Differentiation (2005) 73:425–432 DOI:10.1111/j.1432-0436.2005.00048.x

ORIGINAL ARTICLE

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Lack of a laterality phenotype in *Pkd1* knock-out embryos correlates with absence of polycystin-1 in nodal cilia

Received August 12, 2005; accepted in revised form September 23, 2005

Abstract The invariant asymmetric placement of thoracic and abdominal organs in the vertebrates is controlled by the left-asymmetric activity of the Nodal signaling cascade during embryogenesis. In the mouse embryo asymmetric induction of *nodal* is thought to be dependent on functional monocilia on the ventral node cells and on the *Pkd2* gene, which encodes the calcium channel polycystin-2 (PC2). In humans mutations in *PKD2* and *PKD1* give rise to polycystic kidney disease. The PC1 and PC2 proteins are thought to function as part of a multifactorial complex. Localization of both proteins to the primary renal cilium suggested a function on cilia of the ventral node. Here we investigated *Pkd1* knock-out embryos for laterality defects and found wild-type organ morphogenesis and normal expression of *nodal* and *Pitx2*. While PC2 localized to nodal cilia, no ciliary localization of PC1 was detected in mouse embryos. This finding was confirmed in an archetypical mammalian blastodisc, the rabbit embryo. Thus, absence of PC1 localization to cilia corresponded with a lack of laterality defects in *Pkd1* knock-out embryos. Our results demonstrate a PC1-independent function of PC2 in left–right axis formation, and indirectly support a ciliary role of PC2 in this process.

Key words left–right asymmetry · *Pkd1* · *Pkd2* · polycystin 1 · polycystin 2 · cilia · mouse · rabbit · embryo

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Introduction

Many organs of the vertebrate thorax and abdomen, such as heart, lung, and the gastro-intestinal tract, display marked asymmetries with respect to the embryonic midline (Situs solitus; Capdevila et al., 2000; Levin, 2005). With the exception of mirror-image inversions (Situs inversus totalis), which do not lead to adverse health conditions, defects in correct organ placement invariably result in severe organ malfunctions (Capdevila et al., 2000; Levin, 2005). The first consistent observable molecular asymmetry in vertebrates is presented by the transcription of the growth factor gene *nodal* in the left lateral plate mesoderm (l-LPM; Lowe et al., 1996). Nodal signaling induces three known target genes, *nodal* itself, the feedback inhibitor *Lefty2,* and the homeobox transcription factor *Pitx2* (Hamada et al., 2002; Schier, 2003). Experimental manipulation of the Nodal cascade results in laterality defects and situs abnormalities. In all cases these are preceeded by abberant expression patterns of *nodal*, *Lefty2,* and *Pitx2*, placing the Nodal cascade at the center of left–right (LR) axis development (Schier, 2003; Levin, 2005). A cilia-driven directional fluid flow in the extracellular

space at the mouse node was proposed as mechanism for symmetry breakage upstream of *nodal* (''nodal flow''; Nonaka et al., 1998). Absence of cilia or altered motility correlates with LR defects in embryos with mutant motor or ciliogenic proteins (Hamada et al., 2002; Yost, 2003; Praetorius and Spring, 2005).

We have previously identified the calcium ion channel polycystin-2 (PC2), encoded by the *Pkd2* gene, as a critical LR determinant upstream of Nodal signaling in the mouse embryo (Pennekamp et al., 2002). *Pkd2* knock-out embryos were characerized by right lung isomerism, i.e. mirror-image duplication of the right lung on the left side, randomization of heart looping and placement of stomach and spleen (Pennekamp et al., 2002). Asymmetry of marker gene expression was lost: *nodal* and *Lefty2* were not expressed in the l-LPM, while *Pitx2* was absent anteriorly and induced bilaterally in the posterior two-thirds of the LPM (Pennekamp et al., 2002).

Recent reports in mouse, chick, and zebrafish have demonstrated a role of calcium signaling in vertebrate LR development (McGrath et al., 2003; Tabin and Vogan, 2003; Yost, 2003; Raya et al., 2004; Shimeld, 2004; Sarmah et al., 2005). In mouse a left-asymmetric calcium signal at the node was dependent on PC2 (McGrath et al., 2003). Together with the ciliary localization of PC2 this has led to the proposal of two types of cilia: (a) flow-generating motile cilia at the center of the node, and (b) PC2-dependent mechanosensory cilia at the periphery (McGrath et al., 2003; Tabin and Vogan, 2003). The latter would transform the directional extracellular fluid flow generated by the former into an asymmetric intracellular calcium signal, which in turn would activate the Nodal cascade (2-cilia model of nodal flow; McGrath et al., 2003; Tabin and Vogan, 2003).

PC2 has been proposed to act as a component of a large multiprotein complex, with polycystin-1 (PC1) as the second fixed constituent (Delmas, 2004, 2005). A heteromeric PC1/PC2 complex has been described in tissue culture and in yeast (Qian et al., 1997; Nauli et al., 2003; Delmas, 2005). A functional interaction between PC1 and PC2 is supported by genetic, physiological, and biochemical data in a variety of biological systems, from unicellular eukaryotes (yeast) to the nematode *Caenorhabditis elegans* up to mammalian organisms including humans (Arnaout, 2001; Barr et al., 2001; Newby et al., 2002; Nauli et al., 2003; Delmas, 2005). In the nematode *C. elegans* PC1 and PC2 colocalize to cilia of sensory neurons and act in the same pathway which determines male mating behavior (Barr et al., 2001). Mutations in single genes displayed identical phenotypes indistinguishable from double mutants (Barr et al., 2001). In humans mutations in *PKD1* or *PKD2* result in progressive renal cyst formation, a syndrome with an incidence of 1:1,000 in the general population referred to as autosomal dominant polycystic kidney disease (ADPKD; Arnaout, 2001). Disease manifestations in patients are very similar, although not identical, in particular with respect to age of onset and exact clinical presentation (Arnaout, 2001; Delmas, 2005). The respective *Pkd1* and *Pkd2* knock-out mice share renal cyst formation during embryogenesis, indicating a common role in kidney morphogenesis (Qian et al., 1997; Wu et al., 2000; Lu et al., 2001; Muto et al., 2002; Pennekamp et al., 2002). Based on these studies and on our previous demonstration of PC2 as a critical laterality determinant we wondered whether PC1 was required for LR axis formation during mouse embryogenesis.

Methods

Animals

Staged embryos were isolated from matings of $Pkd1^{+/-}$ heterozygous animals (Muto et al., 2002). Adult animals and embryos were genotyped by PCR. Genomic DNA was isolated from tail biopsy fragments and yolk sacs following standard procedures. The wild-type *Pkd1* allele was amplified using the following primer pair: forward 5′-CAGAGGCATTGATTCTGCTC-3′, reverse 5′-CACAACCTACTTCAGACGCT-3′. Primers for amplification of the knock-out *Neo*-allele were forward 5′-CCTGATGCTCTTCG-3′, and reverse 5′-GATGCCCCATTGAA-3′. In both cases 35 cycles of amplification were used at 95°C/60″, 55°C/30″, 72°C/50″.

Cloning of rabbit *Pkd* gene fragments

For primer design, mouse and human cDNA and/or genomic sequences were aligned. Typically four to six primers were selected per gene and were used in different combinations. PCR was performed on genomic rabbit DNA, and products of the correct size were cloned and sequenced on both strands. Successful conditions were as follows: *Pkd1*, 5′-CCTACCACTGGGACTTTGGGG-3′, 3′-GTGCCTTGCAGGAC ACACACTC-5′, 40 cycles at 95°C/30″, 60°C/1′, 72°C/1′, resulting in amplification of a 855 bp fragment, corresponding to nucleotides 6,575–7,429 of the mouse cDNA (accession number AM049159); *Pkd2*, 5′-TCTCTGGGGAACAAGA CTCATGGA-3′, 3′-CTCACAGGCTGCCAGGAAG AAATC-5′, 40 cycles at 95°C/30″, 58.2°C/1′, 72°C/1′, resulting in amplification of a 835 bp fragment corresponding to nucleotides 674–1,508 of the mouse cDNA (accession number AM049160).

RNA expression analysis by *in situ* hybridization (ISH)

Isolation of mouse and rabbit embryos, and analysis of gene expression by ISH followed standard procedures (Fischer et al., 2002). To visualize the low-level ubiquitous transcription of *Pkd* genes during early rabbit embryogenesis, wash steps were prolonged up to five times, and color development was allowed to continue at room temperature for up to 2 weeks. For histological examination stained embryos were sectioned using a vibratome as described (Fischer et al., 2002).

Protein expression analysis by immuno-histochemistry (IHC)

Embryos were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton in PBS, blocked in CAS Block (Zymed, Berlin, Germany) and subsequently incubated with antibodies as specified. PC1: goat polyclonal anti-PC1 antibody (P-15, 1:50, Santa Cruz,

Heidelberg, Germany), followed by a Cy3-labeled sheep anti-goat IgG antibody (1:200, Sigma, Taufkirchin, Germany), a mouse monoclonal anti-acetylated tubulin antibody (1:700, Sigma), and a Cy2-labeled goat anti-mouse IgG antibody (1:100, Sigma). PC2: Rabbit polyclonal anti-PC2 antibody YCC2 (directed against amino acids 687–962 of recombinant human PC2 protein, 1:400 (Cai et al., 1999), followed by a Cy3-labeled anti-rabbit IgG antibody (1:200, Sigma), a mouse monoclonal anti-acetylated tubulin antibody (1:700, Sigma), and a FITC-labeled goat anti-mouse IgG antibody (1:100, Sigma). Embryos were mounted in Moviol and imaged on a confocal laser-scanning microscope (LSM 5 Pascal; Zeiss, Oberkochen, Germany).

Results

Situs solitus of organ primordia in *Pkd1* knock-out embryos

Pkd1 knock-out embryos, which were generated by a deletion of exons 2–6, are embryonic lethal at day E15.5 (Muto et al., 2002). Asymmetric organ morphogenesis therefore was analyzed on days E12.5, E13.5, and E14.5. Heterozygous mice were interbred and embryos were genotyped by PCR (see "Methods"). A total of 18 homozygous $Pkd1^{-/-}$ embryos were investigated, three at E12.5, 10 at E13.5, and five at E14.5. In parallel 61 heterozygous and wild-type embryos were analyzed. Heart looping resulted in correct placement and asymmetric morphogenesis (Figs. 1A–1C), the lung was characterized by four lobes on the right and one lobe on the left side (Figs. 1A–1C), stomach and spleen were invariably found on the left side (Figs. 1D–1F), lobation of the liver was normal (Figs. 1D–1F), and asymmetric coiling of large and small intestine were unaltered in all three genotypes (not shown). Thus, in all cases asymmetric organ morphogenesis of knock-out embryos (Figs. 1C,1F) was indistinguishable from heterozygous (Figs. 1B,1E) and wild-type specimens (Figs. 1A,1D). In addition, embryonic turning at E10.5 was unaffected in $Pkd1^{-/-}$ embryos (not shown). Taken together no *situs* defects were recorded in *Pkd1* mutant embryos.

Left-asymmetric expression of *nodal* and *Pitx2* in the LPM of *Pkd1* knock-out embryos

As *nodal* and *Lefty2* are only transiently expressed during embryogenesis and are no longer transcribed when organ primordia develop asymetrically (Blum et al., 1999), and because a direct role of *Pitx2* was only demonstrated for lung asymmetry (Gage et al., 1999), we wondered whether asymmetric marker genes expression was altered in $Pkd1^{-/-}$ embryos. *Pitx2* was analyzed in three $Pkd1^{-/-}$ embryos at the six to eight somite stage, and *nodal* in four homozygous mutant embryos at the four to six somite stage. In parallel 20 heterozygotes and wild-type embryos were stained for *Pitx2* and *nodal*. As depicted in Fig. 2, left-asymmetric expression in the LPM was found for *nodal* (Figs. 2A–2C) and *Pitx2* (Figs. 2D–2F) in all cases. As the *Pkd2* knock-out mouse has demonstrated an independent regulation of *Pitx2* in the anterior third of the LPM (Pennekamp et al., 2002), the cranial–caudal extension of the LPM signal was carefully investigated. No differences were detected between whole-mount-stained knock-out (Figs. 2C,2F) and heterozygous, or wild-type embryos (Figs. 2A,2B,2D,2E), as well as in histological vibratome sections (not shown). The lack of a situs defect thus was paralleled by wild-type expression patterns of asymmetric marker genes in $Pkd1^{-/-}$ embryos. Taken

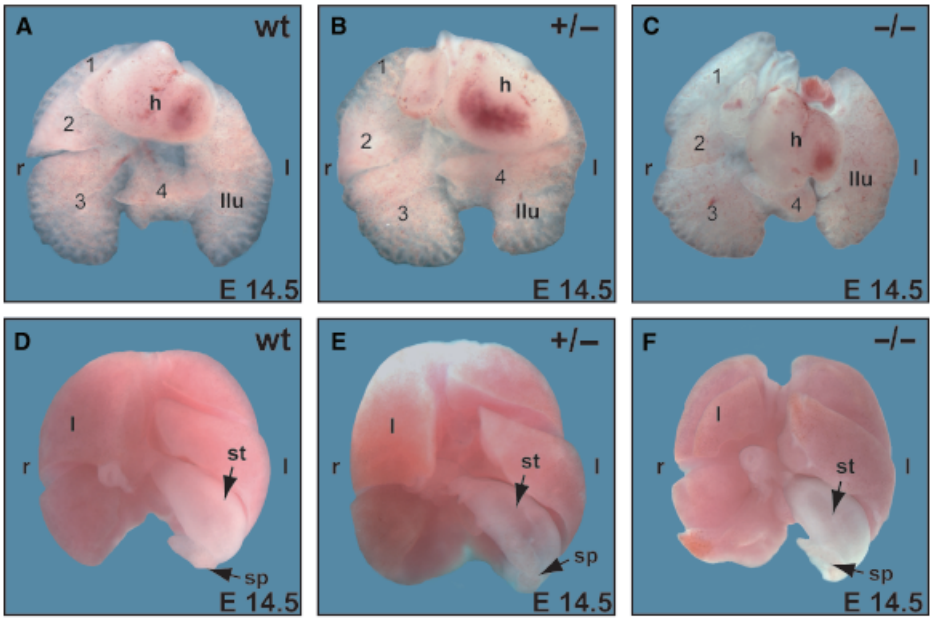

Fig. 1 Situs solitus in *Pkd1* knock-out embryos. Assessment of organ laterality in E14.5 embryos. (**A**–**C**) Normal lobation of the lung and positioning of the heart in wild type (A), heterozygous (B), and *Pkd1* knock-out embryos (C). (**D**–**F**) Left-asymmetric placement of stomach and spleen, and normal liver morphology in wild-type (D), heterozygous (E), and *Pkd1* knock-out embryos (F), h, heart; l, left; llu, left lung; r, right; sp, spleen; st, stomach; wt, wild type, 1–4, lobes of the right lung.

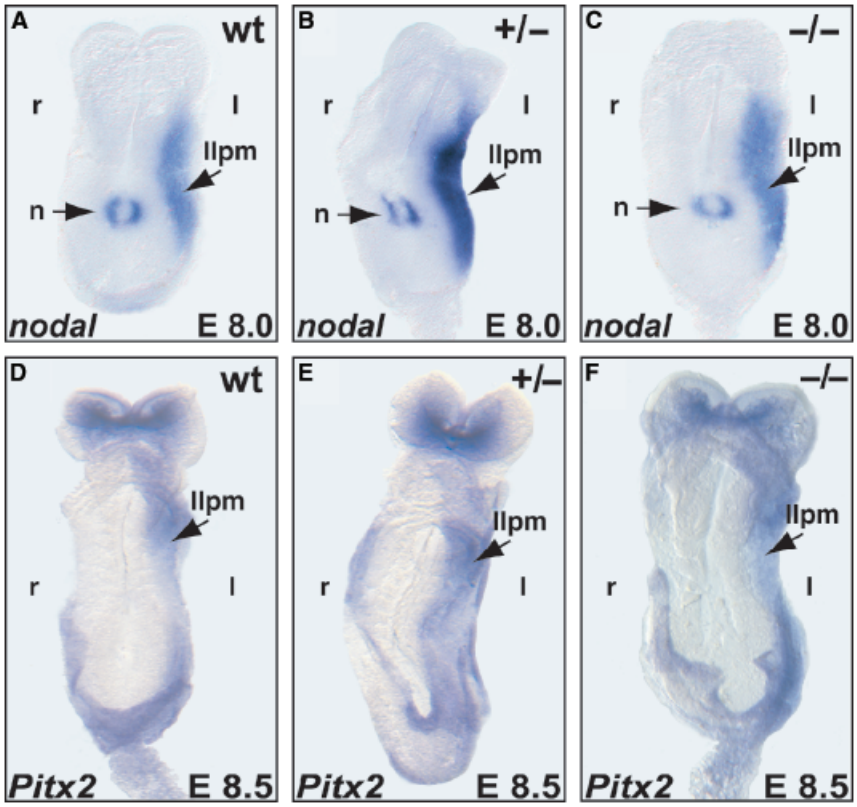

Fig. 2 Normal expression of *nodal* and *Pitx2* in *Pkd1* knock-out embryos. Whole-mount *in situ* hybridisation (ISH) of genotyped embryos. (**A**–**C**) Left-asymmetric transcription of *nodal* in the lateral plate mesoderm (LPM) of wild type (A), heterozygous (B), and *Pkd1* knock-out embryos (C) at the 4–6 somite stage. (**D**–**F**) Unaltered expression of *Pitx2* in the LPM of wild-type (D), heterozygous (E), and *Pkd1* knock-out embryos (F) at E8.5. l, left; llpm, left lateral plate mesoderm; n, node; r, right; wt, wild type.

together, this analysis demonstrated a PC1-independent function of PC2 in LR axis formation.

Ciliary localization of PC2, but not of PC1, at the mouse node

Pkd1, like *Pkd2*, is ubiquitously expressed at low levels from cleavage stages through somitogenesis in the mouse (Guillaume and Trudel, 2000; Pennekamp et al., 2002; and data not shown). Based on the co-localization of PC1 and PC2 on cilia from *C. elegans* sensory neurons to human renal epithelial cells, the surprising absence of a laterality phenotype in $Pkd1^{-/-}$ embryos raised the question of the subcellular localization of PC1. Wild-type mouse embryos from headfold stage to early somitogenesis were therefore analyzed for protein expression, using specific antibodies directed against PC1 and PC2. To visualize nodal cilia, embryos were double stained with an antibody against acetylated tubulin. Figure 3A shows ciliary localization of PC2 at the mouse node in the E7.75 (late headfold) embryo. A clear co-localization of PC2 and tubulin was detected at the periphery of the node. Central cilia frequently lacked a specific PC2 signal. In contrast, no indication of ciliary PC1 localization was detected (Fig. 3C). PC1 signals were restricted to a punctate staining in the surrounding endodermal cells, which was not further characterized (Fig. 3C). Thus, the lack of a laterality phenotype in $Pkd1^{-/-}$ embryos correlated with absent ciliary PC1 localization.

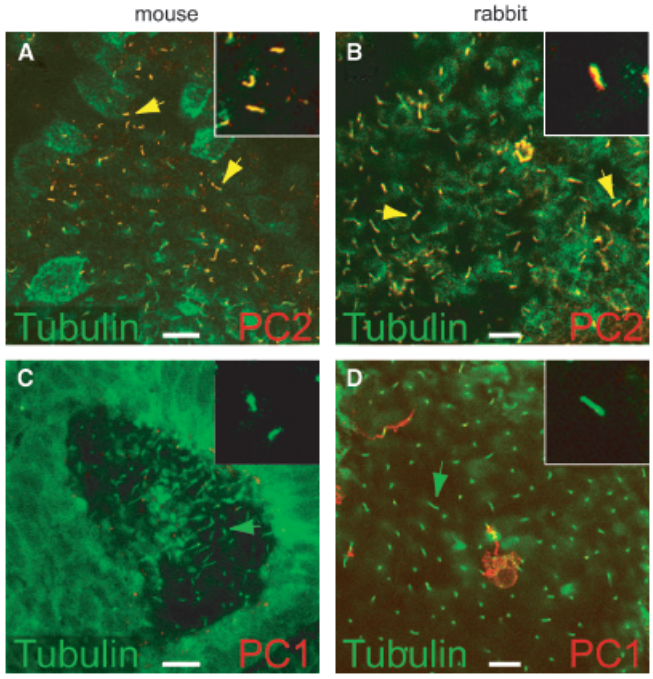

Fig. 3 Selective localization of polycystin-2 (PC2) and not PC1 to monocilia in mouse and rabbbit embryos. Whole-mount immunohistochemistry of wild-type mouse (**A**, **C**) and rabbit embryos (**B**, **D**). Late headfold embryos were double stained for acetylated tubulin (green) and PC1 (red; A, B) or PC2 (red; C, D). Insets show higher magnifications of cilia. Note that a clear PC2 signal was found on cilia in mouse (C) and rabbit (D), while no indications of ciliary localization of PC1 were detectable (A, B).

Cloning and expression analysis of *Pkd1* and *Pkd2* in the rabbit embryo

The cup-shaped headfold mouse egg cylinder does not represent the archetypical mammalian flat blastodisc architecture at the corresponding developmental age (Viebahn et al., 1995). As our previous work has demonstrated fundamental functional differences with respect to FGF8 function in LR development between mouse and rabbit (Fischer et al., 2002), we wondered whether the *Pkd* gene family members were active in the same embryonic tissues in the rabbit blastodisc. Gene fragments of *Pkd1* and *Pkd2* were cloned by RT-PCR. Sequence analysis showed high degrees of conservation (89%–93%) between rabbit, mouse, and human sequences in all cases (not shown). In order to study gene expression patterns, whole-mount ISH was performed on rabbit blastodiscs between node formation (Figs. 4A,4D) and somitogenesis (Figs. 4B,4E). Both genes showed expression at low but clearly detectable levels (compared with absence of signals in embryo hybridized to sense control probes; Figs. 4C,4F). Apparently stronger signal intensities, for example in somites (Figs. 4B,4E) or at the anterior primitive streak (Figs. 4A,4D), were not confirmed in histological sections and thus rather represent differences in tissue thickness (not shown). Taken together, *Pkd* family genes show indistinguishable expression patterns in the mouse egg cylinder and the rabbit blastodisc.

The ubiquitous transcription of *Pkd* genes in rabbit as in mouse precludes predictions as to the temporal and spatial function of the encoded proteins. We therefore wondered whether the differential ciliary polycystin localization in the mouse egg cylinder was also detectable in the rabbit blastodisc. Immunohistochemistry clearly confirmed ciliary localization of PC2 in the corresponding region of the rabbit embryo (Fig. 3C). PC1, as in mouse, was not localized to cilia (Fig. 3D). PC1-specific signals were found in a punctate pattern in the floor plate and endodermal cells, which was not further characterized and may represent localization to intracellular vesicles (not shown). In summary, the analysis of polycystin gene and protein expression in the rabbit confirmed data in mouse, suggesting a conserved ciliary function of PC2 in mammalian LR axis formation.

Discussion

The complete absence of a laterality phenotype in *Pkd1* knock-out embryos was unexpected, despite the fact that PC1 and PC2 display only limited sequence

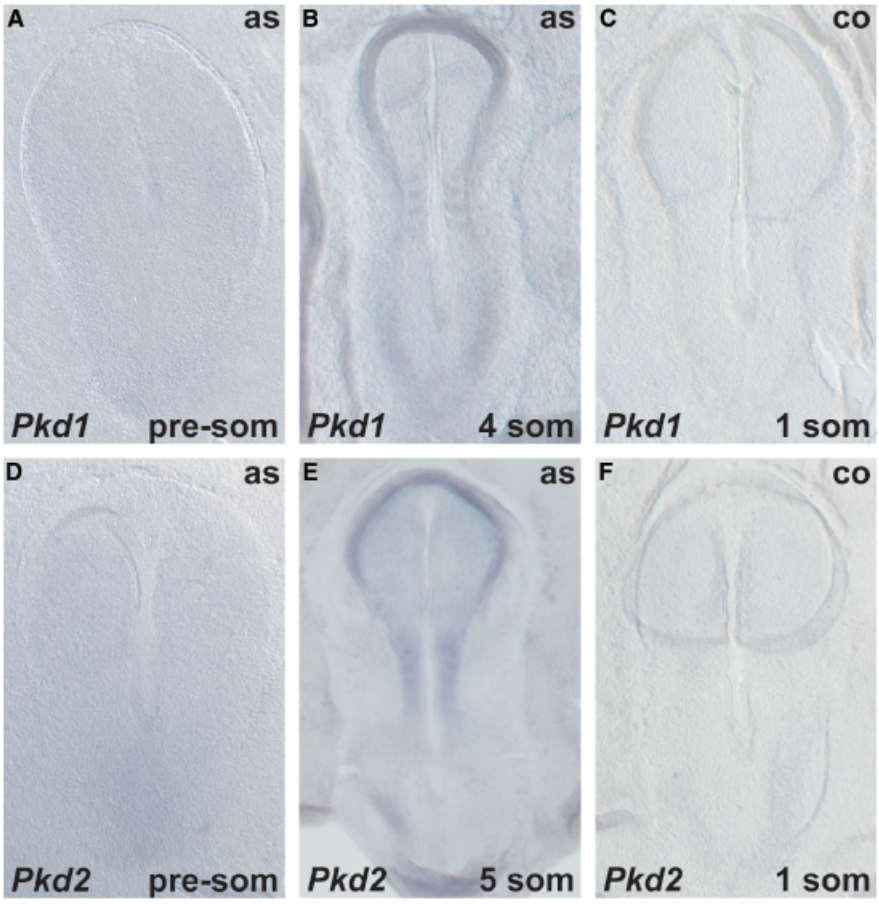

Fig. 4 Transcription of *Pkd1* and *Pkd2* during early rabbit embryogenesis. Whole-mount *in situ* hybridisation of rabbit embryos using antisense (**A**, **B**, **D**, **E**) and sense control probes (**C**, **F**) specific for *Pkd1* (A–C) and *Pkd2* (D–F). (A, D) Presomite embryos. (B, C, E, F) four to five somite embryos, as, antisense probe; co, control sense probe; som, somite.

similarity. Although the exact clinical manifestations of human ADPKD patients vary dependent on the mutated gene (Arnaout, 2001), and PC1 has only tentatively been assigned to the family of transient receptor potential (TRP) channel proteins (Delmas, 2005), mutations in both proteins have resulted in defects in the same biological pathways, i.e. male mating behavior in *C. elegans* (Barr et al., 2001) and renal tubule development and maintenance in vertebrates (Arnaout, 2001; Delmas, 2004, 2005). The demonstrations of co-assembly of a functional ion-channel complex of PC1 and PC2 upon co-expression in chinese hamster ovary cells (Hanaoka et al., 2000), and upon reconstitution at the cell surface of sympathetic neurons (Delmas et al., 2004) had further supported the hypothesis that PC1 was required for the PC2-dependent asymmetric calcium signal as well. Our phenotypic and molecular assessment of laterality in *Pkd1* knock-out embryos, however, clearly precludes a role of PC1 in LR asymmetry, thus rendering LR axis formation the first PC2-dependent biological process in which PC1 is not involved at all.

The ubiquitous low-level transcription and co-expression of *Pkd1* and *Pkd2* throughout early development in the rabbit embryo confirms earlier reports in mouse, in which embryos from cleavage stages up to neurulation were analyzed (Guillaume et al., 1999; Guillaume and Trudel, 2000; Pennekamp et al., 2002; C. Karcher and M. Blum, unpublished results). Differential spatio-temporal expression patterns of *PKD1* and *PKD2* were described, however, during fetal human kidney development (Chauvet et al., 2002). For example, while co-expressed in mesonephric tubules, *PKD2* but not *PKD1* was found in the ureteric bud and uninduced metanephros. These data were taken as indications for interaction of PC1 and PC2 with different partner proteins during nephrogenesis (Chauvet et al., 2002). The weak signal intensities of our non-radioactive whole-mount ISH study probably would not allow the detection of subtle differences. Histological sections of hybridized embryos have not revealed any differential patterns (not shown). While concordant expression of mRNA and protein were reported in human embryos (Chauvet et al., 2002), our immunohistochemical localization of PC2 but not PC1 on nodal cilia highlights the importance of the subcellular distribution of PC proteins. The singular absence of PC1 on nodal cilia contrasts with co-expression of PC1 and PC2 on cilia of renal cells (Pazour et al., 2002; Nauli et al., 2003). As PC1 and PC2 are functionally required on renal cilia for correct kidney development and function, it is tempting to speculate that *Pkd1* knock-out embryos lack a LR phenotype because PC1 has no function on nodal cilia.

The exact role of *Pkd2* in LR development has yet to be determined. The localization of PC2 to nodal cilia in mouse and rabbit strongly supports a function in the context of the proposed "nodal-flow" mechanism of symmetry breakage. In mouse it was recently reported that ciliary motility and the ensuing leftward flow of the extracellular fluid leads to an FGF8-induced transport of vesicular particles containing retinoic acid (RA) and the signaling molecule sonic hedgehog (SHH) to the left side of the node (Tanaka et al., 2005). Upon disintegration of these vesicles an asymmetric calcium signal was induced (Tanaka et al., 2005). Inhibition of FGF8 signaling prevented this calcium signal, which was rescued by exogenous RA and SHH (Tanaka et al., 2005). This scenario predicts that RA and/or SHH signaling should be involved in gating of the PC2 calcium channel, a possibility, which has not been addressed experimentally so far. In the alternative "2-cilia" model PC2 was proposed to act in a mechanotransduction pathway: motile monocilia at the center of the node would produce a directed extracellular fluid flow which would be sensed by cilia at the periphery and be transduced into an intracellular calcium signal via PC2 (McGrath et al., 2003; Tabin and Vogan, 2003; Yost, 2003). The exact PC2 mechanism in LR axis formation remains to be elucidated, and may be more accessible in model systems which allow easier experimental manipulations such as zebrafish and *Xenopus*.

Our results indicate that PC2 acts as a calcium channel protein—be it mechanosensitive or not—on ciliary membranes independent of PC1. Also, and in contrast to renal epithelial cells, ciliary localization of PC2 on node cells should be independent of PC1. PC2 may, however, require other co-factors for LR-specific ciliary functions. A particularly interesting candidate could be Inversin, a large protein containing ankyrin repeats and calmodulin binding sites (Mochizuki et al., 1998; Morgan et al., 1998). Loss-of-function in the *inv*/*inv* mouse mutant results in situs inversion and renal cyst formation (Yokoyama et al., 1993). The homologous human *INVS* gene has also been involved in LR asymmetry, and mutations in *INVS* cause nephronophthisis type 2, an autosomal recessive cystic kidney disease (Otto et al., 2003). The calmodulin binding sites in Inversin were shown to be functionally important for LR development in gain-of-function experiments in the frog *Xenopus* (Yasuhiko et al., 2001). Localization to renal and nodal cilia was demonstrated with a GFP-fusion protein in tissue culture and transgenic mice (Watanabe et al., 2003). Ankyrin repeats are frequently found in TRP channel proteins (Delmas, 2005). They are considered to anchor the channel to the cytoskeleton and thus may represent a tethering mechanism that is required for mechanical gating (Delmas, 2005). PC2 notably lacks ankyrin repeats. It is thus tempting to speculate that PC2 and Inversin may interact in a calcium-dependent manner to constitute a mechanosensory complex at the ciliary membrane in the absence of PC1. Genetic and embryological experiments are underway to explore this possibility in mouse and *Xenopus*.

In conclusion, the analysis of laterality defects in the *Pkd1* knock-out mouse surprisingly has not revealed a PC1 function in LR development. Thus, the determination of the LR axis during embryogenesis is the first biological process in which a completely PC1-independent PC2 function was detected. The detailed analysis of PC2 in this process should not only help to molecularly explore the pathway of vertebrate symmetry breakage, but may also provide an assay system for the systematic dissection of PC2 protein function.

Acknowledgments The anti-PC2 antibody YCC2 was kindly provided by Yiqiang Cai and Stefan Somlo. This work was supported by a DFG grant to M.B. (SFB 495-C7).

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