Molecular Mechanism Responsible for Fibronectin-controlled Alterations in Matrix Stiffness in Advanced Chronic Liver Fibrogenesis*

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Fibrosis is characterized by extracellular matrix (ECM) remodeling and stiffening. However, the functional contribution of tissue stiffening to noncancer pathogenesis remains largely unknown. Fibronectin (Fn) is an ECM glycoprotein substantially expressed during tissue repair. Here we show in advanced chronic liver fibrogenesis using a mouse model lacking Fn that, unexpectedly, Fn-null livers lead to more extensive liver cirrhosis, which is accompanied by increased liver matrix stiffness and deteriorated hepatic functions. Furthermore, Fn-null livers exhibit more myofibroblast phenotypes and accumulate highly disorganized/diffuse collagenous ECM networks composed of thinner and significantly increased number of collagen fibrils during advanced chronic liver damage. Mechanistically, mutant livers show elevated local TGF-β activity and lysyl oxidase expressions. A significant amount of active lysyl oxidase is released in Fn-null hepatic stellate cells in response to TGF-β1 through canonical and noncanonical Smad such as PI3 kinase-mediated pathways. TGF-β1-induced collagen fibril stiffness in Fn-null hepatic stellate cells is significantly higher compared with wild-type cells. Inhibition of lysyl oxidase significantly reduces collagen fibril stiffness, and treatment of Fn recovers collagen fibril stiffness to wild-type levels. Thus, our findings indicate an indispensable role for Fn in chronic liver fibrosis/cirrhosis in negatively regulating TGF-β bioavailability, which in turn modulates ECM remodeling and stiffening and consequently preserves adult organ functions. Furthermore, this regulatory mechanism by Fn could be translated for a potential therapeutic target in a broader variety of chronic fibrotic diseases.

Fibrosis is a part of the wound-healing response that maintains organ structure and integrity following tissue damage. However, excessive fibrosis contributes to a number of diseases such as liver fibrosis (1). Liver fibrosis is defined as an abnormal response of the liver to persistent injury and affects tens of millions of people worldwide (2). It is caused by hepatotropic virus infection and drug and alcohol abuse. Liver fibrosis is of great clinical importance because normal liver architecture is disrupted, and liver function is ultimately impaired. There is no effective treatment for liver fibrosis, and many patients end up with a progressive form of the disease, liver cirrhosis, often requiring a liver transplant (3, 4). The hallmark of liver fibrosis is excessive accumulation of mainly type I collagen-containing extracellular matrices (ECMs) and therefore involves both wound healing and fibrotic processes. ECM production and remodeling in the liver following injury involves the activation of specific cell types, hepatic stellate cells, which transdifferentiate into contractile and proliferative myofibroblasts (5–7).

Collagen is the most abundant scaffolding ECM in tissue/organ stroma and contributes significantly to tissue/organ integrity (8). The lysyl oxidase (LOX) family enzymes are copper-dependent amine oxidases that catalyze the process of covalent cross-linking of collagen by oxidatively deaminating specific lysine and hydroxylysine residues in the telopeptide domains, which increases collagen stiffness (9, 10). Collagen cross-linking stiffens the ECM and has been implicated in cancer cell invasion (11). Collagen cross-linking also accompanies

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3 The abbreviations used are: ECM, extracellular matrix; αSMA, α-smooth muscle actin; ALT, alanine aminotransferase; AFM, atomic force microscopy; BAPN, β-aminopropionitrile; Fn, fibronectin; LAP, latency-associated protein; LOX, lysyl oxidase; LTBP, latent TGF-β-binding protein; MMP, matrix metalloproteinase; mAb, monoclonal antibody; pAb, polyclonal antibody; TEM, transmission electron microscopy.
tissue fibrosis mediated by several profibrogenic cytokines (12). However, the functional contribution of collagen cross-linking to noncancer pathogenesis remains largely unknown.

Transforming growth factor (TGF)-β is a profibrogenic master cytokine responsible for promoting differentiation of tissue-resident fibroblasts into myofibroblasts, up-regulation of ECM production including fibronectin (Fn) and down-regulation of ECM degradation (13). Growing evidence suggests a mechanism by which Fn plays a role in TGF-β signaling (14). Fn is involved in the initial incorporation of LTBP-1 (latent TGF-β-binding protein 1) into the ECM in vitro (15). TGF-β is secreted in a biologically inactive (latent) form in a complex (large latent complex) with TGF-β latency-associated protein (LAP) and LTBPs (16). The extracellular TGF-β activity in response to injury is regulated by the local activation of latent TGF-β complex to active TGF-β. Indeed, elevated TGF-β bioavailability is frequently observed in chronic fibrotic diseases, and the inhibition of local TGF-β activation can protect against the progression of fibrosis in several adult chronic fibrotic diseases (17–20). Despite numerous in vitro studies and animal models, TGF-β/Fn interdependence in the fibrogenic response to advanced chronic liver damage has not yet been addressed in vivo.

Fn is a large dimeric glycoprotein that exists in blood plasma in its soluble form (plasma type) and in its insoluble form (cellular type) as a part of the ECM of almost every tissue in an organism. Plasma Fn is produced solely by hepatocytes in the liver (21, 22). Extensive in vitro functional studies have indicated that Fn plays a key role in a wide range of cellular behaviors (21, 22), and prominent expression of Fn is observed in response to liver injury (23). Based on in vitro findings, it has been postulated that collagen network formation depends on the Fn matrix (24, 25). We therefore hypothesized that removal of Fn from the in vivo system could prevent extensive ECM network formation following tissue damage. To define the functional identity of Fn in adult tissue remodeling, we recently established a null condition for both Fn isoforms in the adult liver. More, it is also unclear how ECM remodeling by myofibroblasts during the development of chronic tissue fibrosis. Further, it is also unclear how ECM remodeling by myofibroblasts results in changes in mechanical tension and supports the activation of pathogenic signaling pathways. Here, we have addressed the molecular mechanism responsible for tissue stiffness in advanced chronic liver fibrogenesis using a mouse model lacking both Fn isoforms in the adult liver.

**Experimental Procedures**

**Maintenance of Mice—**Mice lacking both plasma and cellular type fibronectin in the liver (liver Fn-null mice) were generated by intraperitoneal injections of polyinosinic-polycytidic acid in the Fnαfox/fox/MxCre+ strain as described previously (26). Fn-null livers showed normal liver morphology, and no obvious inflammation or fibrosis was noted after polyinosinic-polycytidic acid injections (26). All mice were maintained and bred at the animal facilities at the Cleveland Clinic (Cleveland, OH) and the University of Liverpool (Liverpool, UK) in accordance with institutional guidelines. The mice were regularly monitored and had free access to standard mouse chow and water. The mice received humane care in accordance with institutional guidelines.

**Induction of Chronic Liver Injury by CCl4—**Chronic liver injury was induced intraperitoneally by administrations of CCl4 solution in olive oil (Fluka) (0.5 ml/kg of body weight as 50% (v/v), twice a week for up to 17 weeks) in sex-matched, 12–15-week-old mice (26, 27). Control and liver Fn-null mice were derived from the same litters. Mice injected only with olive oil as negative controls showed no phenotypes (data not shown).

**Antibodies, Cytokines, and Reagents—**The following antibodies were used for the analyses: rabbit polyclonal antibody (pAb) against mouse fibronectin (Chemicon); rabbit mAb (E184; Epitomics) against αSMA; rabbit pAb against pSmad3, which specifically recognizes the phosphorylated C-terminal serine 423/425 of Smad3 (also cross-reacted with phosphorylated C-terminal serine 465/467 of Smad2) (a kind gift from Dr. Koji Matsuzaki, Kansai Medical University, Osaka, Japan); rabbit pAb against LOX (Sigma-Aldrich); rabbit pAb against cleaved caspase3 (Cell Signaling); mouse mAb against HSC70 (Santa Cruz); mouse mAb against β-actin (clone AC15; Sigma); and affinity-purified rabbit pAbs against mouse LAP of TGF-β1, mouse LTBP-3, human LTBP-4, and human type V collagen (26). The preparation and characterization of rabbit pAbs against bovine type I collagen and type III procollagen have been described elsewhere (28). Rabbit pAb against human lysyl oxidase was generated using its protein residues 22–168 expressed in HEK293 cells as an antigen and affinity-purified as described (29). Rabbit pAb against LTBP-1 was a kind gift from Dr. Lynn Sakai (Research Center, Shriners Hospitals for Children, Portland, OR). FITC- and Cy3-conjugated donkey anti-rabbit IgG, and peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG were from Jackson ImmunoRes Lab. DAPI was from Molecular Probes. EnvisionTM+ System HRP-labeled polymer was from DAKO. Recombiant human TGF-β1 was from R&D Systems. Bovine plasma fibronectin was from Sigma. All inhibitors were from Calbiochem.

**Histological Analysis, Immunohistochemistry and Immunofluorescence, and Transmission Electron Microscopy—**For histological analysis, liver samples were either directly frozen in OCT compound (Tissue-Tek, Sakura Finetek) or fixed overnight in 4% paraformaldehyde in PBS, pH 7.2, and dehydrated in a graded alcohol series before being embedded in paraffin. Sirius Red staining was performed according to standard protocols. Immunohistochemistry and immunofluorescence studies were performed as described previously (30, 31). Double immunohistochemical analysis with paraffin-embedded tissue sections was performed as described (26). Briefly, after the first antigen-antibody reaction, the slides were incubated with dissociation buffer containing 0.1 M glycine HCl (pH 2.2) to dissociate immunoglobulins from antigenic sites. Then the second antigen-antibody reaction was subsequently performed. We carried out the second reaction with-
out antibodies and confirmed the complete dissociation of the first immunoglobulins.

For quantification of fibrotic areas in response to chronic liver injury and LOX signal intensity in hepatic stellate cells, images were captured with the same gain, offset, magnitude, and exposure time. A minimum of five different images were randomly selected, and intensities were quantified using ImageJ software (version 1.48, National Institutes of Health). For cellular signal intensity, the mean intensity in each cell (intensities/areas, 50 cells) was measured, and the average intensity per cell (relative fluorescent units) was calculated as described elsewhere (32). Transmission electron microscopy was performed as described previously (26).
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Polarization Microscopy Analysis—Quantitative polarization microscopic analysis was performed on Sirius Red-stained sections using AbrioTM LS PolScope (CRI Inc., Woburn, MA) (33). In PolScope images, the brightness of each pixel is strictly proportional to birefringence “retardance” of the particular object point and independent of its slow axis orientation (34). Measured retardances and orientations are represented as pseudocolors. Images make it possible to determine specimen anisotropy (retardance and azimuth) at all angles (360°) simultaneously and permit quantitative measurements on the orientation and the degree of organization of the fibers (35). In the present analysis, any azimuth (slow axis orientation) was covered by a 180° range because a fiber bundle has no head or tail. Fiber retardations and orientations were assessed with an automated analyzing system. Fifty measurements were taken in each of the three regions and averaged. The degree of fiber alignment is represented by a calculation of the angular deviation of each orientation distribution. Thus, the smaller the angular deviation, the more aligned the fibers are.

Atomic Force Microscopy (AFM) Analysis—The nanomechanical properties of the liver sections were investigated with AFM (NanoScope VIII MultiMode AFM; Bruker Nano Inc., Santa Barbara, CA). The AFM was equipped with a 150 × 150 × 5-μm scanner (J-scanner) operated with the Peakforce Quantitative Nanomechanical Mapping modality. Liver samples were rapidly frozen in OCT compound (Tissue-Tek; Sakura Finetek), after which 10-μm-thick sections were prepared using a cryostat (Leica CM1950; Leica Microsystems) and adhered to glass slides coated with Vectabond (Vector Laboratories). Cells were directly seeded on glass coverslips and cultured for 7 days. Then the glass slides/cover slips were adhered to metal support stubs using adhesive pads, and the samples were imaged at ambient conditions using a silicon nitride tip. The tip had a nominal tip radius of 8 nm and a 5 N/m spring constant (Bruker TAP150A probe). The preparation method used for the AFM imaging maintained the tissue/cellular structure for imaging without the need for any chemical fixation. The Peakforce Quantitative Nanomechanical Mapping method was calibrated using a reference polymer (PS1; Vishay Measurements Group UK) with a known elastic modulus (2.8 ± 0.1 GPa). The spring constant and deflection sensitivity was calibrated using the reference sample, and the same AFM probe was used for imaging every sample. The data were fitted with the Derjaguin-Muller-Toporov model to extract the elastic modulus, as described previously (36). For tissue samples, 10 × 10-μm images were collected, and the mean elastic modulus was determined using Bruker Nanoscope software, v 1.5. Each image had a resolution of 384 samples/line. The regions selected for AFM were guided by Sirius Red-stained images of serial sections. Smaller scans (2 × 2 μm) were conducted in collagen fibril-rich areas and used to determine the elastic modulus distribution pixel by pixel in these locations. A Gaussian fit was applied to the data. For cell culture samples, collagen fibril areas were selected from 5 × 5-μm scan images, and the mean elastic modulus for collagen fibrils were determined from 0.5 × 0.5-μm scans.

Real Time PCR—Real-time PCR was performed as described previously (37). The following primers were used: ColIa1 forward, 5′-GGCGAGGTGCTGCTT TT-3′; Colla1 reverse, 5′-GGTCCCTGACTCTCACC TCTC-3′; ColIa1 forward, 5′-CGTGAAACATGGAATCTGGGA AA-3′; ColIa1 reverse, 5′-CCATAGCTGAACGTAAACACC-3′; Colf1 forward, 5′-GGAGGAACACACGGGAGG-3′; Colf1 reverse, 5′-CTTGTAGACCTCAGGACATGTC-3′; MMP8 forward, 5′-GGGATTATGGAAATGCGCTGAT-3′; MMP8 reverse, 5′-CGTGTITTCATCTGACCCTTGTA-3′; MMP13 forward, 5′- CCTCTCTTGTGATTACCCIGCTCCT-3′; MMP13 reverse, 5′-CTGTGAGGTCACGTCAGTTA GCT-3′; LOX forward, 5′-CCTATGCGGACAGATAGACT-3′; LOX reverse, 5′-CCA GGTAGCTGGGGTTTACA-3′; LOXL1 forward, 5′-ATGTT CAGCCTGGGAACACT-3′; LOXL1 reverse, 5′-GACGACAGGATTGAAATGCCT CGGAT-3′; LOXL2 forward, 5′-CGTTGAGGTGTCAGTGAG-3′; LOXL2 reverse, 5′-CAAAGAAGCCGA AAAGGATGG-3′; LOXL3 forward, 5′-GCCACGTAGTCTCGGTATG-3′; LOXL3 reverse, 5′-AGGGAGAGGTGGGTTCTCGT CTGCT-3′; LOXL4 forward, 5′-GCCGCTGCAAGATG TGATG-3′; LOXL4 reverse, 5′-GTCGAGTGGTCGTCTGGTGTA-3′; 18S rRNA forward, 5′-GCCGACGACCCATTCG-3′, and 18S rRNA reverse, 5′-ACCCGTGTGGTATGTTGA-3′.

All samples were analyzed in triplicate as a minimum. After the reactions, the specificity of amplifications in each sample was confirmed by dissociation analysis, showing that each sample gave a single melting peak. The relative mRNA levels were normalized to the level of 18S rRNA.

Western Blot Analysis—Western blot analyses were performed as described elsewhere (31). In some immunoblotting analyses, samples were transferred onto an Immobilon-FL PVDF membrane (Millipore) and probed with primary and IRDye 800CW- or IRDye 680-conjugated secondary antibodies (LI-COR Biosciences). Immunoreactive bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences).

TGF-β Bioassay—A mink lung cell line (TMLC) stably transfected with a plasmid encoding TGF-β1 and harboring a reporter construct fused to luciferase was used for the analysis (38). Active TGF-β levels were measured as described elsewhere (32).

Hydroxyproline Assay—Hydroxyproline content was measured as described elsewhere (26).

Hepatic Biochemical Markers—The serum alanine aminotransferase (ALT) level was determined using a standard kit (Genzyme Diagnostics P.E.I., Inc., Canada). Serum total protein (by the Biuret method), albumin (by the BCG method), total bilirubin (by the azobilirubin method), and cholinesterase (by the p-hydroxybenzocholine method) levels were measured using the Hitachi 7180 Auto Analyzer (Japan).

In Vitro Assay Using Mouse Hepatic Stellate Cell Lines—Control and Fn-null mouse hepatic stellate cell lines were established from adult primary hepatic stellate cell cultures under p21-null genetic background as described previously (26) and used for the analysis presented in this study. Briefly, hepatic stellate cells were isolated from adult Fn+/-p21/- mice liver as parental cells, and subsequently those lines were treated with Cre-transducing adenovirus to delete Fn-floxed genes (Fn-null hepatic stellate cells). Established cell lines showed similar morphology and typical activated hepatic stellate cell phenotypes (26). To examine the effects of TGF-β1 on LOX produc-
tion, hepatic stellate cells were cultured for 8 h with DMEM containing 8% Fn-depleted FBS. The medium was then replaced with DMEM without FBS containing 0.2 mM ascorbic acid and 2 pm TGF-β1. Cells were then incubated for 18 h and used for the analysis. To examine the inhibitory effect of collagen cross-linking, cells were treated with β-aminopropionitrile (200 μM BAPN; Fisher Scientific) containing DMEM, 8% Fn-depleted FBS, 0.2 mM ascorbic acid, and 2 pm TGF-β1, then incubated for 7 days, and used for the analysis. We confirmed that the concentration of BAPN used did not affect stellate cell proliferation (data not shown). To examine the effect of Fn on formed collagen stiffness, cells were cultured for 7 days with DMEM containing 8% Fn-depleted FBS, 0.2 mM ascorbic acid, and 10 μg/ml plasma Fn and then used for the analysis. To examine the signaling axis in TGF-β1-mediated LOX production, Fn-null stellate cells were cultured for 24 h with DMEM.
FIGURE 3. Significant hepatic dysfunction and increased liver matrix stiffness in Fn-null livers. A–C, serum levels of the hepatic biochemical markers total bilirubin (A), cholinesterase (B), and albumin (C) during chronic liver injury. The data are means ± S.D. (n = 6 for each group). Note that considerable loss of hepatic functional reserve is demonstrated in mutant livers, as evidenced by significant up-regulation of total bilirubin and down-regulation of cholinesterase levels. *, p < 0.05; **, p < 0.01. D–G, analysis of liver matrix stiffness using AFM at 17 weeks of CCl4 treatment. D, elastic modulus in liver tissues. The data are means ± S.D. Note that elastic modulus are significantly higher in mutant livers (n measured areas) = 9 for each group). *, p < 0.05. E, Gaussian fit of elastic modulus distribution determined pixel by pixel with the Peakforce method in a typical collagen fibril-rich area in each group (2 × 2-μm images were used for this analysis). F and G, AFM images showing a map of mechanical properties (log of the elastic modulus) (F) and topography (height) of collagen fibrils in control and Fn-null liver at 17 weeks of CCl4 treatment (G). Note that mutant livers show thinner and more dispersed fibrils. LivFn, liver Fn.
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A

Control

LivFn-null

LAP

LAP

LTBP-1

LTBP-1

LTBP-3

LTBP-3

LTBP-4

LTBP-4

B

Active TGF-β (RLU)

Control

LivFn-null

8 weeks

17 weeks

C

Control

LivFn-null

pSmad2/3

pSmad2/3

D

Control

LivFn-null

pSmad2/3 positive NPCs

Control

LivFn-null
containing 8% Fn-depleted FBS. The medium was then replaced with DMEM containing 0.2 mM ascorbic acid without FBS and with indicated supplements. Cells were preincubated for 1 h, treated with 2 μM TGF-β1, further incubated for 18 h, and then analyzed. Where indicated, cultures contained 10 μM SIS3 (IC50 = 3 μM) to inhibit Smad3, 10 μM LY294002 (IC50 = 1.4 μM) to inhibit p38 MAP kinase, 10 μM PD169316 (IC50 = 89 nm) to inhibit p38 MAP kinase, 5 μM 420119 (IC50 = 40 nm) to inhibit JNK, and 20 μM PD98059 (IC50 = 2 μM) to inhibit MEK1/2. We confirmed that the concentration of the inhibitors used did not affect stellate cell proliferation (data not shown). Data Presentation and Statistical Analysis—All experiments were performed in triplicate as a minimum, on separate occasions, and the data shown were chosen as representative of results consistently observed. The results are presented as means ± S.D. Differences between selected groups were analyzed using the Mann-Whitney U test. In cases where more than two groups were compared, the Steel-Dwass test was used. A p value of <0.05 was considered significant.

Results

More Extensive Fibrosis/Cirrhosis in Mice Lacking Fn during CCl4-induced Advanced Stages of Chronic Liver Injury—Based on our previous finding that Fn deficiency did not interfere with reconstruction and resolution of collagen organization in initial stages of liver damage (26), we hypothesized that Fn deficiency may not affect the reconstruction of ECM architectures even in advanced stages of chronic liver damage. To address functional roles of Fn during this process, chronic liver fibrosis was induced in a well-established mouse model where injury is brought on by treatment with a liver-damaging agent, CCl4 (0.5 ml/kg of weight, twice a week for 17 weeks) (27). Although increased expression of Fn protein was observed in control livers after injury, its expression in mutant livers (FnLox/lox MxCre+) was remarkably low throughout the experimental period (time 0 through 17 weeks during CCl4 injections). The Fn level in mutant livers at 17 weeks of CCl4 treatment was only ~2% of that in control livers (p < 0.01) as shown by Western analysis (Fig. 1A and data not shown). These data confirmed that neither Fn isofrom was induced in mutant livers during advanced chronic injury. Anatomical and histological analyses revealed no abnormalities in untreated Fn-null livers under standard laboratory conditions (Ref. 26 and data not shown). To determine whether Fn deficiency affects CCl4-induced liver damage during chronic injury, body weight changes, the serum ALT (as a measure for hepatocyte damage) and albumin/globulin ratio (as a measure of net hepatic damage) were examined. These parameters showed similar patterns in both control and Fn-null livers during 17 weeks of CCl4 treatment. Both showed no increase in body weight and a gradual decrease of ALT activity from 4 to 17 weeks and albumin/globulin ratio from 0 to 17 weeks (Fig. 1, B and C), confirming the advanced stages of chronic liver injury in both control and mutant livers (39). Fibrosis was then assessed by Sirius Red staining, which allows visualization of collagen fibers. Histologically, both control and mutant livers developed complete fibrosis patterns throughout the regions such as the P-C bridge (between the portal area and the central vein) and C-C bridge formations (between the central veins) (Fig. 1D), indicating micronodular cirrhosis (27). Unexpectedly, Fn-null livers developed more extended and significant fibrosis. Approximately 12% of the livers were Sirius Red-positive, representing a 3.2-fold increase over control livers (Fig. 1D, p < 0.01). Taken together, these findings indicate that prolonged and persistent chronic damage caused by 17 weeks of CCl4 treatment does establish advanced liver fibrosis/micronodular cirrhosis in both control and Fn-null livers.

Elevated Myofibroblast Activity in Mutant Livers—Myofibroblasts such as activated hepatic stellate cells play a central role in ECM remodeling upon liver injury. Immunofluorescence and Western analysis showed significantly more pronounced expression of α-smooth muscle actin (αSMA), a marker for myofibroblasts in Fn-null livers compared with controls at 17 weeks of CCl4 treatment (p < 0.05; Fig. 2, A and B). There was no obvious apoptosis or cell death in either liver type as shown by cleaved caspase 3 immunostaining and no picnotic nuclei with Hoechst 33342 staining at 17 weeks of CCl4 treatment (Fig. 2C and data not shown).

Significant Hepatic Dysfunction and Increased Liver Matrix Stiffness in Mutant Livers—Next, we examined whether a lack of Fn affects hepatic functions during chronic injury (Fig. 3, A–C). Subsequent analyses of serum hepatic biochemical markers (total bilirubin as a measure of hepatic excretory function; cholinesterase and albumin as a measure of hepatic protein synthesis) revealed no apparent differences between control and mutant livers until 8 weeks of CCl4 treatment. In contrast, in the advanced stage at 17 weeks, mutant mice suffered from a significantly higher level of plasma total bilirubin (0.12 ± 0.02 mg/dl in control (n = 6) versus 0.19 ± 0.04 mg/dl in mutant (n = 6), p = 0.0074) and lower levels of cholinesterase (26.8 ± 1.5 IU/liter in control (n = 6) versus 23.4 ± 1.3 IU/liter in mutant (n = 6), p = 0.025). Serum albumin levels were also decreased (3.37 ± 0.10 g/dl in control (n = 6) versus 3.26 ± 0.15 g/dl in mutant (n = 6)) but without significance (p = 0.39). In addition, liver matrix stiffness as determined by AFM was significantly elevated in Fn-null livers compared with controls at 17 weeks.

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FIGURE 4. Lack of Fn results in elevated accumulation of latent TGF-β complexes and continuous activation of TGF-β during chronic liver injury. A, significantly increased accumulation of LAP and LTBP-1, -3, and -4 (in red) in the ECM of mutant livers at 17 weeks of CCl4 treatment shown by immunofluorescence staining using serial sections. **, p < 0.01. Bar, 100 μm. B, active TGF-β bioassay at 8 and 17 weeks of CCl4 treatment. The data are means ± S.D. (n = 6 for each group). Note that mutant livers show significantly elevated local TGF-β activity at both time points. *, p < 0.05; **, p < 0.01. C, left panels, immunostaining for pSmaud2/3. Sections were counterstained with hematoxylin. Bar, 25 μm. Right panel, analysis of pSmaud2/3-positive cells. The data are means ± S.D. (n = 10 in each group). Note that the number of nuclear pSmaud2/3-positive (in brown) nonparenchymal cells (NPCs; red arrowheads) in mutant livers is significantly higher than controls at 17 weeks of CCl4 treatment. **, p < 0.01. For comparison, hepatocyte nuclei (~2–3-fold larger than those in nonparenchymal cells) are indicated (black arrows). D, left panels, double immunohistochemical staining for pSmaud2/3 (in purple/blue) and αSMA (in brown). Note that the nuclear pSmaud2/3-positive cells express myofibroblast marker αSMA in their cytoplasms (black arrowheads). Bar, 25 μm. Right panel, analysis of pSmaud2/3 and αSMA double-positive myofibroblasts. The data are means ± S.D. (n = 10 in each group). **, p < 0.01. LivFn, liver Fn.
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weeks of CCl₄ treatment (5,128 ± 553.6 MPa in mutant versus 3,313 ± 835.2 MPa in control (n = 9); p < 0.05, Fig. 3, D–G).

Elevated Production of Latent TGF-β Complexes and Increased Local TGF-β Bioavailability in Mutant Livers—Fn is associated with latent TGF-β complexes when incorporated into the ECM (16, 40), and local TGF-β activity is elevated in Fn-null livers in initial stages following liver injury (26, 41). Hence, the effect of Fn deficiency on the production of latent complexes of TGF-β and local TGF-β bioavailability during advanced chronic liver injury was also investigated. Pro-
nounced depositions of LAP and of LTBPl-1, -3, and -4 in the ECM of mutant livers were shown by immunohistochemistry at 17 weeks of CCl4 treatment. Interestingly, fibrillar structures were present even in the absence of Fn (Fig. 4A). Furthermore, the TGF-β activity in mutant livers was significantly higher than in controls at both 8 and 17 weeks of CCl4 treatment (Fig. 4B; \( p < 0.05 \)), indicating constitutively higher levels of active TGF-β in Fn-null livers during chronic liver injury.

TGF-β promotes the initiation of myofibroblast phenotypes and TGF-β1 alone sufficiently initiates stellate cell activation and transdifferentiation into myofibroblasts after liver injury, whereas Fn extra domain A promotes stellate cell motility but not transdifferentiation into myofibroblasts in vitro (26, 42). Latent TGF-β that is activated in response to injury binds to the TGF-β type I receptor, which then phosphorylates the C-terminal regions of Smad 2/3 transcription factors. Activated Smads translocate to the nucleus where they are involved in the regulation of gene expression (43, 44). Indeed, Fn-null livers showed a significantly increased number of nuclear p-Smad2/3-positive nonparenchymal cells (\( p < 0.01 \)) (Fig. 4C). Because constitutively elevated local TGF-β activity was demonstrated in Fn-null livers during chronic liver damage (\( p < 0.05 \); Fig. 4B), the functional link between TGF-β and myofibroblastic phenotypes at 17 weeks of CCl4 treatment was further addressed by double immunohistochemical staining. Fn-null livers showed a significantly increased number of p-Smad2/3 and αSMA double-positive myofibroblasts (31.3 ± 5.5 cells/field in mutant; field = 0.37 mm² (n = 10) versus 9.8 ± 3.3 cells/field in control (n = 10), \( p < 0.01 \)) (Fig. 4D). No nuclear localization of p-Smad2/3 was noted in either noninjured control or Fn-null livers (data not shown).

More Disorganized Collagen Networks with Increased Lysyl Oxidase Expressions in Mutant Livers—Based on these data, we hypothesized that the collagenous ECM fibrillar network formed in the absence of Fn may differ functionally from Fn-mediated collagenous fibrils, and the more extended or fibrotic collagenous network found in mutant livers (Fig. 1D) was associated with the impaired hepatic functions. Collagen network formation occurs as a multistep process and contributes to ECM remodeling and maintenance upon tissue damage (45). Therefore, the expression, assembly, and degradation levels of the major collagens, type I and III, in the advanced stages of chronic damage was next examined. Highly disorganized collagen matrices were induced in Fn-null livers. Immunofluorescent observation revealed that extensive networks of type I and III collagen were diffusely distributed in the ECM (Fig. 5A). Type I collagen mRNA levels was also significantly increased in Fn-null livers (\( p < 0.05 \); Fig. 5B). Collagen networks displayed more disorganized arrangements and significantly larger angular deviation in mutant livers as assessed by Sirius Red-stained samples under the polarized microscope (Fig. 5, C–E). In contrast, control livers formed well organized linear type III and type I collagen networks (Fig. 5, A and C–E). There were no significant differences in the expression of collagen-related matrix metalloproteinase (MMP) MMP8 and MMP13 mRNA levels (Fig. 5F), suggesting that collagen degrading enzymes did not play major roles.

Because type V collagen is an essential element for type III/I collagen fibrillogenesis in vitro when Fn is absent (26), the contribution of type V collagen to advanced stages of chronic liver remodeling at 17 weeks of CCl4 treatment was then determined. Significantly increased deposition and assembly of type V collagen was detected in Fn-null livers in comparison with controls, and the localization of assembled fibrils often overlapped with type I collagen fibrils (Fig. 6A). Increased expression of type V collagen mRNA (~1.5-fold) was also observed in mutant livers compared with controls, although the difference was not statistically significant (Fig. 6B). Subsequent ultrastructural analysis using transmission electron microscopy revealed that the mean diameter of collagen fibrils was identical (47.0 ± 9.7 nm in mutant versus 48.3 ± 8.9 nm in control (\( n = 1,500 \) fibrils in each)) (Fig. 6, C and D). It is known that the diameter of formed type I collagen fibrils is inversely proportional to the type V/type I collagen ratio (46–48). Indeed, a ~33% increase in the number of thinner (30–40 nm) diameter and a ~21% decrease in the number of thicker (50–60 nm) diameter) collagen fibril subpopulations was found in Fn-null livers compared with controls (Fig. 6D, right panel). Such thinner collagen fibril subpopulations were also confirmed by AFM analysis (Fig. 3G). Importantly, a significantly increased number of collagen fibrils was demonstrated in mutant livers compared with controls (48.6 ± 7.0 fibrils/0.25 μm² in mutant versus 41.7 ± 4.6 fibrils/0.25 μm² in control (\( n = 18 \); \( p < 0.01 \); Fig. 6E). The increased fibril number correlated with an increase in hepatic net collagen amount measured by hydroxyproline content (\( p < 0.05 \); Fig. 6F). Thus, these results indicate that elimination of Fn results in thinner and increased number of collagen fibril organizations/assemble and suggest both quantitative and qualitative differences between Fn- and type V collagen-mediated type I collagen fibril network formation following tissue injury.

**FIGURE 5.** Fn-null livers accumulate more extensive and disorganized collagen fibril networks at 17 weeks of CCl4 treatment. A, upper panels, deposition of type I and type III collagens at 0 (untreated) and 17 weeks of CCl4 treatment by immunofluorescent staining. Depositions at 17 weeks are shown using serial sections. Bar, 100 μm. Lower panels, quantification of positive areas at 17 weeks of CCl4 treatment. The data are means ± S.D. (\( n = 4 \) for each group). Note that deposition of type III and type I collagen is significantly increased in mutant livers compared with controls at 17 weeks of CCl4 treatment. **, \( p < 0.01 \), B, real time PCR analysis of Colla1 mRNA levels. Relative mRNA expression levels are shown relative to the control value of 1. The data are means ± S.D. (\( n = 5 \) for each group). Note that collagen mRNA levels in mutant livers are significantly up-regulated. *, \( p < 0.05 \). C–E, analysis of assembled fibrils using polarized microscopy (LS PolScope system). C, immunofluorescent staining for type I collagen (top row) and representative images of retardance and orientation by Sirius Red-stained serial sections (second and third rows, respectively). The level of retardation is denoted by a pixel color, as indicated by the color legend (from purple for the lowest to red for the highest). Note that the maximum magnitude of retardance (red) is identical in the control and mutant liver. Bars, 25 μm. D, representative distribution patterns of fibril bundle orientation in the control and mutant livers. The x axis represents the orientation angle. This is divided into 10° increments, and 0° corresponds to the mean orientation of distributions. The y axis shows the percentage of fibril bundles within 10° orientation angle range. Note that orientation of fibril bundles in mutant livers is less aligned and more disorganized compared with controls. AD, angular deviation. E, the average angular deviation of orientation distributions. The data are means ± S.D. Note that angular deviation is significantly larger in mutant livers. **, \( p < 0.01 \), F, real time PCR analysis of MMP8 and MMP13 mRNA levels. Relative mRNA expression levels are shown relative to the control value of 1. The data are means ± S.D. (\( n = 5 \) for each group). Note that the expression levels are not significantly different. LivFn, liver Fn.
FIGURE 6. Contribution of type V collagen to advanced chronic liver injury and collagen fibril ultrastructure at 17 weeks of CCl4 treatment. A, significantly increased deposition and assembly of type V collagen in mutant livers shown by immunofluorescence staining using serial sections (type V collagen in green; type I collagen in red; Fn in red; DAPI (cell nuclei) in blue) (n = 4 for each group). **, p < 0.01. Bar, 50 μm. B, real time PCR analysis of Col5a1 mRNA levels. Relative mRNA expression levels are shown relative to the control value of 1.0. The data are means ± S.D. (n = 4 for each group). Note that expression levels in mutant livers are ~1.5-fold increased but not significantly different. C–F, ultrastructural analysis of collagen fibrils using transmission electron microscopy. C, electron micrographs of longitudinal (upper panel) and transverse (lower panel) sections. Bar, 100 nm. D, left panel, the average diameter of collagen fibrils (1,500 fibrils for each group: 375 fibrils per animal, n = 4, were calculated). Note that the average fibril diameter is identical between control and mutant livers. Right panel, morphometric analysis of collagen fibril diameter (1,500 fibrils for each group: 375 fibrils per animal, n = 4, were calculated). Note the ~33% increase in the thinner (30–40-nm diameter) and the ~21% decrease in the number of thicker (50–60-nm diameter) collagen fibril subpopulations evident in mutant livers. E, collagen fibril number. The data are means ± S.D. Note that the fibril number is significantly higher in mutant livers (n (measured areas) = 18 for each group. **, p < 0.01. F, hydroxyproline contents. The data are means ± S.D. (n = 4 for each group). Note that net hepatic collagen content in mutant livers is significantly increased. *, p < 0.05. LivFn, liver Fn; n.s., not significantly different.
Collagen cross-linking by LOX family members accompanies tissue fibrosis and stiffens tissues (12). Elevated expression of LOX and its subfamily, LOX-like protein 2 (LOXL2), is detected in both human fibrotic diseases and rodent injury models, and myofibroblasts such as activated hepatic stellate cells are the likely major cellular source of LOX in liver injury (49, 50). Because significantly increased liver tissue stiffness alongside the deterioration of net hepatic function was demonstrated in Fn-null livers at 17 weeks of CCl4 treatment, the functional link between the lack of Fn and LOX expression levels was addressed. LOX and LOXL1–4 mRNA levels were all up-regulated in Fn-null livers compared with controls, and LOX was found to be most up-regulated in mutant livers (~2.64-fold) compared with controls (p < 0.01; Fig. 7A). Therefore, we determined whether elevated TGF-β bioavailability because of the absence of Fn is directly involved in the production of LOX in activated hepatic stellate cells in vitro. The treatment of Fn-null hepatic stellate cells cultured for 18 h with or without 2 pM TGF-β1 treatment. Bar, 25 μm. Right panel, the effect of Smad3 inhibitor SIS3, PI3K inhibitor LY294002, p38 MAP kinase inhibitor PD169316, JNK inhibitor 420119, and MEK1/2 inhibitor PD98059 on TGF-β1 induced LOX expression in Fn-null hepatic stellate cells. The data are from three independent experiments. **, p < 0.01. C, left panels, double immunofluorescence staining for LOX (in green) and DAPI (in blue) in Fn-null hepatic stellate cells cultured for 18 h with or without 2 pM TGF-β1 treatment. Bar, 25 μm. Right panel, the effect of Smad3 inhibitor SIS3, PI3K inhibitor LY294002, p38 MAP kinase inhibitor PD169316, JNK inhibitor 420119, and MEK1/2 inhibitor PD98059 on TGF-β1 induced LOX expression in Fn-null hepatic stellate cells. The data are from three independent experiments. **, p < 0.01.

FIGURE 7. Elevated LOX expression level in Fn-null liver at 17 weeks of CCl4 treatment and its mechanism. A, real time PCR analysis of LOX and LOXL1–4 mRNA levels in control and mutant livers. Relative mRNA expression levels are shown relative to the control value of 1. Error bars represent standard deviation (n = 4 for each group). *, p < 0.05; **, p < 0.01. B, left panels, Western blot analysis of LOX in Fn-null and its parental hepatic stellate cell (HSC) lines with or without treatment of 2 pM TGF-β1 in vitro. Aorta tissue lysates from adult wild-type mice were used as a positive control. Right panel, analysis of induction levels of active LOX in response to TGF-β1 (fold increase). Band intensity was measured by densitometry and normalized to HSC70 (loading control). The data are from three independent experiments. **, p < 0.01. C, left panels, double immunofluorescence staining for LOX (in green) and DAPI (in blue) in Fn-null hepatic stellate cells cultured for 18 h with or without 2 pM TGF-β1 treatment. Bar, 25 μm. Right panel, the effect of Smad3 inhibitor SIS3, PI3K inhibitor LY294002, p38 MAP kinase inhibitor PD169316, JNK inhibitor 420119, and MEK1/2 inhibitor PD98059 on TGF-β1 induced LOX expression in Fn-null hepatic stellate cells. The data are from three independent experiments. **, p < 0.01.
Matrix Stiffness in Advanced Liver Fibrosis

A

![Graph showing elastic modulus (MPa) for different conditions.

NTx# TGF-β1 TGF-β1+BAPN

Fn-null Parental

B

![Images showing collagen type I expression in Fn-null HSC with TGF-β1 and TGF-β1+BAPN.

TGF-β1 TGF-β1+BAPN

Collagen type I

Fn-null HSC

C

![Graph showing elastic modulus (MPa) for Fn-null (+Fn) and Parental conditions.

Fn-null (+Fn) Parental

n.s.
significant elevation of LOX expression in Fn-null hepatic stellate cells in response to TGF-β1 (Fig. 7C). Application of Smad3 inhibitor SIS3 following TGF-β1 treatment significantly inhibited (−42.0%) LOX expression in Fn-null hepatic stellate cells. In addition, P13K inhibitor LY294002 significantly inhibited LOX expression (−38.1%), although to a lesser degree (Fig. 7C). This demonstrates the involvement of both canonical and non-canonical Smad such as P13 kinase-mediated pathways in TGF-β1-mediated LOX expression in hepatic stellate cells.

Fn-null hepatic stellate cells do not organize type I collagen fibril network in culture, and TGF-β1 can induce type I collagen assembly (26). Therefore, the functional link between TGF-β1-induced LOX and structural and mechanical integrity of collagen fibril network in Fn-null hepatic stellate cells was addressed. Indeed, TGF-β1-induced collagen fibril stiffness in Fn-null hepatic stellate cells was significantly higher compared with control (parental) cells (Fig. 8A; p < 0.01). Treatment of Fn-null stellate cells with 2 pm TGF-β1 and LOX inhibitor BAPN (200 μm) resulted in the disruption of TGF-β1-induced fine collagen fibril networks and significantly reduced collagen fibril stiffness (p < 0.01) (Fig. 8, A and B). Importantly, treatment of Fn-null stellate cells with 10 μg/ml plasma Fn recovered collagen fibril stiffness to control (parental) cell levels (Fig. 8C). Thus, taken together, these findings indicate that elevated TGF-β1 bioavailability in Fn-null livers induces more active myofibroblasts and sustains their activated phenotypes. As a consequence of this, these myofibroblasts develop more accumulated, disorganized, and LOX-mediated tightly cross-linked collagenous ECMs during advanced chronic liver damage, which thereby results in the significant deterioration of net hepatic function.

Discussion

Our current comprehensive genetic studies with a Fn-deficient animal model provide the following compelling evidence: 1) Elimination of Fn results in elevated local TGF-β activity and continuously increased intracellular TGF-β signaling in activated hepatic stellate cells (myofibroblasts). This results in the development of more advanced liver fibrosis/cirrhosis in Fn-null livers. 2) Fn-null livers exhibit significantly increased LOX expression in chronic liver injury, and Fn-null hepatic stellate cells are a target for the release of active LOX in response to TGF-β1 through both canonical and noncanonical Smad pathways. Thus, we propose that there are the functional links between Fn-mediated control of TGF-β bioavailability and collagen fibril stiffness regulated by LOX. We suggest that such regulatory mechanisms by Fn could be translated for a potential therapeutic target in other chronic fibrotic diseases.

Our current findings in Fn-null mouse livers are consistent with the fact that elevated TGF-β activity induces enhanced differentiation and activation of quiescent fibroblast cells with a myofibroblastic phenotype (induction of αSMA) in response to tissue damage (5, 7). The expression level of αSMA is directly related to the contractile activity of myofibroblasts, which cause tissue contractures (51). This suggests that persistently elevated TGF-β activity caused by the lack of Fn induces more highly contractile myofibroblasts and leads to greater ECM secretion accompanied with increased LOX-mediated collagen cross-linking. However, collagen network reorganization in adult tissues in response to injury is mediated by both Fn and TGF-β1-induced type V collagen (26), and our results show both quantitative and qualitative differences between Fn- and type V collagen-mediated type I collagen fibril network formation. Therefore, although the contribution of type V collagen-nucleated collagen fibrogenesis in adult tissues is largely undetermined, our findings support the hypothesis that the accumulation of type V collagen-mediated ECMs during persistent chronic damage has an influence on disorganized ECM 3-dimensional architecture. Such phenotypes occurring beyond the early stages following tissue damage could contribute to the critical turning point from normal to abnormal healing in vivo. Consequently, more disorganized and rigid ECMS remarkably impede organ architecture and function. Indeed, accumulating evidence indicates that locally activated TGF-β plays a significant role in the development of liver fibrosis (52–54).

Earlier studies have shown that collagen cross-linking mediated by LOX leads to cancer progression by enhancing ECM receptor integrin signaling (11) and that inhibition of LOX activity partially prevents tissue stiffness following injury (55). Consistent with previous in vitro and in vivo observations (11, 56, 57), our findings indicate that inhibition of collagen cross-linking by BAPN affects TGF-β1-induced structural and mechanical integrity of collagenous ECM networks in Fn-null hepatic stellate cells. We have demonstrated functional links between tissue/organ functions and regulatory mechanisms of LOX underlying chronic fibrotic disease such as liver fibrosis/cirrhosis. BMP1 (bone morphogenic protein 1), an extracellular metalloproteinase, and peristin, a secretory protein in connective tissues, are major activators of LOX and interact with Fn (58, 59). Fn-null embryonic fibroblasts dramatically decrease proteolytic processing of the 45-kDa LOX proenzyme to the 32-kDa active form without changing BMP1 levels compared with control cells, suggesting the regulatory role of Fn matrix in LOX catalytic activity (60). In contrast, we have shown that Fn-null hepatic stellate cells release significant amounts of active LOX in response to TGF-β1 without Fn matrix. Interestingly, this TGF-β1-LOX signaling axis in Fn-null hepatic stellate cells is mediated through both canonical and noncanonical pathways (61). A very recent observation has documented that the expression level of peristin is significantly up-regulated in both acute and chronic liver injury, and TGF-β1 can induce peristin in hepatic stellate cells in vitro (62). Thus, peristin is

**FIGURE 8.** Structural and mechanical integrity of formed collagen fibril networks in Fn-null hepatic stellate cells. A, elastic modulus of collagen fibrils in Fn-null hepatic stellate cells without treatment (Ntx) or with TGF-β1 or TGF-β1 plus 200 μM BAPN, and parental (control) cells without treatment cultured for 7 days. The data are means ± S.D. (n = 7 for Fn-null cells; 6 for parental cells). **, p < 0.01; #, Fn-null stellate cells do not form collagen fibril network in culture without treatment (26). B, immunofluorescence staining for type I collagen (in red, upper panels) and images showing a peak force error by AFM (lower panels) in Fn-null hepatic stellate cells (HSC) cultured for 7 days with TGF-β1 or TGF-β1 plus 200 μM BAPN. Bar in fluorescence staining, 25 μM. Note that BAPN treatment results in the disruption of longitudinal collagen bundle formation. C, elastic modulus of collagen fibrils in Fn-null hepatic stellate cells with 10 μg/ml plasma Fn and parental (control) cells cultured for 7 days. The data are means ± S.D. (n = 6 for each group). n.s., not significantly different.
likely to be involved in TGF-β1-induced LOX activation in Fn-null hepatic stellate cells as cell type-specific phenotypes.

Little insight into the pathophysiological roles of Fn has emerged from studies of genetic changes in humans. There are no documented cases of Fn-null patients, the nearest condition being familial glomerulonephritis in which there are mutations in the type III modules of Fn (63). Although complete Fn-null mice show an embryonic lethal phenotype (64), experimental evidence has documented that skin wounds heal normally in mice lacking plasma type Fn (30); hence an absolute requirement for Fn in response to adult tissue damage has been speculative. The current findings imply that Fn regulates the balance between active and inactive (latent) TGF-β, which in turn modulates ECM production and remodeling following injury and consequently retains adult tissue/organ functions. A number of genetic studies suggest that the absence of, or a mutation in, large latent complex-binding ECMs such as fibrillin-1 and -2 results in increased TGF-β activity and Smad signaling, whereas the absence of, or mutation in, LTBP3 results in decreased activity and signaling (65–70). We have demonstrated that the absence of Fn results in increased TGF-β bioavailability following organ injury. A possible explanation for elevated TGF-β bioavailability is that the deficiency of large latent complex-binding ECMs, as seen in this Fn-null model, decreases the matrix sequestration of large latent complexes, which thereby renders latent TGF-β more accessible for activation with negative consequences for cellular functions (71). In contrast, there were very limited in vivo observations for negative regulation of TGF-β bioavailability by Fn. A recent study reveals that the inhibition of cell-mediated Fn matrix assembly without affecting its production by peptide pU4 shows the normal release of active TGF-β in hepatic stellate cells in vitro (72). However, Fn matrix networks play crucial roles in many important biological events and in adult homeostasis (21, 22). Fn matrix networks promote parenchymal cell survival such as hepatocytes following liver damage (23), and the elimination of Fn extra domain A splicing domain results in a shorter life span (73). Therefore, the regulation of TGF-β bioavailability by Fn with retaining Fn matrix networks would be essential elements for long-term anti-fibrotic strategies in chronic fibrotic diseases to preserve tissue/organ function and homeostasis.

**Author Contributions**—T. Sakai conceived ideas, designed experiments, and supervised the project. A. I., K. S., K. M., T. M., and S. Y. performed experiments. D. K. carried out transmission electron microscopy studies, and R. A. carried out atomic force microscopy studies. T. Sasaki generated anti-type I, III, and V collagen, LAP, LOX antibodies. A. I., K. S., K. M., R. A., T. M., and S. Y., M. W., S. M., and T. Sakai analyzed the data. T. Sakai wrote and edited the manuscript.

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