Part II

Left-Right Axis and Heterotaxy Syndrome

Perspective

Bradley B. Keller

The generation of a unique left-right patterning is essential to higher organisms with complex multicellular organs, and errors in patterning represent the origins of some of the most complex forms of congenital heart disease (CHD). Our understanding of these heterotaxy-related syndromes has evolved from initial clinicopathologic correlates over a century ago to the generation and investigation of model organisms (fly, frog, mouse) and the subsequent identification and validation of patterning-related genes and pathways critical for normal human development and responsible for disease states.

Dr. Shiraishi reviews human heterotaxy syndromes associated with CHD, providing a broad overview of the role of cilia, molecular mechanisms involved in left-right patterning, and associated clinical features. Early asymmetric expression of critical morphogens in left-right patterning (Nodal, Lefty2, Pitx2) is required for normal development, and errors in the expression of these morphogens result in patterning errors. Heterotaxy-related CHD is often associated with unbalanced development of the ventricles, resulting in variations of "single ventricle" physiology and requiring staged surgical palliation to separate the venous and systemic circulations. Children and adults with palliated single ventricle physiology, including heterotaxy patients, face a range of medical complications related to cardiac dysfunction and the consequences of increased central venous pressure and reduced cardiac performance. As highlighted by Dr. Shiraishi, large gaps in our

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understanding of the pathogenesis of heterotaxy syndromes and their optimal medical and surgical management still exist. Hopefully, readers of these chapters will be intrigued and encouraged to pursue solutions.

Dr. Hamada provides an update on the investigation of cilia-mediated flow patterns during early embryogenesis. Motile cilia generate unique extraembryonic flow patterns that mechanically condition non-motile cilia in the ventral node, impact subsequent morphogenesis, and can be explored in fly and mouse model systems. Several key morphoregulatory pathways including agonists and antagonists of noncanonical Wnt signaling and stretch-sensitive Pkd Ca^{2+} channels are clearly involved.

Dr. Shibata and colleagues share some interesting findings on the association of heterotaxy/polysplenia syndrome, single gene mutations in several patterning genes, including *BMPR2*, and pulmonary artery hypertension. These associations require further investigation in model systems where the consequences of CHD including heterotaxy syndrome can be explored in aging animals and in adults with congenital heart diseases.

Thus, the clinical presentation of CHD associated with heterotaxy represents a spectrum of common final pathways and a range of early errors in embryo patterning and morphogenesis, modified during fetal life and then palliated using our current medical and surgical therapies. Investigation of the mechanisms responsible for normal and aberrant patterning and morphogenesis will continue to reveal important genes and pathways that can be used to identify the origins of CHD and may also be important for future targeted therapies, for example, related to pulmonary artery vascular remodeling and hypertension.

Left-Right Asymmetry and Human Heterotaxy Syndrome

6

Isao Shiraishi

Abstract

Heterotaxy syndrome is characterized by a wide variety of cardiac and extracardiac congenital malformations that are primarily induced by disorders of the left-right axis determination during early embryonic development. Prognosis of the disease remains unsatisfactory because the syndrome is often associated with complicated congenital heart diseases. Long-term follow-up of heterotaxy patients, particularly those who underwent Fontan procedure, is now one of the most important issues in pediatric and adult congenital heart disease clinics. Collaborative studies between pediatric cardiologists and basic scientists are essential for improving the prognosis of heterotaxy syndrome.

Keywords

Heterotaxy • Left-right axis • Signal transduction • Heart surgery

6.1 Introduction

Heterotaxy syndrome is a rare but serious congenital disease that occurs approximately 1 to 5,000–7,000 of live birth [1]. Patients are generally subdivided into "bilateral right sided" (right isomerism) or "bilateral left sided" (left isomerism) according to the characteristic morphology of atrial appendages of the heart. However, there is a wide spectrum of pathology with considerable overlap of the anatomical features.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_6

6.2 Molecular and Cellular Mechanisms of Left-Right Determination

The left-right axis determination initiates in the primitive node at E7.5 in mice and develops through the following pathways [2, 3]:

1. Breaking of symmetry as a result of leftward "nodal flow"
2. Transmission of asymmetric signals to the lateral plate mesoderm (LMP)
3. Asymmetric expression of nodal and lefty2 in the LMP
4. Situs-specific morphogenesis mediated by asymmetric expression of Pitx2

6.2.1 Node Cell Monocilia Create Leftward "Nodal Flow" and Activate Asymmetry Signaling Around the Node

The determination of the left-right asymmetry starts as leftward nodal flow generated by rotational movement of monocilia in the primitive node [4, 5]. Clockwise rotation of motile cilia creates unidirectional leftward flow because the rotational axes of cilia tilt caudal direction of the embryos [6, 7].

There are two models why nodal flow is perceived by nodal and perinodal cells. One hypothesis (chemosensory model) is that the nodal flow produces a gradient of left determinant particles (node vesicular parcels) containing hedgehog proteins and nodal [8, 9], which activate downstream signaling of nodal in the left-side perinodal cells.

Alternative hypothesis (mechanosensory model) is that the leftward nodal flow provokes an asymmetrical increase influx of Ca^{2+} ion in the sensory cilia cells through PKD2, a causative gene for human polycystic kidney disease [9, 10]. This Ca^{2+} influx is linked to the activation of nodal in the left-side perinodal cells, which is consequently transferred to the left LPM.

6.2.2 Asymmetry Signaling Transmits to the Left Lateral Plate Mesoderm

Transmission of nodal to the left LMP followed by lefty2 and Pitx2 activation [11] and the consequent heart morphogenesis in the normal subjects, right/left isomerism, and situs inversus is summarized in Fig. 6.1a.

6.2.3 Genes Associated with the Human Heterotaxy Syndrome

Recent human and animal model studies have provided insights into the genetic and developmental etiology of the heterotaxy syndrome. In human, genes that are associated with heterotaxy syndrome are ZIC3, NODAL, CFC1, ACVR2B, LEFTY2, CITED2, and GDF1 [12–14].

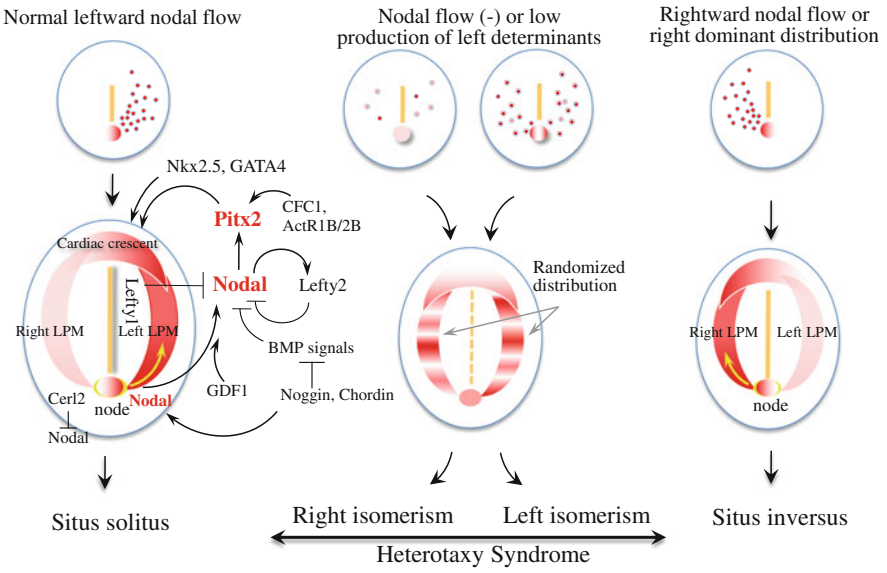

Fig. 6.1 Signal transmission of nodal to the left lateral plate mesoderm followed by Pitx2 activation and the consequent heart morphogenesis in normal and heterotaxy embryos (Adapted and modified from Ref. [1] with permission)

6.3 Clinical Manifestation of the Heterotaxy Syndrome

Factors that deteriorate prognosis of the heterotaxy syndrome are complications with pulmonary venous obstruction, pulmonary arterial distortion, regurgitation of atrioventricular valve, elevated pulmonary vascular resistance, and impaired ventricular function [15].

6.3.1 Right Isomerism

Neonates with right isomerism typically show single atrium, single right ventricle, and univentricular atrioventricular connection often associated with atrioventricular valve regurgitation. First-stage palliation (2–4 weeks after birth) is a control of pulmonary blood flow with pulmonary banding or systemic-pulmonary shunt. Pulmonary venous obstruction due to total anomalous pulmonary venous drainage should be precisely diagnosed and repaired by surgical operation.

The second-stage palliation is the bidirectional Glenn shunt, where the right and/or left superior vena cava is isolated and is connected to the pulmonary artery. This operation is, in general, performed around 6 months after birth.

The third-stage palliation is Fontan procedure. Recently, a modification using extra-cardiac artificial conduit-type total cavo-pulmonary connection (TCPC) is

most often employed, because the long-term prognosis of the conventional atriopulmonary connection is proved to be unsatisfactory characterized by enlargement of the atrium, intractable atrial tachyarrhythmias, and thromboembolisms.

After successful completion of the TCPC, cyanosis disappears and the general conditions of the patients improve. However, number of patients who underwent successful Fontan procedure is approximately 50 % because right isomerism often accompanied with combination of severe and complicated congenital heart diseases [16].

6.3.2 Left Isomerism

Left isomerism is typically associated with atrioventricular septal defect, persistent left superior vena cava, interrupted hepatic portion of the inferior vena cava, and atrioventricular conduction disturbance. In left isomerism, sinus node and atrioventricular nodes are usually hypoplastic, and sinus bradycardia or complete atrioventricular block is frequently accompanied.

6.4 Long-Term Prognosis of Heterotaxy Patients

Although the medical and surgical treatments of the heterotaxy syndrome have remarkably advanced, long-term prognosis of the patients remains unsatisfactory. Right isomerism has been recognized as one of the worst forms of CHD with overall 5-year survival ranging from 30 to 74 %. The results are better in left isomerism with 5-year survival rates ranging between 65 and 84 %, which is still considerably lower than survival for most other forms of CHD [17]. The main reason is that the nature of the Fontan single ventricle physiology is fundamentally imperfect. Representative long-term complications of the Fontan operation are illustrated in Fig. 6.2.

6.4.1 Protein-Losing Enteropathy

Protein-losing enteropathy (PLE), one of the most severe manifestations of the failing Fontan circulation, occurs in 5–10 % of the total postoperative cases [15]. Chronic loss of serum proteins into the gastrointestinal tracts results in systemic edema, ascites, pleural effusion, diarrhea, gastrointestinal bleeding, susceptibility to infections, and ultimately cachexia. The underlying mechanism of PLE remains uncertain. Elevated inflammatory reactions such as TNF-α or IFN-α, dilatation of intestinal lymphatic vessels, and widening between intestinal epithelial cells may be involved in the protein and fluid losses [18]. Steroids, high molecular weight heparin, sildenafil, surgical interventions, for instance, fenestration of atrial-level communications or conversion of the Fontan circuit, are effective. To date,

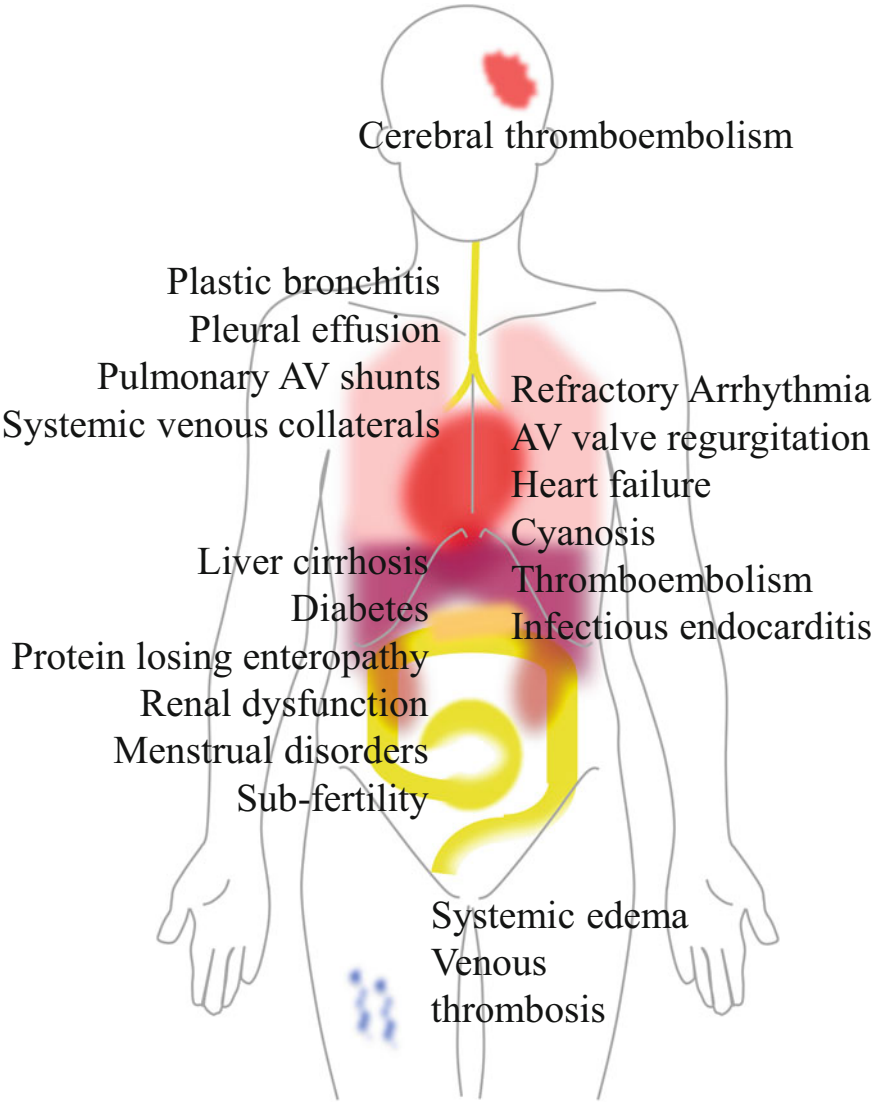

Fig. 6.2 Possible complications of mid- to long term after completion of Fontan procedure for heterotaxy patients (Adapted and modified from Ref. [1] with permission)

cardiac transplantation is considered as the only and complete resolution of PLE pathophysiology.

6.4.2 Arrhythmias

Reentrant atrial tachyarrhythmias are the commonest in patients after Fontan procedure and are often associated with deterioration of hemodynamics, either causally or as a result [15]. Hemodynamic abnormalities such as valve regurgitation or outflow obstruction, if present, should be aggressively treated by surgery.

6.4.3 Heart Failure

Initial feature of heart failure long after the Fontan procedure is characterized by worsening of ventricular relaxation and compliance [15]. These abnormalities may be caused by exposure of hypoxia and volume/pressure overload preceding the Fontan procedure, repetitive surgical operations, and hemodynamic disadvantages of the Fontan circuit. These changes are primarily progressive and consequently lead to failure of the Fontan circuit. Late after the Fontan procedure, systolic dysfunction becomes apparent. Administration of angiotensin converting enzyme inhibitors or β-blockades may be beneficial for particular patients, although the clinical evidence and cellular mechanisms remain to be elucidated.

6.4.4 Hepatic Dysfunction

Hepatic dysfunction, liver fibrosis, and cirrhosis are common complication of patients long after Fontan operation. Recently, cases with hepatocellular carcinoma after Fontan operation have been reported [19, 20]. Careful observation should be necessary to detect the hepatic changes long after Fontan operation.

6.4.5 Management of Failing Fontan Patients

Patients who underwent atrio-pulmonary connection or lateral tunnel procedure are likely to be complicated with thromboembolism or intractable arrhythmias due to enlargement of the right atrium [14]. Surgical intervention with conversion to TCPC is required before such complications become irreversible. Cardiac transplantation may be the only option for patients with severe heart failure, intractable arrhythmias, or recurrent PLE.

6.5 Future Direction and Clinical Implications

In the basic science field, embryonic development of left-right asymmetry has been uncovered by means of mouse genetic engineering. In addition, advanced human genetics have uncovered many responsible genes for heterotaxy syndrome. By means of innovative technologies such as whole genome sequencing or patient-

based human inducible pluripotent stem cells, novel genes will be clarified and analyzed. In the clinical field, anatomical and physiological diagnosis from the fetal period, better clinical managements after birth, and tailor-made surgical operations will improve the prognosis. Cell- or tissue-based regeneration therapies and a new ventricular assist device could improve cardiac function of failing Fontan patients. Multiple approaches including basic and clinical science are necessary to improve the prognosis and quality of life of heterotaxy patients.

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Roles of Motile and Immotile Cilia in Left-Right Symmetry Breaking

7

Hiroshi Hamada

Abstract

Our body possesses three body axes, anteroposterior, dorsoventral, and left-right (L-R) axes. L-R asymmetry is achieved by three consecutive steps: symmetry breaking at the node, differential patterning of the lateral plate by a signaling molecule Nodal, and finally situs-specific organogenesis. Breaking of L-R symmetry in the mouse embryo takes place in the ventral node, where two types of cilia are found. Whereas centrally located motile cilia generate a leftward fluid flow, peripherally located immotile cilia sense a flow-dependent signal. Although Ca^{2+} signaling is implicated in flow sensing, it is still not clear what triggers Ca^{2+} signaling, a determinant molecule transported by the flow or mechanical force induced by the flow.

Keywords

Cilia • Fluid flow • Laterality • Left-right asymmetry

7.1 Introduction

Most of visceral organs in vertebrates including the human are left-right (L-R) asymmetric in their position or shape. The process by which L-R asymmetry is generated can be divided into three steps (Fig. 7.1):

1. The initial breaking of L-R symmetry, which occurs in or near the node and at the late neural-fold stage

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_7

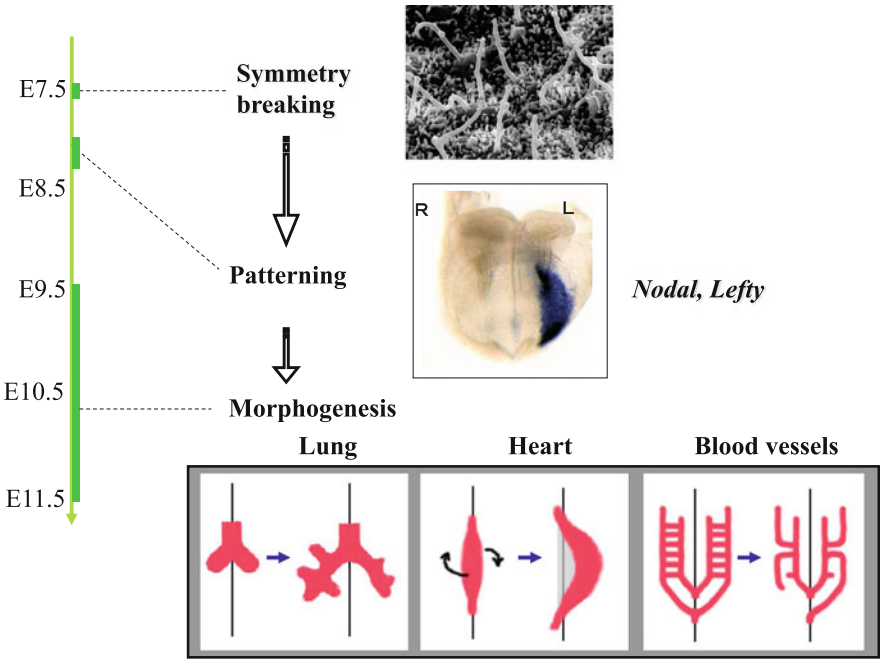

Fig. 7.1 Three steps underlying the generation of L-R asymmetry. Three steps that contribute to the generation of L-R asymmetry are shown: (1) symmetry breaking, (2) molecular patterning of the LPM, and (3) asymmetric organogenesis. The developmental stage (E, embryonic day) corresponding to each step in the mouse is indicated on the *left*

2. Transfer of an L-R-biased signal(s) from the node to the lateral plate mesoderm (LPM), which leads to L-R asymmetric expression of signaling molecules such as the transforming growth factor-β (TGF-β)-related proteins Nodal and Lefty on the left side of the LPM
3. L-R asymmetric morphogenesis of visceral organs induced by these signaling molecules

7.2 Symmetry Breaking by Motile Cilia and Fluid Flow

The breaking of L-R symmetry takes place in the node, an embryonic midline structure located at the anterior tip of the primitive streak in mouse embryos (Fig. 7.2). At the central region of the node, there are about 200 motile cilia that protrude from the ventral side of the node into the node cavity [1] (Fig. 7.2) and rotate in the clockwise direction (when viewed from the ventral side) at a speed of 600 rpm [2]. This rotational movement of the cilia generates the leftward laminar flow of extraembryonic fluid in the node cavity [2], occurs at a speed of ~15–20 μm/s. This leftward fluid flow in the node, referred to as nodal flow, is responsible for symmetry breaking. Many mutant mice that lack nodal flow because the node cilia

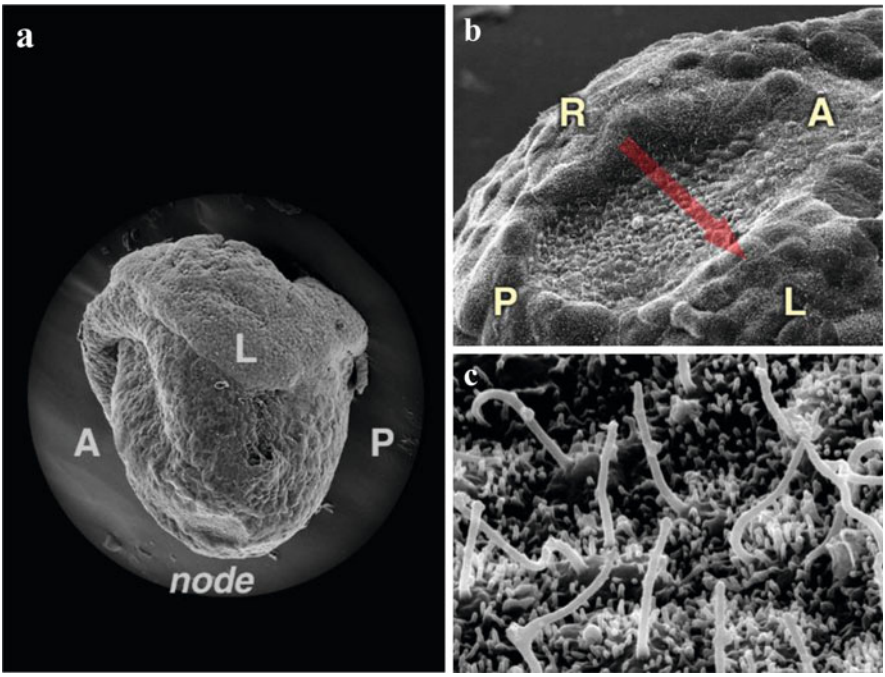

Fig. 7.2 Cilia and fluid flow in the node. A lateral view of the embryonic day 8.0 mouse embryo (**a**). Note that the node is located at the midline. Left-right (L-R) and anteroposterior (A-P) orientations are indicated. A ventral view of the mouse node at lower magnification (**b**). The *red arrow* denotes the leftward flow of extraembryonic fluid. A scanning electron micrograph showing that each cell on the ventral side of the mouse node has a monocilium (**c**)

are either missing or immotile have been identified, all of which exhibit aberrant L-R patterning of the LPM. Furthermore, L-R patterning of the embryo can be reversed when the direction of the flow was experimentally reversed by imposing the rightward artificial flow [3], establishing that the direction of the flow determines L-R.

How is the unidirectional fluid flow generated by rotational movement of the cilia? Hydrodynamic principles predict that the cilia can generate a unidirectional flow if they are tilted toward a specific direction. When the cilia move closer to the surface, the movement of fluid near the surface will be restricted as a result of the "no-slip boundary effect." Conversely, when the cilia move away from the surface, they move the neighboring fluid more effectively. If cilia are tilted toward the posterior side, they will be moving toward the right when they come close to the surface and toward the left when they are far from the surface, thus generating a leftward flow. Observation of these rotating cilia by high-speed video microscopy revealed that they are indeed tilted posteriorly at an average angle of 30° [4, 5]. Recent evidence [6] suggests that, in addition to the "no-slip boundary

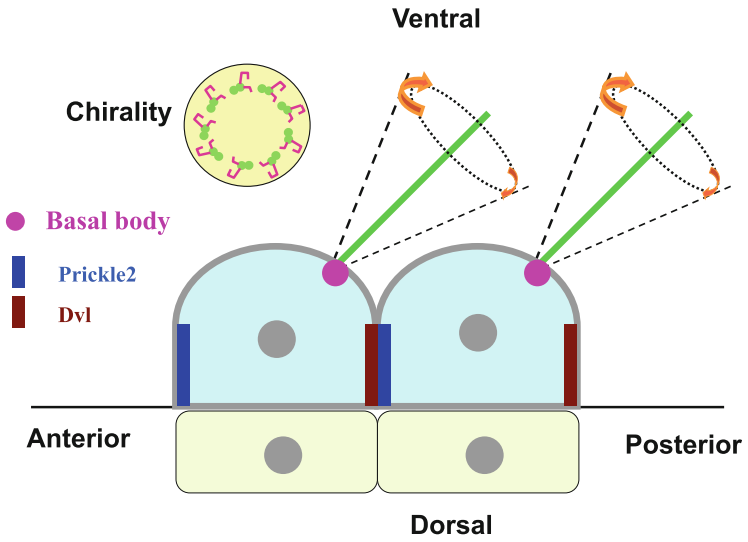

Fig. 7.3 L-R symmetry breaking by preexisting information. Each node cilium (*red bars* on *left*) is posteriorly tilted, likely because the basal body (*green*) is posteriorly shifted within the cell (*blue*). The cilium protrudes from the cell toward the ventral side of the embryo and rotates in a clockwise direction when viewed from the ventral side. Anteroposterior (A-P) and dorsoventral (D-V) orientations are indicated. A schematic representation of a transverse section of a cilium, revealing its chiral structure, is shown on the right. The cilium contains nine pairs of microtubules (*green*) as well as inner and outer arms of dynein (*pink*)

effect," intrinsic asymmetry in rotational stroke may also help generating the unidirectional flow.

Since the L-R axis is the last axis to be determined, symmetry breaking of L-R axis must be achieved by utilizing preexisting positional cues. In fact, two preexisting positional cues are reflected in the cilia: The A-P and D-V axes are thus represented by the posterior tilt and ventral protrusion of the cilia, respectively (Fig. 7.3). The node cilia thus generate the leftward flow by making use both of the preexisting positional cues and their structural chirality.

How is A-P information translated into the posterior tilt of the node cilia? Given its similarity to positioning of the hair in the *Drosophila* wing, a mechanism resembling the planar cell polarity (PCP) pathway involving noncanonical Wnt signaling [7] seems to underlie positioning of the node cilia. Thus, some of the PCP core proteins such as Prickle2 and Vangl1 are localized to the anterior side of node cells [8, 9], whereas Dvl protein is localized to the posterior side [10] (Fig. 7.3). However, it remains unknown what positional cue is responsible for the polarized localization of these PCP core proteins.

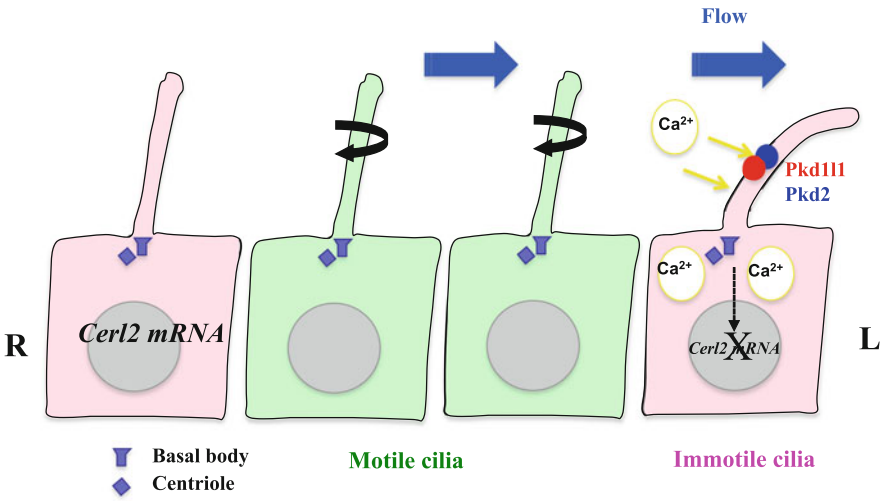

Fig. 7.4 Two types of cilia in the node, motile and immotile. Two types of ciliated cell are present in the node: Those located centrally (*green*) have motile cilia that generate nodal flow, whereas those located peripherally (*pink*) possess immotile cilia that sense the flow. Sensing of the flow requires ciliary localization of a Ca^{2+} channel, the Pkd2-Pkd1l1 complex. The flow-mediated signal results in degradation of *Cerl2* mRNA. In this model, the flow would bend an immotile cilium on the left side

7.3 Sensing of the Fluid Flow by Immotile Cilia

In addition to the motile cilia, there are immotile cilia in the node [11, 12] (Fig. 7.4). Cells located at the central region of the node (pit cells) possess motile cilia, which generate the fluid flow. On the other hand, most cells located at the edge of the node (crown cells) possess immotile cilia [13]. Immotile cilia act as sensors of the fluid flow [13]. Mutant mouse embryo that lack all cilia including those at the node, such as *Kif3a*$^{-/-}$mouse embryos, fail to develop nodal flow and show L-R defects [14]. Such cilium-less embryos are also unable to respond to the artificial flow. However, when immotile cilia are restored in crown cells, the resulting embryo can now respond to the artificial flow [13], demonstrating that immotile cilia sense the flow.

Sensing of the fluid flow by immotile cilia requires a Ca^{2+} channel composed of Pkd2 [15] and Pkd1l1 [16, 17]. Indeed, several Ca^{2+} signaling blockers have been shown to disrupt asymmetric gene expression in crown cells [13]. In particular, the effects of $GdCl_3$ [an inhibitor of stretch-sensitive transient receptor potential (TRP) channels], 2-ABP [an inhibitor of the inositol 1,4,5-trisphosphate (IP_3) receptor], and thapsigargin (an inhibitor of Ca^{2+}-dependent ATPase activity in the endoplasmic reticulum) suggest involvement of a TRP-type channel such as Pkd2 and the IP3 receptor in the sensing of nodal flow. A mutation in *Pkd2* that disrupts the ciliary localization of the encoded protein results in L-R defects similar to those of

Pkd2$^{-/-}$embryos [13, 16], suggesting that Pkd2, together with Pkd1l1, functions in the ciliary compartment of crown cells. Whereas *Pkd2* encodes a Ca^{2+} channel with a short extracellular domain, Pkd1l1 possesses a much larger extracellular domain at its amino terminus. Pkd1l1 may be responsible for sensing of the flow signal and regulating Ca^{2+} channel activity of Pkd2. While oscillations of Ca^{2+} signaling with subtle L>R asymmetry were detected in the node [18], direct observation of L-R asymmetric Ca^{2+} signaling in crown cells has not been successful [13].

A long-standing question since the discovery of nodal flow concerns the action of the flow. Two models have been proposed (Fig. 7.5). According to the chemosensor model (Fig. 7.5a), the flow would transport an unknown molecule toward the left side of the embryo, which will eventually act as the L-R determinant. In an alternative model (two-cilia model or mechanosensor model; Fig. 7.5b), the embryo would sense the mechanical force generated by the flow. Several molecules have been proposed to be the determinant transported by the flow. However, none of them fulfill the requirements for the determinant. On the other hand, many lines of circumstantial evidence, including the recent observation that as few as two rotating cilia are sufficient for the breaking of L-R symmetry [19], favor the latter model. However, it is still not clear what exactly the immotile cilia sense during the symmetry-breaking event.

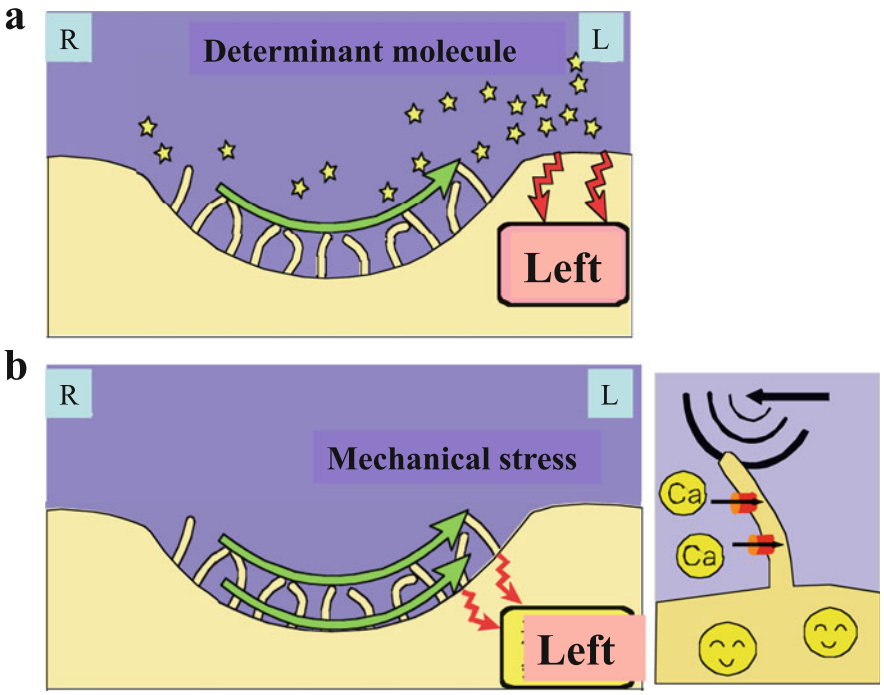

Fig. 7.5 Two models for the mechanism of action of nodal flow. (**a**) Determinant-transporting model. (**b**) Mechanosensory model. *Green arrows* indicate the direction of nodal flow; *yellow stars* denote determinant molecules

7.4 Readouts of the Flow

Cerl2 is the most immediate readout of the flow signal [19, 20]. *Cerl2* encodes a Nodal antagonist, although its precise action is not clear. It is asymmetrically (R > L) expressed in crown cells, and its absence results in randomization of L-R decision making [21]. Whereas expression of *Nodal* is bilateral in crown cells, the R > L expression of *Cerl2* renders Nodal activity in crown cells higher on the left side (Fig. 7.6). The Cerl2-generated asymmetry (R < L) of Nodal activity at the node closely correlates with the asymmetric pattern of *Nodal* expression in LPM [22]. Expression of *Cerl2* is initially symmetric (R = L) at the early headfold stage, but it becomes R > L as the velocity of nodal flow increases, with expression on the left side being downregulated [19, 22]. Finally, $Pkd2^{-/-}Cerl2^{-/-}$double-mutant embryos manifest randomized *Nodal* expression in LPM, resembling the *Cerl2* single mutant [13]. Therefore, *Cerl2* is the main target of the flow signal.

L-R asymmetry of *Cerl2* expression is generated at a posttranscriptional level [23], by degradation of *Cerl2* mRNA via its 3′ untranslated region. Preferential decay of *Cerl2* mRNA on the left is initiated by the leftward flow and further enhanced by the operation of *Wnt-Cerl2* interlinked feedback loops, in which Wnt3

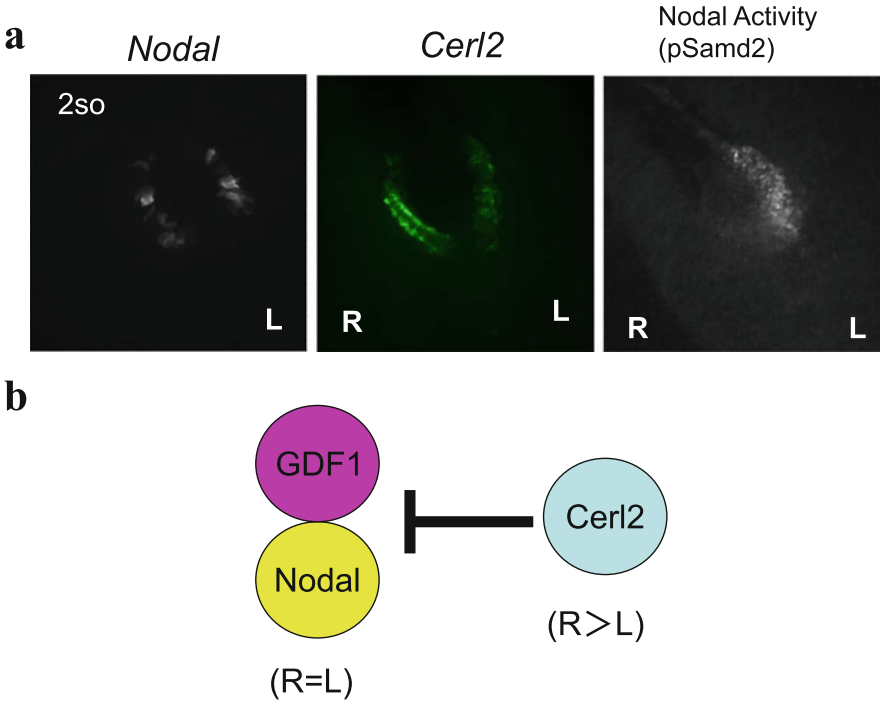

Fig. 7.6 R>L expression of *Cerl2* results in asymmetry in Nodal activity at the node. R>L asymmetric expression of *Cerl2* makes Nodal activity at the node R<L (**a**), Cerl2 is an inhibitor of Nodal (**b**)

upregulates *Wnt3* expression and promotes *Cerl2* mRNA decay, whereas Cerl2 promotes Wnt degradation. Mathematical modeling and experimental data suggest that these feedback loops behave as a bistable switch that is able to amplify in a noise-resistant manner a small bias conferred by fluid flow.

7.5 Future Directions

Although rapid progress has been made in the last 20 years, many important questions remain unanswered. Firstly, how is A-P information translated into the posterior tilt of node cilia? Namely, what is the nature of the A-P information that polarizes node cells along the A-P axis? Secondly, how is the direction of rotation determined for node cilia? Thirdly, how does the nodal flow work? How do motile cilia sense the flow? Do they sense a signaling molecule that is transported by the flow or sense mechanical force? Fourthly, what is the precise role of Ca^{2+} signaling? How does Ca^{2+} signaling induce degradation of Cerl2 mRNA? Finally, to what extent is the mechanism for breaking of L-R symmetry conserved among species? L-R symmetry breaking does not appear to depend on cilia in *Drosophila* and snail [24]. Further development of various approaches (including genetic, cellular, biophysical, and mathematical) will be necessary to answer these questions.

Acknowledgments I thank current and former members of my laboratory for discussion as well as for providing illustrations. The work performed in my laboratory has been supported by CREST, Japan Science and Technology Corporation (JST), and by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Role of Cilia and Left-Right Patterning in Congenital Heart Disease

8

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Abstract

A central role for cilia in the pathogenesis of congenital heart disease was uncovered by our large-scale mouse mutagenesis screen for mutations causing congenital heart disease. This is supported by human clinical studies, which showed a high prevalence of ciliary dysfunction and respiratory symptoms and disease in patients with congenital heart disease. Our mouse studies indicate this involves essential roles for both primary and motile cilia in the pathogenesis of congenital heart disease. As laterality defects were also observed with high prevalence among the congenital heart disease mutants, this further suggested an important role for left-right patterning in the pathogenesis of congenital heart disease. This finding is reminiscent of the high prevalence of heterotaxy among human fetuses with congenital heart disease, indicating the fetal mouse screen may provide a window into the unborn human fetal population. Clinically, congenital heart disease patients with ciliary dysfunction were found to have more respiratory symptoms and disease, a finding with significant clinical implications, as congenital heart disease patients undergoing surgical palliation often have respiratory complications with high morbidity. While this is usually attributed to the heart disease, we propose this may involve intrinsic airway clearance deficits from ciliary dysfunction. Thus the presurgical screening of congenital heart disease patients for respiratory ciliary dysfunction may provide

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_8

opportunities to provide perioperative pulmonary therapy to enhance airway clearance for at-risk patients. Such change in the standard of care may improve outcome, especially for those congenital heart disease patients who must endure multiple rounds of cardiac surgeries.

Keywords
Cilia • Ciliary dysfunction • Respiratory symptoms • Laterality • Heterotaxy

8.1 Introduction

Complex congenital heart disease is clinically well described to be highly associated with heterotaxy, a birth defect involving randomization of left-right patterning [1]. The importance of left-right patterning in congenital heart disease is likely a reflection of the fact that the heart is the most left-right asymmetric organ in the body. This asymmetry is critical for establishing systemic and pulmonary circulation required for efficient oxygenation of blood. While heterotaxy is relatively rare, reported at approximately 1 in 10,000 live births, it is clinically of high importance given it is often associated with complex CHD with high morbidity and mortality [2].

8.1.1 Heterotaxy, Primary Ciliary Dyskinesia, and Motile Cilia Defects

Interestingly, heterotaxy and complex CHD have been reported in ~6 % of patients with primary ciliary dyskinesia (PCD), a sinopulmonary disease that arises from airway mucus clearance defects due to immotile or dyskinetic cilia in the respiratory epithelia [3]. Given PCD is also relatively rare at 1 in 16,000 [4], the co-occurrence of heterotaxy and PCD would suggest a mechanistic link for heterotaxy and PCD. This mechanistic link has now been demonstrated to involve a shared disturbance of motile function. Thus animal model studies have shown motile cilia play an important role in embryonic left-right patterning [5].

In the mouse embryo, motile cilia at the node generate nodal flow that helps specify the left-right axis (Fig. 8.1). The nodal flow is sensed by nonmotile or primary cilia at the node periphery, resulting in activation of calcium signaling at the embryo's left, followed by left-sided activation of the nodal signaling cascade in the left lateral plate mesoderm. As motile cilia in the node and airway are constructed in a similar manner with many of the same proteins, it is not surprising that airway ciliary dysfunction might predict nodal cilia dysfunction. This likely accounts for the high prevalence of heterotaxy among PCD patients.

8.1.2 Motile Respiratory Cilia Defects in Other Ciliopathies

Given primary cilia also has been shown to play a role in the embryonic node to establish the left-right axis, this would suggest that mutations affecting primary

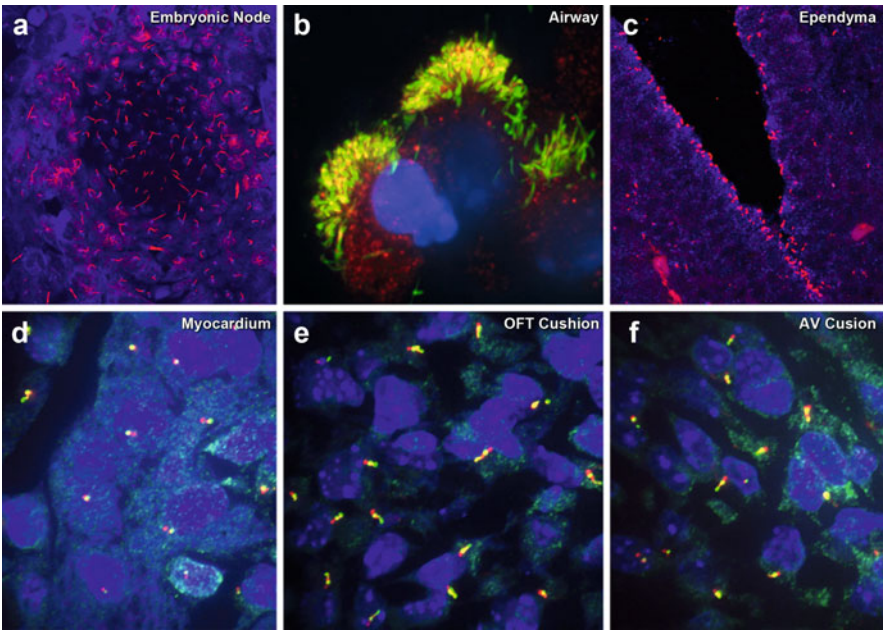

Fig. 8.1 Motile and primary cilia in the mouse embryo. (**a**) Immunostaining with antibodies to acetylated tubulin (*red*) and γ-tubulin (*blue*) was used to visualize motile cilia in the E7.75 mouse embryonic node. (**b–f**) Immunostaining with antibodies to acetylated tubulin and IFT88 visualized cilia in the newborn mouse tracheal airway epithelia (**b**), in E12.5 brain ependyma (**c**), and primary cilia in the myocardium (**d**), outflow tract cushion (**e**), and atrioventricular cushion (**f**) of the E12.5 mouse embryonic heart

cilia function may also contribute to complex CHD associated with heterotaxy. Given the extensive overlap between proteins found in the primary and motile cilia, this would suggest the clinical distinction may be blurred between patients with ciliopathies considered mediated by primary cilia defects vs. those with PCD that have motile cilia defects. Indeed in a recent study, we showed that a patient with cranioectodermal dysplasia, a ciliopathy thought to involve the primary cilia, has presentations consistent with PCD. This includes obstructive airway disease, low nasal NO, and abnormal respiratory ciliary motion [6].

8.1.3 Ciliary Dysfunction in Congenital Heart Disease Patients with Heterotaxy

Even as PCD patients were observed to have a 6 % incidence of heterotaxy, a study of CHD patients with heterotaxy revealed an even higher prevalence of respiratory ciliary dysfunction similar to that seen with PCD [7]. Two tests used for PCD assessment were used to evaluate heterotaxy patients. Ciliary motion analyzed

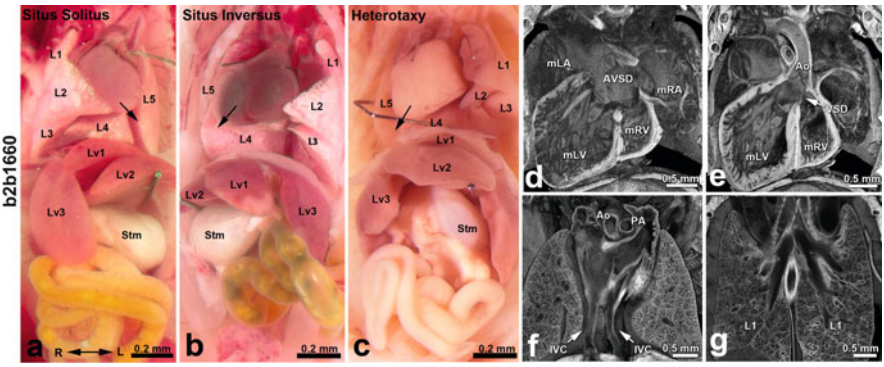

Fig. 8.2 Situs anomalies, congenital heart defects and ciliogenesis defects in laterality mutants. (**a**–**g**) *Ap1b1*^{b2b1660} mutants exhibit situs solitus (**a**), situs inversus (**b**), or heterotaxy (**c**). Situs solitus, characterized by normal left-right visceral organ positioning, the heart apex (*arrow*) points to the left (levocardia), four lung lobes are on the right and one on the left, stomach is to the left, and the dominant liver lobe is on the right. With situs inversus, there is complete mirror reversal of organ situs, while with heterotaxy, visceral organ situs is randomized, such as dextrocardia with levogastria shown in (**c**). The heterotaxy mutant in (**c**) exhibit complex CHD with AVSD (**d**), ventricular septal defect (VSD) (**e**), duplicated inferior vena cava (IVC) (**f**), and left pulmonary isomerism with bilateral single lung lobes (**g**) (From Li et al. [11])

using videomicroscopy of nasal tissue biopsy and nasal nitric oxide (nNO) was measured, which is typically low in patients with PCD. This analysis showed 42 % of the heterotaxy patients have ciliary dysfunction comprising abnormal ciliary motion and low nNO, presentations typically seen with PCD [7].

Interestingly, a mouse mutant exhibiting complex congenital heart defect associated with heterotaxy was identified to have a pathogenic mutation in *Dnah5*, a cilium outer dynein arm component required for motile cilia function and a gene well described to cause PCD [8]. This mutant exhibited mostly immotile cilia in the airway and in the embryonic node [8], accounting for the laterality disturbance and airway clearance defects seen in PCD patients with *DNAH5* mutations. Interestingly, these *Dnah5* mutant mice exhibited either of three different laterality phenotypes: normal situs solitus, mirror symmetric situs inversus totalis, or randomized visceral organ situs known as heterotaxy (Fig. 8.2). It is only with heterotaxy that complex congenital heart disease was observed, indicating that disturbance of the left-right patterning may play an important role in congenital heart disease. As the mouse *Dnah5* mutants with heterotaxy were mostly inviable to term due to their complex congenital heart disease, this would suggest considerable ascertainment bias in the human population. Consistent with this, a study of PCD patients revealed most had either situs solitus or situs inversus, with only a small fraction exhibiting heterotaxy [4].

8.1.4 Respiratory Complications in Heterotaxy Patients with Ciliary Dysfunction

As the central hallmark of PCD is respiratory disease due to mucociliary clearance defects, the question arises as to whether heterotaxy patients may also have respiratory symptoms and disease. Indeed, heterotaxy patients with ciliary dysfunction are observed to have significantly more respiratory symptoms and disease [7]. Furthermore, those undergoing surgical procedures show increased pulmonary morbidity, including increased use of inhaled β-agonist [9]. β-agonist use is typically avoided in cardiac patients given its arrhythmogenic properties. Hence, the increased use of this medication is a strong indicator of serious respiratory complications.

These findings have important clinical translational ramifications, since respiratory complications in heterotaxy patients are usually attributed to the heart disease, and thus any airway clearance defects are not systematically addressed clinically. In light of these findings, a change in the standard of care may be warranted with the presurgical screening of heterotaxy patients for mucociliary clearance defects and providing airway clearance therapy to help reduce postsurgical respiratory complications in those with airway ciliary dysfunction. This may help improve the prognosis for these patients who typically have to endure multiple high-risk cardiac surgeries to palliate their structural heart defects.

8.1.5 Left-Right Patterning and the Pathogenesis of Congenital Heart Disease

The importance of left-right patterning in the pathogenesis of congenital heart disease has also emerged from a large-scale mouse mutagenesis screen. High-throughput screening of ENU-mutagenized mice using fetal echocardiography allowed the ultrasound phenotyping of greater than 80,000 fetuses (Fig. 8.3) [10, 11]. Fetal echocardiography is ideally suited for recovery of mutants with congenital heart defects, as it is an imaging modality developed in the clinical setting for the assessment of cardiac structure and function (Fig. 8.3). Over 200 mutant mouse lines with a wide spectrum of congenital heart defect were recovered. Surprisingly, this included many mutant lines with laterality defects (~30 %), recovered based on the finding of complex CHD in mutants with heterotaxy. Given our screen was focused on congenital heart defects, not left-right patterning defects, this enrichment of laterality mutants would suggest the disturbance of left-right patterning plays an important role in the pathogenesis of congenital heart disease.

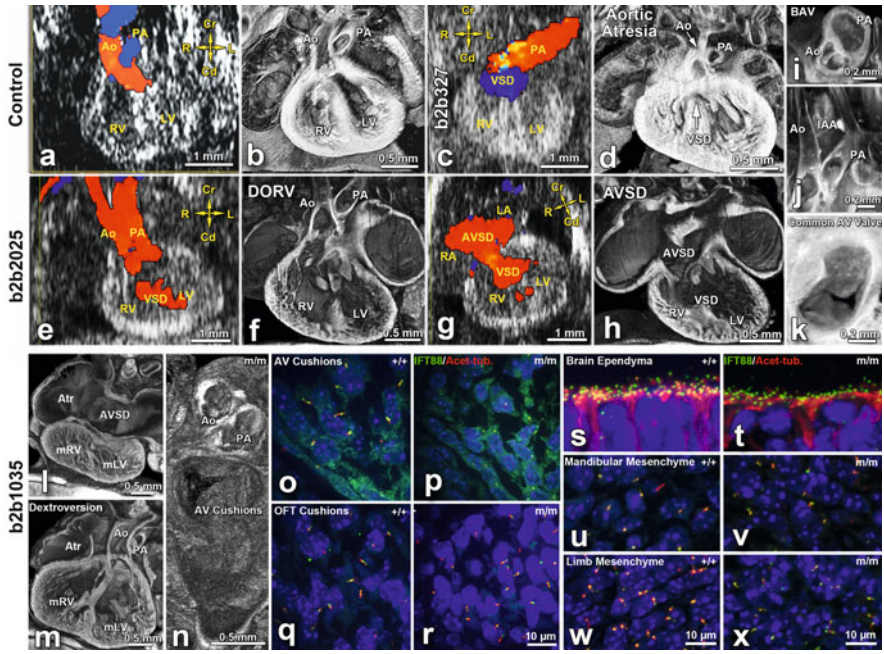

Fig. 8.3 Ultrasound diagnoses of congenital heart disease and cilia defects in mouse mutants with congenital heart disease. Vevo 2100 color flow imaging showed crisscrossing of blood flow, indicating normal aorta (Ao) and pulmonary artery (PA) alignment (**a** Supplemental Movie S1), confirmed by histopathology (**b**). E16.5 mutant (line b2b327) exhibited blood flow pattern indicating single great artery (PA) and ventricular septal defect (VSD) (**c** Supplemental Movie S2), suggesting aortic atresia with VSD, confirmed by histopathology (**d**). Color flow imaging of E15.5 mutant (line b2b2025) with heterotaxy (stomach on right; Supplemental Movie S3c) had side-by-side Ao/PA with Ao emerging from the right ventricle (RV), indicating DORV/VSD (**e**, **f** Supplemental Movie S3a) and presence of AVSD (**g**, **h** Supplemental Movie S3b,S3c). Histopathology also showed bicuspid aortic valve (BAV, **i**), interrupted aortic arch (IAA, **j**), and common AV valve (**k**). (**l**–**n**) *Cc2d2a* mutant exhibits dextrocardia with ventricular inversion (dextroversion) (**m**) and AVSD (**l**) with malformed AV cushions (**n**) but normal outflow cushions. (**o**–**x**) Confocal imaging of E12.5 *Cc2d2a* mutant (m/m) vs. wild-type (+/+) embryo sections showed no cilia in AV cushion (**o**, **p**) but normal ciliation in outflow cushion (**q**, **r**). Fewer and shorter cilia were observed in other mutant embryo tissues (**s**–**x**). *Red*, acetylated tubulin; *green*, IFT88 (From Li et al. [11])

This unexpected finding of a high prevalence of heterotaxy is actually in line with observations in the human fetal population. One clinical study using fetal echocardiography for CHD diagnosis reported that 16 % of human fetuses with congenital heart defects have heterotaxy [12]. This number is likely a minimal estimate, given several clinical studies have shown human fetuses with heterotaxy

and congenital heart defect have very high rates of prenatal/intrauterine death (30–60 %) [13–15]. When combined with the fact that only 28 % of CHD is clinically diagnosed prenatally [16], these findings point to the prevalence of congenital heart disease associated with heterotaxy being significantly underestimated clinically. In our mouse screen, we also noted most mouse fetuses with congenital heart defects associated with heterotaxy died in utero. The recovery of these heterotaxy mutants rests entirely on our screen having been conducted prenatally with fetal ultrasound imaging.

Of importance to note is the fact that many of these mutant lines with heterotaxy actually yielded three distinct phenotypes, similar to what had been observed for the *Dnah5* mutants. Thus animals harboring the same mutation can have normal situs solitus, mirror symmetric situs inversus, or heterotaxy (Fig. 8.2). As with the *Dnah5* mutants, congenital heart defects were usually seen only in mutants with heterotaxy [11]. In a subset of these mutants, videomicroscopy of the tracheal epithelia in these mutants also showed immotile or dyskinetic cilia, suggesting they have mutations affecting motile cilia function and may be PCD mouse models [11].

8.1.6 Ciliome Gene Enrichment Among Mutations Causing Congenital Heart Disease

Whole-mouse exome-sequencing analysis was used to recover the pathogenic CHD-causing mutations in mutants recovered from the large-scale mouse mutagenesis screen. This was made possible given the screen was conducted in a C57BL6 inbred strain background. From this analysis, 91 pathogenic mutations were recovered in 61 genes (Fig. 8.4). Of the 61 genes, 35 (58 %) are in cilia-related or ciliome genes (Fig. 8.5); this included 12 genes (34 %) required for motile cilia function (Fig. 8.4). Indeed 8 of these genes are known to cause PCD, including many alleles of *Dnah5* and *Dnah11* (Fig. 8.5). Interestingly, 23 of the cilia genes are actually primary cilia related (66 %). Of these, half are found in mutant lines with laterality defects and half in lines without laterality defects (Fig. 8.4) [11]. These findings suggest the link between cilia and CHD is broader, not merely a reflection of the role of cilia in left-right patterning. This is further supported by the recovery of 15 pathogenic mutations in genes involved in cilia-transduced cell signaling, including mutations in genes involved in Shh, Wnt, Tgfβ, and calcium signaling (Fig. 8.5), all pathways known to play important role in cardiovascular development [11].

Relevant to this, we note cilia is broadly expressed in the embryonic heart, including in the atrial and ventricular myocardium, the atrioventricular and outflow endocardial cushions (Fig. 8.1d–f). Importantly, in the *Cc2d2a* mutant recovered

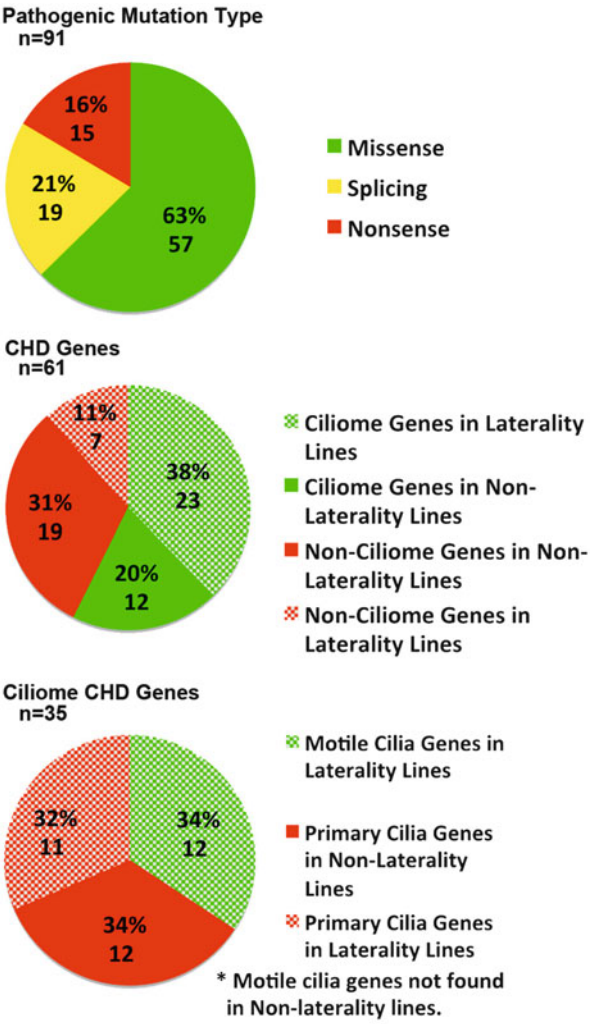

Fig. 8.4 Distribution of pathogenic congenital heart disease causing mutations recovered from large-scale mouse forward genetic screen**.** *Top*: Distribution of pathogenic CHD mutations among different mutation types. *Middle*: Ciliome vs. non-ciliome CHD genes found in laterality vs. nonlaterality CHD mutant lines. *Bottom*: Distribution of ciliome CHD genes affecting motile vs. primary cilia among laterality vs. nonlaterality lines (From Li et al. [11])

from our screen, atrioventricular septal defects were commonly observed, and this was associated with a selective loss of cilia only in the atrioventricular cushions, while the outflow cushions remain unaffected (Fig. 8.5o–r). The overall marked enrichment observed for cilia mutations in the context of a gene agnostic screen would point to the cilia as playing a central role in the pathogenesis of congenital heart disease, a role that is subserved by both motile and primary cilia and goes beyond the role of cilia in left-right patterning.

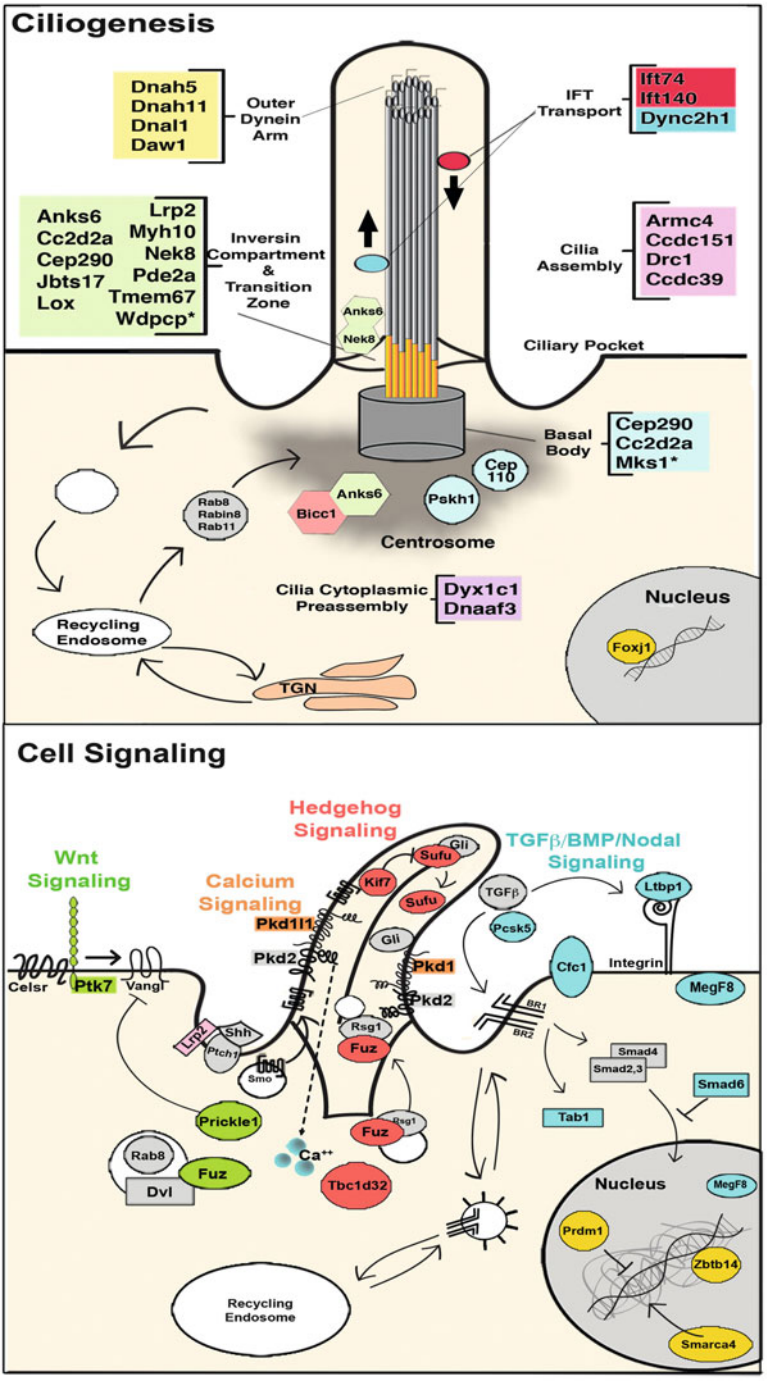

Fig. 8.5 Congenital heart disease genes recovered from mouse mutagenesis screen. Diagrams illustrate biological context of CHD gene function (*color-highlighting* indicates CHD genes recovered; *asterisk* denotes CHD genes recovered from previous screen) (From Li et al. [11])

8.1.7 Ciliary Dysfunction in Congenital Heart Disease Patients Without Heterotaxy

The finding from our mouse studies that cilia defects play a central role in the pathogenesis of CHD would suggest the clinical impact of ciliary dysfunction in congenital heart disease may be broader and have relevance beyond heterotaxy. To examine this question, we conducted a large clinical study of over 200 patients with a broad spectrum of congenital heart disease mostly without heterotaxy to determine the prevalence of ciliary dysfunction [17]. As in our previous study of heterotaxy patients, we assessed for respiratory ciliary dysfunction using videomicroscopy of the nasal epithelia and measured nNO.

This study demonstrated a very high prevalence of ciliary dysfunction, in both the heterotaxy and nonheterotaxy congenital heart disease patients [17]. Moreover, patients with airway ciliary dysfunction had significantly more respiratory symptoms and disease and this did not correlate with heterotaxy status (Table 8.1) [17]. The respiratory symptoms were largely localized to the lower airway and were also significantly associated with PCD symptoms such as chronic otitis media, chronic sinusitis, chronic wet cough, neonatal respiratory distress, pneumonia, and bronchiectasis. Together these findings suggest patients with congenital heart disease of a wide spectrum with or without heterotaxy may have high risk for respiratory ciliary dysfunction. These findings are in agreement with the mouse studies showing a central role for cilia in the pathogenesis of congenital heart disease.

8.1.8 Future Directions and Clinical Implications

We identified a central role for cilia in the pathogenesis of congenital heart disease. This finding uncovered by our mouse mutagenesis screen was unexpected and shows the power of a non-gene-biased phenotype-driven genetic screen to uncover new insights into mechanisms of disease pathogenesis. The important role of cilia in congenital heart disease is supported by the human clinical studies, which also showed a high prevalence of ciliary dysfunction in congenital heart disease patients. Our mouse screen identified primary and motile cilia both playing essential roles in congenital heart disease. The high prevalence of laterality defects among congenital heart disease mutants is reminiscent of the clinicalx observation of a high prevalence of heterotaxy among human fetuses with congenital heart disease. This suggests that observations from our mouse fetal ultrasound screen may provide a window into the unborn human fetal population.

Our finding that congenital heart disease patients with ciliary dysfunction have more respiratory symptoms and disease has important clinical implications. Patients with complex congenital heart disease typically must endure multiple high-risk cardiac surgeries for palliation of their structural heart defects. Not infrequently, respiratory complications with high morbidity are observed postoperatively that can negatively impact outcome. Such respiratory problems are typically

Table 8.1 Multivariate analysis and respiratory symptoms[a]

	All symptoms		PCD symptoms		Upper symptoms		Lower symptoms	
Covariates	exp(b)	*P-value*	exp(b)	*P-value*	exp(b)	*P-value*	exp(b)	*P-value*
HTX vs. non-HTX	1.103	0.51	1.067	0.75	1.223	0.31	0.973	0.90
Abnormal vs. normal CM	1.426	**0.006**	1.423	**0.043**	1.178	0.4	1.786	**0.003**
Low vs. normal nNO	2.030	**0.002**	2.291	**0.005**	1.520	0.22	2.643	**0.001**

From Garrod et al. [17]

Definition of abbreviations: CM ciliary motion, *HTX* heterotaxy, *nNO* nasal nitric oxide, *PCD* primary ciliary dyskinesia

Significant *P* values are bolded

[a]In each log-linear regression model, the regression coefficient exp (β) represents one unit increase in the covariate x as a multiplicative effect on the mean of the outcome. When exp (β) >1, the mean of the outcome increases as x increases; if exp (β) <1, the mean of the outcome decreases as x increases

attributed to the heart disease, and the possibility of intrinsic airway clearance defects due to ciliary dysfunction is never considered and hence not tested nor treated. The observations from the human and mouse studies combined would strongly suggest congenital heart disease patients should be presurgically screened for respiratory ciliary dysfunction, and those with ciliary dysfunction should be provided perioperative pulmonary therapy to enhance airway clearance function. Instituting such change in the standard of care may have significant benefit in improving outcome, especially cumulatively through the multiple rounds of surgery that patients with critical congenital heart disease must endure.

Acknowledgments This work was supported by NIH grant HL-U01098180 and funding from the Pennsylvania Department of Health.

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9 Pulmonary Arterial Hypertension in Patients with Heterotaxy/Polysplenia Syndrome

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Keywords
Gene • TGF-β • BMPR2

Early progressive pulmonary arterial hypertension (PAH) is often observed in patients with heterotaxy/polysplenia especially who have an intracardiac systemic-to-pulmonary shunt. However, its etiology is uncertain and its management is not well established. There was only a Japanese report about PAH in consecutive patients with heterotaxy/polysplenia syndrome [1]. They seemed to develop pulmonary vascular obstructive disease earlier and more severe than expected, even in cases with only pre-tricuspid systemic-to-pulmonary shunt although more detailed analysis is required.

Improved understanding and studies about the molecular genetics of heterotaxy syndrome indicate that this disease can be caused by single gene mutations. Genes currently implicated in human heterotaxy syndrome include *ZIC3*, *LEFTYA*, *CRYPTIC*, and *ACVR2B* [2]. The establishment of left-right asymmetry is regulated by a number of developmental signaling pathways including the notch, which mediate nodal expression surrounding the node [3]. Nodal, a growth regulator produced by the node, is a signaling molecule belonging to the transforming growth factor (TGF)-β superfamily that plays a variety of roles in the early development [4]. Mouse nodal acts through type I (ALK-4, 5, and 7) and type II (ACVR2B) receptors of the TGF-β superfamily. Ligand activation of the receptors requires one or more co-receptors, Cryptic and Cripto. Lefty-1 (homologue of LEFTYA) is a nodal antagonist that is expressed in medial left lateral plate mesoderm [5].

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_9

The mutation in *BMPR2*, which encodes type II receptor of the bone morphogenic protein (BMP), is a well-known genetic cause of PAH [6]. BMP is also belonging to the TGF-β superfamily. Although there has been no report that describes the association between PAH and mutation of genes implicated in heterotaxy syndrome, they might have some effect on the signaling pathway downstream of BMP and, consequently, be relevant to the pulmonary vascular pathogenesis in progressive PAH.

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