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Yasuharu Nagahama,[1,2] Masayuki Sone,[2] Xi Chen,[1] Yoko Okada,[1] Masahiro Yamamoto,[1] Bing Xin,[1] Yasuhiro Matsuo,[1] Mikiko Komatsu,[1] Akira Suzuki,[3] Katsuhiko Enomoto,[4] and Yuji Nishikawa[1]

[1]Division of Tumor Pathology, Department of Pathology, Asahikawa Medical University, Asahikawa, Hokkaido 078-8543, Japan.

[2]Fujii Memorial Research Institute, Otsuka Pharmaceutical Co. Ltd., 1-11-1 Karasaki, Otsu, Shiga 520-0106, Japan.

[3]Division of Embryonic and Genetic Engineering, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan.

[4]Department of Pathology, Akita Red Cross Hospital, Akita, Japan.

Corresponding author: Yuji Nishikawa, Division of Tumor Pathology, Department of Pathology, Asahikawa Medical University, Higashi 2-1-1-1, Midorigaoka, Asahikawa, Hokkaido 078-8510, Japan.

Tel: +81-166-68-2370; Fax: +81-166-68-2379

E-mail: nishikwa@asahikawa-med.ac.jp

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Abbreviations:

BDL, bile duct ligation; β-gal, β-galactosidase; CK19, cytokeratin 19; DAPM, 4,4'-diaminodiphenylmethane; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; G6Pase, glucose 6-phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HNF-4α, hepatocyte nuclear factor-4α; MMP, matrix metalloproteinase; PEPCK, phosphoenolpyruvate carboxykinase; P-HH3, phospho-histone H3; TAA, thioacetamide; TAT, tyrosine aminotransferase; TIMP, tissue inhibitors of matrix proteinases; TNF-α, tumor necrosis factor-α

Abstract

Mature hepatocytes have been suggested to possess a capacity for bile ductular transdifferentiation. However, whether and how hepatocytes contribute to a ductular reaction in chronic liver diseases has not been elucidated. We first examined whether mouse hepatocytes could 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 (β-gal)-positive hepatocytes from Alb-Cre × ROSA26R mice were transplanted into the livers of wild-type mice. We further examined the relative contribution of intrinsic hepatocytes in a ductular reaction in a hepatocyte lineage-tracing model employing poly(I:C)-treated Mx1-Cre × ROSA26R mice. Within collagen gels, hepatocytes demonstrated branching morphogenesis associated with the emergence of bile duct-like phenotype. In the liver repopulation model, many β-gal-positive, hepatocyte-derived bile ductular structures were identified and they markedly increased following liver injury. In Mx1-Cre × ROSA26R mice, relatively minor but significant contributions of hepatocyte-derived bile ductules was observed in both periportal and centrilobular ductular reaction. Interestingly, as the centrilobular ductular reaction progressed, the portal duct/ductules migrated toward the injured area and joined with hepatocyte-derived ductules, leaving the portal tract without biliary structures. Our results indicate that hepatocytes and bile duct/ductules are important sources of ductular reaction and that the intrahepatic biliary system undergoes remarkable remodeling in response to chronic liver injury.

Introduction

Chronic liver diseases are often complicated by a progressive increase in bile ductular structures, which is called a ductular reaction[1,2] A ductular reaction is closely associated with liver fibrosis and is clearly an important tissue alteration to examine as a therapeutic target.[3] However, the cellular origins of a 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 bile ductules (the canals of Hering), proliferate into ductular shapes and eventually contribute to regeneration when the proliferation of hepatocytes is compromised.[4] However, the periportal liver "stem cell niche" has been gradually recognized to be more complex and involve other cell types, such as bile ductular cells and periportal hepatocytes.[5] Although a ductular reaction is typically found in the periportal area, it can also be observed in the centrilobular area where no biliary cells or stem/progenitor cells are present.[6] This observation raises 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 could transdifferentiate into bile ductular cells when cultured within a type-I collagen gel matrix,[7-9] and similar phenotypic plasticity of hepatocytes has been described by other investigators.[10,11] Furthermore, we have recently demonstrated that rat hepatocytes are able to recover their original phenotype following bile ductular transdifferentiation by modulating culture conditions, suggesting that hepatocytic and bile ductular

phenotypes are mutually reversible.[12]

Whether mature hepatocytes can transdifferentiate into bile 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 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet.[13] A hepatocyte lineage-tracing study using an adeno-associated virus vector in mice demonstrated that hepatocytes did not change their phenotype during various liver injuries, including those induced by a DDC diet and bile duct ligation (BDL), or during liver homeostasis.[14] However, more recently, a similar lineage-tracing study demonstrated that hepatocytes could significantly participate in the generation of new ductules during liver injuries induced by a DDC diet and to a lesser degree by BDL.[15] Furthermore, by permanently labeling hepatocytes and bile-duct cells with cell type-specific reporters, the centrilobular ductular reaction that was inflicted by thioacetamide (TAA) was reported to be derived entirely from hepatocytes and not from bile duct/ductules.[16] Given the inconsistency in the conclusions regarding the phenotypic plasticity of hepatocytes, it is necessary to scrutinize the cellular origins of various types of ductular reactions by rigorous pathological examinations in appropriate hepatocyte lineage-tracing systems.

In the present study, we first examined and confirmed that the phenotype of mature mouse hepatocytes was also malleable when cultured within a three-dimensional collagen gel matrix. Then, we attempted to demonstrate that hepatocytes were capable of changing their phenotype to bile

duct/ductular cells in the injured liver by tracing intrahepatically transplanted β-galactosidase (β-gal)-positive 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 contribute to periportal and centrilobular ductular reactions, while the existing bile duct/ductular system exhibits a profound remodeling capacity.

Materials and Methods

Animals.

C57BL/6J (CLEA Japan, Inc., Tokyo, Japan), B6.Cg-Tg (Alb-cre) 21Mgn/J (Alb-Cre; The Jackson Laboratory, Bar Harbor, ME), B6.Cg-Tg (Mx1-cre)1Cgn/J (Mx1-Cre; The Jackson Laboratory),[17] and ROSA26R (provided by Dr. P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA)[18] mice 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 Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences (8th Ed., 2011).

Three-dimensional culture of hepatocytes.

Centrilobular hepatocytes were isolated from the livers of adult C57BL/6J mice (8 to 20 weeks old) by a digitonin-collagenase perfusion technique[19] (Supplemental Fig. 1). We performed a three-dimensional collagen gel culture of mouse hepatocytes as previously reported[8, 20] (Supplemental Fig. 2). After being embedded within the collagen gels, spheroidal aggregates were cultured in Williams' Medium E supplemented with 10 mM nicotinamide, 10% FBS, 10 ng/mL EGF, 10^{-7} M insulin, 10^{-7} M dexamethasone, and 10 ng/mL tumor necrosis factor-α (TNF-α) (PeproTech Inc., Rocky Hill, NJ).

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

In vivo *lineage tracing of hepatocytes.*

For lineage tracing of adult hepatocytes, we applied following two models.

(i) Liver repopulation model

Adult C57BL/6J mice were treated repeatedly with retrorsine (60 mg/kg, i.p., once per week for 5 weeks) to inhibit the proliferative capacity of hepatocytes. Then, the mice were subjected to a 70% partial hepatectomy, and after 24 hours, β-gal-positive hepatocytes, which were freshly isolated from adult Alb-Cre × ROSA26R by the digitonin-collagenase perfusion method, were transplanted

into the spleens (0.5-1 × 10^6 cells/animal).[21] The mice were subjected to chronic liver injury by feeding the animals with a DDC diet (0.1%) for 4 weeks or administering CCl_4 (1 mL/kg, s.c.; three times per week) for 8 weeks.

(ii) Hepatocyte-specific labeling in Mx1-Cre × ROSA26R mice

Male Mx1-Cre × ROSA26R mice (8 to 12 weeks old) were injected intraperitoneally with 250 μg of poly(I:C) (InvivoGen, San Diego, CA) per body three times and at two-day intervals. This dose of poly(I:C) injection caused no apparent organ damage, as assessed by histological studies (data not shown). Three days after the last poly(I:C) administration, the mice were injected with CCl_4 (1 mL/kg, three times per week, s.c.) for 8, 11, and 20 weeks or with TAA (Sigma; 100 mg/kg, three times per week, i.p.) for 21 weeks to induce a ductular reaction in the centrilobular area and were fed a DDC diet (0.1%) for 4 weeks. In addition, the mice were subjected to BDL for 14 days and administration of DAPM (Kanto Chemical Co. Inc., Tokyo, Japan; 40 mg/kg, i.p., at 5-day intervals, i.p.) for 4 weeks.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis.

Total RNA from cells and tissues were extracted and subjected to qRT-PCR analysis for various hepatocytic, bile duct/ductular, and hepatoblastic markers; the reactions were performed in duplicate, and the mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The primer sequences and expected fragment sizes are listed in

Supplemental Table 1. For the analyses of mRNA expression for S100 calcium-binding protein A4 (S100A4), matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitors of matrix proteinases (TIMP)-1, TIMP-3, and TNF-α, we applied TaqMan® Gene Expression Assays.

Western blot analysis.

Protein samples (25 μg of protein per lane) were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The primary antibodies that were used included anti-albumin (Nordic Immunological Laboratories, Tilburg, Netherlands), anti-HNF-4α (K9218, Perseus Proteomics, Tokyo, Japan), anti-CK19 (kind gift of Dr. A. Miyajima, University of Tokyo, Tokyo, Japan), anti- SRY (sex determining region Y)-box 9 (Sox9) (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 PBS overnight at 4 °C. Frozen and paraffin sections were then prepared. In some experiments, to identify the portal veins and to visualize the biliary system in tissue sections of cirrhotic livers, we injected India ink (approximately 100 μL) directly into the portal trunk and common bile duct, respectively, and fractions of the liver tissues were cut and immersion-fixed with 4% paraformaldehyde.

For β-gal histochemistry, 4-μm-thick frozen sections were rinsed in PBS and reacted with

X-gal solution (1 mg/mL X-gal [Sigma], 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide) at 37 °C overnight.

Immunocytochemical and immunohistochemical analyses were performed either by peroxidase or alkaline phosphatase method following an antigen retrieval procedure using the Target Retrieval Solution, pH 6.0 (Dako, Carpinteria, CA). Various chromogens were used: 3,3'-diaminobenzidine tetrahydrochloride (brown; Vector Laboratories, Burlingame, CA), 3-amino-9-ethylcarbazole (reddish brown; DAKO), Histogreen (green; Linaris Biologische Produkte, GmbH, Dossenheim, Germany), and New Fuchsin (red; Nichirei Bioscience, Tokyo, Japan). The following antibodies were used: anti-HNF-4α (P1) (Perseus Proteomics, Tokyo), anti-Sox9 (Millipore), anti-CK19 (gift of Dr. Miyajima), anti-EpCAM (Novus Biologicals), anti-desmin (Abcam), anti-F4/80 (AbD Serotec), anti-LYVE-1 (Abcam), and anti-phospho-histone H3 (Cell Signaling Technology).

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

Statistical analyses.

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

Results

Branching morphogenesis with the emergence of the expression of bile duct/ductular differentiation markers in cultured mouse hepatocytes.

We first examined whether adult mouse hepatocytes could transdifferentiate into bile ductular cells, as was previously shown in rat experiments.[7,8] 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 Fig. 1). When spheroidal aggregates of hepatocytes were embedded and cultured within collagen gels (Fig. 1A; Supplemental Fig. 2), 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 (Fig. 1A). These morphological changes were associated with a dramatic decrease in the mRNA expression of albumin, transthyretin, hepatocyte nuclear factor-4α (HNF-4α) (Fig. 1B), tyrosine aminotransferase (TAT), glucose 6-phosphatase (G6Pase), and phosphoenolpyruvate carboxykinase (PEPCK) (Supplemental Fig. 3), especially in the presence of TNF-α, which suggests the loss of hepatocytic differentiation. In contrast, the expression of mRNAs relevant to bile duct differentiation, such as cytokeratin 19 (CK19), Sox9 (Fig. 1B), and osteopontin (Supplemental Fig. 3), emerged. The mRNA expression of DLK, a marker for hepatoblasts, was not detectable during the entire culture period (Fig. 1B), suggesting that the phenotypic change might be transdifferentiation rather than dedifferentiation.

Western blot analysis showed that albumin expression was relatively well preserved but was decreased when cells were treated with TNF-α, while HNF-4α expression rapidly declined in culture (Fig. 2A). The protein expression of CK19 and Sox9 appeared in cultured hepatocytes, and the expression of the latter was induced earlier and was already apparent in spheroidal cultures (Fig. 2A). Compatible with the above data, immunocytochemical analysis demonstrated a marked reduction of HNF-4α and the emergence of CK19 and Sox9 in cultured hepatocytes (Fig. 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 (Fig. 3A). Transplanted β-gal-positive Alb-Cre × ROSA26R hepatocytes formed colonies of various sizes in the partially hepatectomized livers of retrorsine-treated mice, in which the proliferation of host hepatocytes was suppressed but that of transplanted hepatocytes was stimulated (Fig. 3B). In the background of mild to moderate liver injury, scattered areas of ductular reaction were observed where a small population of increased ductules were β-gal-positive (Fig. 3B). When similarly treated mice were subjected to further liver injury by a DDC diet or CCl_4, an extensive ductular reaction was induced, and a number of β-gal-positive duct/ductules were found, especially proximal to the colonies of transplanted β-gal-positive hepatocytes (Fig. 3C, D). Such β-gal-positive duct/ductules were also positive for

Sox9 (Fig. 3C, D).

Relative contribution of hepatocyte-derived bile ductules in ductular reactions assessed in a hepatocyte-specific gene targeting system using Mx1Cre × ROSA26R mice.

The above *in vitro* and *in vivo* experiments clearly demonstrated that mature hepatocytes possessed the capacity to differentiate into bile duct/ductular cells in response to microenvironmental changes. However, the relative contribution of the proliferation of bile duct/ductules and the transdifferentiation of hepatocytes in the ductular reaction is not known. To examine this, it was necessary to induce injury in intact livers where the resident hepatocytes were specifically and permanently labeled. For this purpose, we applied a poly (I:C)-inducible gene targeting system (Mx1-Cre × ROSA26R).[17, 22, 23] We confirmed the efficient and specific labeling of hepatocytes, which virtually spared the bile duct/ductular cells (Figs 4 and 5A) and other non-parenchymal cells (Figs 4 and 5B), upon the completion of repeated administration of poly (I:C). However, after a long period following labeling (15 months), a small but significant increase in β-gal-positive duct/ductular cells occurred (Fig. 5A), which consisted of 4.2% of the CK19-positive ductular cells (Fig. 5B, C) and suggested an involvement of periportal hepatocytes in the normal homeostasis of duct/ductular cells.

We then examined the cellular origins of a ductular reaction in the periportal area induced by a DDC diet and 4,4'-diaminodiphenylmethane (DAPM). These toxic agents significantly

increased the number of ductular structures composed of CK19-positive cells, which were slightly larger than normal bile ductular cells (Fig. 5A). The reactive bile ductules contained a small number of β-gal-positive cells (Fig. 5A, B), which were estimated to be 1.9% and 4.7% of the CK19-positive ductular cells in the livers of DDC diet-fed and DAPM-treated mice, respectively (Fig. 5C). Increased ductular structures that were induced by BDL also contained a small number of β-gal-positive cells (Supplemental Fig. 4). These data demonstrated that mature hepatocytes also contributed, although not extensively, to the periportal ductular reaction due to various injuries.

Furthermore, we evaluated the cellular origins of ductular reactions in the centrilobular area, where no bile ductular cells exist. Chronic administration of CCl_4 or thioacetamide (TAA), which selectively damages centrilobular hepatocytes, caused inflammation, fibrosis, and a ductular reaction in the centrilobular area (Supplemental Fig. 5). While no hepatic stellate cells, sinusoidal endothelial cells, or Kupffer cells were β-gal-positive in the injured liver (Fig. 4), the CK19-positive ductules in the centrilobular area (zone 3) were composed of β-gal-positive cells and -negative cells (Fig. 6A, B). The β-gal-positive ductules of hepatocyte origin were mainly found in inflamed and fibrosed areas in the vicinity of the central veins, and β-gal-positive and -negative ductules often contacted each other and shared lumens (Fig. 6A). The number of ductules containing β-gal-positive cells per centrilobular area was 4.1 and 1.3 in CCl_4 (20 weeks) and TAA injury, respectively (Fig. 6B). In the early period of CCl_4 injury (8 weeks), 20.6% of ductular cells were β-gal-positive, while this proportion decreased thereafter as the ductular reaction progressed (Fig. 6C). Although TAA induced

an extensive ductular reaction, the proportion of β-gal-positive cells was small (4.3%) (Fig. 6C).

Extensive remodeling of the intrahepatic biliary system in chronic liver injury.

Notably, as the centrilobular ductular reaction progressed, the bile duct/ductules in the portal tract appeared to migrate toward the injured centrilobular area, leaving the portal tract without duct/ductular structures (Fig. 6A, 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 (Fig. 6E). We also found that the hepatic arteries, which were 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 (Fig. 6F). After India ink was injected through the common bile duct, charcoal pigments were found in aberrantly located bile duct/ductules, centrilobular bile ductules, and some bile canaliculi of adjacent hepatocytes, indicating that the biliary system connection was maintained (Fig. 7A).

To elucidate the role of cell proliferation in the increase of ductular structures in centrilobular liver injury, we performed double immunostaining for phospho-histone H3 (P-HH3) and CK19 on β-gal-stained sections and counted proliferating cells in β-gal-negative and -positive duct/ductules. Although P-HH3-positive cells were occasionally found in β-gal-negative duct/ductules in CCl_4 and TAA injuries, small β-gal-positive ductules barely demonstrated P-HH3 positivity (Fig. 7B). Quantification revealed that P-HH3-positive cells composed 0.67% and 1.66%

of the β-gal-negative ductular cells in CCl_4 and TAA injuries, respectively (Fig. 7C). In contrast, β-gal-positive ductules contained only 0.01% of P-HH3-positive cells during CCl_4 injury, and no positive cells were detected during TAA injury (Fig. 7C).

Our results suggested that extensive tissue remodeling processes occurred during migration of ductular cells. We previously identified various proteins involved in tissue remodeling whose mRNA expression was increased in CCl_4-induced cirrhosis by cDNA microarray analysis (Chen and Nishikawa, unpublished observations). We therefore performed qRT-PCR analysis for mRNA expression of such proteins comparing control and CCl_4- or TAA-induced cirrhosis. There were significant increases in S100A4, MMP2, and TIMP-1 mRNA in the injured livers (Fig. 7D).

Discussion

While the phenotype of hepatocytes is considered to be fixed once they are terminally differentiated,[24] recent works have demonstrated phenotypic plasticity of hepatocytes *in vitro*. As an extension of our previous work showing transdifferentiation of rat hepatocytes into bile duct/ductular cells in a collagen gel matrix,[7-9, 12] we demonstrated in this study that mouse hepatocytes also transformed into ductular cells *in vitro*, especially when TNF-α was present in the culture medium. Ductular differentiation of mouse hepatocytes was not associated with the expression of DLK mRNA, which was present in fetal livers and some hepatocellular carcinomas. Thus, the ductular

transformation of mouse hepatocytes within collagen gels could be interpreted to be a result of the process of transdifferentiation rather than dedifferentiation.

The idea that hepatocytes could transform into ductular cells *in vivo* has been a focus of intense debate.[24-26] To provide more evidence of transdifferentiation, we isolated centrilobular hepatocytes that were permanently labeled by β-gal from Alb-Cre × ROSA26R mice and transplanted into the livers of wild type mice. We previously demonstrated that centrilobular and periportal hepatocytes possessed nearly identical capacities for ductular differentiation *in vitro* in rats[8]. Our results demonstrated that hepatocytes could transform into bile duct/ductular cells *in vivo*. While the transformation was readily observed at the periphery of the repopulated hepatocytic colonies, it was markedly enhanced by chronic CCl_4 administration or DDC diet. The robust ductular reaction was also closely associated with inflammation inflicted 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 cytokines, including TNF-α. Interestingly, some hepatocytes near the ductular reaction expressed Sox9 but not CK19, which could represent the intermediate hepatobiliary phenotype.[27]

Hepatocytes and bile duct/ductules could participate in the ductular reaction. The liver repopulation model is not suitable for analyzing the relative contribution of intrinsic hepatocytes and bile duct/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 × ROSA26R mice, in which hepatocytes could be labeled efficiently and specifically by poly(I:C) administration without affecting liver physiology. We found that some of the bile ductules contained cells of hepatocyte origin (β-gal-positive) during periportal and centrilobular liver injuries, although they constituted relatively small fractions of the bile duct/ductules in the injured areas. In an efficient hepatocyte-lineage tracing system in the mouse in which an adeno-associated virus vector was applied, Malato et al.[14] reported that the transformation did not occur in injuries inflicted by acute or chronic CCl_4 administrations, BDL, or DDC diet. In contrast, more recently, using a similar adeno-associated virus-mediated hepatocyte-lineage tracing system, Yanger et al.[15] showed that some of the new ductules that were induced by DDC, and to a lesser degree by BDL, were derived from hepatocytes. While the reason for the discrepancy of these works is unclear, our results supported the latter. Using combinations of tamoxifen-inducible Cre and ROSA26R systems, which label albumin-positive cells or CK19-positive cells, Sekiya and Suzuki[16] reported that virtually all of the CK19-positive ductular cells that appeared in the centrilobular area of mice treated with TAA were of hepatocyte origin and not of bile-duct origin. However, our study has demonstrated that cells participating in a centrilobular ductular reaction induced by TAA, as well as CCl_4, are more complex and include at least transdifferentiated hepatocytes and bile ductules that have migrated from the portal area.

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.[28] Our study strongly suggests that transdifferentiation could participate in homeostasis of the biliary system in intact livers and in the 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.

One of the most unexpected findings from the present study was that the liver showed extensive tissue remodeling in chronic centrilobular liver injury inflicted by repeated administration of CCl_4 or TAA. In humans, severe congestion of the liver has been associated with the centrilobular ductular reaction, which is also known as "reversed lobulation".[29] Desmet suggested that ductular cells in the centrilobular area might originate from hepatocytes in response to the hypoxic condition.[6] Our results show that ductular transdifferentiation of hepatocytes could occur in any area of liver injury that causes inflammation and fibrosis. Furthermore, 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 duct/ductules. Provocatively, hepatic arterial deprivation has been reported to

induce adaptive bile duct/ductular proliferation.[30] The extensive tissue remodeling during chronic centrilobular liver injuries might be related to the changes in the regulatory mechanisms of extracellular matrices. Biliary remodeling during liver regeneration has been shown to be associated with an increase in S100A4, which induces the expression of MMPs.[31] In fact, qRT-PCR analysis comparing control and CCl_4- or TAA-induced cirrhosis demonstrated significant increases in the mRNA of MMP-2 and TIMP-1, 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.[9,32]

In conclusion, our results demonstrated that marked hepatic lobular remodeling occurred following chronic centrilobular injury, involving both ductular transdifferentiation of hepatocytes and proliferation and migration of bile duct/ductular cells (Fig. 8). Because ductular transdifferentiation of hepatocytes has been suggested to be reversible according to the microenvironment,[12] 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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Figure legends

Fig. 1: Branching morphogenesis of mouse hepatocytes with the appearance of the bile ductular phenotype in collagen gel culture. (A) Phase-contrast microscopy. Hepatocytic spheroids immediately after being embedded within a collagen gel matrix (0 d) and cultured for 3, 5, and 10 days in the absence or presence of 10 ng/ml TNF-α. Asterisks denote luminal structures. (B) qRT-PCR analysis for mRNA expression of various genes in freshly isolated hepatocytes (Heps), spheroids, and those cultured within collagen gels (for 5, 10, and 15 days). Total RNA extracted from normal portal tissues (PT) (n = 4), dimethylnitrosamine-induced hepatocellular carcinoma (HCC) (n = 3), and E13.5 fetal liver (n = 3) was used as positive controls for bile duct-specific genes (CK19 and Sox9) and DLK. Each value is the mean of three independent experiments ± SEM (*P<0.05, **P<0.01, ***P<0.005; comparison between control and TNF-α-treated at each time point; unpaired two-tailed t-test).

Fig. 2: Loss of hepatocyte-specific protein expression and the emergence of bile duct-specific protein expression in mouse hepatocytes in collagen gel culture. (A) Western blotting analysis of proteins specific for hepatocytes (albumin and HNF-4α) and bile duct/ductules (CK19 and Sox9) in freshly isolated and cultured hepatocytes. (B) Hematoxylin and eosin (HE) staining and immunocytochemistry for HNF-4α, CK19, and Sox9 of freshly isolated and cultured hepatocytes. Bar, 20 μm.

Fig. 3: Bile ductular transdifferentiation of Alb-Cre × ROSA26R mouse hepatocytes that were transplanted and repopulated in wild-type mouse livers. (A) Schematic diagram of the lineage tracing experiments using a liver repopulation model. (B-D) Combined X-gal histochemistry and immunohistochemistry for CK19 or Sox9 in the livers 8 weeks after hepatocyte transplantation (Tx) (B) and subsequent subjection to liver injuries (C: DDC diet for 4 weeks; D: CCl4 for 8 weeks). Bar, 40 μm.

Fig. 4: Hepatocyte-specific β-gal labeling in Mx1-Cre × ROSA26R mice following poly(I:C) injections. Control (intact) livers three days after the last poly (I:C) injection and livers then treated with CCl4 for 11 weeks. Combined X-gal histochemistry and immunohistochemistry for various liver cell markers. HNF-4α (a marker for hepatocytes; brown), Sox9 and EpCAM (markers for bile duct/ductular cells; red), desmin (a marker for stellate cells; red), LYVE-1 (a marker for sinusoidal endothelial cells; red), and F4/80 (a marker for Kupffer cells; reddish brown). Bar, 20 μm.

Fig. 5: Ductular transdifferentiation of hepatocytes in liver homeostasis and the periportal ductular reaction in a hepatocyte-lineage tracing system using Mx1-Cre × ROSA26R mice. (A) Combined X-gal histochemistry and immunohistochemistry for CK19 in the livers three days (control) and 15 months (aging) after the final poly(I:C) injection and following liver injuries (DDC and DAPM).

Arrowheads denote β-gal-positive bile duct/ductular cells, and N in the DAPM-treated liver indicates the necrotic area. Bar, 40 μm. (B) The number of β-gal-negative and -positive bile duct/ductular cells in the periportal area. (C) The proportion of β-gal-positive cells in CK19-positive bile duct/ductular cells. Each value in B and C is the mean ± SEM of three (control, aging, and DDC) or four animals (DAPM) (***P<0.005; compared with control; one-way factorial ANOVA).

Fig. 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 × ROSA26R mice. (A) Combined X-gal histochemistry and immunohistochemistry for CK19 in livers treated with CCl4 (for 8 and 20 weeks) and TAA (21 weeks). The left and right panels show the centrilobular area (zone 3) and periportal area (zone 1), respectively. PV: portal veins. CV: central veins. Arrowheads denote β-gal-positive bile duct/ductular cells. Bar, 40 μm. (B) The number of β-gal-negative and -positive bile duct/ductular cells in the centrilobular area. (C) The proportion of β-gal-positive cells in CK19-positive bile duct/ductular cells in the centrilobular area. (D) The number of duct/ductular structures within a portal tract. Each value in B-D is the mean ± SEM of three (CCl4 for 8 and 11 weeks and TAA) or four animals (CCl4 for 20 weeks) (***P<0.005; compared with control; one-way factorial ANOVA). (E) Visualization of portal vein branches by retrograde injection of India ink through the portal vein in CCl4-treated livers (20 weeks). HE staining. The blue asterisk denotes the same portal tract. A green arrowhead in C denotes

an interlobular bile duct apparently migrated from the portal tract. (F) Immunohistochemistry for α-smooth muscle actin in intact livers (left) and TAA-treated (21 weeks) livers (right). Green and red arrowheads indicate interlobular bile ducts and hepatic arteries, respectively. PV: portal veins. CV: central veins. Bar, 40 μm.

Fig. 7: Altered bile duct system (reversed lobulation) in the centrilobular ductular reaction. (A) Visualization of the bile duct/ductules by retrograde injection of India ink through the common bile duct in intact (control) and CCl4-treated (for 34 weeks) livers of C57BL/6J mice. Immunohistochemistry for CK19 (green). Yellow arrowheads indicate CK19-positive bile duct/ductules containing charcoal pigments within the lumens; white arrowheads show hepatocytes containing charcoal pigments within the bile canaliculi. (B) Triple staining of X-gal, CK19 (red), and P-HH3 (brown) in the livers of CCl4 (for 20 weeks) (left panels) or TAA (right panels). White arrowheads indicate β-gal-positive bile duct/ductules, and yellow arrows indicate P-HH3-positive bile duct/ductules. PV: portal veins. CV: central veins. Bar, 40 μm. (C) The percentage of proliferating (P-HH3-positive) cells in β-gal-negative and -positive bile duct/ductules. Each value is the mean ± SEM of three (TAA) or four animals (CCl4) (***$P<0.005$; compared with control; one-way factorial ANOVA). (D) qRT-PCR analysis for mRNA expression of S100A4, MMP2, MMP9, TIMP-1, TIMP-3, and TNF-α in the livers of control (n = 4), CCl4-treated (24 weeks; n = 4), and TAA-treated (24 weeks; n = 5) animals. Each value is the mean ± SEM (*$P<0.05$, ***$P<0.005$;

comparison between control and TNF-α-treated at each time point; unpaired two-tailed t-test).

Fig. 8: Schematic representation of duct/ductular cells of two distinct origins in the centrilobular ductular reaction. Our study clearly demonstrates 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, such as chronic inflammation and fibrosis, that are 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.

Figure 1 (Nagahama et al.)

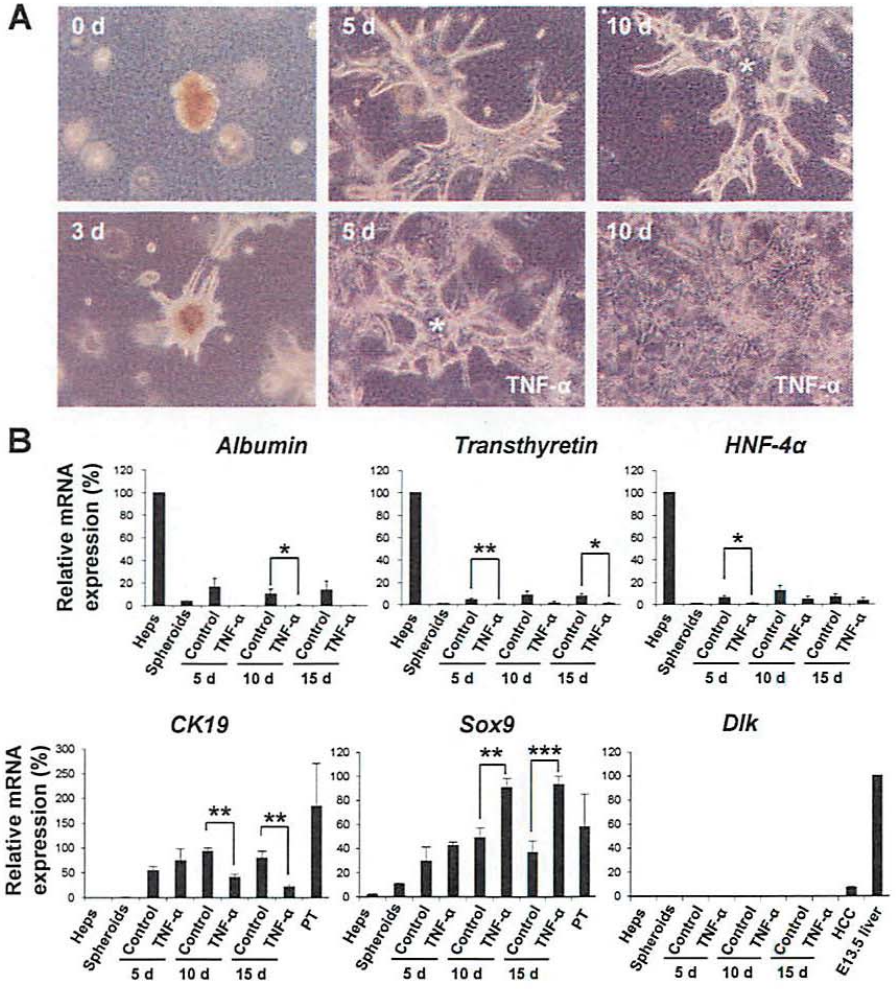


Figure 2 (Nagahama et al.)

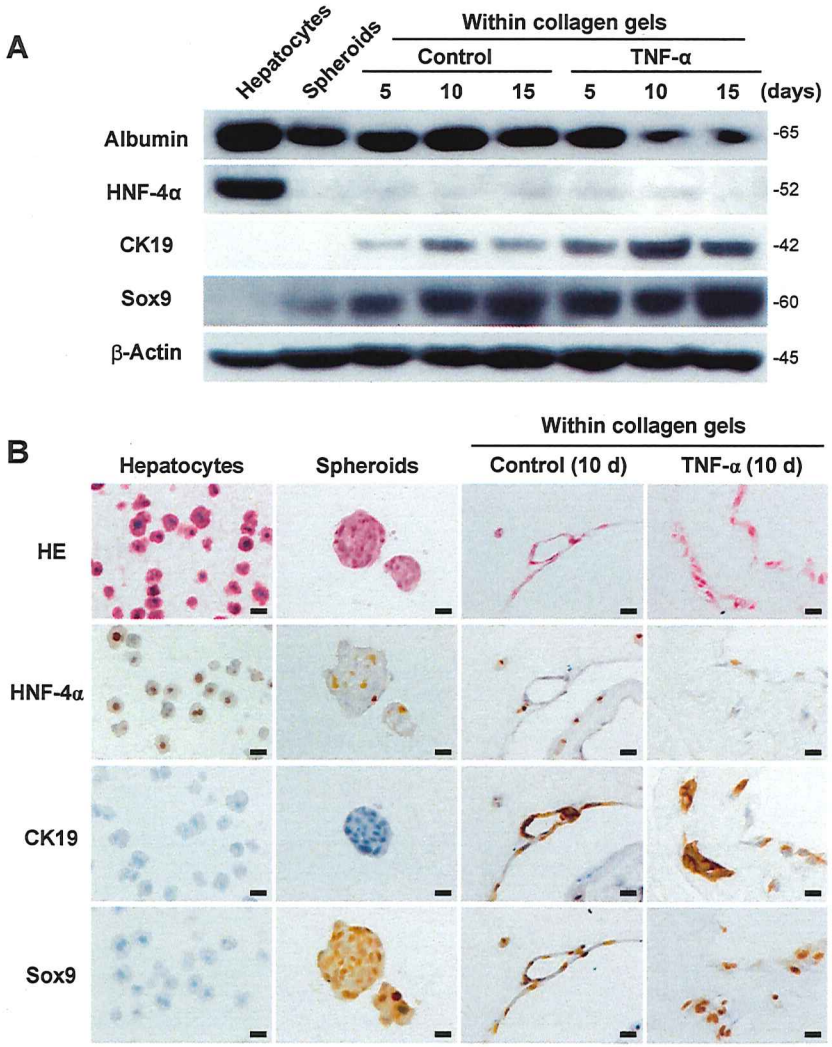
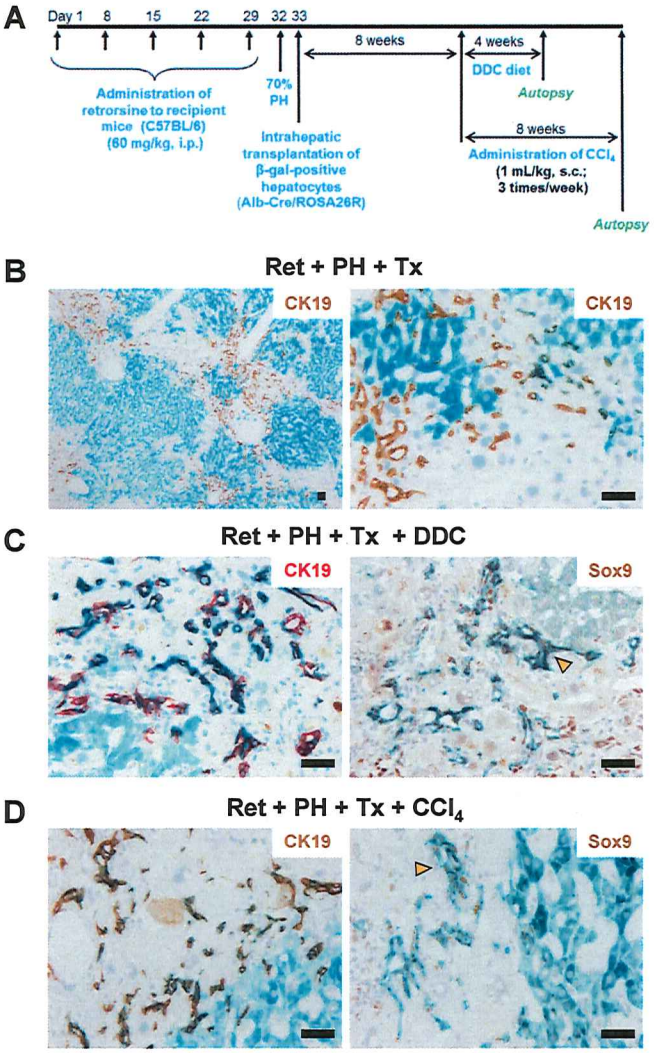


Figure 3 (Nagahama et al.)



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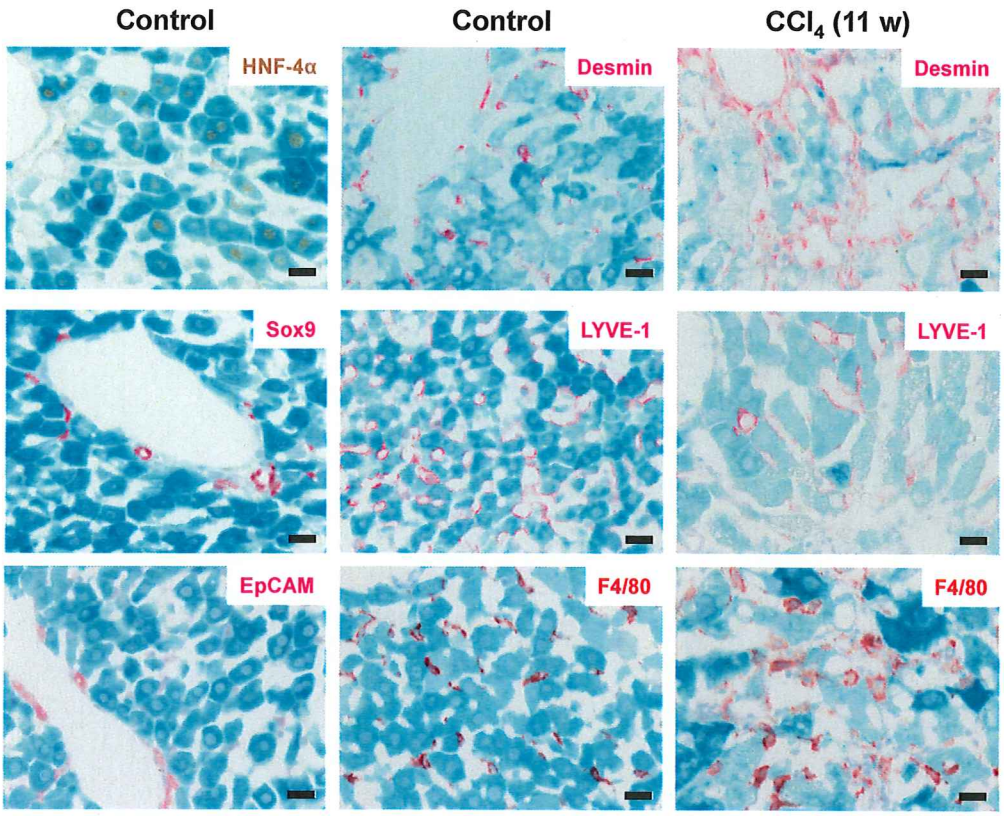

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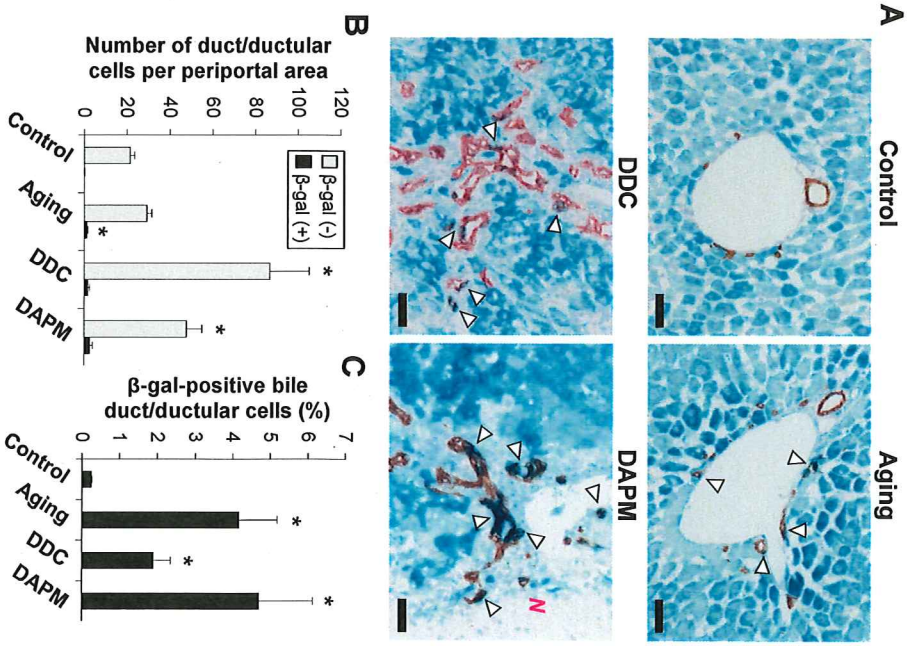

Figure 6 (Nagahama et al.)

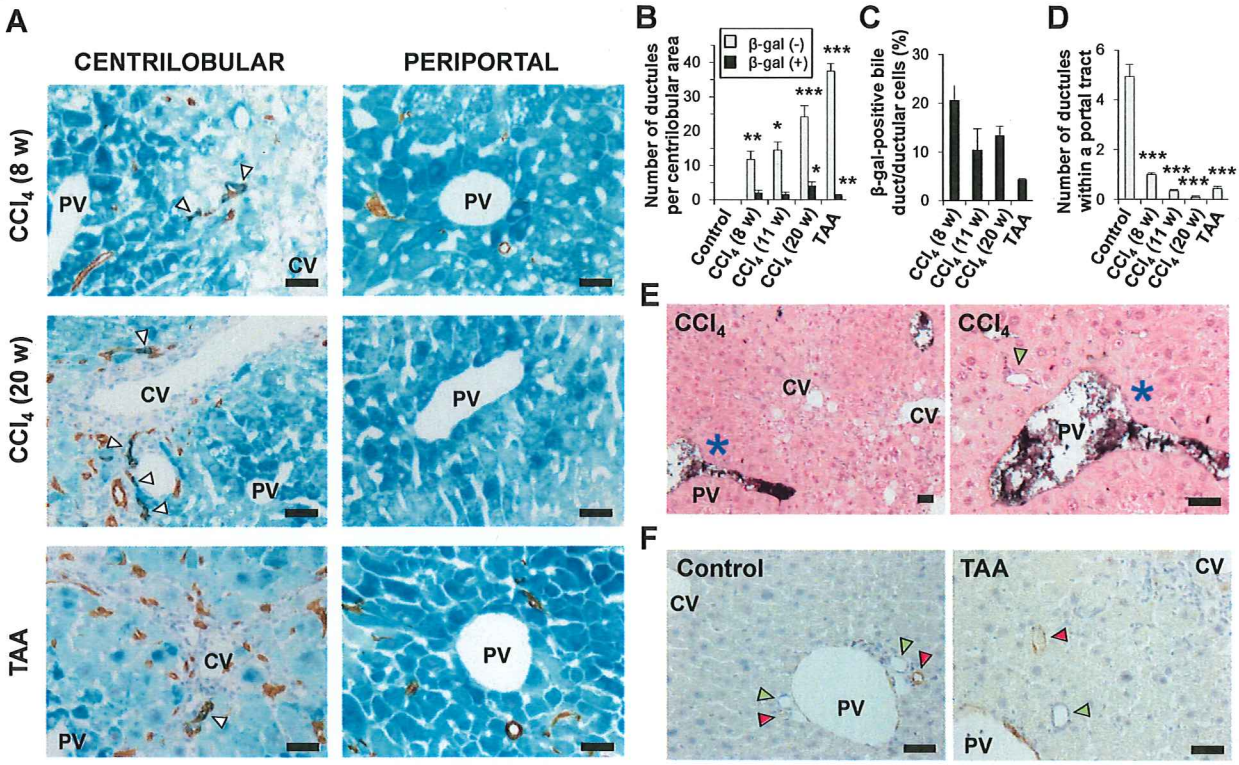

Figure 7 (Nagahama et al.)

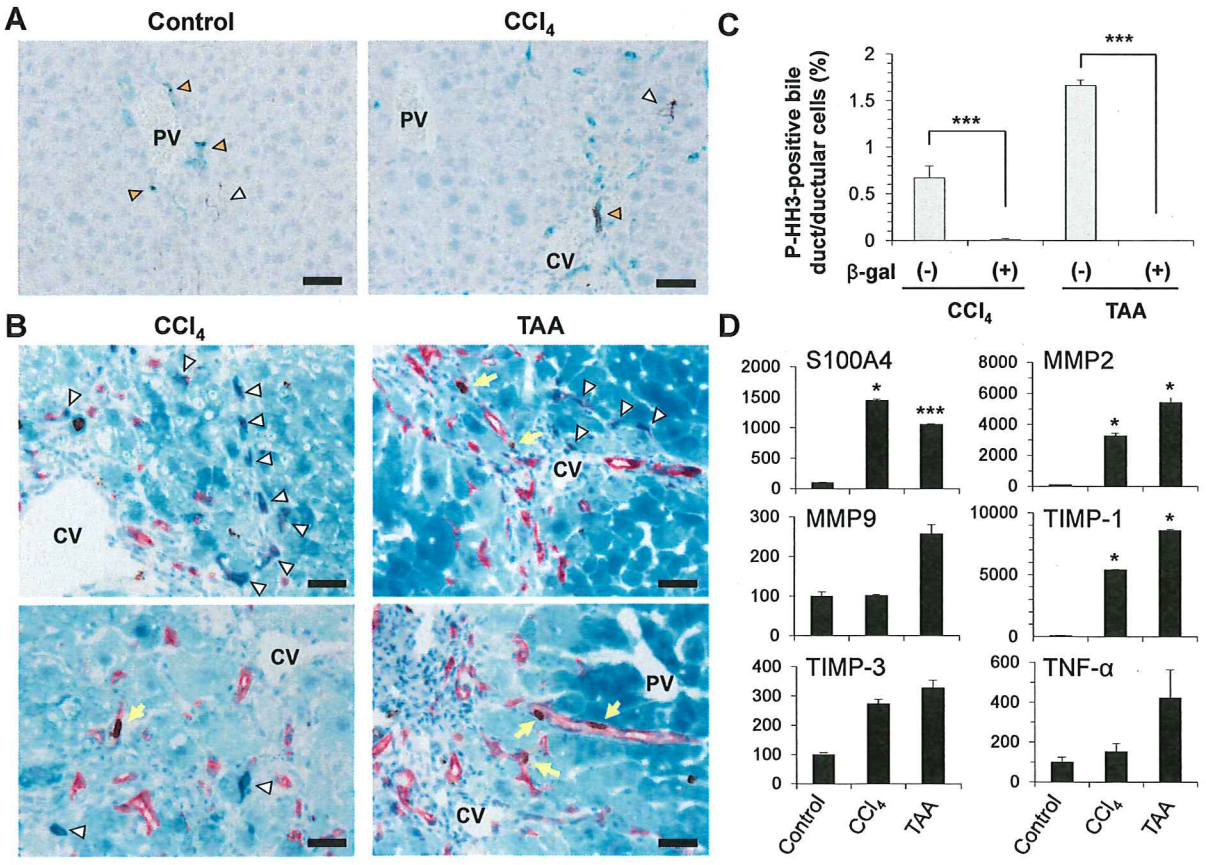

Figure 8 (Nagahama et al.)