GASTROENTEROLOGY 2011;140:1653–1663

A Fibronectin-Independent Mechanism of Collagen Fibrillogenesis in Adult Liver Remodeling

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BACKGROUND & AIMS: Fibrosis is an abnormal extension of the wound healing process that follows tissue damage; it is involved in pathogenesis in a variety of chronic diseases. The formation of extracellular matrix is an essential response in wound healing. Although it has been proposed that collagen organization and assembly depend on the fibronectin matrix in culture, the contribution of fibronectin to these processes remains to be defined in vivo. **METHODS:** We generated a conditional, fibronectin-deficient mouse model of liver injury and explored whether fibronectin would be a suitable target for preventing extensive collagen deposits and scar formation that could lead to liver fibrosis. **RESULTS:** The lack of fibronectin did not interfere with reconstruction of collagen fibril organization in response to liver injury. Signaling by transforming growth factor-β and type V collagen were required for collagen fibrillogenesis during remodeling of adult liver tissue. **CONCLUSIONS: Transforming growth factor-β and type V collagen are targets for regulating the initial fibrogenic response to liver damage.**

Keywords: Liver Disease; Hepatic Stellate Cells; Mouse Model of Fibrosis; Conditional Knockout.

Wound healing is a crucial response to maintain organ structure and integrity after tissue damage. If injuries persist, they underlie the cause of pathogenesis in a variety of chronic fibrotic diseases such as liver fibrosis.[1,2] The essential response in wound healing is the deposition of extracellular matrices (ECMs). The key players in ECM production and remodeling in the liver are hepatic stellate cells (HSCs), which undergo activation and transdifferentiate into myofibroblast-like cells in response to liver damage.[3,4] A paradigm of ECMs in adult liver remodeling is that, similar to other tissues, the initial deposition of plasma-type fibronectin and fibrinogen exudated from plasma to form provisional matrix can stabilize wounded areas and induces the local production of cellular-type fibronectin in activated HSCs.[5] In vitro data suggest that the subsequent formation of an extensive collagen network requires the presence of fibronectin.[6] It also is believed that the profibrogenic cytokine transforming growth factor (TGF)-β is responsible for up-regulating ECM production in activated HSCs.[7]

Fibronectin is a dimeric glycoprotein and exists as 2 types. Plasma fibronectin is a soluble form produced solely by hepatocytes, whereas cellular fibronectin is an insoluble form produced by a variety of cells and incorporated into tissue ECM. Both isoforms are generated from a single gene by alternative splicing.[8] Although considerable in vitro functional studies have indicated that fibronectin plays a key role in cell differentiation, proliferation, migration, and survival,[9,10] knowledge of the functional identity of each fibronectin isoform in adult tissue remodeling remains loosely defined because of the complexity and the lack of model systems.

A recent study suggested a mechanism by which fibronectin plays a role in TGF-β signaling. Fibronectin participates in the initial incorporation of latent TGF-β–binding protein (LTBP)-1 into the ECM in vitro.[11] A cytokine TGF-β is secreted in a biologically inactive (latent) form in a complex with TGF-β latency-associated protein and LTBPs. The tissue concentration of these latent complexes is maintained at a constant level.[12] In response to injury, local latent TGF-β complexes are converted into active TGF-β. There are several mechanisms for activation, such as via chaotropic agents, proteases, integrins (αvβ6, αvβ8), and thrombospondin (TSP)-1, all of which are likely to be tissue-specific.[13,14] However, fibronectin/TGF-β interdependence on the initial fibrogenic response to liver damage has not yet been addressed in vivo and it remains unknown whether the collagen fibrillogenesis could be prevented or attenuated by interfering with fibronectin during the response process. Here, using our mouse model lacking both fibronectin types in the adult liver, we investigated whether fi-

Abbreviations used in this paper: αSMA, α-smooth muscle cell actin; ECM, extracellular matrix; Fn[fl/fl], floxed *fibronectin* gene; HSC, hepatic stellate cell; LTBP, latent transforming growth factor-β–binding protein; pSmad, phosphorylated Smad; TGF, transforming growth factor; TSP-1, thrombospondin-1.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.02.005

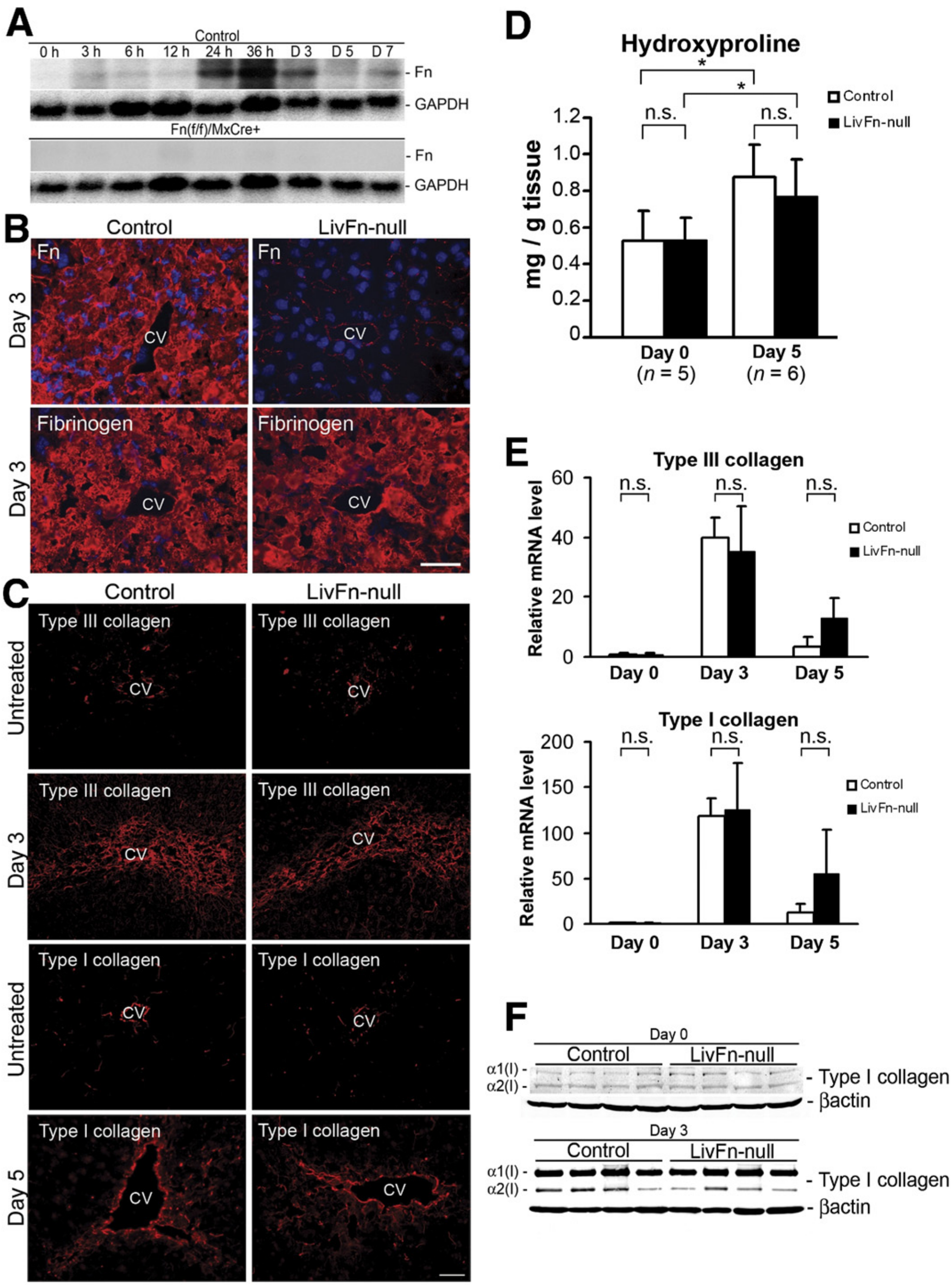


Figure 1. Effect of fibronectin deficiency on hepatic collagen production and assembly after acute liver injury. (*A*) Time course of fibronectin mRNA expression. As a loading control, the blot was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Note that there is no induction of fibronectin (Fn) mRNA in polyinosinic-polycytidic acid–injected Fn(fl/fl)/*Mx-Cre*+ liver throughout the time course. (*B*) Depositions of fibronectin and fibrinogen in control and mutant livers at 72 hours after injury by immunofluorescent staining (fibronectin and fibrinogen in *red*; 4′6-diamidino-2-phenylindole [cell nuclei] in *blue*). CV, central vein. *Bar*, 25 μm. (*C*) Deposition and assembly of type III (untreated and at day 3) and type I (untreated and at day 5) collagen fibrils by immunofluorescent staining. *Bar*, 50 μm. (*D*) Hepatic hydroxyproline content at day 0 (untreated) and day 5 ($n = 5$ at day 0 and $n = 6$ at day 5 for each group). Note that there are no significant differences between control and mutant livers at day 0 and 5, whereas the amounts in both livers at day 5 are significantly higher compared with day 0. *$P < .05$. (*E*) Real-time polymerase chain reaction analysis of *Col3a1* and *Col1a1* mRNAs. Relative mRNA expression levels are shown relative to the control value of 1.0 at day 0 ($n = 5$ for each group). Note that the expression levels in each time point (days 0, 3, and 5) are not significantly different between control and mutant livers. (*F*) Western blot analysis of collagen α1(I) and α2(I) chains at day 0 (untreated) and day 3 after injury. Note that the control and mutant livers show identical expression levels.

bronectin is a suitable molecular target for ameliorating the initial fibrogenic response to liver injury.

Materials and Methods

Generation of Mutant Mice and Animal Studies

Mice carrying a fibronectin-floxed gene (Fn[fl/fl]) were generated (Supplementary Figure 1). Mice lacking both plasma and cellular fibronectin in the liver were established by mating Fn(fl/fl) mice with mice expressing Cre recombinase under the control of the interferon- and polyinosinic-polycytidic acid–inducible *Mx* promoter (*Mx-Cre*) described by Sakai et al.[15] It was confirmed that *fibronectin* gene was deleted from parenchymal as well as nonparenchymal cells (liver fibronectin-null mice) (Supplementary Materials and Methods section and Supplementary Figures 1 and 2). β6 integrin- and TSP-1–knockout mice were kindly provided by Dr Dean Sheppard (University of California, San Francisco, CA) and Dr Jack Lawler (Beth Israel Deaconess Medical Center, Boston, MA), respectively.[16,17] Liver injury was induced by CCl_4.[1,18]

Isolation of Primary HSCs and Generation of Adult HSC Lines

Primary HSCs were isolated principally according to the method described by Schafer et al.[19] Adult control and fibronectin-null HSC lines were generated from HSCs of mice on p53- and p21-null genetic background[20]: HSCs from adult livers of Fn(fl/fl)/p53(−/−) and Fn(fl/fl)/p21(−/−) mice were isolated and treated with a *Cre*-transducing adenovirus to delete the *fibronectin-floxed* genes (Supplementary Materials and Methods section and Supplementary Figure 3).

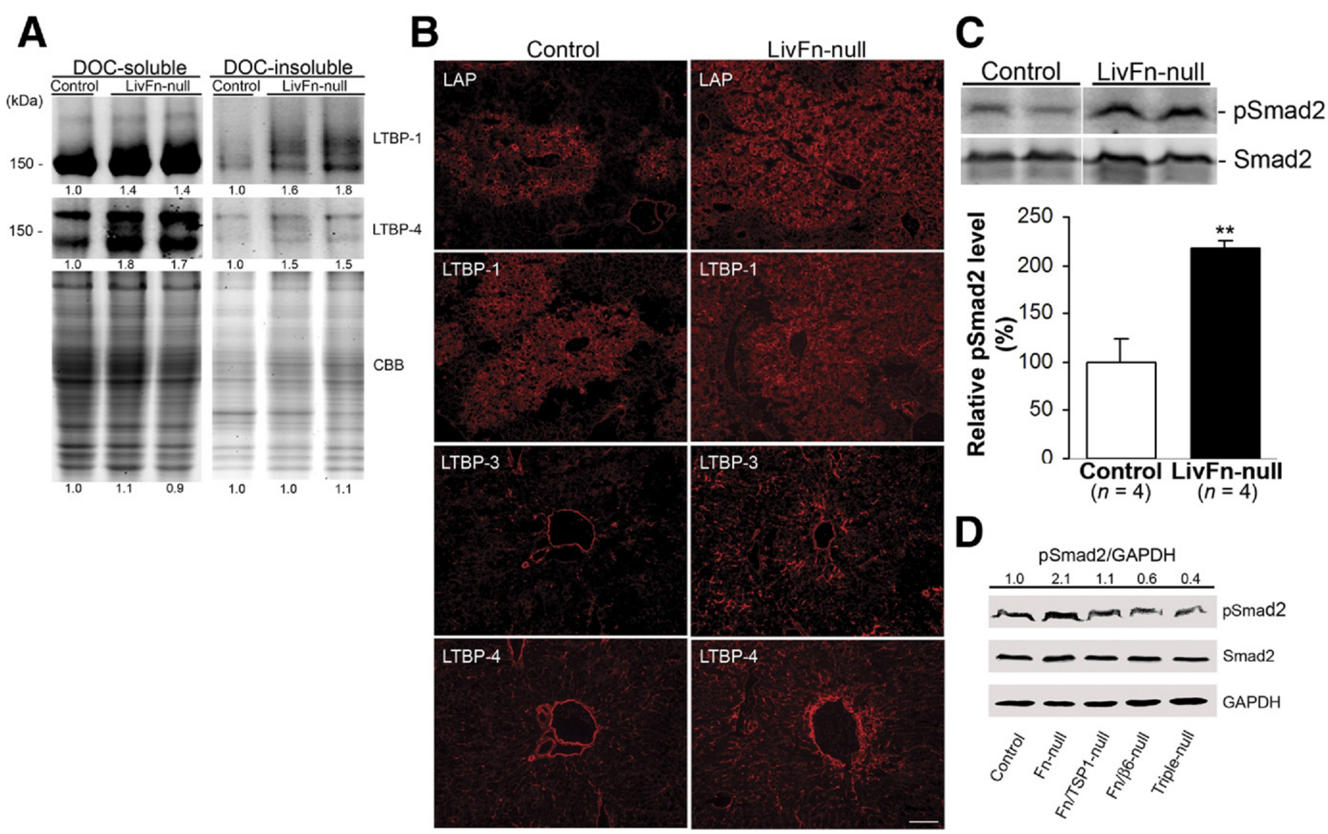
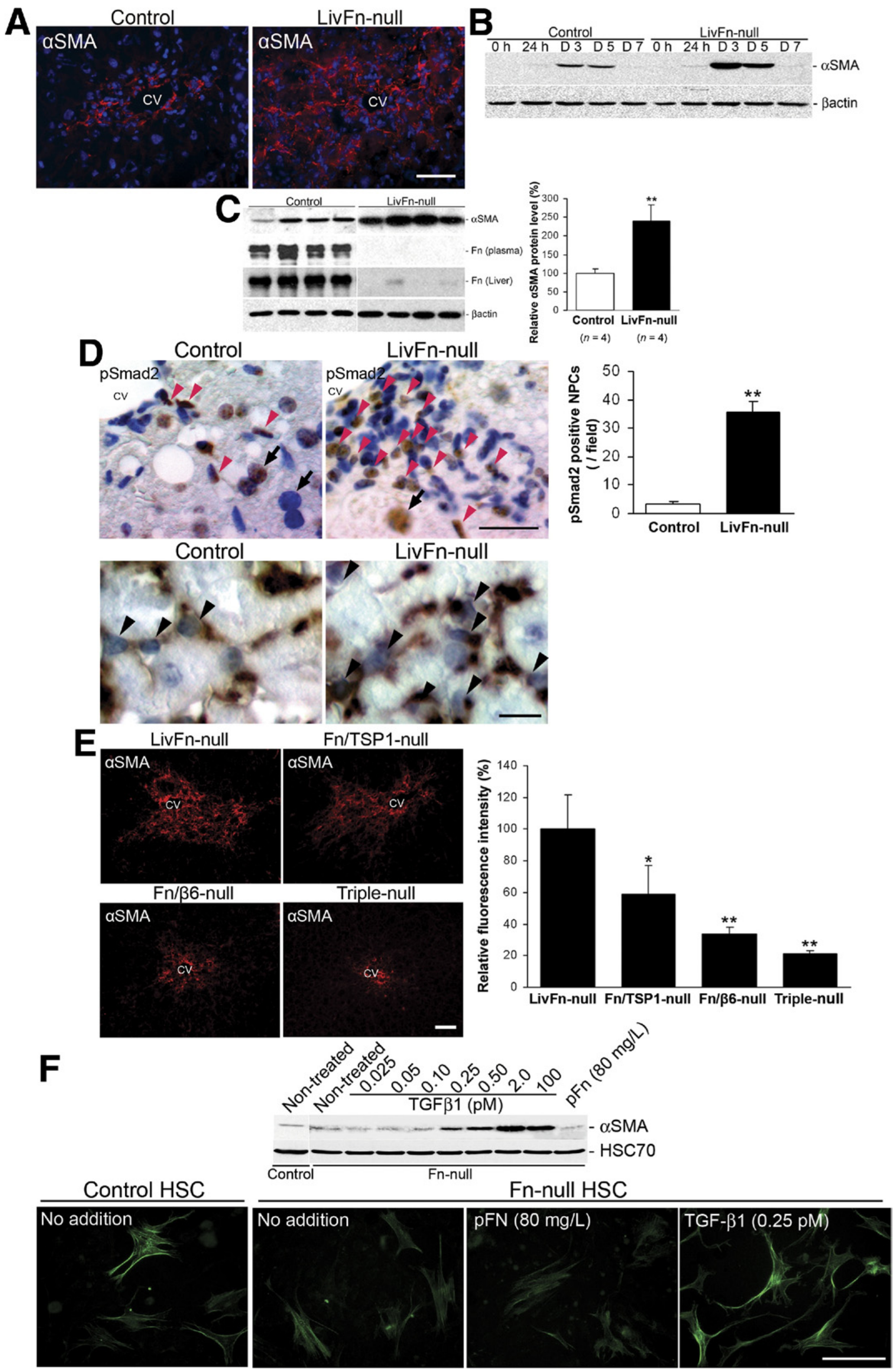


Figure 2. Effect of fibronectin deficiency on latent TGF-β production and TGF-β activation at 40 hours after injury. (*A*) Increased expression of both 2% deoxycholate-soluble and deoxycholate-insoluble LTBP-1 and LTBP-4 in fibronectin-null livers by Western blot analysis under nonreducing conditions. The intensity of the bands was measured by densitometry, and the intensities of each control sample were set to 1.0. The same samples were stained with Coomassie brilliant blue (CBB) to confirm that comparable amounts of protein were loaded. The positions of the molecular weight markers are indicated. (*B*) Increased depositions of latency associated protein (LAP) and LTBP-1, -3, and -4 in fibronectin-null livers by immunofluorescent staining. *Bar*, 100 μm. (*C*) *Upper panels:* representative Western blot analysis of pSmad2 (C-terminal serine 465/467) and total Smad2 expression. *Lower panel:* analysis of pSmad2 intensities. The pSmad2 expression levels are shown relative to the control value of 100 (percentage of control) ($n = 4$ for each group). Note that pSmad2 expression level in fibronectin-null livers is significantly higher compared with controls. $^{**}P < .01$. (*D*) Western blot analysis of pSmad2, total Smad2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control) expression in control, fibronectin-null, fibronectin/TSP-1–double null, fibronectin/β6 integrin-double null, and fibronectin/β6 integrin/TSP-1–triple null (triple-null) livers. Pooled samples from 4 mouse livers from each strain were used for the analysis. The intensity of the bands was measured by densitometry, and each pSmad2 intensity was normalized to GAPDH, then the intensity of the control was set to 1.0. Each pSmad2 intensity is shown relative to the control value.

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. Results are presented as means ± standard deviation. Differences between groups were analyzed using the 2-sided Student *t* test on raw data. A *P* value of less than .05 was considered significant.



For more detailed descriptions of the Materials and Methods used, please see the Supplementary Materials and Methods.

Results

Generation of Liver Fibronectin-Null Mice

To address the role of Fn in remodeling of adult tissue ECMs, we established (liver) fibronectin-null (mutant) mice (Fn(fl/fl)/*Mx-Cre*+) and verified deletion efficiency (Supplementary Figures 1 and 2). Acute liver injury was induced with a well-established model[1,18] of a single treatment with the liver-damaging agent CCl_4 (1 mL/kg body weight). Fibronectin messenger RNA (mRNA) was undetectable in Fn(fl/fl)/*Mx-Cre*+ liver throughout the acute injury period (baseline time 0 through day 7 after injection). In contrast, considerable induction of fibronectin mRNA was noted in control liver (Figure 1*A*). No newly deposited fibronectin protein was obvious in mutant livers throughout the regeneration process, except for a small remnant amount in the sinusoidal ECM as detected with immunohistochemistry (Figure 1*B*; data not shown). These results show that we successfully established an adult mouse model lacking both fibronectin types in the liver during tissue remodeling. Deposition of fibrin(ogen), a counterpart of the provisional matrix with plasma fibronectin, showed no marked differences by immunohistochemistry between control and mutant mice throughout the injury (Figure 1*B*).

Fibronectin Deficiency Does Not Attenuate Collagen Network Formation in Acute Liver Injury

Collagen assembly upon tissue damage occurs as a multistep process. The expression level of collagen mRNAs, processing during collagen protein secretion, and the level of degradation contribute to collagen assembly and maintenance.[21] Because a preformed fibronectin matrix is indispensable for type III and type I collagen-containing fibril formation in culture,[6,22,23] it was hypothesized that the absence of fibronectin could attenuate collagen fibrillogenesis after acute liver injury. However, any difference was not detected between control and fibronectin-null livers in the following: (1) the deposition and assembly of type III and type I collagens; (2) hepatic hydroxyproline content as a measure of net collagen production; (3) expression levels of *Col3a1* and *Col1a1* mRNAs and α1(I) and α2(I) protein chains; and (4) expression levels of collagen-related *matrix metalloproteinases 8* and *13* mRNAs (Figure 1*C–F*; data not shown). These findings indicate that there is an alternative in vivo mechanism for collagen fibrillogenesis in the absence of fibronectin.

Liver Lacking Fibronectin Increases the Production of TGF-β Latent Complexes and Local TGF-β Bioavailability in Response to Injury

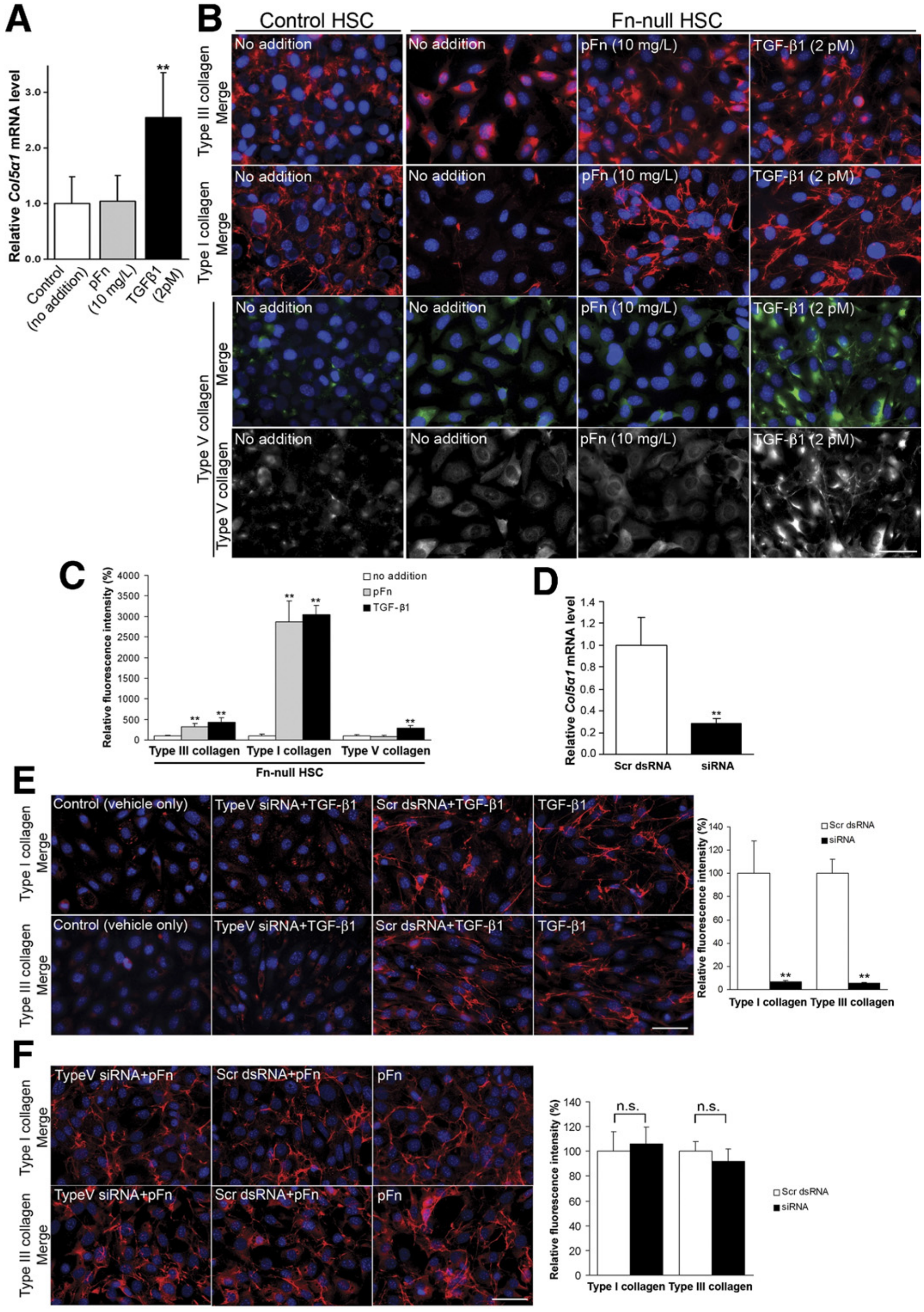
Because fibronectin is associated with latent TGF-β complexes to incorporate into ECM,[7,12,14] we next determined whether lacking fibronectin affected the production of latent complexes of TGF-β after liver injury. The expression of both 2% deoxycholate-soluble and deoxycholate-insoluble fractions (defined as immaturely and maturely assembled matrices, respectively[24]) of LTBP-1 and -4 was higher in fibronectin-null livers after injury (Figure 2*A*). The substantial depositions of latency-associated protein and of LTBP-1, -3, and -4 in the ECM of the mutant liver after injury were confirmed with immunohistochemistry (Figure 2*B*). Although LTBPs are associated with ECM components such as fibronectin and fibrillin,[12,14] they showed fibrillar structures even without fibronectin.

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 expres-

BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Figure 3. The lack of fibronectin increases activation of HSCs after acute liver injury. (*A*) Pronounced induction of hepatic αSMA in fibronectin-null livers at 72 hours after injury by immunofluorescent staining (αSMA in *red*; 4′6-diamidino-2-phenylindole in *blue*). CV, central vein. *Bar*, 25 μm. (*B*) Western blot analysis of the time-course of αSMA induction after injury. Note that a higher induction level of αSMA is indicated in fibronectin-null livers from 3 to 5 days after injury. (*C*) *Left panel:* Western blot analysis of αSMA, and plasma and liver fibronectin protein levels at 72 hours after injury. *Right panel:* analysis of αSMA intensities. The αSMA expression levels are shown relative to the control value of 100 (percent of control) (n = 4 for each group). **$P < .01$. (*D*) *Upper left panels:* immunostaining for pSmad2 at 40 hours after injury. Sections were counterstained with hematoxylin. *Bar*, 25 μm. *Upper right panel:* analysis of pSmad2-positive cells. Note that the number of nuclear pSmad2-positive nonparenchymal cells (*brown*; *red arrowheads*) is significantly higher in fibronectin-null livers. For comparison, hepatocyte nuclei are indicated (*black arrows*). **$P < .01$. *Lower panels:* double-immunostaining for pSmad2 (in *purplish-blue*) and αSMA (in *brown*). Note that the nuclear pSmad2-positive cells express activated HSC marker αSMA in their cytoplasms (*black arrowheads*). *Bar*, 25 μm. (*E*) *Left panels:* induction of αSMA in fibronectin-null, fibronectin/TSP-1– and fibronectin/β6 integrin–double, and fibronectin/β6 integrin/TSP-1–triple knockout livers at 72 hours after injury by immunofluorescent staining. *Bar*, 50 μm. *Right panel:* analysis of intensity in assembled collagen fibrils. Relative fluorescence intensities are shown relative to the control value of 100 (LivFn-null). *$P < .05$; **$P < .01$. (*F*) TGF-β1 effectively induces activation of primary HSCs in vitro. *Upper panel:* Western blot analysis of αSMA at 72 hours after the isolation of primary HSCs from adult control and fibronectin-null intact livers. Primary HSCs were incubated in Dulbecco's modified Eagle medium containing 2% Fn-depleted fetal bovine serum with indicated supplements. HSC70, loading control. *Lower panels:* immunofluorescent staining for αSMA (in *green*) at 72 hours after the isolation of primary HSCs. Note that TGF-β1 (0.25 pmol/L) induces characteristic stellate shapes with long cytoplasmic processes in addition to intense αSMA induction in fibronectin-null HSCs. *Bar*, 100 μm.

sion.[25,26] Therefore, it was next examined whether the increased production of latent TGF-β complexes in fibronectin-null livers altered the local activation of TGF-β after injury. Phosphorylation of Smad2 (pSmad2) in fibronectin-null livers at 40 hours after injury was 2.2-fold higher ($P < .01$) than in controls (Figure 2*C*). To determine the molecular mechanisms underlying increased TGF-β activity in fibronectin-null livers after injury, we generated fibronectin/TSP-1– and fibronectin/β6 integrin–double and fibronectin/β6 integrin/TSP-1–triple knockout mice.[16,17] The induction level of pSmad2 was down-regulated to 52% in fibronectin/TSP-1–null, 29% in fibronectin/β6 integrin–null, and 19% in fibronectin/β6 integrin/TSP-1–null livers in comparison with fibronectin-null livers (Figure 2*D*). These results showed that although both β6 integrin and TSP-1 participate in local



TGF-β activation in acute liver injury, β6 integrin plays a more significant role in its activation than does TSP-1.

Lack of Fibronectin Results in Significantly Increased HSC Activation After Acute Liver Injury

The key event for ECM remodeling in response to liver injury is the activation of HSCs.[3] Both TGF-β and the cellular fibronectin extradomain A segment promote the initiation of HSC activation in vitro, accelerating the induction of the activated HSC marker, α-smooth muscle cell actin (αSMA).[27,28] Because the absence of fibronectin increases local TGF-β activity after injury (Figure 2*C*), we next addressed the functional relationship of fibronectin and TGF-β with HSC phenotypes both in vivo and in vitro. We found that after acute injury fibronectin-null livers had higher αSMA levels than controls, especially in the areas of the central vein (Figure 3*A*–*C*). The number of αSMA-positive cells at day 3 were 173.8 ± 17.1 cells/field in mutant mice (field = 0.24 mm^2, n = 8) vs 69.4 ± 12.4 cells/field in control mice (n = 8; $P < .01$). Fibronectin-null livers also showed a significantly increased number of nuclear pSmad2-positive nonparenchymal cells after injury (35.1 ± 3.8 cells/field in mutant; field = 0.24 mm^2 [n = 10] vs 3.5 ± 1.1 cells/field in control [n = 10]; $P < .01$), and those pSmad2-positive cells expressed αSMA (Figure 3*D*). The αSMA induction was diminished in fibronectin/β6 integrin- and fibronectin/TSP-1–double-knockout livers and was reduced even further in fibronectin/β6 integrin/TSP-1–triple knockout livers (Figure 3*E*). To further determine the extent, if at all, that TGF-β can activate HSCs in the absence of fibronectin, we prepared primary HSCs isolated from adult control and fibronectin-null mice. TGF-β1 stimulation induced αSMA production at concentrations as low as 0.25 pmol/L (6.25 pg/mL). The production surpassed that of nontreated control and cells treated with 80 μg/mL plasma fibronectin. TGF-β1 addition (0.25 pmol/L) also induced characteristic stellate shapes with long cytoplasmic processes (Figure 3*F*). Taken together, our results indicate that TGF-β1 alone sufficiently initiates HSC activation after liver injury.

TGF-β–Induced Collagen V Generates Type III/I Collagen Networks in the Absence of Fibronectin Both In Vitro and In Vivo

To determine whether TGF-β is involved in type III/I collagen network formation in the absence of fibronectin, adult control and Fn-null HSC lines were generated from HSCs of mice on p53- and p21-null genetic backgrounds[20] (Supplementary Figure 3). Because all fibronectin-null cells, including adult fibronectin-null HSCs, do not assemble collagen fibrils in normal cultures,[6,22,23] it was examined which of those factors that regulate activated HSC phenotypes were involved in collagen fibrillogenesis. We found that TGF-β1, which is activated and secreted in response to liver injury[7] and showed increased activity in fibronectin-null livers (Figure 2*C*), was the most potent factor. The addition of TGF-β1 to fibronectin-null HSCs in culture at concentrations as low as 2 pmol/L (50 pg/mL) induced type I collagen fibril network formation. Other cytokines/growth factors such as platelet-derived growth factor–AA (mitogenic factor; up to 800 pmol/L = 20 ng/mL) and amphiregulin (injury-protective factor; up to 3500 pmol/L = 40 ng/mL) had no effect on collagen fibril assembly (Supplementary Figure 4).

There is evidence that TGF-β1 up-regulates *Col5a1* chain mRNA level during osteogenesis.[29] Of the 20 type I/III collagen assembly related molecules examined (Supplementary Table 1), *Col5a1* had the strongest response to TGF-β1: TGF-β1 addition (2 pmol/L) to the media of fibronectin-null HSCs significantly increased *Col5a1*

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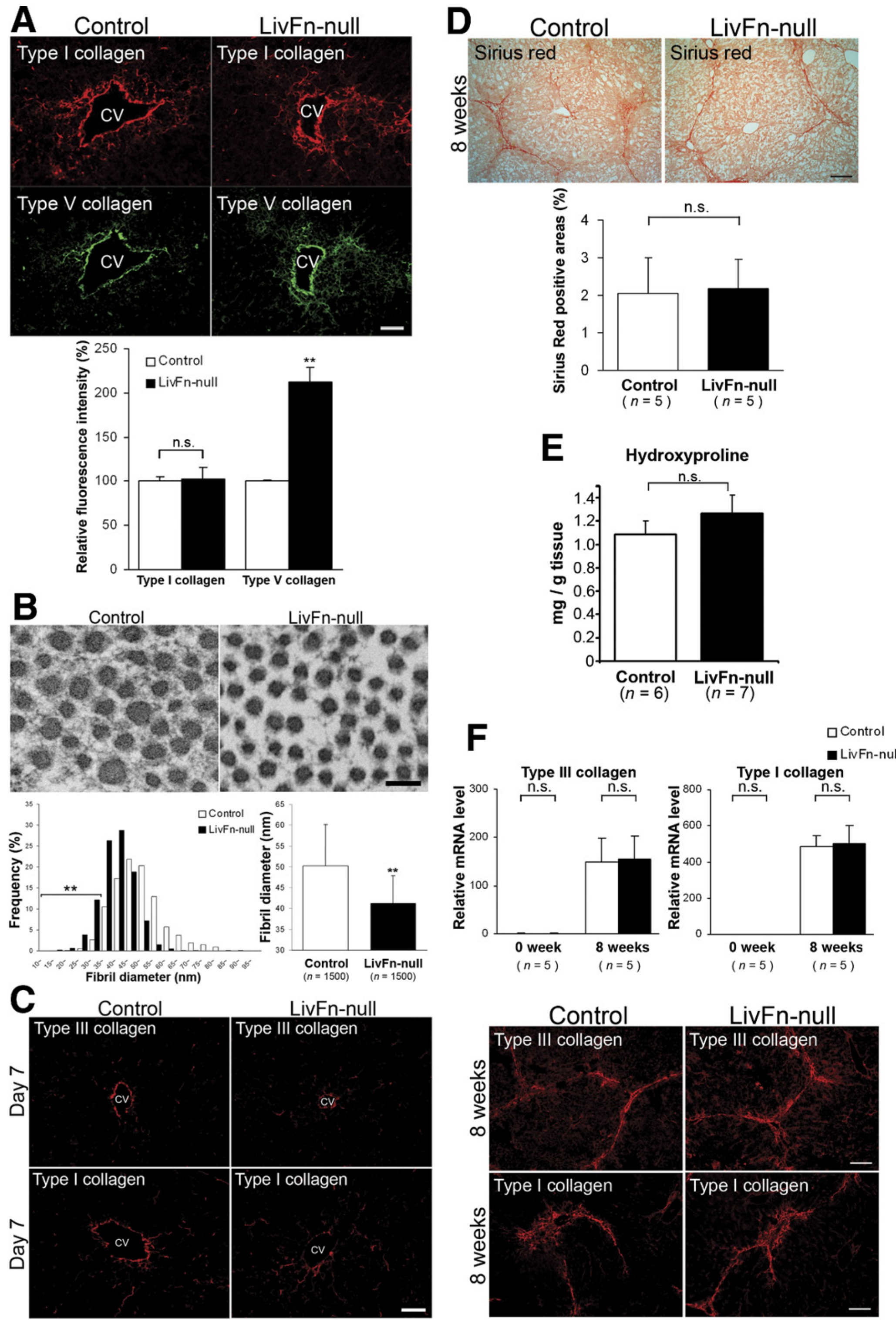
Figure 4. Fibronectin-null HSCs form type V collagen–nucleated type III and type I collagen fibril networks even in the absence of a fibronectin matrix in vitro. (*A*) Real-time polymerase chain reaction analysis of *Col5a1* mRNAs in fibronectin-null HSCs. Cells were incubated for 12 hours with plasma fibronectin (pFn, 10 μg/mL) or TGF-β1 (2 pmol/L). mRNA expression levels are shown relative to the control value of 1.0 (no addition) (n = 4 for each group). Note that TGF-β1 significantly up-regulates *Col5a1* mRNA levels, whereas plasma fibronectin does not have any remarkable effect. $^{**}P < .01$. (*B* and *C*) TGF-β1 induces type III, type I, and type V collagen assembly, whereas plasma fibronectin induces assembly of type III and type I but not type V collagen in fibronectin-null HSCs. (*B*) Double immunofluorescent staining for type III (in *red*), type I (in *red*), or type V collagen (in *green*) and 4′6-diamidino-2-phenylindole (in *blue*) in control and fibronectin-null HSCs. Cells were incubated for 18 hours with plasma fibronectin (pFn, 10 μg/mL) or TGF-β1 (2 pmol/L). For type V collagen staining, the same images for type V single staining (*black and white*) also are shown. *Bar*, 50 μm. (*C*) Analysis of intensity in assembled collagen fibrils in fibronectin-null HSCs shown in panel *B*. Relative fluorescence intensities are shown relative to each control value of 100 (no addition). $^{**}P < .01$. (*D*–*F*) Effect of *Col5a1* siRNA on fibronectin-null HSC phenotypes. (*D*) Real-time polymerase chain reaction analysis of *Col5a1* mRNA levels in fibronectin-null HSCs at 27 hours after transfection. Relative mRNA expression levels are shown relative to the control value of 1.0 (scrambled dsRNA transfectants) (n = 3 for each group). Note that siRNA leads to a significant knockdown (~75%) of *Col5a1* mRNA levels in fibronectin-null HSCs. $^{**}P < .01$. (*E* and *F*) Effect of *Col5a1* siRNA on TGF-β1 (2 pmol/L)-induced (*E*) or plasma fibronectin (10 μg/mL)-mediated (*F*) type I and type III collagen assembly. *Left panels:* deposition and assembly of collagen fibrils (type I and type III collagen in *red*; 4′6-diamidino-2-phenylindole in *blue*) in fibronectin-null HSCs at 45 hours after transfection by immunofluorescent staining. Note that *Col5a1* siRNA almost completely inhibits TGF-β1–induced collagen network formation, whereas it does not remarkably affect plasma fibronectin-mediated collagen network formation. Control (vehicle only), treated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) only. SCR DSRNA, scrambled double-stranded RNA transfectant. *Bar*, 50 μm. *Right panels:* analysis of intensity in assembled collagen fibrils. Relative fluorescence intensities are shown relative to each control value of 100 (cells transfected with scrambled dsRNA and TGF-β1 [2 pmol/L] added [*E*]; cells transfected with scrambled dsRNA and plasma fibronectin [10 μg/mL] added [*F*]). The background fluorescence intensity measured in control (vehicle only) is subtracted from each value. $^{**}P < .01$.

mRNA levels (>2.5-fold) in comparison with controls ($P < .01$; Figure 4*A*). The up-regulation of *Col5a1* mRNA levels was observed at concentrations of TGF-β1 as low as 0.1 pmol/L (data not shown). TGF-β1 (2 pmol/L) was able to induce type V collagen assembly in fibronectin-null HSCs, and underwent type III and type I collagen fibril network formation. In contrast, plasma fibronectin (10 μg/mL) clearly organized type III and type I fibrils but did not induce type V collagen assembly (Figure 4*B* and *C*). When fibronectin-null HSCs were exposed to both TGF-β1 and plasma fibronectin, there was a synergistic effect on type I collagen organization (Supplementary Figure 5), suggesting that TGF-β1 and fibronectin mediate, at least in part, different pathways to induce



collagen assembly. Untreated fibronectin-null and its parental HSC cells expressed similar levels of *Col3a1* or *Col1a1* mRNAs by real-time polymerase chain reaction and the type I collagen secretion by pulse chase analysis (Supplementary Figure 3*D* and *E*). Thus, these findings suggest that the fibronectin-null HSCs have a defect in the process of fibril assembly or their attachment to the cells as they form the matrices. The addition of exogenous pepsin-treated type V collagen alone in fibronectin-null HSCs could form short and thin type I collagen fibrils but at a lesser extent than TGF-β1 (Supplementary Figure 6), suggesting that the intracellular process may play an important role in type V collagen–mediated type I collagen fibril network formation.[30] Matrix metalloproteinase inhibitor GM6001 at a concentration up to 25 μmol/L did not support type III and I collagen assembly in fibronectin-null HSCs (data not shown).

It was further examined whether type V collagen is directly involved in de novo type I and type III collagen network formation. Transfection of *Col5a1* siRNA into fibronectin-null HSCs led to the efficient knockdown of *Col5a1* mRNA levels at 27 hours (~75%; $P < .01$) and maintained approximately 55% reduction 45 hours later compared with scrambled double-stranded RNA transfectants (Figure 4*D*). Accordingly, the siRNA treatment of fibronectin-null HSCs resulted in the reduction of TGF-β–induced *Col5a1* mRNA levels (~50%) and significant inhibition of type I and type III collagen assembly. No obviously assembled fibril networks were detected with immunocytochemistry (~93% inhibition of type I and ~95% inhibition of type III fibril formation, respectively, $P < .01$; Figure 4*E*). In contrast, *Col5a1* siRNA did not affect plasma fibronectin-mediated type III and I collagen assembly even though it reduced *Col5a1* mRNA level to the same extent (~50%) (Figure 4*F*). Scrambled double-stranded RNA had no effect on these phenotypes. Thus, these findings show that type V collagen functions to generate type III/I collagen fibril assembly even in the absence of a fibronectin matrix. Interestingly, this phenotype seems to be specific for adult HSCs. When fibronectin-null embryonic fibroblasts were exposed to plasma fibronectin in culture, type III and type I collagen fibril networks were clearly organized in response to plasma Fn without a type V collagen network formation. In contrast, TGF-β1 at concentrations from 2 to 500 pmol/L supported neither type V collagen nor type III and type I collagen fibril assembly in this cell type (Supplementary Figure 7).

In vivo analysis after acute liver injury revealed significantly more extensive deposition and assembly of type V collagen in fibronectin-null livers at day 5 in comparison with the controls ($P < .01$; Figure 5*A*). The localization of assembled type V collagen fibrils in fibronectin-null livers often overlapped with type I fibrils. Subsequent analysis of collagen fibril ultrastructure showed that the thinner fibril subpopulation (<35 nm diameter) was increased significantly in the fibronectin-null livers at day 5 after injury (16.8% in mutant vs 3.2% in control: 5.3-fold; $P < .01$; Figure 5*B*). As a consequence, the average diameter of fibrils in fibronectin-null livers was smaller than control livers (50.2 $\pm$ 10.0 nm in control mice vs 41.2 $\pm$ 6.7 nm in mutant mice; $P < .01$; Figure 5*B*). Newly organized collagen networks were nearly completely cleared from both control and mutant mice livers by day 7 (Figure 5*C*).

Finally, the initial fibrogenic response to chronic liver injury induced by CCl_4 was examined.[18] It was confirmed that there was no apparent fibronectin protein induction in fibronectin-null livers after injury by Western analysis (Supplementary Figure 8). Histologically, the bridging-fibrosis formation was found in both livers with the same extent and there were no obvious differences in fibrotic areas by Sirius red staining (Figure 5*D*). Serum alanine aminotransferase and total bilirubin levels and the albumin/globulin ratio were not significantly different between control and mutant livers after CCl_4 treatment (Supplementary Figure 9).[31] Furthermore, no difference was detected between control and fibronectin-null livers in hepatic hydroxyproline content (Figure 5*E*), *Col3a1* and *Col1a1* mRNA levels, and deposition and assembly of type III and type I collagens (Figure 5*F*).

Figure 5. Collagen fibril organization in control and fibronectin-null livers in vivo. (*A–C*) Response to acute liver injury. (*A*) *Upper panels:* deposition and assembly of type I (in *red*) and type V (in *green*) collagens at day 5 after injury by immunofluorescent staining. CV, central vein. *Bar*, 50 μm. *Lower panel:* analysis of intensity in assembled collagen fibrils. Relative fluorescence intensities are shown relative to each control value of 100 (control liver). $^{**}P < .01$. (*B*) Ultrastructural analysis of collagen fibrils at day 5 after injury. *Upper panels:* transmission electron micrographs of transverse sections. *Bar*, 100 nm. *Lower left panel:* morphometric analysis of collagen fibril diameter in control and fibronectin-null livers at day 5 after injury (1500 fibrils for each group). Note that there is a significantly increased thinner fibril subpopulation (<35 nm diameter) in fibronectin-null livers. $^{**}P < .01$. *Lower right panel:* the average diameter of collagen fibrils in control and fibronectin-null livers (1500 fibrils for each group). Note that the fibrils in fibronectin-null livers (41.2 $\pm$ 6.7 nm) show significantly smaller diameter than controls (50.2 $\pm$ 10.0 nm). $^{**}P < .01$. (*C*) Deposition and assembly of type III and type I collagen fibrils at day 7 by immunofluorescent staining. *Bar*, 50 μm. (*D–F*) The initial fibrogenic response to chronic liver injury. (*D*) *Upper panels:* Sirius red staining. *Bar*, 100 μm. *Lower panel:* quantification of fibrotic areas ($n = 5$ for each group). (*E*) No significant differences in hepatic hydroxyproline content between control and fibronectin-null livers ($n = 6$ for control; $n = 7$ for mutant). (*F*) *Upper panels:* real-time polymerase chain reaction (PCR) analysis of *Col3a1* and *Col1a1* mRNAs. Relative mRNA expression levels are shown relative to the control value of 1.0 at 0 week (untreated) ($n = 5$ for each group). *Lower panels:* deposition and assembly of type III and type I collagen fibrils by immunofluorescent staining. *Bar*, 50 μm.

Discussion

Based on the experimental evidence showing that the preformed fibronectin matrix is required for in vitro collagen network formation,[6,22,23] we investigated in the present study whether fibronectin was a suitable molecular target for preventing the initial fibrogenic response to liver damage. We found that fibronectin deficiency resulted in increased local TGF-β activity and formed collagen fibril networks similar to control livers after injury.

A number of genetic studies have suggested that the absence of or a mutation in LTBP-binding ECMs such as fibrillin-1 results in increased TGF-β activity and Smad signaling, whereas the absence of or mutation in LTBPs results in decreased activity.[32–36] These results support the hypothesis that the LTBPs and their binding molecules such as fibronectin determine the spatial localization of LTBPs in tissues, thereby regulating the activating mechanisms of TGF-β. We have identified 2 different mechanisms of latent TGF-β activation in response to liver injury: one mediated by TSP-1 and the other, more dominant one, mediated by β6 integrin. Our findings imply that fibronectin regulates the balance of the active and inactive (latent) TGF-β, which in turn modulates ECM production and remodeling, and consequently maintains adult liver homeostasis. Indeed, mouse models of TGF-β1 overexpression show dominant phenotypes such as advanced liver fibrosis.[37,38]

Our present study provides compelling evidence that collagen fibrillogenesis in adult tissues in response to damage is mediated by both fibronectin and type V collagen. Type V collagen-null mouse embryos display defects in collagen fibril formation in the mesenchyme.[30] These results support the hypothesis that both fibronectin and type V collagen play a critical role in collagen fibrillogenesis in embryonic development and adult tissue remodeling, the extent according to the temporal and spatial expression pattern of each molecule. Interestingly, type V collagen–mediated type III/I collagen fibril assembly in response to liver injury seems to be specific for adult HSCs because TGF-β1 supported neither type V collagen nor type III/I collagen fibril assembly in fibronectin-null embryonic fibroblasts. The diameter of formed type I collagen fibrils is inversely proportional to the type V/type I collagen ratio, and adult *Col5a1*(+/−) mouse dermis contains a larger and abnormal population of collagen fibrils.[30,39,40] Indeed, our ultrastructural analysis of collagen fibrils provides evidence that type V–mediated collagen fibrils in fibronectin-null livers contain a significantly increased number of the thinner subpopulation in response to acute liver injury. Taken together, these findings suggest that the excess type V collagen could result in a heterotypic type I collagen assembly. The contribution of type V collagen–nucleated collagen fibrillogenesis in adult tissues is largely undetermined. It remains to be elucidated clinically whether newly reconstructed ECMs induced by initial type V collagen deposition contribute to the critical turning point from normal to abnormal healing. Our current study, therefore, has identified 2 players, TGF-β and type V collagen, that regulate the initial fibrogenic response to liver damage.

Although a prominent expression of fibronectin is observed during tissue repair, the contribution of fibronectin to adult ECM remodeling, particularly to collagen fibrillogenesis, has remained as an unsolved question.[5,9,10,41] Our results have wiped out the long-standing concept that collagen fibril organization requires the prior assembly of fibronectin matrix,[6,22,23] and the further interpretation that fibronectin matrix is probably serving as a scaffold for collagen fibril organization. Our present study has shown that fibronectin scaffold is not always essential for tissue remodeling and a certain cell type can assemble collagen fibril networks in the complete absence of fibronectin in vivo.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.02.005.

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Received August 5, 2010. Accepted February 6, 2011.

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Acknowledgments

The authors are grateful to Dr Claudio Fiocchi for critical reading of the manuscript and valuable suggestions. The authors thank Dr Klaus Rajewsky for *Mx-Cre* mice, Dr Dean Sheppard for β6 integrin-knockout mice, Dr Jack Lawler for thrombospondin-1–knockout mice, Dr Dusko Ilic for p53- and p21-knockout mice, Dr Deane Mosher for fibronectin-null embryonic fibroblasts, and Dr Koichi Matsuzaki and Dr Lynn Sakai for antibodies. The authors also thank Dr Peter Yurchenco for initial electron microscopic investigations, and Dr Shin Hasegawa for support.

Conflicts of interest

The authors disclose no conflicts.

Funding

Some preliminary studies were initiated in the laboratory of Dr Reinhard Fässler (Department of Experimental Pathology, Lund University, Sweden). This work was generated as an independent new project and supported by a National Institutes of Health research grant (DK074538) and The Cleveland Clinic (T. Sakai).