Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis

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Myofibroblasts produce the fibrous scar in hepatic fibrosis. In the carbon tetrachloride (CCL4) model of liver fibrosis, quiescent hepatic stellate cells (HSC) are activated to become myofibroblasts. When the underlying etiological agent is removed, clinical and experimental fibrosis undergoes a remarkable regression with complete disappearance of these myofibroblasts. Although some myofibroblasts apoptose, it is unknown whether other myofibroblasts may revert to an inactive phenotype during regression of fibrosis. We elucidated the fate of HSC/myofibroblasts during recovery from CCL4- and alcohol-induced liver fibrosis using Cre-loxP-based genetic labeling of myofibroblasts. Here we demonstrate that half of the myofibroblasts escape apoptosis during regression of liver fibrosis, down-regulate fibrogenic genes, and acquire a phenotype similar to, but distinct from, quiescent HSCs in their ability to more rapidly reactiviate into myofibroblasts in response to fibrogenic stimuli and strongly contribute to liver fibrosis. Inactivation of HSCs was associated with up-regulation of the anti-apoptotic genes Hspa1a/b, which participate in the survival of HSCs in culture and in vivo.

Chronic liver injury of any etiology produces fibrosis as a result of deregulation of the normal healing process with massive accumulation of extracellular matrix (ECM), including type I collagen (ColI) (1). Myofibroblasts are ColI α-smooth muscle actin (α-SMA)+ cells that produce the ECM scar in fibrosis. One of the most important concepts in clinical and experimental liver fibrosis is reversibility. Removal of the etiological source of the chronic injury in patients (e.g., hepatitis B virus, hepatitis C virus, biliary obstruction, or alcohol) and in rodents [carbon tetrachloride (CCL4) or bile-duct ligation] produces regression of liver fibrosis and is associated with decreased cytokine and ECM production, increased collagenase activity, and the disappearance of myofibroblasts (1, 2). During regression of fibrosis, some myofibroblasts undergo senescence (3) and apoptosis (2). However, the number of apoptotic myofibroblasts and the fate of the remaining myofibroblasts in the recovering liver is unknown.

Hepatic stellate cells (HSCs), the liver pericytes that store retinoids, are a major source of myofibroblasts in hepatotoxic liver fibrosis (4). Liver injury results in activation of quiescent HSCs (qHSCs), which proliferate and undergo phenotypical and morphological changes characteristic of myofibroblasts. Removal of the injurious agent results in the clearance of activated HSCs (aHSCs) by the cytotoxic action of natural killer cells (1) and is linked to up-regulation of ligands of natural killer cell receptors NKG2D, MICA, and ULBP2 in senescent aHSCs (3). Although never demonstrated in vivo, studies in culture suggest that aHSCs can revert to a more quiescent phenotype (5), characterized by expression of adipogenic genes and loss of fibrogenic gene expression (5).

Using genetic labeling of aHSCs/myofibroblasts, we demonstrate here that some aHSCs escape cell death and revert to an inactivated phenotype that is similar to, but distinct from, the original quiescent HSCs, including their ability to more rapidly reactivate into myofibroblasts. Because reversibility of fibrosis has been reported in lungs (6) and kidneys (7), these concepts and approaches may be applicable to study of fibrosis of other organs and provide new targets for anti-fibrotic therapy.

Results

Regression of Liver Fibrosis Is Accompanied by Loss of Myofibroblasts. Our study was designed to determine the fate of aHSCs/myofibroblasts (α-SMA+ColI+) during regression of hepatic fibrosis. For this purpose, reporter Col-GFP mice, expressing collagen-α1(I) promoter/enhancer-driven GFP, were subjected to CCL4-induced liver injury for 2 mo. After cessation of the toxic agent, mice recuperated for 1 or 4 mo, and regression of liver fibrosis was evaluated by measuring collagen deposition and myofibroblast number (Fig. 1 A and B). CCL4-treated mice developed severe fibrosis with activated myofibroblasts (Fig. 1 A and B), which decreased markedly after 1 and 4 mo of recovery. After 1 mo recovery, hydroxyproline levels and expression of the fibrogenic genes collagen-α1(I) and α-SMA were significantly decreased, compared with CCL4-treated mice (7.8 ± 1.2% Col-GFP and 8 ± 1.5% α-SMA; Fig. 1B), confirming that CCL4-activated myofibroblasts disappear during recovery from liver fibrosis. Thus, because Col-GFP mice undergo regression of liver fibrosis after 1 mo of recovery, it is appropriate to study the fate of aHSCs/myofibroblasts for this time period.

Hepatic Stellate Cells Are the Major Source of CCL4-Activated Myofibroblasts. The contribution of aHSCs to liver myofibroblasts in CCL4-treated Col-GFP mice was determined by using flow cytometry of the isolated nonparenchymal liver cell fraction, which contains aHSCs/myofibroblasts, inflammatory cells, and endothelial cells (8). Myofibroblasts were identified by Col-GFP expression, and HSCs were identified by vitamin A expression (1, 4, 8) (detected at 405 nm as an autofluorescent signal quenched by a violet laser) (Fig. 1C, SI Appendix, Fig. S1). GFP+ cells (92 ± 3%) coexpressed vitamin A, demonstrating that aHSCs represent the major population of fibrogenic myofibroblasts in CCL4-injured liver, as predicted by previous qualitative studies (9). Therefore, aHSCs can be genetically labeled on the basis of specific up-regulation of type I collagen expression (SI Appendix, Fig. S2) in CCL4-induced liver fibrosis because other cellular sources do not make a significant contribution to the myofibroblast population.

Some aHSCs Apoptose During Regression of Liver Fibrosis. We hypothesize that the disappearance of aHSCs/myofibroblasts during...
A comparison of the livers of Col-GFP mice that were untreated, CCl4-treated, the livers of Col-GFP mice 7 d after CCl4 cessation, when apoptosis occurred in the liver, or (ii) after HSCs were activated (Fig. 2A). In the recovered liver, these HSCs resided in the peri-sinusoidal space of Disse and exhibited a stellate shape (Fig. 1B, Fig. S4A).

HSCs Transiently Express Collagen Type I During Development. Detection of YFP+ HSCs in Col-α2(I)Cre-YFP and Col-α1(I)Cre-YFP in adult livers before injury (Fig. 2B, SI Appendix, Figs. S4E and S5) may reflect transient collagen gene expression activating Cre during development. To prove this hypothesis, expression of collagen-α1(I) in real time was examined in livers of Col-GFP mice during embryogenesis (SI Appendix, Fig. S5). Indeed, transient expression of collagen-α1(I)-GFPP was detectable in HSCs, identified by vitamin A, Desmin, and GFAP expression, between embryonic day 16.5 (E16.5) and postnatal day 14 (P14) (SI Appendix, Fig. S6A). At P14, 46 ± 8% of HSCs up-regulated collagen-α1(I) in real time but lacked α-SMA expression (SI Appendix, Fig. S6B). These YFP+ HSCs did not exhibit characteristics of myofibroblasts (SI Appendix, Fig. S6C and D), but were more similar to α-SMA+ HSCs than to α-SMA+ HSCs. The fate of embryonic collagen+ HSCs was examined in adult Col-α2(I)Cre-YFP mice (8 wk old). Consistent with our findings, YFP+ HSCs with a history of collagen expression and YFP+ HSCs had identical gene expression profiles characteristic of a quiescent phenotype (SI Appendix, Fig. S6E).

Tamoxifen-Induced Genetic Labeling of αHSCs/myofibroblasts in Adult Mice Confirmed Their Persistence in the Liver After 1 mo of Recovery from CCl4. Tamoxifen-inducible Col-α2(I)Cre-Ert2 mice were generated by crossing Col-α2(I)Cre-Ert2 mice x Rosa26Stop-flx-mTetROnt-mRd-Stop-fox-mRFp mice (SI Appendix, Fig. S2). Genetic labeling of HSCs was achieved in adult CCl4-treated Col-α2(I)Cre-Ert2 mice by daily tamoxifen administration during the last week of CCl4 treatment (SI Appendix, Fig. S7A). Genetically labeled HSCs were visualized by loss of mTRed expression and gain of GFP expression upon Cre-loxP recombination. Desmin+ HSCs (55 ± 6%) expressed GFP after CCl4 and 41 ± 5% of GFP+ HSCs were still GFP+ after 1 mo recovery (Fig. 2C), confirming that CCl4-activated HSCs (and their progeny) persist in the liver after regressive fibrosis. Consistently, GFP+ HSCs expressed Desmin, but not α-SMA (SI Appendix, Fig. S7B). Thus, three independent

regression of liver fibrosis may result from cell death by senescence (3) and apoptosis (2), inactivation, or both (Fig. 1D). Apoptosis of HSCs during regression of liver fibrosis is well documented (2). In agreement, we detected apoptotic αHSCs/myofibroblasts (2.6 ± 0.7%) by colocalization of cleavable caspase-3* and GFP* cells in the livers of Col-GFP mice 7 d after CCl4 cessation, when apoptosis of hepatic cells was highest (SI Appendix, Fig. S5). Overall, early (7 d) recovery from liver fibrosis is accompanied by apoptosis of some αHSCs/myofibroblasts.

Genetically Labeled αHSCs/myofibroblasts Persist in the Liver After 1 mo of Recovery from CCl4. To determine if some liver myofibroblasts survive the regression of fibrosis, Col-α2(I)Cre-YFP mice (collagen-α2(I)Cre x Rosa26Stop-fox-stop-fox-YFP mice; SI Appendix, Fig. S2) were treated with CCl4 (2 mo), allowed to recover (1 mo), and then analyzed for the persistence of genetically labeled YFP* cells (Fig. 2A). HSCs were identified by expression of GFAP and Desmin, and αHSCs/myofibroblasts were detected by expression of α-SMA. HSCs (98 ± 2%) were activated (e.g., upregulated YFP) in response to CCl4 treatment, and YFP expression was detected in 94 ± 4% of myofibroblasts (α-SMA*). Although myofibroblasts had completely disappeared in livers after 1 mo of recovery, YFP+ cells surprisingly persisted. In particular, expression of YFP was detected in 38 ± 8% of Desmin+ and 41 ± 5% of GFP+ cells, consistent with being HSCs that had been previously activated (Fig. 2A).

The immunohistochemistry (Fig. 2A) and flow cytometry (Fig. 2B) of gradient purified HSCs from Col-α2(I)YFP mice identified three HSC phenotypes: (i) qHSCs (vimentin A+ YFP+ α-SMA*), (ii) αHSCs (vimentin A+ YFP+ α-SMA*), and (iii) inactivated HSCs (αHSCs), vimentin A+ YFP+ α-SMA*. After recovery from fibrosis, 56 ± 4% of HSCs coexpressed YFP+ and vitamin A*, indicating that these iHSCs had a history of type I expression but reverted to an inactivated phenotype (Fig. 2B).

Collagen-α2(I) and -α1(I) form a triple helix to produce collagen type I and are coexpressed in αHSCs/myofibroblasts (10). To provide independent confirmation of the above findings, we used Col-α1(I)Cre-YFP mice, generated by crossing collagen-α1(I)Cre mice (SI Appendix, Fig. S4A) with Rosa26stop-fox-flx-fox-YFP mice. As expected, CCl4 treatment of Col-α1(I)Cre-YFP mice produced αHSCs (Desmin+ YFP+ α-SMA* cells; SI Appendix, Figs. S4B and C). Although α-SMA+ myofibroblasts, as detected by flow cytometry of the nonparenchymal cell fraction from CCl4-treated (2 mo) Col-GFP mice (n = 3). (D) Working hypothesis: CCl4 induces activation of HSCs into αHSCs/myofibroblasts. Cre-loxP-based genetic labeling marks the fate of collagen type I-expressing αHSCs/myofibroblasts (SI Appendix, Fig. S1). During recovery from CCl4-liver fibrosis, αHSCs may (i) inactivate (no genetically labeled YFP+ HSCs will remain in the liver) or (ii) inactivate (all YFP+ cells survive) or (iii) some will inactivate (YFP+ HSCs will number <100% of αHSCs).

Fig. 1. Regression of liver fibrosis is accompanied by loss of myofibroblasts. (A) A comparison of the livers of Col-GFP mice that were untreated, CCl4-treated (2 mo), or recovered from CCl4 (1 and 4 mo) with respect to GFP expression, Sirius Red staining, and α-SMA immunohistochemistry. Representative bright-field and fluorescent micrographs are shown using 10× and 20× objectives. (B) Quantiification of the same four groups in A with respect to hydroxyproline content, Sirius Red staining, α-SMA immunofluorescence, GFP expression, collagen-α1(I) mRNA level, and α-SMA mRNA level. *P < 0.01; **P < 0.05. (C) HSCs (vimentin A*) constituting >90% of myofibroblasts (vimentin A*GFP*), as detected by flow cytometry of the nonparenchymal cell fraction from CCl4-treated (2 mo) Col-GFP mice (n = 3). (D) Working hypothesis: CCl4 induces activation of HSCs into αHSCs/myofibroblasts. Cre-loxP-based genetic labeling marks the fate of collagen type I-expressing αHSCs/myofibroblasts (SI Appendix, Fig. S1). During recovery from CCl4-liver fibrosis, αHSCs may (i) inactivate (no genetically labeled YFP+ HSCs will remain in the liver) or (ii) inactivate (all YFP+ cells survive) or (iii) some will inactivate (YFP+ HSCs will number <100% of αHSCs).

Fig. 2A was treated with CCl4 (2 mo), allowed to recover (1 mo), and then analyzed for the persistence of genetically labeled YFP* cells (Fig. 2A). HSCs were identified by expression of GFAP and Desmin, and αHSCs/myofibroblasts were detected by expression of α-SMA. HSCs (98 ± 2%) were activated (e.g., upregulated YFP) in response to CCl4 treatment, and YFP expression was detected in 94 ± 4% of myofibroblasts (α-SMA*). Although myofibroblasts had completely disappeared in livers after 1 mo of recovery, YFP* cells surprisingly persisted. In particular, expression of YFP was detected in 38 ± 8% of Desmin+ and 41 ± 5% of GFP+ cells, consistent with being HSCs that had been previously activated (Fig. 2A).
transgenic mice demonstrated that aHSCs/myofibroblasts revert to an inactive phenotype during regression of fibrosis.

Livers Recovering from Fibrosis Have Fewer HSCs. To quantify the number of HSCs during fibrosis and its regression, we generated GFAPCre-GFP mice (GFAPCre mice x Rosa26lox-Stop-mTRed-STOP-mGFP mice; SI Appendix, Fig. S8A). In uninjured mice, qHSCs were distributed throughout the hepatic acinus and represented 10.6 ± 0.8% of total liver cells. CCI4 induced HSC activation, proliferation (14.3 ± 1.5% of total liver cells), and accumulation of aHSCs in the pericentral area. One month after recovery, the number of HSCs was reduced (5.6 ± 1.8% of total liver cells), and the distribution of HSCs was again similar to qHSCs. On the basis of immunostaining for GFP after recovery from fibrosis in Col-α1(I)Cre-YFP and Col-α1(I)Cre-YFP mice (Fig. 2B; SI Appendix, Fig. S4E), HSCs constitute 2% of total liver cells in the recovered liver (SI Appendix, Fig. S5B).

Genetically Labeled aHSCs/Myofibroblasts Persist in the Liver After 7 wk of Recovery from Alcohol-Induced Liver Fibrosis. We next determined if survival of aHSCs/myofibroblasts occurs during regression of alcohol-induced liver fibrosis. Liver fibrosis (and steatosis) was induced in Col-α1(I)Cre-YFP mice (collagen-α1(I)Cre × Rosa26lox-Stop-boxYFP mice) by intragastric alcohol feeding for 2 mo (SI Appendix, Fig. S9 A and B). Liver fibrosis (and steatosis) regressed in these mice 7 wk after withdrawal from ethanol feeding. Flow cytometry demonstrated that genetic labeling (YFP+) was achieved in 64 ± 5% of myofibroblasts and persisted in 36 ± 4% of vitamin A+ YFP+ HSCs upon recovery from fibrosis (SI Appendix, Fig. S9C). Our findings were confirmed by immunohistochemistry (SI Appendix, Fig. S9 D and E). YFP expression persisted in 38 ± 7% of Desmin+ HSCs/myofibroblasts following regression of liver fibrosis after withdrawal from ethanol despite the disappearance of myofibroblasts (α-SMA expressed in 1.4 ± 1% of YFP+ HSCs/myofibroblasts; SI Appendix, Fig. S9D). Thus, two models of regression of liver fibrosis demonstrate survival of iHSCs.

iHSCs Demonstrate an Increased Response to Repeated Fibrogenic Stimuli. Purified iHSCs had a phenotype similar to that of qHSCs (Desmin+, GFAP+, Synemin+, α-SMA+; SI Appendix, Figs. S9E and S10). However, expression of myofibroblast-specific genes [Col-α1(I), α-SMA, TIMP-1] was induced more strongly in cultured TGF-β1-treated iHSCs than in qHSCs (Fig. 3A). In concordance, Col-GFP mice subjected to two rounds of CCI4 injury separated by a 6-mo interval to allow complete recovery (2 × CCI4) developed more severe fibrosis than littermates treated with one round of CCI4 (1 × CCI4; Fig. 3B). Thus, our in culture and in vivo data indicate that iHSCs with a history of activation are more effectively activated than qHSCs. Adoptively Transferred HSCs (1-mo Recovery), but Not qHSCs, Contribute to Liver Fibrosis in Mice. To test this hypothesis, HSCs were isolated from Col-GFPβ-actin-RFP mice that were uninjured and after 7 d and 1 mo recovery from CCI4-induced fibrosis and adoptively transferred into livers of the newborn Rag2−/−cβTc mice (11) (Fig. 3C). One month later, these Rag2−/−cβTc mice were subjected to CCI4 injury, and fibrotic livers were analyzed for the presence of GFP+ RFP+ HSCs. Highest engraftment (70–78%) was achieved in mice transplanted with HSCs after 7 d or 1 mo recovery (versus 50% for qHSCs; SI Appendix, Fig. S11A). Unlike qHSCs, which were mostly scattered under the capsule or in liver parenchyma and constituted only 0.5 ± 0.2% of total HSCs, HSCs from the recovering livers were incorporated into the myofibroblast population in recipient mice and contributed 19 ± 2.3% and 13 ± 2.0% of total HSCs, respectively (Fig. 3C). Moreover, despite poor engraftment, comparable results were observed in CCI4-treated wild-type mice adoptively transferred with qHSCs or HSCs (2 wk recovery) from Col-α1(I)Cre-YFP mice (SI Appendix, Fig. S11B). Taken together, iHSCs are primed to differentiate into myofibroblasts more rapidly in response to recurrent stimuli.

Inactivated HSCs Gradually Down-Regulate Collagen-α1(I). To further characterize iHSCs, Col-α1(I)Cre-YFP mice were crossed
with Col-GFP mice, and genetically labeled HSCs (YFP*) were analyzed for expression of collagen-α1(I) in real time (YFP*, SI Appendix, Fig. S12), all YFP* HSCs expressed GFP. After 1 mo recovery from fibrosis, YFP* HSCs had decreased GFP expression. Similar results were obtained by flow cytometry (SI Appendix, Fig. S12 B and C), which allowed simultaneous detection of vitamin A, YFP, and GFP expression (12) in isolated HSCs. As expected, qHSCs lacked GFP expression and HSCs expressed GFP in response to CCl4 (87 ± 5%; SI Appendix, Fig. S12B). Following a 2-wk recovery from CCl4, decreased GFP expression was observed in 75 ± 3% of HSCs, of which 92 ± 4% still expressed YFP. The mean fluorescent intensity (mfi) of GFP expression was strongly reduced in YFP* HSCs at this time (~4 × 10^3 mfi, compared with aHSCs of ~6 × 10^4 mfi; SI Appendix, Fig. S12B). GFP expression (~1 × 10^3 mfi) decreased further in 42 ± 4% of HSCs after 1 mo recovery and correlated with the number of YFP* iHSCs (55 ± 3%). Thus, inactivation of HSCs occurs gradually and steadily during recovery from CCl4-induced fibrosis. Interestingly, 45% of HSCs after 1 mo recovery had no history of collagen expression (YFP*) and represent new qHSCs (SI Appendix, Fig. S12B).

### ihHSCs Acquire a New Phenotype Distinct from qHSCs.

To assess changes in global gene expression, inactivated YFP* HSCs (ihHSCs, 1 mo recovery) were evaluated by the whole-mouse genome microarray and compared with qHSCs, aHSCs, and HSCs after 7 d recovery (Fig. 4A). We confirmed that YFP* ihHSCs down-regulated fibrogenic genes (Col-1α1, Col-1α2, Col-1α1, α-SMA, TGFβRI, and TIMP1) during recovery from fibrosis, but failed to obtain a quiescent phenotype [up-regulated peroxisome proliferator-activated receptor γ (PPARγ) and Bambi, but not the other quiescence-associated genes adipose differentiation related protein (Adfp), Adipor1, or GFAP] (5) (Fig. 4B). Unsupervised clustering of gene expression profiles revealed that YFP* ihHSCs (1 mo recovery) express distinct signatures of fibrosis but lack the quiescent phenotype of qHSCs (Fig. 4C). Regression of gene expression with qHSCs, aHSCs, and HSCs after 7 d recovery (Fig. 4D) shows that expression of a set of fibrogenic genes (Sgn1, Ppargc1a, Bambi, Ccl4, and Col1α1) is progressively down-regulated in YFP* ihHSCs during recovery from fibrosis (100% to 0% of qHSCs, 19% to 0% of aHSCs, 8% to 0% of HSCs). Thus, aHSCs, HSCs, and ihHSCs display distinct gene expression profiles during recovery from fibrosis (Fig. 4C).
mo) exhibit an intermediate profile between that of qHSCs and YFP+ HSCs (7 d recovery), but share more similarity to qHSCs than to aHSCs (Fig. 4 C and D). Similar results were obtained using a statistical coefficient analysis comparing expression profiles to qHSCs (Fig. 4C) and un supervised clustering of gene-specific expression profiles (SI Appendix, Fig. S13 A and C).

**Activation of Heat-Shock Proteins 1a/b May Promote Survival of iHSCs at Day 7 of Recovery from Liver Fibrosis.** To understand how YFP+ iHSCs escape apoptosis, we examined the signaling pathways in YFP+ HSCs after 7 d recovery (SI Appendix, Fig. S13B and E). In particular, expression of the anti-apoptotic heat-shock proteins 1a/b (Hspa1a/b) was strongly but transiently induced in these HSCs (Fig. 5A; SI Appendix, Fig. S14A) to the levels comparable to qHSCs, but was dramatically down-regulated in aHSCs and HSCs after 1 mo recovery (Fig. 5A; SI Appendix, Fig. S14A).

We examined if Hspa1a/b would impact survival of cultured HSCs. For this purpose, HSCs were isolated from CCl4-treated Hspa1a/b−/− and wild-type mice (SI Appendix, Fig. S14B) and cultured 5 d on plastic. Hspa1a/b−/− HSCs had a rounded shape and exhibited growth retardation (cell-number ratio knock out: wt: 1:1.7; SI Appendix, Fig. S14C). Moreover, Hspa1a/b−/− HSCs were more susceptible to glycoxid- (13) and TNF-α-induced apoptosis (14) (Fig. 5B; SI Appendix, Fig. S14C). Therefore, up-regulation of Hspa1a/b genes may promote survival of iHSCs during recovery from fibrosis.

**Resolution of CCl4-Induced Fibrosis Is Expedited in Hspa1a/b−/− Mice.** We hypothesized that the loss of survival signals in Hspa1a/b−/− HSCs would result in increased clearance of aHSCs after recovery from CCl4-induced fibrosis. To test this, Hspa1a/b−/− and wild-type mice were subjected to CCl4-induced liver injury. As expected, Hspa1a/b−/− mice developed more severe fibrosis (probably due to increased hepatocyte death) (15) than did the wild-type littersmates (Fig. 5C). However, after stopping CCL4 treatment, regression of liver fibrosis was strongly accelerated in Hspa1a/b−/− mice compared with wild-type mice (decreased 49 vs. 20%, respectively, by Sirius red staining). Hspa1a/b−/− livers also had a greater loss of α-SMA+Desmin+ aHSCs compared with wild-type mice (decreased 68 vs. 40% of Desmin+ positive area, respectively; Fig. 5C). Thus, Hspa1a/b is required so that iHSCs persist in the recovering liver.

**Discussion**

Clinical and experimental hepatic fibrosis regresses dramatically with removal of the underlying etiological agent. Myofibroblasts are a mixed population of type I cells absent from the normal, uninjured liver, rapidly emerge in fibrotic liver to produce the fibrotic scar, and completely disappear with regression of liver fibrosis (1, 2). In hepatotoxic-induced liver fibrosis (such as CCl4 or intragastric alcohol feeding), quiescent hepatic stellate cells (GFAP+Desmin+α-SMA+Col+ qHSCs) under go activation to become the major source of myofibroblasts (GFAP+Desmin+α-SMA+Col+ aHSCs). Our study uses genetic markers to address the fate of these aHSCs/myofibroblasts during regression of liver fibrosis. We demonstrate that aHSCs/myofibroblasts are cleared by two mechanisms: (i) as previously reported, some myofibroblasts undergo cell death by apoptosis (2); and (ii) some myofibroblasts revert to a previously unrecognized inactive phenotype (iHSCs) that is similar to, but distinct from, quiescent HSCs.

Reversal of fibrosis is associated with increased collagenase activity, activation of macrophages/Kupffer cells, and secretion matrix metalloproteinases, and matrix degradation (1). Senescent and apoptosis of activated HSCs plays a significant role in resolution of liver fibrosis by eliminating the cell type responsible for producing the fibrotic scar (2, 3). Here we demonstrate that some aHSCs undergo apoptosis, whereas other aHSCs escape apoptosis, lose expression of fibrogenic genes, and persist in the liver in an inactivated phenotype. This phenomenon was demonstrated using two models of liver fibrosis with different etiologies: CCl4- and alcohol-induced liver injury. These data suggest that inactivation of aHSCs/myofibroblasts is a common feature of regression of liver fibrosis.

Studies in culture suggest that aHSCs can revert to a quiescent phenotype, associated with expression of lipogenic genes (Adip, Adipor1, CREBP, PPAR-γ) (5) and storage of vitamin A in lipid droplets. Depletion of PPAR-γ constitutes a key molecular event for HSC activation, and ectopic overexpression of this nuclear receptor results in the phenotypic reversal of activated HSC to quiescent cells in culture (5). The treatment of activated HSCs with an adipocyte differentiation mixture, overexpression of SREBP-1c, or culturing on basement membrane-like ECM (16) results in up-regulation of adipogenic transcription factors and causes morphologic and biochemical reversal of activated HSCs to quiescent cells (17). Our in vivo cell-fate mapping studies demonstrate that iHSCs survive apoptosis during reversal of liver fibrosis with a new phenotype that is similar to, but distinct from, the original qHSCs.

Our study confirms that HSCs transiently express collagen type I during development (El6.5–P14), but do not spontaneously become myofibroblasts. This observation explains the presence of genetically labeled qHSCs with a history of collagen expression in livers of uninjured adult mice. These genetically labeled qHSCs possess a quiescent phenotype, indistinguishable from qHSCs with no history of collagen expression. In addition, transient activation of HSCs is required for liver regeneration following partial

Fig. 5. Genetically labeled YFP+ HSCs up-regulate prosurvival Hsp1a/b genes at 7 d of recovery. (A) Up-regulation of prosurvival Hsp1a/b genes in YFP+ HSCs at 7 d of recovery. The results are expressed as relative mRNA levels (average of normalized values/multiple probes/gene; *P < 0.001) obtained by Agilent microarray. (B) Apoptosis was induced in Hspa1a/b−/− and wild-type HSCs by glyotoxin (25 nM for 4 h) and TNF-α (20 ng/ml) + actinomycin (0.2 µg/ml) for 18 h. (C) Hspa1a/b−/− and wild-type (WT) mice were gradually subjected to CCl4 injury and recovered for 2 wk, and livers were analyzed by Sirius Red staining for Desmin and α-SMA (positive areas are indicated). Regression of fibrosis and disappearance of fibrogenic myofibroblasts during recovery were calculated in comparison with CCl4 treatment (100%) and are shown as percentage of Sirius Red, Desmin, and α-SMA-positive areas; *P < 0.01, **P < 0.05.
This page contains text discussing the activation and fate of hepatic stellate cells (HSCs). It mentions that certain HSCs down-regulate the fibrogenic genes collagen-α1(1), collagen-α1(2), α-SMA, TGFβRI, and TIMP1 and up-regulate some quiescence-associated genes (PPARγ and Bambi) to levels comparable to qHSCs, but do not reacquire high expression of GAFP, Adip, and Adiponectin (5). These genetically labeled iHSCs constituted ~50% of total HSCs in the liver 1 mo after reversal of liver fibrosis. Interestingly, the remaining HSCs had a history of activation, highly resemble qHSCs phenotypically, and represent new qHSCs generated from residual YFP-qHSCs or from a precursor cell population. Although during development HSCs originate from submesothelial mesenchymal cells, the source of HSC replenishment is unknown. Using bone marrow chimeric mice, several studies have indicated that HSCs originate from endogenous liver cells and not from a bone marrow-derived progenitor cell (8).

Unlike qHSCs, iHSCs completely down-regulate expression of fibrogenic genes, but, in response to TGFβ1, more rapidly activate into myofibroblasts than qHSCs. Consistent with the concept of iHSCs being more fibrogenic than qHSCs, a previously injured and recovered liver develops more fibrosis than a naive liver. We then more directly tested this concept in vivo by adoptive transfer of HSCs into livers of immunodeficient Rag2-/-γc-/- mice. Unlike previous hepatic stellate transfer experiments (20-21), HSCs (1 mo recovery) were transplanted into their natural liver environment, and their response to CCl4 injury was monitored. Here we demonstrate that iHSCs activate and fully integrate into the fibrous scar in recipient mice more efficiently than qHSCs. Thus, in culture and in vivo iHSCs are activated more effectively than naive qHSCs, so that the previously injured liver generates more fibrous scar in response to a repeated injury. It is not clear why some qHSCs escape apoptosis and inactivate, whereas other HSCs die after cessation of the injury. Our study suggests that survival of iHSCs requires the up-regulation of prosurvival signals, such as induction of heat-shock proteins (22). Members of the Hsp70 family of heat-shock proteins, Hspa1a and Hspb1a (22), which play a protective role against stress-induced apoptosis (23), were strongly and transiently up-regulated in iHSCs and 7 d of reversal of fibrosis (when apoptosis of other HSCs is highest) compared with the qHSCs in fibrotic liver. Furthermore, we demonstrate that genetic ablation of Hspa1a/b renders qHSCs more susceptible to TNF-α (14) and glycolxin-induced (13) apoptosis in culture. In concordance, regression of liver fibrosis was strongly accelerated in Hspa1a/b-/- mice and was associated with increased disappearance of α-SMA+Desmin+ HSCs. We can speculate that Hspa1a/b regulate HSC survival, whereas PPAR-γ drives HSC inactivation during reversal from liver fibrosis.

Materials and Methods
See SI Appendix for additional materials and methods, figures, and acknowledgments.

Mice. Expression of collagen type I in real time was studied using reporter Col-GFP (24). Cell fate mapping of aHSCs was studied using collagen-α2(25) and collagen-α1(25) (SI Appendix) and tamoxifen-inducible collagen-α1(26) crossed to Rosa26luc-Stop-Rosa-YFP mice (or Rosa26luc-mT/MedStop-flx-mGFP mice) (Jackson Labs). GAFP+/- mice were used to determine the total number of HSCs.

Liver Fibrosis. Liver fibrosis was induced in mice by intragastric gavage with CCl4 (at 16 × 1.4 dilution in 100 μL of corn oil) over 2 mo (8) or by intragastric ethanol feeding combined with Western diet (for 2 mo) (SI Appendix) (26). Reversal of liver fibrosis was studied 1 mo after CCl4 cessation and 7 wk after withdrawal from alcohol feeding. Recurrent injury in Col-GFP mice was used for 1 mo with CCl4 (8 × 1.4). Liver injury in Rag2-/-γc-/- and Hspa1a/b-/- mice was gradually induced with CCl4 (4 × 1.16; 2 × 1.0; 2 × 1.4) for 1 mo. Collagen content was measured by hydroxyproline and Sirius Red staining.

Isolation of Nonparenchymal Cell Fraction and Primary HSCs. Livers are perfused and digested using pronase/collagenase and the gradient centrifugation method, as previously described (8). Freshly isolated HSCs were analyzed by flow cytometry or cultured in DMEM (Gibco-BRL) + 10% FCS and 2 mM l-glutamine + antibiotics.

Flow Cytometry. Phenotyping of the hepatic nonparenchymal cells was performed on FACS Canto II RUO (BD). Activated myofibroblasts were visualized by GFP expression (488 nm) using argon laser, and vitamin A expression (405 nm) was detected by violet laser. Cell sorting was performed on a MoFlo (Beckman Coulter) for GFP (488 nm, using Lyt-200S laser, iCYP) and apoptosis of aHSCs are described in SI Appendix. Quantitative RT-PCR and apoptosis of aHSCs are described in SI Appendix.

9. Lee KS, Buck M, Houglum K, Chojkier M (1995) Activation of hepatic stellate cells by vitamin A+YFP+ and vitamin A+YFP-ox-YFP mice (or Rosa26luc-mT/MedStop-flx-mGFP mice) (Jackson Labs). GAFP+/- mice were used to determine the total number of HSCs.

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