WNT7B Regulates Cholangiocyte Proliferation and Function During Murine Cholestasis

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We previously identified an up-regulation of specific Wnt proteins in the cholangiocyte compartment during cholestatic liver injury and found that mice lacking Wnt secretion from hepatocytes and cholangiocytes showed fewer proliferating cholangiocytes and high mortality in response to a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, a murine model of primary sclerosing cholangitis. *In vitro* studies demonstrated that Wnt7b, one of the Wnts up-regulated during cholestasis, induces proliferation of cholangiocytes in an autocrine manner and increases secretion of proinflammatory cytokines. We hypothesized that loss of Wnt7b may exacerbate some of the complications of cholangiopathies by decreasing the ability of bile ducts to induce repair. Wnt7b-flox mice were bred with Krt19-cre mice to deplete Wnt7b expression in only cholangiocytes (CC) or with albumin-Cre mice to delete Wnt7b expression in both hepatocytes and cholangiocytes (HC + CC). These mice were placed on a DDC diet for 1 month then killed for evaluation. Contrary to our expectations, we found that mice lacking Wnt7b from CC and HC + CC compartments had improved biliary injury, decreased cellular senescence, and lesser bile acid accumulation after DDC exposure compared to controls, along with decreased expression of inflammatory cytokines. Although Wnt7b knockout (KO) resulted in fewer proliferating cholangiocytes, CC and HC + CC KO mice on a DDC diet also had more hepatocytes expressing cholangiocyte markers compared to wild-type mice on a DDC diet, indicating that Wnt7b suppression promotes hepatocyte reprogramming. *Conclusion:* Wnt7b induces a proproliferative proinflammatory program in cholangiocytes, and its loss is compensated for by conversion of hepatocytes to a biliary phenotype during cholestatic injury. (*Hepatology Communications* 2021;5:2019-2034).

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disorder that is characterized by inflammation and fibrosis of the bile ducts, which can lead to end-stage liver disease and reduced life expectancy. Because the etiology of this disease is not fully understood, effective medical therapies are scarce and, in most cases the only life-extending treatment currently available is liver transplantation. Of the patients fortunate enough to receive a liver transplant, up to 20% will have recurrence of disease.[1-3] Therefore, an effective treatment for PSC is critically needed as the demand for organs available for transplant increases each year.

It is well known the liver is the only internal organ capable of regeneration; however, the bile duct is often overlooked when touting the liver's regenerative ability.

Abbreviations: Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; CC, cholangiocyte; CK-19, cytokeratin-19; DDC, 5-diethoxycarbonyl-1,4-dihydrocollidine; ELISA, enzyme-linked immunosorbent assay; EYFP, enhanced yellow fluorescent protein; HC, hepatocyte; IL, interleukin; KO, knockout; TNFα, tumor necrosis factor alpha; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; PSC, primary sclerosing cholangitis; RT-PCR, reverse-transcription polymerase chain reaction; SCF, stem cell factor; siRNA, small interfering RNA; SMCC, small cell cholangiocyte; Sox9, sex-determining region Y-box transcription factor 9; WT, wild type.

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Much like hepatocytes, cholangiocytes, the epithelial cells that comprise the bile ducts, also exhibit a remarkable ability to regenerate. Following injury, cholangiocytes proliferate to form the tubular structures necessary to ensure bile modification and transport. The restitution of these biliary networks is critical in maintaining liver function and preventing further injury due to cholestasis. During regeneration after acute liver injury, hepatocytes and cholangiocytes typically proliferate to repopulate their own cell types. However, during severe biliary or hepatic injury, regeneration of one of the two epithelial cell compartments can become compromised. This is when a secondary method of regeneration can occur, which is hepatocyte-to-cholangiocyte or cholangiocyte-to-hepatocyte transdifferentiation.[4-7] These two cell populations can act as facultative stem cells for one another, interconverting to maintain tissue integrity.[8] Thus, a therapy that induces a healthy cholangiocyte population, either from hepatocyte-derived cholangiocytes or native cholangiocytes, to proliferate and repair bile ducts could be a crucial treatment for patients suffering from cholestatic liver diseases.

The Wnt/β-catenin signaling pathway is an important regulatory axis in liver physiology and pathology due in large part to its role as a promoter of regeneration and repair.[9] Recent studies have also demonstrated that Wnts are expressed during biliary disease as well. Activation of Wnt signaling is seen in the livers of patients with PSC, and Wnt ligands are associated with proliferating biliary cells in a 3,5-diethoxycarbony l-1,4-dihydrocollidine (DDC) diet, a model for biliary injury and chronic cholestasis.[10,11] Other studies have shown up-regulation of specific Wnts in cholangiocytes after a DDC diet, and in a mouse model of cholangiocarcinoma, these ligands induced cholangiocyte proliferation.[12,13] Our laboratory has also shown that Wnt proteins are expressed in the cholangiocyte population during cholestatic liver injury and that mice lacking Wnt secretion from both hepatocytes and cholangiocytes had fewer proliferating cholangiocytes and high mortality in response to a DDC diet.[14] However, a recent manuscript has shown that cholangiocytes produce Wnts during biliary injury that promote the formation of ductal scars by acting through the Wnt–planar cell polarity pathway to induce fibrogenic cytokine production.[15] Therefore, further mechanistic analyses are needed to determine the role of specific Wnts in the process of biliary injury and repair.

One of the Wnts that has been identified to be up-regulated during cholestasis is Wnt7b, which was shown to induce cholangiocyte proliferation in an autocrine manner independent of β-catenin activity.[13,14] Therefore, we hypothesized that loss of Wnt7b may exacerbate some of the complications caused by cholangiopathies by decreasing the ability of bile ducts to induce repair. To test this hypothesis, we created both cholangiocyte-specific Wnt7b knockouts (CC KOs) or KOs in which Wnt7b is deleted from both hepatocytes and cholangiocytes (HC + CC) and subjected these mice to cholestatic liver injury. We found that both CC KO and HC + CC KO mice had improved biliary injury and decreased inflammation; however, this was not due to changes in ductular mass but rather to increased numbers of hepatocytes expressing biliary markers, which compensates for the inability of cholangiocytes to proliferate due to the lack of Wnt7b.

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Materials and Methods

CELL LINES, TRANSFECTION, AND LUCIFERASE ASSAY

Small cell cholangiocytes (SMCCs)[16] were plated at 2×10^5 cells/well in a six-well plate and transfected with 2.5 μg control or Wnt7b plasmid, described elsewhere,[14] and 50 μM negative or β-catenin small interfering RNA (siRNA; Thermo Fisher Scientific, Waltham, MA) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA), as per the manufacturer's instructions. Cell proliferation was measured 48 hours posttransfection using Colorimetric Cell Viability Kit I (WST-8) (PromoCell, Heidelberg, Germany) or a thymidine incorporation assay, as described.[17] Along with the control and Wnt7b plasmids, cells were also transfected with 2.5 μg of a luciferase TOPFlash plasmid (Upstate Biotechnology, Lake Placid, NY)[14] using Lipofectamine 3000. Luciferase activity was analyzed 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), as per the manufacturer's instructions. For the Wnt7b knockdown study, cells were transfected using Lipofectamine RNAiMax (Invitrogen) and 100 μM of either control or Wnt7b siRNA; TOPFlash activity was measured, as described above.

For the enzyme-linked immunosorbent assay (ELISA) array, SMCCs were plated and transfected with control or Wnt7b plasmid or control or Wnt7b siRNA, as described above. Cell media was changed 24 hours posttransfection, and 48 hours later, the now conditioned media was collected and analyzed using the chemiluminescent Mouse Cytokine ELISA Plate Array I (Signosis, Santa Clara, CA) per the manufacturer's instructions. WST-8, luciferase assay, and ELISA assay results were collected using a Synergy HTX Multi-Mode Microplate Reader from BioTek Instruments.

ANIMAL MODELS

All animals were housed in light- and temperature-controlled facilities and maintained in accordance with the Guide for Care and Use of Laboratory Animals and the Animal Welfare Act. All studies were performed in accordance with the guidelines of the Institutional Animal Use and Care Committee at the University of Pittsburgh School of Medicine and the National Institutes of Health (protocol number 20077675). Control mice ($K19^{CreERT}$/Rosa-$stop^{flox/flox}$- enhanced yellow fluorescent protein [EYFP]) were produced by breeding $K19^{CreERT}$ (Jackson Laboratories, Bar Harbor, ME) with Rosa-$stop^{flox/flox}$-EYFP (Jackson Laboratories). CC KO mice ($K19^{CreERT}$/Rosa-$stop^{flox/flox}$-EYFP/$Wnt7b^{c3}$ mice) were produced by breeding the control $K19^{CreERT}$/Rosa-$stop^{flox/flox}$-EYFP mice with $Wnt7b^{c3}$ mice (Jackson Laboratories). HC + CC KO mice (Albumin [Alb]-Cre/Rosa-$stop^{flox/flox}$-EYFP/$Wnt7b^{c3}$) were produced by breeding the Rosa-$stop^{flox/flox}$-EYFP mice with $Wnt7b^{c3}$ mice, then breeding the Rosa-$stop^{flox/flox}$-EYFP/$Wnt7b^{c3}$ offspring with Alb-$Cre^{+/-}$ mice (Jackson Laboratories). To delete Wnt7b and label cholangiocytes in $K19^{CreERT}$/Rosa-$stop^{flox/flox}$-EYFP/$Wnt7b^{c3}$ mice, three doses of 10 mg/kg tamoxifen were given every other day starting at 21 days old. At 8 weeks of age, all mice were then placed on a 0.1% DDC diet (Animal Specialties and Provisions LLC, Quakertown, PA) or left on standard mouse chow as a control for 1 month. Mice were then killed, and livers and blood serum collected. Livers were divided and fixed in 10% formalin and processed for paraffin embedding or frozen in liquid nitrogen and stored at −80 °C. The number of animals in each group were as follows: wild type (WT) on the control diet, n = 10; WT on the DDC diet, n = 6; CC KO on the control diet, n = 13; CC KO on the DDC diet, n = 8; HC + CC KO on the control diet, n = 4; HC + CC KO on the DDC diet, n = 8. Both male and female mice were used for experimentation. All mice were in a C57Bl6 background and maintained in ventilated cages under 12-hour light/dark cycles with access to enrichment, water, and either standard chow or DDC diet *ad libitum*. Further experimental details are available in the Supporting Materials.

Results

OVEREXPRESSION OF WNT7B IN CHOLANGIOCYTES INDUCES CELL PROLIFERATION INDEPENDENT OF β-CATENIN SIGNALING *IN VITRO*

We have previously shown that Wnt7b along with Wnt7a and Wnt10a are remarkably increased in models of induced cholestasis, such as a DDC diet

and bile duct ligation.[14] We also analyzed expression of these three Wnts in a genetic model of biliary injury, the multidrug resistance protein 2 (Mdr2) KO mouse, which spontaneously develops a phenotype resembling PSC.[18] All three Wnts were upregulated at 7 weeks, the time point at which Mdr2 KO mice develop biliary strictures and periductal fibrosis[19] (Supporting Fig. S1), supporting the relevance of these Wnts in multiple cholestatic models. We had also previously demonstrated that Wnt7b is secreted by cholangiocytes and induces proliferation in biliary cells through noncanonical Wnt signaling mechanisms.[14] To verify these findings, we transfected SMCCs[16] with control or Wnt7b plasmid, treated each group with either β-catenin or control siRNA, and measured cell proliferation. We were able to achieve 6.5-fold higher Wnt7b expression in cells transfected with the Wnt-carrying plasmid compared to control, while β-catenin expression was decreased by 75% compared to control (Supporting Fig. S2A,B). Using both thymidine incorporation and a colorimetric proliferation assay (WST-8), we confirmed that overexpression of Wnt7b promotes cholangiocyte proliferation in an autocrine manner (Fig. 1A-C) and that this process occurs independently of β-catenin activity because knockdown of β-catenin does not affect cell proliferation induced by Wnt7b (Fig. 1B). Furthermore, Wnt7b overexpression in SMCCs reduces rather than induces β-catenin activity, as seen by the decrease in TOPFlash luciferase

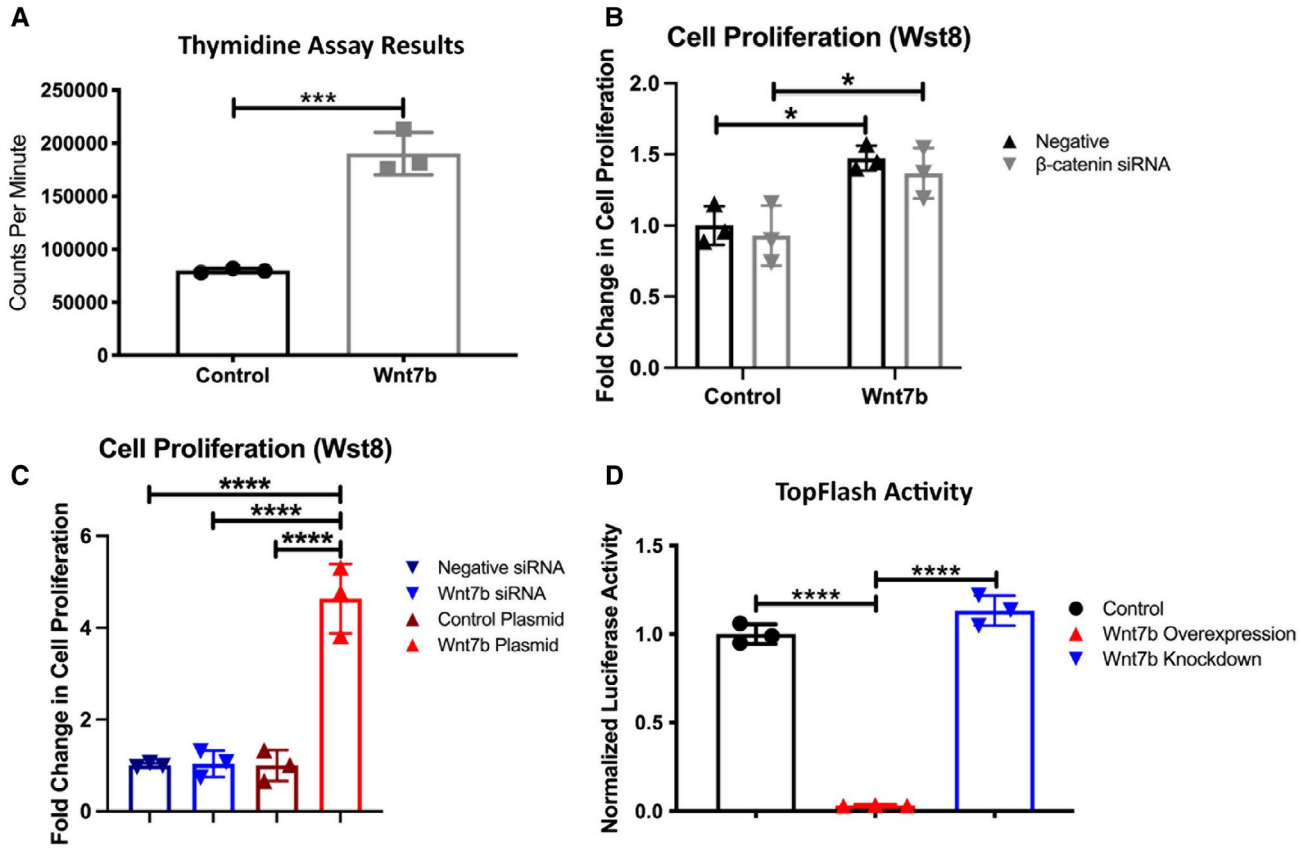

FIG. 1. Overexpression of Wnt7b in SMCC culture induces cell proliferation independent of β-catenin activity, while suppression of Wnt7b has no effect. (A) Thymidine incorporation assay demonstrates that Wnt7b overexpression induces cholangiocyte proliferation *in vitro*. (B) Wst8 colorimetric assay demonstrates that Wnt7b overexpression induces cholangiocyte proliferation, which is not inhibited in the presence of β-catenin siRNA. (C) Suppression of Wnt7b with siRNA does not inhibit cell proliferation. (D) TOPFlash luciferase reporter activity demonstrates that β-catenin activation is not induced by either Wnt7b overexpression or Wnt7b knockdown. Data in A-D represent mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by Student *t* test.

activity compared to cells without Wnt7b expression (Fig. 1C). Interestingly, knockdown of Wnt7b using siRNA (Supporting Fig. S2C) has no effect on either cell proliferation or TOPFlash activity (Fig. 1C, D).

OVEREXPRESSION OF Wnt7b INDUCES AN INFLAMMATORY PHENOTYPE IN CULTURED CHOLANGIOCYTES

Cholangiocyte proliferation, which is observed during cholestasis, is often associated with an "activated" phenotype that is characterized by increased secretion of cytokines and growth factors.[20] As Wnt7b induces cholangiocyte proliferation in an autocrine manner, we wanted to determine if it also induced production of these inflammatory mediators. Using a mouse cytokine ELISA kit, we found that tumor necrosis factor alpha (TNFα), interleukin (IL)-1α, platelet-derived growth factor (PDGF)-BB, and IL-12 were up-regulated significantly in conditioned media from Wnt7b-expressing SMCCs compared to those with control plasmid (Fig. 2). On the other hand, there was no change in any of the cytokines analyzed when Wnt7b was inhibited in SMCCs (Fig. 2). Thus, Wnt7b enhances the immunomodulatory response in cultured cholangiocytes.

Wnt7b DELETION *IN VIVO* IMPROVES BILIARY INJURY AFTER 1 MONTH OF DDC EXPOSURE

In order to generate conditional KOs of Wnt7b, Wnt7b-flox mice were bred with Krt19-Cre mice to deplete Wnt7b expression in only CC or with Alb-Cre mice to delete Wnt7b expression in both HC + CC. Both CC KO and HC + CC KO mice carry the Rosa-stop$^{flox/flox}$-EYFP gene, which was used to assess efficiency of Wnt7b deletion from these cell populations. Approximately 50% of cholangiocytes in the CC KO mice were EYFP+, while 100% of cholangiocytes and hepatocytes were positive for EYFP in the HC + CC KO group (Supporting Fig. S3A). RNAscope for Wnt7b also confirmed a greater than 50% decrease in Wnt7b expression in cholangiocytes from CC KO mice (Supporting Fig. S4). Notably, neither KO group had any phenotypic or biochemical abnormalities at baseline (Fig. 3).

To assess the impact of Wnt7b loss in the setting of cholestatic liver disease, we then fed WT, CC KO, and HC + CC KO mice the control or DDC diet. While Wnt7b expression increased in all genotypes after the DDC diet, CC KO and HC + CC KO mice had significantly less full-length Wnt7b protein than WT mice, confirming loss of Wnt7b from these cell types (Supporting Fig. S3B). Analysis of serum biochemistry after 1 month showed that CC KO and HC + CC KO mice fed the DDC diet had significantly decreased alkaline phosphatase (ALP) levels compared to WT mice on DDC (Fig. 3A), indicating that both types of KO have less biliary injury compared to WT mice. Interestingly, HC + CC KO DDC-treated mice had normalized conjugated and total bilirubin levels compared to both WT and CC KO mice fed the DDC diet, suggesting that the HC + CC KO mice had even less cholestatic injury compared to the other mice. However, hepatic injury was sustained across all three mouse models, as indicated by serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fig. 3B). ALT was equally high in all three mouse models fed the DDC diet. AST also remained high among the three models, but HC + CC KO mice on DDC had significantly decreased AST levels compared to WT mice on DDC. Thus, rather than worsening the phenotype, deletion of Wnt7b protected mice from biliary injury during DDC-induced cholestasis.

Wnt7b KNOCKOUT HAS NO EFFECT ON PARENCHYMAL INJURY OR FIBROSIS

To investigate parenchymal injury between the different genotypes, hematoxylin and eosin stains of liver sections were performed. Both types of KO mice fed the DDC diet had portal damage, including inflammation and ductular response, that was comparable to WT mice fed DDC (Fig 4A). Therefore, Wnt7b knockout had no effect on cholestasis-induced parenchymal injury. To determine if lower ALP levels in Wnt7b KO mice on the DDC diet correlated with decreased portal fibrosis, sirius red staining and quantification were used to assess the fibrotic content of these livers. Fibrosis in CC KO mice on DDC trended lower than in WT mice, while fibrosis in HC + CC KO mice on DDC was comparable to WT mice (Fig. 4B). Additionally, quantification

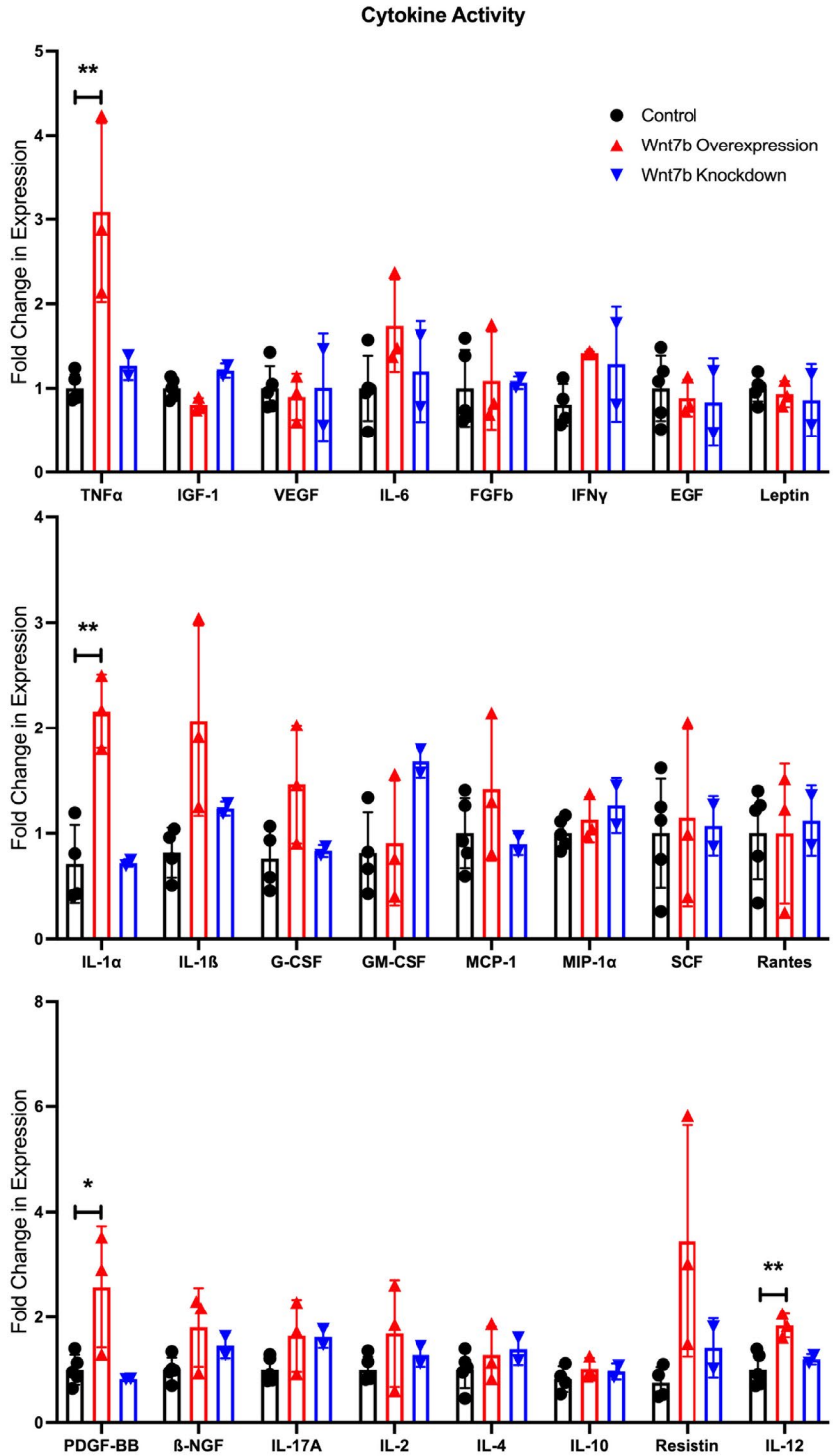

FIG. 2. Wnt7b overexpression in cholangiocyte cell culture promotes up-regulation of commonly altered cytokines. ELISA analysis of (top graph) TNFα, IGF-1, VEGF, IL-6, FGFb, IFNγ, EGF, leptin, (middle graph) IL-1α, IL-1β, G-CSF, GM-CSF, MCP-1, MIP-1α, SCF, Rantes, (bottom graph) PDGF-BB, β-NGF, IL-17A, IL-2, IL-4, IL-10, resistin, and IL-12 show that Wnt7b overexpression promotes up-regulation of common cytokines and growth factors compared to both control and Wnt7b knockdown. Data represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student *t* test. Abbreviations: β-NGF, nerve growth factor β; EGF, epidermal growth factor; FGFb, basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IGF, insulin-like growth factor; MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1 alpha; Rantes, regulated on activation, normal T expressed and secreted; VEGF, vascular endothelial growth factor.

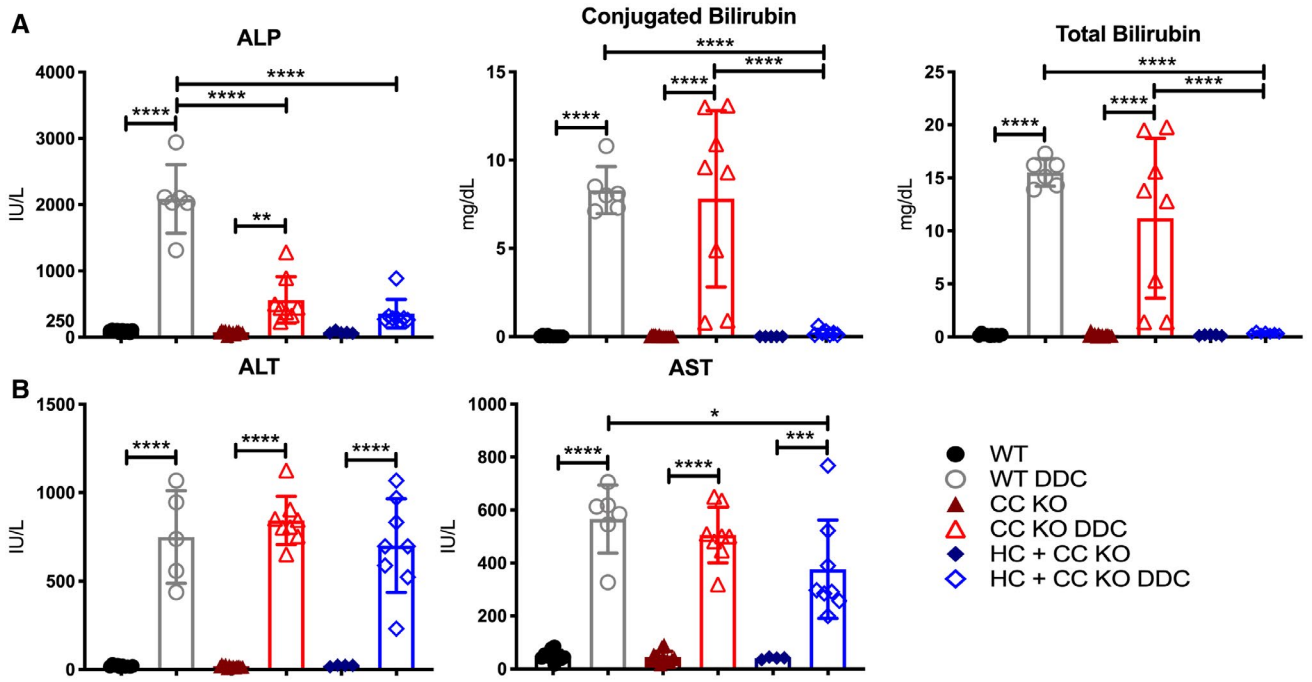

FIG. 3. Blood serum results indicate Wnt7b knockout improves biliary injury after 1 month of DDC exposure. (A) Blood serum levels of ALP, conjugated bilirubin, and total bilirubin show decreased biliary injury in mice lacking Wnt7b in both cholangiocytes only and cholangiocytes and hepatocytes when exposed to a DDC diet for 1 month compared to WT mice. (B) Blood serum levels of ALT and AST indicate no changes in hepatic injury in mice lacking Wnt7b compared to WT mice on the DDC diet for 1 month. Data in A-B represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by one-way ANOVA.

of hydroyproline, which represents the amount of collagen in a sample, also showed no significant differences between WT and either CC KO or HC + CC KO mice on DDC (Fig. 4C). Therefore, we found no direct correlation between serum ALP and ductular fibrosis in our models.

WNT7B REGULATES CHOLANGIOCYTE PROLIFERATION AND DUCTULAR RESPONSE *IN VIVO*

We have previously shown that loss of Wnt secretion from hepatocytes and cholangiocytes leads to fewer cytokeratin 19 (CK19)+Ki67+ cells after DDC treatment; we next assessed the mitogenic role of Wnt7b *in vivo* by quantifying the number of proliferating cell nuclear antigen (PCNA)-positive cholangiocytes after DDC.[14] As expected, both CC KO and HC + CC KO mice on the DDC diet had significantly fewer PCNA-positive cholangiocytes compared to WT mice on the DDC diet (Fig. 5A,B), indicating that loss of Wnt7b directly affects cholangiocyte proliferation. Additionally, we further assessed cell death and injury by terminal deoxynucleotidyl transferase dUTP nick-end labeling and found that neither hepatocyte nor cholangiocyte apoptosis was significantly affected by loss of Wnt7b before or after DDC (Fig. 5C,D).

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis of cholangiocyte markers epithelial cell adhesion molecule, CK-19, and sex-determining region Y-box transcription factor 9 (Sox9) showed that CC KO mice on the DDC diet had sustained expression of cholangiocyte markers compared to WT mice on the DDC diet (Fig. 6A). However, HC + CC KO mice on the DDC diet trended toward decreased cholangiocyte marker expression compared to WT and CC KO mice on the DDC diet (Fig. 6A). These results were also mirrored in histologic images of the early cholangiocyte marker A6. While CC KO mice had sustained ductular reaction compared to WT mice on the DDC diet, HC + CC KO mice on the DDC diet had significantly less ductular response compared to WT mice on the

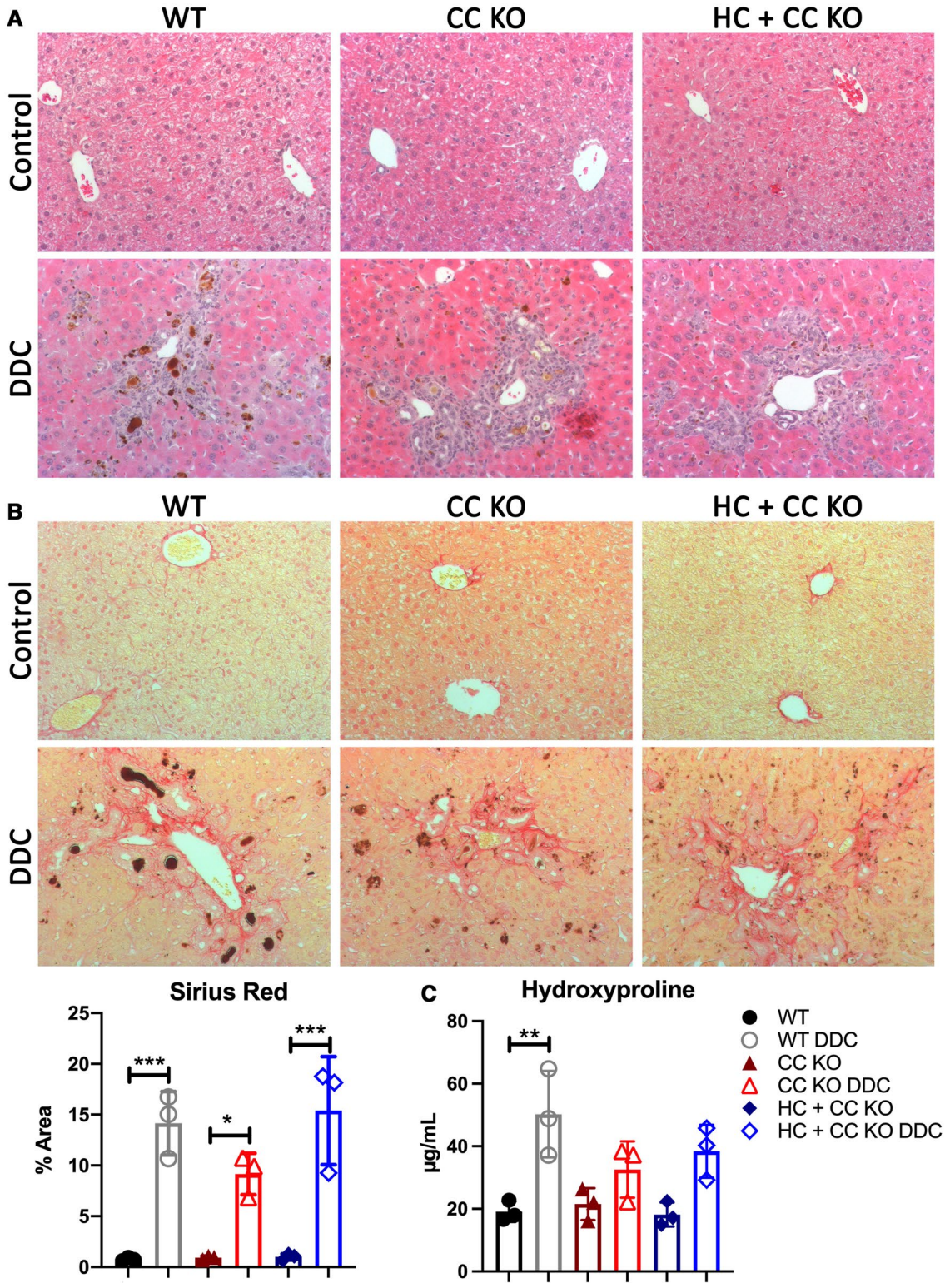

FIG. 4. Wnt7b KO has no effect on ductular response and fibrosis. (A) Hematoxylin and eosin stains of liver sections show no difference in parenchymal injury and ductular response between WT, CC KO, and H + CC KO mice fed a DDC diet (magnification ×200). (B) Sirius red stains of liver sections and quantification of the images show no difference in fibrosis between mice fed the DDC diet; however the CC KO mice trend toward decreased fibrosis (magnification ×200). (C) Hydroxyproline analysis shows no difference in fibrosis between mice fed the DDC diet. Data in B-C represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA.

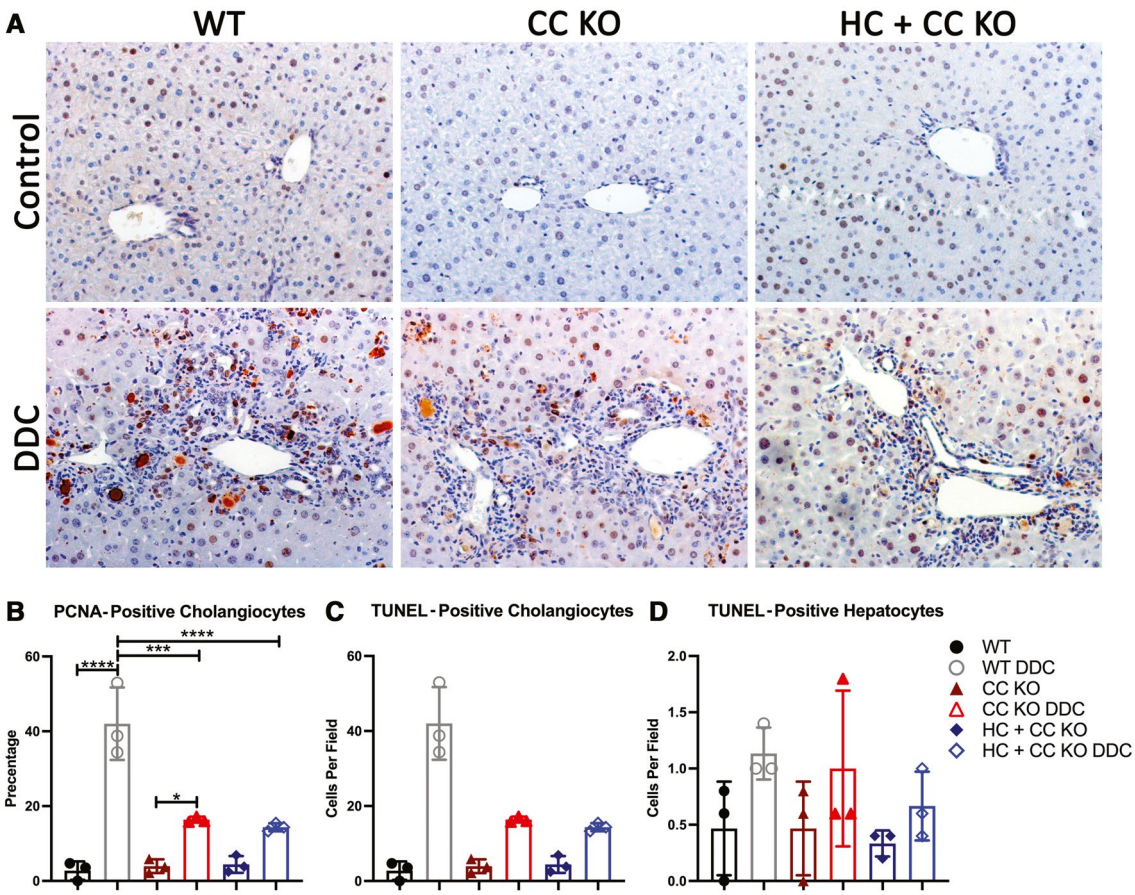

FIG. 5. Wnt7b knockout inhibits cholangiocyte proliferation and does not affect cell death. (A) Representative images (magnification ×200) and (B) quantification of proliferation cell nuclear antigen show that Wnt7b knockout in both the cholangiocyte compartment and the cholangiocyte and hepatocyte compartment inhibits cholangiocyte proliferation in mice fed a DDC diet. Quantification of TUNEL staining shows that neither (C) cholangiocyte nor (D) hepatocyte death is significantly altered by loss of Wnt7b from either CC or HC + CC. Data in B-D represent mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by one-way ANOVA. Abbreviation: TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

DDC diet (Fig. 6B,C). Quantification of immunohistochemistry (IHC) for CK19, a marker of mature cholangiocytes, also confirmed a significant decrease in ductular mass in the HC + CC KO group compared to the WT mice after DDC (Supporting Fig. S5). These results indicate that the suppressed ductular reaction seen in Wnt7b HC + CC KO is a result of decreased cholangiocyte proliferation.

Wnt7b KNOCKOUT PROMOTES HEPATOCYTE-TO-CHOLANGIOCYTE REPROGRAMMING

We also found A6-positive hepatocytes in our mice exposed to the DDC diet. Interestingly, CC KO mice on DDC had more A6-positive hepatocytes than WT mice on the DDC diet, although the number was not significant (Fig. 6D). However, HC + CC KO mice on DDC had a significant amount of A6-positive hepatocytes compared to WT and CC KO mice on the DDC diet (Fig. 6D). These results indicate that loss of Wnt7b may promote hepatocyte expression of cholangiocyte markers or hepatocyte-to-cholangiocyte reprogramming when mice lacking Wnt7b in cholangiocyte and hepatocyte compartments are exposed to a DDC diet.

Cholangiocytes that have undergone a permanent cell-cycle arrest can contribute to the pathogenesis of PSC.[21] To determine if hepatocyte reprogramming had any effect on cellular senescence, we analyzed

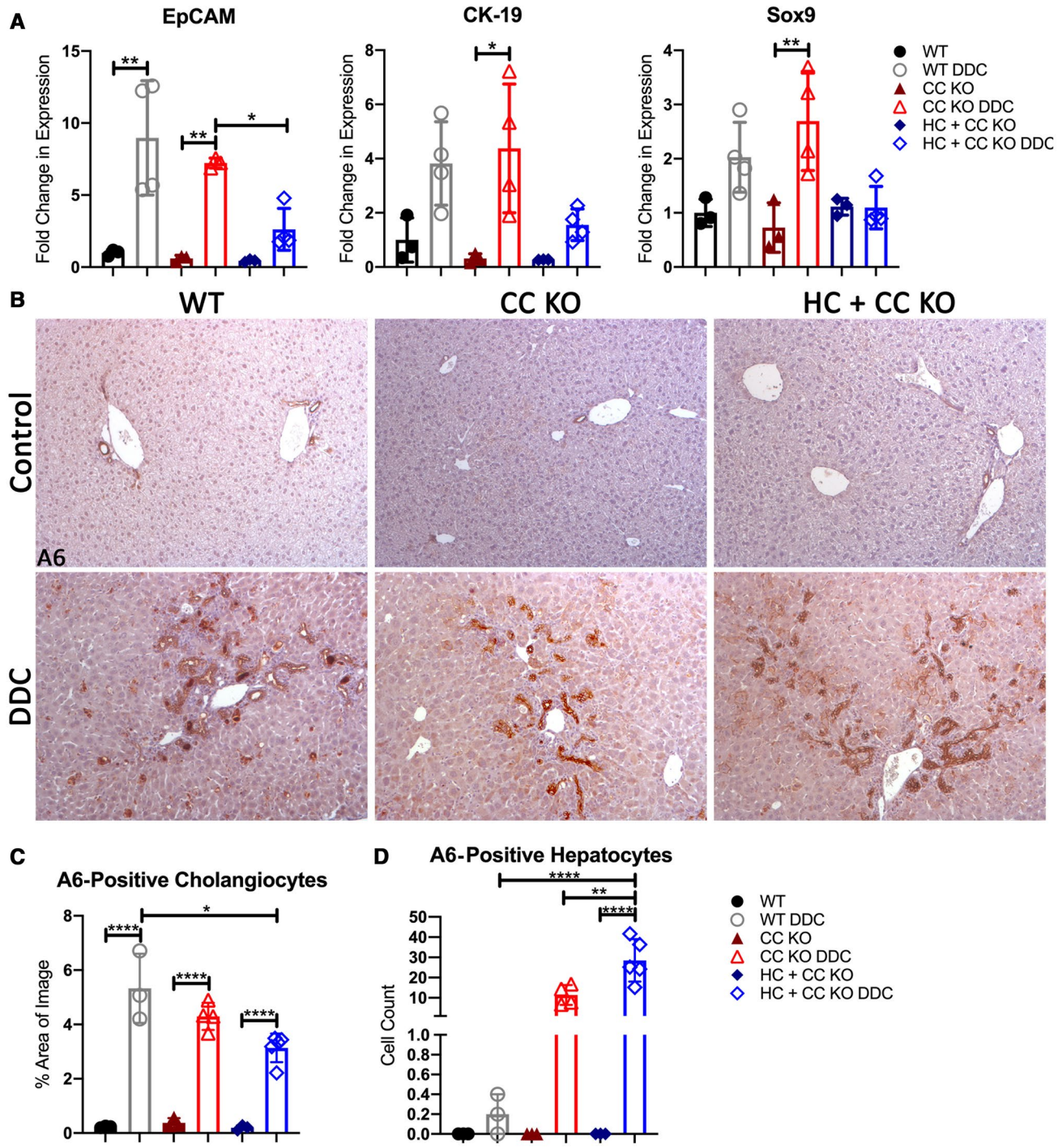

FIG. 6. Wnt7b knockout suppresses ductular reaction and promotes hepatocyte expression of cholangiocyte markers. (A) Quantitative RT-PCR analysis of cholangiocyte markers EpCAM, CK-19, and Sox9 show a significant decrease in EpCAM and a trend toward lower CK-19 and Sox9 in HC + CC KO mice compared to WT mice after DDC. (B) Representative images of cholangiocyte marker A6 show that Wnt7b knockout in both the cholangiocyte compartment and the cholangiocyte and hepatocyte compartment decreases ductular reaction and promotes hepatocyte expression of A6 (magnification ×200). (C) Quantification of A6-positive cholangiocytes shows that HC + CC KO mice on the DDC diet have significantly decreased ductular reaction compared to WT mice on the DDC diet. (D) Quantification of A6-positive hepatocytes shows that that HC + CC KO mice on the DDC diet have significantly more A6-positive hepatocytes compared to WT and CC KO mice on the DDC diet. Data in A, C-D represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ by one-way ANOVA. Abbreviation: EpCAM, epithelial cell adhesion molecule.

the expression of p21 and p16. Although p21 was increased in all genotypes after DDC, HC + CC KO mice had decreased expression compared to WT mice (Supporting Fig. S6A). Similarly, p16, which was increased in both WT and CC KO mice after DDC, was not elevated in HC + CC KO mice (Supporting Fig. S6A). By IHC, we saw numerous p21-positive hepatocytes and cholangiocytes in WT mice after DDC; however, loss of Wnt7b from CC decreased the number of senescent hepatocytes, and loss from both HC and CC significantly decreased the number of both p21-positive hepatocytes and cholangiocytes (Supporting Fig. S6B,C). Thus, an increased number of reprogrammed hepatocytes in HC + CC KO mice correlated with a beneficial effect on cellular senescence.

KO OF Wnt7b FROM BOTH HEPATOCYTES AND CHOLANGIOCYTES RESULTS IN REDUCED BILE ACID ACCUMULATION AND PRODUCTION OF INFLAMMATORY CYTOKINES

To determine the potential functional implications of increased hepatocyte reprogramming, we measured bile acid levels in both total liver and serum. Mice lacking Wnt7b in both hepatocytes and cholangiocytes had significantly lower levels of both hepatic and serum bile acids after DDC compared to either WT or CC KO mice alone (Fig. 7A). To understand if reduced accumulation was due to changes in bile acid metabolism, we next assessed expression of genes involved in bile acid synthesis, detoxification, and transport. Expression of nuclear receptors involved in bile acid synthesis was essentially unchanged in any genotype before or after DDC (Supporting Fig. S7A); however, bile acid uptake was decreased in both WT and HC + CC KO mice after DDC compared to baseline (Supporting Fig. S7C). HC + CC KO mice also showed decreased expression of other genes, such as *small heterodimer partner (SHP)* and *cytochrome P450 (Cyp)2b10*, a bile acid detoxification enzyme, after DDC. (Supporting Fig. S7B,D). Finally, analysis of basolateral bile acid efflux transporters showed decreased expression of *organic solute transporter (Ost)β* and *multidrug resistance-associated protein-3 (Mrp3)* in HC + CC KO mice after DDC compared to both WT and CC KO mice on the control diet, consistent with decreased overall accumulation of bile acids in the liver (Supporting Fig. S7E). Thus, although HC + CC KO exposed to the DDC diet had altered expression of some bile acid metabolism genes, these changes were likely an effect rather than a cause of decreased bile acids and better bile flow, as indicated by lower ALP levels.

Because Wnt7b had induced the expression of several inflammatory modulators *in vitro*, we next assessed the expression of these growth factors and cytokines after the DDC diet. Notably, PDGF-BB was significantly decreased in HC + CC KO mice compared to WT or CC KO mice, as was stem cell factor (SCF) and vimentin, which are both up-regulated in models of biliary injury[22,23] (Fig. 7B). Additionally, IL-1α, IL-6, and TNFα, key cytokines involved in inflammation, were also down-regulated in this model (Fig. 7B). These data support the hypothesis that Wnt7b induces a secretory phenotype in cholangiocytes during cholestasis and its absence reduces biliary inflammation and fibrosis.

β-CATENIN ACTIVATION IS INCREASED IN LIVERS THAT LACK Wnt7b IN HEPATOCYTES AND CHOLANGIOCYTES

Loss of Wnt7b may result in compensatory up-regulation of other Wnts expressed during cholestasis. One of these is Wnt7a, which induces Sox9 expression in a β-catenin-dependent manner.[14] To determine if up-regulation of Wnt7a might be contributing to hepatocyte reprogramming, we examined its expression and found that DDC induced Wnt7a in all three mouse models; however, it was not significantly increased in either CC KO or HC + CC KO mice compared to the WT (Supporting Fig. S8A). Interestingly, however, nonphosphorylated β-catenin, which is an indicator of active canonical Wnt signaling, was higher in HC + CC KO mice on DDC compared to the same mice on the normal diet, whereas phosphorylation of β-catenin at serine 675, which occurs through the cyclic adenosine monophosphate-dependent protein kinase A (PKA) pathway, was unchanged (Supporting Fig. S8B).[24] Thus, the increased number of A6-expressing hepatocytes correlated with activation of canonical β-catenin in HC + CC KO mice (and to a lesser

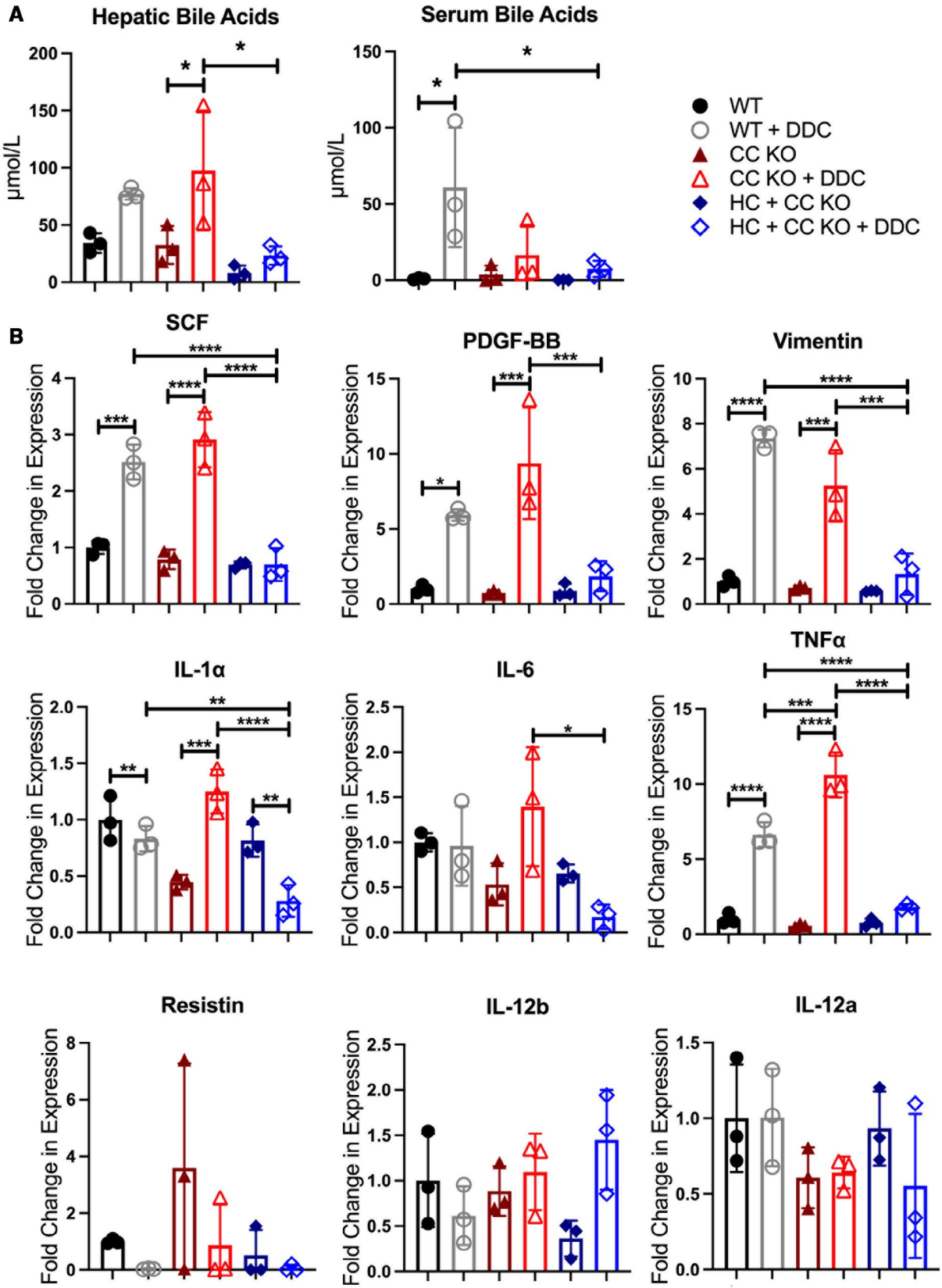
A
Hepatic Bile Acids
Serum Bile Acids
µmol/L
WT
WT + DDC
CC KO
CC KO + DDC
HC + CC KO
HC + CC KO + DDC
B
SCF
PDGF-BB
Vimentin
IL-1α
IL-6
TNFα
Resistin
IL-12b
IL-12a
Fold Change in Expression

FIG. 7. Mice lacking Wnt7b in both their hepatocyte and cholangiocyte compartments have lesser bile acids and decreased expression of commonly altered cytokines after exposure to DDC. (A) HC + CC KO mice on the DDC diet have decreased total hepatic and serum bile acid load compared to WT and CC KO mice on the DDC diet. (B) Quantitative RT-PCR analysis of SCF, PDGF-BB, vimentin, IL-1α, IL-6, and TNFα show that HC + CC KO mice exposed to the DDC diet have significantly decreased expression of these cytokines compared to WT and CC KO mice exposed to the DDC diet. Data in A-B represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by one-way ANOVA.

extent in the CC KO mice), and this phenomenon was independent of Wnt7a.

WNT7B MESSENGER RNA IS UP-REGULATED IN A SUBSET OF PATIENTS WITH PSC

Finally, to demonstrate the clinical relevance of these findings, we analyzed the expression of Wnt7b in livers of patients diagnosed with end-stage PSC. In six out of 16 explanted livers from patients with PSC, we found that expression of Wnt7b messenger RNA was at least 2-fold higher than in normal human livers (Supporting Fig. S9). Additionally, publically available data from 47 healthy and 45 PSC samples were downloaded from the Gene Expression Omnibus database and mined for expression of Wnt7b.[25] The results showed that Wnt7b expression trended higher in patients with PSC, although the results were not significant (two-sided test $P = 0.31$ [for whether healthy expression equals PSC], and one-sided $P = 0.16$ (for whether healthy expression is <PSC). Thus, Wnt7b may be a viable therapeutic target for a subpopulation of patients with PSC.

Discussion

Previous studies have shown that in kidney cells macrophage-derived Wnt7b is critical for regeneration after injury by overcoming a G2 arrest in the cell cycle and preventing apoptosis.[26] Additionally, Wnt7b is expressed in cholangiocarcinoma cells and correlates with disease progression.[13,27] Although *in vitro* studies, we have also shown that Wnt7b plays a role in cholangiocyte proliferation during cholestatic liver injury.[14] As expected, when Wnt7b was deleted from either cholangiocytes alone or hepatocytes and cholangiocytes *in vivo*, these KO mice had decreased numbers of proliferating cholangiocytes during cholestasis. Unexpectedly, despite our initial hypothesis that impaired ductular response would exacerbate cholestatic disease progression in response to DDC, Wnt7b KO mice are actually protected from biliary injury. There is almost a direct relationship between Wnt7b expression and ALP levels in serum; as Wnt7b is deleted from more cell types, biliary injury improves. However, this decrease in injury is not due to the inability of cholangiocytes to proliferate but instead related to the ability of hepatocytes to transdifferentiate into a biliary-like phenotype.

It is well known that hepatocytes are remarkably plastic. When the biliary epithelium is critically injured, hepatocytes can function as "facultative stem cells" and undergo reprogramming from one epithelial cell type to the other to facilitate repair.[8,28] Typically in rodent models, mature hepatocytes transdifferentiate into either fully functional cholangiocytes or a cholangiocyte-like phenotype when there is extensive injury and resident cholangiocytes lose functionality and are incapable of adequately proliferating to compensate for the injury.[6,29-31] Similar results have also been observed in humans. Hepatocytes from both pediatric and adult cholangiopathy patients have been reported to express the ductal/oval cell marker OV-6,[32,33] cholangiocyte-specific cytokeratins,[34-36] and biliary transcription factors.[4,37] These findings from both rodents and humans suggest that the number of hepatocytes expressing biliary markers during biliary injury increases over time. As cholestasis progresses, more hepatocytes compensate for the damage to and loss of the biliary epithelium.

In our models, we found that as more cell types lost Wnt7b, hepatocytes became more likely to express cholangiocyte markers. In WT mice on the DDC diet, biliary injury is repaired through Wnt7b-driven cholangiocyte proliferation and hepatocytes remain hepatocytes. CC KO mice have a few reprogrammed hepatocytes, which once converted to cholangiocytes will self-renew because hepatocyte-derived cholangiocytes in the CC KO mice are able to express Wnt7b (Fig. 8). In HC + CC KO mice, even more hepatocytes become biliary-like because the hepatocyte-derived cholangiocytes do not express Wnt7b; therefore, they cannot self-renew, and more hepatocyte-derived cholangiocytes are needed to help repair the injured bile ducts

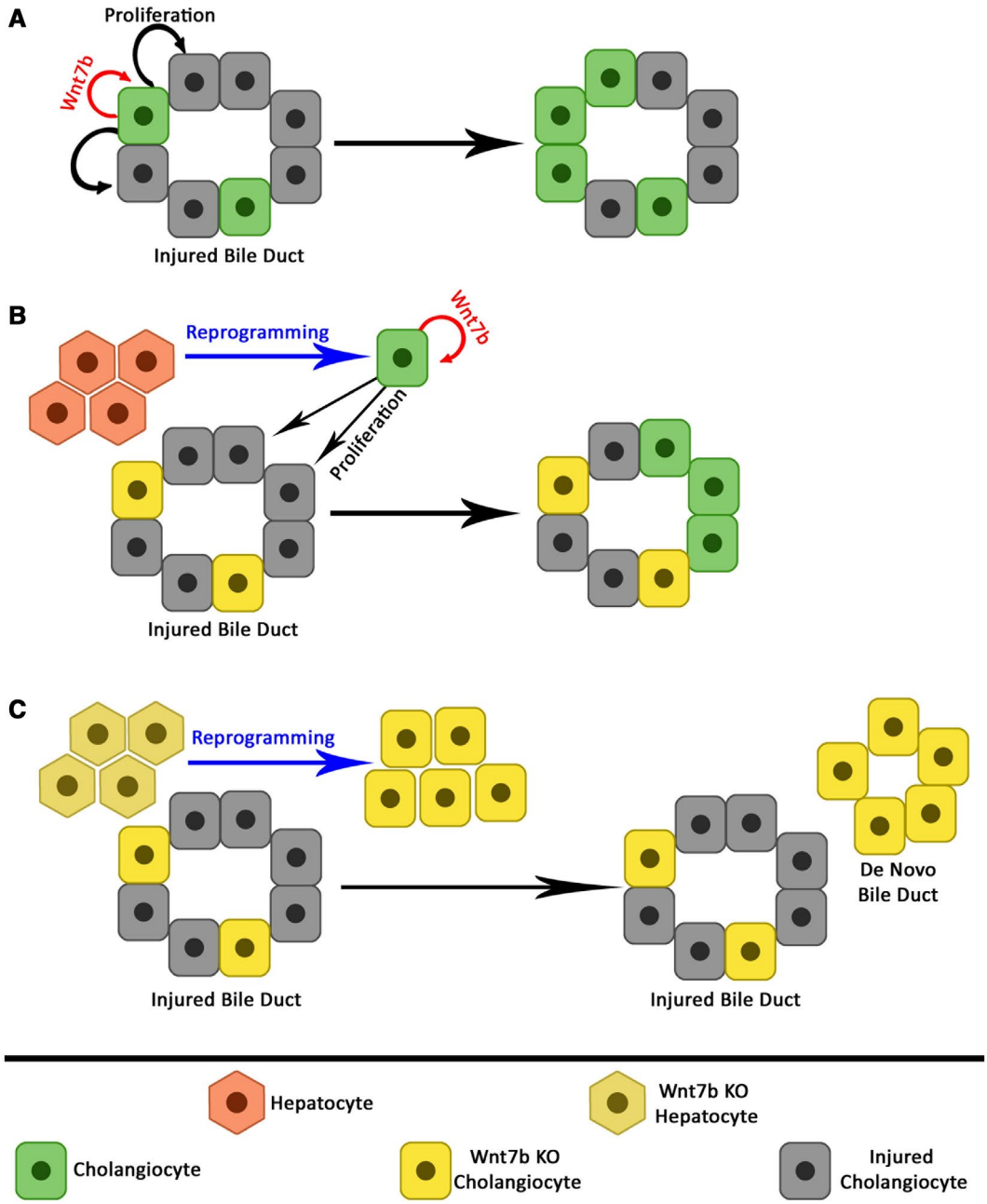

FIG. 8. Schematic of how Wnt7b KO might promote hepatocyte-to-cholangiocyte reprogramming. (A) Cholangiocytes in WT mice exposed to biliary injury attempt to self-renew through Wnt7b expression to repair injured ducts. (B) In CC KO mice, cholangiocytes lacking Wnt7b are unable to proliferate at an efficient rate, so a few hepatocytes begin expressing biliary markers; these cells then self-renew to repopulate the injured ducts. (C) In HC + CC KO mice, cholangiocytes lacking Wnt7b are unable to proliferate, so hepatocytes become cholangiocyte-like. However, these hepatocyte-derived cholangiocytes are also unable to proliferate due to Wnt7b KO, so more hepatocytes must transdifferentiate to alleviate biliary injury.

(Fig. 8). These findings are important because hepatocytes typically do not undergo cellular reprogramming until biliary injury is so extensive that cholangiocytes are unable to compensate. Our study shows that we can induce hepatocytes to begin reprogramming earlier to alleviate injury by blocking cholangiocyte proliferation. In fact, the HC + CC Wnt7b KO mouse might be an ideal model to study hepatocyte reprogramming

during cholestasis because the impaired cholangiocyte proliferation seen in the absence of Wnt7b provides a selective pressure to facilitate this process.

Interestingly, although overexpression of Wnt7b induces cholangiocyte proliferation and acquisition of a reactive phenotype *in vitro*, inhibition of Wnt7b in SMCCs did not inhibit either proliferation or production of inflammatory cytokines and growth factors. This is likely either because the transient duration of siRNA treatment is inadequate to show dramatic changes in cell growth and function over a short time period or because the presence of Wnt7b in the media may have abrogated the effect of siRNA. Nonetheless, it is clear that, *in vivo*, knockout of Wnt7b ameliorates both inflammation and bile acid accumulation after DDC. Although decreased immunomodulatory function in cholangiocytes may be a direct result of Wnt7b loss, the reduction in bile acid levels suggests an improvement in bile flow that may be due to the increased number of biliary-like hepatocytes in the absence of Wnt7b. In lieu of repairing injured cholangiocytes that lack Wnt7b for their own self-renewal, these hepatocytes may be forming *de novo* channels, thus providing a beneficial effect on bile flow or transport. Alternatively, these hepatocyte-derived cholangiocytes may be healthier and less damaged than native cholangiocytes and thus better able to perform vital functions, such as bile modification.

Both Wnt7a and Wnt7b along with Wnt10a are highly expressed in models of cholestasis.[12,14] As Wnt7a induces cellular reprogramming in neighboring hepatocytes in a β-catenin-manner, we hypothesized a compensatory increase in Wnt7a in our Wnt7b KO models (CC or HC + CC) that might account for the increased number of reprogrammed hepatocytes. Interestingly, we did not see a further induction of Wnt7a in either KO model. However, we did note an increase in nonphosphorylated activated β-catenin, which has been shown to induce a biliary phenotype in hepatocytes.[14,38] Therefore, we believe that loss of Wnt7b activates β-catenin through some as-yet unknown mechanism to promote hepatocyte reprogramming. Indeed, TOPflash activity data suggest that overexpression of Wnt7b in cholangiocytes suppresses β-catenin activity, consistent with studies that demonstrated noncanonical Wnts can antagonize β-catenin activation.[39-41] Further mechanistic studies will be needed to determine if the stoichiometry of Wnt7b/Frizzled receptor complex may outcompete binding of canonical Wnts in cholangiocytes during cholestasis.

Beyond its participation in cell-cycle and repair processes, little else is known about the biological function of Wnt7b in the liver. We have shown that overexpression of Wnt7b in a cholangiocyte cell line stimulates secretion of certain cytokines associated with inflammation. During cholestasis, cholangiocytes abandon a differentiated phenotype to repair the bile duct.[42] Under normal conditions, cholangiocytes do not produce an abundance of growth factors and cytokines, but after exogenous or endogenous insult, they acquire the ability to secrete factors that act in an autocrine and paracrine manner to promote remodeling.[20] However, reactive cholangiocytes can also contribute to progression of liver injury by activating hepatic stellate cells and attracting immune cell populations that generate a persistent inflammatory response. Our data suggest that Wnt7b could be one of the switches driving this proliferative/inflammatory phenotype during cholestasis, with unfavorable consequences for disease progression. Modulating this axis to block cholangiocyte proliferation and promote hepatocyte reprogramming could provide a novel treatment that would alleviate biliary injury in a subset of patients with PSC or other cholangiopathies.

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