Galectin-3 Associates with the Primary Cilium and Modulates Cyst Growth in Congenital Polycystic Kidney Disease

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Several lines of evidence implicate the β -galactoside-binding lectin galectin-3 in development and pathological processes in renal collecting ducts: galectin-3 is expressed in the ureteric bud/collecting duct lineage during nephrogenesis, modulates collecting duct growth/differentiation *in vitro*, and is expressed in human autosomal recessive polycystic kidney disease in cyst epithelia, almost all of which arise from collecting ducts. Moreover, exogenous galectin-3 restricts growth of cysts generated by Madin-Darby canine kidney collecting duct-derived cells in three-dimensional culture in collagen. Using the *cpk* mouse model of recessively inherited polycystic kidney disease, we observed widespread galectin-3 mRNA and protein in cyst epithelia. Exogenous galectin-3 reduced cyst formation in suspension culture, and mice-null mutant for *galectin-3* had more extensive renal cysts *in vivo*. Galectin-3 was also detected for the first time in the centrosome/primary cilium, which has been implicated in diverse polycystic kidney disease. Cilia structure/number appeared normal in galectin-3-null mutants. Finally, pacilitaxel, a therapy that retards polycystic kidney disease in *cpk* mice, increased extracellular galectin-3, in which the lectin could potentially interact with cilia. These data raise the possibility that galectin-3 may act as a natural brake on cystogenesis in *cpk* mice, perhaps

via ciliary roles. (*Am J Pathol* 2006, 169:1925–1938; DOI: 10.2353/ajpath.2006.060245)

Galectins are low molecular weight, calcium-independent, β -galactoside-binding lectins.¹ The 30- to 42-kD galectin-3 contains an N-terminal proline-rich domain and a C-terminal carbohydrate recognition domain (CRD), essential for binding simple β -galactosides such as lactosamine and Gal β 1-4GlcNAc and for higher affinity binding to polylactosamine chains.² In the kidney, galectin-3 is expressed in the branching ureteric bud and its collecting duct derivatives during nephrogenesis,³ yet galectin-3-null mutants have almost completely normal kidneys apart from a mild nephron deficit (less than 10% reduction).⁴ Nevertheless, diverse *in vitro* experiments suggest that galectin-3 is important in collecting duct growth and terminal differentiation.^{5,6} Overgrowth of these structures is the most striking renal lesion in human autosomal recessive polycystic kidney disease (ARPKD), which affects 1 in 10,000 to 20,000 and can cause massive kidney distension *in utero* and early neonatal death or progressive renal failure during childhood as well as biliary dysgenesis.^{7,8} We previously demonstrated galectin-3 expression in cysts in human ARPKD and the lectin reduces cyst growth in an *in vitro* model: Madin-Darby canine kidney cells generate cysts when cultured in collagen gel, and exogenous galectin-3 reduces cyst size, whereas galectin-3 blocking antibodies promote cyst expansion.⁹

Human ARPKD is caused by mutations in the *polycystic kidney and hepatic disease (PKHD) 1*,^{10,11} which encodes

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fibrocystin, a transmembrane protein that localizes to the primary cilium/basal body complex.¹² The primary cilium has been the focus of much recent PKD research: this finger-like appendage projects from the cell surface where it senses luminal flow and modulates calcium influx to maintain normal cellular maturation. Almost all of the PKD-associated proteins (eg, polycystins, polaris, inversin, OFD1) immunolocalize to the primary cilium,^{13–16} but galectin-3 has not previously been sought in these structures. The *pck* rat is an orthologous model of ARPKD, but mouse models are not yet widely available and several have liver disease rather than PKD.¹⁷ Phenotypically similar collecting duct cystogenesis does develop, however, in *cpk* (congenital polycystic kidney) mice, which have mutations in *cystin*, and this protein is also expressed in the primary cilium.^{13,14} Heterozygous (+/*cpk*) mice are healthy, and kidneys develop normally, but homozygous *cpk* mice develop cystic dilatation of collecting ducts in the first postnatal week, progressing to massive cyst expansion with destruction of surrounding normal tissues and death by 3 to 4 weeks.¹⁸ The chemotherapeutic agent paclitaxel retards cyst growth in *cpk* mice¹⁹ and some other PKD models.^{20,21}

We hypothesized that galectin-3 might have roles in cyst growth in ARPKD. To begin to explore this question, we investigated galectin-3 protein expression in *cpk* mouse kidneys, comparing it to other ureteric bud/collecting duct lineage markers; then we 1) determined the effects of excess galectin-3 on *cpk* cyst growth in suspension culture, 2) examined whether lack of galectin-3 accelerated early cyst formation *in vivo* using mice on different genetic backgrounds, and 3) assessed the effects of paclitaxel on galectin-3 expression *in vivo* and *in vitro*. These studies demonstrated widespread galectin-3 expression in cyst epithelia, altered cyst growth with different galectin-3 levels, and genotypes *in vivo* and *in vitro* suggestive of a role for galectin-3 as a brake on cyst formation, plus immunolocalization of the lectin to centrosomes and primary cilia. The precise mechanism of action of galectin-3 remains unknown, but preliminary observation of normal cilia structure/number in galectin-3-null mutants and extracellular localization on nonpermeabilized cilia raises the possibility that the effects of galectin-3 may include modulation of ciliary function.

Materials and Methods

Reagents were obtained from Sigma (Poole, UK) unless otherwise stated. Recombinant galectin-3 and mutant galectins were obtained as described.²²

Animals

Animal protocols were approved by the UK Home Office. *Cpk* heterozygotes (Jackson Laboratories, Bar Harbor, ME) on the C57BL-6J background were bred to produce wild-type (+/+), heterozygotes (+/*cpk*), and homozygotes offspring (*cpk/cpk*). Kidneys were collected between 1 and 4 weeks postnatally from five to seven litters each time, hence generating time-matched cystic and

control specimens. Intraperitoneal paclitaxel (150 μ g) in 20 μ l of dimethyl sulfoxide (DMSO) or vehicle alone was administered to some litters at 10 days of age; this dose of paclitaxel was previously shown to retard cyst development and extend the lifespan of *cpk* homozygotes.¹⁹ These litters (10 paclitaxel, six vehicle) were then sacrificed at 3 weeks of age. Kidneys were fixed in 4% paraformaldehyde for immunohistochemistry and *in situ* hybridization, snap-frozen in liquid nitrogen for Western blot, or transported in ice-cold L15 media before cell culture.

We bred *galectin-3*-null mutants²³ with *cpk* heterozygotes to determine the effects of reduced galectin-3 on cystogenesis and analyzed the mice at 1 week of age because we predicted that lack of galectin-3 might accelerate cyst formation. Initial *galectin-3* mutants were on the 129Sv background, which generated double (*cpk* and *galectin-3*) mutants on a mixed C57BL-6J/129Sv background. Subsequently, we backcrossed *cpk* for 10 generations to produce a relatively pure 129Sv background, but nonrenal pathology (see later) confounded these studies; hence, we imported further *galectin-3* mutants on a C57BL-6J background²⁴ to generate double mutants on this pure background. Severity of PKD was assessed by wet kidney weight and kidney to body weight (K/B) ratio. In addition we developed a cystic index to compare PKD severity in the cortex: 0, no cysts; 1, mildly dilated tubules/ducts only; 2, occasional moderately dilated ducts and cysts; 3, more frequent or larger cysts, or cysts extending to the edge of the cortex but with relatively well preserved architecture; 4, frequent large cysts disrupting cortical architecture. Three sections were prepared from each kidney and reacted with *Dolichos biflorus* agglutinin (DBA) and then photographed by a blinded observer and scored by three further blinded observers to generate a mean score.

For genotyping by genomic polymerase chain reaction (PCR), *cpk* primers were 5'-TCCTCCCTCCCTATCTCTCAT-3' and 5'-ATCCAGCAGGCGTAGGGTCTC-3'.¹⁴ These flank the 31-bp deletion in exon 1 of *cystin* and generated 351- or 320-bp products from wild-type and mutant alleles, respectively. *Galectin-3* genotyping for samples on the 129sv and mixed background used two PCR reactions concurrently: galectin-3 primers were 5'-CACGAACGTCTTTTGCTCTCTGG-3' and 5'-TGAAATACCTTACCGAAAAGCTGTCTGC-3', which produced a 305-bp fragment only from wild-type alleles; neomycin primers were 5'-CAAGATGGATTGCACGCAGG-3' and 5'-TATTCGGCAAGCAGGCATCGCCA-3', which generated a 577-bp product only from the mutant, neomycin-containing allele. C57BL-6J *galectin-3* genotyping used two individual primers, with a common downstream primer of 5'-CACTCTCAAAGGGGAAGGCTGACTGTC-3'; wild-type allele, 5'-GTAGGTGAGAGTCACAAGCTGAGGCC-3', which produced a 490-bp fragment; mutant allele, 5'-GGCTGACCGCTTCCTCGTGCTTTACGG-3', which amplified a 300-bp segment from the neomycin gene.

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded kidney sections were probed with antibodies to galectin-3 or the collecting duct marker DBA as described.^{3,25} Other antibodies were aquaporin-2 (Chemicon, Chesham, Hampshire, UK), the archetypal water channel expressed in collecting ducts from early gestation,^{26,27} and calbindin-d28k (calbindin; Chemicon) a cytoplasmic calcium binder expressed in the developing ureteric tree and mature distal tubules/collecting ducts.²⁸ Negative controls comprised preimmune serum or preabsorption with recombinant wild-type protein.

In Situ Hybridization

Galectin-3 probes were generated as described.²⁹ In brief, an 883-bp galectin-3 cDNA insert in Bluescript KS vector was used to transform competent *Escherichia coli* XL1-Blue MRF (Stratagene, La Jolla, CA). Sense and anti-sense probes were labeled with digoxigenin-UTP (Boehringer Mannheim, Lewes, UK). *In situ* hybridization was performed on 7- μ m sections as described.³⁰

Western Blotting

Proteins were extracted from tissues and cysts and quantified as described.^{30,31} Fifty μ g of protein was used per lane, except when preliminary studies demonstrated low galectin-3 levels—galectin-3 was then concentrated using fetuin, which has a high affinity for the CRD.²² Equal volumes of lysate (or medium from cyst cultures) and fetuin-agarose beads were incubated overnight at 4°C, washed repeatedly with phosphate-buffered saline, and then resuspended in sample buffer and boiled to dissociate galectin-3. Western blots were then performed on the supernatant or protein extracts as described.³ Equality of loading/transfer was confirmed initially by Ponceau S staining, then quantified and adjusted with reference to β -actin, a housekeeping protein.³² Controls comprised preabsorption with or loading of recombinant galectin-3.

Cyst and Cell Culture

Cyst culture was performed by dicing and enzymatically digesting kidneys, and the resulting suspension of single cells, small aggregates, and short tubule segments was transferred to agar-coated plates (which prevent adhesion) for 24 hours in a 5% CO₂ air incubator.³¹ Cystogenesis was assessed with additives at the following concentrations, as previously determined^{5,9,19}: recombinant wild-type galectin-3 (10 or 30 μ g/ml), SS mutant galectin-3 with an altered CRD (30 μ g/ml), paclitaxel (10, 35, 50, or 100 μ mol/L), or DMSO (the vehicle for paclitaxel). Ten random fields per well were photographed by a blinded observer after 24 hours, and cyst number and area measured using Scion Image software (Scion Corp., Frederick, MD); three or four wells were used per condition from each kidney to generate average cyst number and size, which were compared with other conditions for

the same kidney (see Statistics).³¹ Inner medullary collecting duct (IMCD) cells were used as described in previous cilia/centrosome studies,³³ and primary *cpk* cultures were generated by plating dissociated kidneys onto standard tissue culture-coated dishes. IMCD cells were grown to confluence and serum starved to induce ciliation whereas *cpk* cells were assessed after 48 hours before cultures became overgrown with fibroblasts. Immunocytochemistry of cysts was performed as described.³¹ High-power views of cysts were generated by digital magnification because it was technically impossible to focus through the cysts on our system without displacing or disrupting them; the resultant loss of fine resolution meant that we were unable to ascertain galectin-3 distribution in cilia in cysts, although these and the associated basal body/centrosome complex were identified in monolayers using antibodies against acetylated α -tubulin and γ -tubulin.^{16,34} Immunocytochemistry of extracellular galectin-3 was obtained by washing and incubating live cells with blocking solution and then anti-galectin-3 antibodies at 4°C before fixation and permeabilization. Efficacy of this technique was confirmed by lack of ciliary acetylated α -tubulin labeling in nonpermeabilized cells (not shown).

Electron Microscopy

Standard processing for scanning electron microscopy was used. Samples were fixed in 3% glutaraldehyde/0.1 mol/L sodium cacodylate/5 mmol/L calcium chloride, manually chipped into 2- to 3-mm pieces under liquid nitrogen, and then passed through distilled water and 1% osmium tetroxide to 100% ethanol. Critical point drying was then performed and samples mounted on aluminum stubs before overnight vacuum drying. They were then gold coated and imaged with a Jeol JSM-35CF scanning electron microscope (Jeol, Welwyn Garden City, UK).

Statistics

Galectin-3 signals were standardized against β -actin to allow semiquantification on Western blot and groups compared using Student's *t*-test. Control, paclitaxel-treated, and galectin-3-treated cyst cultures from the same kidney were compared, plus three to five repetitions in other kidneys. Data were analyzed by two complementary methods: Student's *t*-test and multilevel-modeling (MLwiN 1.10; Centre for Multilevel Modeling, University of Bristol, Bristol, UK, <http://www.cmm.bristol.ac.uk/>), which enabled us to include potential confounders such as interanimal and litter variation.³¹ Virtually identical results were generated by both methods; hence unmodified Student's *t*-test data are reported. For graphical representation (Figures 3 and 4), control cultures were standardized to 100% and experimental results expressed as mean $\pm$ SEM.

Results

Galectin-3 Expression in Normal and Polycystic Kidneys

Our previous research identified galectin-3 almost exclusively in the ureteric bud and collecting duct lineage during development,^{3,5} and this restricted expression pattern was observed here between 1 and 4 weeks in noncystic C57BL-6J mice (Figure 1, A and B; and data not shown). Galectin-3 protein was also detected in the renal pelvis urothelium (Figure 1C). In cystic kidneys, prominent galectin-3 immunoreactivity was detected from mildly dilated collecting ducts through the largest cysts (Figure 1, D and E). Immunostaining was abolished after preabsorption with recombinant galectin-3 (Figure 1G) and, as expected, galectin-3 immunoreactivity was not observed in sections from galectin-3 mutant mice (Figure 1H). Antibody specificity was confirmed using Western blot: clear bands were obtained in cystic kidneys at the expected size of 30 kd (with weaker 60-kd dimers), but fetuin-agarose concentration had to be used to consistently detect these in normal littermates. Antibody preincubation with recombinant protein abolished these bands, and recombinant galectin-3 gave similarly sized bands (Figure 1I). Higher overall galectin-3 levels were confirmed in time-matched cystic kidneys (Figure 1J; $P < 0.05$), but this might be secondary to the increased proportion of collecting duct-derived epithelia with corresponding loss of normal surrounding tissues as cysts develop.³⁵

Because galectin-3 can be secreted, which raised the possibility of synthesis in some cells and uptake by others, we used *in situ* hybridization to localize galectin-3 mRNA. Signal was observed in a subset of tubules in normal kidneys, which phenotypically resembled the collecting ducts positive by immunohistochemistry, but more prominent transcripts were detected in cyst epithelia in cystic kidneys (Figure 1, K and L). No signal was detected using the sense probe (Figure 1M).

Widespread galectin-3 expression in cysts might simply represent nonspecific re-expression of ureteric bud/collecting duct markers as epithelia dedifferentiate toward an embryonic-like phenotype. Hence, we compared expression with other collecting duct proteins including aquaporin-2 and calbindin (Figure 2). Similar expression patterns were observed for galectin-3 and aquaporin-2 in normal kidneys, with positive immunoreactivity restricted to collecting ducts (Figure 2, A and B).

Calbindin expression was more widespread, including some distal tubule segments (Figure 2C). In *cpk/cpk* mice, immunostaining for aquaporin-2 and calbindin was observed in some but not all cysts. In addition, aquaporin-2 immunostaining was often heterogeneous within individual cysts, whereas calbindin immunoreactivity appeared much stronger in smaller cysts (Figure 2, E and F). These data contrast with prominent, consistent galectin-3 immunoreactivity across the whole range of cyst sizes (Figure 2D).

Galectin-3 Expression and Modulation of Cyst Numbers in Vitro

Suspension cultures started with a mixture of single cells, small aggregates, and tubules, and these components appeared unchanged after 24 hours of culture of noncystic kidneys, whereas dissociated cystic kidneys (re)generated numerous cysts up to 100 μ m in diameter (Figure 3, A–D). Most cysts were associated with cell aggregates rather than tubular structures and reacted with DBA (data not shown), consistent with collecting duct origin, whereas more than 95% of cells lining the cysts expressed galectin-3 (Figure 3E); minimal background immunofluorescence was observed in the cysts using preimmune serum (Figure 3F). A significant reduction in cyst numbers was recorded at 24 hours with 10 or 30 μ g/ml exogenous wild-type galectin-3 ($P < 0.05$, Figure 3G); there was no significant difference between the two doses of galectin-3, and the effect was reproducible, with average reductions of cyst numbers from 60 to 80% using a total of eight polycystic animals. Similar concentrations of mutant SS galectin-3, which has an altered CRD, had no effect on cyst numbers, and less than 5% of cells were nonviable in any of the cultures as assessed by trypan blue uptake (data not shown).

Effects of Galectin-3 Mutation on Cyst Formation in Vivo

Based on the above experiments, we predicted that up-regulated galectin-3 might act as a brake on cyst formation in *cpk* mice; hence we generated compound *cpk/galectin-3* mutants and examined these at 1 week of age to ensure we did not miss accelerated cyst formation. Initial experiments were on the mixed C57BL-6J/129Sv background: 266 offspring were generated and geno-

Figure 1. Renal galectin-3 protein and mRNA expression. All samples from 3-week-old mice. **A–C, K:** Wild-type kidneys; **D–G, I, M:** *cpk/cpk* cystic kidneys, with wild-type galectin-3; **H:** *cpk/cpk* cystic kidneys in galectin-3-null mutant (KO); **A–F, H:** galectin-3 immunohistochemistry; **G:** immunohistochemistry with preimmune serum; **F:** DBA-labeled; **K–M:** *in situ* hybridization with galectin-3 probes as labeled. **A:** Low-power view demonstrated galectin-3-positive collecting ducts in cortex (one prominent duct in longitudinal section is **arrowed** as an example) and medulla. **B:** Higher power view of cortex demonstrated galectin-3 expression in ducts (**arrows**) between PAS-positive (but galectin-3-negative) proximal tubules. Using adjacent sections, these galectin-3-labeled ducts also bound DBA, confirming that they were collecting ducts (data not shown). **C:** Strongly positive galectin-3 immunohistochemistry in the urothelium (**arrows**). **D:** Galectin-3-positive cysts throughout cortex and medulla of cystic kidney. **E and F:** Prominent galectin-3 expression in cyst (cy) epithelia, which also bound DBA, hence confirming collecting duct origin. **G and H:** Positive signal was not detected in cystic epithelia using either preimmune serum (**G**) or in galectin-3-null mutant mice (**H**). **I:** Galectin-3 Western blot from wild-type and *cpk/cpk* kidneys demonstrated a 30-kd major and a 60-kd minor band consistent with monomers and dimers, which was abolished by preabsorption with excess recombinant galectin-3 protein (preabs). Recombinant galectin-3 was used as a positive control (gal-3). **J:** Band densitometry confirmed a significantly increased amount of galectin-3 in cystic versus wild-type kidneys ($n = 5$; $*P < 0.05$). **K:** Anti-sense galectin-3 probe revealed a weak signal in subset of tubules (**arrows**). **L:** Prominent signal in cystic epithelia (cy). **M:** No significant signal in cystic epithelia using the sense probe (cy). Scale bars: 200 μ m (**A, D**); 30 μ m (**B, C**); 50 μ m (**E–H**); 20 μ m (**K–M**).

Figure 2. Galectin-3 and collecting duct proteins in wild-type and cystic kidneys. All samples from 3-week-old mice. **A–C:** Wild-type kidneys; **D–F:** *cpk/cpk* kidneys. Immunostaining was for galectin-3 (*gal-3*), aquaporin-2 (*aq-2*), and calbindin (*calb*). All counterstained with PAS. **A and B:** Similar distribution of immunoreactive galectin-3 and aquaporin-2 in collecting ducts (arrows) in normal kidneys; **C:** more widespread calbindin expression in distal tubules as well as collecting ducts. **D:** Prominent cytoplasmic galectin-3 expression in collecting ducts/cysts of all sizes, from small undilated tubules through to large cysts. **E and F:** Aquaporin-2 and calbindin expression was variable: immunoreactivity was observed in some but not all cysts, aquaporin-2 distribution was often heterogeneous within individual cysts, and there was intense calbindin immunostaining of tubules and small cysts but reduced intensity in larger cysts (*). Scale bar = 50 μ m.

typed; 63 were wild type at the *cpk* locus, 142 were heterozygote, and 61 homozygous *cpk* mutants; these ratios are not significantly different from the 1:2:1 expected. From the latter 61, 15 had wild-type *galectin-3*, 35 were heterozygotes, and 11 were null mutants; although there were fewer *galectin-3*-null mutants, the altered ratio was not significant. For noncystic mice (ie, wild-type and heterozygous *cpk*), *galectin-3* genotype had no effect on either absolute kidney weight or K/B ratio (data not shown). For the cystic mice (ie, *cpk/cpk*), *galectin-3*-null mutants had a significantly higher mean kidney weight (65 mg versus 46 mg, $P < 0.03$; Figure 3H) and K/B ratio (1.72 versus 1.15%, $P < 0.02$; Figure 3I) than *galectin-3* wild-type mice. Next, we performed two further sets of breeding experiments. First, we backcrossed the *cpk* allele for 10 generations to produce a virtually pure 129Sv background: florid cyst formation was observed with consistent K/B ratios approaching 2.5% at 1 week (data not shown), but there was an unfortunate confounder because *cpk/cpk* mice also developed massive pancreatic cysts and liver inflammation leading to premature death and perturbed genotype frequencies. Secondly, we generated double mutants on the pure C57BL-6J background: 118 progeny were examined at 1 week of age, with genotypes at the expected Mendelian ratio. For cystic mice, absolute kidney weights were similar (null mutant, 34 mg, versus wild type, 30 mg; $P = 0.8$) but K/B ratios suggested a trend approaching significance (0.92% versus 0.79%, $P = 0.07$). Both genotypes had frequent medullary cysts, but we detected marked differences in cortical cyst development: prominent DBA-positive cysts extended to the edge of the cortex in the majority of *galectin-3*-null mutants, whereas these were rare in wild types. The cortical cystic index was significantly increased in *galectin-3*-null mutants versus wild types ($P = 0.01$; Figure 3, J–L).

Effects of Paclitaxel on Galectin-3 Expression in Cystic Kidneys and in Vitro Cyst Culture

In Vivo

A single dose of 150 μ g of paclitaxel at 10 days reproducibly prolonged the lifespan of cystic (wild-type *galectin-3*) mice beyond 45 days, whereas vehicle (DMSO)-treated mice died at ~4 weeks of age, confirming previous observations.¹⁹ Paclitaxel-treated *cpk/cpk* mice had much better preservation of renal architecture than mice administered vehicle alone: although dilated collecting ducts were observed, very large cysts were rarely seen in the cortex, and relatively normal appearing glomeruli and proximal tubules were present (data not shown). Using confocal microscopy, galectin-3 expression was detected in more than 95% of cells in cyst epithelia of vehicle-treated mice (Figure 4A), predominantly in a cytoplasmic distribution although a weak speckled nuclear signal was occasionally observed (Figure 4D). Galectin-3 again immunolocalized to the epithelia of dilated tubules and small cysts in paclitaxel-treated mice (Figure 4B): cytoplasmic staining was observed in

some of these cells (Figure 4E, arrow), whereas another subset had more prominent apical immunostaining (Figure 4E, arrowheads), which was only rarely observed in control-treated sections. Positive signal was not observed with preimmune serum (Figure 4, C and F). Galectin-3 protein levels appeared qualitatively lower in paclitaxel versus DMSO-treated control animals (Figure 4G), which might reflect the reduced percentage of cystic epithelia overall and/or potential redistribution of galectin-3. Stronger galectin-3 bands were observed in cyst fluid in paclitaxel-treated mice than their DMSO-treated littermates (Figure 4H).

In Vitro

Confocal imaging demonstrated cytoplasmic galectin-3 immunoreactivity in control cyst cultures (Figure 4I), whereas immunostaining was prominent on the external surface and less evident in the cytoplasm of cysts generated in medium with added paclitaxel (Figure 4J). This external surface can be considered equivalent to the apical membrane *in vivo* because cyst polarity is reversed in suspension¹⁹; cilia were visible on this surface, but resolution was insufficient to ascertain cilia galectin-3 expression in different conditions. Doses of 35 and 50 μ mol/L paclitaxel (30 and 42 μ g/ml, respectively) caused a significant reduction in the number of cysts in suspension culture at 24 hours ($P < 0.02$ and $P < 0.05$, respectively; Figure 4K). Additional experiments in which both paclitaxel and galectin-3 were added did not cause a greater inhibition of cyst numbers or size (data not shown). Western blotting demonstrated that paclitaxel was associated with a tendency to decreased galectin-3 levels in the cell-associated fraction of cyst cultures but increased concentration in conditioned medium consistent with galectin-3 secretion (Figure 4L).

Cilia and Centrosome-Associated Galectin-3 Expression

We used epithelial monolayer culture to seek galectin-3 expression in the cilia/centrosome complex. Our initial focus was IMCD cells because of previous cilia/centrosome studies,³³ but similar results were noted with *cpk* primary cultures. Diffuse cytoplasmic galectin-3 was consistently detected throughout IMCD monolayers but, using z-plane scanning, localized immunoreactive protein was also observed in cilia, mitotic spindle poles, and areas consistent with centrosomes/basal bodies, co-localizing with acetylated α -tubulin (Figure 5, A–C). Parallel sections using preimmune serum were negative (Figure 5D). At higher power, discontinuous punctate galectin-3 expression was frequently detected in primary cilia (Figure 5, E–H). Centrosomal distribution was confirmed by co-localization with γ -tubulin (Figure 5, I–K). The punctate galectin-3 pattern might be consistent with intraflagellar transport, but using unpermeabilized cells we demonstrated that at least some of the galectin-3 was extracellular (ie, on the outside of cilia; Figure 5, L–N). Cilial galectin-3 was also observed consistently after paclitaxel

treatment, but we were unable to quantify whether the levels and distribution differed in the present study (data not shown).

Cilia in *cpk*/galectin-3 Mutant Kidneys

Changes in cilia structure might explain the differences in cyst progression in galectin-3 mutants; hence, we examined gross cilia structure and number in C57BL-6J and mixed background mice using confocal and electron microscopy. Confocal images of kidney sections revealed frequent acetylated α -tubulin-positive cilia in wild-type mice, *cpk* mutants with normal *galectin-3* genotype, and *cpk* mutants lacking *galectin-3*, and we did not observe gross differences in thickness or length (Figure 6, A and B; and data not shown). Small and large cysts were examined using electron microscopy: cilia were observed arising from more than 90% of cells lining cyst epithelia in both *galectin-3* wild-type and null mutant animals (Figure 6, C–H), and cilia length and distribution appeared identical although this was not formally quantified. These preliminary observations suggest that galectin-3 genotype does not perturb gross ciliary structure, but further work is needed to accurately measure size and distribution in all of the strains and genotypes.

Discussion

Our results identify prominent galectin-3 expression in the *cpk* model of ARPKD and raise the possibility that levels of this lectin may be functionally important in cyst growth.

Galectin-3 in *cpk* Collecting Duct Cysts

Galectin-3 is expressed widely during development²⁹ but has a restricted distribution during nephrogenesis: in humans, galectin-3 immunolocalizes to the mesonephric duct, then in the ureteric bud, which branches from it, and finally in bud derivatives, the collecting ducts³; mouse development is similar, with prominent cytoplasmic staining in developing collecting ducts and basal immunolocalization in maturing ducts.⁵ Here we observed continuing collecting duct-specific expression in mice up to 4 weeks after birth well after nephrogenesis finishes, which is consistent with a recent report in the ddY mouse strain.³⁶ Such lineage-specific expression patterns are intriguing because *galectin-3*-null mutants

are viable and have grossly normal kidney development,³⁷ albeit with a 10% decrease in nephron numbers.⁴ These minor defects suggest that other mechanisms must compensate for loss of normal galectin-3 expression *in vivo*, but what about pathological situations when galectin-3 should be widely expressed? One example is peritonitis: galectin-3 was initially reported as “Mac-2” in activated macrophages during experimental peritonitis, and unsurprisingly, null mutants have inflammatory defects in the response to peritonitis.²⁴ Likewise, in the kidney, lack of the lectin makes mice more susceptible to both diabetic and age-related glomerulopathy.^{38,39} These data raise the possibility that galectin-3 might have a protective effect in some renal diseases, but what about PKD?

We previously observed galectin-3 up-regulation in human ARPKD and herein demonstrated widespread galectin-3 expression in *cpk* mice by immunohistochemistry, *in situ* hybridization, and Western blotting. This pattern was not replicated by other collecting duct markers, including aquaporin-2 and calbindin: consistent cytoplasmic galectin-3 expression occurred in all cyst sizes, whereas aquaporin-2 and calbindin were not detected in all cysts, aquaporin-2 immunostaining was often heterogeneous even within individual cysts and calbindin appeared much stronger in smaller cysts. These data suggest that there is not a common mechanism involving widespread expression of collecting duct proteins during cystogenesis.

Galectin-3—A Brake on Renal Cystogenesis?

What possible functions might widespread galectin-3 expression have in PKD? There are at least four possibilities: 1) it may cause increased cyst formation, perhaps by acting as a cell survival factor,⁴⁰ paralleling pax-2 overexpression in both *cpk* and human cystic kidney disease^{41,42}; 2) it may restrict cystogenesis, because the lectin does retard cyst growth in Madin-Darby canine kidney collagen gel cyst cultures,^{9,43} restricts growth and branching of the ureteric bud/collecting duct lineage in organ culture,⁵ and can promote differentiation *in vitro*⁶; 3) it may have no effect on cyst growth but have other functions such as adaptation to acidosis⁴⁴; or 4) it may have no function at all. We explored these functions using two approaches: first, suspension culture using cells from our *cpk* mice, a technique previously used in human

Figure 3. Exogenous galectin-3 reduced cyst growth *in vitro*, whereas the lack of the lectin accelerated cystogenesis on two genetic backgrounds *in vivo*. A–G: Suspension culture experiments; samples from mice on pure C57BL-6J background; G–J: *cpk* and *galectin-3* mutant breeding results. **A** and **B**: Representative images of suspension cystogenesis in dissociated wild-type kidneys at the start of culture and after 24 hours. Single cells, aggregates, and partially digested tubules were observed at both times; cysts were very rarely seen after culture of these normal kidneys. **C** and **D**: Dissociated *cpk/cpk* kidneys appeared similar to those from wild-type littermates at the start of culture but multiple cysts were visible by 24 hours. **E**: Prominent cytoplasmic galectin-3 expression in cysts (L indicates lumen) as assessed by confocal microscopy; **F**: preimmune serum. **G**: Ten and thirty μ mol/L galectin-3 caused decreased cyst numbers versus control (0) cultures after 24 hours ($n = 5$, $^*P < 0.05$); there was no significant difference between the two doses. **H** and **I**: Initial experiments compared wet kidney weight or kidney to body weight (K/B) ratio in *cpk/cpk* mice on a mixed C57BL-6J/129Sv background with either wild-type (W) or null mutant (M) *galectin-3* genotypes: both kidney weight and K/B ratio were larger in mice lacking *galectin-3*; mean kidney weight was 65 mg in *galectin-3* mutants versus 46 mg in wild-type mice ($^*P < 0.03$), whereas K/B ratio was 1.72% for mutants and 1.15% for *galectin-3* wild-type mice ($^*P < 0.02$). **J** and **K**: DBA-labeled sections of *galectin-3* wild-type and null mutant cystic sections on the pure C57BL-6J background: cysts were consistently larger in the *galectin-3*-null mutants, bound DBA (ie, still derived from collecting ducts) and extended further into the cortex, often reaching the outer rim. (Note **J** was assessed as a cortical cystic index of two and **K** of three). **L**: Cortical cyst score was significantly increased in 1-week-old *cpk/cpk galectin-3*-null mutant mice on a pure C57BL-6J background (mean 2.83 versus 2.01 for *galectin-3* wild-type; $^*P = 0.01$). Scale bars: 80 μ m (**A–D**); 20 μ m (**E**, **F**); and 200 μ m (**J**, **K**).

Figure 4. Effects of paclitaxel *in vivo* and *in vitro*; all samples are from *cpk* cystic kidneys. **A–D:** Galectin-3 confocal immunohistochemistry on control, vehicle-treated mice (**A, C, D, F**); or mice treated with paclitaxel (**B, D**); **C** and **F:** preimmune serum replaced anti-galectin-3 antibody; scanning voltage/offset was equalized to allow clearer comparison of these sections. Paclitaxel-treated kidneys contained fewer smaller cysts and renal architecture was better preserved. Galectin-3 distribution was predominantly cytoplasmic in control cyst (cy) epithelia (**A** and **C**); expression persisted in cysts and tubules (t) from paclitaxel-treated animals (**B** and **D**), although signal intensity appeared reduced compared with vehicle-treated kidneys and some cells had more prominent apical (arrowheads) rather than cytoplasmic (arrows) immunolocalization. Low levels of background signal were observed in sections exposed to preimmune serum. **G:** Western blot demonstrated less galectin-3 in whole kidney homogenates from paclitaxel-treated cystic mice versus vehicle-treated mice at 3 weeks, but this result may be biased by the decreased percentage of cystic epithelia in the paclitaxel group. **H:** Galectin-3 bands appeared stronger in cyst fluid from paclitaxel mice; one potential explanation is that this agent may cause galectin-3 secretion into the cysts. **I** and **J:** Digitally magnified images of galectin-3 immunocytochemistry on cysts grown in control medium (**I**) or with 50 μ M/L paclitaxel (**J**); prominent cytoplasmic galectin-3 immunoreactivity was observed in controls whereas positive signal was more prominent on the external surface of paclitaxel-treated cysts. Cilia were detected on the external surface of some cysts but could not be visualized at sufficient resolution to determine ciliary galectin-3 expression; cy indicates the cyst lumen, arrows indicate the internal surface, and arrowheads the external surface. **K:** 35 and 50 μ M/L paclitaxel significantly reduced both cyst number and mean cyst area compared with DMSO (vehicle) control cultures ($n = 4$; $*P < 0.05$; $**P < 0.02$). **L:** Representative Western blot of galectin-3 protein in the cell-associated fraction (cells) or paired conditioned media (medium) from cultures treated with vehicle (0) or 50 μ M/L paclitaxel (50), ie, lane 1 of the cell blot came from the same culture as lane 1 of the medium blot and so forth. Galectin-3 was prominent in the cell preparation but barely detected in the media in control cultures, whereas the lectin was more prominent in the medium when exposed to 50 μ M/L paclitaxel. Scale bars: 20 μ m (**A–C**); 5 μ m (**D–F**, and **I, J**).

Figure 5. Galectin-3 is expressed in cilia and centrosomes *in vitro*. Confocal immunocytochemistry of IMCD (A–D, L–N) and *cpk* (E–K) epithelial monolayers. Galectin-3 is FITC (green)-labeled, and co-stained with TRITC (red)-labeled acetylated α -tubulin (A–G, K–M) or γ -tubulin (J and K). Note parallel cultures stained with preimmune and preabsorbed galectin-3 were completely negative (not shown). A–C: Diffuse cytoplasmic galectin-3, plus co-localization with acetylated α -tubulin; appearances consistent with centrosomes (arrowheads), mitotic spindle poles (narrow arrows), and basal bodies (thick arrows). D: Control sections (inset) using preimmune serum demonstrated low levels of background fluorescence, and cilia were not visible. E–G: Primary cilia appeared structurally normal and galectin-3 expression was detected in/on them (arrowheads) in a discontinuous pattern. H: Further punctate galectin-3 in a long cilium. I–K: Galectin-3 in the centrosome, co-localized with γ -tubulin (arrows). L–N: Galectin-3 immunoreactivity was still detected even when immunostained before permeabilization, suggesting that some galectin-3 protein is extracellular; yellow line indicates edge of cell. Acetylated α -tubulin staining was completely negative without permeabilization (data not shown). Scale bars: 10 μ m (A, B, D); 5 μ m (C); 0.5 μ m (E, F); 0.25 μ m (G, H, L, M); and 0.1 μ m (I–K).

ADPKD and several rodent PKD models,^{19,20,31} and second, a genetic approach using *cpk/galectin-3* mutants.

Dissociated cystic kidneys (but not wild-type organs) generated cysts in suspension culture and galectin-3 significantly reduced cyst numbers in this system. The suspension model is not perfect (see below) but Woo and colleagues¹⁹ did demonstrate that ADPKD-dissociated

Figure 6. Cilia in *cpk* mice with wild-type and null mutant *galectin-3*. All images from cystic mice. Left column depicts sections from mice with wild-type *galectin-3* genotype and right column from *galectin-3*-null mutant mice on the mixed C57BL/6J/129Sv background. A and B: Galectin-3 FITC (green); acetylated α -tubulin TRITC (red). Similar size and distribution of cilia observed using confocal microscopy; galectin-3 immunoreactivity was not detected in sections from *galectin-3*-null mutants. C–H: Scanning electron microscopy demonstrated cilia arising from more than 95% of cells lining the cysts. Gross differences in cilia size and appearance were not observed between the different genotypes and background strains. Scale bars: 2 μ m (A, B); 80 μ m (C, D); 8 μ m (E, F); and 4 μ m (G, H).

cells formed cysts much more readily than normal cells in this milieu, so it is rather good system at distinguishing PKD from normal renal epithelia. The growth regulatory/anti-cyst effects in organ culture and Madin-Darby canine kidney studies were attributed to binding of wild-type galectin-3 CRD to extracellular structures such as GalNAc α 1,3(Fuc α 1,2)Gal β 1,4 GlcNAc, thereby enhancing cell-matrix and cell-cell interactions at basal and lateral surfaces. Adhesion partners include laminins, integrins, and hensen.^{6,45} Anti-cyst effects are not necessarily via the same mechanism in suspension, however, because cyst morphology is inside-out with microvilli on the external surface in suspension.^{46,47} These data reiterate the need for further studies *in vivo*, but it is noteworthy that the other results reported here suggest that galectin-3 has access to the morphologically luminal surface (and cilia) in both *cpk* mice and in suspension because the

lectin was detected in cyst fluid *in vivo* and exogenous galectin-3 would have immediate access to these structures in suspension culture.

We explored whether lack of galectin-3 accelerated cystogenesis *in vivo* using compound *cpk/galectin-3* mutants. Similar breeding strategies demonstrated the importance of *pax-2* and EGF signaling in *cpk* and other murine PKD.^{42,48} Our initial experiments using mice on a mixed genetic background demonstrated that severity of PKD was significantly worse at 1 week of age in null mutant *galectin-3* mice compared with wild-type animals, as assessed by K/B ratio and cyst size/distribution on histology (Figure 3, H and I; and data not shown). These mice appeared healthy and had indistinguishable weights from their littermates at this early stage of PKD; hence it is unlikely that there were major differences in acidosis, which is one of the potential functions of galectin-3,⁴⁴ although we did not assess acid-base status here. There was large variability in PKD severity between the offspring, however, raising the possibility of disease-modifying quantitative trait loci⁴⁹; hence, the next logical experiment involved backcrossing the mice until we had a relatively pure 129Sv background. This was done for 10 generations, and florid kidney cysts were observed by 1 week, but *cpk/cpk* mice also developed massive pancreatic cysts and liver inflammation irrespective of galectin-3 genotype; moreover, many mice died prematurely. These pathologies will be the focus of a separate article, but concentrating on the kidney phenotype, we then imported a different galectin-3 mutant already on the C57BL-6J background. One-week-old pups on this background were smaller than 129Sv, and the K/B ratio was even more diminished; hence it might have been too early in the PKD disease process to expect major differences in cyst formation. Nevertheless, there were consistent changes in histology with a significantly higher cystic index in galectin-3-null mutants. More experiments are needed to assess cyst progression at later stages, but these data already confirm that lack of galectin-3 has effects on early cyst formation on at least two genetic backgrounds. Taken together with our, and previous, studies on cyst culture, we suggest that galectin-3 may be acting as a natural brake on cyst formation, although endogenous expression is clearly insufficient on its own to block cyst formation completely. This may parallel the beneficial effects of galectin-3 in experimental diabetic nephropathy, in which there is up-regulation of the lectin by the diabetic milieu and accelerated glomerulopathy in galectin-3-null mutants.^{38,39}

Paclitaxel and Redistribution of Galectin-3

Paclitaxel retards cyst growth in certain ARPKD models *in vivo* and *in vitro*.^{19,21,50} One action of paclitaxel is to stabilize microtubules, hence potentially affecting diverse functions including membrane vesicle trafficking, exo/endocytosis, and cilia formation^{51,52}; there also appears to be a correlation between this function and efficacy in preventing cyst formation *in vivo*.⁵³ Paclitaxel has numerous other potential actions, however, including induction

of cytokines, indirect cytotoxicity, and hyperphosphorylation of bcl-2. Because galectin-3 and paclitaxel both reduce cyst formation *in vitro*, we explored the possibility that these effects may be linked. Reiterating previous observations,¹⁹ one 150- μ g paclitaxel dose at 10 days retarded cyst development and preserved renal architecture at 21 days. This effect was associated with decreased whole kidney galectin-3 levels versus vehicle-treated littermates, which was statistically significant when factored for β -actin (data not shown). One potential explanation for this difference could be the reduced proportion of collecting duct epithelia in the paclitaxel-treated kidneys, but altered distribution might also contribute because there was less cytoplasmic staining and prominent apical signal in paclitaxel-treated mice. Increased galectin-3 was also detected in cyst fluid, contrasting markedly with the decreased levels in the whole kidney. *In vitro*, paclitaxel decreased cyst numbers, again with decreased cytoplasmic and prominent apical (external surface) immunostaining, which was associated with increased galectin-3 in the conditioned medium. These data suggest that paclitaxel stimulates galectin-3 secretion, which is consistent with previous preliminary studies in monolayer culture of baby hamster kidney cells (R.C.H., personal observation), and may explain why the combination of exogenous galectin-3 plus paclitaxel did not have an additive effect.

Galectin-3 in the Primary Cilium/Centrosome

The primary cilium/centrosome complex has become central to PKD research and we detected galectin-3 in this location. At least some of the lectin was extracellular, as assessed using nonpermeabilized cells, whereas gross cilia structure/distribution was unchanged in *galectin-3*-null mutants (pending more formal review of size/distribution across different genotypes). Intriguingly, exogenous galectin-3 in suspension and paclitaxel *in vivo* and *in vitro*, which both decreased cyst formation, appear likely to deliver galectin-3 to a position where it can associate with the primary cilium. Hence, it is possible that galectin-3 in this location might modulate ciliary signaling to maintain epithelial stabilization and maturation. Unfortunately, our current analysis of galectin-3 distribution within individual cilia was too imprecise to detect changes in distribution/quantity of ciliary lectin with either exogenous galectin-3 or paclitaxel, so this should be one area to consider in future studies. Other potential avenues might include orthologous ARPKD models (when mutants with renal cysts become available), and it would also be intriguing to investigate *orpk* mice: paclitaxel does not reduce *orpk* cyst formation,⁵⁴ but would galectin-3 be equally ineffective in these mice with malformed cilia?

Conclusion

In conclusion, we report widespread expression of galectin-3 in the *cpk* model of ARPKD, that exogenous galectin-3 reduced cyst numbers in suspension culture

and *galectin-3*-null mutant mice on different genetic backgrounds had more severe PKD at 1 week of age. In addition, we demonstrated altered galectin-3 distribution after administration of the anti-cyst drug paclitaxel consistent with increased galectin-3 secretion. Finally, we report expression of galectin-3 in the primary cilium/centrosome for the first time. These data raise the possibility that galectin-3 may act as a natural brake on cystogenesis in *cpk* mice, perhaps via ciliary roles.

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