Endothelial Notch Activation Reshapes the Angiocrine of Sinusoidal Endothelia to Aggravate Liver Fibrosis and Blunt Regeneration in Mice

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Liver sinusoidal endothelial cells (LSECs) critically regulate liver homeostasis and diseases through angiocrine factors. Notch is critical in endothelial cells (ECs). In the current study, Notch signaling was activated by inducible EC-specific expression of the Notch intracellular domain (NIC). We found that endothelial Notch activation damaged liver homeostasis. Notch activation resulted in decreased fenestration and increased basement membrane, and a gene expression profile with decreased LSEC-associated genes and increased continuous EC-associated genes, suggesting LSEC dedifferentiation. Consistently, endothelial Notch activation enhanced hepatic fibrosis (HF) induced by CCl4. Notch activation attenuated endothelial nitric oxide synthase (eNOS)/soluble guanylate cyclase (sGC) signaling, and activation of sGC by 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) reversed the dedifferentiation phenotype. In addition, Notch activation subverted the hepatocyte-supporting angiocrine profile of LSECs by down-regulating critical hepatocyte mitogens, including Wnt2a, Wnt9b, and hepatocyte growth factor (HGF). This led to compromised hepatocyte proliferation under both quiescent and regenerating conditions. Whereas expression of Wnt2a and Wnt9b was dependent on eNOS-sGC signaling, HGF expression was not rescued by the sGC activator, suggesting heterogeneous mechanisms of LSECs to maintain hepatocyte homeostasis. Conclusion: Endothelial Notch activation results in LSEC dedifferentiation and accelerated liver fibrogenesis through eNOS-sGC signaling, and alters the angiocrine profile of LSECs to compromise hepatocyte proliferation and liver regeneration (LR). (HEPATOLOGY 2018; 68:677-690).

Liver fibrosis (LF) is a common pathological process of end-stage liver diseases. Although LF is characterized by abnormal deposition of extracellular matrix, diminished hepatocyte regeneration is responsible for compromised liver function accounting for death.1 Signals and mechanisms coordinating fibrogenesis and liver regeneration (LR) have not yet been fully clarified, hampering precision intervention of this pathology.

LSECs are specialized ECs lining liver sinusoids. These highly endocytic ECs exhibit a characteristic phenotype of nondiaphramed fenestrae and the lack of basal membrane, facilitating high-efficient material exchange between blood and the space of Disse (SD).2-4 Normally, shear stress and vascular endothelial growth factor (VEGF) derived from hepatocytes and hepatic stellate cells (HSCs) maintain the LSEC phenotype, at least partly, through eNOS-sGC signaling.5-8 During liver fibrogenesis, LSECs undergo...
DEDifferentiated LSECs lose their fenestrae and express ECM to develop a basement membrane, leading to HSC activation and hepatocyte damage. LSEC dedifferentiation has been recognized as an initial pathological marker and is essential for LF, because blocking LSEC dedifferentiation with a sGC activator abrogates fibrogenesis in rodent models.\(^{(12,13)}\)

In addition, LSECs also function as a niche to maintain hepatocyte homeostasis by angiocrine factors.\(^{(14-17)}\) Gata4-dependent LSECs are required for liver development and hepatocyte proliferation.\(^{(4)}\) LSECs secrete Wnt ligands and HGF to support replenishment and functional zonation of hepatocytes under steady state.\(^{(18-21)}\) LSECs also contribute to LR.\(^{(14-17,22-24)}\) In mice, partial hepatectomy (PHx)-induced LR is accompanied by downregulation of angiopoietin-2 (Angpt2) and transforming growth factor (TGF)-β1, and increased secretion of Wnt2a and HGF through the VEGF receptor (VEGFR) 2/inhibitor of differentiation-1 (Id1) pathway in LSECs, leading to enhanced hepatocyte proliferation on day 2.\(^{(19,25)}\) VEGFR2-Id1 signaling and re-expressed Angpt2 also promote LSEC-mediated angiogenesis on day 4 post-PHx to re-establish hepatic circulation.\(^{(19,25)}\) Moreover, stromal-derived factor-1 and its receptors, C-X-C chemokine receptor (CXCR)4 and CXCR7, control a balance between LR and fibrosis.\(^{(26)}\) However, a relationship between LSEC dedifferentiation and angiocrine and the underlying mechanisms have not been completely elucidated.

Notch signaling is evolutionarily highly conserved and regulates vasculogenesis, angiogenesis, and vascular remodeling.\(^{(27,28)}\) Triggering Notch receptors activates proteolytic cleavages catalyzed by γ-secretase, liberating the Notch intracellular domain (NIC). NIC enters nucleus to activate transcription of downstream molecules, such as the hairy and enhancer of split (Hes) family members, through the transcription factor, recombination signal-binding protein Jκ (RBP-J).\(^{(27)}\) In quiescence, Notch2 is highly expressed by LSECs, whereas Jagged1, delta-like ligand (Dll)1, and Dll4 are expressed at low level.\(^{(3)}\) Previous studies have shown that deficiencies in Notch1 or RBP-J in adult mice disrupted sinusoidal and hepatocyte homeostasis.\(^{(29-31)}\) However, the overall consequence of Notch activation in LSECs has not been assessed.

In this study, we report that activation of Notch signaling is...
signaling in ECs led to LSEC dedifferentiation and enhanced LF, and altered LSEC angiocrine spectrum that attenuated LR.

**Materials and Methods**

**MICE**

Mice were maintained in a specific pathogen-free facility on the C57BL/6J background. ROSA-STOPfloxed-NIC mice (murine Notch1 NIC [1749-2293] followed by IRES-GFP in the ROSA26 locus; Jackson Laboratory, Bar Harbor, ME) were crossed with the CDH5-CreERT mice (kindly provided by R.H. Adams). Littermates were genotyped by PCR to obtain CDH5-CreERT (control; Ctrl) and CDH5-CreERT-NIC (NICeCA) mice. Male mice (6 weeks old) were injected intraperitoneally with tamoxifen (100 mg/kg; Sigma-Aldrich, St. Louis, MO) totally for seven injections (Fig. 1A). Mice were kept for 1 more week to minimize potential influence from tamoxifen before further analyses. In some cases, from the third injection of tamoxifen, mice were injected simultaneously with 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1; 5 mg/kg; Sigma-Aldrich) for 7 times in total (Fig. 1A). PHx was performed as described. To induce LF, mice were injected intraperitoneally with 0.6 mL/kg of CCl4 (Sigma-Aldrich) twice a week for 6 weeks, with olive oil as a control. To measure portal pressure, mice were anesthetized and the portal vein was punctured with a 25-G needle connected to a sensor and the MedLab-U/4C501 Bioinformation Collecting and Processing System (Zhong Shi Di Chuang Sci&Technol Dev Co., Ltd, Beijing, China). Stable pressure of portal vein was recorded and processed automatically.

All animal experiments were reviewed and approved by the Animal Experiment Administration Committee of the Fourth Military Medical University (Xi’an, China) to ensure ethical and humane treatment of animals. The study followed the guideline outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health.

**FIG. 1.** Endothelial Notch activation disrupted liver homeostasis. (A) Genotypes of mouse models and schedules for tamoxifen and YC-1 injection. (B) Liver sections from NICeCA and control mice were analyzed with H&E staining, TEM, anti-Ki67 IHC, and TUNEL. Ki67+ hepatocytes are indicated with arrows. (C) Sinusoid areas and width of SD. (D) The numbers of Ki67+ and TUNEL+ hepatocytes were compared between NICeCA and control mice. (E) Serum level of ALT and AST in NICeCA and control mice was determined. Bars = means ± SD, n = 6; *P < 0.05; **P < 0.01; ***P < 0.001.
Academy of Science and published by the National Institute of Health (publications 86-23, revised 1985).

Human liver cirrhosis (LC) and hepatic hemangiooma samples were obtained from patients in the Department of Hepatobiliary Surgery, Xi-Jing Hospital. All subjects involved were given informed consent for use of their tissues for research purposes. The use of human tissues for this study was approved by the Ethics Committee, Xi-Jing Hospital.

HISTOLOGY

Liver samples were fixed in 10% buffered formalin or in 4% paraformaldehyde (PFA). Formalin-fixed samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) routinely. PFA-fixed samples were embedded with optimum cutting temperature compound and sectioned at 8-μm thickness. For immunohistochemistry (IHC), sections were incubated with primary antibodies at 4°C overnight, washed, and incubated with horseradish peroxidase–conjugated secondary antibodies for 2 hours at room temperature. Sections were then developed using a 3,3′-diaminobenzidine substrate kit (Zhongshan Jingiao Biotech, Beijing, China). Immunofluorescence (IF) was performed using fluorescent antibodies and counterstained with Hoechst 33258 (Sigma-Aldrich). Antibodies are listed in Supporting Table S1. Fluorescein isothiocyanate (FITC) or DyLight 594–conjugated Isolectin B4 (Vector Laboratories, Burlingame, CA) was used in staining with the same protocol. Apoptotic cells were stained with a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit (Promega, Madison, WI). Digital images were taken under a fluorescence microscope (BX51; Olympus, Tokyo, Japan) or laser-scanning confocal fluorescence microscope (FV-1000; Olympus). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out as described. (29)

CELL ISOLATION AND CULTURE

Hepatocytes and liver nonparenchymal cells were isolated as described, with modifications. (29) Briefly, mice were anesthetized and perfused with Hank’s buffer without Ca^{2+} from the portal vein, followed by Hank’s buffer with Ca^{2+} and Mg^{2+} containing 0.2 mg/mL of collagenase IV (Sigma-Aldrich). The liver was removed and finely minced with a tissue homogenizer in perfusion buffer containing 0.2 mg/mL of collagenase IV and 10 μg/mL of DNase I (Roche, Basel, Switzerland). Single-cell suspension was generated by passing through a 70-μm cell mesh and resuspended in Hank’s buffer. Hepatocytes were pelleted by low-speed centrifugation at 50g for 3 minutes and resuspended in Hank’s buffer, followed by three washes with Hank’s buffer to remove cell debris. Supernatants were further centrifuged at 400g for 8 minutes to recover LSECs. Pelleted cells were resuspended in 4 mL of 17.6% iodoxanol (Axis-Shield Diagnostics Ltd., Tarrytown, NY) and 4 mL of 8.2% iodoxanol and 2 mL of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) were orderly loaded on the top of the cell suspension. The cell suspension was then centrifuged at 1,400g for 20 minutes in a swing-out rotor without brake. LSECs were recovered from the interface between the 8.2% and 17.6% iodoxanol solutions, mixed with an equal volume of DMEM and centrifuged at 400g for 8 minutes. Cells were resuspended and incubated with anti-CD146 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 minutes at 4°C in dark. Cells were then washed with the washing buffer (phosphate-buffered saline [PBS] containing 2 mM of ethylenedimainetetraacetic acid and 0.5% bovine calf serum [bovine serum albumin]; pH 7.2) and resuspended in 1 mL of the washing buffer. The cell suspension was then applied onto the magnetic column of a MACS separator, and washed with 500 μL of the washing buffer for three times. Finally 1 mL of the washing buffer was loaded on the MACS column, and LSECs were immediately flushed out.

Hepatocytes were cultured in collagen I–coated plates with hepatocyte medium (ScienCell, San Diego, CA), supplemented with 5% fetal bovine serum (GIBCO, Rockville, MD), 1% hepatocyte growth supplement, 2 mM of l-glutamine, 100 μg/mL of streptomycin, and 100 U/mL of penicillin. HGF (50 ng/mL; Sigma-Aldrich) or Wnt2a-containing conditional medium (CM; collected from HEK293T cells transfected with an Wnt2a-expressing vector) was added and incubated for 20 hours. Hepatocyte proliferation was determined with anti-Ki67 IF. LSECs were cultured in collagen I–coated 24-well plates with EC medium supplemented with EC growth solution (ScienCell). GSI IX (Sigma-Aldrich), mD1R, (34) or YC-1 was added at concentrations of 75 μM, 5 μg/mL, and 30 μM, respectively.

ENDOCYTOSIS

Endocytosis of LSECs in vivo was assayed using FITC/formaldehyde–treated serum albumin (FITC-FSA), according to published protocols. (35)
GENE EXPRESSION PROFILING

RNA was extracted, sequenced, and analyzed by custom service provided by RiboBio (Guangzhou, China) using an Illumina HiSeq3000 (Illumina, San Diego, CA). Briefly, total RNA was extracted from LSECs isolated from NICeCA or control mice using TRIzol (Invitrogen), according to the manufacturer’s protocol. RNA integrity was evaluated using the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA), and each sample had the RINe above 7.0. mRNA was isolated and fragmented to approximately 200 base pairs (bp) in length and subjected to complementary DNA (cDNA) synthesis followed by adaptor ligation and enrichment with a low-cycle PCR using the TruSeq RNA LT/HT Sample Prep Kit (Illumina). Purified library products were evaluated using the Agilent 2200 TapeStation and Qubit2.0 (Life Technologies, Carlsbad, CA) and then diluted to 10 pM for cluster generation in situ on the HiSeq3000 pair-end flow cell followed by sequencing (2×150 bp) on HiSeq3000. Original data were deposited in the Gene Expression Omnibus database (accession no.: GSE102419). Bioinformatic analysis was performed using the OmicShare tools at www.omicshare.com/tools.

RT-PCR

Total RNA was prepared by using TRIzol and reverse-transcribed into cDNA with the PrimeScrip RT reagent kit (TaKaRa Biotechnology, Dalian, China). Quantitative real-time PCR (qPCR) was performed using the SYBR premix ExTaqII (TaKaRa Biotechnology) and Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, CA), with β-actin as a reference control. Primers are listed in Supporting Table S2.

WESTERN BLOTTING

Total protein was extracted with the radioimmuno-precipitation assay lysis buffer, containing 10 mM of phenylmethanesulfonyl fluoride, and quantified with a bicinchoninic acid assay protein assay kit (Thermo Fisher Scientific, Rockford, IL). Samples were separated with sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Blottings were incubated with primary antibodies followed by HRP-conjugated secondary antibodies (Supporting Table S1) and developed with enhanced chemiluminescence (Clinx Science Instruments, Shanghai, China).

ENZYME-LINKED IMMUNOSORBENT ASSAY AND BIOCHEMISTRY

HGF in culture supernatants was analyzed with an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience/Thermo Fisher Scientific). Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined with a Chemistry Analyzer (AU400; Olympus). Hydroxyproline was determined with a kit (Jiancheng Bioengineering Institute, Nanjing, China).

STATISTICAL ANALYSIS

Statistical analysis was performed with the SPSS (version 12.0; SPSS, Inc., Chicago, IL) program. Comparisons between groups were undertaken using the unpaired Student t test. Results are expressed as means ± SD. P < 0.05 was considered statistically significant.

Results

ENDOTHELIAL Notch ACTIVATION DAMAGED LIVER HOMEOSTASIS

In tamoxifen-induced NICeCA mice (Fig. 1A), expression of Hes1 and hairy/enhancer-of-split related with YRPW motif protein 1 (Hey1), two target genes of canonical Notch signaling, was up-regulated significantly in LSECs isolated from NICeCA mice, suggesting Notch activation (Supporting Fig. S1C). No gross abnormality was noticed systemically or in liver of NICeCA mice (Supporting Fig. S1D,E). However, in quiescent NICeCA mice, the area of liver sinusoids increased significantly (Fig. 1B,C) with widened SD as shown by TEM (Fig. 1B,C). Ki67+ hepatocytes decreased whereas TUNEL+ hepatocytes increased as compared to the control (Fig. 1B,D), suggesting reduced proliferation and increased apoptosis. The level of serum ALT and AST increased (Fig. 1E). These data suggested that EC-specific Notch activation impaired liver homeostasis under steady state in mice.
ENDOTHELIAL Notch ACTIVATION LED TO LSEC DEDIFFERENTIATION

Density of LSECs decreased, as shown by anti-Lyve-1 IF and isoelectin staining (Fig. 2A), accompanied by increased apoptosis as detected by TUNEL (Fig. 2A). Endocytic capacity of LSECs, as shown by uptake of FITC-FSA, decreased remarkably in NIC<sup>eCA</sup> mice, suggesting LSEC dysfunction (Fig. 2B; Supporting Fig. S2).

Gene expression profiling showed that LSECs from NIC<sup>eCA</sup> mice expressed decreased LSEC-associated genes, but increased continuous EC-associated genes (Fig. 2C).<sup>(4)</sup> qRT-PCR confirmed that expression of capillary EC markers laminin, CD31, and endothelin-1 (ET-1) was up-regulated in LSECs with activated Notch signaling (Fig. 2D). Ultrastructure under SEM indicated that the number of fenestrae in LSECs decreased significantly in NIC<sup>eCA</sup> mice (Fig. 2E). IF indicated that Notch activation led to increased expression of laminin by LSECs (Fig. 2E). In summary, these data demonstrated that Notch activation resulted in LSEC dedifferentiation.

ENDOTHELIAL Notch ACTIVATION AGGRAVATED CCl<sub>4</sub>-INDUCED LF

Induction of LF in NIC<sup>eCA</sup> and control mice CCl<sub>4</sub> showed that NIC<sup>eCA</sup> mice manifested significantly aggravated fibrogenesis, as evidenced by increased ECM deposition detected by Sirius Red staining (Fig. 3A), and elevated tissue hydroxyproline level (Fig. 3A). IF showed increased Desmin (Fig. 3B) and alpha-smooth muscle actin (a-SMA; Fig. 3B) staining, suggesting enhanced HSC activation after endothelial Notch activation. In fibrotic NIC<sup>eCA</sup> mice, less liver sinusoid fenestrae (Fig. 3C) and a higher level of
capillary marker CD31 (Fig. 3C) were observed. Consistent with enhanced fibrogenesis, elevated expression of fibrosis-related genes, including collagen-1, α-SMA, TGF-β, platelet-derived growth factor beta (PDGFβ), and tissue inhibitor of metalloproteinase-1 (TIMP-1), in LSECs was detected (Fig. 3D). We also observed higher serum ALT and AST (Fig. 3E), and higher portal pressure (Fig. 3F), in NICCA mice, consistent with increased hepatocyte injury and portal resistance. Moreover, to access the clinical relevance of our findings, we collected liver biopsies from patients with or without cirrhosis, and performed IHC to detect nuclear Notch1 intracellular domain (N1IC) in LSECs. The result showed that in the liver of patients with cirrhosis, the number of LSECs with nuclear N1IC increased significantly as compared to that of patients without cirrhosis, suggesting a role of Notch activation in LSECs during LC (Supporting Fig. S3A,B). These data collectively indicated that endothelial Notch activation resulted in aggravated LF associated with LSEC dedifferentiation.

**Notch ACTIVATION-INDUCED LSEC CAPILLARIZATION WAS eNOS-sGC DEPENDENT**

Further analysis of RNA-sequencing (RNA-seq) data suggested that a set of genes in eNOS-sGC signaling, which participates in maintaining LSEC phenotypes, were changed upon Notch activation (Fig. 4A). Then, we examined expression of molecules involved in this pathway. The result showed that mRNA level of VEGFR2, VEGFR3, and eNOS decreased, whereas expression of Angpt2 increased significantly in LSECs isolated from NICCA mice (Fig. 4B). Western blotting confirmed that in LSECs from NICCA mice, Hes1 was up-regulated whereas VEGFR2, eNOS, and phosphorylated eNOS decreased (Fig. 4C,D). Blockade of Notch signaling by γ-secretase inhibitor (GSI) in vitro showed decreased expression of eNOS and VEGFR2, and increased Angpt2, whereas VEGFR3 mRNA level remained unchanged (Fig. 4E). Treatment of normal
LSECs with a Notch ligand protein, mD1R, reduced expression of VEGFR2 and eNOS, and up-regulated Angpt2 (Supporting Fig. S4A). Expression of VEGFR1 and sGC components, including guanylate cyclase (GUCY)1A2, GUYC1A3, and GUYC1B3, was not influenced significantly by Notch activation and blockade (Supporting S4B,C). These data suggested that Notch activation might result in LSEC dedifferentiation through repressing expression and/or activity of eNOS-sGC.

NICeCA and control mice were injected with YC-1, an sGC activator (Fig. 1A). LSEC dedifferentiation induced by Notch activation, characterized by decreased fenestrae and increased laminin expression, was abrogated by YC-1, as shown by SEM and immunofluorescence staining (Fig. 5A-C). Laminin was colocalized with Lyve-1, confirming that it was likely derived from LSECs (Fig. 5A). The reduced number of Lyve-1+ LSECs induced by Notch activation was also rescued by YC-1 (Fig. 5D). qRT-PCR confirmed the down-regulation of laminin in LSECs from NICeCA mice injected with YC-1 (Fig. 5E). These results suggested that Notch activation led to a down-regulated eNOS-sGC pathway, which was responsible for LSEC dedifferentiation.

**ENDOTHELIAL Notch ACTIVATION IMPEDED LR POST-PHx**

Normal mice were subjected to PHx, and LSECs were isolated on days 1, 2, 4, 6, and 8 after the operation or from the sham group. qRT-PCR showed that the mRNA levels of Dll4, Notch1, Hes1, and Hey1 were markedly reduced on day 2 post-PHx, but recovered eventually and reached a normal level on day 8 (Fig. 6A). To examine whether endothelial Notch activation influences LR, we performed PHx in NICeCA and control mice. The result showed that NICeCA mice exhibited significantly retarded LR post-
FIG. 5. YC-1 reversed LSEC dedifferentiation caused by endothelial Notch activation. (A) NIC\textsuperscript{CA} and control mice were injected intraperitoneally with YC-1 (Fig. 1A). Liver sections were analyzed by SEM, antilaminin, and anti-Lyve-1 IF. (B-D) LSEC fenestrae (B), laminin\textsuperscript{1} pixels (C), and Lyve-1\textsuperscript{1} pixels (D) were quantitatively compared. (E) LSECs were isolated from mice in (A), and expression of laminin was determined using qRT-PCR, with \(\beta\)-actin as an internal control. Bars = means \(\pm\) SD, n = 6; \(* P < 0.05; ** P < 0.01; *** P < 0.001.\) Abbreviation: n.s, not significant.

FIG. 6. Endothelial Notch activation resulted in compromised hepatocyte proliferation during LR. (A) Normal mice were subjected to PHx. LSECs were isolated on indicated days (S, sham-operated), and expression of Dll4, Notch1, Hes1, and Hey1 was determined using qRT-PCR. (B) NIC\textsuperscript{CA} and control mice were subjected to PHx. Liver/bodyweight ratio was determined on days 2, 4, 6, and 8 post-PHx. (C,D) Liver samples were collected on days 2, 4, and 8 post-PHx, sectioned, and analyzed with anti-Ki67 IHC (C). The number of Ki67\textsuperscript{1} cells was quantitatively compared (D). (E) Liver sections on PHx day 4 were analyzed by anti-Lyve-1 IF and SEM. Lyve-1\textsuperscript{1} pixels and LSEC fenestrae were compared. Bars = means \(\pm\) SD, n = 6; \(* P < 0.05; ** P < 0.01; *** P < 0.001.\)
PHx, as shown by lowered liver weight/body weight ratio at each regeneration time point as compared to the control (Fig. 6B). Anti-Ki67 IHC showed that Notch activation in ECs significantly reduced hepatocyte proliferation in the process of PHx-induced LR (Fig. 6C,D). In addition, incomplete LSEC regeneration (Fig. 6E) and sustained capillarization (Fig. 6E) were observed in NICeCA mice on day 4 post-PHx. Therefore, EC-specific Notch activation hampered LR post-PHx.

**LSECs WITH Notch ACTIVATION FAILED TO SUPPORT HEPATOCYTE PROLIFERATION DUE TO ATTENUATED Wnt LIGANDS AND HGF PRODUCTION**

Comparison of gene expression profiles of LSECs showed that several hepatocyte mitogens, including Wnt2a, Wnt9b, and HGF, were down-regulated remarkably in NICeCA LSECs (Fig. 7A). qRT-PCR confirmed down-regulation of HGF, Wnt2a, and Wnt9b in LSECs from NICeCA mice (Fig. 7B). Furthermore, ELISA and western blotting also indicated that Notch activation down-regulated HGF and Wnt2a in LSECs at the protein level (Fig. 7C). These data suggested that EC-specific Notch activation impaired hepatocyte proliferation, likely through down-regulating paracrine Wnt ligands and HGF in LSECs.

Next, we cultured primary hepatocytes with CM from control or NICeCA LSECs and examined hepatocyte proliferation. The result showed that CM from wild-type LSECs (Ctrl-CM) promoted hepatocyte proliferation, but CM from NICeCA LSECs exhibited significantly reduced ability to stimulate hepatocyte proliferation as detected by anti-Ki67 IF (Fig. 7D,E). Of note, addition of HGF significantly restored the ability

![FIG. 7.](image-url)
of NIC<sub>eCA</sub> LSECs to stimulate hepatocyte proliferation (Fig. 7D,E). Similarly, Wnt2a, which was derived from transfected HEK293T cells (Supporting Fig. S5), also partially rescued hepatocyte proliferation in culture with CM from NIC<sub>eCA</sub> LSECs (Fig. 7F). These results indicated that activation of Notch signaling in LSECs impaired hepatocyte proliferation, likely by down-regulating HGF and Wnt ligands.

ENDOTHELIAL Notch ACTIVATION PERTURBED LSEC ANGIOCRINE FUNCTION THROUGH HETEROGENEOUS MECHANISMS

Next, we injected NIC<sub>eCA</sub> and control mice with the sGC activator, YC-1. The result showed that YC-1 rescued the morphological change of sinusoids in NIC<sub>eCA</sub> mice, as shown by H&E staining (Fig. 8A). Furthermore, YC-1 reversed decreased hepatocyte proliferation (Fig. 8A,B) and increased hepatocyte apoptosis (Fig. 8A,C), as well as increased hepatocyte damage in mice with endothelial Notch activation (Fig. 8D). Moreover, we examined the mRNA level of HGF, Wnt2a, and Wnt9b in isolated LSECs after YC-1 administration. The result showed that expression of Wnt2a and Wnt9b was up-regulated remarkably by YC-1 (Fig. 8E). However, expression of HGF was not rescued, but even further down-regulated in LSECs from NIC<sub>eCA</sub> mice after YC-1 administration (Fig. 8E). These data indicated that Notch activation regulated angiocrine function in LSECs by heterogeneous mechanisms: down-regulating Wnt2a and Wnt9b through repressing eNOS-sGC, and down-regulating HGF through other undefined signaling.

Discussion

Inducible disruption of Notch1 or RBP-J using Mx-Cre resulted in abnormal sinusoidal remodeling, intussusceptive angiogenesis, and hepatic angiosarcomas. To discriminate roles of Notch signaling in hepatocytes and LSECs, Cuervo et al. specifically disrupted RBP-J or Notch1 in LSECs, which resulted in altered sinusoidal structure and disturbed microcirculation. It was observed that loss of function of Notch signaling leads to LSEC capillarization. However, because LSECs are vulnerable to environmental insults such as shear stress, the capillarization phenotype of LSECs in these loss-of-function models could be a consequence of tortuous sinusoids. In the current study, we utilized a gain-of-function model of Notch signaling. Our gene expression profiling and morphological and LSEC functional data have indicated that endothelial Notch activation results in LSEC dedifferentiation. In the presence of CCl<sub>4</sub>-induced liver...
damage, LSEC dedifferentiation promotes liver fibrogenesis, as demonstrated by many studies, likely through increased HSC activation attributed to up-regulation of TGF-β and PDGF-β.\(^{(10-13)}\)

Notch activation-induced LSEC dedifferentiation could be attributed to attenuated eNOS-sGC signaling, which has been proven to maintain the differentiation of LSECs.\(^{(2,12,13)}\) Indeed, in our study, an sGC activator efficiently rescued LSEC dedifferentiation induced by Notch activation. In NIC\(^{CA}\) mice, expression of eNOS was down-regulated at both mRNA and protein level. Moreover, activation of Notch signaling down-regulated VEGFR2 in LSECs. VEGFR2 is a receptor tyrosine kinase that can activate protein kinase B (Akt) that phosphorylates eNOS to maintain the differentiation of LSECs.\(^{(36)}\) Therefore, Notch activation could skew down eNOS-sGC signaling through two mechanisms, in LSECs: directly down-regulating eNOS expression and reducing eNOS phosphorylation by down-regulating its upstream kinases, VEGFR2. This is quite different from the situation in continuous ECs. In cancer vasculature, inhibition of Notch signaling resulted in the down-regulation of eNOS.\(^{(37)}\) Furthermore, it has been reported that Notch signaling up-regulates certain subunits of sGC in continuous ECs.\(^{(38,39)}\) This inconsistency could be attributed to epigenetic difference between LSECs and continuous ECs, which show different gene expression profiles.\(^{(4)}\) The mechanism for Notch activation-mediated eNOS down-regulation in LSECs is currently unknown. Bioinformatic analysis of the molecular interaction network suggests that Notch/RBP-J signaling might regulate eNOS through Hes1 or Hey1 (Supporting Fig. S6A).\(^{(27)}\) In embryonic stem cells, chromatin immunoprecipitation sequence has shown that Hey1 binds to the eNOS promoter (Supporting Fig. S6B). More experiments are required to validate mechanisms for Notch activation-mediated down-regulation of eNOS in LSECs.

In addition to dedifferentiation, activation of Notch signaling modified the angiocrine spectrum of LSECs remarkably. Of note, several hepatocyte mitogens, including HGF, Wnt2a, and Wnt9b, were down-regulated significantly in LSECs upon Notch activation. Our in vitro culture assay has indicated that normal LSEC-derived conditional medium promotes hepatocyte proliferation, and this ability is abrogated by endothelial Notch activation. Addition of exogenous HGF or Wnt2 can rescue the effect of Notch activation, suggesting that Notch activation in LSECs damages hepatocyte proliferation by reducing the expression of HGF and Wnt ligands. This could be the reason of reduced hepatocyte proliferation of NIC\(^{CA}\) mice under both steady state and regeneration. In addition, Notch activation also up-regulates Angpt2, an EC-derived decoy ligand of Tie2, and competes with pericytes-derived Angpt1 to stimulate Tie2 receptor tyrosine kinase of ECs.\(^{(40)}\) Aberrant expression of Angpt2 in LSECs with forced Notch activation might further damage LR by modulating hepatocyte proliferation and angiogenesis in phase I and phase II regeneration, respectively.\(^{(25)}\)

Notch signaling might regulate the expression of different angiocrine factors with different mechanisms. Our data of profiling, qRT-PCR, and ELISA/western blotting indicated that HGF and Wnt levels were reduced in NIC\(^{CA}\) LSECs, accompanied with reduced hepatocyte proliferation. Although both HGF and Wnt rescued compromised pro-proliferation ability of NIC-LSECs in vitro, YC-1 rescued hepatocyte proliferation and Wnt expression, but not HGF expression in vivo, favoring a more important role of Wnt ligands. Therefore, the down-regulation of Wnt ligands (specifically Wnt2a and Wnt9b in our study) in LSECs with Notch activation is dependent on eNOS-sGC, and therefore associated with LSEC capillarization. On the other hand, Notch activation represses HGF expression in LSECs, most likely through other way(s) than eNOS-sGC. The mechanism of this disparity is unclear. Recently reports have suggested that bone marrow (BM)-derived LSEC progenitor cells (BM-SPCs) are dominant producers of HGF after liver injury. It is possible that Notch signaling regulates quiescent LSECs and BM-SPCs in different ways, leading to differential expression and regulation of Wnt and HGF.\(^{(41,42)}\) More studies are needed to clarify how Notch signaling differentially regulates Wnt2a/9b and HGF.

In summary, hepatocytes undergo cell division to maintain homeostasis that is continuously challenged by endogenous and environmental stress signals.\(^{(19)}\) Under steady state, hepatocytes secrete VEGF that, together with shear stress signal, maintains the differentiated state of LSECs through VEGFR2/Akt/eNOS.\(^{(5-8)}\) Highly differentiated LSECs produce nitric oxide catalyzed by eNOS to guarantee low tension and maximum permeability of liver microcirculation, and repress HSC activation.\(^{(32,13,43)}\) Quiescent LSECs also secrete Wnt2a/9b, which is downstream to eNOS-sGC signaling as demonstrated in the current study, to support hepatocyte proliferation.\(^{(18-21)}\) Our data have shown that Notch, which responds to
various environmental stimuli such as VEGF and inflammation,(44,45) serves as a negative regulator of this circuit: Notch activation down-regulates eNOS-sGC signaling, leading to increased LSEC dedifferentiation, increased HSC activation and fibrosis, and reduced expression of Wnt2a/9b. Notch activation represses the expression of HGF by LSECs and/or BM-SPCs through mechanisms other than eNOS-sGC (Supporting Fig. S7). During LR, Notch signaling was down-regulated through days 2 and 4 post-PHx to allow hepatocyte proliferation and vascular regeneration.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29834/supplinfo.