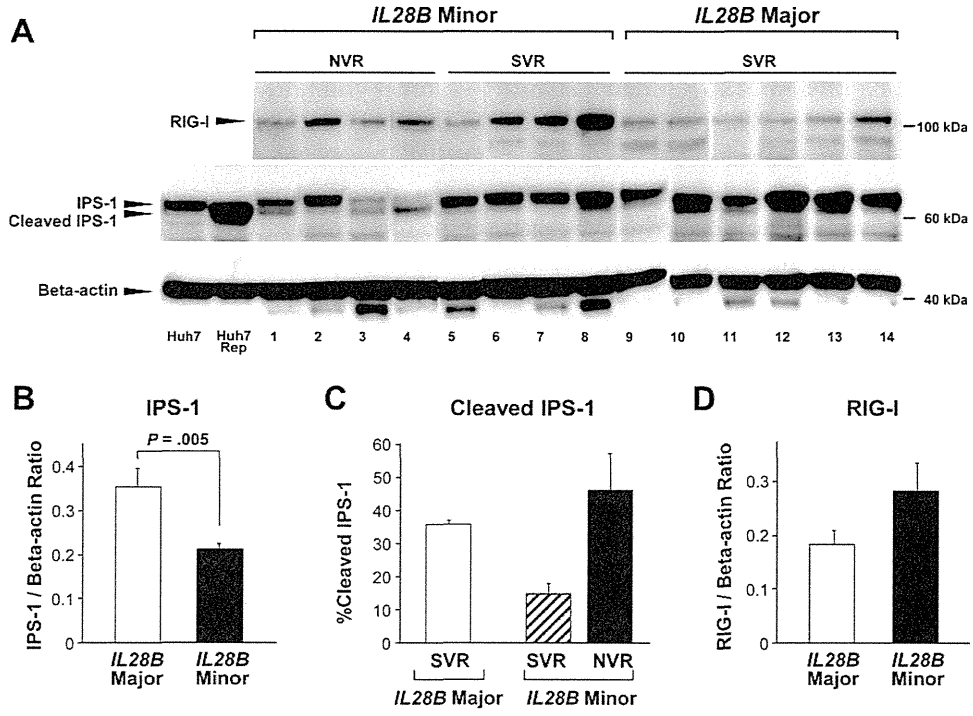

Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and β-actin are indicated by arrows. Naive Huh7 cells were used for a positive control for full-length IPS-1 (lane Huh7), and cells transfected with HCV-1b subgenomic replicon (Reference #20) were used for a positive control for cleaved IPS-1 (lane Huh7 Rep). (B) Total IPS-1 protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error. *P*-value was determined by Mann-Whitney *U* test. (C) Percentage of cleaved IPS-1 products in total IPS-1 protein according to treatment responses stratified by *IL28B* genotype. Error bars indicate standard error. (D) RIG-I protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error.

expression, total hepatic IPS-1 protein expression was significantly lower in *IL28B* minor patients than in *IL28B* major patients (Fig. 5B). With regard to *IL28B* minor patients, the percentage of cleaved IPS-1 protein in total IPS-1 in SVR was lower than that in NVR (Fig. 5C). In contrast to IPS-1 protein expression, hepatic RIG-I protein expression was higher in *IL28B* minor patients than that in *IL28B* major patients (Fig. 5D).

Discussion

In the present study we found that the baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the genetic variation of *IL28B* and final virological outcome in CH-C patients treated with PEG-IFNα/RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFNα/RBV combination therapy is evident, mechanisms responsible for this association remain unknown. *In vitro* studies have suggested that cytoplasmic viral sensors, such as RIG-I and MDA5, play a pivotal role in the regulation of IFN production and augment IFN production through an amplification circuit.[7,8] Our results indicate that expressions of *RIG-I* and *MDA5* and a related amplification system may be up-regulated by endogenous IFN at a higher baseline level in *IL28B* minor patients. However, HCV elimination by subsequent exogenous IFN is insufficient in these patients, as reported,[19] suggesting that *IL28B* minor patients may have adopted a different equilibrium in their innate immune response to HCV. Our data are further supported by recent reports of an association between intrahepatic levels of IFN-stimulated gene expression and PEG-IFNα/RBV response as well as with *IL28B* genotype.[21-23]

In contrast to cytoplasmic viral sensor (*RIG-I, MDA5*, and *LGP2*) and modulator (*ISG15* and *USP18*) expression, the adaptor molecule (*IPS-1*) expression was significantly lower in *IL28B* minor patients. Moreover, western blotting further confirmed IPS-1 protein downregulation in *IL28B* minor patients by revealing decreased protein levels. Because IPS-1 is one of the main target molecules of HCV evasion,[9,18]

transcriptional and translational *IPS-1* expression are probably suppressed by HCV with resistant phenotype, which may be more adaptive in *IL28B* minor patients than in *IL28B* major patients. When we analyzed the proportion of full-length or cleaved IPS-1 to the total IPS-1 protein in a subgroup of *IL28B* minor patients, cleaved IPS-1 product was less dominant in SVR than in NVR, whereas uncleaved full-length IPS-1 protein was more dominant in SVR than in NVR. Therefore, the ability of HCV to evade host innate immunity by cleaving IPS-1 protein and/or host capability of protection from IPS-1 cleavage is probably responsible for the variable treatment responses in *IL28B* minor patients.

Our results indicated a close association between *IL28B* minor patients with higher γ-GTP level and higher frequency of HCV core double mutants, which are known factors for NVR. In contrast, no significant association was observed between *IL28B* genotype and age, gender, or liver fibrosis, which are also known to be unfavorable factors for virological response to PEG-IFNα/RBV. Therefore, certain factors other than the *IL28B* genotype may independently influence virological response. To elucidate whether gene expression involving innate immunity independently associates with a virological response from the *IL28B* genotype, we performed further analysis in a subgroup and conducted a multivariate regression and ROC analyses. Our multivariate and ROC analyses demonstrate that higher expressions of *RIG-I* and *ISG15* as well as a higher ratio of *RIG-I/IPS-1* are independently associated with NVR, and quantification of these values is more useful in predicting final virological response to PEG-IFNα/RBV than determination of *IL28B* genotype in each individual patients. However, the SVR rates in our patients were similar among *IL28B* genotypes, which suggests more SVR patients with the *IL28B* minor allele were included in the present study than those in the general CH-C population. Hence, our data did not necessarily exclude the possibility of the *IL28B* genotype in predicting NVR, although our multivariate analysis could not identify the *IL28B* minor allele as an independent factor for NVR. Interestingly, an association between *IL28B* genotype and expressions of *RIG-I* and *ISG15* as well as *RIG-I/IPS-1* expression ratio is still observed even in patients with the same subgroup of virological response (Fig. 3).

In the present study, although hepatic *IFNλ* expression was observed to be higher in *IL28B* minor and NVR patients, it was not statistically significant. Because *IL28B* shares 98.2% homology with *IL28A*, our primer could not distinguish the expression of *IL28B* from that of *IL28A*, and moreover, we could not specify which cell expresses *IFNλ* (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFNλ* in relation to treatment response need further clarification by specifying type of IFNλ and uncovering the producing cells.

In the present study we included genotype 1b patients because it is imperative to designate a virologically homogenous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have reported that the *RIG-I/IPS-1* ratio was significantly higher in NVR with HCV genotype 2.[19] However, our preliminary results indicated that baseline hepatic *RIG-I* and *ISG15* expression and the *RIG-I/IPS-1* expression ratio is not significantly different among *IL28B* genotypes in patients infected with genotype 2 (Supporting Figure). This may be related to the rarity of NVR with HCV genotype 2 and the lower effect of *IL28B* genotype on virological responses in patients infected with HCV genotype 2.[24] The association among treatment responses in all genotypes, the different status of innate immune responses, and *IL28B* genotype needs to be examined further.

Differences in allele frequency for *IL28B* SNPs among the population groups has been reported. The frequency of *IL28B* major allele among patients with Asian ancestry is higher than that among patients with European and African ancestry.[25] Because *IL28B* polymorphism strongly influences treatment responses within each population group,[5] our data obtained from Japanese patients can be applied to other population groups. However, the rate of SVR having African ancestry was lower than that having European ancestry within the same *IL28B* genotype.[5] Hence, further study is required to clarify whether this difference among the population groups with the same *IL28B* genotype could be explained by differences in expression of genes involved in innate immunity.

In a recent report, an SVR rate of telaprevir with PEG-IFNα/RBV was only 27.6% in *IL28B* minor patients.[26] Because new anti-HCV therapy should still contain PEG-IFNα/RBV as a platform for the therapy, our findings regarding innate immunity in addressing the mechanism of virological response and predicting NVR remain important in this new era of directly acting anti-HCV agents, such as telaprevir and boceprevir.

In conclusion, this clinical study in humans demonstrates the potential relevance of the molecules involved in innate immunity to the genetic variation

of *IL28B* and clinical response to PEG-IFNα/RBV. Both the *IL28B* minor allele and higher expressions of *RIG-I* and *ISG15* as well as higher *RIG-I/IPS-1* ratio are independently associated with NVR. Innate immune responses in *IL28B* minor patients may have adapted to a different equilibrium compared with that in *IL28B* major patients. Our data will advance both understanding of the pathogenesis of HCV resistance and the development of new antiviral therapy targeted toward the innate immune system.

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Wnt5a Signaling Mediates Biliary Differentiation of Fetal Hepatic Stem/Progenitor Cells

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non-canonical Wnt signaling; Calcium/calmodulin-dependent kinase II (CaMKII); hepatic maturation; primitive bile ductal structure; hepatic nuclear factor 1β (HNF1β)

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Abbreviations:

Ab, antibody; ALB, albumin; CaMKII, calcium/calmodulin-dependent kinase II; CK, cytokeratin; CPS, carbamoyl phosphate synthetase; DAPI;

4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; E, embryonic day; EGF, Epidermal growth factor; FCS, fetal calf serum; HGF, hepatocyte growth factor; HNF, hepatic nuclear factor; MRP, multidrug resistance-associated protein; P, postnatal day; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PKC, Protein kinase C; RT, reverse transcriptase; TCF, T-cell factor; TAT, tyrosine aminotransferase; TGF, transforming growth factor; TO, tryptophan-2,3-dioxygenase.

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Abstract

The molecular mechanisms regulating differentiation of fetal hepatic stem/progenitor cells, called *hepatoblasts*, which play pivotal roles in liver development, remain obscure. Wnt signaling pathways regulate the development and differentiation of stem cells in various organs. While a β-catenin-independent non-canonical Wnt pathway is essential for cell adhesion and polarity, the physiological functions of non-canonical Wnt pathways in liver development are unknown. Here we describe a functional role for Wnt5a, a non-canonical Wnt ligand, in the differentiation of mouse hepatoblasts. Wnt5a was expressed in mesenchymal cells and other cells of wild-type mid-gestational fetal liver. We analyzed fetal liver phenotypes in Wnt5a-deficient mice using a combination of histological and molecular techniques. Expression levels of Sox9 and the number of HNF1β-positive HNF4α-negative biliary precursor cells were significantly higher in Wnt5a-deficient liver relative to wild-type liver. In Wnt5a-deficient fetal liver, *in vivo* formation of primitive bile ductal structures was significantly enhanced relative to wild-type littermates. We also investigated the function of Wnt5a protein and downstream signaling molecules using a three-dimensional culture system that included primary hepatoblasts or a hepatic progenitor cell line. *In vitro* differentiation assays showed that Wnt5a retarded the formation of bile duct-like structures in hepatoblasts, leading instead to hepatic

maturation of such cells. While Wnt5a signaling increased steady-state levels of phosphorylated Calcium/calmodulin-dependent protein kinase II (CaMKII) in fetal liver, inhibition of CaMKII activity resulted in the formation of significantly more and larger-sized bile duct-like structures *in vitro* compared with those in vehicle-supplemented controls.

Conclusions: We demonstrate that Wnt5a-mediated signaling in fetal hepatic stem/progenitor cells suppresses biliary differentiation. We also suggest that activation of CaMKII by Wnt5a signaling suppresses biliary differentiation.

Introduction

Hepatic stem cells are multipotent stem cells located within ductal plates in fetal and neonatal livers, and canals of Hering in pediatric and adult livers.[1] The extrahepatic stem cell niches are peribiliary glands within the bile ducts in humans.[2] Hepatic stem/progenitor cells, called hepatoblasts in fetal liver, proliferate actively and give rise to hepatocytes and cholangiocytes.[3, 4] Lineage commitment of such cells can be traced by several cell surface markers, including NCAM, ICAM-1, and EpCAM, in humans.[1, 5] While our group[6] and others[7] demonstrated roles for transcription factors regulating the biliary differentiation of hepatic stem/progenitor cells, the molecular mechanisms behind these events have yet to be fully elucidated.

The Wnt-family secreted ligands and the corresponding Frizzled-family cell surface receptors play a crucial role in the differentiation, proliferation, and self-renewal of stem cells in various organs.[8] Wnt signaling pathways involve interactions between a complex set of molecular cognates that includes 19 different Wnt ligands and 10 Frizzled (Fzd) receptors in humans and mice (reviewed at http://www.stanford.edu/~rnusse/wntwindow.html). Upon binding to Fzd receptors on the surface of a target cell, Wnt proteins activate one of two classes of downstream pathways distinguishable by their dependency on β-catenin. Examples of canonical β-catenin-dependent pathways include β-catenin-dependent activation of T-cell factor

(TCF) by either Wnt1 or Wnt3.[8] In contrast, Wnt4 and Wnt5a activate non-canonical β-catenin independent pathways that include downstream molecules such as Calcium/calmodulin-dependent protein kinase II (CaMKII), Rho-kinase, Rac1, Calcineurin, and Protein kinase C (PKC).[9]

In liver development, β-catenin is known to regulate the maturation, expansion, and survival of hepatoblasts, and its deletion results in increased apoptosis of hepatoblasts in mid-gestational fetal livers.[10] While the function of non-canonical Wnt signaling in liver development is currently unknown, β-catenin-independent Wnt pathways have been shown to function predominantly as regulators of cell polarity and mobility in other organs.[9] In systemic Wnt5a-deficient (KO) mice, the size of caudal structures, lung morphogenesis, and intestinal elongation are also abnormal.[11-13]

Recent reports demonstrate that Wnt5a regulates hematopoietic, mesenchymal, and neural stem cell functions.[14-16] Wnt5a has been shown to increase the repopulation of short- and long-term hematopoietic stem cells by maintaining these cells in a quiescent G0 state.[14] Wnt5a maintains mesenchymal stem cells and promotes osteoblastogenesis in preference to adipogenesis in bone marrow,[15] and also improves the differentiation and functional integration of stem cell-derived dopamine neurons.[16] In healthy adult mouse liver, Wnt5a is expressed in mature hepatocytes and cholangiocytes.[17] Nonetheless, the physiological functions of Wnt5a and the signaling

cascades that it initiates during liver development and in hepatic stem/progenitor cells are unknown.

In this study, we investigated the function of Wnt5a and its downstream targets in the development of murine fetal hepatic stem/progenitor cells. Analysis on Wnt5a KO mice demonstrated that loss of Wnt5a abnormally promotes the formation of bile ductal structures in fetal liver *in vivo*. Wnt5a-supplementation not only retarded the formation of bile-duct like structures, but also promoted hepatic maturation of hepatic stem/progenitor cells *in vitro*. CaMKII activity, which showed Wnt5a-dependence in fetal liver, suppressed the formation of bile-duct like structures. These data indicate that Wnt5a-mediated CaMKII signaling plays an essential role in the differentiation of murine fetal hepatic stem/progenitor cells.

Materials and Methods

Animals

Systemic Wnt5a KO mice in C57BL/6 background were originally generated by Yamaguchi et al.[11] Wnt5a KO mice and wild type (WT) littermates were produced by crossbreeding Wnt5a heterozygous mice. All animals were treated based on the guidelines of the Institute of Medical Science, University of Tokyo and those of Tokyo Medical and Dental University.

In vitro bile duct-like differentiation assay of primary hepatoblasts

Bile duct-like differentiation assays were performed as previously described[6] with some modifications. Fetal hepatic cells of E14.5 liver were dissociated with collagenase[4] and Dlk^+ cells were isolated from the resulting population using a magnetic cell sorter (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and then cultured in collagen gel (Nitta Gelatin, Osaka, Japan). After 30 minutes of incubation at 37°C on basal-layer collagen, 1 or 2×10^4 cells were suspended in 1 ml DMEM/F12, mixed with 1 ml collagen gel solution, and plated onto basal-layer collagen in 6-well culture dishes. Plated cells were cultured for 7 days with an additional 2 ml DMEM supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO), 1×insulin/transferrin/selenium, 20 ng/ml epidermal growth factor (EGF, PeproTech,

Rocky Hill, NJ), 20 ng/ml hepatocyte growth factor (HGF, PeproTech) and 25 ng/ml tumor necrosis factor α (PeproTech).

In vitro bile duct-like differentiation assay of hepatic progenitor cell line

The HPPL liver progenitor cell line has been reported to exhibit characteristics of differentiated cholangiocytes in three-dimensional culture.[18, 19] As in the previous report, we maintained HPPL cells in DMEM/F-12 containing 10% FCS, 1× insulin/transferrin/selenium, 10 mM nicotinamide, 10^{-7} M Dex, and 5 ng/ml HGF and EGF and suspended cells in a mixture of type I collagen and Engelbreth-Holm-Swarm sarcoma gel (EHS gel, Becton Dickinson, Bedford, MA) at a density of 4×10^4 cells/ml. Cell suspension was added to each cell culture insert (Millipore, Billerica, MA) and after incubation at 37°C for 2 h, 500 μl of DMEM/F-12 with growth factors was added above and below the insert and the cells were cultured for seven days. To test the effects of inhibitors of CaMKII, Rho-kinase, Rac1, Calcineurin, and PKC on HPPL differentiation, KN93, KN92, KN62, Y-27632, NSC23766, Cyclosporin A, and Go6976 (see *Materials* in Supplementary file) were added individually to the culture medium when each three-dimensional culture was initiated. Independent analyses were performed in triplicate and 5 fields were randomly selected for counting the cysts that indicate bile duct-like differentiation of cells.

In vitro hepatic maturation assay of primary hepatoblasts

To induce hepatic differentiation, primary hepatoblasts from WT E14.5 mice were cultured as previously described.[6] Briefly, 2.5×10^5 MACS-isolated Dlk^+ cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1× non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-7} M Dex in each well of a 6-well gelatin-coated dish. After 5 days, the resulting cells were supplemented with medium containing 20% EHS gel for an additional 2 days prior to analysis.

Materials and methods providing details of materials, cell isolation, Hematoxylin-eosin (HE) staining, RT-PCR analysis, immunostaining, immunoblot analysis, Wnt5a-blocking experiments, microarray analysis, and statistics procedures are described in the Supplementary file.

Results

Expressions of Wnt5a and Frizzled receptors during liver development

We first analyzed *Wnt5a* expression during liver development using quantitative RT-PCR. *Wnt5a* expression was detected in fetal and neonatal livers of WT mice and showed a gradual increase during liver development (Fig. 1A). To investigate Wnt5a expression in mid-gestational fetal liver, we purified the fractions of hepatoblasts, mesenchymal cells, mesothelial cells, endothelial cells, and hematopoietic cells from E14.5 liver using FACS. Quantitative RT-PCR analysis indicated that *Wnt5a* was expressed in hepatoblasts, mesenchymal cells, mesothelial cells, endothelial cells, and hematopoietic cells. The expression level of Wnt5a was significantly higher in mesenchymal cells than in hepatoblasts and other types of cells in mid-gestational fetal liver (Fig. 1B). Frizzled is a family of cell surface receptors for Wnt ligands. Adult hepatocytes from 12-week-old mice served as the control. RT-PCR analysis of E14.5 hepatoblasts resulted in detection of 9 of 10 Fzd receptors (all except *Fzd9*), whereas E14.5 hematopoietic cells expressed 9 of 10 Fzd receptors (all except *Fzd2,* Fig. 1C and supplementary Fig. 1).

Loss of Wnt5a promotes the formation of bile duct in fetal liver

Since one of the reported phenotypes of systemic Wnt5a KO mice was postpartum

death[11], we investigated the function of Wnt5a in liver development using mid- to late-gestational fetuses. We determined that although average liver weight in Wnt5a KO E18.5 fetal mice was significantly lower than in WT littermates, the average ratio of liver:body weight in the KO mice was not significantly different from the ratio in WT mice (supplementary Fig. 2).

Histological analysis of E18.5 livers showed that the number of luminal spaces around the portal vein, which we interpret to be primitive bile ducts, was greater in Wnt5a KO mice than in WT mice (Fig. 2A). To further investigate these changes in bile duct development, expression of *Sox9* (a representative transcriptional factor expressed in biliary precursor cells)[20] was analyzed. Expression levels of *Sox9* were significantly higher in Wnt5a KO E16.5 fetal livers relative to WT livers (Fig. 2B). The Notch pathway plays an essential role in the morphogenesis of bile duct structures.[21] Expression levels of *Notch1*, *Notch2*, and *Jagged1* were significantly higher in Wnt5a KO E16.5 fetal livers relative to WT livers (supplementary Fig. 3A). Numbers of $Hes1^{+}$ cells in E18.5 livers were significantly greater in Wnt5a KO mice than in WT mice (supplementary Fig. 3B). Expression levels of *Cyclin D1* and *c-Myc* (target transcripts of canonical β-catenin-dependent Wnt pathway) in Wnt5a KO livers were equal to those in WT livers (supplementary Fig. 4A). We tried to assess the protein level of Sox9, however, immunostaining analysis of Sox9 did not work well probably due to technical

problems (data not shown).

During normal liver development, hepatoblasts located around the portal vein develop as hepatocyte nuclear factor (HNF)1β^{+}HNF4α^{-} biliary precursor cells.[22] In normal E16.5 fetal livers, monolayer rings of biliary precursor cells, termed ductal plates, can be detected.[23] Two-day older WT E18.5-stage fetal livers contained primitive ductal structures (PDS) consisting of multiple HNF1β^{+}CK19^{+}-cell lumina (Fig. 2C).

Immunohistological analysis revealed that numbers of HNF1β^{+}HNF4α^{-} biliary precursor cells in E16.5 livers (Fig. 2D) and in PDS formed by these cells in E18.5 livers (Fig. 2E) were significantly higher in Wnt5a KO mice relative to WT mice. Double staining of CK19 and entactin (a component of basement membrane) confirmed that the number of PDS formed by CK19^{+} cells was also significantly higher in E18.5 Wnt5a KO liver relative to the WT (Fig. 2F). These results demonstrate clearly that loss of Wnt5a excessively promotes the formation of bile ducts in fetal liver.

Expression analysis of fetal livers in Wnt5a KO mice

Expression of genes coincident with hepatic maturation was also analyzed in Wnt5a KO fetal livers using quantitative RT-PCR. In E16.5 fetal livers, ALB and HNF4α mRNA levels were nearly equal between WT and Wnt5a KO mice. Similarly, we observed no significant differences between WT and Wnt5a KO E18.5 fetal livers

with regard to copy numbers of tyrosine aminotransferase (TAT), carbamoyl phosphate synthetase 1 (CPS1), glucose-6-phosphatase (G6Pase), or HNF4α mRNAs (supplementary Figs. 5A and B). These data suggest that the maturation of hepatoblasts to hepatocytes is not impaired in Wnt5a KO mice.

Proliferation of fetal liver cells in Wnt5a KO mice was analyzed by immunoblot and immunostaining. Immunoblot analysis revealed that PCNA production in Wnt5a KO livers was almost equal to that in WT livers (supplementary Fig. 4B). Numbers of $CK19^{+}PCNA^{+}$ cells in E18.5 were almost equal to those in WT livers (supplementary Fig. 4C). Changes in gene expression in Wnt5a KO livers were analyzed using cDNA microarray analysis (supplementary Fig. 5C and supplementary Table 5). Cluster analysis revealed that several molecules associated with amino acid metabolism and cell migration were upregulated or downregulated in Wnt5a KO fetal livers compared with those in WT livers.

Wnt5a retards formation of bile duct-like structures from primary hepatoblasts

In collagen gel-embedding culture, mouse primary hepatoblasts differentiate into bile duct-like branching structures, coincident with the expression of biliary cell-specific genes such as CK19 (Fig. 3A, left panel).[6] To investigate the effects of Wnt5a on differentiation of hepatoblasts into biliary cells *in vitro*, we cultured primary

hepatoblasts derived from E14.5 WT fetal livers, and assessed the formation of bile duct-like branching structures.

We observed that cells in cultures derived from E14.5 WT fetal liver formed approximately 10 colonies (consisting of >100 cells in large branching structures) per 1×10^4 cells (Fig. 3A, right panel); colonies with medium (50-99 cells) or small (10-49 cells) branching structures also were noted. In cultures supplemented with Wnt5a, there were significant decreases in the average number colonies with large- and medium-sized branching structures relative to vehicle-only controls.

Wnt5a suppresses cyst formation derived from HPPL in three-dimensional culture

To assess the potential of hepatic stem/progenitor cells for bile-duct like luminal formation, we used an HPPL three-dimensional culture system.[19] HPPL is established from mouse E14 Dlk^+ hepatoblasts, and differentiates into hepatic and cholangiocytic lineages.[18] In this system, HPPL cells form cysts that exhibit characteristics of differentiated cholangiocytes producing CK19, E-cadherin, and other characteristic markers. We categorized HPPL-derived colonies into one of three classes: colonies without clear lumina, small cysts (50-100 μm diameter with clear lumina), and large cysts (>100 μm diameter with clear lumina). As previously described,[19] immunocytostaining of cultured cells showed that colonies without clear lumina

produced both the hepatic marker ALB and the biliary marker CK19, suggesting incomplete terminal differentiation. Cells in the luminal walls of small and large cysts, in contrast, produced CK19 but not ALB, indicating their differentiation to a cholangiocyte lineage (supplementary Fig. 6). Vehicle-only controls or cultures treated with Wnt3a did not show a significant difference in overall number of cysts. In contrast, cultures supplemented with Wnt5a displayed significantly fewer cysts, due both to an absence of large cysts and a significantly reduced number of small cysts (Fig. 3B). Wnt5a is expressed in HPPL cells (supplementary Fig. 7A). We verified the specificity of effect of Wnt5a by blocking experiments. Cultures supplemented with anti-Wnt5a Ab resulted in a significant increase in numbers of HPPL-derived cysts relative to control Ab, and blocked the effects of Wnt5a supplementation (Fig. 3C). Numbers of HPPL-derived cysts were higher in cultures supplemented with Wnt5a-specific inhibitor relative to vehicle-only controls (supplementary Fig. 7B).

Immunoblot analysis indicated that CK19 production in HPPL-derived colonies were significantly down-regulated in cultured cells supplemented with Wnt5a relative to vehicle-supplemented controls, whereas the levels of ALB, AFP and PCNA did not change (Fig. 3D). Expression analysis of HPPL-derived colonies revealed that HNF1β, Notch1 and multidrug resistance-associated protein 3 (MRP3, a key primary active transporter in biliary cells) were significantly downregulated in cultured cells