The Ion Channel Polycystin-2 Is Required for Left-Right Axis Determination in Mice

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Summary

Generation of laterality depends on a pathway which involves the asymmetrically expressed genes nodal, Ebaf, Leftb, and Pitx2 [1–3]. In mouse, node monocilia are required upstream of the nodal cascade [4]. In chick and frog, gap junctions are essential prior to node/organizer formation [5, 6]. It was hypothesized that differential activity of ion channels gives rise to unidirectional transfer through gap junctions, resulting in asymmetric gene expression [3, 6]. PKD2, which if mutated causes autosomal dominant polycystic kidney disease (ADPKD) in humans, encodes the calcium release channel polycystin-2 [7–11]. We have generated a knockout allele of Pkd2 in mouse. In addition to malformations described previously [12], homozygous mutant embryos showed right pulmonary isomerism, randomization of embryonic turning, heart looping, and abdominal situs. Leftb and nodal were not expressed in the left lateral plate mesoderm (LPM), and Ebaf was absent from floorplate. Pitx2 was bilaterally expressed in posterior LPM but absent anteriorly. Pkd2 was ubiquitously expressed at headfold and early somite stages, with higher levels in floorplate and notochord. The embryonic midline, however, was present, and normal levels of Foxa2 and shh were expressed, suggesting that polycystin-2 acts downstream or in parallel to shh and upstream of the nodal cascade.

Results and Discussion

Expression and Disruption of Mouse Pkd2

In situ hybridization experiments were performed to analyze the distribution of Pkd2 gene transcripts during early embryogenesis. In agreement with a previous report [13], we found ubiquitous expression of Pkd2 from the two-cell to the compacted blastocyst stage (data not shown). Low-level ubiquitous mRNA expression was retained during egg cylinder, early, and late headfold stages (Figures 1A and 1B). Stronger Pkd2 signals were evident in the floorplate and notochord (Figure 1C). L-R asymmetries were not found at any stage.

Gene targeting of Pkd2 is shown in Figures 1D and 1E. A true null allele was generated, as Pkd2 mRNA was not expressed in homozygous mutant embryos due to stop and polyadenylation signals in both LacZ and neo cassette of the targeted allele (Figure 1F). While heterozygous mice, in agreement with studies published previously [12, 14], were normal and fertile, no homozygous mutant animals were born. Pkd2−/−LacZ−/− embryos died between E12.5 and birth. As reported by Wu et al. [12], homozygous mutant embryos were characterized by cardiac defects and renal failure (see the Supplementary Material available with this article online for a figure showing nonlaterality phenotypes).

Laterality Defects in Pkd2 Mutant Mice

Mutant embryos displayed multiple laterality defects which are summarized in Table 1. Wild-type and Pkd2−/−LacZ−/− embryos showed asymmetric lobation of the lung, with four lobes on the right and one lobe on the left side (Figure 2A and data not shown). In contrast, most homozygous mutant embryos showed right isomerism of the lung, with four lobes on both sides (Figures 2B and 2C). Three Pkd2−/−LacZ−/− embryos (6%) showed normal lobation, and two embryos (4%) had complete inversion of lung situs (data not shown).

In addition, alterations of cardiac position were detected in mutant embryos. In wild-type embryos, the apex of the heart points toward the left (levocardia). This was found in all heterozygous and in about one third of homozygous mutant embryos (Table 1, Figure 2A). Dextrocardia (inversion of heart situs) was observed in another third (Figure 2C), and in the remaining embryos the apex pointed toward the midline (mesocardia; Figure 2B). Regardless of the situs, all hearts of mutant embryos displayed severe structural defects (see Supplementary Material). Within the abdominal cavity, about one third of homozygous mutant embryos had stomach and spleen on the left side, as in wild-type and heterozygous embryos (Figure 2D), but most Pkd2 knockout embryos displayed inversion of abdominal situs with a right-sided stomach and pancreas (Figure 2E and data not shown). The spleen was absent or severely reduced in size (hyposplenia), and the liver frequently showed abnormal lobation and/or midline positioning (data not shown).

Laterality defects were paralleled by abnormal heart looping and embryonic turning at E8.5–E10.5 (Figures 2F and 4A–4D and Table 1). In wild-type and Pkd2−/−LacZ−/− embryos, the heart tube always looped correctly to the right (Figure 4A and data not shown), while, among Pkd2−/−LacZ−/− embryos, roughly one third each dis-

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Figure 1. Expression of Pkd2 and Generation of Mutant Mice

(A–C) Pkd2 mRNA is ubiquitously expressed at headfold and early somite stages. Note the enhanced Pkd2 signal in the notochord (nt) and floorplate (fp) at E10.5 (C). (A) Posterior and (B) frontal view of wild-type embryos following nonradioactive whole-mount in situ hybridization. (C) Transverse section of a Pkd2+/−/LacZ−/− embryo following LacZ staining, photographed in dark field illumination.

(D) The targeting vector used to mutate Pkd2 (top), the wild-type Pkd2 gene locus (middle), and the targeted Pkd2 allele (bottom) are shown. Exons are indicated by blue boxes. The first exon contains the ATG start codon to which lacZ (gray) was fused. The coding region of exon 1 and part of intron 1 were deleted in the targeting vector which in addition contained a neomycin-resistance cassette (neo) for positive and the thymidine kinase gene (tk) for negative selection. The probe used for Southern hybridization and the sizes of the expected fragments obtained after EcoRV digestion are indicated. E, EcoRV; H, HindIII; K, KpnI; P, PstI; S, SalI.

(E) Southern blot analysis of EcoRV-digested yolk sac DNA.

(F) Absence of Pkd2 mRNA in homozygous mutant embryos. Northern blot analysis of total RNA isolated from genotyped E16.5 embryos (left).

played normal looping (Figure 4B), left-sided looping (Figure 4C), or heart tubes retaining a midline position (Figure 4D). Axial rotation, by which embryos achieve the characteristic fetal position with the tail positioned on the right side of the head, was randomized in homozygous Pkd2 mutants (Figure 2F and Table 1).

Normal Midline Development in Pkd2 Mutant Embryos

Midline defects frequently cause laterality disturbances in vertebrates [15, 16]. Upregulation of Pkd2 in the notochord and floorplate indicated a role for Pkd2 in midline development. However, analysis of the midline marker genes shh and Foxa2 revealed normal expression in homozygous mutant embryos (Figures 3A and 3B). Histological examination verified that floorplate and notochord were present and unaltered (Figures 3A and 3B). Pkd2 therefore seems to act downstream or in parallel to shh in the pathway of L-R axis formation.

Altered Gene Expression of L-R Pathway Genes

Phenotypic alterations of laterality reminiscent of those described above for homozygous mutant Pkd2 embryos have previously been reported in knockout mouse mu-

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<tr>
<th>Table 1. Summary of Laterality Defects and Marker Gene Expression in Pkd2 Knockout Embryos</th>
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<td>SS (Normal)</td>
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<td>LPM Pitx2</td>
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LI, left isomerism; RI, right isomerism; SI, situs inversus; SS, situs solitus.
Pkd2

nodal

factor

Pitx2

Pitx2

in all mutant embryos analyzed at the 3 to 5 somite out embryos the anterior LPM was negative for high-related TGF

nodal

nodal

Pkd2

feedback inhibitors of the nodal signaling pathway [3]. isomerism that we detected in Pitx2 and early somite stages (Figure 3C; [17, 18]). In Bilateral expression of asymmetrically expressed in mesendodermal cells at asymmetric expression of the nodal marker genes (A–C) Frontal view of dissected lungs and hearts of wild-type (A) and Pkd2+/−/LacZ−/− (B and C) embryos at E16.5. In normal embryos (A), the apex of the heart (h) points to the left (levocardia). In mutant specimen, the apex of the heart is either normal (data not shown), positioned in the middle (B; mesocardia), or points to the right (C; dextrocardia). The left lung (llu) of wild-type embryos consists of one lobe, whereas the right lung has four lobes: cranial (1), medial (2), caudal (3), and accessory (4). Pkd2+/−/LacZ−/− embryos display right lung isomerism (four lobes on either side; B and C).

(D and E) Ventral view of liver (ll), stomach (st), and spleen (sp) of Pkd2+/−/LacZ−/− embryos at E16.5. In wild-type embryos, stomach and spleen are positioned on the left side (data not shown). Pkd2+/−/LacZ−/− embryos show either normal (D) or inverted (E) location of the stomach. In embryos with inverted stomach, the spleen is either greatly reduced in size (hyposplenia, data not shown) or absent (E).

(F) Mutant embryos exhibit randomized turning. Ventral views of Pkd2+/−/LacZ−/− embryos at E10.5. The tail is located either on the right side of the head (n), as in wild-type embryos, or, due to inversion of turning, on the left side of the head (i). The heart ventricles in (B) and (C), the stomachs in (D) and (E), and the tails in (F) are outlined by dots. l, left; r, right, n, normal; i, inverted.

Figure 2. Laterality Defects of Pkd2 Mutant Embryos

(A–C) Frontal view of dissected lungs and hearts of wild-type (A) and Pkd2+/−/LacZ−/− (B and C) embryos at E16.5. In normal embryos (A), the apex of the heart (h) points to the left (levocardia). In mutant specimen, the apex of the heart is either normal (data not shown), positioned in the middle (B; mesocardia), or points to the right (C; dextrocardia). The left lung (llu) of wild-type embryos consists of one lobe, whereas the right lung has four lobes: cranial (1), medial (2), caudal (3), and accessory (4). Pkd2+/−/LacZ−/− embryos display right lung isomerism (four lobes on either side; B and C).

(D and E) Ventral view of liver (ll), stomach (st), and spleen (sp) of Pkd2+/−/LacZ−/− embryos at E16.5. In wild-type embryos, stomach and spleen are positioned on the left side (data not shown). Pkd2+/−/LacZ−/− embryos show either normal (D) or inverted (E) location of the stomach. In embryos with inverted stomach, the spleen is either greatly reduced in size (hyposplenia, data not shown) or absent (E).

(F) Mutant embryos exhibit randomized turning. Ventral views of Pkd2+/−/LacZ−/− embryos at E10.5. The tail is located either on the right side of the head (n), as in wild-type embryos, or, due to inversion of turning, on the left side of the head (i). The heart ventricles in (B) and (C), the stomachs in (D) and (E), and the tails in (F) are outlined by dots. l, left; r, right, n, normal; i, inverted.

tants affecting nodal pathway-related genes [15]. Consequently, we analyzed the expression of the asymmetric marker genes nodal, Ebafl, Leftb, and Pitx2 (summarized in Table 1). In wild-type embryos, nodal is asymmetrically expressed in mesendodermal cells at the primitive node and in the left LPM between headfold and early somite stages (Figure 3C; [17, 18]). In Pkd2+/−/LacZ−/− embryos, expression of nodal mRNA at the node was unchanged (Figure 3D), while left LPM expression was absent in 10/12 embryos (Figure 3D). Two mutant embryos showed bilateral expression of nodal (data not shown). Ebafl and Leftb encode two highly related TGFβ-type secreted proteins [19]. Both are direct response genes of nodal and act as negative feedback inhibitors of the nodal signaling pathway [3]. In wild-type mouse embryos, Ebafl is asymmetrically expressed in the left prospective floor plate and Leftb in the left LPM between the 3 to 6 somite stage (Figure 3E; [19]). Using an Ebafl probe which simultaneously detected Leftb transcripts, we failed to detect signals in all mutant embryos analyzed at the 3 to 5 somite stage (Figure 3F). The bicoid-type homeobox transcription factor Pitx2 is asymmetrically expressed in the left LPM and on the left side of the linear heart tube at early somite stages (Figure 4A; [3]). Surprisingly, in about two thirds of mutant embryos, Pitx2 was bilaterally expressed in the LPM (Figures 4B–4D). In four cases (19%), Pitx2 was absent from the LPM but expressed normally in the head, and three embryos (14%) showed normal expression of Pitx2 (data not shown). This marker gene analysis demonstrates that Pkd2 is required for the asymmetric expression of the nodal cascade in the left LPM.

Bilateral expression of Pitx2 was previously reported in knockout embryos for Ebafl, SPC4, and Smad5 [20–22]. In contrast to Pkd2+/−/LacZ−/− embryos, nodal and Leftb were bilaterally expressed in these mutants as well. Most significantly, SPC4 [21] and Ebafl [20] mutant embryos were characterized by left pulmonary isomerism, suggesting that bilateral symmetrical expression of Pitx2 was inconsistent with the right pulmonary isomerism that we detected in Pkd2+/−/LacZ−/− embryos. Close examination of Pitx2 in situ hybridization signals revealed that in Pkd2+/−/LacZ−/− embryos Pitx2 expression did not extend as far anteriorly as in heterozygous and wild-type embryos (compare Figure 4A with Figures 4B–4D). Serial transverse sections confirmed that in knockout embryos the anterior LPM was negative for Pitx2 transcripts, and signals in the heart were greatly reduced or absent (Figures 4A’–4A’’ and 4B’–4B’’; data not shown). At E10.5–E11, Pitx2 was expressed in the left lung bud of Pkd2+/−/LacZ−/− embryos (Figure 4E), while no signals were detectable in homozygous mutant Pkd2 lung buds (Figure 4F). Thus, as in all other cases known,
lack of Pitx2 expression in the budding lung was linked to right pulmonary isomerism in the Pkd2 mutant.

The marker gene expression observed in Pkd2 knockout embryos challenges some aspects of our present understanding of L-R axis specification. For example, it is currently held that nodal induces Pitx2 in the LPM. Gene expression patterns of nodal and Pitx2, however, were not strictly linked in some other mouse mutants acting upstream of the nodal cassette as well, like in shh, FGF8, or SPC4 mutant embryos [21, 23]. Furthermore, a mirror-image uncoupling of nodal and Pitx2 expression was previously observed in Ebaf knockout embryos, where nodal was bilaterally expressed in the LPM, yet bilateral Pitx2 was restricted to a domain anterior of the septum transversum, and expression in the gut was normal left-sided [20]. Thus, expression in the body wall and in the medial and posterior part of the gastrointestinal tract seems to be induced independently of nodal, while the anterior domain of Pitx2 activity including the lung primordium might be under control of the nodal signaling cascade. Uncoupling of Pitx2 activity and nodal signaling was also reported in the zebrafish, where complete removal of nodal in Cyclops/Squint double mutants did not fully block asymmetric Pitx2 transcription in the diencephalon [24]. The 65% inverted gut asymmetry is intriguing and correlates surprisingly well with the frequency of bilateral Pitx2 expression. In Ebaf mutant embryos where gut Pitx2 was normal, no inversions of gut rotation were reported [20]. A critical role of Pitx2 gene dosage on gut situs was inferred from analysis of hypomorphic Pitx2 alleles [25]. In addition, right-sided misexpression of Pitx2 resulted in gut rever-

Figure 3. Expression of shh, Foxa2, nodal, and Ebaf/Leftb in Pkd2 Mutant Embryos

(A and B) The embryonic midline (floorplate, fp; notochord, nt) was present and expressed normal levels of shh (A and A') and Foxa2 (B and B') mRNA. The approximate planes of sections are indicated in (A) and (B); fg, foregut. (C and D) nodal mRNA was present around the node (n) but absent from the left LPM of Pkd2+/--LacZ/+/- embryos at the 3 to 6 somite stage. (E and F) Ebaf/Leftb was not expressed in the left prospective floorplate (l-pfp) and LPM of Pkd2+/--LacZ/+/- embryos at the 3 to 6 somite stage. Nonradioactive whole-mount in situ hybridization of Pkd2+/--LacZ/+/- (C and E) and Pkd2+/--LacZ/+/- (A, B, D, and F) embryos. (C–F) Ventral views of embryos. I, left; r, right.
Figure 4. Pitx2 Expression in Pkd2 Mutant Embryos

(A–D) Pitx2 is bilaterally expressed in the LPM of homozygous mutant embryos. However, the anterior limits of bilateral Pitx2 expression in Pkd2−/−LacZ/− embryos were more posterior compared to the heterozygous embryo shown in (A) (arrows in [B]–[D]) and histological transverse sections of embryos (A and B). The approximate planes of sections shown in (A′–A′′) and (B′–B′′) are indicated. Note that in Pkd2−/−LacZ/−LacZ/−LacZ/− embryos, heart looping was normal (B), inverted (C), or aberrant (D), such that the heart tube stayed in the midline (see also left view of heart in inset of panel [D]). The outline of the hearts is marked by black dots in (A)–(D). (E and F) Pitx2 was absent from the left lung bud (llb) of homozygous mutant embryos (E), while normal left-asymmetric expression was found in heterozygous embryos at E10.5 (F). Planes of sections are indicated in insets. Nonradioactive whole-mount in situ hybridization of Pkd2−/−LacZ/− (A and E) and Pkd2−/−LacZ/−LacZ/−LacZ/− (B–D and F) embryos.

l, left; llb, left lung bud; lpm, lateral plate mesoderm; myc, myocardium; r, right.

Both inv and polaris are required for the structural and functional integrity of monocilia on ventral cells of the mouse node. It was hypothesized that bilateral symmetry in mouse is broken by unidirectional rotation of node monocilia resulting in asymmetric distribution of secreted factor(s) [4]. In polaris mutants, cilia are absent [26], while cilia in inv are characterized by aberrant rotation [29]. Interestingly, polaris and pkd2 localize to cilia of male-specific sensory neurons in C. elegans and are required for the highly stereotypic male mating behavior [30, 31]. Polaris mutant worms lack sensory cilia, demonstrating a conserved ciliogenic role for this protein [32, 33], while pkd2 mutants retain structurally normal cilia [31]. It remains to be seen if Pkd2 in mouse localizes to and functions on node monocilia and whether its primary mode of action is via the regulation of inv function.

Supplementary Material

Supplementary Material including the Experimental Procedures and a figure showing the nonlaterality phenotypes of Pkd2 knockout embryos is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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