GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Activation of Hepatic Stellate Cells Requires Dissociation of E-Cadherin—Containing Adherens Junctions with Hepatocytes

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Hepatic stellate cells (HSCs) are resident mesenchymal cells in the space of Disse interposed between liver sinusoidal endothelial cells and hepatocytes. Thorn-like microprojections, or spines, project out from the cell surface of HSCs, crossing the space of Disse, to establish adherens junctions with neighboring hepatocytes. Although HSC activation is initiated largely from stimulation by adjacent cells, isolated HSCs also activate spontaneously in primary culture on plastic. Therefore, other unknown HSC-initiating factors apart from paracrine stimuli may promote activation. The dissociation of adherens junctions between HSCs and hepatocytes as an activating signal for HSCs was explored, establishing epithelial cadherin (E-cadherin) as an adhesion molecule linking hepatocytes and HSCs. *In vivo*, following carbon tetrachloride–induced liver injury, HSCs lost their spines and dissociated from adherens junctions in the early stages of injury, and were subsequently activated along with an increase in YAP/TAZ expression. After abrogation of liver injury, HSCs reconstructed their spines and adherens junctions. *In vitro*, reconstitution of E-cadherin—containing adherens junctions by forced E-cadherin expression quiesced HSCs and suppressed TAZ expression. Additionally, increase of TAZ expression leading to the activation of HSCs by autocrine stimulation of transforming growth factor- β , was revealed as a mechanism of spontaneous activation. Thus, we have uncovered a critical event required for HSC activation through enhanced TAZ-mediated mechanotransduction after the loss of adherens junctions between HSCs and hepatocytes. (*Am J Pathol* 2021, 191: 438–453; <https://doi.org/10.1016/j.ajpath.2020.12.007>)

Hepatic stellate cells (HSCs) are resident mesenchymal cells localized within the space of Disse, interposed between liver sinusoidal endothelial cells (LSECs) and hepatocytes. Quiescent HSCs store vitamin A lipid droplets in their cytoplasm and encircle the endothelial cells with their long, branching cellular process cells to form hepatic sinusoids. Characteristically, many thorn-like microprojections, or spines, project out from the cell surface of HSCs, crossing the space of Disse, to establish adherens junctions with neighboring hepatocytes. In response to hepatic injury of any etiology, quiescent HSCs transdifferentiate into activated myofibroblasts to produce extracellular matrix (ECM), including collagen and fibronectin, and proliferate, migrate

toward regions of injury, and lose vitamin A lipid droplets. After cessation of injury, activated HSCs either undergo apoptosis or revert to an inactivated phenotype,^{1,2} which reduces production of ECM. However, persistent activation of HSCs during chronic liver injury due to viral infection, alcohol, or in nonalcoholic steatohepatitis provokes

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accumulation of collagen in the space of Disse, leading to capillarization of sinusoids, followed by panlobular fibrosis. Furthermore, cirrhosis, the most advanced form of fibrosis, is a critical risk factor for hepatic carcinogenesis.³ Therefore, clarifying the extracellular signals that suppress activated HSCs is an important step toward identifying novel therapeutic targets for cirrhosis and hepatocellular carcinoma, although none has yet been identified.

Canonically, HSC activation has been conceived as a two-phase event: initiation followed by perpetuation. Initiation refers to early changes in gene expression and phenotype that render cells responsive to stimulants for perpetuation.⁴ The initiation of HSC activation results primarily from paracrine stimulation produced by neighboring cells, including hepatocytes, macrophages, LSECs, inflammatory cells, platelets, and HSCs themselves.⁵ After initiation, several features of the perpetuation phase, including proliferation, ECM production, chemotaxis, and increased contractility may occur, depending on the type of stimulus. For instance, platelet-derived growth factor is the most potent HSC mitogen identified,^{6,7} and transforming growth factor beta (TGF- β) induces the production of collagen and other ECM molecules and down-regulates the degradation of ECM by matrix metalloproteinases in HSCs.^{8–10} However, isolated HSCs activate spontaneously in the absence of these exogenous initiation factors, and culture on uncoated plastic or other stiff matrices.^{11,12} This raises the prospect that initiating factors other than paracrine signals may also contribute to cellular activation.

Although HSCs surround LSECs in normal liver, their contact with LSECs is smooth and does not constitute direct cell adhesion.¹³ Conversely, HSCs extend protrusions called spines, that establish adherens junction with hepatocytes.¹⁴

The current study focused on the biological significance of contact between HSCs and hepatocytes through these adherens junctions. It was found that early activation of HSCs requires dissociation of these adherens junctions after injury to hepatocytes, which constitutes a critical event required for HSC activation through enhanced mechanotransduction.

Materials and Methods

Ethics Statement

All experiments were conducted in compliance with the guidelines for the care and use of laboratory animals and approved by Osaka City University.

Liver Injury Model

Liver injury was induced by intraperitoneal injections of carbon tetrachloride (CCl₄) to 6- to 7-week-old Wistar rats or 8- to 10-week-old C57BL/6j mice (0.5 μ L/g body weight, dissolved in corn oil at a ratio of 1:3).

Table 1 Real-Time Quantitative PCR Primer Sequences

Gene name	Primer sequences
<i>Tgfb1</i>	Forward 5'-TCGAGGGCGAGAGAAGTTTA-3' Reverse 5'-AAAAAGAATGTCCCGGCTCTC-3'
<i>Pdgfrb</i>	Forward 5'-CGCCTGCAAGTGTGAGACAAT-3' Reverse 5'-CGAATGGTCACCCGAGCTT-3'
<i>Tnfa</i>	Forward 5'-TCCCAGGTTCTCTTCAAGGGA-3' Reverse 5'-GGTGAGGAGCAGTAGTCGG-3'
<i>Il1b</i>	Forward 5'-TTGACGGACCCAAAAGATG-3' Reverse 5'-TGGACAGCCAGGTCAAAG-3'
<i>Il6</i>	Forward 5'-TGATGCACTTGAGAAAACA-3' Reverse 5'-ACCAGAGGAAATTTCAATAGGC-3'
<i>Ctgf</i>	Forward 5'-CCGCCAACCAGCAAGATC-3' Reverse 5'-ACCGACCCACCGAAGACA-3'
<i>Acta2</i>	Forward 5'-CGAAACCACCTATAACAGCATCA-3' Reverse 5'-GCGTTCTGGAGGGGCAAT-3'
<i>Col1a1</i>	Forward 5'-CCAAGGGTAACAGCGGTGAA-3' Reverse 5'-CCTCGTTTTCTTCTTCTCCG-3'
<i>Col1a2</i>	Forward 5'-TGTTGGCCCATCTGGTAAAGA-3' Reverse 5'-CAGGGAATCCGATGTTGCC-3'
<i>Cdh1</i>	Forward 5'-GAGGTCTACACCTTCCCGGT-3' Reverse 5'-CCACTTTGAATCGGGAGTCT-3'
<i>Cdh2</i>	Forward 5'-CAGGGTGGACGTCATTGTAG-3' Reverse 5'-AGGGTCTCCCACTGATTC-3'
Human	
<i>TGFB1</i>	Forward 5'-CCCTGGACACCAACTATTGC-3' Reverse 5'-GCAGAAGTTGGCATGGTAGC-3'
<i>PDGFRB</i>	Forward 5'-GTGCTCACCATCATCTCCCT-3' Reverse 5'-ACTCAATCACCTTCCATCGG-3'
<i>CTGF</i>	Forward 5'-CAGGCTAGAGAAGCAGAGCC-3' Reverse 5'-TGGAGATTTTGGGAGTACGG-3'
<i>ACTA2</i>	Forward 5'-GACCGAATGCAGAAGGAGAT-3' Reverse 5'-CACCGATCCAGACAGAGTATTT-3'
<i>COL1A1</i>	Forward 5'-AAGAGGAAGGCCAAGTCGAG-3' Reverse 5'-CACACGTCTCGGTCATGGTA-3'
<i>COL1A2</i>	Forward 5'-GAAAAGGAGTTGGACTTGGC-3' Reverse 5'-AGCAGGTCCCTTGAAACCTT-3'
<i>CDH1</i>	Forward 5'-GGGTGACTACAAAATCAATC-3' Reverse 5'-GGGGGCAGTAAGGGCTCTTT-3'
<i>CDH2</i>	Forward 5'-CACTGCTCAGGACCCAGAT-3' Reverse 5'-TAAGCCGAGTGATGGTCC-3'
<i>SERPINH1</i>	Forward 5'-AGAGTAGAATCGTGTGCGCG-3' Reverse 5'-CTGAGAAGCAGGAGGGAGC-3'
<i>SERPINE1</i>	Forward 5'-AGAAACCCAGCAGCAGATTC-3' Reverse 5'-TGGTGCTGATCTCATCCTTG-3'
<i>SNAI1</i>	Forward 5'-ACCCCAATCGGAAGCCTAACT-3' Reverse 5'-GGTCGTAGGGCTGCTGGAA-3'
<i>MMP2</i>	Forward 5'-TGATGTCCAGCAGTGGAT-3' Reverse 5'-GGAAAGCCAGGATCCATTTT-3'
<i>TGFBR2</i>	Forward 5'-AGCATCACGGCCATCTGTG-3' Reverse 5'-TGGCAAACCGTCTCCAGAGT-3'

Transmission Electron Microscopy

The tissue blocks prepared with perfusion fixation method were postfixed with phosphate-buffered 2% osmium tetroxide, dehydrated, and embedded in Poly/Bed 812. Thin sections were examined in a transmission electron

microscope (model 100CX, JEOL Ltd., Tokyo, Japan) with an accelerating voltage of 80 kV.

HSC Isolation and Culture

HSCs were isolated as previously described¹⁵ from C57BL/6j mice by using pronase/collagenase perfusion followed by Nycodenz gradient centrifugation. Isolated primary HSCs were cultured in Dulbecco's modified Eagle medium (Wako, Osaka, Japan) containing 10% fetal bovine serum, 100 mg/mL penicillin, and 100 U/mL streptomycin.

The human HSC line HHStECs (lot number 10326) was purchased from ScienCell Research Laboratories (Carlsbad, CA). These cells were maintained by using the Stellate Cell Medium set (catalog number 5301) at 37°C in a humidified 5% carbon dioxide atmosphere.

To generate an epithelial cadherin (E-cadherin)-coated dish, recombinant E-cadherins (1.5 µg/mL; R&D Systems, Minneapolis, MN) were coated in a 24-well plate with phosphate-buffered saline for 2 hours followed by blocking with 1% bovine serum albumin containing phosphate-buffered saline for 3 hours. Mouse primary HSCs or HHStECs were then cultured in the plate.

Three-Dimensional Spheroid Culture

HHStECs or mouse primary HSCs were seeded 5.0×10^3 per well in a 96-well, low attachment spheroid culture plate (Sumitomo Bakelite, Tokyo, Japan). For analysis, at least six spheroids were pooled. For neutralization of E-cadherin, E-cadherin monoclonal antibody (SHE78-7, Thermo Fisher Scientific, Waltham, MA) was added into the culture medium at the concentration of 10 µg/mL. For the analysis of TGF-β signaling in the presence or absence of E-cadherin-mediated adherens junctions, empty vector-transfected (control), or E-cadherin overexpressed HHStEC cells were cultured as spheroid and stimulated by 10 ng/mL of TGF-β. Proteins were then isolated at 0, 5, 10, 30, and 60 minutes after TGF-β stimulation.

Plasmid Transfection

To generate transient overexpression of E-cadherin or TAZ, HHStECs were transfected with an empty plasmid, hE-

cadherin-pcDNA3 (gift from Barry Gumbiner; plasmid number 45769, Addgene, Cambridge, MA) or HA-TAZ (gift from Kunliang Guan; plasmid number 32839, Addgene) using lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer's protocol.

Quantitative Real-Time and Direct Reverse Transcription PCR of mRNA

RNA was extracted from cells by using TRIzol reagent (Thermo Fisher Scientific) and Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA). Real-time quantitative PCR (qPCR) was performed by using cDNA generated from RNA and the SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific). The qPCR reaction was conducted by using the SYBR green PCR master mix (Thermo Fisher Scientific) in the Thermal Cycler Dice Real Time System 2 (Takara Bio, Shiga, Japan). The values were quantified by using the comparative CT method and were normalized to 18S ribosomal RNA. Data are expressed as the ratio to the average of the control group. The primers used in this study are listed in Table 1.

Western Blot

Cells were homogenized with radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1% SDS) containing the protease inhibitor cocktail cOmplete Mini (Roche, Basel, Switzerland) and phosphatase inhibitors (1 mmol/L sodium fluoride, 1 mmol/L β-glycerol phosphate, and 1 mmol/L sodium vanadate). Protein samples were subjected to 8% to 15% SDS-PAGE and were transferred to polyvinylidene difluoride membranes using standard Western blot techniques. After blocking with 5% skim milk, the membranes were probed with primary antibodies diluted at 1:1000 and horseradish peroxidase-conjugated secondary antibodies diluted at 1:5000. Immunoreactive bands were visualized by using the ImmunoStar Zeta or ImmunoStar LD system and were detected with an LAS3000 or LAS4000 device (GE Healthcare, Milwaukee, WI). WB Stripping Solution (Nacalai Tesque, Kyoto, Japan) was used to remove the antibodies from the Western blot.

Figure 1 Morphologic changes of spine-mediated adherens junctions in hepatic stellate cells (HSCs) with hepatocytes during liver inflammation. **A–E:** Transmission electron microscopy of liver in 6- to 7-week-old Wistar rats. **A:** A representative image of the relationship between hepatocytes and HSCs in a normal liver is shown in the **left panel**. The **middle** and **right panels** show the enlarged images of the asterisk-marked region of the **left panel**. HSCs extend spines toward hepatocytes (**yellow arrows**). Some tips of spines have high electron density, suggesting that the spine constitutes an adherens junction with hepatocytes. **B:** Shown is the enlarged image of the region where a hepatocyte and an HSC are in contact. **C:** The **top left panel** shows the toluidine blue staining image around a central vein (CV) under a light microscope. The representative image around a CV at 12 hours after carbon tetrachloride (CCl₄) injection is shown in the **bottom left panel**. The **right panel** shows the enlarged figure of the **bottom left panel**. The spines in HSCs have disappeared while the microvilli extending from the hepatocytes remain (**black arrows**). **D** and **E:** Features of the perpetuation phase of HSCs at 12 hours after CCl₄ injection. **D:** A mitotic figure of the HSC is shown by a **red arrow**. **E:** *De novo* synthesis of collagen fibrils by the HSC are shown by **red arrowheads**. **F–G:** Transmission electron microscopy of normal mouse liver. **F:** A representative image of the relationship between hepatocytes and HSCs in a normal liver of 8- to 10-week-old C57BL/6j mice is shown in the **left panel**. The enlarged image focusing on the site of adherens junctions between hepatocytes and HSCs (**yellow arrows**) is shown in the **right panel**. **G:** The representative image of the mouse liver at 6 hours after CCl₄ injection is shown in the **left panel**. The right panel shows the enlarged image of the loss of adherens junctions in HSCs with hepatocytes. Scale bars: 2 µm (**A** and **G, left panels**) and 500 nm (**A, middle and right panels; E; and F, right panel**); 100 nm (**B**); 1 µm (**C** and **D; F, left panel; and G, right panel**). VA, vitamin A droplet.

Figure 2 Hepatic stellate cells (HSCs) reconstruct adherens junctions with regenerated hepatocytes in the recovery stage. Transmission electron microscopy of the liver of 6- to 7-week-old Wistar rats 7 days after carbon tetrachloride (CCl_4) injection. **A:** HSCs store lipid droplets as a feature of quiescent and reconstructed adherens junctions with hepatocytes (red arrows). The inset figure shows the toluidine blue staining image under a light microscope. **B:** An HSC, which has not yet recovered the adhesion with hepatocytes, extends regenerating spines and establishes an adherens junction *de novo* with a regenerating, mitotic hepatocyte. **C:** Fibrous structure linking a hepatocyte and an HSC is shown as a yellow arrow. An enlarged image is shown in the right panel. Scale bars: 1 μm (A); 100 nm (B); 200 nm (C).

membrane. The quantification of Western blot bands was performed by using ImageJ software version 1.52n (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Data are expressed as means $\pm$ SEM of at least three different experiments.

Flow Cytometry

Isolated HSCs were stained with Alexa Fluor 647 anti-human/mouse CD324 (DECMA-1; BioLegend, San Diego, CA) or isotype control (RTK2071; BioLegend). HSCs emit autofluorescence under UV excitation due to their vitamin A content.¹⁶ Therefore, the expression of E-cadherin in UV autofluorescence-positive cells was characterized by using FACS LSR II (BD Biosciences, San Diego, CA).

Histological, Immunohistochemical, and Immunofluorescence Analysis

For histological evaluation, mouse liver were fixed with 4% paraformaldehyde and embedded into paraffin. Then, mouse liver section were sliced (4 μm) and stained with hematoxylin and eosin. For immunohistochemical analysis, they were deparaffinized, hydrated, heated to 110°C in citrate buffer for 20 minutes, depleted of endogenous peroxidase activity, and then blocked with Blocking One (Nacalai

Tesque) for 1 hour. Next, the slides were treated with primary antibodies overnight at 4°C. The slides were incubated with secondary antibody (Bioss, Inc., Woburn, MA) for 1 hour at room temperature, and the reaction was visualized by diaminobenzene substrate (Vector Laboratories, Burlingame, CA). All specimens were counterstained with hematoxylin. For immunofluorescence analysis, after hybridization with primary antibodies, the slides were incubated with Alexa Fluor 488 or 594 conjugated donkey anti-mouse or rabbit IgG (Invitrogen, Carlsbad, CA). DAPI (Dojindo, Kumamoto, Japan) was used as a nuclear marker. For quantitative analysis, three images were taken at every indicated time point, and cells positive for Ly6G per field of each view were counted.

For immunoelectron microscopy, 4% paraformaldehyde fixative-fixed, frozen mouse liver slices were cut into 4 μm with a cryostat (HM 560; Microm, Walldorf, Germany). The immunostaining procedure was similar to that described in the previous paragraph. After visualizing by diaminobenzene, they were postfixed with 2% osmium tetroxide (Wako) for 1 hour on ice and dehydrated in an ethanol series before embedding in a Quetol 812 mixture. Ultrathin sections were stained with lead citrate solution and observed in Talos F200C G2 (Thermo Fisher Scientific) with an accelerating voltage of 200 kV.

Primary Antibodies

The following antibodies were used for Western blot analysis and immunofluorescence: anti-E-cadherin (24E10), anti-phospho-Smad2 (Ser465/467), anti-Smad2 (D43B4), anti-YAP (D8H1X), anti-TAZ (V386), and anti-platelet-derived growth factor-receptor β (28E1), purchased from Cell Signaling Technology (Danvers, MA). Anti-neural cadherin (H-2), anti-placental cadherin (D-6), anti-vascular endothelial cadherin (F-8), and anti-heat shock protein 47 (G-12) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- α -smooth muscle actin (1A4; DAKO, Glostrup, Denmark), anti-Ly6G (1A8; R&D systems), anti-glyceraldehyde-3-phosphate dehydrogenase (Millipore, Billerica, MA), and anti-cytoglobin (kindly provided by Prof. Norifumi Kawada, Osaka City University) were also used for Western blot and immunohistochemical analyses.

ELISA of Cellular Supernatants

E-cadherin or TAZ was overexpressed in HHStC cells and cultured as spheroids. Forty-eight hours later, the levels of TGF- β in the supernatant of spheroid culture were determined by using a human TGF- β ELISA kit (BioLegend).

Statistical Analysis

All data are expressed as means $\pm$ SD. All data were analyzed by using analysis of variance with a post hoc Dunnett's test or unpaired *t*-test.

Results

Activated HSCs Lose Adherens Junctions with Hepatocytes

The morphologic changes of adherens junctions between hepatocytes and HSCs in rat liver during CCl₄-induced acute liver injury were investigated first. In normal liver, several spine-mediated adherens junctions with hepatocytes projected out both from the surface of the dendritic processes as well as from the cell bodies (Figure 1A). A high magnification of an adherens junction between the spine of an HSC and hepatocyte is shown in Figure 1B. The adhesive power of the junction is illustrated by the subjunctional cytoplasm of the hepatocyte being pulled up to form a spine. Actin-like microfilaments in both cells aligned together within each subjunctional region.

At 12 hours, individual HSCs in the injured areas were surrounded by hepatocytes with ballooning degeneration (upper left image of Figure 1C). Some microvilli of the degenerating hepatocytes remained in close contact with HSCs. Importantly, however, neither spines nor adherens junctions were observed (Figure 1C), whereas mitotic figures (Figure 1D) were observed in HSCs.

Characteristically, Golgi vacuoles located close to the cell membrane of HSCs were filled with collagen fibrils, some of which appeared to be secreted into the extracellular space (Figure 1E).

Based on the findings from the rat experiments, morphologic studies were validated in a mouse CCl₄ model. Similar to findings in rat liver, spine-mediated adherens junctions with hepatocytes were also identified between hepatocytes and HSCs in normal mouse liver (Figure 1F), which disappeared within 6 hours after CCl₄ injection (Figure 1G).

HSCs Reconstitute Adherens Junctions with Regenerated Hepatocytes during Recovery

Next examined was the relationship between HSCs and hepatocytes in rat liver 7 days after a single CCl₄ injection, at the stage of subsiding injury. Damaged hepatocytes had recovered, and inflammatory cell infiltration had subsided. HSCs reconstituted their adherens junctions with hepatocytes with increased lipid droplets, a feature of HSC quiescence⁴ (Figure 2A). Interestingly, the HSCs which had not yet restored their adhesion to hepatocytes, extended regenerating spines and established an adherens junction *de novo* with a regenerating, mitotic hepatocyte (Figure 2B). In the same region, fibrous structures linking hepatocytes and HSCs were observed at the tip of the spine (Figure 2C).

E-Cadherin Mediates Adhesion between Hepatocytes and HSCs

Cadherins constitute a large superfamily of cell–cell adhesion molecules.^{17,18} The classic cadherins consist of E-cadherin, neural cadherin, placental cadherin, and vascular endothelial cadherin.¹⁹ Thus, we explored the cadherin(s) responsible for hepatocyte–HSC adherens junctions. Almost all of the isolated mouse HSCs contained lipid droplets (left upper panel of Figure 3A) and expressed cell-specific markers such as cytoglobin²⁰ and heat shock protein 47,²¹ whereas none of the HSCs expressed albumin and CK18, the specific markers of hepatocytes (right upper panel of Figure 3A). These data confirmed the high purity of isolated mouse HSCs. In addition, murine HSCs expressed E-cadherin but not other cadherins (bottom panel, Figure 3A). The expression of E-cadherin in HSCs was examined by flow cytometry. Because they contain vitamin A, quiescent HSCs have autofluorescence when excited by UV.²² As shown in the upper panel of Figure 3B, 99.1% of isolated cells were UV-positive, which expressed E-cadherin (middle and bottom panel, Figure 3B). Similar to a previous report of rat HSCs,¹² this study also confirmed that the expression of cadherin in mouse HSCs switched from E-cadherin to neural cadherin along with morphologic changes and spontaneous activation (Figure 3C). Moreover,

desmin²³ or cytoglobin-positive HSCs expressed E-cadherin by immunofluorescence (Figure 3D).

Generally, E-cadherin is expressed primarily in epithelial cells, including hepatocytes. Although HSCs are a type of mesenchymal cells, previous studies have shown that E-cadherin co-localizes with glial fibrillary acidic protein, a classic marker of quiescent HSCs in rat normal liver.^{12,24} Therefore, we assessed immunohistochemical staining for E-cadherin in normal mouse liver, and detected it at the surrounding cytoglobin-positive cells as well as between hepatocytes (Figure 3E), indicating that hepatocytes express E-cadherin even on the sinusoidal surface. Considering that cadherins are homophilic cell-adhesion molecules, these findings indicate that E-cadherin might contribute to the adhesion between hepatocytes and HSCs.

For a more detailed analysis, the localization of E-cadherin was visualized by using immunoelectron microscopy. As expected, the immunostain for E-cadherin was confirmed at the adherens junctions between hepatocytes and HSCs (Figure 3F) as well as hepatocyte–hepatocyte adherens junctions (Figure 3G). Taken together, our findings support the conclusion that E-cadherin is expressed in HSCs and mediates adhesion with hepatocytes.

Initiation of HSC Activation Is Independent of Stimulation by Exogenous Initiation Factors

We next analyzed the temporal relationship between the disappearance of the adherens junctions and the enhancement of exogenous factors for HSC initiation using a mouse CCl₄ model. Hematoxylin and eosin staining and immunohistochemical staining for a neutrophil marker, Ly6G, revealed that the infiltration of neutrophils, which secrete exogenous activation factors of HSCs such as reactive oxygen species,²⁵ was present at 12 and 24 hours but not at 6 hours after CCl₄ when E-cadherin–mediated adherens junctions disappeared (Figure 4, A and B). As indicated by qPCR, mRNA expression levels in the liver for exogenous stimuli that encode important drivers of HSC activation,

including *Tgfb1*, *Pdgf*, *Tnfa*,²⁶ *Il1b*,^{27,28} and *Il6*,²⁹ were still not changed within 6 hours after CCl₄ injection (Figure 4C). In addition, the expression of *Acta2*, which encodes α -smooth muscle actin, a specific marker of HSC activation, tended to increase 3 hours after CCl₄ injection.

The rodent experiments revealed that the HSCs had already lost adherens junctions with hepatocytes in the early stage of injury, when few, if any, exogenous initiating factors are produced. However, TGF- β signaling, which is a typical driver of perpetuation of the HSC-activated phenotype, is not always regulated by its level of expression but rather through its activation from its latent form. We therefore analyzed the effect of E-cadherin–mediated adherens junctions on the response to TGF- β signaling. E-cadherin–overexpressed HHStECs were cultured as spheroids that reconstruct E-cadherin–mediated adherens junctions and were stimulated by TGF- β . Phase-contrast microscopy showed a clear outline of each cell in the control. In contrast, a fused image of the cell contour was observed after overexpression of E-cadherin. In addition, whereas the expression of TGF- β receptor type II was not altered (Figure 4D), phosphorylation of Smad2 and Smad3 was suppressed in HHStECs compared with control cells after reconstitution of E-cadherin–mediated adherens junctions (Figure 4E). Additionally, the expression of *SNAI1* and *CDH2* genes and α -smooth muscle actin protein, which are downstream of TGF- β signaling, was significantly decreased in the presence of homophilic E-cadherin binding (Figure 4, F and G). These findings support our hypothesis that the initiation of HSCs by the disappearance of E-cadherin–mediated adherens junctions occurred independently from the stimulation by exogenous initiation factors.

Mechanism of HSC Initiation following the Loss of Adherens Junctions

We next investigated the pathway initiating activation of HSCs during the loss of adherens junctions with hepatocytes. In

Figure 3 Epithelial cadherin (E-cadherin) mediates adhesion between hepatocytes and hepatic stellate cells (HSCs). **A:** The image of the phase-contrast microscope of isolated mouse HSCs (mHSCs) is shown in the **top left panel**. The high purity of isolated mHSCs was confirmed by Western blot (**top right panel**). Western blot analysis of cadherins in mHSCs just after isolation is shown in the **bottom panel**. Two representatives are shown here. HepG2 is positive control for the cadherins. Albumin and cytokeratin 18 (CK18) are specific markers of hepatocytes. Cytoglobin and heat shock protein 47 (HSP47) are specific markers of HSCs. **B:** Flow cytometry analysis of primary mouse HSCs. HSCs are gated by UV-positive cells (**top panel**). Histogram data show the expression of E-cadherin in primary mouse HSCs. A representative image of at least three independent experiments is shown. HSCs were stained with isotype antibody (**middle panel**) or anti-E-cadherin antibody (**bottom panel**). **C:** Isolated mHSCs were cultured and observed by phase-contrast microscopy at the indicated time points. HSCs on day 1 contain abundant lipid droplets. However, the amount of lipid droplets was decreased, and the cell bodies began spreading with time. The **bottom panel** shows Western blot analysis of cultured mHSCs at the indicated time points. Representative images of three independent experiments are shown. **D:** Isolated mHSCs were cultured on glass chamber slides. After 24 hours' culture, HSCs were immunostained for E-cadherin and quiescent HSC markers desmin and cytoglobin for 24 hours followed by DAPI staining for 15 minutes. **E:** Normal mouse liver sections were immunostained for E-cadherin and cytoglobin, a specific marker of HSCs, for 24 hours followed by DAPI staining for 15 minutes. E-cadherin was detected by immunostaining at the surrounding cytoglobin-positive cells (**arrows**) as well as between hepatocytes (**arrowheads**). **F–G:** Immunoelectron microscopic images of E-cadherin in normal mouse liver. The expression of E-cadherin was confirmed at the adherens junctions between hepatocytes and HSCs (**F, arrows**) as well as hepatocyte–hepatocyte adherens junctions (**G, arrows**). $n = 4$ (**A** and **B**). Scale bars: 50 μ m (**A; C; D, top panels**; and **E**); 2 μ m (**F, left panel**), 1 μ m (**F, right panel**; and **G**). Ecad, epithelial cadherin; FSC, forward scatter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ncad, neural cadherin; Pcad, placental cadherin; VEcad, vascular endothelial cadherin.

A

B

C

D

E

F

G

normal differentiated epithelial tissues, contact inhibition suppresses cell activity through homophilic E-cadherin binding to tightly regulate tissue morphogenesis,^{30,31} which involves YAP/TAZ signaling.³² When epithelial cells proliferate, YAP/TAZ localizes to the nucleus where it is transcriptionally active. In contrast, when the epithelial cells contact other cells through E-cadherin, YAP/TAZ is phosphorylated, underlying the effect of contact inhibition.³² Immunohistochemical staining for YAP and TAZ revealed that TAZ expression around central veins was increased in mouse liver 12 hours after a CCl₄ injection compared with that in normal mouse liver. However, most of the staining was cytoplasmic, not nuclear, and not transcriptionally active (Figure 5A). Moreover, although the expression of TAZ was not observed in desmin-positive cells in untreated mouse liver, TAZ expression increased and localized in the nuclei of desmin-positive cells 12 hours after CCl₄ injection (Figure 5B).

We next examined the YAP/TAZ pathway in isolated HSCs from mouse liver 12 hours after CCl₄ injection. First, we verified the purity of isolated HSCs from damaged liver after CCl₄ injection. As shown in the upper panel of Figure 5C, the expression of cytoglobin, but not albumin, was confirmed in HSCs from both untreated and CCl₄-injected mice. We then investigated YAP and TAZ expression in HSCs by Western blot analysis. YAP and especially TAZ expression was dramatically increased, along with α -smooth muscle actin, which is an activation marker of HSCs, compared with those of untreated mice (bottom panel of Figure 5C). These results indicate that the expression of TAZ increased in HSCs that had lost their adherens junctions with hepatocytes.

Recent studies show that the Hippo pathway is involved in HSC activation.^{33,34} Therefore, hypothesized that the adherens junctions between hepatocytes and HSCs maintain HSC quiescence through contact inhibition that suppresses YAP/TAZ signaling. To verify this hypothesis, we mimicked E-cadherin-mediated contact inhibition in culture. Mouse primary HSCs, which express E-cadherin for

several days after isolation, were cultured on E-cadherin-coated dishes to create E-cadherin-mediated adherens junctions. Observation by phase-contrast microscope revealed that mouse HSCs on E-cadherin-coated dishes maintained a quiescent morphology, including multiple thin elongate processes that extended radially from the cell body,³⁵ whereas HSCs on uncoated dishes displayed an activation phenotype that included cell spreading and loss of dendritic process³⁶ (Figure 5D). In addition, the expression of TAZ and activation markers of HSCs was decreased in mouse HSCs cultured on E-cadherin-coated dishes compared with HSCs cultured on uncoated dishes (Figure 5E). As with mouse primary HSCs, culture of E-cadherin overexpressing human HHStECs on E-cadherin-coated dishes suppressed the expression of TAZ in a dose-dependent manner, which was paralleled by down-regulation of activation markers of HSCs (Figure 5F). Even in E-cadherin-overexpressing HHStECs, which were cultured as spheroids to reconstruct E-cadherin-mediated adherens junctions, suppression of HSC activation markers down-regulated TAZ expression (Figure 5G).

We next examined the influence of inhibiting E-cadherin-mediated adherens junctions using an E-cadherin neutralizing antibody. The addition of an E-cadherin neutralizing antibody to spheroids of cultured mouse HSCs induced activation markers of HSCs and the enhancement of downstream gene expression of the YAP/TAZ pathway (Figure 5H). This outcome suggests that the homophilic binding of E-cadherin regulates HSC activation by down-regulating the Hippo pathway.

TAZ Directly and Indirectly Regulates HSCs Activation

Previous studies have implicated TAZ in HSC activation^{34,37}; however, the direct regulation of TAZ in HSCs has not been fully elucidated. Therefore, we overexpressed TAZ in HHStECs to investigate whether TAZ directly regulated HSC activation. TAZ overexpression led to the up-regulation of the

Figure 4 The initiation of hepatic stellate cells (HSCs) occurred independently from the stimulation of exogenous initiation factors. **A:** Liver sections at the indicated time points after carbon tetrachloride (CCl₄) injection were stained with hematoxylin and eosin. Infiltration of inflammatory cells was present at 12 hours and 24 hours (arrows) but not at 6 hours after CCl₄ administration. **Inset** figures show the enlarged image. **B:** Immunohistochemical staining for Ly6G, a specific marker of neutrophils, is shown in the **left panel**. For quantitative analysis, three images captured at every indicated time point, and the number of positive cells for Ly6G per field of each view, were counted (**right panel**). **C:** mRNA expression of exogenous stimulation factors for HSC activation and *Acta2* in liver. CCl₄ (5 μ L/g body weight) was injected into mice, and mRNA was isolated from the whole liver at the indicated time points after CCl₄ injection. Gene expression for acute inflammatory cytokines that contribute to HSC initiation, including *Tnfa*, *Il1b*, *Il6*, *Tgfb*, and *Pdgf*, and an activation marker of HSCs, *Acta2*, were analyzed by real-time quantitative PCR (qPCR). **D:** Human HSC line (HHStEC) cells transfected with epithelial cadherin (E-cadherin) or control cells were cultured as spheroids for 48 hours. The **top left panel** shows control cells and the **top right panel** shows E-cadherin-transfected HHStEC cells. Then, the expression of transforming growth factor beta (TGF- β) receptor 2 was examined by qPCR (**bottom panel**). **E–G:** The influence of E-cadherin-mediated adherens junctions on TGF- β -Smad2/3 pathway. **E:** HHStEC cells transfected with E-cadherin or control cells were cultured as spheroid for 48 hours. The HHStEC cells were then stimulated with 10 ng/mL TGF- β for each indicated time. Proteins were isolated at the indicated time points, and the phosphorylation of Smad2 and Smad3 was evaluated by using Western blot analysis. **F:** HHStEC cells transfected with E-cadherin or control cells were cultured as spheroids for 24 hours. The HHStEC cells were then cultured with 10 ng/mL TGF- β for 48 hours. The gene expression of *SNAI1* and *CDH2* were analyzed according to qPCR. **G:** α -Smooth muscle actin (α -SMA) expression was investigated by using Western blot analysis. A representative image of three independent experiments is shown in the **left panel**. For quantification, the band density of α -SMA was analyzed and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) density (**right panel**). Data are expressed as means $\pm$ SD (**B–G**). $n = 3$ (**A** and **C**); $n = 6$ (**D**); $n = 4$ (**F**). * $P < 0.05$ versus 0h; [†] $P < 0.01$. Scale bars: 100 μ m (**A** and **B**); 50 μ m (**D**). O/E, overexpressed.

perpetuation markers of HSCs, suggesting that the enhancement of TAZ expression due to the loss of adherens junctions is an initiation factor in HSC activation (Figure 6A). Interestingly, in addition to YAP/TAZ pathway—modulated genes such as *ACTA2* and *CTGF*, the downstream genes of TGF- β signaling, including *PDGFRB* and *COL1A1*,^{38,39} were induced by TAZ overexpression, which was also associated with the increase in *TGFBI* gene expression (Figure 6B). Conversely, the expression of *TGFBI* in HHStECs was significantly decreased by reconstitution of E-cadherin—mediated adherens junctions (Figure 6C). TGF- β , which is mainly produced by activated HSCs in mouse fibrosis models,⁴⁰ acts as an autocrine-positive regulator for ECM production^{40,41} and up-regulates *TGFBI* gene expression in HHStECs; the expression level of its receptor *TGFBR2* was not changed (Figure 6D). Moreover, after the enhancement of YAP/TAZ expression, TGF- β expression in isolated mouse HSCs was increased along with their spontaneous activation (Figure 6E). A previous study reported a decrease in TGF- β expression after YAP and TAZ siRNA treatment in NIH3T3 cells.⁴² We therefore hypothesized that TGF- β expression increased as a result of HSC activation by YAP/TAZ enhancement, followed by the up-regulation of TGF- β —modulated genes in an autocrine manner. To verify this hypothesis, we examined the expression level of TGF- β in the culture supernatants of E-cadherin or TAZ-overexpressed HHStECs. The reconstruction of E-cadherin—mediated adherens junctions decreased TGF- β secretion. Conversely, overexpression of TAZ stimulated TGF- β production in the HHStECs (Figure 6F), indicating that besides direct regulation, TAZ indirectly activates HSCs via enhancement of TGF- β production in an autocrine manner.

These results clarify the relationship among E-cadherin—mediated adherens junctions between HSCs and hepatocytes, the role of TAZ, and the activation of HSCs. In normal liver, homophilic E-cadherin binding suppresses TAZ expression, which contributes to maintaining HSC quiescence. In contrast, the loss of this binding in injured

liver leads to an increase of TAZ expression and HSC activation (Figure 6G).

Discussion

This study identifies E-cadherin at the spines of quiescent HSCs as a key component of the adherens junctions between hepatocytes and HSCs. This interaction critically regulates HSC activation through discrete intracellular events that engage YAP/TAZ. Concurrently, isoform switching from E-cadherin to neural cadherin leads to the formation of a complex with platelet-derived growth factor—receptor.⁴³ The activation of HSCs by the loss of adherens junctions may lead to loss of contact inhibition, thereby promoting migration and responsiveness to extracellular signals. Based on morphologic observations, Gressner and Lahme's⁴⁴ prediction from over two decades ago that spines in HSCs represent sites of contact inhibition is reinforced by our current findings. Although our studies focused on changes in the Hippo pathway and its effectors (YAP/TAZ), there are also other signals downstream of E-cadherin—mediated contact inhibition, including Wnt and growth signaling pathways.⁴⁵ In addition to rodents, the loss of the adherens junctions has been observed in human liver. Using electron microscopy, Mak and Lieber⁴⁶ investigated human alcoholic steatosis and cirrhosis. In alcoholic steatosis, HSCs with abundant retinoid droplets comprised the adherens junctions linking the cells to surrounding hepatocytes. In the cirrhotic liver, however, HSC lipid content decreased, and their adherens junctions with hepatocytes disappeared, along with the accumulation of collagen in the space of Disse. Although a cause—effect relationship between the loss of adherens junctions with hepatocytes and initiation of HSCs activation in human liver is not fully established, it is clear that HSC loss of adherens junctions with hepatocytes is a feature of the fibrotic liver.

Our findings have also uncovered a mechanism underlying spontaneous activation of rodent HSCs when placed in culture

Figure 5 Homophilic binding of epithelial cadherin (E-cadherin) attenuates hepatic stellate cell (HSC) activation by down-regulation of Hippo pathway signaling. **A:** Liver sections of normal liver or 12 hours after carbon tetrachloride (CCl₄) injection were immunostained for YAP and TAZ. A representative image around a central vein (CV) is shown. **B:** Liver sections of untreated or CCl₄-injected mice were immunostained for desmin and TAZ for 24 hours followed by DAPI staining for 15 minutes. The expression of TAZ was absent from HSCs expressing the HSC-specific marker desmin in untreated mouse liver (**top panels**). In contrast, TAZ expression was increased and localized in the nuclei of desmin-positive cells 12 hours after CCl₄ injection (**white arrows, bottom panels**). **C:** The purity of isolated HSCs from untreated or CCl₄-injected mice was confirmed by Western blot analysis. The expression of cytoglobin but not albumin was observed in the HSCs (**top panels**). The expression of YAP, TAZ, and α -smooth muscle actin (α -SMA) in HSCs isolated from untreated or CCl₄-injected mice was examined by Western blot analysis (**bottom panels**). Two representative images are shown here. **D:** Isolated mouse HSCs (mHSCs) were cultured on uncoated or E-cadherin—coated dishes for 48 hours. The morphology of HSCs was examined by using a phase-contrast microscope. **E:** Isolated mouse HSCs were cultured on uncoated or E-cadherin—coated dishes for 48 hours. The expression of TAZ and the HSC activation marker α -SMA were examined by using Western blot analysis. **F:** Human HSC line cells transfected with E-cadherin or control cells were cultured on E-cadherin—coated dishes for 48 hours. The expression of HSC activation-related molecules was investigated by using Western blot analysis. **G:** Human HSC line cells transfected with E-cadherin or control cells were cultured as spheroid for 48 hours. The expression of HSC activation-related molecules was investigated by using Western blot analysis. **H:** The inhibitory effect of E-cadherin—mediated adherens junctions was examined by using an E-cadherin neutralizing antibody. Isolated mHSCs were cultured as spheroid to reconstruct E-cadherin—mediated binding in the presence or absence of E-cadherin neutralization antibody (10 μ g/mL). The gene expression of activation markers of HSCs, including downstream of YAP/TAZ pathway such as *Ctgf* and *Acta2*, were then examined by real-time quantitative PCR. Data are expressed as means $\pm$ SD (**H**). $n = 4$ (**C**); $n = 3$ (**D** and **H**). * $P < 0.05$ versus cont. Scale bars: 100 μ m (**A**); 25 μ m (**B**); 50 μ m (**D**). Ecad ab, E-cadherin neutralization antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP47, heat shock protein 47; mHepatocyte, mouse hepatocyte; Ncad, neural cadherin; O/E, overexpressed; Pcad, placental cadherin.

on plastic. The initiation of HSC activation by the up-regulation of TAZ induced TGF- β expression, consistent with previous findings that secretion of TGF- β by isolated HSCs in culture gradually increases in a time-dependent manner.⁴⁷ In addition to HSCs, other cells, including macrophages and LSECs, secrete TGF- β during liver injury. Our current results indicate that TGF- β activity diminished in HSCs that steadily establish their adherens junctions with hepatocytes, even in the presence of TGF- β secreted by these cells. A previous study showed that E-cadherin inhibits Smad2/3 phosphorylation.²⁴ Furthermore, the inhibition of YAP/TAZ signaling reduces the phosphorylation of Smad2/3,^{37,48,49} suggesting that E-cadherin-mediated adhesion of HSCs to hepatocytes not only maintains their quiescence but also attenuates TGF- β signaling.

We suggest that a group of LSECs and hepatocytes in contact with one HSC could be considered as a stellate cell unit or “stellon” and propose that the stellon constitutes a functional unit distinct from the hepatic lobule. Viewing our data in the context of a stellon provides a different concept that aligns with the cellular architecture of the liver. Specifically, one HSC surrounds several LSECs and adheres to 20 to 40 hepatocytes via its spines.¹⁴ Even if one or two hepatocytes are deleted from a hepatic cord, the initiation of HSC activation might not occur because other adherens junctions remain intact. However, in persistent liver injury, the loss of most adherens junctions between HSCs and hepatocytes through broad injury of hepatocytes promotes initiation of HSC activation. HSCs lose their adherens junctions to detach from hepatocytes, which allows them to acquire YAP/TAZ-dependent migration, proliferation, and the production of ECM.

Human HSCs lines, HHStECs and LX2, are often used for *in vitro* analysis of HSC biology. However, E-cadherin was barely expressed in these cell lines. Consistent with this finding, the cells were in a state of sustained activation. Thus, these lines would not be suitable to explore mechanisms of initiation of HSC activation. We simulated quiescence in these cell lines by overexpressing E-cadherin or maintainance in spheroid culture. Using this culture model enabled us to seek novel factors driving early HSC activation. In contrast, therapeutically re-expressing E-cadherin

could reconstitute adherens junctions to inhibit HSC activation.

We observed that HSCs extend their spines to hepatocytes in the recovery phase of hepatic injury. E-cadherin regulates actin filaments and cell shape,⁵⁰ indicating that the HSC spine reflects the cell's dynamics. Interestingly, we have discovered a previously unrecognized structure linking hepatocyte and HSCs. We considered that this fibrous structure possibly contributes to the re-establishment of a linkage between HSCs and hepatocytes. The elucidation of the components and function of the adherens junction by HSCs could reveal important insights into mechanisms of early HSC activation and hepatic fibrosis, and potentially identify novel antifibrotic targets.

The initial trigger for the disappearance of spines in HSCs is still unclear. Many intracellular events occur within hepatocytes immediately after CCl₄ administration. Interestingly, HSCs restore their spines during the recovery period from liver injury. We compared the gene expression in the liver of a CCl₄-injected rat with that of a normal rat by microarray analysis and identified cell protrusion dynamics-related genes such as *coll1*⁵¹ and *flna*,⁵² which were confirmed to be localized in activated HSCs (data not shown). Further study is needed to clarify how HSCs recognize this response.

In conclusion, our results suggest that E-cadherin-mediated adherens junctions with hepatocytes maintain HSC quiescence, and detachment from this junction contributes to the activation of HSCs and downstream signaling events that promote hepatic fibrosis.

Author Contributions

Conceptualization, investigation, visualization, and writing (original draft), H.U.; investigation and formal analysis, H.Y.; conceptualization and formal analysis, T.M.; investigation, N.K. and K. Inoue; investigation and writing (original draft), Y.H.; conceptualization, investigation, and writing (original draft), K.W.; resources and investigation, T.S.; conceptualization and writing (review and editing), S.L.F.; and conceptualization, supervision, writing (original draft), and project administration, K. Ikeda.

Figure 6 The relationship among epithelial cadherin (E-cadherin)-mediated adherens junctions between hepatocytes and hepatic stellate cells (HSCs), TAZ expression, and the activation of HSCs. **A** and **B**: The direct effect of TAZ overexpression in HSCs on its activation was examined. Human HSC line (HHStEC) cells overexpressed with TAZ or control cells were cultured as spheroid for 48 hours. The influence of TAZ overexpression on HSC activation was investigated by Western blot analysis (**A**) and real-time quantitative PCR (qPCR) (**B**). **C–F**: The relationship among E-cadherin-mediated adherens junction, YAP/TAZ pathway, and autocrine activation of HSCs by transforming growth factor beta (TGF- β). **C**: HHStEC cells overexpressing E-cadherin or control cells were cultured as spheroids for 48 hours. Gene expression of *TGFB1* was analyzed by qPCR. **D**: HHStEC cells were stimulated by TGF- β (10 ng/mL) for 48 hours. Gene expression of *TGFB1* and *TGFB2* was then analyzed by qPCR. **E**: Mouse HSCs were cultured on plastic dishes, and protein or mRNA was isolated at the indicated time points and then evaluated by using Western blot analysis (**left panel**) and qPCR (**right panel**). **F**: E-cadherin or TAZ was transfected in HHStEC cells. The cells were cultured as spheroids for 48 hours. The levels of TGF- β in culture medium were then analyzed by ELISA. **G**: Proposed mechanism of activation of HSCs by dissociation of E-cadherin-containing adherens junctions with hepatocytes. In normal liver, homophilic E-cadherin binding suppresses TAZ expression, which contributes to maintaining HSC quiescence. In contrast, the loss of this binding in injured liver leads to an increase of TAZ expression and autocrine stimulation by TGF- β , resulting in HSC activation. Data are expressed as means $\pm$ SD (**B–F**). $n = 3$ (**A** and **B**); $n = 6$ (**C** and **F**); $n = 4$ (**D** and **E**); * $P < 0.05$ versus control; † $P < 0.05$ versus day 1; ‡ $P < 0.05$. α -SMA, α -smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; O/E, overexpressed.

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