GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Blockade of Hedgehog Signaling Attenuates Biliary Cystogenesis in the Polycystic Kidney (PCK) Rat

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Caroli disease represents a hepatic manifestation of autosomal recessive polycystic kidney disease, and belongs to a class of cholangiociliopathies. The role of Hedgehog signaling, a major pathway regulated by primary cilia, in biliary cystogenesis in Caroli disease remains unknown. Using the polycystic kidney (PCK) rat as an animal model of Caroli disease, this study investigated the involvement of Hedgehog signaling in its pathogenesis. *In vitro* experiments revealed that PCK cholangiocytes overexpressed Smoothened, Gli1, and Gli1's target molecule cyclin D1. The nuclear expression of Gli1, Gli2, and Gli3 was observed in PCK cholangiocytes by immunocytochemistry. An immunohistochemical analysis using liver sections confirmed the overexpression of Smoothened and cyclin D1, and the nuclear expression of the Gli proteins in the biliary epithelium of PCK rats as well as human Caroli disease. The treatment of PCK cholangiocytes with cyclopamine inhibited cell proliferative activity that was associated with the inhibition of nuclear translocation of Gli1 and Gli2, and reduced cyclin D1 expression. The *in vivo* administration of cyclopamine to PCK rats decreased abnormally elevated serum liver enzymes, and significantly attenuated bile duct dilation as well as kidney cyst formation. These results suggest that cholangiocyte hyperproliferation is causally associated with the aberrant activation of Hedgehog signaling, and the inhibition of the signaling has potential as a therapeutic strategy for biliary cystogenesis in Caroli disease. (*Am J Pathol* 2018, 188: 2251–2263; <https://doi.org/10.1016/j.ajpath.2018.06.014>)

Caroli disease is characterized by the progressive, multiple cystic dilation of intrahepatic bile ducts, and is frequently associated with portal fibrosis corresponding to congenital hepatic fibrosis (CHF).¹ Caroli disease with CHF is recognized as an extrarenal manifestation of autosomal recessive polycystic kidney disease (ARPKD). The complications of this liver disease include recurrent cholangitis related to Caroli disease and those associated with portal hypertension due to CHF, with both being indications for liver transplantation.² ARPKD is caused by mutations in the *PKHD1* gene, which encodes a protein known as fibrocystin (also called polyductin), and the gene and protein interact with autosomal dominant polycystic kidney disease (ADPKD) genes and proteins.¹

Fibrocystin has been identified in the same complex as polycystin-2, and localizes to primary cilia as well as to the basal body.³ Primary cilia are solitary, nonmotile organelles on the apical cell surface of most mammalian cells involved

in mechano-, osmo-, and chemoreception. Primary cilia have also been implicated in planar cell polarity, cell cycle control, and numerous essential signaling pathways. ARPKD and ADPKD are examples of ciliopathies that represent genetic diseases caused by dysfunctions in cilia or cilium-anchoring structures. Hepatic cysts and fibrosis frequently occur as part of the phenotype of multiple ciliopathies, which have, thus, been termed cholangiociliopathies.^{4,5}

Cholangiocyte primary cilia are important for normal physiology, and defects in their structure and/or function lead to disorders such as cholangiocyte hyperproliferation and altered fluid secretion and absorption, resulting in liver cyst formation.⁶ Many studies have been performed using

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various animal models of ARPKD and ADPKD in an attempt to clarify the complex molecular and cellular mechanisms underlying cyst formation in the liver and kidneys.

Several different knockout mouse models of ARPKD have been described with a number of different *Pkhd1* mutations, and the polycystic kidney (PCK) rat is a spontaneous rodent model of ARPKD and Caroli disease with CHF harboring the *Pkhd1* mutation.^{7,8} Shortened and abnormal ciliary structures have been observed in cholangiocytes of the PCK rat, which are associated with aberrant intracellular levels of the second messenger, cyclic adenosine monophosphate (cAMP), coupled with decreased intracellular calcium concentrations increasing both proliferation and secretion.^{9,10}

Signaling pathways regulated by primary cilia involve Sonic Hedgehog (Shh) and Wnt signaling, which have been the most intensively studied pathways over the last few decades. Hedgehog (Hh) signaling is a major regulator of cell differentiation, cell proliferation, and tissue polarity, and its aberrant activation is a leading cause of a number of tumors.^{11,12} In mammalian cells, Hh signaling involves complex relationships between Hh ligands (Shh, Indian hh, and Desert hh) and two plasma membrane proteins, Patched1 (Ptch1) and Smoothened (Smo). Canonically, binding of the Hh ligand to Ptch1 leads to the derepression and translocation of Smo to cilia, resulting in the activation of glioma-associated oncogene homolog (Gli) transcription factors.

Gli transcription factors consist of three different isoforms (Gli1, Gli2, and Gli3). Gli1 is exclusively a transcriptional activator, whereas Gli2 and Gli3 have dual activities, with Gli2 functioning as a major pathway activator and Gli3 mostly as a repressor. Gli transcription factors regulate the expression of target genes including those involved in Hh pathway feedback (eg, Gli1) and cell proliferation (eg, cyclin D1). Gli2 transactivates the expression of Gli1 by binding to the Gli-protein-binding site in the promoter region of the *Gli1* gene, and *Gli1* itself is a transcriptional target of the Hh pathway, leading to self-activation. Thus, increases in *Gli1* mRNA expression and the translocation of Gli2 from the cell cytoplasm to nuclei are regarded as indicators of the activation of canonical Hh signaling.¹² Suppressor of fused (Sufu) controls the response of Gli to Hh ligands.

Despite the close relationship between primary cilia and Hh signaling, limited information is currently available on the role of Hh signaling in renal cystic diseases, including ADPKD.¹³ The involvement of Hh signaling in biliary cystogenesis in Caroli disease with CHF as well as kidney cyst formation in ARPKD remains largely unknown.¹⁴ Using the PCK rat as an animal model of Caroli disease with CHF, the present study investigated the involvement of Hh signaling in cholangiocyte hyperproliferation, and aimed to develop a novel therapeutic strategy for the disease.

Materials and Methods

The protocols of the animal studies were approved by the committee of the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Permit Number: AP-173887). The study using human materials was performed with the approval of the ethics committee of Kanazawa University Graduate School of Medicine (Permit Number: 1985-3).

Cell Culture and Treatments

Cholangiocytes were isolated, purified, and cultured from the intrahepatic large bile ducts of normal (CrI:CD) and PCK rats (Charles River Laboratories Japan, Yokohama, Japan) as described previously.^{15–17} Cells were set on cell culture dishes covered with standard medium, composed of Dulbecco's modified Eagle's medium/F-12 (Gibco; Thermo Fisher Scientific, Waltham, MA) containing 10% bovine growth serum (HyClone; GE Healthcare Life Sciences, Little Chalfont, UK), 5 μ mol/L forskolin (Wako Pure Chemical Industries, Osaka, Japan), 20 ng/mL of epidermal growth factor (Millipore; Merck, Darmstadt, Germany), and 1% antibiotic-antimycotic (Gibco; Thermo Fisher Scientific) at 37°C in an atmosphere of 5% CO₂. To block Hh signaling, a small molecule inhibitor, cyclopamine (Sigma-Aldrich; Merck), was used as an antagonist of Smo.

Three-Dimensional Cell Culture

Biliary cyst formation was examined using the three-dimensional cell culture system with BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, Franklin Lakes, NJ). Following the treatment with cyclopamine (Sigma-Aldrich; Merck), morphological changes were recorded using a digital camera under phase-contrast microscopy. Five fields at $\times 40$ magnification were recorded for each experimental group, and the diameters of well-developed cysts were measured.

RT-PCR and Quantitative RT-PCR

Total RNA was extracted from cultured cholangiocytes and whole-liver tissues using an RNA extraction kit (RNeasy Mini Kit; Qiagen, Tokyo, Japan) and Isogen (Nippon Gene Co., Tokyo, Japan), respectively, and was then used to synthesize cDNA with reverse transcriptase (ReverTra Ace; Toyobo Co., Osaka, Japan). PCR amplification was performed using *Taq*DNA polymerase (Takara EX Taq; Takara Bio, Otsu, Japan). Primer sequences and PCR conditions are given in Table 1. PCR products were subjected to agarose gel electrophoresis and then stained with ethidium bromide.

Quantitative RT-PCR (RT-qPCR) was performed according to a standard protocol using the SYBR Green PCR Master MIX (Toyobo Co.) and Mx3005P Real-Time QPCR System (Agilent Technologies, Santa Clara, CA). The

Table 1 Primer Sequences and PCR Conditions

Gene	Sequences	Annealing temperature (°C)	PCR cycles	Product size (bp)
<i>Shh</i>	F: 5'-GCGTAACGCTACGAGAGGAG-3' R: 5'-CTCGCTTCCGCTACAGATTG-3'	50	40	238
<i>Ptch1</i>	F: 5'-CATCAGCCTCCCTTGAAGTC-3' R: 5'-GCCAGAATGCCCTTCAGTAG-3'	65	40	171
<i>Smo</i>	F: 5'-CTGTCTCCCATGATGGACCT-3' R: 5'-CCACAGGTTGGCTTGTCTT-3'	65	40	188
<i>Sufu</i>	F: 5'-ATTCGAGATCGATCCACACC-3' R: 5'-GTCTTTGCCAGACAGCCTTC-3'	65	30	184
<i>Gli1</i>	F: 5'-CAGCTCAAAGCTCAGCTCCT-3' R: 5'-ATGCAAAGCCAGATCCAAAC-3'	65	40	199
<i>Gli2</i>	F: 5'-GGAGCCCAACACAGCTAGTC-3' R: 5'-CTGTGTCTTGAGATGGCTGA-3'	60	40	182
<i>Gli3</i>	F: 5'-CCTACCAGGGACTCAGGACA-3' R: 5'-GCCTCCATTTTGATGCTGTT-3'	65	40	186
<i>Ccnd1</i>	F: 5'-CGTGGCCTCTAAGATGAAGG-3' R: 5'-CTGGCATTTTGGAGAGGAAG-3'	65	25	185
<i>Acta2</i>	F: 5'-CATCCACGAAACCACCTATA-3' R: 5'-CTGTTTGCTGATCCACATCT-3'	60	30	262
<i>Col1a1</i>	F: 5'-TGGTGACAAGGGTGAGACAG-3' R: 5'-ACCGTTGAGTCCATCTTTGC-3'	60	35	193
<i>Gapdh</i>	F: 5'-GAGTCAACGGATTGCTCGT-3' R: 5'-TTGATTTTGGAGGATCTC-3'	60	30	240

F, forward; R, reverse.

primers listed in Table 1 were used, and cycling conditions were 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fold differences relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated. Each assay was conducted in five sets.

Cell Proliferation Assay

The proliferative activity of cholangiocytes was assessed using the WST-1 assay (Roche Diagnostics, Mannheim, Germany). Cholangiocytes were treated with appropriate concentrations of cyclopamine (Sigma-Aldrich; Merck). At the intervals indicated, WST-1 reagent was added and incubated for 1 hour before reading the plate. Each assay was conducted in eight sets.

Apoptosis Assay

Apoptosis was examined in cultured cholangiocytes using the single-stranded DNA apoptosis enzyme-linked immunosorbent assay (ELISA) kit (Chemicon; Merck) according to the manufacturer's protocol. Apoptosis was assessed by measuring absorbance at 405 nm using a microplate reader as previously described.¹⁸ Each assay was conducted in eight sets.

As an additional experiment, DNA fragmentation attributable to apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Paraffin-embedded cell blocks were prepared from cultured cholangiocytes. Deparaffinized sections were digested with 10 µg/mL proteinase K solution for 15 minutes, and were then stained using a commercial kit

Table 2 Primary Antibodies Used for Immunostaining

Primary antibody	Clone	Source	Dilution
Sonic Hedgehog	Polyclonal	Neuromics Antibodies, Edina, MN	1:50*
Patched1	Polyclonal	Santa Cruz Biotechnology, Dallas, TX	1:50 [†]
Smoothed	Polyclonal	Bioss Antibodies, Woburn, MA	1:50 [†]
Sufu	Polyclonal	Proteintech Group, Rosemont, IL	1:50
Gli1	Polyclonal	Bioss Antibodies, Woburn, MA	1:50*
Gli2	Polyclonal	LifeSpan BioSciences, Seattle, WA	1:100 [‡]
Gli3	Polyclonal	GeneTex, Irvine, CA	1:50 [‡]
Cyclin D1	SP4	Nichirei Biosciences, Tokyo, Japan	Prediluted*
Ki-67	SP6	Nichirei Biosciences	Prediluted*

In immunohistochemical staining, antigen retrieval was performed by heating in Tris-ethylenediaminetetraacetic acid buffer (pH 9.0) with a pressure cooker (*), by incubating 20 mg/mL of proteinase K at room temperature for 6 minutes ([†]), and by microwaving in 10 mmol/L citrate buffer (pH 6.0) ([‡]).

Gli, glioma-associated oncogene homolog; Sufu, suppressor of fused.

(In Situ Apoptosis Detection Kit; Takara Bio). After color development with 3,3'-diaminobenzidine tetrahydrochloride, sections were counterstained with hematoxylin.

ELISA

Proteins were extracted from cultured cholangiocytes using the Cytoplasmic & Nuclear Protein Extraction Kit (101Bio, Mountain View, CA), and total protein was measured spectrometrically. The cytoplasmic and nuclear protein levels of Smo and cyclin D1, respectively, were assessed using commercially available ELISA kits (MyBioSource, San Diego, CA) according to the manufacturer's protocol. Each assay was conducted in eight sets.

Immunocytochemistry

Cholangiocytes seeded in slide chambers were fixed with 4% paraformaldehyde, and were permeabilized for 10 minutes with 0.1% Triton X-100. After blocking, cells were incubated with the primary antibodies listed in Table 2. Alexa Fluor 488 (Abcam, Tokyo, Japan) was used as a secondary antibody, and nuclei were stained with DAPI. Sections were observed under immunofluorescence confocal microscopy.

Immunohistochemistry

Livers were removed from 3-week-old, 2-month-old, and 10-month-old rats, and kidneys were obtained from

Figure 1 Detection of Hedgehog signaling-related molecules in cultured cholangiocytes of polycystic kidney (PCK) rats. **A:** Detection of Sonic Hedgehog (*Shh*), Patched1 (*Ptch1*), Smoothened (*Smo*), suppressor of fused (*Sufu*), glioma-associated oncogene homolog (*Gli1*), *Gli2*, and *Gli3* mRNAs in the cultured cholangiocytes of normal and PCK rats by RT-PCR. **B** and **C:** The analysis using quantitative RT-PCR shows that *Smo* (**B**) and *Gli1* (**C**) mRNA are more strongly expressed in PCK cholangiocytes than in normal cholangiocytes. **D:** An enzyme-linked immunosorbent assay analysis confirms that the Smo protein is overexpressed in PCK cholangiocytes. **E:** Confocal fluorescence immunocytochemistry for normal and PCK cholangiocytes showing the overexpression of Smo and the nuclear localization of Gli1 and Gli3 in PCK cholangiocytes. The nuclear localization of Gli2 is observed in normal and PCK cholangiocytes. Data are expressed as means $\pm$ SD (**B–D**). * $P < 0.05$, ** $P < 0.01$. Original magnification, $\times 1000$ (**E**).

Figure 2 Effects of the treatment with cyclopamine on cultured cholangiocytes of polycystic kidney (PCK) rats. **A** and **B**: The effects of the treatment with cyclopamine on cell proliferative activity were assessed by the WST-1 assay for normal (**A**) and PCK (**B**) cholangiocytes. **C**: Confocal fluorescence immunocytochemistry of glioma-associated oncogene homolog (Gli)1 and Gli2 for PCK cholangiocytes, showing that the nuclear expression of Gli1 and Gli2 is diminished following the treatment. **D**: Images of three-dimensional cell cultures of normal and PCK cholangiocytes. **E**: The results of measurements of cyst sizes in gels, showing that the treatment reduces the cystic overgrowth of PCK cholangiocytes. **F** and **G**: The treatment reduces the expression of cyclin D1 at the mRNA (**F**) and protein (**G**) levels in PCK cholangiocytes, as assessed by quantitative RT-PCR and enzyme-linked immunosorbent assay (ELISA), respectively. **H**: Cholangiocyte apoptosis is not significantly induced by the treatment, as evaluated by the single-stranded DNA apoptosis ELISA kit. Apoptosis was assessed by measuring optical density (OD) at 405 nm using a microplate reader. The plus and minus signs (**E–H**) represent the presence and absence of 10 $\mu\text{mol/L}$ cyclopamine treatment, respectively. **I**: The analysis using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method also shows no increase in the number of TUNEL-positive cells following the treatment in normal and PCK cholangiocytes (arrows). The periods of the cyclopamine treatment were 3 (**C**) and 5 (**D–I**) days. Data are expressed as means $\pm$ SD (**F–H**). * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 1000$ (**C** and **I**); $\times 40$ (**D**).

2-month-old rats. Liver tissues from patients with Caroli disease with CHF ($n = 5$; the mean age of the patients, 58 years) were obtained at the time of surgery. As controls, normal or subnormal human livers were used ($n = 5$; the mean age of the patients, 62 years). Liver and kidney tissues were fixed with 10% formalin neutral buffer solution (pH 7.4) and embedded in paraffin. After

deparaffinization, the sections were incubated with the primary antibodies listed in Table 2. Sections were then incubated with a secondary antibody using Histofine Simple Stain MAX PO (Nichirei Biosciences, Tokyo, Japan). Color development was performed using 3,3'-diaminobenzidine tetrahydrochloride, and sections were counterstained with hematoxylin.

Figure 3 Immunohistochemical expression of Hedgehog-related proteins in the rat liver. **A:** Representative images of the immunohistochemical results of Sonic Hedgehog (Shh), Patched1 (Ptch1), Smoothened (Smo), glioma-associated oncogene homolog (Gli)1, Gli2, and Gli3 obtained from 2-month-old normal and polycystic kidney (PCK) rats. Smo is overexpressed in the bile duct epithelium of PCK rats and is accompanied by the nuclear expression of Gli1, Gli2, and Gli3. The nuclear expression of Gli2 in the bile duct epithelium is also observed in normal rats. **Boxed areas in left panels** are shown at higher magnification in **right panels**. **B:** The results of immunohistochemistry for cyclin D1, showing that cyclin D1 is frequently expressed in the bile duct epithelium of PCK rats. **C:** The cyclin D1-labeling index of the bile duct epithelium is significantly higher in 3-week-old, 2-month-old, and 10-month-old PCK rats than in normal rats of the same ages. $**P < 0.01$. Original magnification: $\times 200$ (A, left panels); $\times 1000$ (A, right panels, and B).

Figure 4 Immunohistochemical expression of Hedgehog-related proteins in the rat kidney. Representative images of the immunohistochemical results of Smoothed (Smo), glioma-associated oncogene homolog (Gli)1, Gli2, and Gli3 obtained from 2-month-old normal and polycystic kidney (PCK) rats. Overexpression of Smo and frequent nuclear expression of Gli1, Gli2, and Gli3 is observed in the cyst-lining epithelium of PCK rats. **Insets** show boxed areas at higher magnification. Original magnification: $\times 200$; $\times 1000$ (**insets**).

In Vivo Administration of Cyclopamine

Normal (Crl:CD) and PCK rats were purchased from Charles River Laboratories Japan. Twenty male rats were used. At 4 weeks of age, rats were divided into control and experimental groups. The experimental groups (five rats per group) were intraperitoneally administered 10 mg/kg cyclopamine (LC Laboratories, Woburn, MA) dissolved in 10% ethanol daily between 4 and 8 weeks of age. The dosage was selected based on a previous study.¹⁹ Control groups received vehicle (10% ethanol) alone. At 8 weeks of age, rats were weighed, and blood was obtained for hematological analyses. The liver and kidney were weighed and immersed in 10% formalin neutral buffer solution (pH 7.4), and tissues were then embedded in paraffin. A part of the liver tissues was also used for the RT-qPCR analysis.

Histological Assessment

Whole tissue sections of the liver and kidney were used to measure cystic areas and liver fibrosis. The areas of

cystically dilated bile ducts and kidney cysts were assessed using hematoxylin and eosin–stained sections, and liver fibrosis was evaluated using Picrosirius red staining. Digital images of sections were reproduced on a computer, and an image analysis was performed using Adobe Photoshop

Figure 5 Immunohistochemical expression of Hedgehog-related proteins in livers of patients with Caroli disease with congenital hepatic fibrosis (CHF). **A:** The results of immunostaining using liver tissues from patients with Caroli disease with CHF, showing that Smoothed (Smo) is overexpressed, and glioma-associated oncogene homolog (Gli)1 and Gli3 localize in the nuclei of the biliary epithelial cells of dilated bile ducts in Caroli disease with CHF. Similar to the results obtained using rats, the nuclear expression of Gli2 in the bile duct epithelium is observed in normal livers and livers with Caroli disease with CHF. **Insets** show boxed areas at higher magnification. **B:** The bile duct epithelium of Caroli disease with CHF is frequently labeled by cyclin D1 using immunohistochemistry. **C:** The cyclin D1-labeling index of the bile duct epithelium is significantly higher in livers with Caroli disease with CHF than in normal livers. Data are expressed as means $\pm$ SD (**C**). $^{**}P < 0.01$. Original magnification: $\times 100$ (**A**); $\times 600$ (**A, insets**); $\times 400$ (**B**).

software version 7.0 (Adobe Systems, San Jose, CA). A color threshold was applied at a level that separated the cystic area from noncystic tissue, and Picrosirius red–stained material from the background to calculate the volume of the cystic area and fibrosis. Areas of interest were expressed as a percentage of the total tissue area.

The immunohistochemical expression of Ki-67 and cyclin D1 in the bile duct epithelium was quantitatively evaluated. The number of positive cells was counted in a high-power field under a light microscope, and the percentages of biliary epithelial cells positive for Ki-67 and cyclin D1 were expressed as the Ki-67–labeling index and the cyclin D1–labeling index, respectively.

Statistical Analysis

The data were presented as means $\pm$ SD. The significance of differences was assessed using a *t*-test or the *U*-test. $P < 0.05$ was considered to be significant.

Results

Detection of Hh Signaling–Related Molecules in PCK Cholangiocytes

In the cultured cholangiocytes of normal and PCK rats, the expression of mRNAs for *Shh*, *Ptch1*, *Smo*, *Sufu*, *Gli1*, *Gli2*, and *Gli3* was detected by RT-PCR (Figure 1A). Among these mRNAs, an analysis using RT-qPCR showed that the expression of mRNAs for *Smo* and *Gli1* was significantly increased in PCK cholangiocytes than in normal cholangiocytes (Figure 1, B and C). The ELISA analysis confirmed that protein extracts from PCK cholangiocytes contained significantly elevated levels of the Smo protein (Figure 1D). Confocal immunofluorescence microscopy showed the overexpression of the Smo protein in PCK cholangiocytes (Figure 1E). The nuclear expression of Gli1 and Gli3 was observed in PCK cholangiocytes, but it was weak or negligible in normal cholangiocytes (Figure 1E). By contrast, the nuclear expression of Gli2 was observed in normal and PCK cholangiocytes (Figure 1E).

Effects of the Treatment with Cyclopamine on PCK Cholangiocytes

Normal and PCK cholangiocytes were treated with cyclopamine, an antagonist of Smo, and cell proliferative

activity was assessed. In normal cholangiocytes, the treatment with cyclopamine did not significantly affect cell proliferative activity (Figure 2A). By contrast, the overgrowth of PCK cholangiocytes was significantly inhibited by the treatment in a dose-dependent manner, with 10 $\mu\text{mol/L}$ cyclopamine exerting the greatest effect (Figure 2B). In subsequent *in vitro* experiments, cyclopamine was used at a dosage of 10 $\mu\text{mol/L}$.

The treatment with cyclopamine diminished the nuclear expression of Gli1 and Gli2 in PCK cholangiocytes (Figure 2C). The nuclear expression of Gli2 was also reduced in normal cholangiocytes treated with cyclopamine (data not shown). In three-dimensional cell cultures, the treatment significantly inhibited the excessive cystic growth of PCK cholangiocytes, whereas the growth of normal cholangiocytes was unaffected (Figure 2, D and E).

A comparison of the mRNA expression levels of cyclin D1 between untreated normal and PCK cholangiocytes revealed that they were significantly higher in PCK cholangiocytes (Figure 2F). Following the treatment with cyclopamine, the expression of cyclin D1 mRNA and protein was significantly reduced in PCK cholangiocytes (Figure 2, F and G).

Under untreated conditions, the frequency of apoptosis was significantly higher in PCK cholangiocytes than in normal cholangiocytes, and an apoptotic response was not significantly induced in cholangiocytes following the cyclopamine treatment, as assessed using the ssDNA ELISA kit (Figure 2H). Similarly, the analysis using the TUNEL method identified only a few TUNEL-positive cells in cyclopamine-treated normal and PCK cholangiocytes (Figure 2I).

Immunohistochemical Expression of Hh-Related Proteins in the Liver and Kidney

The immunohistochemical expression of Shh and Ptch1 was observed in the bile duct epithelium of normal and PCK rats; however, positive signals were faint or weak (Figure 3A). Consistent with the results of *in vitro* experiments, Smo was overexpressed in the bile duct epithelium of PCK rats, and this was accompanied by the occasional expression of Gli1 and Gli3 in their nuclei (Figure 3A). The nuclear expression of Gli2 in the bile duct epithelium was observed in normal and PCK rats (Figure 3A). The overexpression of Smo and frequent nuclear expression of Gli1, Gli2, and Gli3 was also noted in the cyst-lining epithelium of PCK kidney (Figure 4).

Figure 6 Effects of *in vivo* administration of cyclopamine to polycystic kidney (PCK) rats. Total body (A), liver/body (B), and kidney/body (C) weights of normal and PCK rats with or without the *in vivo* administration of cyclopamine. D–F: Representative images for the histomorphometric analysis of the areas of the dilated bile duct lumen (D, colored in green), liver fibrosis (E, colored in red; Picrosirius red staining), and kidney cyst (F, colored in blue) of PCK rats. G–I: The histomorphometric analysis of the biliary cystic (G), liver fibrosis (H), and kidney cyst (I) areas shows that the sizes of the biliary cystic area and kidney cyst area are significantly reduced by the treatment. J and K: The immunohistochemical expression of Ki-67 in the bile duct epithelium of PCK rats is reduced (J), and a quantitative analysis of the Ki-67–labeling index confirms this result (K). L and M: The immunohistochemical expression of cyclin D1 (L) and the cyclin D1–labeling index (M) of the bile duct epithelium of PCK rats are also significantly reduced following the treatment. The plus and minus signs (A–C, G–I, K, and M) represent the presence and absence of *in vivo* cyclopamine treatment, respectively. Data are expressed as means $\pm$ SD (A–C, G–I, K, and M). * $P < 0.05$; ** $P < 0.01$. Original magnification: $\times 40$ (D and E, right panels); $\times 20$ (F, right panels); $\times 1000$ (J and L). Scale bars, 5 mm (D–F).

The immunohistochemical expression of cyclin D1 was markedly increased in the bile duct epithelium of PCK rats, whereas it was weakly expressed in normal rats (Figure 3B). The quantitative analysis showed that the cyclin D1-labeling index of the bile duct epithelium was markedly higher in 3-week-old, 2-month-old, and 10-month-old PCK rats than in normal rats of the same ages (Figure 3C). In PCK rats, the nuclear expression of Gli1 and Gli2 in the bile duct epithelium tended to be higher in 2-month-old rats compared with those in 3-week-old rats and 10-month-old rats, showing age-related differences. The results were consistent with those of cyclin D1 expression. By contrast, the nuclear expression of Gli2 in the bile duct epithelium of normal rats appeared not to be significantly different among the ages.

The results of immunostaining using liver tissues from patients with Caroli disease with CHF were similar to those of PCK rats, with Smo being overexpressed, and Gli1, Gli2, and Gli3 localizing in the nuclei of dilated intrahepatic bile duct epithelium (Figure 5A). The nuclear expression of Gli2 was also noted in the bile duct epithelium of normal livers (Figure 5A). The cyclin D1-labeling index of the bile duct epithelium was significantly higher in livers with Caroli disease with CHF than in normal livers (Figure 5, B and C).

Effects of *in Vivo* Administration of Cyclopamine to PCK Rats

Normal and PCK rats were intraperitoneally administered cyclopamine at 10 mg/kg daily for 4 weeks starting at 4 weeks of age. After the treatment, no significant reductions in total body weight were observed (Figure 6A). Laboratory data showed that serum alanine transaminase and alkaline phosphatase levels were higher in untreated PCK rats than in untreated normal rats, and the treatment significantly reduced these levels in PCK rats (Table 3).

Cyclopamine did not affect the liver or kidney weight of PCK rats (Figure 6, B and C). However, as expected, the histomorphometrical analysis showed that it significantly inhibited the cystic dilation of intrahepatic bile ducts and kidney cyst formation in PCK rats (Figure 6, D, F, G, and I). Accordingly, the Ki-67-labeling index and cyclin D1-labeling index of the bile duct epithelium were

significantly reduced by the treatment (Figure 6, J–M). The analysis using Picrosirius red-stained sections showed that the severity of liver fibrosis was not significantly reduced in PCK rats following the treatment ($P = 0.09$) (Figure 6, E and H). In addition, the expression of mRNAs for alpha-smooth muscle actin and procollagen type 1 in the whole liver was not significantly different between PCK rats with and without cyclopamine treatment as assessed by RT-qPCR (data not shown).

Discussion

The present study showed the overexpression of Smo and Gli1 as well as the nuclear translocation of Gli1, Gli2, and Gli3 in PCK cholangiocytes, and revealed the involvement of the aberrant activation of Hh signaling in biliary cystogenesis in PCK rats. The activation of Hh signaling has been implicated in various inflammatory diseases and malignant tumors. In the biliary tract, the activation of Hh signaling accompanied by the overexpression of Hh-related molecules, including Smo, Gli1, and Gli2, and/or the nuclear translocation of Gli proteins have been reported in chronic liver diseases, such as primary biliary cholangitis and primary sclerosing cholangitis, and biliary tract cancers, including cholangiocarcinoma and gallbladder cancer.^{20–23}

The mechanisms underlying the overexpression of Smo were not elucidated in the present or previous studies.^{20–22} Because the expression of Smo is known to be post-transcriptionally regulated by microRNAs, a possible mechanism involved in the overexpression of Smo is that mediated by microRNAs. In specific cell types, microRNAs, such as miR-125b and miR-326, directly target *Smo* mRNA, and the weaker expression of these microRNAs has been shown to correlate with the overexpression of Smo.²⁴ MicroRNA alterations have been reported in PCK cholangiocytes.²⁵ In this context, further studies are warranted.

The nuclear expression of Gli2, but not Gli1 was observed in normal cholangiocytes in the present study. The treatment with cyclopamine for normal cholangiocytes inhibited the nuclear expression of Gli2, but had no effect on cell proliferative activity or cyclin D1 expression. In the normal biliary tract, Hh signaling may

Table 3 Laboratory Data of Normal and PCK Rats with or without the Cyclopamine Treatment

Blood chemistry	Normal untreated	Normal cyclopamine	PCK untreated	PCK cyclopamine
Total protein (g/dL)	7.0 ± 0.1	6.1 ± 0.3	6.3 ± 0.1	6.0 ± 0.2
Albumin (g/dL)	4.6 ± 0.1	4.2 ± 0.1	4.0 ± 0.1	3.8 ± 0.1
Aspartate aminotransferase (IU/L)	98 ± 27	73 ± 5	119 ± 12	107 ± 6
Alanine transaminase (IU/L)	41 ± 2	43 ± 8	59 ± 5 [†]	48 ± 3*
Alkaline phosphatase (IU/L)	1166 ± 86	1170 ± 95	1595 ± 98 [†]	1410 ± 84*
Blood urea nitrogen (mg/dL)	22 ± 1	18 ± 1	21 ± 1	19 ± 0
Creatinine (mg/dL)	0.32 ± 0.03	0.31 ± 0.01	0.32 ± 0.01	0.29 ± 0.01

Data are expressed as means ± SD.

* $P < 0.05$ (versus untreated PCK rats).

[†] $P < 0.01$ (versus untreated normal rats).

be constantly activated via the nuclear translocation of Gli2, and this activation may be associated with biliary physiology, but does not significantly contribute to the cell proliferative activity of cholangiocytes. The role and the molecular mechanism of Hh signaling during maintenance of bile duct homeostasis are not fully understood and remain to be further investigated, as described in a recent article.²⁶

Similar to the present results, the knockdown of *Gli1* mRNA, but not *Gli2* mRNA was previously shown to significantly reduce the cell proliferative activity of cholangiocarcinoma cells, indicating that Gli1 rather than Gli2 mediates this activity.²¹ Gli1 possesses an activator domain, whereas Gli2 contains activator and repressor domains.¹² Although the cascade of events occurring between Smo derepression and Gli activation is still poorly understood, Gli1 and Gli2 proteins are not expected to share a completely common set of genes as their downstream targets.

In this study, the nuclear expression of Gli3 in PCK cholangiocytes appeared to be increased. Gli3 acts mostly as a repressor, but it can also have positive effects. In the presence of Hh ligands, Smo interacts with signaling components which modify Gli2 (and probably Gli3) to make it a transcriptional activator and prevent the proteolytic processing of Gli3 to the repressor form. Consequently, the ratio between full-length active/truncated repressive Gli isoforms (GliA/GliR ratio) raises with transcriptional activation of Hh target genes.

It has been reported that histone deacetylase 6 (HDAC6) is overexpressed in medulloblastoma cells, and HDAC6 inhibits the proteolytic processing of Gli3 that leads to Hh signaling activation.²⁷ The overexpression of HDAC6 has been also reported in PCK cholangiocytes as well as ADPKD cells.^{28,29} From these results, it is speculated that Gli3 seen in the nuclei of cholangiocytes of PCK rats and human Caroli disease represents the active isoform of Gli3. Although the differences in the expression of GliA and GliR proteins in normal and diseased cells could not be determined in this study, we consider that the balance between GliA/GliR is dysregulated in cholangiocytes of PCK rats and human Caroli disease, and the GliA/GliR ratio may be abnormally activated.

Canonical Hh signaling requires cilia, and noncanonical, Smo-independent pathways have also been linked to Hh signaling. Noncanonical Hh signaling has been implicated in the biology of cholangiocarcinoma cells, which often do not express cilia.³⁰ Smo-independent Gli activation may occur via MAP kinase-, PI3K/AKT-, and mTOR-dependent pathways.³¹ Because the activation of signaling pathways involving MAP kinases, PI3K/AKT, and mTOR has been documented in detail in PCK cholangiocytes,^{15,32} the activation of Gli observed in PCK cholangiocytes may be associated with these signaling pathways. However, the inhibitory effects of cyclopamine on the cell proliferative activity of and Gli activation in PCK cholangiocytes indicate that the activation of Hh signaling depends on Smo,

and, thus, it may be causally related to abnormal cholangiocyte cilia.

Cyclopamine was the first Hh inhibitor to be identified, and inactivates Smo by directly binding to its heptahelical bundle. Several Smo inhibitors are currently being tested in clinical trials for the treatment of multiple cancer types. In most cases, the anticancer effects of cyclopamine are related to the induction of apoptosis in cancer cells.³³ When cyclopamine was administered to a rat model of ischemia reperfusion injury accompanied by cholestatic liver damage induced by bile duct ligation, cyclopamine-treated rats showed less liver damage, and less cholangiocyte proliferation and apoptosis.³⁴ In the present study, cyclopamine did not significantly induce apoptosis in PCK cholangiocytes. This may be beneficial from a therapeutic perspective because cholangiocytes in PCK rats and Caroli disease with CHF do not possess a neoplastic nature.

Despite advances in our understanding of the mechanisms underlying biliary cystogenesis and the discovery of potential therapeutic targets, drug treatments for these patients are limited. Novel approaches mainly focus on the cAMP signaling pathway and evaluating the effects of somatostatin analogs as well as several new targets (eg, HDAC6, Cdc25A phosphatase, peroxisome proliferator-activated receptor γ , and matrix metalloproteases).^{35–38} In addition to these targets, the present results show that the inhibition of Hh signaling might be beneficial. However, cyclopamine has limitations as a drug candidate because it causes many potent side effects, including weight loss, dehydration, and death in mouse models. Thus, other Smo inhibitors and Gli inhibitors that modulate targets acting downstream of Smo may be useful.³⁹ In addition, a combination of drugs that inhibit the Hh signaling and HDAC6 may be more beneficial.⁴⁰

In conclusion, the present study shows that Hh signaling is aberrantly activated in PCK cholangiocytes. The resultant induction of cyclin D1 may lead to cholangiocyte hyperproliferation, which contributes to the cystic dilation of intrahepatic bile ducts. The immunohistochemical analysis showed that the activation of Hh signaling may also be involved in the pathogenesis of Caroli disease with CHF in humans. Importantly, the *in vivo* inhibition of Hh signaling decreased abnormally elevated serum liver enzymes, and attenuated bile duct dilation and kidney cyst formation in PCK rats. These results suggest that the inhibition of Hh signaling, which targets Smo and/or Gli, has potential as a therapeutic strategy for biliary cystogenesis in Caroli disease with CHF.

Acknowledgments

Y.S. and K.H. conceived and designed the study; Y.S. performed the experiments, with technical support from M.Y., and wrote the manuscript; M.S. analyzed data and provided intellectual comments; and all authors interpreted data, and critically reviewed and approved the manuscript.

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