The Zebrafish as a Model to Study Polycystic Liver Disease

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Abstract

In the polycystic liver diseases (PLD), genetic defects initiate the formation of cysts in the liver and kidney. In rodent models of PLD (i.e., the PCK rat and *Pkd2*^{WS25/-} mouse), we have studied hepatorenal cystic disease and therapeutic approaches. In this study, we employed zebrafish injected with morpholinos against genes involved in the PLD, including *sec63*, *prkcsh*, and *pkd1a*. We calculated the liver cystic area, and based on our rodent studies, we exposed the embryos to pasireotide [1 μ M] or vitamin K3 [100 μ M] and assessed the endoplasmic reticulum (ER) in cholangiocytes in embryos treated with 4-phenylbutyrate (4-PBA). Our results show that (a) morpholinos against *sec63*, *prkcsh*, and *pkd1a* eliminate expression of the respective proteins; (b) phenotypic body changes included curved tail and the formation of hepatic cysts in zebrafish larvae; (c) exposure of embryos to pasireotide inhibited hepatic cystogenesis in the zebrafish models; and (d) exposure of embryos to 4-PBA resulted in the ER in cholangiocytes resolving from a curved to a smooth appearance. Our results suggest that the zebrafish model of PLD may provide a means to screen drugs that could inhibit hepatic cystogenesis.

Introduction

POLYCYSTIC LIVER DISEASE (PLD), characterized by formation of multiple fluid-filled cysts of biliary origin, is associated with autosomal dominant polycystic liver disease (ADPLD), autosomal dominant polycystic kidney disease (ADPKD), and autosomal recessive polycystic kidney disease (ARPKD). ADPKD (caused by mutation in two genes, *pkd1a* and *pkd2*) and ARPKD (caused by mutation in *pkhd1*) result in cyst formation in both the kidney and liver. ADPLD, a rare progressive disorder, is caused by mutations in two genes, *sec63* or *prkcsh*, leading to cyst formation predominantly in the liver.¹⁻³

Multiple rodent (mice and rats) models and *in vitro* experimental systems (i.e., cultured renal and hepatic epithelial cell lines derived from diseased animals) of ADPKD and ARPKD have been developed to study the mechanisms of hepatorenal cystogenesis and to test therapeutic interventions.³⁻⁵ Recently, the zebrafish has emerged as an excellent system to study renal cystogenesis in PKD.^{6,7} The zebrafish model presents several advantages, including relatively easy and economical maintenance, short generation time, external fertilization, large numbers of rapidly developing embryos produced per mating, and development of transparent embryos. The transparency enables the use of fluorescent trackers, and the large numbers of embryos produced enables rapid screening of variables, including potential therapeutic

compounds. However, to our knowledge, the zebrafish has not been used to study liver cystogenesis that arises as a result of manipulation of genes related to PLD (i.e., *pkd1a*, *pkd2*, *pkhd1*, *prkcsh*, or *sec63*). Moreover, no rodent or fish models of ADPLD exist to date.

In the present study, we developed zebrafish models to study hepatic cystogenesis in (a) ADPLD by causing mutations of genes that lead to hepatic cystogenesis in humans (i.e., *sec63* and *prkcsh*), and (b) ADPKD by mutating *pkd1*, the gene responsible for development of liver and kidney cystic diseases in more than 95% of human cases. These zebrafish models were also used to test the effects of several therapeutic agents on hepatic cyst growth: (i) the synthetic somatostatin analog, pasireotide; (ii) vitamin K3 (VK3); and (iii) 4-phenylbutyrate (4-PBA). We previously showed that both pasireotide and VK3 affected hepatic and renal cystogenesis in PCK rats and *Pkd2*^{WS25/-} mice.^{3,5,8} We also studied the chemical chaperone, sodium 4-PBA, which suppresses endoplasmic reticulum (ER) stress by chemically enhancing the ER capacity to cope with the expression of misfolded proteins. Both proteins implicated in cystic liver disease (*sec63* and *prkcsh*) reside in the ER and are involved in the processing of glycoproteins to prevent protein misfolding or retention of proteins in the ER. Mutations in *sec63* or *prkcsh* likely affect a specific set of proteins that mediate cholangiocyte growth, proliferation, and secretion, thereby contributing to cystogenesis.

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After interfering with expression of PLD-related genes (i.e., *sec63*, *prkcsh*, and *pkd1a*), we observed (a) gross phenotypic changes in the larvae; (b) formation of cysts in the liver; (c) diminution of the cystic areas after treatment with pasireotide (also shown by us to be effective in rodent models), but not VK3; and (d) a reduction in swelling and convolution in the ER of cholangiocytes after 4-PBA. These data suggest that the zebrafish is a useful additional model system to study hepatic cystogenesis and to perform therapeutic screening.

Materials and Methods

Fish maintenance and breeding

All work was approved by Institutional Animal Care and Use Committee and performed in the Zebrafish Core Facility (Mayo Clinic). Embryos were obtained from Segrest Farms (Gibsonton, FL) and wild-type adults were raised under standard laboratory conditions.⁹ Wild-type adults were mated in groups of one male and two females and uninjected controls were reserved from each harvested group.

Morpholino construction and injection

We used the following splice-blocking morpholinos designed and purchased from Gene Tool LLC, (Philomath, OR): *sec 63* (CGTACTGAACTGCTGTCCGGCCAT), *prkcsh* (TCAACAA TAGATGCACGCAGGTCAT), *pkd1a* (GTCTGTTCCTGAGA -CAGTACCGG), and a scrambled antisense sequence control (CCTCTTACCTCAGTTACAATTTATA).

The scrambled control is a negative control morpholino oligo that targets a human beta-globin intron mutation. In addition, we designed 5-base-mismatched controls for each oligo: *sec63* (CGTAgTcAAAgTGCTcTCCcGCCAT), *prkcsh* (TCAAgAA TAcATcCACcCAcGTcAT), and *pkd1a* (GTCTcTTCCTcAcA CAcTACCcG).

Morpholinos were stored at a stock concentration of 1 mM in RNA-free water and injected at a 1:10 dilution in *Danio* solution. Morpholino was injected into the yolk of one-cell-stage embryos using PLI-100 microinjection controller (Harvard Apparatus, Holliston, MA) and micromanipulator (Narishige, Greenvale, NY) as previously described.¹⁰

Western blots of zebrafish embryos

To confirm the specificity and knockdown effect of the designed morpholinos, western blots were performed. Embryos were removed from their chorions in batches of ~100 by placing them in 1 mg/mL of pronase and swirling occasionally for 20 min. Dechorionating was completed by gentle trituration with a Pasteur pipette, after which the chorions float and were decanted followed by rinsing three times in cold Ringer's solution. The yolks were removed, and the dechorionated, deyolked embryos were rinsed in cold Ringer's solution. Embryos were resuspended in sodium dodecyl sulfate (SDS) sample buffer (200 μ L sample buffer per 150 embryos) and homogenized for uniform consistency, boiled, and loaded on a 10% SDS gel. Gels were transferred to nitrocellulose and blotted with antibodies to *sec63*, *prkcsh*, and *pkd1a* (1:200 rabbit polyclonal; Novus Biologicals, Littleton, CO). Appropriate goat anti-rabbit secondary antibodies were used (1:1000; Life Technologies, Carlsbad, CO). Bands were observed with the ECL plus western blotting detection kit (GE Health Care, Piscataway, NJ).

Ultrastructural analysis

For electron microscopy, zebrafish larvae (4 days post-fertilization [dpf]) were sacrificed in 1.0% tricaine in embryo water. Samples were incubated in 1% osmium tetroxide for 30 min, dehydrated, dried in a critical-point dryer, mounted onto specimen stubs, and sputter-coated with the gold-palladium alloy. Specimens were examined with an SEM Hitachi S-4700 microscope (Hitachi, Pleasanton, CA).

Histologic analysis

Zebrafish larvae (4 dpf) were anesthetized in 0.5% tricaine to enable observation of any phenotypic changes caused by the injection of morpholinos. Approximately 200 larvae in each morpholino group and wild type (WT) were analyzed, and phenotypic alterations noted and photographed. In a separate experiment, zebrafish larvae (4 dpf) were sacrificed in 1.0% tricaine in embryo water. A slit was cut in the ventral portion of the abdomen using a sharp scalpel. The fish were placed in Dietrich's solution (30% ethanol, 10% formalin, and 2% acetic acid, pH 2.7) for decalcification and fixation at room temperature for 96 h. The fixed and decalcified fish were embedded in paraffin and serially sectioned to obtain 4 μ m sections. Paraffin sections of the fish were stained with hematoxylin and eosin (H&E) and the liver was photographed to measure the area. The Liver and cystic area was determined using Meta-Morph software (Universal Imaging, West Chester, PA) installed on a Pentium IBM-compatible computer. After image acquisition using a light microscope and color digital camera (Nikon DXM 1200; Nikon Corporation, Kanagawa, Japan), the hepatic cystic area was expressed as a percentage of the total liver area. Measurements were performed on three serial sections within each liver, and the data were averaged from five individual fish from each group.

Treatment protocol

Zebrafish embryos were divided into four groups: WT, *sec63*, *prkcsh*, and *pkd1a*. In each group, 200 embryos were injected with either the control buffer (WT) or the respective morpholino. The injected embryos were placed in a 96-well plate and received treatment with (a) pasireotide (1 μ M), (b) VK3 (100 μ M), and (c) 4-PBA (0.05 mM; Sigma Chemical, St. Louis, MO). At 4 dpf, the larvae were sacrificed with tricaine, decalcified, and fixed in Dietrich's solution, embedded, sectioned, and stained for H&E (as described above) for histologic measurements of the liver and cystic area. The 4-PBA treatment group of larvae was processed for transmission electron microscopy, and the ER was ultrastructurally observed.

Statistical analysis

All values are reported as mean $\pm$ standard error (SE). Statistical analysis was performed using Student's *t*-test and results were considered statistically significant at $p < 0.05$.

Results

Protein expression is absent after injection of specific morpholinos

Western blots of zebrafish embryos using antibodies to *sec 63*, *prkcsh*, or *pkd1a* revealed single bands migrating at the

FIG. 1. Morpholinos against *sec63*, *prkcsh*, and *pkd1a*-eliminated protein expression. The figure shows a western blot of zebrafish embryos (200 μ L sample buffer with 150 embryos per lane) on a 10% SDS gel (WT, wild-type embryos injected with *Danio* buffer; Scr, scrambled control; MO, morpholinos). A protein band was visible at the appropriate molecular weight for *sec63*, (77,000 kDa), *prkcsh* (90,000 kDa), and *pkd1a* (115,000 kDa).

appropriate molecular weights in the lanes containing WT (embryos injected with *Danio* buffer) and the scrambled control (Scr), but protein expression specifically knocked down and absent in the lanes loaded with the morpholino-injected embryos (Fig. 1).

Morpholinos induced phenotypic body changes and hepatic cysts

Light microscopic images of zebrafish larvae (4 dpf) demonstrate that depletion of proteins associated with cystic liver disease leads to phenotypic changes, including abnormal body curvature and a kink in the mid tail region (Fig. 2). The phenotypic tail kink was more evident in the fish with morpholino *prkcsh* or *pkd1a*. For each condition, in excess of 200 larvae were observed and photographed. Phenotypic abnormalities were found in greater than 95% of the larvae. Embryos injected with a respective 5-base-mismatched morpholino showed no phenotypic alteration in body curvature or tail shape. By light microscopic observation of H&E-stained zebrafish, the presence of multiple

cysts of varying shapes and sizes was observed in the livers of 4 dpf zebrafish larvae from embryos injected with morpholinos to *sec63* and *prkcsh* with less notable changes in *pkd1a* and absent in the wild-type embryos injected with a scrambled control morpholino (Fig. 3). We applied a calculation of the cystic area expressed as a percentage of the total liver area, and found that embryos injected with the morpholino to *sec63* had a cystic area of $55\% \pm 2\%$ of the total liver area. *Prkcsh* had the highest percentage of cysts with $67\% \pm 5\%$ of total liver. *Pkd1a* was less affected with $36\% \pm 4\%$ cystic area as a percent of the total liver area. Each of the morpholino cystic area calculations was statistically significant ($p < 0.05$) compared with WT. Scanning electron microscopy (Fig. 4) showed the formation of cysts in the morpholino-treated larvae compared with no cysts in the WT.

Treatment with pasireotide inhibited hepatic cystogenesis and treatment with 4-PBA reversed ER abnormalities

H&E staining of zebrafish liver allowed calculation of comparative changes in the cystic area in WT versus morpholino-injected embryos receiving treatment with pasireotide or VK3 (Fig. 5). After treatment with pasireotide or VK3, the cystic area as a percentage of the total liver area at 4 dpf was calculated. In the groups of embryos injected with morpholinos to *sec63*, *prkcsh*, and *pkd1a*, a statistically significant ($p < 0.05$) decrease in the cystic area was observed after treatment with pasireotide; however, VK3 did not reach statistical significance. Pasireotide decreased the liver cystic area by $83\% \pm 3\%$ in *sec63*-injected embryos, $76\% \pm 2\%$ in *prkcsh*, and $60\% \pm 4\%$ in *pkd1a*.

Concurrent with the statistically significant increase in the cystic area observed in each morpholino group, we also observed swollen and convoluted ER in cholangiocytes in the morpholino groups compared with control (Fig. 6). After exposure of morpholino-treated larvae to 4-PBA, we observed by transmission electron microscopy (Fig. 6) a reversal of the swollen and convoluted ER in the embryos injected with morpholinos to *sec63*, *prkcsh*, and *pkd1a*, but saw no changes in cystic areas (Supplementary Fig. S1; Supplementary Data available online at www.liebertonline.com/zeb). In experiments in which we exposed morpholino-treated larvae to pasireotide, we saw changes in cystic areas (Fig. 5), but we did

FIG. 2. Morpholinos induced phenotypic body changes of body curvature and kinked tail. Light microscopic images of zebrafish larvae (4 dpf) demonstrate that depletion of proteins associated with cystic liver disease led to phenotypic changes, including abnormal body curvature, while mismatched morpholinos showed no effect (magnification $\times 30$).

FIG. 3. Morpholinos caused an increase in the cystic area of the liver. Each of the morpholinos led to a statistically significant increase in the cystic area compared with WT (magnification $\times 60$).

FIG. 4. Morpholinos induced hepatic cystogenesis. Scanning electron micrographs show the presence of liver cysts (*) in *sec63*, *prkcsh*, and *pkd1a* in zebrafish larvae (4 dpf) compared with control. *Left panels:* WT (magnification $\times 400$; bar = $50\ \mu\text{m}$); *sec63* (magnification $\times 1000$; bar = $25\ \mu\text{m}$); *prkcsh* (magnification $\times 200$; bar = $100\ \mu\text{m}$); *pkd1a* (magnification $\times 400$; bar = $50\ \mu\text{m}$). *Right panels:* magnification $\times 10,000$; bar = $2\ \mu\text{m}$.

FIG. 5. Exposure of embryos to pasireotide inhibited hepatic cystogenesis. Treatment of morpholino-injected embryos revealed that the liver cysts and cystic areas were significantly reduced in response to pasireotide but not vitamin K3.

not observe a reversal of the convoluted ER in these embryos (Supplementary Fig. S2).

Discussion

In this work, we describe in detail the use of the zebrafish as a model system to study cystic liver disease. Our results show that (i) morpholinos against the genes involved in PLD (i.e., *sec63* and *prkcsh*) and ADPKD (i.e., *pkd1a*) eliminate protein expression of these genes; (ii) knock down of these genes is associated with both gross phenotypic body changes and the formation of cysts of the intrahepatic biliary system in zebrafish larvae; (iii) exposure of embryos with knock down of these genes to drugs previously shown by us to inhibit hepatorenal cystogenesis in rodent models (i.e., pasireotide) also inhibited hepatic cystogenesis in the zebrafish model; (iv) following exposure of embryos with knock down of genes to the chemical chaperone 4-PBA, we showed a resolving of the swelling and convolution of the ER associated with ER stress in cholangiocytes that occurred in the knock down compared

with the nontreated larvae; and (v) treatment of the morpholino-injected embryos with pasireotide did not rescue the ER phenotype and treatment with 4-PBA did not affect liver cystogenesis. Based on these preliminary observations, we believe that the zebrafish may serve as an additional model to study the pathogenesis of PLD and to screen potential pharmacologic therapies.

Others have utilized the zebrafish model to study cystic disease exclusively in the kidney. Drummond and coworkers identified a relatively large set of genetic loci associated with cystic pronephroi in zebrafish.¹¹ The results of a large-scale retroviral insertional mutagenesis screen have identified 10 zebrafish genes that when mutated cause pronephric cysts. *Pkd1a/b* and *pkd2* were also found to regulate extracellular matrix formation in the kidney and serve as primary defects underlying ADPKD. Body curvature has been used extensively as a surrogate marker for kidney cyst formation in large-scale high-throughput screens in zebrafish,⁶ thereby enabling the discovery of potential drug candidates and dissection of signaling pathways. Sun and colleagues have

FIG. 6. Treatment with 4-phenylbutyrate (4-PBA) reduced endoplasmic reticulum (ER) stress. Morpholino-injected zebrafish larvae (4 dpf) displayed swollen and convoluted shaped ER (*) in *sec63* and *prkcsh* but fewer in *pkd1a* compared with WT. After treatment with 4-PBA (0.05 mM) the ER returned to the shape and size observed in WT. *Left panels:* magnification $\times 40,000$; bar = $0.5 \mu\text{m}$. *Right panels:* magnification $\times 100,000$; bar = 200 nm .

described a chemical modifier screen that identifies histone deacetylase inhibitors as drug candidates for PKD treatment.¹² Screening first by body curvature and laterality, they showed that valproic acid was able to reduce the progression of cyst formation in the kidney. These findings were then reproduced in a mouse model of ADPKD.¹²

In the *jcpc* mouse model of PKD, mutations in the bicaudal C gene (*Bicc1*) have been shown to be responsible for the cystic phenotype; however, the function of *Bicc1* is unknown. A study by Bryda and colleagues¹³ used antisense morpholinos to *Bicc1* in zebrafish, thereby supporting the use of zebrafish as an alternative *in vivo* model to study PKD pathogenesis. *Sec 10* knock down in zebrafish has allowed the study between *Sec 10* and ciliary proteins in the formation of renal cysts and PKD phenotypes. The zebrafish therefore represents an op-

portunity to study phenotypes in a nonmammalian model and extend the findings of PKD in the kidney to that of hepatocystogenesis. Our work has extended these efforts to the liver and represents a systematic assessment of the zebrafish to study cystic liver disease.

The use of 4-PBA or tauroursodeoxycholic acid has been shown to relieve ER stress in various conditions, including liver injury, and resolution of fatty liver.^{14,15} Our findings revealed mis-shapen ER in the *sec63* and *prkcsh* knock down livers more so than *pkd1a*. The findings of *sec63* affecting liver pathology and ER in the zebrafish are consistent with findings published by Talbot *et al.*¹⁶ Both *sec63* and *prkcsh* are involved in carbohydrate processing, folding, and translocation of newly synthesized glycoproteins in cholangiocytes and export of unfolded proteins to the cytoplasm during ER-associated degradation. It

has been postulated that 4-PBA or tauroursodeoxycholic acid resolve defective ER degradation and may alleviate translocation issues leading to cytoplasmic retention and cyst formation.¹⁶ Recent discovery of genes involved in glycoprotein processing having a potential role in PLD opens up the possibility for a comprehensive mapping of the role of protein glycosylation in liver pathology.^{17,18} This finding is consistent with the observation that both *sec63* and *prkcsH* gene mutations are found in PLD patients. Most of the mutations in PLD are truncating and proteins transcribed by these genes are involved in oligosaccharide processing, implicating a new avenue for identification of novel therapeutic drugs.

In conclusion, we have shown that the zebrafish can represent a model of PLD with distinctive hepatic and pathogenic features that in many ways resemble the human liver pathology. 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. Zebrafish provide a powerful system capable of detailed developmental genetic analysis and are well suited for the study of hepatic disorders. This, combined with the growing number of liver disease models, provides an exciting framework for understanding development, disease, and potential screening of new therapies. Finally, sharing of chemical libraries between zebrafish researchers with diverse biological interests should lead to an unprecedented wealth of new insights.¹⁹ The National Institutes of Health (NIH) maintains a clinical collection compound library (www.nihclinicalcollection.com), which represents a compendium of Food and Drug Administration-approved drugs that we plan to review and implement in our future experiments with specific knock down morpholinos involved in cystic liver disease.

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Author Disclosure Statement

No competing financial interests exist.

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