GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Induction of Steatohepatitis and Liver Tumorigenesis by Enforced *Snail* Expression in Hepatocytes

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Snail is a transcription factor that regulates many cellular events involved in development, homeostasis, and disease. In hepatocellular carcinoma (HCC), *Snail* induces epithelial-to-mesenchymal transition that confers invasive properties on tumor cells during HCC progression and malignancy. *Snail* activation observed in HCC mouse models suggests its involvement not only in progression, but also onset of HCC. However, it remains unclear whether *Snail* directly contributes to HCC initiation or whether it supports HCC initiation promoted by other oncogenes. In this study, we generated mouse models for liver-specific and hepatocyte-specific overexpression of *Snail* to show the independent roles of *Snail* in liver homeostasis and disease. Enforced *Snail* expression resulted in liver and hepatocyte enlargement, inflammatory cell infiltration in the liver, lipid accumulation in hepatocytes, substantial increases in serum alanine aminotransferase and bile acids, yellow discoloration of tissues caused by bilirubin accumulation, and liver tumorigenesis. *Snail* overexpression suppressed mRNA expression of the tight junction components claudins and occludin and that of proteins associated with bile acid metabolism, leading to disruption of the biliary canaliculus formed among hepatocytes and excretion of abnormal amounts of unusual bile acids from hepatocytes. In conclusion, enforced *Snail* expression in hepatocytes is sufficient for induction of steatohepatitis and liver tumorigenesis through disruption of the biliary canaliculus and bile acid homeostasis in the liver. (*Am J Pathol* 2020, 190: 1271–1283; <https://doi.org/10.1016/j.ajpath.2020.02.005>)

Snail (also known as *Snai1* and *Sna*) is a zinc-finger transcription factor involved in many aspects of cellular processes, including cell motility, differentiation, proliferation, and survival, as well as in embryonic development, tissue homeostasis, and disease.¹ For example, *Snail* expression normally is repressed during kidney development because *Snail* disrupts epithelial homeostasis in the kidney by repressing Cadherin-16 expression,² and aberrant activation of *Snail* in the adult mouse kidney induces epithelial-to-mesenchymal transition and kidney fibrosis.^{2,3} During skin development, *Snail* is expressed specifically in the hair buds, and sustained expression of *Snail* in the adult mouse skin results in epidermal hyperproliferation and differentiation defects.⁴ Intestinal epithelial cells that compose the adult mouse intestines also express *Snail*, and abnormal expression levels of *Snail* in the intestinal epithelium lead to disruption of intestinal homeostasis.⁵ Furthermore,

pathologic activation of *Snail* in various types of epithelial tumors can direct tumor progression by inducing epithelial-to-mesenchymal transition and loss of cell adhesion, leading to the acquisition of invasive properties.⁶

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In the liver, Snail is expressed in quiescent mature hepatocytes,^{7,8} and glycogen synthase kinase-3 β —dependent Snail degradation acts as a fundamental cue for initiation of hepatocyte proliferation during liver regeneration after transient acute liver failure.⁸ In contrast, hepatocytes up-regulate Snail expression in response to chronic hepatic damage and contribute to the progression of liver fibrosis.⁷ Increased Snail activity also is related to hepatocellular carcinoma (HCC) progression and malignancy through down-regulation of E-cadherin (E-cad) and induction of epithelial-to-mesenchymal transition and invasive properties in tumor cells.^{9,10} *Snail* gene expression is regulated by various signaling pathways, including Wnt, epidermal growth factor, transforming growth factor- β , and Notch.¹¹ In an HRAS^{G12V}-driven HCC mouse model, transforming growth factor- β —induced Snail activation under suppression of p53 or activation of transcriptional coactivator with PDZ domain—binding motif promoted liver tumorigenesis,¹² suggesting that Snail is involved not only in progression, but also initiation, of HCC. However, it remains unknown whether Snail acts as a central player for HCC induction or as a promoter of HCC initiation induced by other oncogenes. Moreover, the underlying mechanism for HCC development in response to Snail activation remains unclear.

To address these issues, Snail was activated in the liver without any oncogenic events and the independent roles of Snail in liver homeostasis and disease were studied. Our data show that enforced *Snail* expression in hepatocytes is sufficient for induction of steatohepatitis and liver tumorigenesis in the absence of any additional cues for HCC development by disrupting the biliary canaliculus and bile acid homeostasis in the liver. These results suggest that Snail activation and Snail-mediated disruption of the biliary canaliculus and bile acid homeostasis in the liver act as an inducer of liver injury and contribute to the onset and progression of liver disease.

Materials and Methods

Mice

C57BL/6 mice (Clea Japan, Tokyo, Japan), *Alb-Cre* mice¹³ (The Jackson Laboratory, Bar Harbor, ME), *Alb-CreER^{T2}* mice¹⁴ (a gift from Pierre Chambon and Daniel Metzger, Institute of Genetics and Molecular and Cellular Biology, Strasbourg, France), and *R26R^{YFP/YFP}* mice¹⁵ (a gift from Frank Costantini, Columbia University, New York, NY) were used. In addition, a *CAG-floxed neo-Snail* mouse strain was generated to induce Cre-mediated overexpression of a mouse *Snail* full-length cDNA tagged with a hemagglutinin (HA) epitope¹⁶ (a gift from Antonio García de Herreros, Universitat Pompeu Fabra, Barcelona, Spain). The CAG promoter¹⁷ (a gift from Jun-ichi Miyazaki, Osaka University, Osaka, Japan) was used to drive transgene expression. For induction of Cre activity, 10-week-old *Alb-*

CreER^{T2}; *CAG-floxed neo-Snail* mice were given a single subcutaneous injection of tamoxifen (TM) (7.5 mg/mouse; Sigma-Aldrich, St. Louis, MO) dissolved in olive oil (Nacalai Tesque, Kyoto, Japan) at a concentration of 50 mg/mL. The experiments were approved by the Kyushu University Animal Experiment Committee, and the care and use of the animals were performed in accordance with institutional guidelines.

Immunostaining

For frozen tissue sections, liver tissues were embedded directly in Tissue-Tek OCT optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan) and sectioned. The frozen tissue sections were fixed with 4% paraformaldehyde for 5 minutes at room temperature. For paraffin-embedded tissue sections, liver tissues were fixed with Zinc Formalin Fixative (Polysciences, Warrington, PA), dehydrated in ethanol and xylene, embedded in paraffin wax, and sectioned. After deparaffinization and rehydration of the sections, antigen retrieval was performed by microwaving in 0.01 mol/L citrate buffer (pH 6.0). For immunohistochemistry, the sections were incubated with 0.3% hydrogen peroxide in methanol for 20 minutes at room temperature to quench endogenous peroxidase activity. After washing in phosphate-buffered saline and blocking with Block Ace (DS Pharma Biomedical, Osaka, Japan), the sections were incubated with the following primary antibodies: mouse anti-HA (1:100; Covance, Princeton, NJ); mouse anti-hepatocyte nuclear factor 4 α (Hnf4 α ; 1:500; PPMX, Tokyo, Japan); mouse anti-E-cad (1:1000; BD Biosciences, San Jose, CA); mouse anti-N-cadherin (1:1000; BD Biosciences); rabbit anti-cytokeratin 19¹⁸ (1:2000); rabbit anti-claudin-3 (Cldn3) (1:300; Abcam, Cambridge, MA); mouse anti-carcinoembryonic antigen—related cell adhesion molecule 1 (1:500; eBioscience, San Diego, CA); mouse anti- β -catenin (1:500; BD Biosciences); rat anti-CD45 (1:500; Novus Biologicals, Centennial, CO); rabbit anti-phosphorylated histone H3 (1:500; Millipore, Burlington, MA); and goat anti-green fluorescent protein/yellow fluorescent protein (1:2000; Abcam). After washing, the sections were incubated with a horseradish peroxidase—conjugated goat anti-rabbit IgG antibody (1:1000; Dako, Carpinteria, CA) for immunohistochemistry or with Alexa 488— and Alexa 555—conjugated secondary antibodies (1:1000; Thermo Fisher Scientific, Waltham, MA) specific to the species of the primary antibodies with DAPI for immunofluorescence staining.

Gene Expression Analysis

Total RNA was prepared from the liver tissues using a Nucleospin RNA II Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions, and cDNAs were synthesized from the total RNA as described.¹⁹ Quantitative PCR (qPCR) analyses using TaqMan probes (Applied

Figure 1 Overexpression of the *Snail* gene in the mouse liver. **A:** Experimental procedure for overexpression of a *Snail*-HA fusion gene in specific cell types. After cell-specific Cre-mediated recombination, the loxP-flanked neo-stop cassette is removed, leading to the expression of the *Snail*-HA fusion gene under the regulation of the CAG promoter. **B:** Quantitative PCR analyses of *Snail* were performed on total RNA obtained from the livers of 8-week-old wild-type (WT) (1) and *Alb-Cre;CAG-floxed neo-Snail* (2) mice. All data were normalized by the values in the livers of WT mice, and the fold differences are shown. **C:** Western blot analyses were conducted for the livers of 8-week-old WT (1) and *Alb-Cre;CAG-floxed neo-Snail* (2) mice, and representative data are shown. β-actin was used for normalization. **D:** Co-immunofluorescence staining for hemagglutinin (HA) and hepatocyte nuclear factor 4α (Hnf4α) in the livers of 4-week-old WT and *Alb-Cre;CAG-floxed neo-Snail* mice. Representative fluorescence images of Hnf4α-positive hepatocytes are shown. DNA was stained with DAPI. **E:** Co-immunofluorescence staining for N-cadherin (N-cad) and E-cadherin (E-cad) in the livers of 8-week-old WT and *Alb-Cre;CAG-floxed neo-Snail* mice. Representative fluorescence images of the periportal (top row) and pericentral (bottom row) zones of the hepatic lobule are shown. DNA was stained with DAPI. **F:** Immunohistochemical staining of the cholangiocyte marker cytokeratin 19 (CK19) in the livers of 8-week-old WT and *Alb-Cre;CAG-floxed neo-Snail* mice. Representative images are shown. The graph shows the percentages of CK19-positive cells residing around each portal vein (PV) in the livers of 8-week-old WT and *Alb-Cre;CAG-floxed neo-Snail* mice. Ten PVs were selected randomly in three tissue sections from the liver of each mouse. Data are expressed as means ± SD. $n = 3$ (B and F). ** $P < 0.01$. Scale bars: 20 μm (D); 100 μm (E and F). CV, central vein.

Biosystems, Waltham, MA) were conducted as described previously.²⁰ TaqMan probes for *Snail* (Mm00441533_g1), *occludin* (*Ocln*) (Mm00500912_m1), and *Cldn3* (Mm00515499_s1) were used. The TaqMan gene expression assay IDs (Applied Biosystems) are shown in parentheses after the gene names. As a normalization control, TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase control reagents (Applied Biosystems) were used. To examine the expression of *Cyp8b1*, *Cyp7b1*, and *Hsd3b7*, qPCR was conducted using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan), according to the manufacturer's instructions. qPCR primers for *Cyp8b1* (forward: 5'-TGGAAGCCAAGAAGTCGTTC-3' and reverse: 5'-TCCTCCTGTACCACCCTGAG-3'), *Cyp7b1* (forward: 5'-CAATGACCCGGAAATCTTCGA-3' and reverse: 5'-AGTTCTCTGCCACACTTTCA-3'), *Hsd3b7* (forward: 5'-ATGGTGAAGGTCATCAGGTCATGA-3' and reverse: 5'-CCAGTATGTGCATCCAAGCAACAT-3'), and *GAPDH* (forward: 5'-TGTGTCCGTCGTGGATCTGA-3' and reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3') were used. The values for *GAPDH* were used as normalization controls.

Western Blot Analysis

Western blot analyses were conducted as described previously.⁸ The following primary antibodies were used: goat anti-Snail (1:200; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-HA (1:200; Covance); and mouse anti- β -actin (1:5000; Abcam).

Measurements of Total Bile Acid, Bilirubin, Alkaline Phosphatase, Alanine Aminotransferase, and Aspartate Aminotransferase

Mouse serum was collected from the femoral artery. The amounts of total bile acid, bilirubin, and alkaline phosphatase in the serum were measured using a Total Bile Acids Assay Kit (Diazyme Laboratories, Poway, CA), Quanti-Chrom Bilirubin Assay Kit (Bioassay Systems, Hayward, CA), and LabAssay alkaline phosphatase Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. The amounts of alanine aminotransferase and aspartate aminotransferase in the serum were measured using a Transaminase CII Test Kit (FUJIFILM Wako Pure Chemical Corporation).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

Paraffin-embedded liver tissue sections were stained using an Apoptosis In Situ Detection Kit (FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer's instructions.

Gene Expression Microarray and Data Analysis

Total RNA was extracted from the liver of 13-week-old wild-type and *Alb-Cre;CAG-floxed neo-Snail* mice using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Microarray analyses were performed as described previously (Cell Innovator, Fukuoka, Japan).²⁰ Functional enrichment analyses of the differentially expressed genes were performed using the Database for Annotation, Visualization, and Integrated Discovery.^{21,22} Microarray data are available on the NCBI Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE141110).

Oil Red O Staining

Frozen sections of liver tissues were fixed with 4% paraformaldehyde for 5 minutes at room temperature, washed with phosphate-buffered saline, and incubated with 60% Oil red O (Muto Pure Chemicals, Tokyo, Japan) in water for 30 minutes at room temperature. After washing with 60% isopropanol for 1 minute and additional washing with phosphate-buffered saline, the sections were incubated with hematoxylin (Muto Pure Chemicals) for 5 minutes.

Electron Microscopy and Analysis of the Serum Bile Acid Composition

Transmission electron microscopy and scanning electron microscopy were performed by BML (Tokyo, Japan) and the Laboratory for Research Support, Medical Institute of Bioregulation (Kyushu University, Fukuoka, Japan), respectively. Bile acid composition in mouse serum was analyzed by the Junshin Clinic Bile Acid Institute (Tokyo, Japan).

Statistical Analysis

Statistical significance was analyzed using an unpaired *t*-test. Kaplan–Meier survival curves were analyzed statistically by

Figure 2 *Snail* overexpression in the mouse liver causes hepatomegaly, steatohepatitis, and liver tumorigenesis. **A:** Representative liver morphologies in 4-week-old (4w), 8w, 12w, 24w, 36w, and 48w wild-type (WT) and *Alb-Cre;CAG-floxed neo-Snail* mice. **Arrowheads** indicate tumors formed in the livers of *Alb-Cre;CAG-floxed neo-Snail* mice. **B:** Liver-to-body-weight ratios in male and female 4w, 8w, 12w, 24w, 36w, and 48w WT and *Alb-Cre;CAG-floxed neo-Snail* mice. **C:** Kaplan–Meier survival curves of male and female WT and *Alb-Cre;CAG-floxed neo-Snail* mice. There is no significant difference between male and female WT mice ($P = 0.5432$) or between male and female *Alb-Cre;CAG-floxed neo-Snail* mice ($P = 0.0528$). **D:** Representative images of hematoxylin and eosin (H&E)-stained and Oil red O-stained liver tissues obtained from 8w WT mice and 4w, 8w, 12w, 24w, and 36w *Alb-Cre;CAG-floxed neo-Snail* mice. **Arrowheads** indicate a representative area of inflammatory cell infiltration. Data are expressed as means $\pm$ SD (**B**). $n = 3$ (**B**). * $P < 0.05$, ** $P < 0.01$. Scale bars: 1 cm (**A**); 100 μ m (**D**, H&E); 200 μ m (**D**, Oil red O). CV, central vein; PV, portal vein.

the log-rank test. Differences at $P < 0.05$ were considered statistically significant.

Results

Generation of Transgenic Mice Overexpressing *Snail* in the Liver

To achieve overexpression of *Snail* in the liver, a mouse strain expressing Cre recombinase under the control of the mouse albumin enhancer/promoter (*Alb-Cre* mice)¹³ was crossed with a *CAG-floxed neo-Snail* mouse strain. The *CAG-floxed neo-Snail* mice were generated in this study to induce Cre-mediated overexpression of an HA-tagged mouse *Snail* full-length cDNA (Figure 1A). As expected, the double-transgenic mice, such as *Alb-Cre;CAG-floxed neo-Snail* mice, expressed a large amount of *Snail* mRNA and Snail protein in the liver (Figure 1, B and C), and the HA tag was observed in Hnf4 α -positive hepatocytes (Figure 1D). The liver zonation in the expression patterns of E-cad and N-cadherin indicated specific expression of E-cad and N-cadherin in hepatocytes residing around the portal and central veins, respectively (Figure 1E). In the liver of *Alb-Cre;CAG-floxed neo-Snail* mice, the number of E-cad-positive hepatocytes was decreased, and N-cadherin-positive hepatocytes replaced E-cad-positive hepatocytes in the periportal zone of the hepatic lobule (Figure 1E). These data show that Snail down-regulated the expression of its direct target gene *E-cad* in periportal hepatocytes. In addition to the effect of *Snail* overexpression on hepatocytes, the number of cholangiocytes composing the intrahepatic bile ducts was decreased significantly in *Alb-Cre;CAG-floxed neo-Snail* mice (Figure 1F). Global gene expression profiles in the liver of wild-type and *Alb-Cre;CAG-floxed neo-Snail* mice showed different gene expression patterns in both types of livers, including reduction of the cholangiocyte markers cytokeratin 19, *Klf5*, *Prom1* (also known as *CD133*), and *Onecut1* (also known as *Hnf6*) in the liver of *Alb-Cre;CAG-floxed neo-Snail* mice (Supplemental Figure S1, A and B). Because Cre recombinase is expressed not only in mature hepatocytes, but also bipotent hepatoblasts during liver development, cholangiocytes differentiated from hepatoblasts also expressed the transgenes, as well as hepatocytes (Supplemental Figure S2). Thus, the decrease in the number of cholangiocytes in the liver of *Alb-Cre;CAG-floxed neo-Snail* mice suggests that Snail activation inhibits cholangiocyte differentiation from hepatoblasts.

Liver-Specific *Snail* Overexpression Induces Steatohepatitis and Lethal Tumorigenesis

The liver of *Alb-Cre;CAG-floxed neo-Snail* mice became massively enlarged compared with that of wild-type mice, regardless of age or sex, and small tumors developed in the liver of the double-transgenic mice at approximately 24

weeks after birth, and subsequently grew to form large tumors (Figure 2, A and B). Survival curves showed that both male and female *Alb-Cre;CAG-floxed neo-Snail* mice died earlier than wild-type mice and that no double-transgenic mice survived until 80 weeks after birth (Figure 2C). Histologic analyses showed that the hepatocytes in *Alb-Cre;CAG-floxed neo-Snail* mice increased in size and accumulated large amounts of lipids in their cytoplasm, and that inflammatory cell infiltration occurred in the *Snail*-induced fatty liver (Figure 2D and Supplemental Figure S3A). Consistent with these data, gene ontology enrichment analysis showed that the extracted genes whose expression levels were significantly different between the liver of wild-type and *Alb-Cre;CAG-floxed neo-Snail* mice were highly enriched in genes associated with lipid metabolism (Supplemental Figure S1A). Although hepatic lipid accumulation was observed uniformly in the liver, hepatocyte enlargement initially occurred in the pericentral zone and gradually expanded to the entire hepatic region (Figure 2D and Supplemental Figure S3B). Meanwhile, cell proliferation and apoptosis were not induced in the liver of *Alb-Cre;CAG-floxed neo-Snail* mice (Supplemental Figure S3, C and D), suggesting that the liver hypertrophy in these mice arose from an increase in hepatocyte size. Taken together, these data show that enforced expression of *Snail* in the liver can induce steatohepatitis and liver tumorigenesis independently in the absence of any oncogenic stimuli.

Snail Overexpression in the Liver Results in a Defect of Biliary Excretion

In the serum of *Alb-Cre;CAG-floxed neo-Snail* mice, the amounts of alanine aminotransferase, a sensitive indicator of liver damage, and of total bile acid and bile pigment bilirubin were increased significantly compared with those in wild-type mice (Figure 3, A–C). The accumulation of bilirubin in the blood and tissues of *Alb-Cre;CAG-floxed neo-Snail* mice resulted in yellow discoloration of their tissues, known as jaundice or hyperbilirubinemia (Figure 3D). Moreover, *Alb-Cre;CAG-floxed neo-Snail* mice excreted abnormal amounts of unusual bile acids in the urine (Table 1), and some genes involved in bile acid synthesis were down-regulated in their livers (Figure 3E). In contrast, the expression levels of genes encoding bile acid transporters and nuclear receptors were increased or invariable, respectively, in the livers of *Alb-Cre;CAG-floxed neo-Snail* mice compared with that of wild-type mice (Supplemental Figure S1B). Interestingly, as shown in Figure 2D, the hepatocyte enlargement and hepatic lipid accumulation observed in *Alb-Cre;CAG-floxed neo-Snail* mice were more severe in the livers of 8-week-old mice compared with 4-week-old mice. Indeed, the amounts of serum alanine aminotransferase in *Alb-Cre;CAG-floxed neo-Snail* mice at 8 weeks after birth were substantially higher than those at 4 weeks after birth (Figure 3A). However, significant increases in serum total bile acid and bilirubin

Figure 3 Liver-specific *Snail* overexpression induces abnormal biliary excretion and liver injury. **A–C:** Amounts of alanine aminotransferase (ALT) (**A**), total bile acid (TBA) (**B**), and bilirubin (**C**) in the serum of 4-week-old (4w) and 8w wild-type (WT) and *Alb-Cre;CAG-floxed neo-Snail* mice. **D:** Representative images of subcutaneous and visceral tissues and serum of 12-week-old WT and *Alb-Cre;CAG-floxed neo-Snail* mice. **E:** Quantitative PCR analyses of total RNA obtained from the liver of 8w WT and *Alb-Cre;CAG-floxed neo-Snail* mice. All data were normalized by the values for the livers of WT mice, and the fold differences are shown. Red lines indicate means. Data are expressed as means $\pm$ SD (**A–C** and **E**). $n = 3$ (**A–C**); $n = 10$ WT mice (**E**); $n = 7$ *Alb-Cre;CAG-floxed neo-Snail* mice (**E**). Scale bars = 1 cm. * $P < 0.05$, ** $P < 0.01$.

already were detected in 4-week-old *Alb-Cre;CAG-floxed neo-Snail* mice (Figure 3, B and C), in which the degree of liver damage remained relatively low. Because an increase in bile acids is a cue for liver injury,²³ our data suggest that defective biliary excretion occurred before the development of hepatic damage in the liver of young *Alb-Cre;CAG-floxed neo-Snail* mice and became a key effector for induction of subsequent liver injury.

Hepatocyte-Specific *Snail* Overexpression Is Sufficient for Induction of Steatohepatitis and Liver Tumorigenesis

The number of cholangiocytes was decreased markedly in the liver of *Alb-Cre;CAG-floxed neo-Snail* mice (Figure 1F). Thus, the defect in biliary excretion in these mice may arise from a significant decrease in intrahepatic bile ducts and induce *Snail*-dependent liver injury. To examine this possibility, a mouse strain expressing an inducible form of Cre recombinase (*CreER^{T2}*) from the albumin genomic locus (*Alb-CreER^{T2}* mice)¹⁴ was crossed with the *CAG-floxed neo-Snail* mouse strain generated in this study. In the double-mutant mice, administration of TM allowed hepatocyte-specific

overexpression of *Snail*. The intrahepatic bile ducts in the TM-administered *Alb-CreER^{T2};CAG-floxed neo-Snail* mice appeared normal compared with those in wild-type mice (Figure 4, A and B). However, other phenotypic characteristics found in TM-administered *Alb-CreER^{T2};CAG-floxed neo-Snail* mice were similar to those in *Alb-Cre;CAG-floxed neo-Snail* mice, such as hepatocyte and liver enlargement accompanied by inflammatory reaction, hepatic lipid accumulation, and liver tumorigenesis (Figure 4, A and C). Moreover, the amounts of alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, total bile acid, and bilirubin in the serum of TM-administered *Alb-CreER^{T2};CAG-floxed neo-Snail* mice were increased substantially (Figure 4D). These data show that defective biliary excretion and liver injury result from a property change in hepatocytes, rather than in cholangiocytes, as a result of *Snail* overexpression.

Disruption of the Biliary Canalculus as a Result of *Snail* Overexpression in Hepatocytes

Next, the changes that occurred in the *Snail*-overexpressing hepatocytes were studied for induction of the defect in biliary excretion during the early stage of liver injury.

Table 1 Variety of Bile Acids Contained in the Urine of 8-Week-Old WT and *Alb-Cre;CAG-floxed neo-Snail* Mice

Bile acids	Bile acid abbreviations	WT			<i>Alb-Cre;CAG-floxed neo-Snail</i>		
		Sample 1, $\mu\text{mol/L}$	Sample 2, $\mu\text{mol/L}$	Sample 3, $\mu\text{mol/L}$	Sample 1, $\mu\text{mol/L}$	Sample 2, $\mu\text{mol/L}$	Sample 3, $\mu\text{mol/L}$
Total bile acids	TBA	0.58	0.32	0.35	388.16	57.67	59.90
Taurocholic acid	TCA	ND	ND	ND	8.90	3.08	4.71
Cholic acid	CA	0.02	0.05	ND	0.04	ND	ND
Taurocholic acid 3-sulfate	TCA3S	ND	ND	ND	19.39	16.72	7.90
Cholic acid 3-sulfate	CA3S	ND	ND	ND	0.11	0.11	ND
Chenodeoxycholic acid	CDCA	ND	ND	ND	ND	ND	0.04
Taurochenodeoxycholic acid 3-sulfate	TCDCAS	ND	ND	ND	ND	ND	2.09
Tauroursodeoxycholic acid 3-sulfate	TUDCA3S	ND	ND	ND	ND	ND	0.12
Tauroursodeoxycholic acid 7-N-acetylglucosaminide	TUDCA-7NAG	ND	ND	ND	0.75	0.25	0.23
Ursodeoxycholic acid 7-N-acetylglucosaminide	UDCA-7NAG	0.45	0.24	0.30	ND	ND	0.03
Deoxycholic acid 3-sulfate	DCA3S	ND	ND	ND	ND	ND	0.07
Taurolithocholic acid 3-sulfate	TLCA3S	ND	ND	ND	ND	ND	0.09
Tauro- α -muricholic acid 3-sulfate	α -MCA	0.02	ND	ND	ND	ND	ND
α -muricholic acid 3-sulfate + tauro- β -muricholic acid 3-sulfate	T- α -MCA3S + T- β -MCA3S	ND	ND	ND	31.55	7.22	8.96
Tauro- β -muricholic acid	T- β -MCA	ND	ND	ND	24.79	8.77	6.46
β -muricholic acid	β -MCA	0.07	0.03	ND	1.25	0.22	0.23
β -muricholic acid 3-sulfate	β -MCA3S	ND	ND	ND	9.11	1.26	1.21
ω -muricholic acid	ω -MCA	ND	ND	0.05	ND	ND	0.06
Taurocholic acid-1 β -ol	TCA-1 β -ol	ND	ND	ND	4.97	15.07	4.47
Cholic acid-6 α -ol	CA-6 α -ol	ND	ND	ND	ND	ND	0.05
3 β ,7 α ,12 α -trihydroxy-5-cholenoyl-aurine	T- Δ 5-3 β ,7 α ,12 α -(OH)3	ND	ND	ND	ND	ND	0.33
3 β ,7 α ,12 α -trihydroxy-5-cholenoyl-aurine 3-sulfate	T- Δ 5-3 β ,7 α ,12 α -(OH)3-3-S	ND	ND	ND	ND	ND	0.33
3 β ,7 α -dihydroxy-5-cholenoyl-aurine	T- Δ 5-3 β ,7 α -(OH)2	ND	ND	ND	ND	ND	0.09
3 β ,7 α -dihydroxy-5-cholenoyl-aurine 3-sulfate	T- Δ 5-3 β ,7 α -(OH)2-3-S	ND	ND	ND	2.71	1.17	1.96
3 β ,7 α -dihydroxy-5-cholenic acid 3-sulfate	Δ 5-3 β ,7 α -(OH)2-3-S	ND	ND	ND	1.71	0.73	1.10
3 β -hydroxy-5-cholenoyl-aurine 3-sulfate	T- Δ 5-3 β -OH-3-S	ND	ND	ND	ND	ND	0.19
7 α ,12 α -dihydroxy-3-oxo-4-cholenoyl-aurine	TCA- Δ 4-3-one	ND	ND	ND	6.97	2.07	3.07
7 α ,12 α -dihydroxy-3-oxo-4-cholenic acid	CA- Δ 4-3-one	ND	ND	ND	ND	ND	0.03
12 α -hydroxy-3-oxo-4-cholenoyl-aurine	TCDCAS- Δ 4-3-one	ND	ND	ND	0.23	ND	0.42
Norcholeic acid	nor-CA	ND	ND	ND	0.83	1.01	0.26
3 α ,7 α -dihydroxycholestanic acid	C27-DHCA	0.02	ND	ND	ND	ND	ND
Cholic acid 7-sulfate	CA-7S	ND	ND	ND	1.33	ND	ND
Taurocholic acid 7-sulfate	TCA-7S	ND	ND	ND	273.51	ND	15.36
Cholic acid 3-glucuronide	CA3G	ND	ND	ND	ND	ND	0.04

Urine samples were obtained from three mice each.

ND, not detected; WT, wild-type.

Previous studies showed that Snail directly suppresses mRNA expression of the tight junction (TJ) components claudins and *Ocln*.^{24,25} Consistently, our qPCR analyses showed that the expression levels of *Cldn3* and *Ocln* in the

liver of *Alb-Cre;CAG-floxed neo-Snail* mice were much lower than the levels in wild-type mice (Figure 5A). Furthermore, immunofluorescence analyses showed the absence of Cldn3 and abnormal localization of

Figure 4 Phenotypic alterations in mice overexpressing *Snail* in the liver arise from a property change in hepatocytes, rather than in cholangiocytes. **A:** The livers were obtained from tamoxifen (TM)-administered 26-week-old wild-type (WT) mice and TM-administered 26-week-old and 72-week-old *Alb-CreER^{T2};CAG-floxed neo-Snail* mice. Representative liver morphologies, representative images of hematoxylin and eosin (H&E)-stained and Oil red O-stained liver tissues, and representative immunohistochemical images of cytokeratin 19 (CK19) are shown. **B:** Percentages of cells immunoreactive for CK19 residing around each portal vein (PV) in the livers of TM-administered 26-week-old WT (1) and *Alb-CreER^{T2};CAG-floxed neo-Snail* (2) mice. Ten PVs were selected randomly in three tissue sections from the liver of each mouse. **C:** Liver-to-body-weight ratios in TM-administered 26-week-old WT (1) and *Alb-CreER^{T2};CAG-floxed neo-Snail* (2) mice. **D:** Amounts of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bile acid (TBA), and bilirubin in the serum of TM-administered 26-week-old WT (1) and *Alb-CreER^{T2};CAG-floxed neo-Snail* (2) mice. Data are expressed as means $\pm$ SD. $n = 3$ (B–D). Scale bars: 1 cm (liver images); 100 μ m (liver tissue sections). * $P < 0.05$, ** $P < 0.01$.

anti-carcinoembryonic antigen-related cell adhesion molecule 1 (also known as biliary glycoprotein) normally localized at hepatocyte TJs with *Cldn3*, in the liver of not only adult (8-week-old), but also young (4-week-old), *Alb-Cre;CAG-floxed neo-Snail* mice (Figure 5B). In addition to *Cldn3* and *Ocln*, the expression levels of genes

encoding other TJ components also were down-regulated in the liver of *Alb-Cre;CAG-floxed neo-Snail* mice (Supplemental Figure S1C). Because hepatocytes form the biliary canaliculus by adhering to adjacent hepatocytes through TJs, our data suggest that Snail-induced suppression of the integral membrane proteins localized at TJs

Figure 5 *Snail* overexpression in the mouse liver causes disruption of the biliary canaliculus formed among hepatocytes. **A:** Quantitative PCR analyses on total RNA obtained from the livers of 8-week-old (8w) wild-type (WT) and *Alb-Cre;CAG-floxed neo-Snail* mice. All data were normalized by the values for the livers of WT mice, and the fold differences are shown. **B:** Co-immunofluorescence staining for claudin-3 (*Cldn3*) and anti-carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM-1*) in the livers of 4w and 8w WT and *Alb-Cre;CAG-floxed neo-Snail* mice. Representative fluorescence images are shown. DNA was stained with DAPI. **C:** Representative transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images of liver tissues obtained from 8w WT and *Alb-Cre;CAG-floxed neo-Snail* mice. **Arrowheads** indicate the tight junction and biliary canaliculus formed among hepatocytes, respectively, both of which were observed in the livers of WT mice, but not in the livers of *Alb-Cre;CAG-floxed neo-Snail* mice. Instead, many lipid droplets and abnormal mitochondria were observed within hepatocytes in the livers of *Alb-Cre;CAG-floxed neo-Snail* mice. **D:** Co-immunofluorescence staining for *Cldn3* and *CEACAM-1* in the livers of TM-administered 22-week-old WT and *Alb-CreER^{T2};CAG-floxed neo-Snail* mice. Representative fluorescence images are shown. DNA was stained with DAPI. Data are expressed as means $\pm$ SD. $n = 3$ (A). Scale bars: 20 μ m (B and D); 10 μ m (C). * $P < 0.05$.

disrupted the structure of the biliary canaliculus and led to the defect in biliary excretion. In fact, transmission electron microscopy and scanning electron microscopy observations showed that the biliary canaliculus disappeared from the liver of *Alb-Cre;CAG-floxed neo-Snail* mice through disruption of hepatocyte TJs (Figure 5C). The resulting extension of the intercellular space formed between hepatocytes in the liver of *Alb-Cre;CAG-floxed neo-Snail* mice may allow bile acid diffusion into the blood circulatory system. In addition to the structural defect in hepatocyte TJs, ultrastructural analyses showed characteristic mitochondrial abnormalities in hepatocytes of *Alb-Cre;CAG-floxed neo-Snail* mice, suggesting induction of oxidative stress based on an increase in reactive oxygen species production for liver damage (Figure 5C). Disruption of the hepatocyte TJs, characterized by loss of Cldn3 and abnormal localization of anti-carcinoembryonic antigen-related cell adhesion molecule 1 in the liver, also was observed in TM-administered *Alb-CreER^{T2};CAG-floxed neo-Snail* mice (Figure 5D). Taken together, our data show that enforced *Snail* expression in hepatocytes causes disruption of the biliary canaliculus through suppressed expression of TJ components and leads to a defect in biliary excretion from an early stage of liver injury.

Discussion

In this study, we showed that enforced *Snail* expression in hepatocytes is able to induce steatohepatitis and liver tumorigenesis independently through disruption of the biliary canaliculus and bile acid homeostasis in the liver. Thus, *Snail* may act not only as an epithelial-to-mesenchymal transition inducer in HCC for tumor progression and invasion,^{9,10} but also a key player for HCC induction. Our findings suggest that *Snail* activation and *Snail*-mediated disruption of the biliary canaliculus and bile acid homeostasis in the liver are involved in the pathogenesis of liver disease. Indeed, *Snail* up-regulation is detected in mouse hepatocytes responding to chronic hepatic injury, leading to progression of liver fibrosis,⁷ and in human hepatocytes residing in the liver of hepatitis B virus-infected patients.²⁶ In this study, the pathologic changes found in the liver of both *Alb-Cre;CAG-floxed neo-Snail* mice and *Alb-CreER^{T2};CAG-floxed neo-Snail* mice, such as fatty liver and subsequent steatohepatitis, appear similar to the signs and symptoms of chronic liver disease such as alcoholic and nonalcoholic fatty liver disease.²⁷ Interestingly, it was reported that transforming growth factor- β signaling is activated in the liver after alcohol intake^{27,28} and that *Ocln* increases resistance to alcohol-induced liver damage.²⁹ Thus, it can be suggested that transforming growth factor- β -induced *Snail* activation and *Snail*-induced *Ocln* down-regulation occur in response to alcohol intake and increase susceptibility to alcohol-induced hepatic injury.

TJs that produce hepatocyte polarity in the liver and form the biliary canaliculus structure among hepatocytes are composed of the integral membrane proteins claudins, *Ocln*, and junctional adhesion molecules, and many peripheral membrane proteins, including zonula occludens-1, -2, and -3, which bind intracellularly to claudins, *Ocln*, and junctional adhesion molecules.³⁰ Enforced *Snail* expression in hepatocytes is sufficient for disruption of the TJs formed among hepatocytes. Because *Snail* directly suppresses the expression of *claudins* and *Ocln*,^{24,25} it is suggested that the disruption of hepatocyte TJs in the liver of both *Alb-Cre;CAG-floxed neo-Snail* mice and *Alb-CreER^{T2};CAG-floxed neo-Snail* mice results from significant decreases in the TJ components claudins and *Ocln*. However, because TJs are composed of many proteins, it is difficult to conclude that only decreases in claudins and *Ocln* within hepatocytes disrupt the formation and maintenance of TJs. Indeed, TJ components other than claudins and *Ocln* also were reduced in the liver of *Alb-Cre;CAG-floxed neo-Snail* mice. Thus, it is suggested that *Snail* directly or indirectly can suppress the mRNA expression of TJ components, and thus the expression levels of *Snail* in hepatocytes may be important for maintenance of the physiological function of hepatocyte TJs in the liver. In addition to disruption of the biliary canaliculus, enforced *Snail* expression in the liver results in down-regulated expression of genes associated with bile acid synthesis, such as *Cyp8b1*, *Cyp7b1*, and *Hsd3b7*, and induces excretion of abnormal amounts of unusual bile acids from hepatocytes. Human *CYP8B1*, *CYP7B1*, and *HSD3B7* are the genes responsible for 12 α -hydroxylase deficiency, oxysterol 7 α -hydroxylase deficiency, and 3 β -hydroxy- Δ 5-C27-steroid dehydrogenase/isomerase deficiency, respectively, which are observed in diseases involving inborn errors of bile acid metabolism.³¹ Thus, *Snail* contributes to the maintenance of bile acid homeostasis by regulating the expression levels of key genes involved in bile acid metabolism in hepatocytes.

Bile acids induce mitochondrial dysfunction, oxidative stress, and reactive oxygen species generation in hepatocytes,^{32,33} and large amounts of bile acids are implicated as a major cause of liver injury.²³ Thus, increased and abnormal biliary excretion as a result of *Snail*-induced disruption of the biliary canaliculus and bile acid homeostasis in the liver may lead to hepatic damage mediated by oxidative stress through bile acid-induced mitochondrial alterations in hepatocytes. Indeed, the hepatocytes in *Alb-Cre;CAG-floxed neo-Snail* mouse livers contain many morphologically abnormal mitochondria. In the human fatty liver, the number of hepatocytes containing abnormal mitochondria is significantly higher than that in the normal liver.³⁴ Moreover, mitochondrial dysfunction and oxidative stress are observed in liver tissues from patients with alcoholic and nonalcoholic steatohepatitis.³⁵ In addition, bile acids may contribute to tumor initiation and progression through a mechanism involving oxidative stress and DNA damage.²³ Thus, it has been suggested that the retention of

large amounts of bile acids in *Alb-Cre;CAG-floxed neo-Snail* mice and *Alb-CreER^{T2};CAG-floxed neo-Snail* mice allows the induction of liver tumorigenesis. Although bile acids induce apoptosis of hepatocytes,²³ the present data showed that an increase in bile acids caused by enforced *Snail* expression in the liver did not affect the induction of hepatocyte apoptosis. These results suggest that *Snail* suppresses the expression of genes not only for TJ components and bile acid synthesis, but also apoptosis in hepatocytes. Indeed, *Snail* is known to have a role in resistance to apoptosis,³⁶ as well as many other functions. The present study shows that the amount of *Snail* in hepatocytes is deeply related to the function of hepatocytes and should be controlled properly for the maintenance of liver homeostasis. Suppression of *Snail* up-regulation in hepatocytes that reside in the damaged liver may contribute to the prevention of liver disease progression.

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Author Contributions

S.M. performed all experiments and collected and analyzed data; and A.S. conceived, designed, and managed the project and wrote the paper.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.02.005>.

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