Kidney Injury Accelerates Cystogenesis via Pathways Modulated by Heme Oxygenase and Complement

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ABSTRACT

AKI accelerates cystogenesis. Because cystogenic mutations induce strong transcriptional responses similar to those seen after AKI, these responses may accelerate the progression of cystic renal disease. Here, we modulated the severity of the AKI-like response in Cys1cpk/cpk mice, a model that mimics autosomal recessive polycystic kidney disease. Specifically, we induced or inhibited activity of the renoprotective enzyme heme oxygenase (HO) and determined the effects on renal cystogenesis. We found that induction of HO attenuated both renal injury and the rate of cystogenesis, whereas inhibition of HO promoted cystogenesis. HO activity mediated the response of NFκB, which is a hallmark transcriptional feature common to both cystogenesis and AKI. Among the HO-modulated effects we measured, expression of complement component 3 (C3) strongly correlated with cystogenesis, a functionally relevant association as suggested by Cys1cpk/cpk mice with genetically induced C3 deficiency. Because both C3 deficiency and HO induction reduce cyst number and cyst areas, these two factors define an injury-stimulated cystogenic pathway that may provide therapeutic targets to slow the formation of new renal cysts and the growth of existing cysts.


Polycystic kidney disease (PKD), the most common inherited cause of ESRD, affects 12.5 million people worldwide (approximately 600,000 in the United States). Autosomal dominant PKD (ADPKD; MIM 173900 and MIM 173910) occurs in 1:400–1:1000 individuals and is caused by mutations in the PKD1 or PKD2 genes.1–4 Autosomal recessive PKD (ARPKD; MIM 263200) occurs in 1:20,000 live births and is caused by defects in the PKHD1 gene.5,6 Although these gene mutations are essential for development of each PKD phenotype, their effects are regulated by nongenetic factors. For example, a tamoxifen-inducible Cre-driven knock-out of Pkd1 before postnatal day 12 (P12) induces rapid cystogenesis resembling ARPKD in mice, whereas induction of the same genetic knockout after P14 leads to extremely slowly progressing cystic disease.7 However, even in the latter case, rapid cystogenesis can be achieved in response to insults that cause AKI, such as ischemia reperfusion6 or administration of a nephrotoxic drug.9 AKI reportedly accelerates renal cystogenesis in several other PKD models.10–12

Although AKI is an important trigger for cystogenesis, it remains unknown whether milder chronic renal injury, such as that caused by PKD gene defects, also accelerates cystogenesis. The similarity of the renal injury responses during AKI and PKD gene defect-induced cyst formation, as reflected by

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genome-wide transcriptional analyses,\textsuperscript{13,14} suggests a central role of PKD gene mutation-induced injury in renal cystogenesis. For example, of 22 genes found to be most highly overexpressed in mice with rapidly versus slowly progressing cystic kidney disease,\textsuperscript{14} 18 were also found to be overexpressed in kidneys of mice subjected to ischemia-reperfusion injury\textsuperscript{13} (i.e., Hp, C3, Lcn2, Saa3, Cts, Sprr2f, Lrg1, E430024C06Rik, Ccl6, Lyz, Niban, 1200016E24Rik, Socs3, Serpine1, Clqb, Timp1, Cd14, and F13a). Likewise, transcriptome analysis indicates substantial overlap between biologic processes associated with cystogenesis and with AKI. For example, the overlap identified by the National Institutes of Health Database for Annotation, Visualization, and Integrated Discovery Bioinformatics resources\textsuperscript{15} includes responses to wounding (Gene Ontology term GO:0009611) and stress (GO:0009611), organ development (GO:0048513), positive regulation of biologic processes (GO:00048518), response to external stimulus (GO:0009605), and vasculature development (GO:0001944). These transcriptional parallels suggest that injury triggered by PKD mutations induces renal responses qualitatively similar to those seen in AKI.

To fully ascertain the PKD gene mutation-induced AKI-like response and to investigate its possible role in PKD pathogenesis, we experimentally slowed or hastened the injury responses in the Cys\textsuperscript{1}cpk mouse model of ARPKD.\textsuperscript{16}

**RESULTS**

**Heme Oxygenase Activity Modulates Severity of Renal Injury in Cys\textsuperscript{1}cpk/cpk Mice**

Heme oxygenase (HO) is a potent renoprotective factor. It effectively decreases both renal injury-induced oxidative stress and activation of molecular pathways that lead to adverse AKI outcomes, and it does so in various forms of renal injury (summarized in Nath \textit{et al.}\textsuperscript{17}). Because approaches for HO activity manipulation are well established\textsuperscript{18} and increased HO expression in cystic kidneys has been confirmed by independent analyses,\textsuperscript{14,19} we used experimental induction versus inhibition of HO activity as a strategy to modulate the severity of cystogenic mutation-induced renal injury. First, we confirmed that HO activity can be experimentally manipulated in the Cys\textsuperscript{1}cpk model. Compared with control Cys\textsuperscript{1}cpk/cpk mice treated with the Tris-HCl vehicle, HO induction \textit{via} treatment of mice with cobalt protoporphyrin (CoPP) increased HO activity by 80% (HO+ group; $P=0.006$), whereas HO inhibition using tin mesoporphyrin (SnMP) reduced HO activity by 50% (HO\textsuperscript{−} group; $P=0.027$) (Figure 1A). Similar to its effects in other forms of renal injury, induction of HO activity had renoprotective effects in the Cys\textsuperscript{1}cpk/cpk mice, as reflected by maintenance of less abnormal kidney weight (Figure 1B) and decreased expression of renal injury markers. For example, expression of neutrophil gelatinase-associated lipocalin 2 (NGAL)-encoding gene (Lcn2) decreased by 44% ($P=0.013$; Figure 1C).

**HO Activity Modulates Cystogenesis in Cys\textsuperscript{1}cpk/cpk Kidneys**

Induction of HO activity reduced cystogenesis rates in Cys\textsuperscript{1}cpk/cpk kidneys, whereas its inhibition had converse effects. Specifically, on postnatal day 12 (P12), 1 week after induction of HO activity with CoPP, the Cys\textsuperscript{1}cpk/cpk kidneys were smaller compared with control mice. For example, the kidney weight/body weight ratio (KW/BW), a commonly used correlate of cystic disease severity,\textsuperscript{14,20,21} was decreased by 12% ($P=0.038$; Figure 1B). These findings were corroborated by morphometric correlates of reduced cystic disease severity in CoPP-treated animals (Figure 1, D and E). Although the overall average cyst area was decreased by 18% with marginal significance (overall $P=0.052$), cortical cyst/noncystic tissue area ratio was reduced by 37% ($P=0.016$). A comparable but nonsignificant trend was observed in the medulla. Similar to the overall cyst area index, HO induction reduced overall cyst number by 23% ($P=0.013$).

In contrast to the cystogenesis-reducing effects of HO activity induction, HO activity inhibition (with SnMP) had the opposite, cystogenesis-promoting effects. Specifically, the Cys\textsuperscript{1}cpk/cpk mice were administered CoPP and SnMP at P5. SnMP was then administered at P7 and P9, and these mice were sacrificed at P12. Inhibition of HO activity for 1 week was associated with larger kidney size (e.g., KW/BW ratio increased by 45%) ($P=0.003$; Figure 1B), indicating an accelerated rate of renal cystogenesis. The cystic disease progression was most prominent in the renal cortex, where the cyst/noncystic tissue area ratio was increased by 67% ($P=0.004$; Figure 1D). A comparable (but nonsignificant) trend was observed in the medulla. Similar to the cyst area indices, HO inhibition increased the number of renal cysts (Figure 1D), particularly in the cortex as indicated by 31% increase in cortical cyst number index ($P=0.049$); the overall cyst number increased by 15% with marginal significance ($P=0.090$). A similar but nonsignificant trend was present in the medulla.

Taken together, these data point to the renal cortex as the major site of HO-modulated cystogenesis.

**Prioritization of Putative Regulators of the HO-Mediated Cystogenic Effects**

As a first step toward identifying putative mechanisms by which HO activity might modulate the pace of cystogenesis, we compared transcription profiles derived from cases of rapidly progressive renal cystogenesis\textsuperscript{14} versus AKI.\textsuperscript{13} This comparison of the published literature and our own re-analyses of these transcriptional data using the same bioinformatic approach\textsuperscript{14} revealed significant overlap between cystogenesis and AKI, dominated by NF\textsuperscript{kB}-regulated genes such as C3, Lcn2, Saa3, Lyz, Serpine1, Cd14 and Ccl2. To ascertain whether HO activity regulates the expression of these NF\textsuperscript{kB}-regulated genes in Cys\textsuperscript{1}cpk/cpk kidneys, we determined the expression of major NF\textsuperscript{kB} targets in Cys\textsuperscript{1}cpk/cpk kidneys with induced versus inhibited HO activity. In addition to Lcn2 (an AKI marker NGAL-encoding gene), we analyzed expression of AKI markers Ccl2 (an MCP-1-encoding gene),\textsuperscript{22} Cd14,\textsuperscript{13,23} and C3.
Cys1 P renal cystic disease progression (expression in this model correlated strongly with the pace of and/or predictors of renal cystogenesis.20,25,26 For all of these generated from these crosses revealed reduced rates of

tissues harvested from the studied Cys1/cp/cp mice at P12.

Among the NFκB-regulated genes we studied, the magnitude of transcriptional changes triggered by induction versus inhibition of HO activity in Cys1/cp/cp kidneys was greatest for C3 (Figure 1C). In addition, C3 expression most strongly correlated with the rate of cystogenesis as reflected by KW or KW/BW (r=0.725 and 0.729; both P<0.001; Figure 2A).

To validate these associations, we examined renal C3 expression in a different Cys1/cp/cp model in which the pace of cystogenesis was induced by different admixtures of two distinct genetic backgrounds: C57BL/6J and CAST/Ei.21 Similarly to the renal C3 expression in HO-modulated cystogenesis, C3 expression in this model correlated strongly with the pace of renal cystic disease progression (e.g., for KW/BW, r=0.919; P<0.001; Figure 2B). In contrast, relative expression of C3 in Cys1/cp/cp versus wild-type Cys1/+/+ kidneys with uniform rate of the disease progression (all C57BL/6J background) remained relatively unchanged with progressing age and cystic disease severity (Figure 2C), unlike expression of Cd14 or Ccl2 that progressively increases with age of Cys1/cp/cp mice.20

The above associations of C3 expression with the HO-modified pace of cystogenesis in Cys1/cp/cp kidneys point to C3 as a possible nexus between PKD and cell injury-mediated pathways. In addition, the levels of C3 overexpression in this cystic model and AKI (36 hours after 30-minute ischemia) are similar, with a four-fold14 versus five-fold increase.13 Because local C3 production is essential for the pathogenesis of adverse AKI and other renal injury outcomes,24,27,28 renal C3 expression and the ensuing amount of available C3 substrate may represent a limiting factor in renal activation of the complement system and its subsequent adverse injury effects. In cystic kidneys, C3 that is locally produced and activated14 may have similar adverse effects. Therefore, the above-described associations between C3 expression levels and cystic disease progression could be functionally relevant. We recognize that even a strong association between C3 expression and cystic disease severity does not prove a causal effect, but our data nevertheless suggest that C3 is a putative regulator of cystogenesis-promoting effects that PKD mutation-induced renal injury exerts downstream to HO (Figure 2D).

C3 Accelerates Renal Cystogenesis in Cys1/cp/cp Kidneys

To directly evaluate the biologic contribution of C3 to renal cystogenesis, we crossed the Cys1/cp/cp mice with C3-deficient mice (C3−/−; Figure 3A). Phenotypic analyses of unique mutants generated from these crosses revealed reduced rates of cystogenesis in C3 hypocomplementemic (C3−/− and C3+/+) versus normocomplementemic (C3+/+) kidneys as reflected by KW/BW (Figure 3B). For example, compared with C3+/+ mice, KW/BW was increased by 11% on average in the presence of one C3 allele and by 33% in the presence of two C3 alleles (P=0.040). Body weights were not significantly affected by C3 deficiency. In contrast, the C3 effects on KW/BW were absent in unaffected wild-type (Cys1/++) kidneys. Similarly to KW/BW, kidney length/body length ratios, another phenotypic correlate of cystic disease severity,21 were also significantly reduced in C3 hypocomplementemic versus C3 normocomplementemic Cys1/cp/cp mice (data not shown).

Morphometric analyses of stained kidney sections (Figure 3C) supported the phenotypic data (Figure 3D). Compared with the C3 hypocomplementemic mice, C3+/+ mice had ratios of cortical cyst/tissue area increased by 64% (P=0.031), medullary cyst/tissue area ratios increased by 74% (P<0.001), cortical cyst number/tissue area ratio increased by 25% (P=0.031), and medullary cyst number/tissue area ratio increased by 28% (P=0.001). Differences between C3−/− and C3+/+ data were insignificant.

Taken together, these data are consistent with cystogenesis-promoting effects of C3 in both medulla and cortex.

Fluid Flow Effects on C3 Expression and Activation in Cystic Tubules

To uncover mechanisms that regulate C3 expression in renal tubule cells with cystogenic defects, we developed a cell line model 176–6c that allows conditional induction of a PKD-like phenotype. Specifically, this model of principal cells of collecting ducts contains an inducible deletion of kinesin 3a (Kif3a−) that leads to PKD-like phenotype with a well-characterized structural primary cilia defect29; uninduced Kif3a sufficient (Kif3a+) cells can be used as closely matched controls (Figure 4, A and B). Exposure of cilia-deficient Kif3a− cells to laminar flow reduced the content of activated C3 fragments (e.g., αC3/β-actin ratio by 78%; iC3b/β-actin ratio by 75%; both P<0.001; Figure 4, B and C). The flow also reduced the content of activated C3 fragments in control Kif3a+ cells (e.g., αC3/β-actin ratio by 50%; iC3b/β-actin ratio by 37%; both P<0.001; Figure 4, B and C). Similarly reduced was C3 mRNA expression in these cells (e.g., C3/Actb expression by 35%; P=0.007). These data suggest that C3 expression might be enhanced in tubules with diminished fluid flow force such as dilated or cystic tubules, forming a positive feedback loop in the cystogenic pathway. Consistent with this hypothesis, we found increased content of C3 and its activated fragments in aspirates from Cys1/cp/cp cysts versus plasma or urine (Figure 5A) as well as urine from ARPKD patients versus matched normal controls (Figure 5, B and C).

DISCUSSION

Our studies point to a PKD mutation-induced injury as a key cystogenesis-accelerating process whose effects are mediated
Figure 1. HO modulates cystogenesis in Cys\textsuperscript{fpk/cpk} kidneys. (A) HO activity in Cys\textsuperscript{fpk} mice can be efficiently induced with CoPP (HO+) and inhibited by SnMP (HO-) compared with mice injected with the Tris-HCl vehicle (ctrl). (B) Induction of HO activity reduced phenotypic measures of cystic disease severity: KW and KW/BW of Cys\textsuperscript{fpk/cpk} mice. Inhibition of HO activity had opposite effects. (C) Induction of HO activity consistently reduced expression of several renal injury markers. Among the prioritized genes commonly
by modifiers of renal injury severity, namely HO and C3 (Figure 2D). Consequently, our findings support the emerging concept of renal injury as an integral pathogenic component of naturally progressing cystic kidney disease.14 Importantly, this study demonstrates that cystogenesis-promoting injury effects are not restricted to relatively severe forms of injury that lead to AKI, but include also a milder but chronic form of injury triggered directly by a genetic cystogenic defect. Additional support for such a concept is provided by previous studies that demonstrated cystogenesis-reducing effects of AKI-protective strategies including TNF inhibition,30 administration of NGAL,31 statins,32 hydration,33 and alkalization therapies.34 Because these interventions likely also reduced the severity of renal injury that is triggered by the cystogenic mutations, their cystogenesis-inhibiting effects should be at least in part mediated by the reduction of cystogenic mutation-induced injury responses.

Reduction of injury-induced oxidative stress (e.g., by HO induction) is a promising strategy for limiting adverse kidney injury outcomes (reviewed by Haugen and Nath15 and by Tracz et al.36). Similarly, our results suggest that HO, presumably through reduction of oxidative stress, might have similar injury- and cystic burden-reducing effects in PKD. The opposite, cystogenesis-promoting effects of HO inhibition are consistent with cystogenesis-promoting effects of other oxidative stress-inducing strategies such as inhibition of glutathione synthesis.37 Our observation that HO-modulated cystogenic effects were most pronounced in the renal cortex is consistent with the highest capacity for increased HO expression in response to stress, i.e., by proximal tubular cells.

Although oxidative stress can promote renal injury via multiple pathways, their respective roles in renal cystogenesis are relatively unexplored. An obvious candidate for cystogenic effects of injury, an injury-induced increase in renal cell proliferation,39 may not be primarily responsible for the injury-induced acceleration of renal cystogenesis. This view is supported by the lack of cystogenesis acceleration after AKI in a hyperproliferative Cux1-overexpressing Kif3a knockout model (B.K. Yoder, personal communication, 2012). Alternatively, injury may trigger specific changes in a cellular milieu that increase the responsiveness of these cells to the adverse cystogenic mutation effects. These changes may include loss of cellular characteristics associated with full terminal differentiation and re-appearance of developmentally more immature characteristics that may limit injury and promote tissue regeneration. In PKD, such chronic repair may promote cystogenic pathway activation and facilitate progression of renal cystic disease (summarized by Weimbs40). Because C3 also plays a central role in regeneration (e.g., of liver41), it may accelerate cystogenesis by promoting both exacerbation of injury as well as acceleration of distinct regenerative responses. Support for this hypothesis is provided by a reported increase in renal levels of activated C3 split products in ARPKD and its models such as the Cys1cpk/cpk14 and Bicc1bpk/bpk mouse.14

Local, that systemic, regulation of the C3-mediated responses in cystic kidneys is suggested by the strong localization of C3 into cystic and noncystic renal tubular cells in ARPKD and Cys1cpk/cpk kidneys,14 by the capacity of the renal tubular epithelium to produce as well as activate C314 and by the suggested role of fluid flow force in this regulation (Figures 4 and 5). Also, C3 plays a central role in differentiation, attachment and survival of monocytes/macrophages,42,43 and their M2-polarization44 (a hallmark feature of accelerated cystogenesis in the Cystf98 model of ARPKD14), and depletion of macrophages by liposomal clodronate attenuates cystogenesis in orthologous ADPKD models by reducing proliferation of cystic tubules.45 In addition to the macrophage effects, C3 may also induce pro-cystogenic30,46 TNF release47 and possibly directly activate c-Src48 in renal tubule cells expressing CR3 receptors (e.g., proximal tubule and collecting duct cells; data not shown). Taken together, these effects point to C3 as a possible nexus linking cystogenic pathways relevant to ARPKD and ADPKD.

Because both C3 deficiency and HO induction have protective effects in AKI,17,49 the pathway defined by these two factors may also link established cystogenic pathways with those underlying the injury-accelerated cystogenesis.10–12 Particularly attractive mediators of such effects are macrophages, a cell type essential for pathogenesis of both AKI50 and ADPKD.35 Specifically, in the Cystf98 model injury reduction (i.e., by HO induction) lowered macrophage activity, as reflected by decreased expression of genes encoding macrophage markers CD14 (Figure 1), macrophage activation marker CD32 (Fcg2rb) and an M2-marker Ccr5 (both by 40%; P=0.041 and P=0.025); reduction of other M2 markers (e.g., Arg1 by 28%, CD163 by 30%) did not reach statistical significance. Similar but statistically nonsignificant results were observed in C3 deficient Cys1cpk/cpk kidneys (data not shown). Taken together, these data suggest that at least part of the observed cystogenesis-regulating effects of HO- and C3-defined pathways are mediated by macrophages and their effects are relevant to both acute as well as chronic injury-induced pathways of renal cystogenesis.

The injury-associated pathways, including those involving HO, C3 and macrophages, do not directly cause PKD because dysregulation of either of them (i.e., HO inhibition and C3 or macrophage upregulation) does not alone trigger renal cyst formation. However, they likely play a central role in establishing the proper cystogenic milieu that allows translation of a PKD gene defect into the cystic phenotype. This is consistent overexpressed in cystic and AKI kidneys, transcriptional changes induced by HO activity manipulation in Cys1cpk/cpk kidneys were highest for C3. (D) Representative tissue sections demonstrate cystogenesis-modulating effects of induction versus inhibition of HO activity. Bar, 1 mm. (E) Morphometric analyses demonstrate that HO activity induction had most prominent cystogenesis-inhibiting effects in renal cortex, whereas HO activity inhibition had opposite effects. All results represent mean values; bars indicate SEM.
with observations of reduced capacity of PKD mutations to induce renal cystic phenotype after the developmental switch\(^7\) and recovery of such capacity after AKI.\(^8\),\(^9\) Therefore, we suggest a model in which PKD mutations impair the process of renal tubule elongation,\(^{51}\) whereas positive regulators of renal injury (i.e., macrophages and C3) enhance the cystogenic milieu including renal tubule cell proliferation (renoprotective HO has opposite effects). However, because changes in HO or C3 activity alone do not apparently abrogate all mechanisms regulating epithelium-like cell susceptibility to cystogenic defects, even complete absence of biologically powerful factors such as C3 cannot fully attenuate the cystic disease progression alone. In the case of C3 knockout, it is also possible that the absence of C3 in early developmental stages leads to enhanced importance of C3 pathway alternatives. For example, noncomplement-derived C5-cleaving enzymes that are abundant in neutrophils\(^{52}\),\(^{53}\) may replace the C3b-generated C5 convertases. This is consistent with relatively mild effects of complete C3 deficiency in mouse models and human patients.

Although the exact molecular mechanisms underlying the cystogenic effects of C3 remain unknown, our observation of reduced cystogenesis in C3-deficient Cys\(^{1^+}\) mice, together with previously described associations of a hypomorphic C3 variant\(^{54}\),\(^{55}\) with slower rates of disease progression in ADPKD patients,\(^{56}\) suggest that C3 could be a therapeutic target for a broad spectrum of hepatorenal/fibrocystic disorders. However, this requires further validation, particularly in ARPKD and ADPKD or their animal models. Our study also adds to the body of published evidence pointing to C3 as a key component of a common renal injury pathway and thus a potential target for intervention in additional forms of acute and chronic renal injury.

In summary, cystogenesis-inhibiting effects of renoprotective HO induction or C3 deficiency in the Cys\(^{1^+}\) model of ARPKD indicate that a PKD gene defect-induced renal injury plays a key role in renal cystogenesis. It remains to be determined whether injury-preventing or inhibiting strategies are also effective in PKD patients.

**CONCISE METHODS**

**Cys\(^{1^+}\) Mice**

The Cys\(^{1^+}\) mouse is an ARPKD phenocopy that closely resembles the human disease course with its renal and extrarenal manifestations,\(^{57}\),\(^{58}\) unlike orthologous mouse ARPKD models that lack the
typical ARPKD-like renal cystic phenotype. The Cys1cpk mutation, which arose spontaneously in the C57BL/6J-Pldnpa strain, involves a tandem deletion that leads to the truncation of a novel, cilia-associated protein called cystin.\textsuperscript{16} In the studied mice, the Cys1cpk mutation was expressed on C57BL/6J genetic background, with exception of mice with variable rates of cystic disease progression that were generated in an (C57BL/6J-Cys1cpk/+ x CAST/Ei)F1 intercross.\textsuperscript{21} Identification of Cys1cpk/cpk mutants was done with a Cys1cpk allele-specific assay.\textsuperscript{16,21} All protocols were approved by the University of Alabama at Birmingham (UAB) Animal Care and Use Committee. The UAB is fully accredited by the American Association of the Accreditation of Laboratory Animal Care.

### Experimental Modulation of HO Activity

HO activity was chemically manipulated using well established protocols\textsuperscript{18} with proven effects on renal injury-induced $O_2^-$ production.\textsuperscript{19} Both metalloporphyrins, CoPP and SnMP, were obtained from Frontier Scientific (Park City, UT). Their solutions were prepared fresh each week by dissolving the metalloporphyrin powder in 10 mmol/L Tris base, with subsequent adjustment of the pH to 7.8 with 0.1 N HCl. The HO activity was induced with 3 mg/kg of CoPP injected intraperitoneally to the affected mice at P5. For HO inhibition, the mice were also injected with CoPP as described above at P5; however, they also received 2 mg/100 g of SnMP intraperitoneally at P5, P7, and P9. Control animals received intraperitoneal injections of metalloporphyrin-free solution of Tris-HCl (pH 7.8) at P5, P7, and P10. Mice were sacrificed at P12. At that time we scored phenotypic marks of renal cystogenesis including kidney and body weights using established protocols.\textsuperscript{21} One of the kidneys from each animal was snap-frozen and stored in liquid nitrogen, and the other was fixed in 10% buffered formalin for histologic evaluation.

HO activity in treated and control mice was measured by bilirubin generation in microsomal preparations from spleen according to established protocols.\textsuperscript{60,61} Briefly, spleen microsomes were incubated with rat liver cytosol, a source of bilirubin reductase (3 mg), as well as hemin (20 $\mu$M/L), glucose-6-phosphate (2 mM/L), glucose-6-phosphate dehydrogenase (0.2 U), and NADPH (0.8 mM/L) for 1 hour at 37°C in the dark. The formed bilirubin was extracted with chloroform, and the change in OD (464–530 nm) was measured (extinction coefficient, 40 mM/L $\cdot$ cm$^{-1}$ for bilirubin). Enzyme activity was expressed as picomolar quantities of bilirubin formed per 60 minutes per milligram of protein.

### Gene Expression Analyses

RNA and cDNA were prepared from whole kidneys as previously described.\textsuperscript{14} Gene expression studies were performed with TaqMan probes arranged into custom-designed low-density arrays ( Applied Biosystems, Foster City, CA). TaqMan assays relevant to this study include the following: Mm01324473_g1 (Lcn2), Mm00441243_g1 (C3-Mm00437876_g1 (C3), and Mm00607939_s1 (Actb; a gene encoding $\beta$-actin). Ct values were determined with 7000 SDS RQ software (version 1.1) and subsequently standardized using a Ct value for Actb as reference. Actb is a suitable reference gene for this model based on equivalence testing, a two one-sided $t$ test,\textsuperscript{62} of available genome-wide expression studies and TaqMan Mouse Endogenous Control
Array data for the Cys1<sup>cpk</sup> model. The standardized Ct values were used to determine significance of studied associations.

**Morphometric Analyses**

Paraffin-embedded kidneys were sectioned through the long axis and the hilus and were stained with hematoxylin and eosin. Histomorphometry was performed by an experienced veterinary pathologist (T.R.S.), without knowledge of experimental classifications, using a Nikon E600 microscope equipped with a SPOT Insight digital camera (Diagnostic Instruments, Sterling Heights, MI) and Image-Pro Plus v6.2 image analysis software (Media Cybernetics Inc, Bethesda, MD). RGB color images made at 31 objective magnification were divided into separate images of the cortex and medulla. The demarcation between the cortex and medulla was readily distinguished in most cases, but cysts that extended into both the cortex and medulla such that their origin could not be determined were excluded from analysis by deleting them from the image. Cortical and medullary cyst numbers and cyst and tissue areas were quantified by converting the images to grayscale, thresholding them to produce a black image on a white background, and determining the number and areas of cysts, represented as white objects within the image, and the total area of the tissue, represented as the area of the image including cysts, automatically using the count/size and macro functions of Image-Pro Plus. Results were expressed as ratios of cyst number and area to total area of cortex or medulla.

**C3-Deficient Mice**

The C3-deficient model (B6.129S4-C3<sup>tm1Crr</sup>/J; C3<sup>−/−</sup>) contains C3 gene disruption by PGK/Neo cassette and an approximately 600-bp

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**Figure 4.** Fluid flow inhibits C3 expression and activation by renal tubules in vitro. (A) SV40-immortalized principal cells of collecting duct with tamoxifen-inducible Kif3a knockout: the left panel shows no induction (Kif3a+, cilia+), whereas the right panel shows that primary cilia staining (Ac-α-tubulin: red) is absent after tamoxifen induction (Kif3a−, cilia−). (B) Exposure of these cells to laminar fluid flow (0 versus 10 dyn/cm²) (positive or negative fluid flow) reduced the content of activated C3 fragments (αC3 and iC3b) in both Kif3a+ and Kif3a− cells, but did not significantly alter β-actin content in these cells. (C) The effects of flow on relative (β-actin adjusted) levels of total C3, αC3, and iC3b were highly significant (all P<0.001; F<sub>1,4</sub>=522, F<sub>1,4</sub>=158, and F<sub>1,4</sub>=218). In the case of total C3 and iC3b, the flow-induced inhibition was stronger in cilia-deficient Kif3a− cells (F<sub>1,4</sub>=25.5, P=0.007; and F<sub>1,4</sub>=20.0, P=0.011). All results represent mean values; bars indicate SEM.

**Figure 5.** Biologically active C3 split products are present in cyst fluid and urine. (A) C3 and its split products are present in cyst fluid aspirates from Cys1<sup>cpk/cpk</sup> kidneys in levels that exceed those found in plasma (same amount of sample was applied to each well); urinary C3 was undetectable (unless urine was immunoprecipitated). (B) C3d, a biologically active cleaved fragment of C3, is present in urine of ARPKD patients and their age- and sex-matched controls (the same amount of urine was used for immunoprecipitation and subsequent loading on the gel). (C) After adjustment for urine creatinine, urinary C3d levels were higher in ARPKD patients versus matched controls.
deletion involving a coding region segment (AA 620–741 of pro-C3). These C3<sup>−/−</sup> mice were backcrossed to C57BL/6J for seven generations. We obtained the mice from the Jackson Laboratory (Bar Harbor, ME) and used them to generate C3 deficient C57<sup>fl/fl</sup>/C3<sup>−/−</sup> mice in an (C3<sup>−/−</sup> × Cys1<sup>flk/flk</sup>) F1 intercross. These unique mutants were sacrificed at P12, biometrical measurements were obtained, and morphometric analyses were performed similarly as described above for studies of cystogenic HO effects. An allele status for C3 was determined with a PCR assay per the distributor’s protocol. Cys1 allele status was determined with Cys1<sup>flk</sup> allele-specific assay.<br><br><strong>Cell Culture Studies</strong><br><br>Renal tubular epithelium-like cell line 176–6c was derived from individual collecting ducts microdissected from mice with temperature sensitive-SV40 (the ImmortoMouse transgene H-2kb-tsA58) and Kif3a knockout driven by tamoxifen-inducible Cre recombinase expressed from the actin promoter (CAGG-creER).<sup>65</sup> The origin of this cell line was confirmed by selective expression of key markers associated with principal cells of collecting ducts e.g., Aqp2 and Avpr2 but not Cdm or Calbi), and its expression profile closely resembled that of the IMCD-K2 cell line, a well established model of principal cells of collecting ducts (gift from Eric Schwiebert, UAB). After induction (1 μg/ml) of a portion of this cell line with 4-OH-tamoxifen (Sigma, St. Louis, MO), the cells were grown for >1 week in tamoxifen-free media at 33°C in 5% CO<sub>2</sub>; the uninduced control portion of the cell line was maintained with a PCR assay per the distributor’s protocol. Cys1<sup>flk</sup> mice were generated as described above, and remnant urine samples were collected according to protocols approved by the UAB Institutional Review Board. Creatinine was measured by ELISA (R&D Systems, Minneapolis, MN).

**Statistical Analyses**

Statistical evaluations were performed with the SPSS 11.5 statistical software package (SPSS Inc). Homogeneity of variance assumption of ANOVA was tested with Levene’s test. If the assumption was not violated, ANOVA was performed to test group differences and significant overall differences were followed up with Dunnett’s test comparing each intervention group to the controls. If the ANOVA assumption of the homogeneity of variance was violated, group differences were tested with the nonparametric Kruskal–Wallis test. The dose effect was analyzed with a linear regression. Subsamples of generated mice analyzed in gene expression and morphometric studies were sex balanced and representative of the full sample. We determined that the number of mice in these subsample groups (n = 6) is sufficient to characterize significant cystogenesis-modulating effects based on our previous studies.<sup>14,20</sup> C3 expression levels in cell culture experiments were compared by mixed ANOVAs. Differences in urinary C3d levels between matched samples were evaluated by paired t tests.

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**DISCLOSURES**

None.

**REFERENCES**


