bend of the outflow tract, that is, in its distal (truncal) part. The morphogenesis of the valves from the ridges proceeds by “excavation” on the distal surface [62, 63] but is mechanistically poorly understood.

The topography of the distal end of the formed heart merits discussion. In particular the apparent “rotation” of the junction of the outflow tract with the great arteries [59–61, 64] and the “absorption” or “retraction” of the distal boundary of the myocardial wall of the outflow tract [59, 61, 65] have attracted attention. The rotation issue originates from the fact that the 4th branchial arch lies cranial of the 6th, whereas the pulmonary semilunar valve lies cranial of the aortic semilunar valve. Traditionally it is said that the valve anlagen rotate, and that the absorption of the outflow tract accounts for the compensatory detordation [43, 59, 64, 66–68]. However, when analyzing this riddle, it must be kept in mind that the course of the respective bloodstreams does not change with development [45]. Furthermore, marks on the outflow tract myocardium retain their topographical position during development [23, 69], ruling out a local displacement of cells.

To “rotate” the distal myocardial boundary must therefore move proximally, that is, myocardium must disappear distally. In human embryos the configuration of the distal boundary changes from circular at 4 weeks to saddle-shaped at 5 weeks by the disappearance of myocardium overlying the intercalated endocardial ridges [48, 70]. We have observed in rat embryos that this disappearance of myocardium is preceded by fragmentation of the myosin-staining pattern in these cells. The cells of the intercalated ridges themselves also become intensely desmin positive (Ya et al., submitted). Because

the mitotic activity of the outflow tract myocardium is low compared to the rest of the heart ([33, 57]; Ya et al., submitted), and because no apoptotic activity is observed in this part of the outflow tract ([71–73]; Ya et al., submitted), we assume that the myocardial cells transdifferentiate to contribute to the arterial wall. Myocardial transdifferentiation to fibroblastic cells has been reported at this location in chicken embryos [74], while retroviral labeling of the cardiac neural crest of this species at the 10-somite stage shows that a small collar of the aortic wall immediately above the semilunar valve does not originate from the neural crest ([74a]; R.E. Poelmann and A.C. Gittenberger-De Groot, personal communication). The process of myocardial transformation/remodeling of the distal outflow tract continues well beyond the end of the embryonic period as can be deduced from the observation that (a) the semilunar attachment of the leaflets crosses the ventriculoarterial junction [75], whereas the development of the valves is entirely *within* the muscular confines of the outflow tract; (b) from the observation that the fibrous continuity between aorta and mitral valve develops only *after* the completion of the embryonic period [76]; and (c) from the finding that the origin of the coronary arteries from the aorta is covered with myocardium in the 8th week, whereas this is no longer the case in the formed heart (Lamers et al., submitted; Ya et al., submitted).

Disappearance of the distal myocardium easily explains the apparent rotation of the junction of the outflow tract and the great arteries (Fig. 2): the bloodstreams retain their spiraling course, but the ventriculoarterial junction descends toward the ventricle. The saddle-shaped configuration of the ventriculoarterial junction explains the difference in the plane through the aortic and pulmonary semilunar valves. Furthermore, the myocardial transformation has been found to be most extensive dextrodorsally, that is, near the future aortic valve, explaining the difference in level of the aortic and pulmonary semilunar valves in the formed heart. Finally, we have observed that the aortic and pulmonary semilunar valves, when first identifiable, are at their definitive position (Lamers et al., submitted), implying that they are predetermined to develop just downstream of the “bayonet” bend of the outflow tract (see also [72]), and that all the myocardium downstream of that position transforms into arterial-wall cells.

The formation of the outlet portion of the muscular ventricular septum

Temporally the septation of the distal portion of the outflow tract precedes that of the proximal portion which begins at approx. 6.5 weeks. Because the lower boundary of the ridges still coincides with the transition of the trabeculated portion of the right ventricle into the nontrabeculated myocardial wall of the outflow tract, we do not believe that trabeculation advances distally, as proposed by Pexieder [46]. Soon after fusion is completed, the

cells of the condensed mesenchymal pillars, which have also fused at this level, become pycnotic [50, 71–73], bind annexin V, and contain fragmented genomic DNA (Ya et al., submitted), that is, undergo apoptosis. The appearance of these apoptotic cells is followed by migration of myocytes from the adjacent outflow tract wall into the septum [49, 72, 77]. This process occupies most of the 8th week of development. At the same time the cardiomyocytes of the remaining part of the outflow tract resume their mitotic activity. It has been argued that the myocardial walls of the proximal outflow tract become apposed as a result of the disappearance of the mesenchymal cells and subsequently fuse. Although disappearance of the intervening mesenchymal cells certainly contributes, the invading myocytes have a highly typical, slender phenotype with few, poorly organized myofibrils (Lamers et al., submitted), which distinguishes them from those in the adjacent myocardial wall of the outflow tractum.

The insertion of the lower boundary of the septal endocardial ridge on the right side of the muscular ventricular septum and that of the parietal ridge on the dextrolateral wall of the atrioventricular canal leaves, after the fusion of both ridges, only a hole across the dorsal portion of the interventricular septum to be closed to complete septation. This hole is largely sealed off by the fused superior and inferior endocardial cushions of the atrioventricular canal. We have observed the last trace of the communication between left and right embryonic ventricles in a 7.5-week-old human embryo (Carnegie stage 22) as a tiny channel that ventrally accompanied the right bundle branch of the ventricular conduction system. The origin of this channel was found on the left side just ventral of the fused cushions (future atrioventricular portion of the membranous septum) and on the right side just ventral of the attachment of the inferior endocardial cushion on the muscular septum. The latter remnant of the interventricular communication therefore delineates the right bundle branch as the dorsal boundary of the muscular outlet septum. Perhaps it should be mentioned here that the atrioventricular endocardial cushions do not contribute to the interventricular portion of the membranous septum. This part of the septum develops as a result of the delamination of the septal leaflet of the tricuspid valve from the muscular ventricular septum [78] but reaches ventrally as far as the structure that determines its shape, the inferior atrioventricular cushion (the future septal leaflet of the tricuspid valve), that is, up to the right bundle branch [49]. The right bundle branch is therefore a landmark structure in the right ventricle.

The entire proximal portion of the endocardial ridges of the outflow tract becomes populated with myocardial cells and forms the outlet portion of the muscular ventricular septum [49]. Its right ventricular boundary runs from the atrioventricular portion of the membranous septum via the lower boundary of the medial papillary muscle (under which the right bundle branch is located), includes the supraventricular crest and the anterosuperior leaflet of the tricuspid valve [49], and extends ven-

trally along what is called the septal band. In some hearts one can still see a raphe running from the medial papillary muscle toward the commissure of the left and right cusps of the pulmonary semilunar valve that we believe to be the remnant of the fusion line between the two ridges. The boundary of the outflow tract with the left ventricle is formed by the descending branch of the left coronary artery. In the left ventricle the outflow tract myocardium initially only surrounds the base of the semilunar valves, but most of it disappears as a result of mesenchymal transformation, giving rise to, among others, the well-known fibrous continuity between aorta and mitral valve [76]. In 40% of hearts an anterolateral muscle bundle persists in the left ventricle as remnant of the outflow tract musculature [79]. We have arrived at this description after extensive study of human embryonic hearts that were stained immunohistochemically or visualized by scanning electron microscopy ([49]; Lamers et al., submitted). In particular, the staining pattern of creatine kinase M has proven to be a sensitive marker for outflow tract derived myocardium up to 10 weeks of development. When dissecting an adult heart, the boundary between the muscular interventricular septum s.s. and its outlet portion can still be identified in a cross section by the more compact structure of the latter. Our description concurs with that given earlier by Goor et al. [80].

Congenital malformations of the outflow tract and the ventriculoarterial junction in man

Several overviews of the extant congenital malformations of the outflow tract and the ventriculoarterial junction are available (e.g., [67, 81–84]). The major groups are double-outlet right ventricle (DORV), “infundibular” ventricular septal defects, transposition of the great arteries, common arterial trunk, and tetralogy of Fallot. Attempts at an embryological explanation of the origin of these malformations are almost as old as heart embryology itself [85]. Nevertheless, in the absence of informative animal models many of these morphogenetic views had to remain speculative because of the time elapsing between the putative embryological event underlying the origin of the defect or malformation and its anatomic presentation at birth. Furthermore, the frequently changing views on many of the morphogenetic events in heart development did invoke repetitious adaptations of the hypothetical causes of the syndromes. Moreover, the habit of illustrating one’s findings by modifying preexisting drawings (for an interesting commentary, for example, of the much cited work of Kramer [43] see [25]), or by using overly schematic drawings that are only distantly reminiscent of the actual morphology of the embryonic heart (for an example of the ventriculoarterial connection see [86]) has done little to bridge the gap between embryology and congenital pathology of the heart. As a result pathologists became dissatisfied with embryology [87] and developed a descriptive nomenclature of the

congenital malformations that was unbiased by speculative morphogenetic views [88–90].

Notwithstanding these cautionary notes, considerable progress has been made in understanding the morphogenetic processes that underlie both normal development and that of the malformed heart. Insight obtained from animal models increasingly contributes to these interpretations, because usually only these models can provide specimens at the stage of development that the malformation comes into being. Below we argue that most congenital malformations of the ventriculoarterial junction stem from disturbances that result in developmental arrest or in abnormal pattern formation (“real” teratology). Abnormal pattern formation can in turn originate from problems with laterality (situs inversus, incorrect looping of the heart tube, isomerisms, and discordant alignment of segments) or from aberrant or incomplete formation of structural elements, for example, in neurocristopathies.

Malformations of the ventriculoarterial junction resulting from developmental arrest

Double-outlet right ventricle

DORV appears to be an almost perfect example of a malformation that can be traced to a delay and subsequent cancellation of a normal morphogenetic process, leading to the more or less complete persistence of the embryonic configuration [48, 91–93]. Until the 5th week of develop-

Fig. 3 Schematic diagram depicting the developmental changes in shape of the “primary” interventricular junction between the embryonic left and right ventricles. As a result of the remodeling of the myocardium that accompanies cardiac septation, and that results mainly from growth of the embryonic right ventricle, the originally flat junction transforms into a bent structure with three segments: one surrounding the right atrioventricular connection (1), a second surrounding the connection between the embryonic left ventricle and the subaortic outlet (2), and finally, a third segment that surrounds the remaining portion of the interventricular foramen, and that closes during septation (3). In DORV segment 2 does not or only partially develop. (Modified from [48])

Table 1 Animal models of outflow tract development

Name	Gene	Species	Mutation	Expression pattern	Cardiac phenotype	Other defects	Ref.
–	–	Syrian hamster	Spontaneous recessive	?	Bicuspid aortic valve; abnormal origin coronary arteries	–	[112]
–	Hox-A3 (=1.5)	Mouse	Targeted disruption; recessive	Brain; neural crest derivatives	Aortic and pulmonary valve malformation	Persistent patent ductus arteriosus; branchial arch artery anomalies; defective pharyngeal pouch derivatives	[147]
–	Retinoic acid receptor (RAR/RXR)	Mouse	Targeted disruption	Newborn	RXR α : hypoplasia of ventricular wall; typical cardiac vitamin A-deficiency syndrome (common trunk and branchial arch anomalies) only in RAR α plus RAR β , RAR γ , or RXR α double mutants	Double mutants: eye, glands, skeleton, pharyngeal pouch derivatives	[109, 110, 135]
Splootch Pax3 (Sp ^{1H})		Mouse	Radiation-induced; recessive	?	Common trunk; shape semilunar valve leaflets “normal,” shape, and fusion AV cushions normal	Lumbosacral rhachischizis; reduced number of spinal ganglia (caudally); frequent absence pharyngeal pouches	[111]
–	Endothelin-1 (Edn1)	Mouse	Targeted disruption + antibodies; recessive	Endothelium branchial arch arteries + OFT ridges	VSD; DORV	Branchial arch artery anomalies; coarctation; Et-1 is partially supplied via placenta	[120]
–	Sox4	Mouse	Targeted disruption; recessive	14.5	Common trunk; no formation of semilunar valve leaflets; AV cushion normal	Branchial arch artery anomalies	[107] ^a
–	Neurotrophin 3 (Ntf3)	Mouse	Targeted disruption; recessive	NB ^b	ASD; VSD; common trunk; DORV; pulmonary stenosis; thickened valve leaflets	Sensory and sympathetic neurons reduced; premature closure of arterial duct	[128]
Neuro-fibro-matosis (Nf1)		Mouse	Targeted disruption; recessive	14.5	VSD; common trunk; AV cushions fuse; but not populated by fibroblasts; semilunar valve anlagen present	hyperplasia of sympathetic ganglia; small eyes	[131]
Patch (Ph)	α -PDGFR receptor	Mouse	Spontaneous; recessive	15	ASD; VSD; tricuspid atresia; common trunk; DORV; valve abnormalities; abnormal origin of coronary arteries	Branchial arch artery anomalies; cleft face; ectopia cordis; hemorrhaging	[132]
–	–	Dog (Keshond)	Spontaneous recessive	Viable	Conotruncal hypoplasia > infundibular VSD; if large; with pulmonary stenosis (Fallot)		[133, 148]
–	–	Chicken	Removal cardiac neural crest at HH stage 8–11		DORV; Fallot; coronary artery anomalies; VSD; common trunk; TGA; tricuspid atresia; double inlet left ventricle	Branchial arch artery anomalies; ectopia cordis; reduced or absent pharyngeal pouch derivatives	[127, 129]
–	Connexin43	Mouse	Targeted disruption	NB	Abnormal looping (persisting A-loop); criss-cross heart; abn. delamination anterosuperior leaflet tricuspid valve		[138] ^a
iv/iv	–	Mouse	Spontaneous	NB	Abnormal looping (50% L-loop); common SV; A; AVC; subaortic VSD; DORV; TGA; Fallot	Situs inversus	[137]

^a Ya et al., submitted^b Probably supplemented maternally

ment blood from the embryonic left ventricle must pass through the interventricular foramen and the embryonic right ventricle to reach the outflow tract (Fig. 1). Because the myocytes surrounding the interventricular foramen contain sulfate-3-glucuronyl residue carrying glycolipids, they can be identified immunohistochemically [94]. We have demonstrated that, as a result of the differential growth of the embryonic right ventricle and the ensuing myocardial remodeling, the originally circular interventricular foramen becomes divided into three separable segments (Fig. 3): (a) that surrounding the passage from the embryonic left ventricle to the outflow tract, (b) that surrounding the right atrioventricular passage, and (c) that surrounding the remaining part of the interventricular foramen (now called foramen II) [48]. Dextroposed ("overriding") aorta and DORV can be traced to varying degrees of insufficient growth of the myocardium surrounding the segment of the interventricular foramen that forms the passage from the embryonic left ventricle to the outflow tract, that is, to a developmental arrest in morphogenesis. It is therefore not surprising that dextroposed aorta and DORV are frequently found together with other cardiac malformations (see also Table 1).

Infundibular ventricular septal defects

Ventricular septal defects (VSDs) that can be traced to lack of fusion of the endocardial ridges of the outflow tract or, for that matter, to the endocardial cushions of the atrioventricular canal (for classifications see [84, 95, 96]), are by definition examples of developmental arrest. However, it must be kept in mind that a sizable portion of them arise as a hemodynamic shunt, which prevents fusion to occur at the predetermined stage of development. After this committed stage has passed, fusion appears to be no longer possible. Infundibular VSDs include both the distally located subarterial defects and the more proximal perimembranous defects (distal to the medial papillary muscle). Aorticopulmonary window can probably be considered as a lack of fusion of the distal (supravulvar) portion of the ridges and may as such also belong to this group [97]. In accordance with this hypothesis, its presentation is usually not associated with the neural crest syndrome (see "common trunk").

Malformations of the ventriculoarterial junction resulting from abnormal pattern formation

Transposition of the great arteries

In transposition of the great arteries (TGA), the aorta is connected to the morphologically right ventricle, whereas the pulmonary trunk is connected to the morphologically left ventricle. Two conditions are possible, namely that pulmonary venous blood has access to the 3rd and 4th branchial arch arteries (congenitally corrected transposition), or that it has access to the 6th branchial arch

arteries (complete transposition). Complete TGA usually results from the presence of a ventriculoarterial discordance, whereas congenitally corrected TGA requires a combination of an atrioventricular and a ventriculoarterial discordance.

Congenitally corrected transposition is thought to develop if the atrioventricular junction "rotates" to the left instead of to the right (forming an L- rather than the normal D-loop). In such hearts the atria occupy their expected topographic position, but the morphologically left ventricle becomes situated on the right side and the morphologically right ventricle on the left side (atrioventricular discordance). Since only the direction of looping is reversed, the topographic relations at the junction of the outflow tract with the branchial arches do not change. Even though the morphologically right ventricle retains its access to the ventrocranial channel in the outflow tract and the morphologically left ventricle that to the dorsocaudal channel, the morphologically right ventricle is connected to the 3rd and 4th branchial arch arteries and the morphologically left ventricle to the 6th arch arteries, because the spiraling course of the endocardial ridges is absent in the L-loop configuration (Fig. 2C). It should furthermore be noted that, because of the reverse looping, ventricular structures with a caudal position in D-looped hearts become cranial structures in L-looped hearts. Thus, the ventricular conduction system (atrioventricular node and bundle) occupies a cranial position in hearts with a L-loop configuration [98].

It is not known what determines the spiraling course of the endocardial ridges in the outflow tract. In Sox4 deficiency, an animal model of hearts with a D-loop configuration in which the neural crest cells migrate along an abnormal route into and through the endocardial tissue of the outflow tract, we have found both ventrocranial origin of the pulmonary trunk (normal spiraling course of ridges; pulmonary trunk crosses aorta) and dorsocaudal origin (abnormal straight course of ridges; pulmonary trunk does not cross aorta; Ya et al., submitted). In other animal models the same straight course and even an inversion of the spiraling course of both ridges has been reported [99]. The deviant course of both ridges leads primarily to a ventral (leftward) displacement of the base of the parietal endocardial ridge ([99]; Ya et al., submitted). As a result, the infundibular outlet septum "rotates" leftward on the ventricular septum, so that the right ventricle gains preferential access to the caudal (aortic) channel, and the left ventricle to the cranial (pulmonary) channel. This description in the embryo fits that most frequently observed in human neonates: situs solitus with a malaligned infundibular (outlet) septum and the aorta in a right-sided ventral position relative to the pulmonary trunk [67, 81].

Common trunk

Common trunk is certainly an ill-defined outflow tract malformation. Some opt for a purely descriptive defini-

tion and use the term common trunk when a "single arterial trunk that leaves the heart by way of a single arterial valve and gives rise directly to the coronary, systemic and one or both pulmonary arteries" [82, 100]. Others take a more ontogenetic view and define common trunk as the most severe form of a "trunco-conal septal defect" [97], while still others have been intrigued by its similarity with Fallot's tetralogy and claim that pulmonary atresia plus partial or complete absence of the aortopulmonary septum are its landmark features [101, 102].

We know of only two descriptions of common trunk in embryos [103, 104]. For this reason the morphogenetic events underlying the development of common trunk are mostly based on deductions from observations in human neonates. The classical embryological interpretation of common trunk is a complete or partial failure of downgrowth of the aortopulmonary septum, together with incomplete development of the endocardial ridges of the outflow tract (e.g., [82, 97, 100, 103, 105]). Some consider the abnormal development of the ridges to be secondary to faulty development of the aortopulmonary septum (e.g., [82]). The extensive population of the ridges of the outflow tract by neural crest cells (see "the aortopulmonary septum"), the prevalence of common trunk in animal models in which genes that are expressed in neural-crest derivatives are disrupted, and the lack of such concordance between deficiencies of the outflow tract and the atrioventricular canal in these models (Table 1), certainly support this view.

However, the view that the infra-valvular defects can be traced to faulty development of the proximal (conal) portions of the endocardial ridges and the supra-valvular (aortopulmonary) defects to faulty development of the distal (truncal) portions of the ridges [105] can also muster some support. Firstly, as a result of the local transdifferentiation of myocardium into aortic wall cells (see "The formation of the semilunar valves") the distal portion of the ridges comes to lie at and distal to the semilunar valves. Thus defective development and/or fusion of the distal ridges can account for aortopulmonary window topographically. Furthermore, although DiGeorge syndrome, which is thought to be due to an abnormal development of the neural crest, is associated with a high incidence of common trunk, association with aortopulmonary septal defect or anomalous origin of the pulmonary artery from the ascending aorta is rare [106].

Our own view on the developmental origin of common trunk is based on a study of the hearts of Sox4-disrupted embryos [107; Table 1] that we recently completed (Ya et al., submitted). Sox4-deficient hearts suffer from a defective transformation of the endocardial ridges into semilunar valves and from a lack of fusion of these ridges. The spectrum of outflow tract malformations that we observed, ranged from two pulmonary artery orifices in the dorsal truncal wall (type 2 [108]/type A2 [101]/type I [97]), via a single pulmonary ostium in the left lateral truncal wall (type 1 [108]/type A1 [101]/type II [97]) to a defect in the proximal (conal) portion of the outlet septum, that is, with two valve anlagen (type IV

[97]). Pulmonary artery underdevelopment was correlated with absence of the arterial duct. We did not observe isolated supra-valvular defects (aortopulmonary window, type III [97]). Sox4 deficiency was, in addition, characterized by an abnormal number and position of the intercalated ridges relative to the parietal and septal ridges, by a configuration of the ridges typical for transposition of the great arteries, and by variable size of the aorta relative to the pulmonary trunk. The position of the septal and parietal ridges could be established unambiguously by determining the position of the typical mesenchymal condensations in the endocardial ridges. An abnormal position of the parietal and septal ridges probably causes a correspondingly abnormal position of the aortopulmonary septum. Underdevelopment of the pulmonary or, more rarely, the aortic portion of the outflow tract was associated with local absence of an intercalated ridge. In our only common trunk with a single ascending vessel, the typical rod(s) of condensed mesenchyma were not identified.

Common trunk in Sox4-deficient mice originates from a deficiency of the endocardial tissue. The associated lack of differentiation of the semilunar valves is not seen in common trunk caused by the dysfunction of neural crest cells (e.g., resulting from deficiency of nuclear retinoic acid receptors [109, 110] or the Pax3 transcription factor [111]). This deduction in turn implies that the neural crest does not play a prominent role in semilunar valve development, as is indeed also suggested by the isolated presence of bicuspid aortic valve in a strain of Syrian hamsters [112].

Together these findings indicate that common trunk results from underdevelopment of the future pulmonary or aortic portion of the outflow tract and that the abnormal position of the mesenchymal condensations in the developing ridges reflects the aberrant route along which neural crest derived myofibroblasts populate the endocardial tissue of the embryonic outflow tract, which in turn eventually leads to malalignment of the outlet septum. Our study therefore unexpectedly revealed that the structural changes in the hearts of these embryos correspond closely to those described by Van Praagh and Van Praagh over 20 years ago [101, 113]. In particular, they suggest that tetralogy of Fallot, which in essence is an underdevelopment of the subpulmonary infundibulum [114, 115], is indeed a "first cousin" of common trunk [102]. Common arterial trunk is a typical feature of DiGeorge syndrome [106], which is most often associated with (micro)deletions of chromosome 22q11. The hypothesis of a common link in the signal-transduction pathway leading to common trunk and to tetralogy has been strengthened substantially by the recent finding that cases of familial tetralogy also map to chromosome 22q11 [116, 117].

Animal models of congenital defects in the outflow tract of the heart

Although the spatiotemporal expression pattern of many genes in the developing heart has been mapped (for recent reviews see [7, 12]), only few human gene defects that cause malformations of the ventriculoarterial junction have been identified. Supravalvular aortic stenosis, a pediatric vasculopathy that is caused by a disruption of the elastin gene [118], coincides topographically with the distal boundary of the outflow tract. Conceivably the transdifferentiation of the myocardial wall into the (elastic) wall of the aorta does not proceed normally, leading to the development of the local stenosis. Disruption of the fibrillin gene causes Marfan's syndrome [119], a generalized connective tissue disease with prominent clinical manifestations of the aortic root. To gain a broader understanding of congenital cardiac pathology the development of adequate animal models for inheritable human malformations remains therefore essential. Firstly, only if animal models are available can a reliable series of temporally consecutive stages of development be analyzed morphologically and/or functionally. This becomes even more important if disruption of a gene is lethal at a relatively early developmental stage, or if the product of the disrupted gene can be provided to the fetus to a variable extent via the placenta (see e.g., [120]). Furthermore, it is not always a priori clear to what extent data on signal transduction or the regulation of gene expression that are obtained in vitro can be extrapolated to truly three-dimensional processes such as morphogenesis.

Table 1 shows the currently known malformations of the outflow tract that are due to single, or in the case of nuclear retinoic acid receptors double, gene defects. We did not include trisomies because (cardiac) malformations associated with chromosomal anomalies can probably be traced to multiple genes. Analysis of this table reveals that in no case thus far has the outflow tract myocardium itself been specifically involved, probably because malfunction of the "primary" myocardium would have led to very early embryonic death. Furthermore, a remarkable spectrum of malformations in each of the horizontal rows is seen, although only a single gene is affected. This broad phenotypic presentation of the disruption of a gene is probably characteristic for genes that are either links in a signal-transduction pathway [endothelin-1, neurotrophin3, neurofibromin, α -platelet-derived growth factor (PDGF) receptor, connexin43], or transcription factors (Hox-A3, Sox4). Most likely the downstream members of these signal transduction pathways or the target genes of the transcription factors continue to be active to a variable extent, but as a consequence of a lack of regulation these downstream activities are no longer (tightly) controlled, thus allowing the emergence of large interindividual differences in phenotype. Since the downstream genes that interfere with normal development remain to be identified in virtually all cases, it seems best, in the context of the present review,

to order the syndromes according to the topography of symptoms.

Malformations that are confined to the semilunar valves

The bicuspid aortic valve (BAV) syndrome in the Syrian hamster is associated in 45–50% of the cases with coronary artery anomalies (V. Sans-Coma, personal communication) but not with other malformations. Human BAV, the most frequent congenital cardiac anomaly (0.4–1% in autopsies [121]), is often familial [122, 123]. Human BAV has also been found in association with an anomalous origin of (one of the) coronary arteries [124, 125], and with coarctation of the aorta, in particular in patients with Turner's syndrome [126].

Malformations that affect the entire outflow tract

The combination of cardiac defects with malformations of structures originating from the branchial arches clearly suggests involvement of the neural crest in the genesis of the syndromes observed after inactivation of the transcription factors RAR [109], RXR [110], Hox-A3 [127], Sox-4 [107], and Pax3 [111] and of the peptide hormones endothelin-1 [120] and neurotrophin3 [128]. For comparison, we have therefore included the syndrome observed after *surgical* removal of the cardiac neural crest (localized between the otic placode and somite 3) [127, 129]. A remarkable feature of these syndromes resembling neural crest ablation is that, with the exception of the nuclear retinoic acid receptors and neurotrophin3 deficiency, they are topographically confined to the outflow tract portion of the heart. In fact it is probably the neural crest contribution that is responsible for the observed differences in the behavior of the endocardial ridges of the outflow tract and the endocardial cushions in the atrioventricular canal. Of this group, deficiency of the homeobox gene Hox-A3 preferentially affects more cranial structures (semilunar valves), whereas deficiency of neurotrophin3 also affects more caudal structures such as the atrial septum. The presence of atrial septal defects (ASDs) in neural crest associated malformations probably relates to neural crest cells populating the so-called spina vestibuli [130], a band of extracardiac mesenchyma entering the heart at the venous pole.

Disruption of the neurofibromin gene [131] which in man causes neurofibromatosis, the α PDGF-receptor gene [132], and the gene responsible for the cardiac malformations in the Keeshond [133], affects the development of all endocardial cushion/ridge derived structures in the heart (Table 1). Despite this phenotypic similarity the expression pattern of the deficient genes is quite different: α PDGF-receptor gene is almost expressed exclusively in the endocardial tissues [132], whereas the type II transcript of the neurofibromin1 gene is expressed homogeneously in the myocardium but at mouse embryonic day 12 only [134]. The consequences of inactivation

of the gene responsible for the cardiac defects in Keshondon appear to be confined to more distal (cranial) endocardial structures than those due to disruption of the α PDGF receptor.

Disruption of a gene encoding one of the nuclear retinoic acid receptors demonstrates the high degree of functional redundancy that often exists among members of a family of genes. Thus deficiency for either the RAR α , RAR β , or RAR γ type of retinoic acid receptor does not lead to the cardiac malformations that accompany vitamin A deficiency [109]. Although deficiency for RXR α , a member of the other family of nuclear retinoic acid receptors, confers some predisposition to the vitamin A deficiency syndrome [110, 135], its characteristic cardiac features, persistent truncus arteriosus, and branchial arch anomalies are seen frequently only in animals with combined deficiencies (RAR α plus either RAR β , RAR γ , or RXR α) [109, 110]. Interestingly, the endocardial ridges look unaffected in early embryos and, as in the case of Pax3-deficient "Splootch" mice [111], valve leaflets do develop. This observation suggests that in those persistent truncus arteriosus cases in which the primary deficiency is in the neural crest, valve leaflet development is hardly affected, whereas in those cases in which the primary deficiency is in the endocardial ridges, such as in Sox4-deficiency, it is. In man, semilunar valve leaflets are present in common trunk [82, 104, 136], suggesting that it usually stems from a neurocristopathy.

Malformations resulting from an abnormal looping of the cardiac tube

Both disruption of the gene responsible for situs inversus [137] and the connexin43 gene [138] cause abnormalities in the looping of the cardiac tube. In both cases the initial, ventrally oriented growth of the cardiac loop proceeds normally, but the subsequent component in the transverse plane either randomly forms rightward (normal D-loop) and leftward (L-loop; iv/iv syndrome), or develops only after a 2- to 3-day delay (connexin43 deficiency). If the rightward bend of the cardiac loop does not form, that is, if the right ventricle retains a ventral position, a so-called "criss-cross" configuration [139, 140] develops in connexin43 deficient embryos: the atrioventricular cushions are rotated 90°, the position of the ventricular septum is horizontal, and the endocardial ridges in the outflow tract follow a parallel instead of a spiral course, as in transposition. The abnormal delamination of the anterosuperior leaflet of the tricuspid valve from the myocardialized outlet septum (see [49]) that dominates the presentation of connexin43 deficiency at birth, and that is responsible for the neonatally lethal obstruction of the pulmonary outlet [138] appears to be a secondary effect of the kinking of the outflow tract at its junction with the ventrally positioned right ventricle (Ya et al., submitted).

The iv/iv syndrome is characterized by the simultaneous presence of abnormalities in the proper configuration of the cardiac loop (random development of either

D- or L-loop [67, 137]) and in the proper manifestation of laterality (presence of atrial and pulmonary isomerisms [141]). The association of the inactivation of the connexin43 gene with impaired cardiac looping is therefore particularly intriguing, since mutations in this gene have been linked to laterality problems (visceroatrial heterotaxia) in human neonates [142], although connexin43 is certainly not the only gene involved [143]. These observations show that abnormal cardiac looping should be considered a laterality problem.

Loss of function mutation of the homeobox gene Nkx2-5 impairs formation of the cardiac loop [144], but is not considered here because its early death precludes establishing its influence in outflow tract development.

Prospects

It is to be anticipated that the number of genetically modified animals with cardiac malformations will grow rapidly in the coming years. Once a sufficient number of modifications with a comparable phenotype is available, it should be possible to predict the signal-transduction pathway or the structural elements responsible for this phenotype, and complement the series of modifications accordingly. As the example of the retinoic acid receptors has demonstrated, to arrive at this level of insight it will also be necessary to deal with the extensive level of functional redundancy within gene families by creating double or triple mutants. On the other hand, if deficiency of a morphogenetic factor is responsible for early embryonic death, the creation of "conditional" knockouts [145, 146], in which gene inactivation can be induced at will at a particular developmental stage, is indicated. Moreover, to understand the extremely complex manifestations of congenital heart malformations, crossings between mice with phenotypically distinct syndromes may be necessary. However, in addition to proceeding in this highly technological direction, it will also be necessary to more precisely describe the respective deficiencies if we are to arrive at an accurate inventory of syndromes that share a morphogenetic or pathogenetic mechanism. Developmental cardiology will therefore benefit from an intensive collaboration between molecular biologists and embryologists.

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