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 supplemented with Wnt5a relative to vehicle-supplemented controls (Fig. 3E). HNF1β and Sox9 were significantly upregulated in cultured cells supplemented with anti-Wnt5a Ab relative to control Ab (supplementary Fig. 7C), whereas the levels of hepatocytic markers did not change (supplementary Fig. 7D). Consistent with our *in vivo* results, these data indicate that Wnt5a suppresses bile duct-like cyst formation of fetal hepatic progenitor cells *in vitro*.

Wnt5a induces the expression of hepatic maturation markers in primary hepatoblasts in vitro

We evaluated the potential of primary hepatoblasts for hepatic maturation using an *in vitro* hepatic differentiation assay.[24] Phase-contrast microscopy after addition of EHS gel identified several morphological changes within cells, including formation of highly condensed cytosol, and clear, round nuclei typical to mature hepatocytes (Fig. 4A, middle panel). Since similar gross morphological changes were also seen in cells cultured in the presence of Wnt5a (right panel), we used quantitative RT-PCR to measure the effect of Wnt5a on expression of hepatic maturation marker genes in stem/progenitor cells. Expression of TAT and CPS1 in cultured cells increased significantly with supplemental Wnt5a (Fig. 4B), whereas changes in TO, G6Pase, and HNF4α mRNA levels were not significantly different. These results indicate that Wnt5a contributes, in part, to primary hepatoblast maturation. Taken together, our *in vitro* data demonstrate that Wnt5a retards biliary differentiation and promotes hepatic differentiation of hepatoblasts.

Inhibition of CaMKII activity promotes the formation of bile-duct like cysts derived from HPPL

While Wnt5a is known to stimulate several signaling cascades, including CaMKII, Rho-kinase, Rac1, Calcineurin, and PKC, the specific cascade triggered by Wnt5a in hepatic stem/progenitor cells is unknown. To address this question, we analyzed the effects of specific inhibitors of these candidate molecules in HPPL-derived cysts, where Wnt5a is expressed (Supplementary Fig. 7A). Relative to controls, inhibitors specific to CaMKII (KN93 and KN62) resulted in a significant increase in numbers of both small and large bile-duct like cysts derived from HPPL (Figs. 5A and 5B). In contrast, other inhibitors, including Y-27632 (Rho-kinase inhibitor), NSC23766 (Rac1 inhibitor), Cyclosporin A (Calcineurin inhibitor) and Go6976 (PKC inhibitor), had no effect on the number or size of HPPL-derived cysts (Fig. 5C). We examined the expression of biliary markers in HPPL-derived cysts treated with CaMKII inhibitor (KN62). Expression of MRP3, a key primary active transporter in biliary cells, in HPPL-derived cysts increased significantly with supplemental CaMKII inhibitor (Fig. 5D). There were no significant differences in mRNA levels of ALB, HNF4α, and β-catenin-related molecules between HPPL-derived cysts treated with CaMKII inhibitor and those treated with vehicle (supplementary Fig. 8). The protein level of AFP in HPPL-derived cysts treated with CaMKII inhibitor was lower than that in vehicle-supplemented controls, whereas the levels of CK19 and PCNA did not change (Fig. 5E). These data indicate that CaMKII activity suppresses the formation of HPPL-derived cysts, whereas activities of other Wnt5a-mediated candidates did not influence the efficacy of cyst formation.

Phosphorylation of CaMKII in primary hepatoblasts

To investigate the activation state of CaMKII in fetal and neonatal WT livers, we used immunoblots of liver homogenates derived from E14.5, E16.5, E18.5, postnatal day (P) 1, P7, and P14 mice to measure CaMKII phosphorylation levels. Phosphorylation at threonine-286, specifically, has been reported to maintain CaMKII in an active state.[25] Phosphorylation of PKC, a kinase that did not affect cyst formation in HPPL cells, was also examined. While we detected both phosphorylated CaMKII (p-CaMKII) and PKC (p-PKC) in each fetal and neonatal liver homogenate, levels of phosphorylated CaMKII increased gradually over time (supplementary Fig. 9A, top panel), similar to the pattern of Wnt5a expression during liver development (Fig. 1A).

In contrast, developmental changes in the steady-state levels and phosphorylation of PKC in these samples (supplementary Fig. 9A, lower panels) did not correspond to Wnt5a expression patterns.

Using immunostaining of FACS-purified primary hepatoblasts with anti p-CaMKII Ab, we detected p-CaMKII in >90% of FACS-purified primary hepatoblasts (supplementary Fig. 9B, upper panels); p-PKC was also detected with anti p-PKC antibodies in these cells (supplementary Fig. 9B, lower panels). These data demonstrate that both CaMKII and PKC are in an active state in primary hepatoblasts.

Wnt5a regulates the phosphorylation of CaMKII in fetal liver

To verify whether CaMKII activation is controlled by Wnt5a, levels of p-CaMKII in HPPL grown in the absence or presence of Wnt5a were examined. Immunoblot analysis showed that Wnt5a stimulation increased the level of phosphorylated CaMKII, with p-CaMKII levels peaking 3 hours after Wnt5a supplementation and then decreasing to baseline levels after 12 hours (Fig. 6A and supplementary Fig. 10A). Similar to a previous report,[15] total CaMKII protein levels in HPPL also increased after CaMKII activation. Ratios of p-CaMKII/CaMKII also increased, peaking 3 hours after Wnt5a supplementation (supplementary Fig. 10B). In contrast, Wnt5a had no effect on p-PKC and p-Rac1 levels in HPPL (supplementary Figs. 10C and D) nor on nuclear translocation of NFAT (representative downstream molecule of Calcineurin; data not shown).

We also tested the combined effect of Wnt5a plus a CaMKII inhibitor (KN62) on cyst formation in HPPL-derived cells. The number and size of cysts in HPPL-derived cells decreased with Wnt5a alone, and increased with CaMKII inhibitor alone. When used in combination (HPPL treated with both CaMKII inhibitor plus Wnt5a), the number and size of cysts was similar to CaMKII inhibitor alone, and significantly higher than cells treated with Wnt5a alone (Fig. 6B and C).

We also used immunoblots to compare p-CaMKII levels in WT and Wnt5a KO fetal liver homogenates. Levels of p-CaMKII were significantly lower in Wnt5a KO relative to WT fetal livers (Fig. 6D); quantification using densitometry revealed that p-CaMKII levels in Wnt5a KO livers were also significantly lower than those in littermate WT livers (supplementary Fig. 10E), indicating that Wnt5a mediates an increase in CaMKII phosphorylation in fetal liver.

Discussion

This study provides the first evidence of a physiological role for Wnt5a in liver development, in that Wnt5a was observed to suppress the formation of bile ducts derived from hepatoblasts. Our data showed increased expression of Sox9, Notch1, Notch2, and Jagged1 in Wnt5a KO livers (Fig. 2B and supplementary Fig. 3A), as well as abnormally increased formation of primitive ductal structures (Fig. 2E and F). In Wnt5a KO livers, the numbers of $HNF1\beta^{+}HNF4\alpha^{-}$ biliary precursor cells and primitive ductal structures were increased around the portal vein only (zone 1), whereas such cells were not observed in zone 2 or 3 (Fig. 2D-F). At E14.5 stage, $HNF1\beta^{+}HNF4\alpha^{-}$ biliary precursor cells were not detected in Wnt5a KO livers similar to WT livers (supplementary Fig. 11A). These results suggested that lineage commitment of hepatoblasts into biliary cells is determined by the microenvironment around the portal vein, depending on the presence or absence of Wnt5a protein. The lung and intestine of systemic Wnt5a KO were abnormal, while tissue structures of pancreas and kidney were almost normal (supplementary Fig. 12). Immunostaining analysis showed that $p75NTR^{+}$ cells were detected in E18.5 Wnt5a KO livers, similar to WT livers (supplementary Fig. 11B). These results implied that development of mesenchymal cells in E18.5 Wnt5a KO livers is not impaired compared with that in littermate WT livers. Wnt5a expression was significantly higher in mesenchymal cells than in hepatoblasts or other types of cells in mid-gestational WT fetal liver (Fig. 1B). Thus, the microenvironment around the portal vein, which consists of mesenchymal cells, other types of cells, and extracellular matrices, regulates appropriate cell fate decision of hepatoblasts, whereas loss of Wnt5a in such developmental niche leads to abnormally increased formation of primitive ductal structures (Fig. 7). Further investigation of this hypothesis will require conditional deletion of Wnt5a-downstream molecules in hepatoblasts at late-gestational fetal stages.

Maturation of hepatoblasts to a hepatocyte lineage is regulated by several factors, including oncostatin M, HGF, and extracellular matrices.[24] Our data showed that hepatic maturation of primary hepatic stem/ progenitor cells was promoted in cultures supplemented with Wnt5a (Fig. 4A and B). On the other hand, no significant changes in hepatocyte-marker expression were detected in Wnt5a KO relative to WT livers. It may be that there is functional redundancy among different Wnt-family ligands *in vivo*, since several non-canonical-signaling Wnt ligands (Wnt4, Wnt5a, and Wnt11) are expressed in normal fetal liver.[26] In support of the hypothesis that other non-canonical Wnt ligands may compensate for Wnt5a, supplementary Figure 13A shows that Wnt4 expression levels in liver increase significantly in Wnt5a KO versus WT littermates. These data strongly support our hypothesis that the effect of Wnt5a on hepatic maturation is compensated by other non-canonical Wnt ligands, such as Wnt4.

CaMKII, a serine/threonine protein kinase present in essentially every tissue, regulates important functions including modulation of ion channel activity, cellular transport, and cell morphology in neural tissues.[27] A Wnt5a-CaMKII pathway has been reported to induce osteoblastogenesis by attenuating adipogenesis in mesenchymal bone marrow stem cells.[15] Our results show that in liver, inhibition of CaMKII activity promoted bile duct-like cyst formation (Fig. 5A and B), and that phosphorylation of CaMKII is dependent on Wnt5a stimulation (Fig. 6). While these results provide strong support to our hypothesis that Wnt5a stimulates CaMKII in hepatoblasts, we have not identified which molecules function downstream of CaMKII.

CaMKII has been reported to activate the TGFβ-activated kinase 1(TAK1)-Nemo-like kinase (NLK) pathway, and that resulting phosphorylation of TCF inhibits β-catenin dependent transcription.[28] On the other hand, CaMKII-TAK1-NLK signaling induces bone-marrow mesenchymal stem cells to undergo osteoblastogenesis depending on specific downstream signaling cascades.[15] Our expression analysis showed that expression levels of *Cyclin D1* and *c-Myc* (the direct target molecules of β-catenin activation) did not change in Wnt5a KO mice *in vivo* (supplementary Fig. 4) nor in HPPL-derived cysts treated with CaMKII inhibitor *in vitro* (supplementary Fig. 8), compared with the respective control samples. Preliminary data (not shown) demonstrated that the levels of TAK1 mRNA and protein during development did not correlate with those of Wnt5a and p-CaMKII in whole liver lysates. Moreover, Wnt5a stimulation did not increase the level of activated β-catenin in HPPL (supplementary Fig. 13B and C). These results suggest that the Wnt5a-CaMKII pathway does not activate β-catenin in hepatoblasts. On the other hand, Wnt5a stimulation increased the level of stabilized p53 (phosphorylated at Ser15) in HPPL (supplementary Fig. 13B and D), suggesting that stabilization of p53 is associated with Wnt5a-CaMKII signaling. Further study will be needed to clarify this issue.

Recent studies have shown pathological roles for Wnt5a in various organs: Addition of recombinant Wnt5a significantly reduced the migratory capacity of colorectal cancer cell line.[29] Whereas increased Wnt5a expression correlates with advanced stages of gastric cancer with poor prognosis,[30] there is no definitive data about Wnt5a in the progression of hepatocellular carcinomas. In this study, we reveal one function of Wnt5a in fetal liver in the suppression the biliary differentiation of hepatic stem/progenitor cells. To clarify the pathological role of Wnt5a in liver disease, inducible systemic Wnt5a KO mice or liver-specific CaMKII KO mice would be needed in future studies. Any future evidence demonstrating a role for Wnt5a in adult hepatic stem/progenitor cells and cancer stem cells may lead to studies of Wnt5a signaling as a therapeutic target against abnormal bile ductal formation in the liver or cholangiocellular carcinoma.

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Acknowledgement

We thank Prof. A. Miyajima (the University of Tokyo) for the gift of anti-CK19 antibody. We thank K. Itoh, K. Okada, and A. Yanagida (Institute of Medical Science, the University of Tokyo) for excellent technical assistance.

Figure Legends

Figure 1. Expression analyses of Wnt5a and Frizzled receptors during liver development. (A) Quantitative RT-PCR analysis of Wnt5a in fetal and neonatal livers. Embryonic E14, E16, E18 and postnatal P7, and P14 indicate Wnt5a expression in whole livers derived from wild-type (WT) mice at these days of development, respectively. Values represent the ratio of Wnt5a at each stage relative to expression of this RNA in E14.5 fetal liver following normalization of template copy number to β-actin. Bars represent mean ± SD of 3 separate experiments. (B) Quantitative RT-PCR analysis of Wnt5a. Lane a, $CD45^-Ter119^-CD71^-Dlk^{high}$ cells from E14.5 liver (hepatoblasts); b, $CD45^-Ter119^-CD71^-PDGFR^+$ cells from E14.5 liver (mesenchymal cells); c: $CD45^-Ter119^-CD71^-PCLP\text{-}1^+$ cells from E14.5 liver (mesothelial cells); d, $CD45^-Ter119^-CD71^-Flk1^+$ cells from E14.5 liver (endothelial cells); e, $CD45^+Ter119^+CD71^+$ cells from E14.5 liver (hematopoietic cells). Lanes a, b, c, d, and e were normalized by numbers of β-actin copies quantified by TaqMan-PCR analysis;

equal numbers of copies were applied as templates. Wnt5a expression was significantly higher in mesenchymal cells than in hepatoblasts, mesothelial cells, endothelial cells and hematopoietic cells. Bars represent mean ± SD of 3 separate experiments. *$p<0.05$. (C) Expression of Frizzled (Fzd) family. Lane 1, hepatoblasts ($CD45^-Ter119^-Dlk^{high}$ cells) purified from E14.5 liver; 2, hematopoietic cells ($CD45^+Ter119^+$ cells) from E14.5 liver; 3, adult hepatocytes from 12-week-old mouse liver; 4, negative control (distilled water); 5, samples without reverse-transcriptase reaction (negative controls for false-positive amplification of genomic DNA); 6, positive control. RT-PCR products of Fzd receptors are indicated. Images shown are representative of 3 separate experiments.

Figure 2. Loss of Wnt5a excessively promotes the formation of bile duct in fetal liver. (A) Representative images depicting luminal spaces around the portal vein in E18.5 Wnt5a knock out (KO) and littermate WT livers stained with hematoxylin and eosin. (B) Quantitative RT-PCR analysis of the cholangiocyte marker Sox9 is depicted as the ratio of Sox9 copy number in E16.5 Wnt5a KO livers relative to WT livers (all normalized to β-actin). Steady-state levels of Sox9 mRNA were significantly higher in Wnt5a KO livers relative to WT livers. (C) Representative images of immunostained sections from E18.5 WT livers. Left panel; double immunostaining using CK19 (red)

and entactin (green) antibodies. Right panel; double immunostaining using HNF1β (green) and HNF4α (red) antibodies. Inset black-and-white frames depict high-power-field images of cells with positive staining for CK19 (left panel) and HNF1β (right panel). (D, E) Left 2 panels; immunostaining of HNF1β (green) and HNF4α (red) in E16.5 (D) and E18.5 (E) livers. Right panel (D); number of HNF1β^{+}HNF4α^{-} cells in 10 random fields examined in WT and Wnt5a KO livers. Right panel (E); number of primitive ductal structures (PDS) in 10 random fields examined in WT and Wnt5a KO livers. (F) Left panel; immunostaining of CK19 (red) and entactin (green) in E18.5 livers. Right panel; numbers of PDS in 10 random fields of WT and Wnt5a KO livers. Arrowheads indicate PDS. Images shown are representative of 3 independent experiments. Bars in dot-plot graphs represent mean ± SEM of values shown. $^{*}p<0.05$. $^{**}p<0.01$. Scale bars: 50 μm.

Figure 3. Wnt5a suppresses formation of bile duct-like structures derived from hepatic stem/progenitor cells. (A) Bile duct-like branching structures derived from primary hepatoblasts. Left panel; representative view of bile duct-like branching structures consisting of >100 cells derived from primary hepatoblasts. Colonies were immunostained with CK19 (green) and counterstained with DAPI (blue). Scale bar: 100 μm. Right panel; numbers of colonies demonstrating branching structures in cultures

supplemented with 100 ng/ml Wnt5a or vehicle only. Numbers of small (consisting of 10-49 cells), medium-sized (50-99 cells), and large (>100 cells) branching structures per one well were counted. (B) Numbers of bile duct-like cysts derived from the hepatic stem/progenitor cell line (HPPL) in 5 random fields per well in cultures supplemented with 100 ng/ml Wnt5a, 100 ng/ml Wnt3a, or vehicle only (left panel). There were significantly fewer small cysts (50-100 μm diameter with clear lumina) and large cysts (diameter >100 μm with clear lumina) in cultures supplemented with Wnt5a relative to vehicle only. Right panel; representative views of cysts in HPPL three-dimensional cultures supplemented with either vehicle, Wnt3a or Wnt5a. Scale bars: 50 μm. (C). Numbers of bile duct-like cysts derived from HPPL in 5 random fields per well in cultures supplemented with either control IgG, anti-Wnt5a antibody (Ab), or both anti-Wnt5a Ab plus recombinant Wnt5a protein. Cultures treated with anti-Wnt5a Ab resulted in a significant increase in total numbers of bile-duct like cysts derived from HPPL, and blocked the effect of Wnt5a supplementation. (D). Immunoblot analysis of CK19, Albumin (ALB), α-fetoprotein (AFP), and proliferating cell nuclear antigen (PCNA) in HPPL-derived cysts treated with Wnt5a. CK19 production in HPPL-derived cysts treated with Wnt5a was downregulated relative to that with vehicle-supplemented controls, whereas protein levels of ALB, AFP, and PCNA did not change. Lane 1-3 and Lane 4-6 are vehicle-supplemented controls and Wnt5a-supplemented