GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Contributions of Hepatocytes and Bile Ductular Cells in Ductular Reactions and Remodeling of the Biliary System after Chronic Liver Injury

Yasuharu Nagahama,^{*†} Masayuki Sone,[†] Xi Chen,^{*} Yoko Okada,^{*} Masahiro Yamamoto,^{*} Bing Xin,^{*} Yasuhiro Matsuo,^{*} Mikiko Komatsu,^{*} Akira Suzuki,[‡] Katsuhiko Enomoto,[§] and Yuji Nishikawa^{*}

From the Division of Tumor Pathology,^{*} Department of Pathology, Asahikawa Medical University, Asahikawa; the Fujii Memorial Research Institute,[†] Otsuka Pharmaceutical Co., Ltd., Otsu; the Division of Embryonic and Genetic Engineering,[‡] Medical Institute of Bioregulation, Kyushu University, Fukuoka; and the Department of Pathology,[§] Akita Red Cross Hospital, Akita, Japan

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Address correspondence to Yuji Nishikawa, M.D., Ph.D., Division of Tumor Pathology, Department of Pathology, Asahikawa Medical University, Higashi 2-1-1-1, Midorigaoka, Asahikawa, Hokkaido 078-8510, Japan. E-mail: nishikwa@asahikawa-med.ac.jp.

Mature hepatocytes are suggested to possess a capacity for bile ductular transdifferentiation, but whether and how hepatocytes contribute to ductular reaction in chronic liver diseases has not been elucidated. We examined whether mouse hepatocytes can transdifferentiate into bile ductular cells *in vitro*, using a three-dimensional collagen gel culture method, and *in vivo*, using a liver repopulation model in which β -galactosidase-positive hepatocytes from *Alb-Cre* $\times$ *ROSA26R* mice were transplanted into the liver of wild-type mice. We further examined the relative contribution of intrinsic hepatocytes in ductular reaction in a hepatocyte lineage-tracing model using *Mx1-Cre* $\times$ *ROSA26R* mice treated with polyinosinic-polycytidylic acid. Within collagen gels, hepatocytes exhibited branching morphogenesis associated with the emergence of bile duct-like phenotype. In the liver repopulation model, many β -galactosidase-positive, hepatocyte-derived bile ductular structures were identified; these markedly increased after liver injury. In *Mx1-Cre* $\times$ *ROSA26R* mice, relatively minor but significant contributions of hepatocyte-derived bile ductules were observed in both periportal and centrilobular ductular reaction. As the centrilobular ductular reaction progressed, the portal ducts or ductules migrated toward the injured area and joined with hepatocyte-derived ductules, leaving the portal tract without biliary structures. We conclude that hepatocytes and bile ducts or ductules are important sources of ductular reaction and that the intrahepatic biliary system undergoes remarkable remodeling in response to chronic liver injury. (*Am J Pathol* 2014, 184: 3001–3012; <http://dx.doi.org/10.1016/j.ajpath.2014.07.005>)

Chronic liver diseases are often complicated by ductular reaction, which is a progressive increase in bile ductular structures.^{1,2} Ductular reaction is closely associated with liver fibrosis and thus is an important tissue alteration to examine as a therapeutic target.³ However, the cellular origins of ductular reaction remain elusive. Experimental studies using rodents have suggested that hepatic stem-progenitor cells or transit-

amplifying cells, which are believed to reside at the boundary of hepatocytes and the canals of Hering (ie, bile ductules), can proliferate into ductular shapes and eventually contribute to

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regeneration when the proliferation of hepatocytes is compromised.⁴ However, the periportal liver stem-cell niche is gradually coming to be recognized as more complex and involving other cell types, including bile ductular cells and periportal hepatocytes.⁵ Although ductular reaction typically occurs in the periportal area, it may also occur in the centrilobular area, where no biliary cells or stem–progenitor cells are present.⁶ Furthermore, bile duct–specific cytokeratins have been detected in damaged hepatocytes in the centrilobular area in alcoholic liver disease.⁷ Such observations raise the possibility that, in some types of ductular reaction, ductular transdifferentiation of mature hepatocytes might be involved.^{1,6}

Our previous studies showed that mature rat hepatocytes can transdifferentiate into bile ductular cells when cultured within a type-I collagen gel matrix,^{8–10} and similar phenotypic plasticity of hepatocytes has been described by other investigators.^{11,12} Furthermore, we have recently demonstrated that, under modulated culture conditions, rat hepatocytes can recover their original phenotype after bile ductular transdifferentiation.¹³ This suggests that hepatocytic and bile ductular phenotypes are mutually reversible.

Whether mature hepatocytes can transdifferentiate into bile duct or ductular cells (hereafter, duct/ductular cells) *in vivo* has been a matter of debate. In a liver repopulation model using fumarylacetoacetate hydrolase–deficient mice, transplanted and repopulated hepatocytes did not substantially contribute to the ductular reaction induced by a diet with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC).¹⁴ In a hepatocyte lineage–tracing study using an adeno-associated virus vector in mice, hepatocytes did not change their phenotype in the presence of various type of liver injury, including those induced by a DDC diet or bile duct ligation (BDL), or during liver homeostasis.¹⁵ More recently, however, a similar lineage-tracing study demonstrated that hepatocytes can significantly participate in the generation of new ductules in the presence of liver injury induced by a DDC diet and to a lesser degree in the presence of injury induced by BDL.¹⁶ Furthermore, in a study using permanent labeling of hepatocytes and bile-duct cells with cell type–specific reporters, the centrilobular ductular reaction induced by thioacetamide (TAA) was derived almost entirely from hepatocytes, and not from bile ducts or ductules.¹⁷ Given the disparate findings in the literature regarding phenotypic plasticity of hepatocytes, it is necessary to scrutinize the cellular origins of various types of ductular reactions by rigorous pathological examination in appropriate hepatocyte lineage–tracing systems.

In the present study, we first examined and confirmed that the phenotype of mature mouse hepatocytes is also malleable when cultured within a three-dimensional collagen gel matrix. We then attempted to demonstrate that hepatocytes are capable of changing their phenotype to bile duct/ductular cells in the injured liver, by tracing intrahepatically transplanted β -galactosidase–positive (β -gal⁺) hepatocytes. Furthermore, the extent of the contribution of ductular transdifferentiation of hepatocytes in the ductular reaction of various types of liver

injury was examined in a hepatocyte lineage–tracing system using *Mx1*-Cre mice. Our results demonstrate that hepatocytes can indeed transdifferentiate into bile duct/ductular cells and thus contribute to periportal and centrilobular ductular reactions, and that the existing bile duct/ductular system exhibits a profound remodeling capacity.

Materials and Methods

Animals

C57BL/6Jcl mice (CLEA Japan, Tokyo, Japan), B6.Cg-Tg(*Alb*-Cre)21Mgn/J mice (*Alb*-Cre; Jackson Laboratory, Bar Harbor, ME), B6.Cg-Tg(*Mx1*-Cre)1Cgn/J mice (*Mx1*-Cre; Jackson Laboratory),¹⁸ and ROSA26R mice (provided by Dr. Philippe Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA)¹⁹ were used in this study. The protocols for animal experimentation were approved by the Animal Research Committee, Asahikawa Medical University, and all animal experiments adhered to the criteria outlined in the current edition of the Guide for the Care and Use of Laboratory Animals.²⁰

Three-Dimensional Culture of Hepatocytes

Centrilobular hepatocytes were isolated from the liver of adult C57BL/6Jcl mice (8 to 20 weeks old) by a digitonin–collagenase perfusion technique²¹ (Supplemental Figure S1). We performed a three-dimensional collagen gel culture of mouse hepatocytes, as described previously^{9,22} (Supplemental Figure S2). After the cells were embedded within the collagen gels, spheroidal aggregates were cultured in Williams' medium E supplemented with 10 mmol/L nicotinamide, 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 10^{−7} mol/L insulin, 10^{−7} mol/L dexamethasone, and 10 ng/mL tumor necrosis factor- α (TNF- α) (PeproTech, Rocky Hill, NJ).

After completion of culture, the gels were washed three times with phosphate-buffered saline and then were either snap-frozen for biochemical analyses or fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or subjected to immunocytochemistry.

In Vivo Lineage Tracing of Hepatocytes

For lineage tracing of adult hepatocytes, we applied two models: a liver repopulation model and a hepatocyte-specific labeling model using *Mx1*-Cre $\times$ ROSA26R mice treated with polyinosinic–polycytidylic acid [poly(I:C)].

Liver Repopulation Model

Adult C57BL/6Jcl mice were treated repeatedly with 60 mg/kg retrorsine *i.p.* (once weekly for 5 weeks) to inhibit the proliferative capacity of hepatocytes. The mice were then subjected to a 70% partial hepatectomy; after 24 hours, β -gal⁺ hepatocytes, freshly isolated from adult *Alb*-Cre $\times$ ROSA26R by the digitonin–collagenase perfusion method, were transplanted

into the spleen (0.5 to 1×10^6 cells per animal).²³ The mice were subjected to chronic liver injury induced either by feeding with a 0.1% DDC diet for 4 weeks or by administration of 1 mL/kg CCl₄ s.c. (three times per week) for 8 weeks.

Hepatocyte-Specific Labeling in *Mx1*-Cre × *ROSA26R* Mice
Male *Mx1*-Cre × *ROSA26R* mice (8 to 12 weeks old) were each injected intraperitoneally with 250 µg of poly(I:C) (InvivoGen, San Diego, CA), three times at 2-day intervals. This dose of poly(I:C) injection caused no apparent organ damage, as assessed by histological studies (data not shown). At 3 days after the last poly(I:C) administration, the mice were injected with 1 mL/kg CCl₄ s.c., three times per week for 8, 11, and 20 weeks or with 100 mg/kg TAA i.p. (Sigma-Aldrich, St. Louis, MO), three times per week for 21 weeks to induce ductular reaction in the centrilobular area and were fed a 0.1% DDC diet for 4 weeks. In addition, the mice were subjected to BDL for 14 days and administration of 40 mg/kg 4,4'-diaminodiphenylmethane (DAPM) i.p. (Kanto Chemical, Tokyo, Japan), at 5-day intervals for 4 weeks.

RT-qPCR Analysis

Total RNA from cells and tissues was extracted and subjected to quantitative real-time RT-PCR (RT-qPCR) analysis for mRNA expression of various hepatocytic, bile duct/ductular, and hepatoblastic markers, as well as several proteins involved in tissue remodeling. The reactions were performed in duplicate, and the mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as a housekeeping gene. The specific primers used were as follows: albumin (*Alb*), forward 5'-TTGCCAAGTACATGTGTGAA-3' and reverse 5'-GGCTC-TTCTACAAGAGGCTG-3'; cytokeratin 19 (*Krt19*), forward 5'-GACTTCCTATAGCTATCGCC-3' and reverse 5'-AGTA-GGAGGCGAGACGATCA-3'; delta-like 1 (*Dlk1*), forward 5'-ATGTCTGCAGGTGCCATGTTG-3' and reverse 5'-GGC-TTGACAGACACTCGAAG-3'; glucose 6-phosphatase (*G6pc*), forward 5'-TGTCTGTGATTGCTGACCTG-3' and reverse 5'-AAAGTGAGCAGCAAGGTAGA-3'; hepatocyte nuclear factor-4α (*Hnf4a*), forward 5'-GGCATGGATA-TGGCCGACTAC-3' and reverse 5'-GACAGCTTCCTT-CTTCATGCC-3'; secreted phosphoprotein 1 (alias osteopontin) (*Spp1*), forward 5'-GAGGAGAAGCTTTA-CAGCCT-3' and reverse 5'-AAGTGTCTGCTTGTG-TACTA-3'; phosphoenolpyruvate carboxykinase (*Pepck*), forward 5'-CCCAAGAGCAGAGAGACACA-3' and reverse 5'-GTAGTGAGTTCCCACCATAT-3'; Sox9, forward 5'-TGCAGCACAAGAAAGACCAC-3' and reverse 5'-TCTCAATGTTGGAGATGACG-3'; tyrosine amino-transferase (*Tat*), forward 5'-ACAGCTTGTCTCGTGGT-CAAC-3' and reverse 5'-TAAGGTACATGGCTCCA-GATG-3'; transthyretin (*Ttr*), forward 5'-GAGTA-GAACTGGACACCAAATC-3' and reverse 5'-AGAA-TGCTTACGGCATCTTCC-3'; *Gapdh* mRNA, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TC-CACCACCCTGTTGCTGTA-3'. We used TaqMan (Life

Technologies, Carlsbad, CA) gene expression assays for analysis of mRNA expression for S100 calcium-binding protein A4 (*S100a4*), matrix metalloproteinase-2 (*Mmp2*), matrix metalloproteinase-9 (*Mmp9*), TIMP metalloproteinase inhibitor 1 (*Timp1*), TIMP metalloproteinase inhibitor 3 (*Timp3*), and TNF-α (*Tnfa*).

Western Blot Analysis

Protein samples (25 µg protein per lane) were subjected to SDS-PAGE and then were transferred to polyvinylidene difluoride membranes. The primary antibodies used were anti-albumin (Nordic Immunological Laboratories, Tilburg, The Netherlands), anti-HNF-4α (K9218; Perseus Proteomics, Tokyo, Japan), anti-CK19 (kind gift of Dr. Atsushi Miyajima, University of Tokyo, Tokyo, Japan), anti-SRY (sex determining region Y)-box 9 (Sox9) (EMD Millipore, Billerica, MA), and anti-β-actin (Novus Biologicals, Littleton, CO).

Microscopical Analyses

Livers were perfused with 4% paraformaldehyde and soaked in 30% sucrose in phosphate-buffered saline overnight at 4°C. Frozen and paraffin-embedded sections (4 µm thick) were then prepared. In some experiments, to identify the portal veins and to visualize the biliary system in tissue sections of cirrhotic liver, India ink (approximately 100 µL) was injected directly into the portal trunk and common bile duct, respectively, and fractions of the liver tissues were cut and immersion-fixed with 4% paraformaldehyde. In addition, to obtain images of the three-dimensional architecture of the bile ducts or ductules and blood vessels, 1-mm-thick slices of the fixed liver, in which the biliary system and portal vein were engorged with India ink and vermilion ink, respectively, were rendered transparent with an aqueous clearing reagent (SCALEVIEW-A2; Olympus, Tokyo, Japan) and then examined under an inverted microscope.

For β-gal histochemistry, frozen sections were rinsed in phosphate-buffered saline and reacted with X-Gal solution [1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma-Aldrich), 5 mmol/L potassium ferrocyanide, and 5 mmol/L potassium ferricyanide] at 37°C overnight.

Immunocytochemical and immunohistochemical analyses were performed using either the peroxidase or alkaline phosphatase method after antigen retrieval using target retrieval solution, pH 6.0 (Dako, Carpinteria, CA). Several different chromogens were used: 3,3'-diaminobenzidine tetrahydrochloride (brown; Vector Laboratories, Burlingame, CA), 3-amino-9-ethylcarbazole (reddish brown; Dako), Histogreen (green; Linaris Biologische Produkte, Dossenheim, Germany), and New Fuchsin (red; Nichirei Biosciences, Tokyo, Japan). The following antibodies were used: anti-HNF-4α (P1) (Perseus Proteomics), anti-Sox9 (EMD Millipore), anti-CK19

(gift of Dr. Atsushi Miyajima), anti-EpCAM (Novus Biologicals), anti-desmin (Abcam, Cambridge, UK), anti-F4/80 (AbD Serotec, Düsseldorf, Germany; Raleigh, NC), anti-LYVE-1 (Abcam), and anti-phospho-histone H3 (Cell Signaling Technology, Danvers, MA).

For quantification analyses, the numbers of bile duct/ductular structures or cells were counted on 50 photographs of each liver sample imaged with a 40× objective.

Statistical Analysis

Statistical analyses were performed using Student's *t*-test or analysis of variance on more than three independent experiments or animals.

Results

Branching Morphogenesis with Emergence of Expression of Bile Duct–Ductular Differentiation Markers in Cultured Mouse Hepatocytes

We first examined whether adult hepatocytes can trans-differentiate into bile ductular cells in mouse, as was previously shown in rat experiments.^{8,9} To avoid possible contamination

of bile duct/ductular cells and the putative hepatic stem–progenitor cells, we selectively isolated hepatocytes from the centrilobular zone (Supplemental Figure S1). When spheroidal aggregates of hepatocytes were embedded and cultured within collagen gels (Figure 1A and Supplemental Figure S2), the hepatocytes soon migrated into the gels and formed spiny processes, and after 5 days demonstrated branching morphogenesis with scattered luminal structures that was markedly enhanced by TNF- α (Figure 1A). These morphological changes were associated with a dramatic decrease in the mRNA expression of *Alb*, *Ttr*, *Hnf4a* (Figure 1B), *Tat*, *G6pc*, and *Pepck* (Supplemental Figure S3), especially in the presence of TNF- α , which suggests the loss of hepatocytic differentiation. By contrast, the expression of mRNAs relevant to bile duct differentiation emerged, such as *Krt19*, *Sox9* (Figure 1B), and *Spp1* (encoding osteopontin) (Supplemental Figure S3). The mRNA expression of *Dlk1*, a marker for hepatoblasts, was not detectable during the entire culture period (Figure 1B), suggesting that the phenotypic change might be trans-differentiation rather than dedifferentiation.

Western blot analysis showed that albumin expression was relatively well preserved (but was decreased when cells were treated with TNF- α), whereas HNF-4 α expression rapidly declined in culture (Figure 2A). Protein expression

Figure 1 Branching morphogenesis of mouse hepatocytes with the appearance of the bile ductular phenotype in collagen gel culture. **A:** Representative phase-contrast microscopy images (10× objective) of hepatocytic spheroids immediately after embedding within a collagen gel matrix (0 day) and cultured for 3, 5, and 10 days in the absence or presence of 10 ng/mL TNF- α . Note luminal structures (asterisks). **B:** RT-qPCR analysis for mRNA expression of various genes in freshly isolated hepatocytes, spheroids, and those cultured within collagen gels for 5, 10, and 15 days. Total RNA extracted from normal portal tissues ($n = 4$), diethylnitrosamine-induced hepatocellular carcinoma ($n = 3$), and E13.5 fetal liver ($n = 3$) were used as positive controls for bile duct–specific genes (*Krt19* and *Sox9*) and *Dlk1*. Data are expressed as means $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and † $P < 0.005$ versus control, unpaired two-tailed *t*-test. Enn.n, embryonic day; Heps, hepatocytes; HCC, hepatocellular carcinoma; PT, portal tissue.

of CK19 and SOX-9 appeared in cultured hepatocytes; expression of SOX-9 was induced earlier, being apparent already in spheroidal cultures (Figure 2A). Compatible with these findings, immunocytochemical analysis revealed a marked reduction of HNF-4 α and the emergence of CK19 and SOX-9 in cultured hepatocytes (Figure 2B).

Emergence of Hepatocyte-Derived Bile Ductular Structures in the Ductular Reaction in Liver Repopulation Experiments

To evaluate the transdifferentiation capacity of mature hepatocytes *in vivo*, we then performed lineage-tracing

experiments using a liver repopulation model (Figure 3A). Transplanted β -gal⁺ Alb-Cre $\times$ ROSA26R hepatocytes formed colonies of various sizes in the partially hepatectomized liver of retrorsine-treated mice, in which the proliferation of host hepatocytes was suppressed but that of transplanted hepatocytes was stimulated (Figure 3B). In a setting of mild to moderate liver injury, scattered areas of ductular reaction were observed in which a small population of increased ductules were β -gal⁺ (Figure 3B). When similarly treated mice were subjected to further liver injury induced by a DDC diet or CCl₄, an extensive ductular reaction occurred; a number of β -gal⁺ ducts or ductules were found, especially proximal to the colonies of transplanted

Figure 3 Bile ductular transdifferentiation of *Alb-Cre* $\times$ *ROSA26R* mouse hepatocytes that were transplanted and repopulated in wild-type mouse liver. **A**: Schematic of the lineage tracing experiments using a liver repopulation model. **B–D**: Combined X-Gal histochemistry and immunohistochemistry for CK19 or SOX-9 in liver at 8 weeks after hepatocyte transplantation (**B**) and subsequent submission to liver injury induced by a DDC diet for 4 weeks (**C**) or by CCl_4 treatment for 8 weeks (**D**). Scale bar = 40 μm . DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; PH, partial hepatectomy; Ret, retorsine; Tx, transplantation; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

β -gal⁺ hepatocytes, which were also positive for SOX-9 (Figure 3, C and D).

Relative Contribution of Hepatocyte-Derived Bile Ductules in the Ductular Reaction Assessed in a Hepatocyte-Specific Gene Targeting System

These *in vitro* and *in vivo* experiments clearly demonstrated that mature hepatocytes possess the capacity to differentiate into bile duct/ductular cells in response to microenvironmental changes. However, the relative contribution of the proliferation of bile ducts or ductules and the transdifferentiation of hepatocytes in the ductular reaction remained unknown. To examine this, it was necessary to

induce injury in intact liver in which the resident hepatocytes were specifically and permanently labeled. We therefore applied a poly(I:C)—inducible gene targeting system (*Mx1-Cre* $\times$ *ROSA26R*).^{18,24,25} We confirmed the efficient and specific labeling of hepatocytes, which almost entirely spared the bile duct/ductular cells (Figures 4 and 5A) and other non-parenchymal cells (Figures 4 and 5B), on the completion of repeated administration of poly(I:C). However, after a long time (15 months) had elapsed since labeling, a small but significant increase in β -gal⁺ duct/ductular cells occurred (Figure 5A), reaching 4.2% of the CK19⁺ ductular cells (Figure 5, B and C), which suggested involvement of periportal hepatocytes in the normal homeostasis of duct/ductular cells.

We then examined the cellular origins of ductular reaction in the periportal area induced by a DDC diet or DAPM. These toxic agents significantly increased the number of ductular structures composed of CK19⁺ cells, which were slightly larger than normal bile ductular cells (Figure 5A). The reactive bile ductules contained a small number of β -gal⁺ cells (Figure 5, A and B), which were estimated to make up 1.9% and 4.7% of the CK19⁺ ductular cells in the liver of DDC diet-fed and DAPM-treated mice, respectively (Figure 5C). The increased ductular structures induced by BDL also contained a small number of β -gal⁺ cells (Supplemental Figure S4). These findings demonstrated that mature hepatocytes also contribute, although not extensively, to the periportal ductular reaction due to various injuries.

We also evaluated the cellular origins of ductular reactions in the centrilobular area, where no bile ductular cells are present. Chronic administration of CCl_4 or TAA selectively damaged centrilobular hepatocytes, causing inflammation,

Figure 4 Hepatocyte-specific β -gal labeling in *Mx1-Cre* $\times$ *ROSA26R* mice after polyinosinic–polycytidylic acid [poly(I:C)] injections in control (intact) liver at 3 days after the last poly(I:C) injection and in liver treated with CCl_4 for 11 weeks. Combined X-Gal histochemistry and immunohistochemistry for various liver cell markers: HNF-4 α (hepatocytes; brown); SOX-9 and EpCAM (bile duct/ductular cells; red); desmin (stellate cells; red); LYVE-1 (sinusoidal endothelial cells; red); and F4/80 (Kupffer cells; reddish brown). Scale bar = 20 μm .

Figure 5 Ductular transdifferentiation of hepatocytes in liver homeostasis and the periportal ductular reaction in a hepatocyte lineage-tracing system using *Mx1-Cre* $\times$ *ROSA26R* mice. **A:** Combined X-gal histochemistry and immunohistochemistry for CK19 in the liver at 3 days (control) and 15 months (aging) after the final poly(I:C) injection and after liver injuries induced by DDC diet or DAPM. β -Gal⁺ bile duct/ductular cells are indicated by arrowheads; note the necrotic (N) area in the DAPM-treated liver. **B:** The number of β -gal⁻ and β -gal⁺ bile duct/ductular cells in the periportal area. **C:** The proportion of β -gal⁺ cells in CK19⁺ bile duct/ductular cells. Data are expressed as means $\pm$ SEM. $n = 3$ (control, aging, and DDC) or $n = 4$ (DAPM) mice. * $P < 0.05$ versus control, one-way factorial analysis of variance. Scale bar = 40 μ m. DAPM, 4,4'-diaminodiphenylmethane.

fibrosis, and ductular reaction in the centrilobular area (Supplemental Figure S5). Although no hepatic stellate cells, sinusoidal endothelial cells, or Kupffer cells were β -gal⁺ in the injured liver (Figure 4), the CK19⁺ ductules in the centrilobular area (zone 3) were composed of β -gal⁺ and β -gal⁻ cells (Figure 6, A and B). The β -gal⁺ ductules of hepatocyte origin were found mainly in inflamed and fibrosed areas in the vicinity of the central veins, and β -gal⁺ and β -gal⁻ ductules often were in contact with each other and shared lumens (Figure 6A). The number of ductules containing β -gal⁺ cells per centrilobular area was 4.1 and 1.3 in CCl₄ (20 weeks) and TAA (21 weeks) injury, respectively (Figure 6B). In the early period of CCl₄ injury (8 weeks), 20.6% of ductular cells were β -gal⁺, but this proportion decreased thereafter as the ductular reaction progressed (Figure 6C). Although TAA induced an extensive ductular reaction, the proportion of β -gal⁺ cells was small (4.3%) (Figure 6C).

Extensive Remodeling of the Intrahepatic Biliary System in Chronic Liver Injury

Notably, as the centrilobular ductular reaction progressed, the bile ducts or ductules in the portal tract appeared to migrate toward the injured centrilobular area, leaving the portal tract without duct/ductular structures (Figure 6, A and D). The vascular structures in the uninjured area were confirmed to be portal vein branches by retrograde injection of India ink from the portal trunk (Figure 6E). The hepatic arteries, which are usually confined within the portal connective tissue, were often located in the midst of liver lobules, suggesting migration of hepatic arteries toward the centrilobular direction (Figure 6F).

To visualize three-dimensional alterations of the intrahepatic bile duct system in the centrilobular ductular reaction, we retrogradely injected India ink and vermilion ink into the common bile duct and the portal vein trunk, respectively, of control and CCl₄-injured liver, and then treated fixed liver slices with a clearing reagent until they became transparent. In control liver, India ink was accumulated in bile ducts or ductules around the portal veins; the portal veins contained vermilion ink (Figure 7A). By contrast, in CCl₄-injured liver, pigment-containing bile ducts or ductules were present as extensive networks within the lobules, most of them reaching the centrilobular area (Figure 7A). In CCl₄-injured liver of poly(I:C)-treated *Mx1-Cre* $\times$ *ROSA26R* mice, India ink pigments infused into the common bile duct were found inside the aberrantly located bile ducts or ductules, some of which were composed of β -gal⁺ and β -gal⁻ cells, indicating that the biliary system connection was maintained (Figure 7, B and C). Furthermore, examination of serial sections revealed the actual anatomical connection between periportal bile ducts and centrilobular bile ducts or ductules (Figure 7C).

To elucidate the role of cell proliferation in the increase of ductular structures in centrilobular liver injury, we performed double immunostaining for phosphorylated histone H3 (p-HH3) and CK19 on β -gal-stained sections and counted proliferating cells in β -gal⁻ and β -gal⁺ ducts or ductules. Although p-HH3⁺ cells were occasionally found in β -gal⁻ ducts or ductules in CCl₄ and TAA injured tissue, small β -gal⁺ ductules barely demonstrated p-HH3 positivity (Figure 8A). Quantification revealed that p-HH3⁺ cells made up 0.67% and 1.66% of the β -gal⁻ ductular cells in CCl₄ and TAA injuries, respectively (Figure 8B). By contrast, β -gal⁺ ductules contained only 0.01% of p-HH3⁺ cells in the presence of CCl₄ injury, and no positive cells were detected in the presence of TAA injury (Figure 8B).

These results suggest that extensive tissue remodeling processes occur during migration of ductular cells. We have previously identified various proteins involved in tissue remodeling whose mRNA expression was increased in CCl₄-induced cirrhosis by cDNA microarray analysis (X.C. and Y.N., unpublished observations). We therefore performed RT-qPCR analysis for mRNA expression of such proteins, comparing control and CCl₄-induced or

Figure 6 Contribution of the ductular transdifferentiation of hepatocytes and migration of existing bile duct/ductular cells in the centrilobular ductular reaction in a hepatocyte lineage—tracing system using *Mx1-Cre* $\times$ *ROSA26R* mice. **A:** Combined X-Gal histochemistry and immunohistochemistry for CK19 in liver treated with CCl₄ (for 8 and 20 weeks) or TAA (21 weeks) in the centrilobular (zone 3) and periportal (zone 1) areas. β -Gal⁺ bile duct/ductular cells are indicated by arrowheads. **B:** The number of β -gal⁻ and β -gal⁺ bile duct/ductular cells in the centrilobular area. **C:** The proportion of β -gal⁺ cells in CK19⁺ bile duct/ductular cells in the centrilobular area. **D:** The number of duct/ductular structures within a portal tract. **E:** Visualization of portal vein branches by retrograde injection of India ink through the portal vein in CCl₄-treated liver (20 weeks). The same specimen is shown at two magnifications, and in each the portal tract is indicated by an asterisk. An interlobular bile duct (arrowhead) apparently migrated from the portal tract. H&E stain. **F:** Immunohistochemistry for α -smooth muscle actin in intact and TAA-treated (21 weeks) liver. Note interlobular bile ducts (green arrowheads) and hepatic arteries (red arrowheads). Data are expressed as means $\pm$ SEM. $n = 3$ mice (CCl₄, 8 and 11 weeks; TAA) or $n = 4$ mice (CCl₄, 20 weeks). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control, one-way factorial analysis of variance. CV, central veins; PV, portal veins. Scale bar = 40 μ m.

TAA-induced cirrhosis. There were significant increases in *S100a4*, *Mmp2*, and *Timp1* mRNA in the injured liver (Figure 8C).

Discussion

Although some investigators have proposed that the phenotype of hepatocytes is fixed once they are terminally differentiated,²⁶ this notion has been challenged by recent studies demonstrating phenotypic plasticity of hepatocytes.^{9,11,16} With the present study, as an extension of our previous work showing transdifferentiation of rat hepatocytes into bile duct/ductular cells in a collagen gel matrix,^{8–10,13} we have demonstrated that mouse hepatocytes also transform into ductular cells *in vitro*, especially when TNF- α is present in the culture medium. Ductular differentiation of mouse hepatocytes was not associated with the expression of *Dkl1* mRNA, which was present in fetal liver and in some hepatocellular carcinomas. Thus, the ductular transformation of mouse hepatocytes within collagen gels could be interpreted

as resulting from the process of transdifferentiation rather than dedifferentiation.

The idea that hepatocytes can transform into ductular cells *in vivo* has been a subject of intense debate.^{26–28} To look for further evidence of transdifferentiation, we isolated centrilobular hepatocytes (permanently labeled by β -gal) from *Alb-Cre* $\times$ *ROSA26R* mice and transplanted them into the liver of wild-type mice. We previously demonstrated that centrilobular and periportal hepatocytes of rat possess nearly identical capacities for ductular differentiation *in vitro*.⁹ Our present results demonstrated that hepatocytes can transform into bile duct/ductular cells *in vivo*. The transformation was readily observed at the periphery of the repopulated hepatocytic colonies, and it was markedly enhanced by chronic CCl₄ administration or a DDC diet. The robust ductular reaction was also closely associated with inflammation induced by retrorsine and a partial hepatectomy and was further augmented by additional injurious stimuli. The inflammatory background might promote ductular transdifferentiation through an increase in extracellular matrices and various inflammatory

Figure 7 The three-dimensional structures of the centrilobular ductular reaction and its connection to the existing biliary system. **A:** Three-dimensional visualization of the bile ducts or ductules by retrograde injection of India ink and vermilion ink through the common bile duct and the portal vein trunk, respectively, in intact (control) and CCl_4 -treated (20 weeks) liver. **B and C:** Combined X-Gal histochemistry and immunohistochemistry for CK19 of the liver in a hepatocyte lineage-tracing system, using *Mx1-Cre* $\times$ *ROSA26R* mice, in which India ink was injected through the common bile duct. **B:** Intact (control) and CCl_4 -treated (20 weeks) liver. The boxed area in the middle panel is shown in detail with a 100 $\times$ oil immersion objective in the bottom panel. Note the presence of India ink pigments (arrow) inside the lumen of the CCl_4 -induced aberrant bile ductule, which is composed of $\beta\text{-gal}^+$ and $\beta\text{-gal}^-$ cells. **C:** Five serial sections (4 μm thick) of CCl_4 -treated (20 weeks) liver. The periportal bile ducts (red arrowheads) and the centrilobular bile ductules (yellow arrowhead) are continuous. $\beta\text{-Gal}^+$ bile duct/ductular cells are indicated by white arrowheads. The boxed area (section 5) is shown in detail with a 100 $\times$ oil immersion objective in the panel to the right. Note intraluminal India ink pigments (arrow). Scale bar = 40 μm .

cytokines, including $\text{TNF-}\alpha$. Interestingly, some hepatocytes near the ductular reaction expressed SOX-9 but not CK19, which could represent an intermediate hepatobiliary phenotype.²⁹

No definitive functional significance for the ductular transdifferentiation of hepatocytes has been elucidated. However, it is noteworthy that similar phenomena occur during the development of the intrahepatic bile duct system in which albumin-producing hepatoblasts form bile duct/ductular cells along the developing portal connective tissue.³⁰ Our present findings strongly suggest that transdifferentiation can participate in homeostasis of the biliary system in intact liver and in ductular reaction in various liver diseases. We speculate that this cellular response might have a crucial role in maintaining and establishing the connection of the bile conduit system between hepatocytes and bile ductular cells.

Hepatocytes and bile ducts or ductules can participate in ductular reaction. The liver repopulation model is not

suitable for analyzing the relative contribution of intrinsic hepatocytes and bile ducts or ductules, because repopulation by the transplanted hepatocytes would affect the structures of the host hepatic lobules and retrorsine administration and a partial hepatectomy would alter the proliferative activity of the host cells. In the present study, we used *Mx1-Cre* $\times$ *ROSA26R* mice, in which hepatocytes can be labeled efficiently and specifically by poly(I:C) administration without affecting liver physiology. Some of the bile ductules contained cells of hepatocyte origin (ie, $\beta\text{-gal}^+$) in the presence of periportal and centrilobular liver injuries, although they constituted relatively small fractions of the bile ducts or ductules in the injured areas. In an efficient hepatocyte-lineage tracing system in mouse, in which an adeno-associated virus vector was applied, Malato et al¹⁵ reported that the transformation did not occur with injuries induced by acute or chronic CCl_4 administrations, BDL, or DDC diet. By contrast, in a more recent

Figure 8 Migration and proliferation of bile ducts or ductules in the centrilobular ductular reaction. **A:** Triple staining of X-Gal, CK19 (red), and p-HH3 (brown) in the liver of CCl₄ (20 weeks; *n* = 4) or TAA (*n* = 3) in a hepatocyte lineage—tracing system using *Mx1-Cre* × *ROSA26R* mice. β-Gal⁺ bile ducts or ductules are indicated by arrowheads; p-HH3⁺ bile ducts or ductules are indicated by arrows. **B:** The percentage of p-HH3⁺ cells, indicative of proliferation, in β-gal⁻ and β-gal⁺ bile ducts or ductules. **C:** RT-qPCR analysis for relative mRNA expression of *S100a4*, *Mmp2*, *Mmp9*, *Timp1*, *Timp3*, and *Tnf* in the liver of control (*n* = 4), CCl₄-treated (24 weeks; *n* = 4), and TAA-treated (24 weeks; *n* = 5) animals. Data are expressed as means ± SEM. ****P* < 0.001, one-way factorial analysis of variance (**B**). **P* < 0.05; †*P* < 0.005 versus control, unpaired two-tailed *t*-test (**C**). Scale bar = 40 μm.

study using a similar adeno-associated virus—mediated hepatocyte-lineage tracing system, Yanger et al¹⁶ showed that some of the new ductules induced by a DDC diet, and to a lesser degree those induced by BDL, were derived from hepatocytes. Although the reason for the discrepancy between these studies is unclear, our results are in accord with those of Yanger et al.¹⁶ Using combinations of tamoxifen-inducible Cre and *ROSA26R* systems, which label albumin-positive cells or CK19⁺ cells, Sekiya and Suzuki¹⁷ reported that almost all of the CK19⁺ ductular cells that appeared in the liver of the mice treated with TAA were of hepatocyte origin and not of bile-duct origin. Our present findings, however, indicate that cells participating in a centrilobular ductular reaction induced by TAA (or by CCl₄) are more complex and include at least transdifferentiated hepatocytes and bile ductules that have migrated from the portal area.

Although less extensively described than periportal ductular reaction, centrilobular ductular reaction is also involved in various human liver diseases. Severe congestion of the liver has been associated with this reaction, which is also known as reversed lobulation.³¹ Desmet⁶ suggested that ductular cells in the centrilobular area might originate from hepatocytes in response to the hypoxic conditions. Centrilobular hepatocyte injury and fibrosis associated with ductular reaction are also characteristics of alcoholic liver disease and nonalcoholic steatohepatitis.^{7,32} One of the most unexpected findings from the present study was that the liver showed extensive tissue remodeling in chronic centrilobular liver injury induced by repeated administration of CCl₄ or TAA. Our results show for the first time that periportal ductular cells and interlobular bile ducts can proliferate and migrate to the centrilobular area and establish the connection between the transdifferentiated hepatocytes. This tissue remodeling also involves intralobular migration of hepatic arteries, which are known to play important roles in the circulation of bile ducts or ductules. Provocatively, hepatic arterial deprivation has been reported to induce adaptive proliferation of bile duct/ductular cells.³³ It is of note that similar migration of hepatic arteries (centrizonal arteries) has been observed in patients with nonalcoholic steatohepatitis,

Figure 9 Schematic representation of two distinct origins for duct/ductular cells in the centrilobular ductular reaction. The present findings demonstrate the phenotypic plasticity of mature hepatocytes, as well as the extensive remodeling capacity of the existing bile duct system. Centrilobular hepatocytes transdifferentiate into bile duct/ductular cells in response to microenvironmental changes (eg, chronic inflammation and fibrosis) induced by chronic liver injury. Concomitantly, bile duct/ductular cells, which were originally located in the portal tract, proliferate and migrate toward the injured area, eventually establishing connections with transdifferentiated hepatocytes.

especially in the presence of advanced centrilobular fibrosis and ductular reaction.³⁴

The extensive tissue remodeling in the presence of chronic centrilobular liver injuries might be related to the changes in the regulatory mechanisms of extracellular matrices. Biliary remodeling during liver regeneration is associated with an increase in S100-A4, which induces the expression of MMPs.³⁵ In the present study, RT-qPCR analysis comparing control and CCl₄-induced or TAA-induced cirrhosis demonstrated significant increases in the mRNA of *Mmp2* and *Timp1*, as well as of *S100a4*. Furthermore, soluble factors, such as inflammatory cytokines released from the inflamed centrilobular area might be involved in the remodeling process.^{10,36}

In conclusion, our present results demonstrate that marked hepatic lobular remodeling occurs after chronic centrilobular injury, involving both ductular transdifferentiation of hepatocytes and proliferation and migration of bile duct/ductular cells (Figure 9). Because ductular transdifferentiation of hepatocytes has been suggested to be reversible, depending on the microenvironment,¹³ clear recognition of the presence of bile ductules of different cellular origins in the injured liver should be important for a more precise understanding of the pathology of chronic liver diseases and the development of new therapeutic interventions.

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Supplemental Data

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