Polycystin-2 expression is developmentally regulated

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1Department of Pathology, Renal Pathology Laboratory, Columbia Presbyterian Medical Center, New York 10032; and 2Department of Medicine, Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York 10461

Markowitz, Glen S., Yiqiang Cai, Li Li, Guanqing Wu, Llewellyn C. Ward, Stefan Somlo, Vivette D. D'Agati. Polycystin-2 expression is developmentally regulated. Am. J. Physiol. 277 (Renal Physiol. 46): F17–F25, 1999.—PKD2 encodes a protein of unknown function that is mutated in 15% of autosomal dominant polycystic kidney disease (ADPKD) families. We used polyclonal antisera against PKD2 to examine the pattern of Pkd2 expression in staged mouse embryos. Staining for Pkd2 was documented as early as the 6th embryonic day (day E6) in the embryonic ectoderm and endoderm. Low-intensity staining is seen in metanephric ureteric bud at day E12.5. By day E15.5, the adult pattern of expression is established with low level staining in proximal tubules and high level, basolateral staining in distal tubules. Pkd2 expression is first detected in the medullary collecting ducts at postnatal day 14. Outside of the kidney, Pkd2 expression is widely distributed in utero and more restricted postnatally. The greatest intensity of staining is seen in the fetal but not adult adrenal cortex and in red blood cell precursors. Expression also is seen in multiple endocrine organs, in cardiac, skeletal, and smooth muscle, and in multiple mesenchymal tissues. The diffuse distribution and early expression of Pkd2 suggest a fundamental developmental role. The persistent strong expression in adult kidney is consistent with a more organ-specific function in the maintenance of the mature metanephric tubule.

polycystin-2; renal development; autosomal dominant polycystic kidney disease

AUTOSOMAL DOMINANT polycystic kidney disease (ADPKD) is a common hereditary disorder characterized by renal tubular cysts. Progressive cyst formation and enlargement leads to loss of renal function, hypertension, and in ~50% of cases, end-stage renal disease by the age of 60 yr. Extranadal manifestations of ADPKD include cyst formation in the liver, pancreas, and spleen, as well as mesenchymal manifestations, such as mitral valve prolapse, colonic diverticulosis, and intracranial berry aneurysms (8).

ADPKD is a genetically diverse entity caused by mutations of at least three distinct chromosomal loci. The majority of patients with ADPKD (~85%) (16) have mutations in the PKD1 gene, located in 16p13.3 (1, 7, 13). This gene encodes polycystin-1, a 4,303-amino acid integral membrane glycoprotein with a 2,500-amino acid extracellular domain, 11 transmembrane spanning segments (12), and an intracellular COOH terminus that contains a coiled-coil domain (17). A role for polycystin-1 in cell-cell and/or cell-matrix interactions has been proposed (1, 7, 13).

The second gene for ADPKD, PKD2, is located in 4q21–23 and encodes a 968-amino acid integral membrane protein, polycystin-2 (14). Polycystin-2, which is mutated in ~15% of cases of ADPKD, contains six membrane spanning domains and intracellular COOH and NH2 termini. Pkd2 shares homology with PKD1 as well as a family of voltage-activated calcium channel subunits and the capacitative calcium entry channels. Evidence suggests that polycystin-2 interacts directly with polycystin-1 (17, 20). This direct interaction is consistent with the clinical observation that patients with mutations of either PKD1 or PKD2 develop an identical phenotype of renal and extrarenal disease, albeit the severity of disease resulting from mutation in PKD2 is milder (15, 19). Evidence for a third genetic locus comes from kindreds with ADPKD in which genetic linkage to PKD1 or PKD2 has been excluded (2, 5, 6, 21).

Recently, new insights into the expression and role of PKD2 have been uncovered (23). Polycystin-2 is expressed in the tubules throughout the kidney, with highest expression levels in the medullary thick ascending limb and distal tubules. These are the same nephron segments from which the majority of renal cysts arise in a mouse model of ADPKD produced by inactivating Pkd2. This model confirmed a cellular recessive mechanism requiring “second hits” to the normal Pkd2 allele, as has been proposed for human PKD1 based on studies demonstrating loss of heterozygosity in cytlining epithelial cells from ADPKD kidneys (3, 18).

Although the established pattern of PKD1 expression suggests a tight temporal and spatial regulation, no data are currently available on the developmental immunohistochemical staining profile of Pkd2. Northern blot analyses of whole organ homogenates have indicated that Pkd2 is expressed in most fetal and adult organs (14). The Pkd2−/− mice are embryonic lethal, dying between embryonic days E13.5 and E15.5, thereby confirming an essential developmental role for PKD2 (24). To investigate the role of Pkd2 expression in mouse development, we have analyzed the expression of polycystin-2 protein in embryonic and adult mice using a polyclonal antibody against Pkd2. The findings support a basic cellular function of Pkd2 in many developing tissues and a more specific role in the maintenance of differentiated tubular architecture in the mature kidney.

METHODS

Generation of PKD2 antibodies. A fusion protein, C2, was generated from amino acids 687–962 of the cytoplasmic...
COOH-terminal portion of human polycystin-2 (23). PCR primers were designed with BamH I and EcoR I linkers. The primer sequences are as follows: 5'-CGC GGA TCC GTG AAA TCT GAC TTT GCA CA-3' (forward) and 5'-CGC GAA TTC AAT TTC CAC CTG CAC CT-3' (reverse). Recombinant proteins were obtained by subcloning PCR-generated fragments into the pGEX2T expression vector (Pharmacia Biotech, Piscataway, NJ). Subclones were sequence to confirm nucleotide and reading frame fidelity to PKD2. Recombinant proteins were expressed in bacteria and purified with glutathione-agarose according to the manufacturer's protocol (Pharmacia Biotech). New Zealand rabbits were immunized with the purified fusion protein (HRP, Denver, PA). Antiserum YCC2 raised against C2 fusion protein was tested by ELISA, and only high titer antisera were used for subsequent studies. Antibody YCC2 was affinity purified using C2 polycystin-2 fragment coupled to HiTrap affinity columns (Pharmacia Biotech). Antibody specificity was confirmed by Western blot, immunoprecipitation, peptide competition, and cell transfection studies (4). In addition, an independently derived NH2-terminal antiserum, YCB9 (23), demonstrated a staining specificity and pattern identical to that seen with YCC2 (4).

PKD2 Immunohistochemistry. C57BL/6J whole mouse embryos from 12 different developmental time points ranging from day E6 to E18.5 were fixed in 10% phosphate-buffered Formalin and embedded in paraffin. In addition, all major organs were isolated from three mice at postnatal ages 1 day, 14 days, and 4 mo. Tissues were fixed in Formalin and embedded in paraffin. For each specimen, four to six paraffin sections were cut at 3 µm, rehydrated in graded alcohols and xylene, quenched for 30 min in 1% hydrogen peroxide in 80% ethanol, and then blocked with 10% normal goat serum for 20 min. Sections were then stained with the YCC2 antibody at dilutions of 1:400 and 1:800 overnight at 4°C. Subsequently, slides were washed in PBS, treated with 1% goat anti-rabbit secondary antibody for 30 min, washed, and then treated with 1% avidin-biotin-peroxidase complex for 30 min. Finally, slides were washed, incubated in diaminobenzidine color reagent for 2 min, washed, and counterstained with hematoxylin. Negative controls included serial sections stained with either 1) preimmune serum or 2) an equal concentration of rabbit IgG standard. Low level background staining was present in some control slides, and thus the grading of positivity reflects the difference between primary antibody and control staining as assessed in serial sections. Furthermore, punctate granularity with distinct subcellular distribution was required for staining to be considered positive. In selected slides, specific staining could be abrogated by preadsorption of antibody with the fusion protein (4).

PKD2 Western blots of whole organ homogenates. For Western immunoblots of whole organ homogenates, a single adult mouse was killed, and all major organs were harvested. Individual organ homogenates were prepared in SBE buffer (250 mM sucrose, 1 mM EGTA, and 10 mM HEPES-KOH, pH 7.5) containing aprotonin (90 µg/ml), benzamidine (1 mM), leupeptin (4 mg/ml), phenylmethylsulfonfyl fluoride (PMSF, 0.8 mM), p-tosyl-L-arginine methyl ester (20 µg/ml), N-α-benzoyl-L-arginine methyl ester (20 µg/ml), and soybean inhibitor of trypsin (20 µg/ml). Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of total proteins (80 µg) were solubilized from each organ in sample buffer (125 mM Tris, pH 6.8, 200 mM dithiothreitol, 6% SDS, 20% glycerol, and 0.2% bromophenol blue) and subjected to electrophoresis on SDS-PAGE gels without boiling. Fractionated proteins were electrotransferred to Polyscreen PVDF membrane (DuPont NEN Research Products, Boston, MA) and immunoblotted by PKD2 polyclonal antisera (YCC2, 1:4,000). The blotted membranes were incubated with peroxidase-conjugated secondary antibody (anti-rabbit IgG) and detected by enhanced chemiluminescence (ECL, DuPont NEN Research Products).

RESULTS

Early expression of Pkd2. At day E6, the earliest stage of development studied, strong Pkd2 expression was seen in ectoderm, parietal endoderm, and columnar cells of the extra-embryonic endoderm (Fig. 1A). Positivity was also identified in the surrounding decidualized endometrium but not in endometrial glands or stroma distant from the implantation site. By day E9.5, Pkd2 expression was identified in condensing mesenchyme of the somites (Fig. 1B) as well as in cardiac myocytes.

Expression of Pkd2 in the developing kidney. Pkd2 expression was undetectable in the mesonephros (Fig. 2A). In early metanephric development, there was low level expression in the ureteric bud, with no detectable expression in the condensing mesenchyme (Fig. 2B; Table 1). Similarly, Pkd2 was undetectable in the comma and S-shaped bodies and in developing glomerular vesicles of the nephrogenic zone. By day E15.5, the

![Fig. 1. Early embryonic expression of Pkd2. A: at embryonic day E6, Pkd2 staining is seen in primitive endoderm and ectoderm and in the immediately surrounding decidua (×300). B: at day E10.5, Pkd2 is expressed in the condensing mesenchyme of developing somites (×300).](image-url)
inner cortex revealed low level punctate cytoplasmic expression in proximal tubules and strong basolateral expression in distal tubules (Fig. 2C). Medullary collecting tubules lacked Pkd2 expression. The segment-specific staining patterns in proximal and distal tubules persisted throughout development and after birth (Figs. 2, D–F, and 3, A–E), whereas medullary collecting ducts first displayed Pkd2 expression after birth.
both in utero and postnatally (Fig. 4). The islets of Langerhans were found to express Pkd2 in fetal mice, in the mature mouse, both endocrine and mesenchymal tissues (Fig. 4). Expression of Pkd2 in endocrine tissues of endodermal and ectodermal origin. Pkd2 expression was not identified in many of the epithelial structures examined. Gastrointestinal organs transiently expressed Pkd2 in utero, although this positivity was lost after birth (Fig. 5D). Epithelial structures with no detectable Pkd2 expression included pulmonary parenchyma, the exocrine pancreas, bile ducts, hepatic parenchyma, and skin. Similarly, nondecidualized endometrium and fallopian tube epithelia showed no evidence of Pkd2 expression postnatally (although in uterine tissue examination was inadequate). Interestingly, in the adult mouse, retinal pigment epithelium and ducts within glandular epithelium of the paranasal sinuses displayed moderate to high Pkd2 expression. Within the ducts of the paranasal sinuses, the staining had a distinctly basolateral distribution, similar to that seen in the distal tubule of the kidney.

Expression of Pkd2 in hematopoietic cells. Nucleated red blood cell (RBC) precursors (normoblasts) displayed high-intensity expression of Pkd2 (Fig. 5C). This staining was seen from day E12 to birth throughout the liver, the predominant site of hematopoiesis in utero. Similarly, staining was seen in RBC precursors in the spleen both in utero and briefly in the immediate postnatal period. After birth, the major site of hematopoiesis shifts to the bone marrow, where this high-intensity expression of Pkd2 persisted in RBC precursors into adult life. This expression was confirmed by immunostaining of bone marrow aspirate smears.

Expression of Pkd2 in neural tissues. Positive staining for Pkd2 was identified in the neural tube at day E9.5; this staining was no longer detectable over background levels by day E11.5 and was not identified in the developing forebrain or spinal cord. The only neural tissue with Pkd2 expression both in fetal and adult tissue was the paraspinal ganglia, which maintained moderate levels of expression throughout development (Fig. 5B).

Western blot of Pkd2 in mouse whole organ homogenates. Pkd2 was identified in most adult mouse tissues by Western blot (Fig. 6). The highest levels of expression were identified in the kidney, lung, ovary, and uterus. Intermediate levels of expression were present in the brain, heart, liver, spleen, pancreas, and stomach. Pkd2 protein was identified at low levels in the gastrointestinal tract and skeletal muscle.

**DISCUSSION**

The aim of this study was to determine the whole body expression patterns of Pkd2 in murine development using an affinity-purified polydonal antibody to the COOH terminus of PKD2. Our findings indicate that Pkd2 expression is highly developmentally regulated, with particularly high expression levels in kidney. It is absent from the mesonephros and becomes switched on in metanephric development, where it is present at low levels in the ureretic bud, but not in the

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**Table 1. Pkd2 expression in renal development**

<table>
<thead>
<tr>
<th>Cell Type/Structure</th>
<th>In Utero Expression</th>
<th>Postnatal Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesonephros (all cells)</td>
<td>negative</td>
<td>NA</td>
</tr>
<tr>
<td>Metanephros</td>
<td>trace</td>
<td>negative</td>
</tr>
<tr>
<td>Ureteric bud</td>
<td>trace</td>
<td>negative</td>
</tr>
<tr>
<td>Condensing mesenchyme</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Comma-S-shaped bodies</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Proximal tubules</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Distal tubules/MTAL</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Cortical collecting ducts</td>
<td>NA</td>
<td>2+</td>
</tr>
<tr>
<td>Medullary collecting ducts</td>
<td>negative</td>
<td>1+</td>
</tr>
<tr>
<td>Glomeruli</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Interstitial cells</td>
<td>negative</td>
<td>negative</td>
</tr>
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Scale of scoring: negative, trace, 1-3+. MTAL, medullary thick ascending limb; NA, not applicable/not available.

(Fig. 3F). Glomeruli, interstitial cells, and small vessels were negative. In contrast to the diffusely granular cytoplasmic staining seen in the majority of organs throughout the body, renal distal tubular expression of Pkd2 was distinctly basolateral (Fig. 3C). Notably, the intensity of Pkd2 expression in distal tubules exceeded that of any other organ or tissue type. In the proximal tubule, the diffuse cytoplasmic staining observed in maturing proximal tubules was replaced by more basolateral staining in the adult kidney (Fig. 3D).

Expression of Pkd2 in mesenchymal tissues. Many developing mesenchymal tissues were found to express Pkd2 in utero, although this expression was often lost after birth (Table 2). At day E13.5 Pkd2 was highly expressed in developing cartilage of the vertebral bodies (see Fig. 5A) and bone (mandible) with subsequent reduced expression in mature cartilage and no detectable staining in mature bone. By contrast, both smooth muscle and skeletal muscle displayed reactivity in utero that persisted postnatally (Fig. 4, A–C). Of note, Pkd2 expression was greater in vascular smooth muscle than in smooth muscle of the visceral organs and the highest expression of any smooth muscle was found in the pulmonary arteries (Fig. 4B). The earliest expression in mesenchymal tissue was identified in cardiac myocytes (Fig. 4A), where the intensity of cardiac staining was strongest at day E9.5 and subsequently fell to lower expression levels that persisted postnatally.

Expression of Pkd2 in endocrine tissues. In contrast to the minimal expression of Pkd2 in most epithelial tissues, Pkd2 positivity was generally strong in endocrine tissues (Fig. 4, D–F; Table 2). The intensity of staining in the fetal adrenal cortex (Fig. 4D) was among the most intense Pkd2 staining seen in any tissue; this positivity was abruptly lost postnatally at a time when fetal adrenal cortex is replaced by adult adrenal cortex. Although the pituitary gland was not identified in fetal mice, in the mature mouse, both chromophilic and chromophobic cells stained with moderate intensity for Pkd2 (Fig. 4E). Within the pancreas, the islets of Langerhans were found to express Pkd2 both in utero and postnatally (Fig. 4F). Postnatal staining for Pkd2 was also seen in testicular germ cells and Sertoli cells as well as in the corpus luteum of the ovary.

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uninduced mesenchyme. Although no expression could be detected in the condensing mesenchyme at the tips of the ingrowing ureteric buds or in the S-shaped bodies, there was strong expression in the maturing distal tubules and to a lesser degree in the proximal tubules of the elongating nephron. Whereas medullary collecting tubules displayed no detectable Pkd2 expression in fetal kidney, moderate collecting duct expres

Fig. 3. Pkd2 expression in adult kidney (4 mo). A: whole mount of kidney showing staining limited to distal nephron segments of cortex, medullary thick ascending limbs of inner stripe of outer medulla, and collecting tubules of papillary tip (×10). B: high-power view (×315) of cortex showing strong basolateral staining of distal convoluted tubules and macula densa with weaker punctate staining in proximal tubules. Staining is not identified in the glomerulus. C: distal tubules ("D") display distinctly basolateral staining. A thinner line of basal positivity is seen in a cortical collecting duct ("C") (×475). D: proximal tubules have delicate basolateral positivity; a distal tubule is present at top left for comparison (×475). E: abrupt transition is seen at the junction of inner stripe of outer medulla and inner medulla corresponding to the transition from medullary thick ascending limbs (positive) to thin limbs of Henle (negative) (×200). F: collecting ducts of papillary tip have strong granular basolateral expression of Pkd2 (×475).
The subcellular pattern of Pkd2 expression is cytoplasmic and punctate/granular in the majority of organs sampled. The two major exceptions are the renal tubules, where staining is distinctly basolateral, and RBC precursors, where staining is perinuclear. This distribution suggests a distinct subcellular compartmentalization that may be plasma membrane associated in the case of renal tubules or endoplasmic reticulum/Golgi associated in the case of RBC precursors. Both the plasma membrane and subcellular organelar membranes are specialized cellular compartments engaged in processes that require transmembrane signaling and ion transport. This observation is of particular interest in light of the structural predictions that Pkd2 represents a transmembrane protein with homology to calcium channels and the hypothesis that it is involved in cell signaling pathways.

Pkd2 expression was documented in many organs and tissue types of the developing mouse embryo. With the exception of the metanephros, Pkd2 expression tended to be higher and more generalized in utero and more restricted postnatally. Earliest expression was seen at day E6 in all three germ cell layers, as well as in the surrounding decidualized endometrial stroma. These findings are in agreement with the description of Pkd1 expression as early as the morula stage (11), suggesting a basic role for Pkd1 and Pkd2 in early tissue patterning. Similarly, the expression of Pkd2 in early condensing mesenchyme at day E9.5 is consistent with our previous observations that Pkd1 is involved in tissue condensation and mesenchymal-to-epithelial conversion (11). Both proteins are particularly highly expressed in paraspinal ganglia, neural crest derivatives, and endocrine glands such as the adrenal cortex and medulla, with moderate expression levels in vascular and visceral smooth muscle and cardiac muscle (11). All of these tissues share a high level of autonomic innervation. Since neuromuscular and hormonal signaling are calcium-dependent processes, this tissue distribution is consistent with the hypothesis that Pkd2 may play a role in subcellular processes regulated by calcium-dependent transport or signaling events.

The extrarenal distribution of Pkd2 bears many similarities and some differences to the previous immunohistochemical reports of Pkd1 distribution in murine development. Geng et al. (9) have shown Pkd1 expression in cardiac myocytes, intestinal villi, pulmonary bronchioles, hepatic bile ducts, pancreatic ducts, choroid plexus, and ependyma. Another study has documented Pkd1 immunostaining in biliary epithelium, hepatocytes, central nervous system, choroid plexus, neural ganglia, cardiac myocytes, intestinal villi, bronchial epithelium, and skeletal muscle (10). Although we also noted Pkd2 staining in many of these organs and cell types, several major differences were observed. Of particular note is the absence of detectable Pkd2 expression by immunohistochemistry in the biliary or pancreatic ductal epithelium, sites that are predicted to express Pkd1 and Pkd2 based on the frequency of cyst formation in these structures in human ADPKD. This discrepancy can be best explained by low levels of Pkd2 expression in these tissues/cell types, levels that fall below the detection threshold for immunohistochemistry. This interpretation is supported by our detection of Pkd2 protein expression by Western blot analysis in both liver and pancreas.

The results of our immunohistochemical studies and Western blot analysis are in general agreement. By
Western blot, the highest expression of Pkd2 was seen in kidney, lung, ovary, and uterus. This high expression in kidney was confirmed by immunohistochemistry. The lungs are highly vascular organs, and some of the high pulmonary expression levels can be attributed to the observed distribution of Pkd2 in the pulmonary arterial system. The high expression in uterus and ovary are consistent with the generalized observation that Pkd2 expression is strong in endocrine tissues and autonomically innervated smooth muscle. The moderate intensity of Pkd2 expression in heart and pancreas by Western blot matches our immunohistochemical-

Fig. 4. Pkd2 expression in muscle and endocrine organs. A: at day E10.5, cardiac myocytes are diffusely positive for Pkd2 (×100). B: at day E17.5, Pkd2 staining in the lung is confined to the smooth muscle of the pulmonary vasculature (×200). C: at day E16.5, there is granular Pkd2 staining in skeletal myocytes (×320). D: at day E16.5, fetal adrenal cortex is strongly positive for Pkd2 (×320). E: at day 14, mature pituitary gland expresses Pkd2 (×320). F: at day 14, pancreatic islets of Langerhans express Pkd2. No expression is seen in the exocrine pancreas (×320).
findings in these organs. The discrepancy between the moderate expression of Pkd2 observed in spleen, stomach, liver, and brain by Western blot and the negative immunostaining results for Pkd2 in the parenchyma of these organs may be explained by the lower sensitivity of immunohistochemistry as well as to the detection of RBC precursors in liver and spleen. The low level of expression by Western blot in skeletal muscle despite significant immunohistochemical staining may relate to the notoriously rapid degradation of skeletal muscle in the course of fresh tissue dissection.

In conclusion, Pkd2 expression in the developing mouse is highly temporally and spatially regulated. Expression in all germ cell layers and surrounding decidua as early as day E6, as well as in the condensing mesenchyme by day E9.5, suggest a role for Pkd2 in early tissue condensation events, likely through cell-cell, cell-matrix interactions required for complex tissue patterning. These findings indicate a basic function of Pkd2 in cellular or subcellular processes. A more specific role for Pkd2 in maintenance of mature renal tubules appears to have evolved in the metanephros. The discrepancy between the widespread tissue distribution of Pkd2 in embryonic development and the relatively organ-restricted phenotype of ADPKD suggest probable redundancy of function with respect to this more basic cellular function(s) and the emergence of a more tissue-specific and essential role for Pkd2 in the development and maintenance of the mature metanephric tubule.

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