HPPL-derived cysts, respectively. (E) Expression analysis of HPPL-derived cysts treated with Wnt5a. Expression levels of HNF1β, multidrug resistance-associated protein 3 (MRP3), and Notch1 in HPPL-derived cysts in medium supplemented with Wnt5a were significantly lower than those in HPPL-derived cysts in medium supplemented with vehicle, indicating that Wnt5a retarded biliary maturation of HPPL cysts. Results represent the mean ± SD of 3 separate experiments. *$p<0.05$. **$p<0.01$.

Figure 4. Expression of hepatic maturation markers under the culture supplemented with Wnt5a. (A) Phase contrast images of the cultured primary hepatoblasts induced to mature to hepatocytes with EHS gel alone or EHS gel plus 100 ng/ml Wnt5a. (B) Expression levels of tyrosine amino transferase (TAT), carbamoyl phosphate synthetase 1 (CPS1), tryptophan-2,3-oxygenase (TO), glucose-6-phosphatase (G6Pase), and hepatocyte nuclear factor (HNF) 4α are depicted as the ratio of copy mRNA number in cells treated with EHS gel alone or EHS gel plus 100 ng/ml Wnt5a for 7 days relative to control cells. Hc, primary adult hepatocytes (positive control). All samples were normalized by numbers of β-actin copies quantified by TaqMan-PCR analysis; equal numbers of copies were applied as templates. Results represent the mean ± SD of 3 independent experiments. *$p<0.05$.

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Figure 5. Inhibitors of Calcium/calmodulin-dependent kinase II (CaMKII) increased the number and size of bile duct-like cysts derived from HPPL. (A) Inhibitors specific for CaMKII activity (KN62 and KN 93) were added at the beginning of HPPL three-dimensional culture. Numbers of total cysts, small cysts and large cysts increased significantly in medium supplemented with KN62 or KN93. Cultures treated with DMSO alone (vehicle) or KN92 (an inactive analogue of KN93) served as negative controls for KN62 (vehicle) and KN93, respectively. (B) Representative DAPI-stained (blue, left panels) or phase contrast confocal microscopy images (right panels) of bile duct-like cysts. (C) Numbers of total cysts were not changed by the inhibitors of Rho kinase (Y-27632), Rac1 (NSC23766), Calcineurin (Cyclosporin A, CyA), or Protein kinase C (PKC, Go6976). Vehicle-only treatments (distilled water or DMSO) served as negative controls for Y-27632 (in distilled water), NSC23766 (in distilled water), CyA (in DMSO), and Go6976 (in DMSO). (D) Expression of multidrug resistance-associated protein 3 (MRP3) in HPPL cysts. MRP3 expression was significantly increased in medium supplemented with CaMKII inhibitor (KN62), suggesting that CaMKII inhibitor promoted biliary maturation of HPPL cysts. (E) Immunoblot analysis of CK19, AFP and PCNA in HPPL-derived cysts treated with vehicle (DMSO) or CaMKII inhibitor (KN62). The level of AFP in HPPL-derived cysts treated with CaMKII inhibitor was lower than that in vehicle-supplemented controls,

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whereas the levels of CK19 and PCNA did not change. Results represent the mean ± SD of 3 independent experiments. *p<0.01. **p<0.05. ***p<0.01. Scale bars: 100 μm.

Figure 6. Phosphorylation of CaMKII is regulated by Wnt5a stimulation in fetal liver. (A) Immunoblot analysis of p-CaMKII, p-PKC, and p-Rac1 in HPPL at pretreatment (0), and then 0.5, 1, 3, 6, 12, and 24 hours after stimulation by Wnt5a. Homogenate of whole E14.5 embryo served as a positive control (PTC). Wnt5a treatment increased the levels of both total CaMKII and p-CaMKII in HPPL, but did not change the levels of p-PKC and p-Rac1. (B) Representative phase-contrast images of cysts derived from HPPL supplemented either with vehicle (DMSO), 100 ng/ml Wnt5a, CaMKII inhibitor (KN62), or 100 ng/ml Wnt5a plus CaMKII inhibitor. (C) Numbers of bile duct-like cysts derived from HPPL in 5 random fields per well in cultures supplemented with vehicle (DMSO), Wnt5a, CaMKII inhibitor (KN62), or Wnt5a plus CaMKII inhibitor. The effect of Wnt5a on HPPL cysts was cancelled by KN62 treatment. (D) Immunoblot analysis of p-CaMKII in E16.5 WT and Wnt5a KO livers demonstrating a decrease in p-CaMKII level in Wnt5a KO livers. Mice 1-5 and Mice 6-10 are E16.5 WT and Wnt5a KOs, respectively. Results are represented as mean ± SD of 3 individual experiments. *p<0.05. Scale bars: 100 μm.

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Figure 7. Schema for the biliary differentiation of hepatoblasts in Wnt5a KO liver. Wnt5a is expressed in mesenchymal cells and other types of cells in mid-gestational fetal liver, and increases the level of CaMKII activation in hepatoblasts. The microenvironment around the portal vein, which consists of mesenchymal cells, other types of cells, and extracellular matrices, regulates appropriate differentiation of hepatoblasts into biliary cells, whereas loss of Wnt5a in such microenvironment leads to downregulation of CaMKII activation in hepatoblasts and abnormally increased formation of bile ducts.

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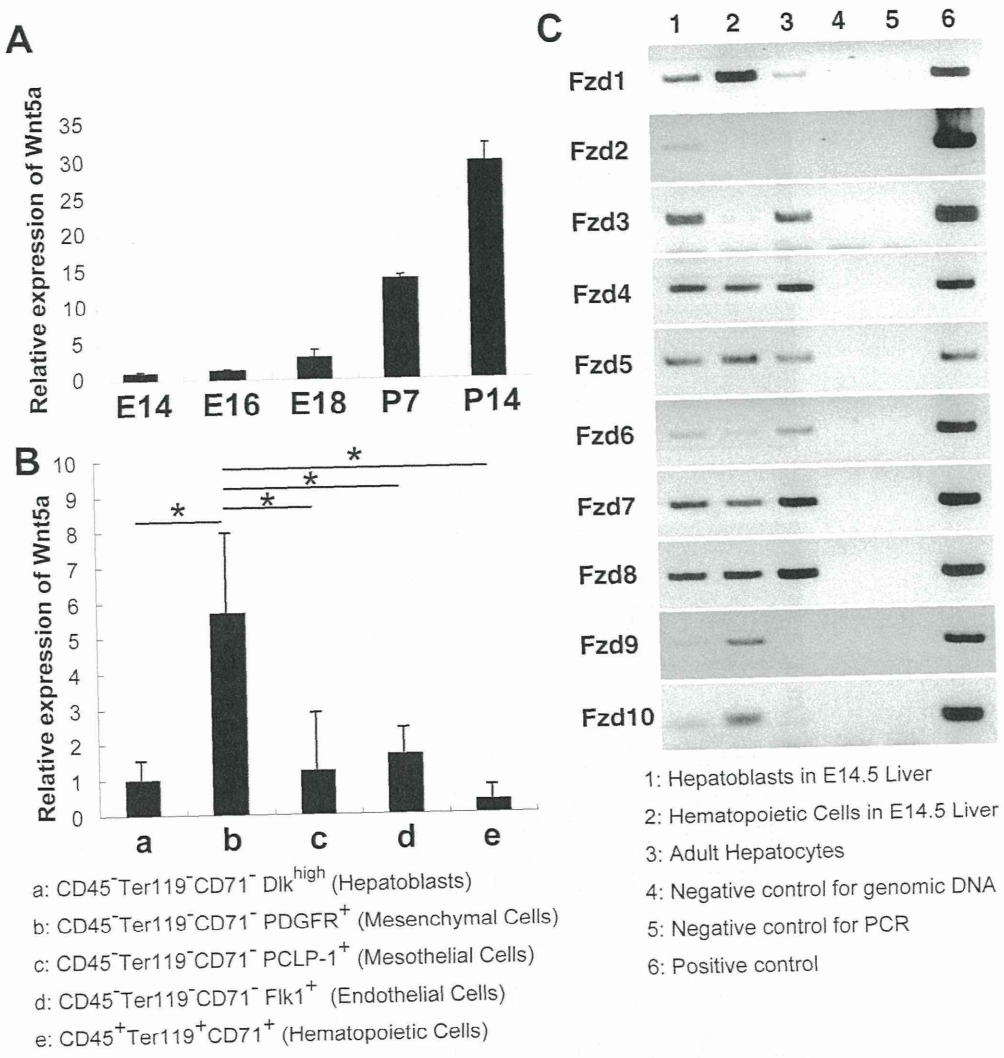

a: $CD45^{-}Ter119^{-}CD71^{-}$ Dlk^{high} (Hepatoblasts)

b: $CD45^{-}Ter119^{-}CD71^{-}$ $PDGFR^{+}$ (Mesenchymal Cells)

c: $CD45^{-}Ter119^{-}CD71^{-}$ $PCLP\text{-}1^{+}$ (Mesothelial Cells)

d: $CD45^{-}Ter119^{-}CD71^{-}$ $Flk1^{+}$ (Endothelial Cells)

e: $CD45^{+}Ter119^{+}CD71^{+}$ (Hematopoietic Cells)

1: Hepatoblasts in E14.5 Liver

2: Hematopoietic Cells in E14.5 Liver

3: Adult Hepatocytes

4: Negative control for genomic DNA

5: Negative control for PCR

6: Positive control

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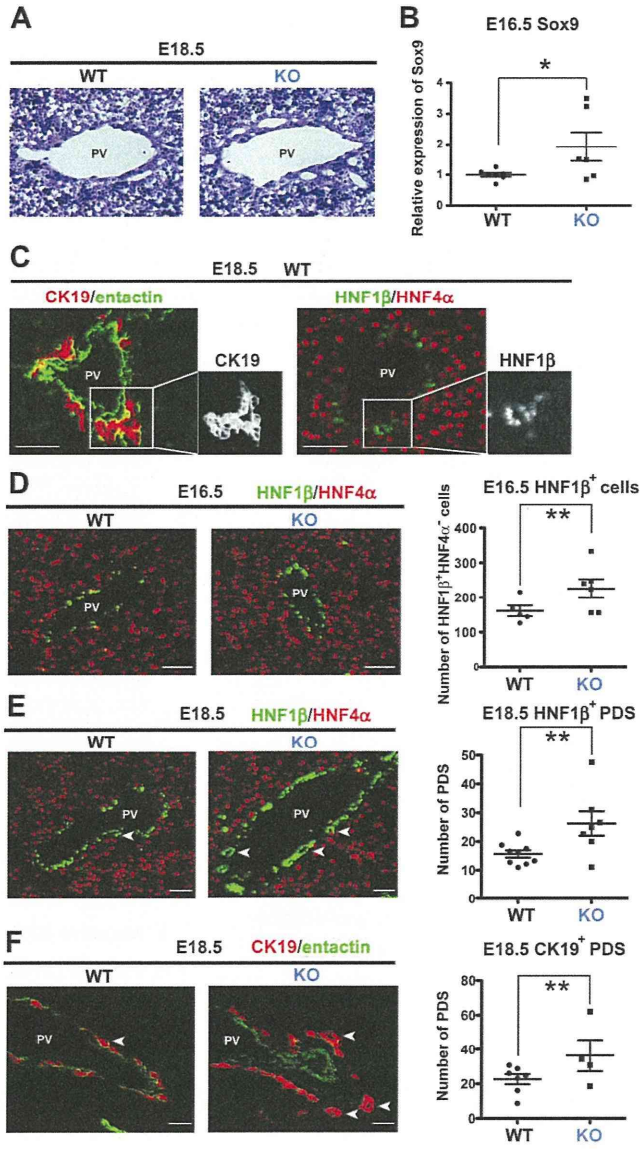

Kiyohashi *et al.* Figure 2

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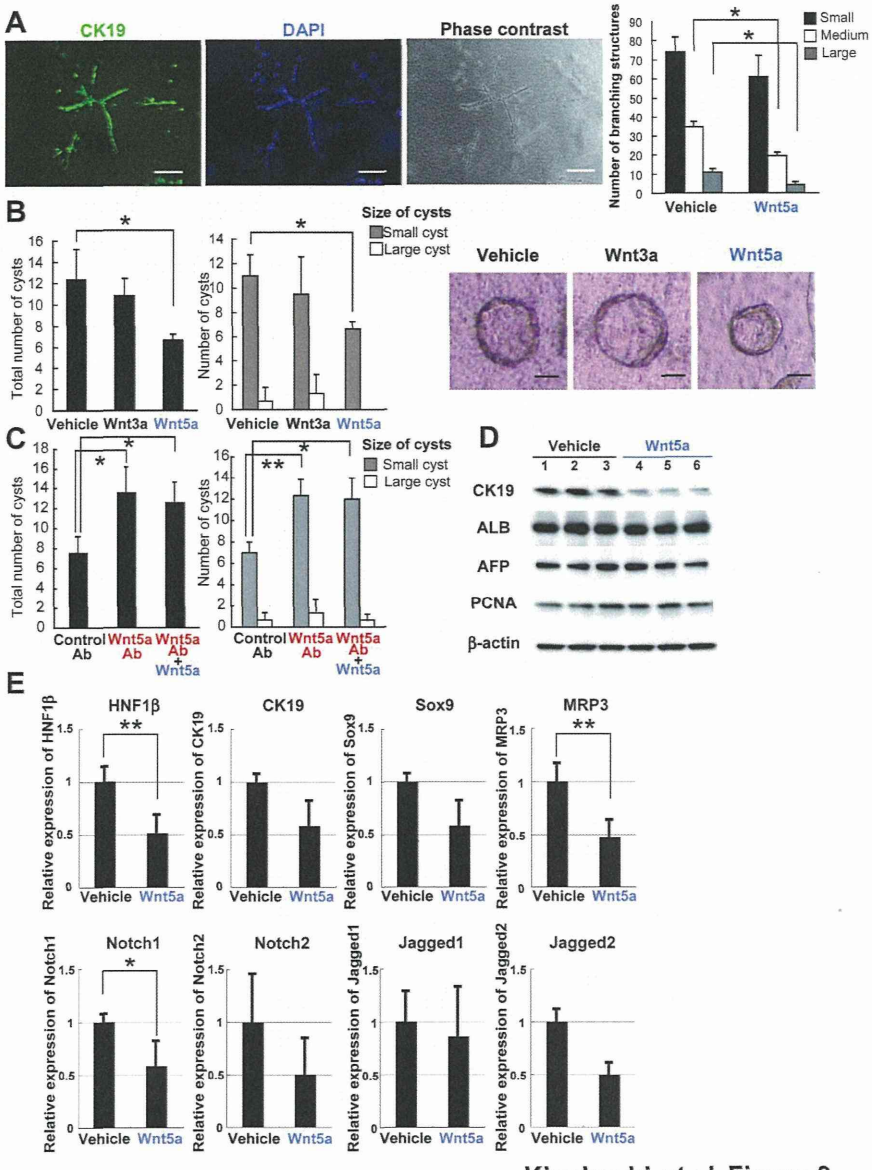

Kiyohashi *et al.* Figure 3

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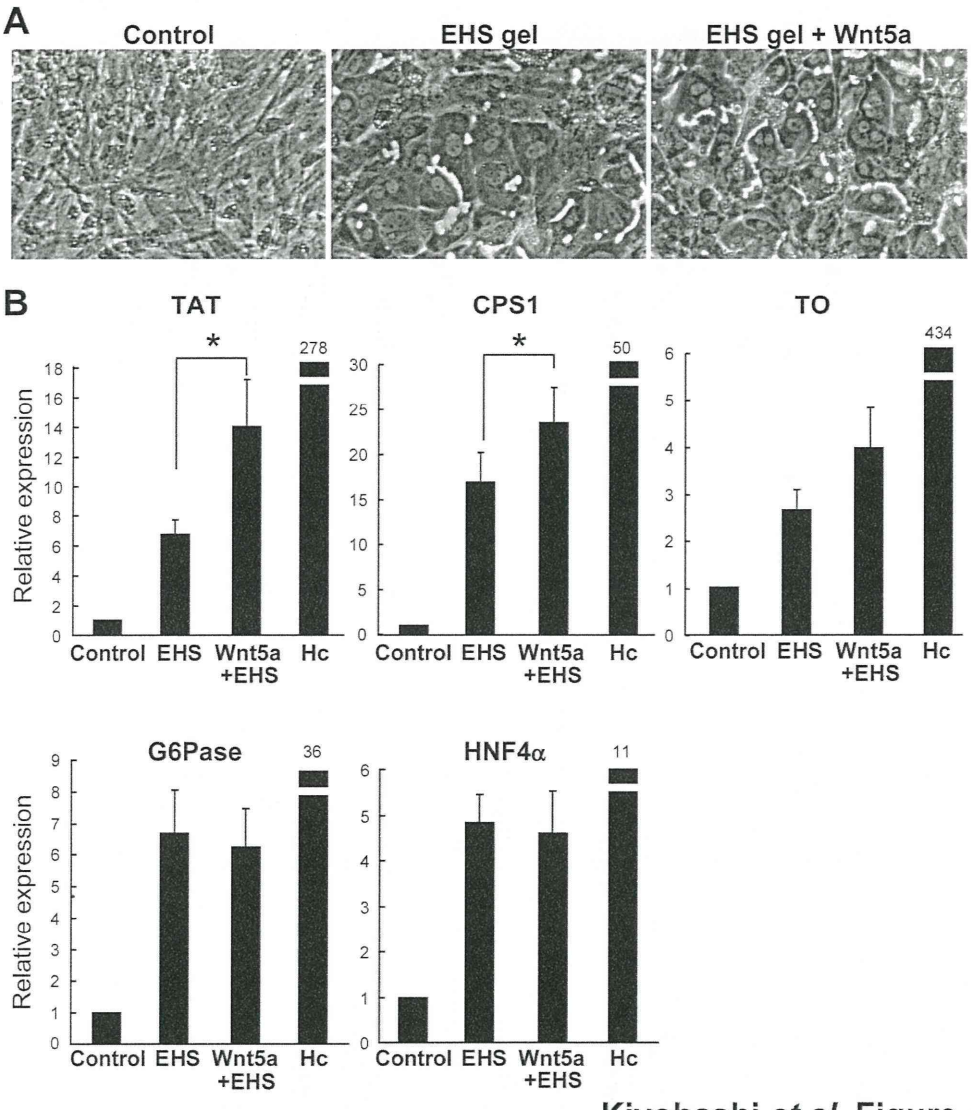

Kiyohashi *et al.* Figure 4

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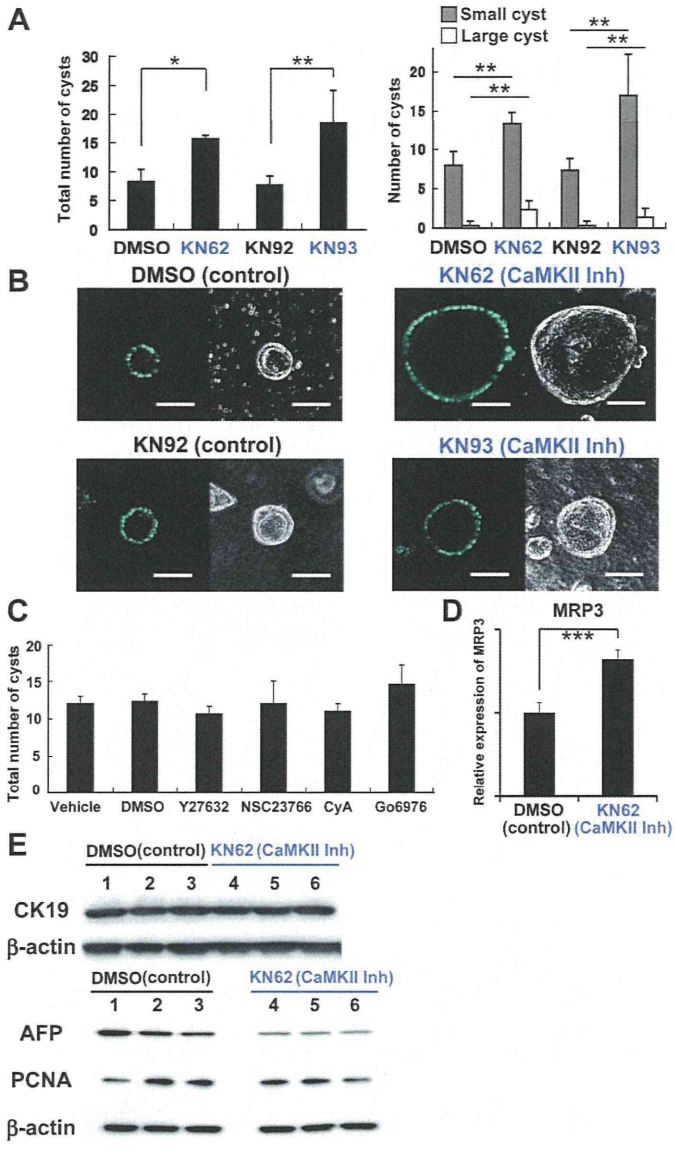

Kiyohashi *et al.* Figure 5

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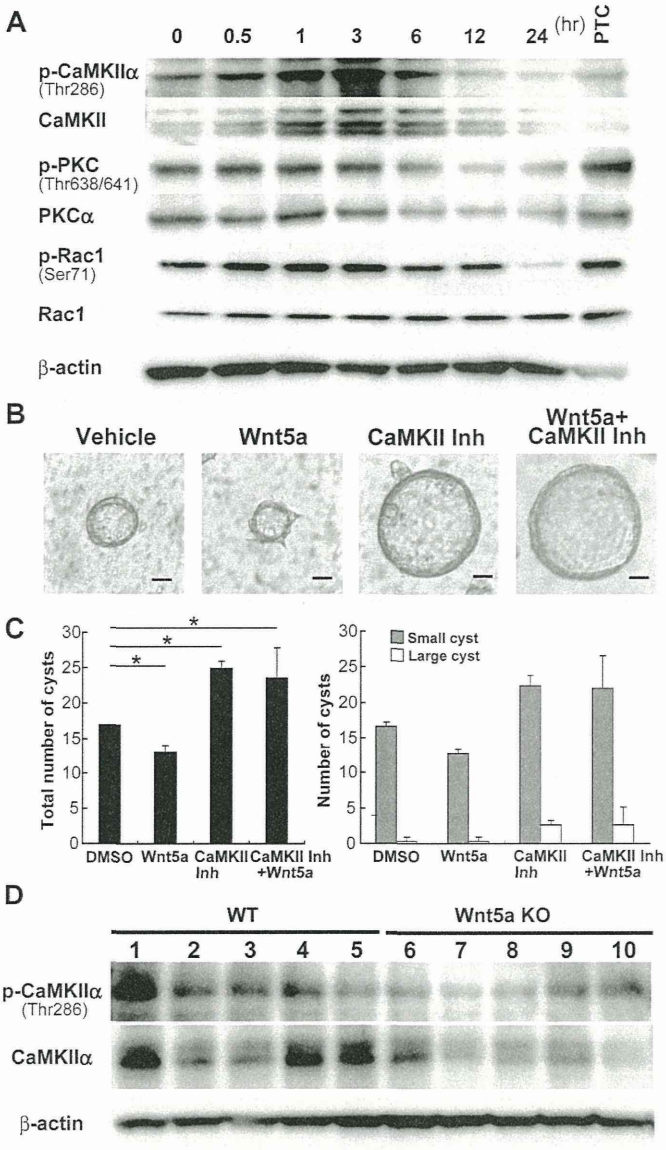

Kiyohashi *et al.* Figure 6

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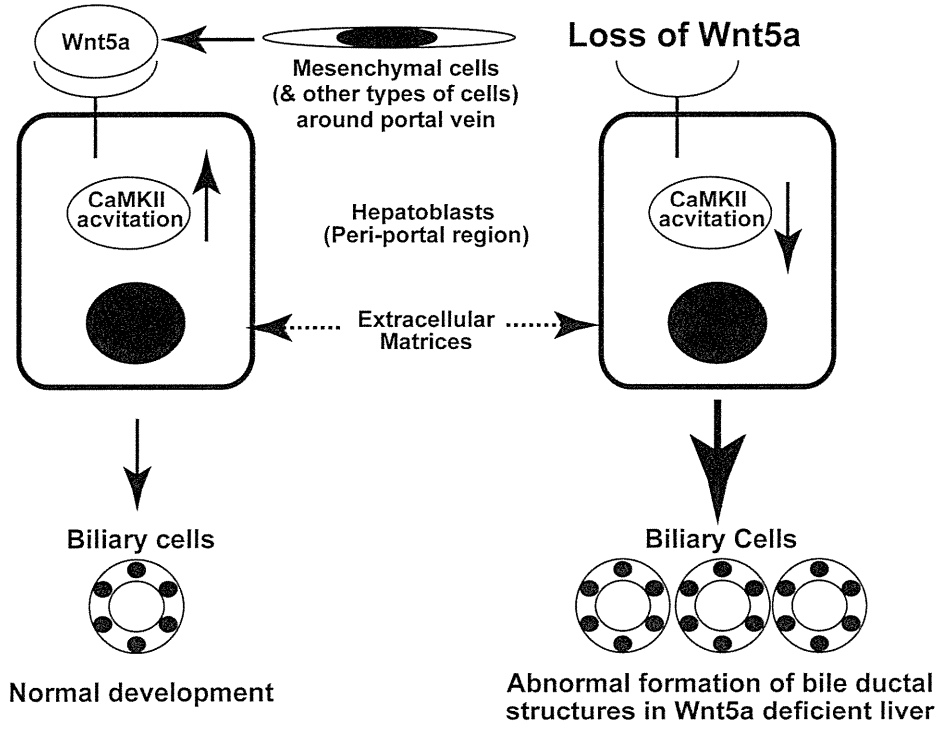

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Supplementary Materials and Methods

Materials.

Reagents and suppliers were: Dulbecco's modified Eagle's medium (DMEM), DMEM/F-12, fetal calf serum (FCS), penicillin/streptomycin/L-glutamine (100×), dexamethasone (Dex), non-essential amino acid solution, dimethyl sulfoxide. Sigma (St. Louis, MO); Epidermal growth factor (EGF), hepatocyte growth factor (HGF) and tumor necrosis factor (TNF) α, PeproTech (Rocky Hill, NJ); recombinant mouse Wnt3a, R&D Systems (Minneapolis, MN); recombinant mouse Wnt5a, Millipore (Billerica, MA); EHS (Engelbreth-Holm-Swarm sarcoma) gel and laminin, Becton Dickinson (Bedford, MA); 1× insulin/transferrin/selenium (ITS), Invitrogen (Carlsbad, CA); KN93 (CaMKII inhibitor), KN92 (inactive analogue of KN93), KN62 (CaMKII inhibitor), NSC23766 (Rac1 inhibitor), Y-27632 (Rho-kinase inhibitor), Go6976 (PKC inhibitor), and Cyclosporin A (calcineurin inhibitor), Calbiochem (San Diego, CA); rat anti-Dlk monoclonal antibody, Medical and Biological Laboratories (Nagoya, Japan); nicotinamide, Wako Pure Chemicals (Osaka, Japan).

Isolation of liver cells.

Fetal hepatic cells of E14.5 liver were dissociated by collagenase treatment as described.[1] Dissociated liver cells were incubated with fluorescein isothiocyanate

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(FITC)-conjugated anti-Dlk antibody (Ab), phycoerythrin (PE)-Cy7-conjugated anti-CD45 Ab (eBioscience, San Diego, CA), PE-Cy7-conjugated anti-CD71 Ab (eBioscience), and PE-Cy7-conjugated anti-Ter119 Ab (Pharmingen, San Diego, CA) for 60 min at 4°C. As well as with these markers, E14.5 fetal liver cells were co-stained with one of PE-conjugated anti-platelet-derived growth factor (PDGF) receptor α, PE-conjugated anti-Flk-1, and PE-conjugated anti-podocalyxin-like protein 1 (PCLP1), one Ab at a time. The stained cells were purified by use of a MoFlo™ fluorescence-activated cell sorter (FACS; DAKO, Glostrup, Denmark).

Adult hepatocyte isolation was performed following a 2-step collagenase digestion.[2] The parenchymal-cell (mature hepatocyte) fraction was separated from non-parenchymal cells by low-speed centrifugation (50 *g*, 1 min). Dead cells were removed by centrifugation in 50% Percoll solution (GE Healthcare UK Ltd, Buckinghamshire, UK); hepatocytes were then washed with PBS and collected.

Hematoxylin-eosin (HE) staining

For hematoxylin-eosin (HE) staining, liver samples were fixed in phosphate-buffered saline (PBS) containing 10% formalin for 24 h and embedded in paraffin. The samples were sectioned 5-μm thick and stained with HE.

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RT-PCR analysis.

The protocol for RT-PCR was previously described.[3] Briefly, total RNA samples were extracted from liver tissues with Trizol reagent (Invitrogen). For molecular analyses of HPPL-derived cysts, cells were dissociated using 0.05% collagenase solution and were collected. Then, total RNA samples were obtained using RNeasy Micro Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of RNA sample using Superscript II RNase H reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA samples were normalized by the number of β-actin copies (quantified by TaqMan PCR), with equal copies applied as PCR templates. The resulting cDNA was amplified using the GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, CA) at 95 °C for 3 min followed by 40 cycles at 94 °C for 10 s, 58 °C for 10 s and 72 °C for 30 s. Primers that spanned the introns of target genes were designed using the website Primer3 (http://frodo.wi.mit.edu/). Primer sequences are listed in supplementary Table 1. Amplified products were separated by electrophoresis on 1.2% agarose gels and stained with ethidium bromide.

For quantitative RT-PCR analysis, the expression of each gene was quantified using a Universal Probe Library system (Roche Diagnostics, Indianapolis, IN) and an ABI 7500 real time PCR system (Applied Biosystems). The resulting cDNA was

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amplified at 95 °C for 10 min followed by 40 cycles at 94 °C for 15 s and 60 °C for 60 s. Primers were designed using the website Universal Probe Library Assay Design Center (Roche). Primer sequences are listed in supplementary Table 2.

Immunohistological analysis

Protocol for immunohistological analysis was previously described.[1] Briefly, fresh livers snap-frozen in OCT compound were sectioned to 8-μm thick slices. Thawed sections were fixed in PBS containing 4% paraformaldehyde (PFA, Nakalai Tesque, Kyoto, Japan) and permeabilized using 0.1% Triton X-100/PBS. After washing with PBS, samples were incubated overnight in primary antibody (Ab; supplementary Table 3) solution at 4 °C. After washing sections 3× with PBS, they were then incubated for 60 min at room temperature in an appropriate secondary Ab solution. Microwave treatment (500 W 10 min) in 10 mM Na-citrate buffer (pH 6.0) was required for staining tissues for PCNA and Hes1. For immunostaining of PCNA, M.O.M. immunodetection kit (Vector, Burlingame, CA, USA) was used according to the manufacturer's instructions. The primary Ab against PCNA was detected using biotin-conjugated anti-mouse IgG Ab, then incubated with streptoavidin-FITC. Immunostaining of Hes1 was performed as previously described. For each analysis, addition of an appropriate immune serum provided a negative control. Nuclei were stained with 4',

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6-diamidine-2'-phenylindole dihydrochloride (DAPI, Sigma). For each mouse, we counted the number of immunostained cells and primitive ductal structures in 10 randomly selected fields and present these data as the total number observed per mouse.

Immunoblot analysis

A detailed western was previously described.[4] Briefly, tissue samples were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate) containing a cocktail of inhibitors of proteases (Roche Diagnostics, Indianapolis, IN) and phosphatases (Nacalai Tesque, Kyoto, Japan). After sodium dodecyl sulfate/polyacrylamide gel electrophoresis of liver homogenates (20 or 50 μg protein) and transfer to PVDF membranes, blots were incubated with primary Abs (supplementary Table 4), followed by incubation with peroxidase-labeled secondary Ab (GE Healthcare, Uppsala, Sweden) and visualization using the ECL Western Blotting Analysis System (GE Healthcare). Images of immunoblot chemiluminescence were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

Wnt5a-blocking experiments

HPPL cells were suspended in a mixture of type I collagen and EHS gel at a

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density of 4×10^4 cells/ml. Cell suspension was added to each cell culture and after incubation at 37°C for 2 h, 500 μl of DMEM/F-12 with Wnt5a inhibitors was added above and below the insert and the cells were cultured for seven days. Cells were treated with one of following compounds; 2 μg/ml Wnt5a Ab (R&D systems, Minneapolis, MN), 2 μg/ml control goat IgG (Sigma), both 2 μg/ml Wnt5a Ab plus 100 ng/ml recombinant Wnt5a (R&D systems), 100 μM Box5 (Wnt5a inhibitor, Millipore)[5], and both 100 μM Box5 plus 100 ng/ml recombinant Wnt5a.

Microarray analysis

For oligo DNA microarray analysis, total RNA samples were extracted from liver tissues of E18.5 WT or Wnt5a KO mice with Trizol reagent. We used 3D-Gene Mouse Oligo chip 24k (23,522 distinct genes, Toray Industries, Tokyo, Japan) in this assay. Total RNA was labeled with Cy3 or Cy5 using the Amino Allyl MessageAMP II aRNA Amplification Kit (Applied Biosystems). The Cy3- or Cy5-labeled aRNA pools were hybridized for 16 h. The hybridization signals obtained using 3D-Gene Detected signals for each gene, were normalized by the global normalization method (Cy3/Cy5 ratio median=1). Genes with Cy3/Cy5 normalized ratios of >2.0 or <0.5 were defined as upregulated or downregulated genes, respectively. Hierarchical cluster analysis was performed using global normalization data according to Pearson product-moment

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correlation coefficient. Pathways that showed significant gene expression changes were analyzed using GenMAPP ver2.1 (MAPP finder, http://www.genmapp.org/) software. The microarray data was deposited to Gene Expression Omnibus of NCBI (http://www.ncbi.nlm.nih.gov/geo/, GEO accession number, GSE41803).

Immunocytostaining of cultured cells

Cultured cells within gels were fixed with 4% paraformaldehyde /PBS and permeabilized using 0.5% Triton X-100/PBS. After blocking with 10% donkey serum/PBS, the gels were incubated with anti-CK19 Ab followed by Alexa 488-conjugated anti-rabbit IgG Ab. Nuclei were stained with DAPI. For each analysis, addition of an appropriate immune serum provided a negative control. Immunostained bile-duct like branching structures were counted under the fluorescent microscope (Olympus, Tokyo, Japan) or confocal lasermicroscope (Olympus).

Immunocytostaining of FACS-sorted cells

Dissociated liver cells derived from WT E14.5 fetuses were incubated with FITC-conjugated anti-Dlk Ab, allophycocyanin (APC)-conjugated anti-CD45 Ab (Pharmingen) and APC-conjugated anti-Ter119 Ab (Pharmingen) for 60 min at 4°C. After washing with PBS containing 3% FCS, the stained cells were sorted directly onto slideglasses coated by poly-L-lysine by use of a MoFlo™ FACS. Sorted cells were

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fixed with 4% PFA/PBS and permeabilized using 0.5% Triton X-100/PBS. After blocking with 10% donkey serum/PBS, the cells were incubated with primary Ab followed by Alexa 546-conjugated anti-rabbit IgG Ab (Invitorgen). Nuclei were stained with DAPI. For each analysis, addition of an appropriate immune serum provided a negative control.

Statistics

We used Microsoft Excel software (Microsoft, Redmond, WA, USA) to calculate standard deviations, standard error and the statistical significance of differences between samples (2-tailed Student's *t* test). P values <0.05 were considered statistically significant.

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Supplementary Tables

Supplementary Table 1. Primer list for RT-PCR

Wnt5a	F	5'-ATTGTCCCCCAAGGCTTAAC-3'	Fzd6	F	5'-AGATGACATGGTTCTCTGATC-3'
	R	5'-GTCTCTCGGCTGCCTATTTG-3'		R	5'-CATGATTTGTGGTCGCTCCTG-3'
Fzd1	F	5'-AGCCGAAAAAGTATGGCTGA-3'	Fzd7	F	5'-GTTGCTACTTCATGGTGGCAG-3'
	R	5'-GCACCTTCACCAGAGGGTAG-3'		R	5'-TAGCACACTCCACTGAGTAGG-3'
Fzd2	F	5'-TATGCAATGAGCGCTTCTCAG-3'	Fzd8	F	5'-ACCAGAGCCTTGACAACCTAC-3'
	R	5'-AAGGATGTACCGATGAACAGG-3'		R	5'-ATGCCCACTACTAGGCACATG-3'
Fzd3	F	5'-ACAGAGTTCGGATTGAGATCC-3'	Fzd9	F	5'-TCTGCTATGTAGCCAGCATGG-3'
	R	5'-CAGGAGTGACTGAGCAAAGTC-3'		R	5'-TGAGCATGAAGACAGCCACAG-3'
Fzd4	F	5'-TGAGACTAGTGGATGCCGATG-3'	Fzd10	F	5'-AGTGGGCAGATGTATGTTATC-3'
	R	5'-TGCCATGTTTGAGTCATCTGC-3'		R	5'-TCAAGATAGTCTTCACAGCCG-3'
Fzd5	F	5'-ATCGCAGGTTATGCACAGTAC-3'	β-actin	F	5'-AGACTTCGAGCAGGAGATG-3'
	R	5'-AGTGCTGTTCATACAGGTAGC-3'		R	5'-ACTCATCGTACTCCTGCTTG-3'

Fzd, Frizzled; F, forward primer; R, reverse primer.