Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Hepato-Renal Pathology in *Pkd2*^{ws25/-} Mice, an Animal Model of Autosomal Dominant Polycystic Kidney Disease

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Polycystic liver diseases, the most important of which are autosomal dominant and autosomal recessive polycystic kidney diseases, are incurable pathological conditions. Animal models that resemble human pathology in these diseases provide an opportunity to study the mechanisms of cystogenesis and to test potential treatments. Here we demonstrate that *Pkd2*^{ws25/-} mice, an animal model of autosomal dominant polycystic kidney disease, developed hepatic cysts. As assessed by micro-computed tomography scanning of intact livers and by light microscopy of hepatic tissue, hepatic cystic volumes increased from 12.82 ± 3.16% (5- to 8-month-old mice) to 21.58 ± 4.81% (9- to 12-month-old mice). Renal cystogenesis was more severe at early stages of disease: in 5- to 7-month-old mice, cystic volumes represented 40.67 ± 5.48% of kidney parenchyma, whereas in older mice cysts occupied 31.04 ± 1.88% of kidney parenchyma. Mild fibrosis occurred only in liver, and its degree was unchanged with age. Hepatic cysts were lined by single or multiple layers of squamous cholangiocytes. Cystic cholangiocyte cilia were short and malformed, whereas in renal cysts they appeared normal. In *Pkd2*^{ws25/-} mice, mitotic and apoptotic indices in both kidney and liver were increased compared with wild-type mice. In conclusion, *Pkd2*^{ws25/-} mice exhibit hepatorenal pathology resembling human autosomal dominant polycystic kidney disease and represent a useful model to study mechanisms of cystogenesis and to evaluate treatment options. (Am J Pathol 2010; 176:1282-1291; DOI: 10.2353/ajpath.2010.090658)

The polycystic liver diseases are a group of genetic disorders that may occur alone or in combination with polycystic kidney disease. The polycystic liver diseases are characterized by the presence of hepatic cysts with or without hepatic fibrosis.¹⁻³ We recently proposed the term “cholangiociliopathies” for these conditions because they are linked to abnormalities in the function and/or structure of cholangiocyte primary cilia.^{2,4} The cholangiociliopathies include autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD), Meckel-Gruber syndrome, Bardet-Biedl syndrome, nephronophthisis, and Joubert syndrome. The most common polycystic liver diseases are ARPKD and ADPKD.² Presently there is no effective treatment for polycystic liver diseases. The availability of animal models of these conditions provides the opportunity to assess the molecular mechanisms of hepatorenal cystogenesis and to evaluate potential therapeutic interventions. Ideally, these models should be genetically orthologous with and exhibit the phenotype of human polycystic liver diseases.³

A number of animal models to study the mechanisms of cystogenesis and to evaluate potential therapeutic strategies for hepatorenal pathology have been described.⁵ A number of these models (ie, *cpk*,^{6,7} *bpk*,^{8,9} and *kat*¹⁰ mice, and *Han:SPRD-cy*¹¹ and PCK rats¹²⁻¹⁴) are spontaneous mutants while others were generated by chemical mutagenesis (*jcpk* mice),^{8,15} transgenic technologies (*orpk*¹⁶ and *inv*^{17,18} mice), or gene specific targeting (*Pkd1* and *Pkd2* mice).^{3,19-23} In several models (eg, *cpk* and *bpk* mice and the PCK rat), the hepatic and renal pathology resemble the human phenotype (ie, cellular origin of the hepatic and renal cysts, their localization, onset of disease, etc). Some models are genetically

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orthologous to their human counterparts (eg, the PCK rat and *inv* mice), while others display features of ADPKD or ARPKD, but so far no human gene syntenic to the gene mutated in animal models has been found. The majority of animal models that exhibit liver pathology (ie, *cpk*, *bpk*, *jcpk*, *orpk*, and *inv* mice, and PCK rat) are the models of ARPKD. To date, the creation of mouse models of ADPKD with a liver phenotype has been less successful. *Pkd1* or *Pkd2* heterozygote mice usually have no distinct hepatorenal cystic phenotype, while homozygotes with mutations in either *Pkd1* or *Pkd2* genes die *in utero* or perinatally.^{3,23,24}

In recent reviews,^{3,5,25,26} all available animal models to study renal and hepatic pathology have been carefully analyzed. Based on these analyses, it appears that the best models to test experimental therapies for potential treatment of hepatorenal ciliopathies are the *bpk*, *cpk*, and *Pkd2^{ws25/-}* mice, and the PCK rat.³ Both *bpk* and *cpk* mice resemble human ARPKD and are caused by mutations in genes (*Bicc1* and *Cys1*, respectively) that do not have human genetic orthologs.¹⁵ In the PCK rat, the gene, *Pkhd1*, is orthologous to the human *PKHD1*.²⁷ We have recently described in detail the hepatic pathology of the PCK rat¹⁴ and have used this model extensively to understand the mechanisms of hepatic cystogenesis and to test possible therapies.²⁸

The *Pkd2^{ws25/-}* mouse, an animal model of ADPKD, was generated by inducing two mutations in mouse homolog *Pkd2*—true null mutation (*ws183*, designated *Pkd2^{-/-}*) and an unstable recombinant-sensitive allele (*ws25*; designated *Pkd2^{ws25}*).²⁰ *Pkd2^{ws25/-}* mice develop liver cysts slowly and progressively, and to date, appear to be one of the best models to study the mechanisms of hepatic cystogenesis in ADPKD. Wu and colleagues²⁰ examined to a very limited degree hepatic phenotype in 10- to 11-week-old *Pkd2^{ws25/-}* mice (ie, number of cysts present in affected livers) while describing the development of this animal model.

In the present paper, we study in detail the liver and kidney pathology in *Pkd2^{ws25/-}* mice throughout the course of disease progression (at ages of 5 to 8 months and 9 to 12 months) with a focus on the cystic and fibrotic volumes over time, the rate of cell proliferation, apoptosis, and ciliary morphology.

Materials and Methods

Animals

Pkd2^{ws25/-} mice were developed by cross-breeding of *Pkd2^{+/-}*: *Pkd2^{ws25/+}* mice and screened by Southern blot as previously described.²⁰ No liver or kidney cysts were detected in *Pkd2^{ws25/+}* mice (*n* = 20) and they were excluded from further analysis. In total, we examined 31 *Pkd2^{ws25/-}* (17 females and 14 males) and 11 wild-type (5 females and 6 males) mice. *Pkd2^{ws25/-}* mice were divided into two groups: 5 to 8 months old (*n* = 15; 8 females and 7 males) and 9 to 12 months old (*n* = 16; 7 females and 9 males). All studies were performed after approval of the Mayo Institutional Animal Care and Use Committee. Ani-

mals were anesthetized with pentobarbital (50 mg/kg body weight, i.p.). Livers and kidneys of 6 wild-type (3 females and 3 males) and 20 *Pkd2^{ws25/-}* mice [10 females (five from each age group) and 10 males (five from each age group)] were perfused and fixed with 4% paraformaldehyde/2% glutaraldehyde in 0.1 mol/L PBS. Median and right liver lobes and left kidney from every wild-type and *Pkd2^{ws25/-}* mouse were paraffin-embedded, and sectioned at 4 μ m thick. These sections were used for immunohistochemistry and confocal microscopy. Left and caudate liver lobes and right kidney from every wild-type and *Pkd2^{ws25/-}* mouse were processed and used for transmission and scanning electron microscopy.

Immunohistochemistry

Tissue sections of median (*n* = 3) and right (*n* = 3) liver lobes (six sections per mouse) and tissue sections of left kidney (*n* = 5) of each wild-type (*n* = 6) and *Pkd2^{ws25/-}* (*n* = 20) mice were deparaffinized, hydrated in ethanol, and stained with H&E, Picrosirius red, and CK-19 (diluted 1:50; Sigma, St. Louis, MO). Five random non-overlapping fields of each tissue section were analyzed. H&E sections were used to measure cystic volumes and Picrosirius red collagen stained sections to measure fibrotic volumes. Since we did not observe any significant differences in liver and kidney weights between male and female *Pkd2^{ws25/-}* mice, for statistical analysis of hepatic and renal cystic and fibrotic volumes, they were combined. Hepatic and renal cystic and fibrotic volumes were detected using MetaMorph software (Universal Imaging, West Chester, PA) installed on a Pentium IBM-compatible computer (Del OptiPlex) following image acquisition using light microscope and color digital camera (Nikon DXM 1200). Hepatic and renal cystic/fibrotic volumes were calculated as a percentage of total liver or kidney parenchyma, respectively.

Transmission and Scanning Electron Microscopy

Left and caudate liver lobes and right kidney from each wild-type (*n* = 6) and *Pkd2^{ws25/-}* (*n* = 20) mice were cut into small pieces (2 to 4 mm³). For transmission electron microscopy, samples were post-fixed in 1% osmium tetroxide for 1 hour, rinsed in distilled water, dehydrated, embedded in Spurr's resin, and sectioned at 80 nm. Samples for scanning electron microscopy were incubated in 1% osmium tetroxide for 30 minutes, dehydrated, dried in a critical point drying device, mounted onto specimen stubs, and sputter-coated with gold—palladium alloy. Five random nonoverlapping fields of left or caudate liver lobes and five random nonoverlapping fields of right kidney of each wild-type and *Pkd2^{ws25/-}* mice were analyzed. Scanning electron micrographs were used to measure the length of cilia by Image J (NIH, Bethesda, MD). The number of cilia analyzed is provided in the Results section. Transmission electron micrographs were used to measure the height (ie, straight distance between apical and basolateral mem-

branes) and width (ie, straight distance between cell junctions) of normal (44 cells analyzed) and *Pkd2^{ws25/-}* (76 cells analyzed) cholangiocytes by Image J. Transmission electron microscopy was performed with a Joel 1200 electron (Joel USA Inc., Peabody, MA) and scanning electron microscopy with Hitachi S-4700 microscope (Hitachi Inc., Pleasanton, CA).

Three-Dimensional Reconstruction of the Hepatic Cysts

Wild-type ($n = 3$, all males) and *Pkd2^{ws25/-}* ($n = 9$, 5 females and 4 males) mice were injected via the tail vein with Fenestra LC (ART Advanced Research Technologies Inc., Montreal, Canada) imaging agent. The agent was then allowed to accumulate in the liver for 3+ hours. Each liver lobe was fixed in 10% buffered formalin, transferred to a solution of glycerin in water with increasing concentrations (30% to 100%), suspended in a thin-walled plastic cylinder with clear Bioplastic polymer poured around. The liver parenchyma and hepatic cysts were scanned and reconstructed in three dimensions using a technique previously published by us.¹⁴ Specimens were scanned using a micro-computed tomography (CT) scanner. Quantitative analysis was performed using Analyze (Biomedical Imaging Resource; Mayo Foundation, Rochester, MN).

Immunofluorescence Confocal Microscopy

Immunofluorescence confocal microscopy was performed with Zeiss LSM 510 confocal microscope using $\times 40$ Plan-

Apochromat oil objectives. Tissue sections of median or right liver lobes ($n = 3$) and left kidney ($n = 3$) of each wild-type ($n = 6$) and *Pkd2^{ws25/-}* ($n = 20$) mice and ADPKD patients ($n = 3$; four liver sections per liver) were incubated overnight at 4°C with the antibody to proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:50) or acetylated α -tubulin (Sigma, dilution 1:200) and then with corresponding fluorescent secondary antibody (Invitrogen Molecular Probes Eugene, OR, dilutions 1:100) for 1 hour at room temperature. Coverslips were mounted using ProLong Gold antifade reagent. Nuclei were stained with 4,6-diamidino-2-phenylindole (Invitrogen). Adobe Photoshop was used to observe and quantitate PCNA-positive nuclei and terminal deoxynucleotidyl transferase dUTP nick-end labeling assay with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Inc; Temecula, CA) to detect apoptosis. For mitotic and apoptotic indices, 1000 nuclei of cholangiocytes and renal tubular cells in randomly selected fields of liver and kidney sections were counted. The mitotic and apoptotic indices were calculated as a percentage of cells positive for PCNA or terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Statistical Analysis

All values are reported as mean $\pm$ SE. Statistical analysis was performed by Student's *t*-test and results were considered statistically significant at $P < 0.05$.

Table 1. Gross Anatomy, Cystic, and Fibrotic Volumes, Mitotic and Apoptotic Indices in *Pkd2^{ws25/-}* Mice

Parameters	Wild-type	<i>Pkd2^{ws25/-}</i> mice	
		5–8 month	9–12 month
Body weight (g)			
Male	33.57 $\pm$ 2.04	35.72 $\pm$ 1.19	34.46 $\pm$ 0.86
Female	31.64 $\pm$ 1.55	32.93 $\pm$ 2.06	33.18 $\pm$ 1.53
Liver weight (g)			
Male	1.48 $\pm$ 0.13	2.13 $\pm$ 0.25*	2.76 $\pm$ 0.25*†
Female	1.58 $\pm$ 0.19	2.07 $\pm$ 0.22*	2.61 $\pm$ 0.71*†
Liver weight (% of body weight)			
Male	4.70 $\pm$ 0.33	6.04 $\pm$ 0.59*	8.05 $\pm$ 0.91*†
Female	4.74 $\pm$ 0.22	6.29 $\pm$ 0.36*	8.11 $\pm$ 0.79*†
Kidney weight (g)			
Male	0.48 $\pm$ 0.08	0.73 $\pm$ 0.09*	0.85 $\pm$ 0.13*†
Female	0.46 $\pm$ 0.03	0.64 $\pm$ 0.07*	0.92 $\pm$ 0.17*†
Kidney weight (% of body weight)			
Male	1.45 $\pm$ 0.13	2.06 $\pm$ 0.12*	2.57 $\pm$ 0.38*†
Female	1.48 $\pm$ 0.17	1.98 $\pm$ 0.41*	2.69 $\pm$ 0.25*†
Hepatic cystic volume by micro-CT (% of total liver parenchyma)	—	15.84 $\pm$ 0.86	23.26 $\pm$ 2.93†
Hepatic cystic volume by light microscopy (% of total liver parenchyma)	—	12.82 $\pm$ 3.16	21.58 $\pm$ 4.81†
Hepatic fibrotic volume (% of total liver parenchyma)	0.92 $\pm$ 0.32	2.71 $\pm$ 0.64*	2.12 $\pm$ 0.89*
Renal cystic volume (% of total kidney parenchyma)	NA	40.67 $\pm$ 5.48	31.04 $\pm$ 1.88†
Renal fibrotic volume (% of total kidney parenchyma)	1.56 $\pm$ 0.19	2.01 $\pm$ 0.34	2.16 $\pm$ 0.59
Mitotic index (% of PCNA positive nuclei out of total 1000 nuclei)			
Liver	1.12 $\pm$ 0.17	18.77 $\pm$ 4.39*	20.26 $\pm$ 3.49*
Kidney	0.85 $\pm$ 0.15	2.86 $\pm$ 0.62*	2.55 $\pm$ 0.46*
Apoptotic index (% of nuclei positive for terminal deoxynucleotidyl transferase dUTP nick-end labeling, out of total 1000 nuclei)			
Liver	0.56 $\pm$ 0.38	3.41 $\pm$ 1.14*	3.86 $\pm$ 1.13*
Kidney	2.86 $\pm$ 1.37	13.55 $\pm$ 2.11*	21.02 $\pm$ 2.92*†

*Statistically significant ($P < 0.05$), as compared with wild-type.

†Statistically significant ($P < 0.05$), as compared with 5- to 8-month-old *Pkd2^{ws25/-}* mice.

Results

Liver Pathology

Body weights were comparable in wild-type and *Pkd2^{ws25/-}* mice (Table 1). In contrast, in *Pkd2^{ws25/-}* mice, cystic livers were markedly enlarged often occu-

pying the greater part of the abdominal cavity. In wild-type mice, liver weights [1.48 ± 0.13 g (males) and 1.58 ± 0.19 g (females)] represented $\sim 4.7\%$ of total body weight. In 5- to 8-month-old *Pkd2^{ws25/-}* mice, liver weights were significantly increased [2.13 ± 0.25 g (males, $P < 0.01$) and 2.07 ± 0.22 g (females, $P < 0.001$)], representing $\sim 6\%$ of total body weight. Further liver enlargement was observed in 9- to 12-month-old *Pkd2^{ws25/-}* mice [2.76 ± 0.25 g (males, $P < 0.001$ compared with wild-type mice) and 2.61 ± 0.71 g (females, $P < 0.004$ compared with wild-type mice)] accounting for $\sim 8\%$ of total body weight. No significant differences in liver weights between male and female *Pkd2^{ws25/-}* mice were found. Macroscopically, multiple cysts filled with fluid were observed in *Pkd2^{ws25/-}* mice (Figure 1A). The presence of liver cysts of different sizes and shapes was further confirmed by light (Figure 1B) and scanning electron (Figure 1C) microscopy. Ultrastructural examination of the hepatic cysts by transmission electron microscopy showed that they possess microvilli and have well-developed cellular junctions (Figure 1D). The majority of the cystic cholangiocytes were squamous in appearance. Compared with cholangiocytes lining intrahepatic bile ducts in wild-type mice, cystic cells were enlarged with thickened basement membrane. The height (ie, the distance between apical and basolateral membranes) was comparable in normal (1.97 ± 0.35 μm) and cystic cholangiocytes (2.18 ± 0.51 μm) while width (ie, distance between two cell junctions) was significantly larger (5.91 ± 0.25 μm , $P < 0.0001$) in *Pkd2^{ws25/-}* cholangiocytes compared with wild-type mouse cholangiocytes (2.55 ± 0.22 μm). The epithelial cells lining liver cysts in *Pkd2^{ws25/-}* mice stained positively for the epithelial cell

Figure 1. Liver pathology in normal and *Pkd2^{ws25/-}* mice. **A:** At the gross level, livers in *Pkd2^{ws25/-}* mice are significantly enlarged with multiple cysts that could be observed macroscopically. Light microscopy (H&E staining, **B**) and scanning electron microscopy (**C**) shows the presence of multiple cysts of different dimensions and shapes in the liver parenchyma. **D:** Transmission electron microscopy demonstrates that cholangiocytes lining liver cysts in *Pkd2^{ws25/-}* mice were squamous in shape. **E:** Epithelial cells lining liver cysts are positive for CK19, an epithelial cell marker. Light, scanning and transmission micrographs are representative images of livers from 9- to 12-month-old *Pkd2^{ws25/-}* mice. WT, wild-type mice. **Arrows** indicate bile ducts, **asterisks** indicate liver cysts. Scale bars: 10 mm (**A**) and 200 μm (**C**).

Figure 2. Hepatic cysts in *Pkd2^{ws25/-}* mice and human patients with ADPKD are lined by single-layered (upper panels) or multilayered (bottom panels) epithelial cells. Cholangiocytes are stained with acetylated α -tubulin (red) and nuclei are stained with 4,6-diamidino-2-phenylindole (blue). L, cyst lumen. Magnification, original $\times 100$ (*Pkd2^{ws25/-}* mice) and $\times 40$ (ADPKD patients).

Figure 3. Hepatic cystic volumes in *Pkd2^{ws25/-}* mice assessed by micro-CT scanning and three-dimensional reconstruction. **A:** Micro-CT images of intact liver lobes of wild-type (WT) and two age groups of *Pkd2^{ws25/-}* mice were used to determine cystic volumes. **B:** Bar graphs demonstrate that cystic volume was increased over time. Liver parenchyma is white and liver cysts appear as dark gray spots. Scale bars = 1 cm. **P* < 0.05 compared with 5- to 8-month-old *Pkd2^{ws25/-}* mice.

marker, CK-19, indicating that hepatic cysts arise from cholangiocytes (Figure 1E). Majority of hepatic cysts in *Pkd2^{ws25/-}* mice were lined by single layer of cholangiocytes (Figure 2, upper left panel). Approximately 10% of liver cysts in *Pkd2^{ws25/-}* mice were multilayered (Figure 2, left bottom panel). Thus we examined the presence of multilayered cysts in human patients with ADPKD and found that ~ 20% of hepatic cysts in ADPKD patients have multiple layers of epithelial cells (Figure 2, right panels).

Assessment of the Total Liver and Total Hepatic Cystic Volume

To assess the volume of hepatic cysts, intact liver lobes of wild-type and *Pkd2^{ws25/-}* mice were scanned using a micro-CT scanner and then reconstructed in three dimensions, ie, a technique that we previously used to analyze liver pathology in the PCK rat, an animal model of ARPKD.¹⁴ Both liver parenchyma (white) and multiple cysts (black spots) could be easily recognized on CT

images (Figure 3A). In wild-type mice, no cysts were present and parenchymal volume was set equal to 100%. In *Pkd2^{ws25/-}* mice, numerous black spots representing hepatic cysts were observed. The total cystic volumes in these animals were calculated as a percentage of the total parenchymal volumes. In 5- to 8-month-old *Pkd2^{ws25/-}* mice, liver cysts occupy $15.84 \pm 0.86\%$ of total liver parenchyma while in 9- to 12-month-old *Pkd2^{ws25/-}* mice, the cystic volumes increased to $23.26 \pm 2.93\%$ (*P* < 0.05) of total liver parenchyma (Figure 3B and Table 1).

Hepatic Cystic and Fibrotic Scores

To confirm and extend results of micro-CT scanning and to assess the severity of hepatic fibrosis in *Pkd2^{ws25/-}* mice, light microscopy was used. In 5- to 8-month-old *Pkd2^{ws25/-}* mice, cysts occupy approximately $12.82 \pm 3.16\%$ of liver parenchyma, while in older animals, cysts occupy up to $21.58\% \pm 4.81\%$ (*P* < 0.05, Figure 4A). These data are comparable with the results obtained by

Figure 4. Hepatic cystic and fibrotic volumes in 5- to 8-month-old and 9- to 12-month-old *Pkd2^{ws25/-}* mice. **A:** Representative light microscopic (H&E staining) images and quantitative assessments (bar graphs) of the hepatic cystic volumes in *Pkd2^{ws25/-}* mice demonstrate that fraction of the liver parenchyma occupied by cysts was increased with age. **B:** Representative light microscopic images (Picrosirius red staining) and quantitative assessment (bar graphs) show presence of mild fibrotic deposits around liver cysts. Magnification, original $\times 10$. **P* < 0.05, ***P* < 0.0001.

Figure 5. Scanning electron microscopy (A, B) and transmission electron microscopy (C) show that in *Pkd2^{ws25/-}* mice, cholangiocyte primary cilia are short and malformed compared with wild-type (WT). Bar graphs demonstrate that cilia were $\sim$ two times shorter in *Pkd2^{ws25/-}* mice. Scale bars: 2 μm (A), 1 μm (B), and 500 nm (C). * $P < 0.05$.

micro-CT technique. Consistent with the human pathology, *Pkd2^{ws25/-}* mice exhibit mild fibrosis. In 5- to 8-month-old *Pkd2^{ws25/-}* mice, hepatic fibrotic tissue occupied $2.71 \pm 0.64\%$ ($P < 0.0001$) of total liver parenchyma, as compared with $0.92 \pm 0.32\%$ in wild-type mice. The severity of fibrotic deposits was unchanged during disease progression (Figure 4B and Table 1).

Cholangiocyte Cilia

We examined the structure of cholangiocyte cilia in wild-type and *Pkd2^{ws25/-}* mice. Since there were no significant differences in ciliary length between the two age groups of the *Pkd2^{ws25/-}* mice, for statistical analysis we combined both groups. The cystic cholangiocyte cilia ($n = 123$) were much shorter ($1.56 \pm 0.89 \mu\text{m}$; $P < 0.05$) relative to normal ones [$(n = 56)$, $3.26 \pm 1.29 \mu\text{m}$, Figure 5A]. A closer examination of cystic cilia by scanning electron microscopy showed that $\sim 25\%$ of cilia in cystic cholangiocytes had morphological malformations—ie, aberrant structures, bulbs at the ciliary tip (Figure 5B). This observation was further confirmed by transmission electron microscopy (Figure 5C).

Kidney Pathology

In *Pkd2^{ws25/-}* mice, kidneys were enlarged with numerous macroscopically visible cysts (Figure 6A). Compared with wild-type kidneys [0.48 ± 0.08 g (males) and 0.46 ± 0.03 g (females)], the two age groups of *Pkd2^{ws25/-}* mice had significantly increased kidney weights. In 5- to 8-month-old *Pkd2^{ws25/-}* mice, kidney weights were increased to 0.73 ± 0.09 g (males, $P < 0.0001$) and 0.64 ± 0.07 g (females, $P < 0.005$) accounting for $\sim 2\%$ of total body weight. In 9- to 12-month-old *Pkd2^{ws25/-}* mice, kidney weights were further increased to 0.85 ± 0.13 g (males, $P < 0.005$) and 0.92 ± 0.17 g (females, $P < 0.05$) accounting for $\sim 2.6\%$ of total body weight. Light (Figure 6B) and scanning electron (Figure 6C) micrographs show in greater detail the renal cysts in *Pkd2^{ws25/-}* mice.

In contrast to hepatic cysts, renal cystogenesis appears to be more severe at an early stage of disease. In 5- to 8-month-old *Pkd2^{ws25/-}* mice, cystic volumes represented $40.67 \pm 5.48\%$ of total kidney parenchyma while in older *Pkd2^{ws25/-}* mice (ie, 9 to 12 months), renal cystic volumes decreased to $31.04 \pm 1.88\%$ ($P > 0.05$; Figure 7A and Table 1). Renal fibrotic volumes were not

Figure 6. Renal pathology in wild and *Pkd2^{ws25/-}* mice. **A:** Macroscopically, kidneys in mutant mice were significantly enlarged with identifiable cysts scattered on kidney surface. Light microscopy (H&E staining, **B**) and scanning electron microscopy (**C**) shows the presence of renal cysts (asterisks). Scale bars = 10 mm (**A**, **C**). Light and scanning micrographs are representative images of kidneys from 9- to 12-month-old *Pkd2^{ws25/-}* mice.

different in *Pkd2^{ws25/-}* mice, as compared with wild-type mice (Figure 7B and Table 1).

Finally, we examined the morphology of renal cilia in wild-type and *Pkd2^{ws25/-}* mice by scanning electron microscopy (Figure 8, A and B). In wild-type mice, primary cilia ($n = 25$) in renal epithelial cells were $2.67 \pm 0.13 \mu\text{m}$ in length. In *Pkd2^{ws25/-}* mice, renal cilia ($n = 51$) had a tendency to be shorter ($2.05 \pm 0.15 \mu\text{m}$) although the observed changes were marginal ($P = 0.053$).

Figure 7. Renal cystic and fibrotic volumes in *Pkd2^{ws25/-}* mice. **A:** Representative light microscopic (H&E staining) images and quantitative assessments (bar graphs) of the renal cystic volumes in *Pkd2^{ws25/-}* mice show that in 5- to 8-month-old *Pkd2^{ws25/-}* mice renal cysts occupy a larger fraction of kidney than in 9- to 12-month-old mice. **B:** Representative light microscopic images (Picrosirius red staining) and quantitative assessment (bar graphs) demonstrate that percentage of fibrotic deposits appears to be non-significant in both age groups of the cystic mice compared with wild-type (**B**). Magnification: $\times 4$ (**A**) and $\times 10$ (**B**). * $P < 0.05$.

Proliferation and Apoptosis of Cystic Cholangiocytes and Renal Epithelial Cells

One of the major mechanisms involved in progressive cyst growth is enhanced proliferation of epithelial cells that line cysts in both liver and kidney. Therefore, we assessed the rate of cholangiocyte and renal epithelial cell proliferation by PCNA expression. In 5- to 8-month-old of *Pkd2^{ws25/-}* mice, $18.77 \pm 4.39\%$ nuclei of cystic cholangiocytes ($P < 0.0001$) were PCNA-positive compared with $1.12 \pm 0.17\%$ in wild-type mouse cholangiocytes. The percentage of PCNA-positive nuclei did not change significantly (ie, $20.26 \pm 3.49\%$) with age (Figure 9). In renal epithelial cells of 5- to 8-month-old *Pkd2^{ws25/-}* mice, percentage of PCNA-positive nuclei was increased to $2.86 \pm 0.62\%$ ($P < 0.01$), as compared with wild-type mouse kidney ($0.85 \pm 0.15\%$) but, like in hepatic cysts, it did not change with age ($2.55 \pm 0.46\%$; Figure 9 and Table 1). Finally, the analysis of apoptotic indices in cystic cholangiocytes of 5- to 8-month-old *Pkd2^{ws25/-}* showed that they were increased to $3.41 \pm 1.14\%$ ($P < 0.01$), compared with wild-type mouse cholangiocytes ($0.56 \pm 0.38\%$), and did not change over time ($3.86 \pm 1.13\%$, Table 1). In contrast, in renal epithelia, apoptotic indices were increased gradually with disease progression being much higher in 9- to 12-month-old cystic mice ($21.02 \pm 2.92\%$, $P < 0.05$) than in younger animals ($13.55 \pm 2.11\%$; Table 1).

Discussion

In this work, we describe in detail the hepatorenal phenotype over time of *Pkd2^{ws25/-}* mice, an animal model of ADPKD. We have shown that compared with wild-type mice: (i) liver weights are increased over the course of disease progression; (ii) kidney weights are enlarged in younger *Pkd2^{ws25/-}* mice but no further increase in kidney weights occurs in older mice; (iii) hepatic cysts are lined by single or multiple layers of epithelial cells with

Figure 8. Scanning electron microscopy (A) and quantitative assessment of length of cilia in renal epithelial cells (B) in wild-type (WT) and *Pkd2^{ws25/-}* mice. Scale bars = 2 μm .

microvilli and well-developed cellular junctions; (iv) the majority of the hepatic cystic cells are squamous in appearance; (v) epithelial cells lining liver cysts are positively stained for the epithelial cell marker, CK19, suggesting that hepatic cysts arise from cholangiocytes; (vi) hepatic cystic volumes increases over time while fibrotic volumes remain similar; (vii) renal cystogenesis appears to be more severe at early stages of disease development; (viii) no significant renal fibrosis was observed; (ix) cholangiocyte cilia in hepatic cysts are short and malformed compared with wild-type cells and their length is not age-dependent; (x) cilia in renal epithelial cells lining renal cysts appear to be shorter although these changes are not statistically significant; and (xi) mitotic and apoptotic indices are significantly increased in cystic cholangiocytes and renal cystic epithelial cells and do not change during the course of disease progression.

Hepatic and renal cystogenesis in *Pkd2^{ws25/-}* mice results from a somatic inactivation of the second *Pkd2* allele. Our work supports and extends observations that *Pkd2^{ws25/-}* mice have a severe and reproducible phenotype of human ADPKD.²⁰ Previous work has shown that all mice develop renal and liver cysts at early time points of disease development (e., 10 to 11 weeks of age).²⁰ Thus, we looked at the hepatic and renal pathology in these animals over an extended time frame and in greater detail.

Ultrastructurally, most of the cholangiocytes lining liver cysts in *Pkd2^{ws25/-}* mice have a squamous shape with well-developed microvilli and cell junctions. This is consistent with previous observations in kidney showing cystic renal epithelial cells become flattened with age, while at early stages of cyst development cystic cells are predominantly cuboidal.²⁹

Hepatic cysts were surrounded by fibrotic deposits. As in human ADPKD, the degree of fibrosis in *Pkd2^{ws25/-}* was mild. Interestingly, no changes in hepatic fibrosis were found with age. In contrast to the hepatic phenotype, we did not see significant fibrosis around renal cysts in both age groups of *Pkd2^{ws25/-}* mice.

Substantial evidence suggests hepatic and renal cystogenesis is associated with structural (ie, shortened and/or malformed cilia) and/or functional (ie, impaired expression and/or function of ciliary-associated proteins) abnormalities in primary cilia. Indeed, we found primary cilia in cholangiocytes lining liver cysts in *Pkd2^{ws25/-}* mice are malformed and shortened compared with wild mouse cholangiocytes. These data are in agreement with our previous study showing that cilia in cystic cholangiocytes of the PCK rat, an animal model of ARPKD, have similar abnormal structural and functional characteristics.¹⁴ Architecture of renal cilia had been previously analyzed in younger *Pkd2^{ws25/-}* mice (1 to 2 months of age). At this point of renal cystogenesis (designated as

Figure 9. Proliferation of cholangiocytes and renal epithelial cells in cystic epithelia of *Pkd2^{ws25/-}* mice measured by PCNA expression. Bar graphs show that the percentage of PCNA-positive nuclei was significantly increased in cells lining hepatic and renal cysts compared with wild-type (WT) mice. No significant differences in the rate of proliferation were noticed between two age groups of *Pkd2^{ws25/-}* mice. PCNA-positive nuclei are stained in green; nuclei are stained with 4,6-diamidino-2-phenylindole (blue). BD, bile duct lumen; asterisks indicate hepatic or renal cyst lumen. Confocal micrographs are representative images of liver and kidneys from 5- to 8-month-old *Pkd2^{ws25/-}* mice. Magnification, $\times 60$ (liver) and $\times 40$ (kidney). * $P < 0.05$ compared with wild-type.

early or intermediate stage), epithelial cells have normal appearing primary cilia.²⁹ Consistent with this observation, in renal epithelial cells, despite the fact that primary cilia had a tendency to be shorter, the observed changes (ie, $P = 0.053$) were not statistically significant. The relevance of this observation to renal cystogenesis is unclear.

While cyst growth in liver and kidney is linked to a number of different mechanisms, cell proliferation and apoptosis appears to be the most important. Our data demonstrate that the rates of proliferation and apoptosis in cystic cholangiocytes were significantly increased in *Pkd2*^{ws25/-} mice, as compared with wild-type mice and did not change significantly during the disease progression while cystic volumes continued to increase. These data suggest that together with cell proliferation and apoptosis, other mechanisms may contribute to hepatic cyst expansion. Indeed, we have recently demonstrated that increased fluid secretion plays an important role in liver cystogenesis.³⁰ In contrast to liver cyst, renal cystogenesis appears to be more severe in younger *Pkd2*^{ws25/-} mice and decreases with age. Consistent with this observation, we found that the rate of apoptosis in renal cystic epithelial cells was significantly higher in 9- to 12-month-old cystic mice, as compared with 5- to 8-month-old animals while the rate of cell proliferation was similar in both age groups of *Pkd2*^{ws25/-} mice. These data suggest that increased rate of apoptosis might be responsible for decreased renal cystic volume over time.

In conclusion, *Pkd2*^{ws25/-} mice represent a model of ADPKD with distinctive hepatic and renal pathogenic features that in many ways resemble the liver and kidney human pathology (ie, progressive cyst growth, dysregulated cell proliferation, and ciliary abnormalities). Our data suggest that this model will be useful to study mechanisms of cyst formation and progressive growth, and to evaluate treatment options of inherited cystic diseases.

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