Genetic Labeling Does Not Detect Epithelial-to-Mesenchymal Transition of Cholangiocytes in Liver Fibrosis in Mice

DAVID SCHOLTEN,*§ CHRISTOPH H. ÖSTERREICHER,* ANJALI SCHOLTEN,* KEIKO IWAIASKO,* GUOQIANG GU,§ DAVID A. BRENNER,* and TATIANA KISSELEVA*

*Department of Medicine, University of California, San Diego, La Jolla, California; †Department of Medicine III, University Hospital Aachen, Aachen, Germany; and §Department of Cell and Developmental Biology, Vanderbilt University, Medical Center, Nashville, Tennessee

BACKGROUND & AIMS: Chronic injury changes the fate of certain cellular populations, inducing epithelial cells to generate fibroblasts by epithelial-to-mesenchymal transition (EMT) and mesenchymal cells to generate epithelial cells by mesenchymal-to-epithelial transition (MET). Although contribution of EMT/MET to embryogenesis, renal fibrosis, and lung fibrosis is well documented, role of EMT/MET in liver fibrosis is unclear. We determined whether cytokeratin-19 positive (K19+) cholangiocytes give rise to myofibroblasts (EMT) and/or whether glial fibrillary acidic protein positive (GFAP+) hepatic stellate cells (HSCs) can express epithelial markers (MET) in response to experimental liver injury.

METHODS: EMT was studied with Cre-loxP system to map cell fate of K19+ cholangiocytes in K19CreERT or fibroblast-specific protein-1 (FSP-1)CreERT mice, generated by crossing tamoxifen-inducible K19CreERT mice or FSP-1Cre mice with Rosa26f/f-YFP mice. MET of GFAPGFP or FSP-1CreERT-labeled HSCs was studied in GFAPGFP or FSP-1CreERT-labeled HSCs. MET was determined whether cytokeratin-19 positive (K19+) cholangiocytes expressed yellow fluorescent protein (YFP). All mice developed liver fibrosis. However, specific immunostaining of K19CreERT cholangiocytes showed no expression of EMT markers α-smooth muscle actin, desmin, or FSP-1. Moreover, cells genetically labeled by FSP-1CreERT expression did not coexpress cholangiocyte markers K19 or E-cadherin. Genetically labeled GFAPGFP HSCs did not express epithelial or liver progenitor markers in response to liver injury.

RESULTS: On Cre-loxP recombination, >40% of genetically labeled K19+ cholangiocytes expressed yellow fluorescent protein (YFP). All mice developed liver fibrosis. However, specific immunostaining of K19CreERT cholangiocytes showed no expression of EMT markers α-smooth muscle actin, desmin, or FSP-1. Moreover, cells genetically labeled by FSP-1CreERT expression did not coexpress cholangiocyte markers K19 or E-cadherin. Genetically labeled GFAPGFP HSCs did not express epithelial or liver progenitor markers in response to liver injury.

CONCLUSION: EMT of cholangiocytes identified by genetic labeling does not contribute to hepatic fibrosis in mice. Likewise, GFAPCreERT-labeled HSCs showed no coexpression of epithelial markers, providing no evidence for MET in HSCs in response to fibrogenic liver injury.

Keywords: EMT; Mesenchymal-to-Epithelial Transition; MET.

Liver cirrhosis, an outcome of chronic liver injury, is characterized by excessive accumulation of extracellular matrix (ECM) proteins, mostly type I collagen. Hepatic stellate cells (HSCs) are considered a major source of ECM but not the only source of myofibroblasts in the injured liver. Hepatic myofibroblasts may also originate from portal fibroblasts, interphase (septal) myofibroblasts, and, to a smaller extent, bone marrow–derived mesenchymal cells. Another mechanism implicated in the fibrogenesis of parenchymal organs is epithelial-to-mesenchymal transition (EMT), when epithelial cells acquire features of mesenchymal cells. During this process, epithelial cells detach from the epithelial layer; lose their polarity, expression of epithelial markers (eg, cytokeratin-19 [K19]), CK7, E-cadherin, and tight junction proteins (zonula occludens-1); increase their motility; and obtain a (myo)fibroblastic phenotype. Epithelial cells transitioning into (myo)fibroblasts up-regulate fibroblast specific protein-1 (FSP-1; a Ca2+-binding S100 protein), which has become a universal marker of EMT during fibrogenesis and cancer. EMT plays an important role in organogenesis during embryonic development. In adult tissues, EMT is a mechanism that facilitates metastasis and cancer development. In addition, several studies suggest that EMT occurs in response to injury and chronic inflammation and facilitates fibrosis. EMT in chronic injury has been best characterized in fibrosing kidneys. With the use of the gGT-LacZ transgenic mice, which allows the identification of tubular epithelial cells in fibrotic kidneys, Iwano et al demonstrated that more than one third of renal interstitial fibroblasts are derived by EMT. Additional studies have linked EMT to lung fibrosis, rheumatoid arthritis, and retinopathy. Meanwhile, the contribution of EMT to organ fibrogenesis remains unclear.

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; Ab, antibody; BDL, bile duct ligation; Col2(I), collagen α2(I); ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; FSP-1, fibroblast-specific protein-1; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HSC, hepatic stellate cell; K19, cytokeratin-19; MET, mesenchymal-to-epithelial transition; Pan-Ck, pancytokeratin; RT-PCR, reverse transcription polymerase chain reaction; YFP, yellow fluorescent protein.

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bution of EMT-derived cells to a population of hepatic myofibroblasts in fibrotic liver is unknown. Typical myofibroblasts, independent of their origin or localization, are identified by the specific morphology (production of the stress fibers), the ability to secrete ECM (fibronectin and collagen types I and III), and the expression of α-smooth muscle actin (α-SMA). EMT of hepatocytes has been reported in patients and in mice with liver fibrosis. Using genetic fate labeling of albumin hepatocytes, Zeisberg et al reported that a population of hepatic FSP-1 fibroblasts is derived from mature hepatocytes in response to liver injury. However, only minimal expression of α-SMA was observed in these FSP-1 cells (<10%). In concordance with this data, genetic crossing of albumin-Cre with Rosa-LacZ mice labeled HSCs generated by crossing of GFAPCre mice with collagen α(I)-green fluorescent protein (GFP) reporter mice, in which transition of LacZ hepatocytes into collagen-expressing myofibroblasts was monitored in vivo, detected no EMT-derived myofibroblasts in fibrotic liver. EMT of cholangiocytes has been linked to primary biliary fibrosis and nonalcoholic fatty liver disease in patients, rats, and mice. Those studies, specifically in human beings, relied mostly on immunohistochemical analysis, and their conclusions were based on the co-localization of epithelial and myofibroblastic markers in the same cells. Generation of cholangiocyte-specific mice (expressing Cre under the control of inducible K19 promoter and EMT-specific mice (expressing Cre under the FSP-1 promoter) now makes it possible to trace the fate of cholangiocytes and to study their possible EMT in response to injury.

Because EMT reflects inherent cell plasticity, it is closely associated with another process, mesenchymal-to-epithelial transition (MET), which is characterized by (trans)differentiation of mesenchymal cells into epithelial cells. It has been suggested that in response to chronic injury, HSCs can undergo MET and under certain circumstances acquire an epithelial phenotype. In the course of hepatic fibrosis, HSCs show unique plasticity by undergoing activation from a quiescent to a myofibroblast phenotype. In their quiescent state, HSCs reside in the space of Disse and store vitamin A. They express neural markers, such as glial fibrillary acidic protein (GFAP), synemin, and synaptophysin, and mesenchymal/mesodermal markers, such as desmin and vimentin. In response to chronic liver injury, quiescent HSCs activate, lose vitamin A droplets, and transform into myofibroblasts. On activation, HSCs change their structure, migrate to the site of injury, down-regulate neural markers, and up-regulate mesenchymal markers, eg, collagen α(I), α-SMA, and fibronectin. HSC precursors probably originate by embryonic EMT. Because of the expression of GFAP in quiescent HSCs, MET can be studied in genetically labeled HSCs generated by crossing of GFAPCre mice with reporter mouse. Moreover, new monoclonal antibodies (Abs), recognizing a broad variety of hepatic epithelial progenitor cells in mice, provide new reagents to study the mechanism of MET and liver regeneration in fibrotic liver.

Materials and Methods

Mice

GFAP-Cre mice, ROSA26-Stop/YFP mice, Rosa-26/Fsp-1 Cre mice were obtained from The Jackson Laboratory (Bar Harbor, ME). K19CreERT mice were crossed to homozygosity with Rosa26Cre reporter mice and treated with tamoxifen (5 mg/100 µL corn oil × 9; Sigma T5648-1G; Sigma, St. Louis, MO) to achieve Cre-LoxP recombination. FSP-1Cre mice are a gift of Dr Nielson (Vanderbilt University School of Medicine, Nashville, TN). Collagen α(II) [Col2(II)]Cre mice were obtained from Dr Yang. See Supplementary Materials for details.

Induction of Liver Injury and Measurement of Collagen Deposition

Twelve-week-old mice were subjected to bile duct ligation (BDL) or treated with CCl₄ as previously described, and hydroxyproline content was measured as described. Liver sections were stained with Sirius red, and positive area was measured in 5 fields/mouse and quantified with ImageJ (National Institutes of Health, Bethesda, MD).

RT-PCR and Real-Time Quantitative PCR

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed with the use of standard conditions with an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA), specific primers, and SYBRGreen. Concentration-time product values of each sample were normalized to 18s mRNA expression. Values were expressed as fold induction in comparison with untreated or sham controls.

Immunofluorescence and Immunohistochemistry

Formalin-fixed frozen liver tissues were stained with anti-GFP Ab (Abcam, Cambridge, MA), anti-α-SMA Ab (Abcam), antidesmin Ab (Thermo Scientific, Waltham MA), anti-FSP-1 Ab (gift from Dr Neilson), anti-GFAP Ab (Abcam), anti-pancytokeratin (Pan-CK; DakoCytomation, Carpinteria, CA), Troma III (Development Studies Hybridoma Bank, University of Iowa, Iowa City, IA), fluorescein isothiocyanate–conjugated monoclonal anti–α-SMA (Sigma) or the appropriate isotype control, followed by secondary Alexa Fluor Abs and nuclei counterstaining with 4,6-diamidino-2-phenylindole. Immunohistochemistry was performed with anti–α-SMA Ab (Abcam) and anti-GFP Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by diaminobenzidine staining (Vector Laboratories, Burlingame, CA) and counterstaining with hematoxylin.
HSC Isolation and Immunostaining

A collagenase-pronase perfusion method was used to isolate HSCs as previously described. Isolated HSCs were fixed in 4% buffered formalin and stained with anti-GFP Ab and antidesmin Abs.

Statistics

All data are shown as mean ± SEM. Differences between multiple groups were compared using 1-way analysis of variance with post hoc Bonferroni correction (SPSS 15.0 software; SPSS Inc, Chicago, IL). Differences between the 2 groups were compared with a 2-tailed unpaired t test (SPSS 15.0 software; SPSS Inc). P values less than .05 were considered significant.

Results

Study Design

This study was designed to determine whether chronic liver injury induces (1) cholangiocytes to contribute to a myofibroblast population by EMT and (2) HSCs to undergo MET to enforce the regeneration of epithelial cells (hepatocytes and cholangiocytes) and to serve as a facultative source of hepatic progenitors. A genetic approach, based on the Cre-loxP system, was used to label the cells of interest before the change of their cellular fate. To study the role of EMT in hepatic fibrosis, cholangiocyte-specific K19CreERT mice, in which tamoxifen-inducible CreERT was knocked into the endogenous K19 locus, were crossed with ROSA26CreERT reporter mice (Figure 1A). Double transgenic K19CreERT offspring, homozygous for Cre and yellow fluorescent protein (YFP), were treated with tamoxifen (5 mg/mouse; Figure 1C) to maximally label K19+ cholangiocytes with YFP. To identify the cells transitioning into the new phenotype by EMT, FSP-1Cre mice were crossed with Rosa26CreERT reporter mice to generate FSP-1YFP mice, in which the cells expressing FSP-1 are permanently labeled by YFP expression (Figure 1B). In turn, to study MET, quiescent HSCs were labeled by crossing GFAPCre mice with Rosa26CreERT reporter mice (generating GFAPCre+ mice), whereas activated HSCs were labeled by crossing Col2(I)Cre mice with Rosa26CreERT reporter mice (generating Col2(I)Cre mice; Figure 1B).

Induction of Liver Fibrosis to Study EMT in Cholangiocytes

To study the role of EMT in hepatic fibrosis, cholangiocyte-specific K19CreERT mice were subjected to liver injury by BDL for 21 days or by the administration of CCl4 (0.5 mL/g × 16 times) for 2 months (Figure 1C). Similarly, FSP-1Cre mice, GFAPCre+, and Col2(I)Cre mice were subjected to the BDL or CCl4 with the use of the same treatment protocol.

All mice developed liver fibrosis (Figure 2A). Hydroxyproline content was increased approximately 3-fold in the livers of BDL-operated K19CreERT mice, compared with the sham-operated littermates. Sirius red staining reached 9% in BDL livers compared with 1.4% in sham-operated K19CreERT mice. Elevated levels of collagen α1(I) (6.8-fold), α-SMA (5.3-fold), and FSP-1 protein (6-fold) mRNA expression were detected in livers of the BDL versus sham-operated mice (Figure 2A and B). Similar results were obtained in the CCl4-treated K19CreERT mice, as shown by hydroxyproline content (4.4 times than in control mice), Sirius red staining (11% versus 1.4% in control mice), immunohistochemistry, and RT-PCR (Figure 2A and C). Therefore, we concluded that the liver injury induced by the BDL or CCl4 resulted in fibrosis so that EMT or MET could be induced in these mice.

Induction of Cre/loxP Recombination in Mice to Study EMT/MET

Tamoxifen-inducible Cre-loxP recombination was analyzed in K19CreERT mice before or after liver injury and was compared with untreated mice (no tamoxifen). As expected, only K19CreERT mice that received tamoxifen expressed YFP, as detected by specific immunostaining with anti-GFP Ab (Figure 3A and Supplementary Figure 1). Next, the efficiency of Cre-loxP recombination was estimated in control or liver-injured K19CreERT mice. As expected, K19CreERT cholangiocytes were stained positive with anti–Pan-CK Ab (Figure 3A) and localized specifically in the bile ducts, identified by H&E or immunostaining with Troma III Ab (Supplementary Figures 2 and 3). The percentage of labeled cholangiocytes was calculated in comparison to the total Pan-CK-positive cholangiocytes (100%), and constituted 32% ± 2% in the sham-operated K19CreERT mice (Figure 3A and C). Obstruction of the common bile duct causes proliferation of the ductular epithelial cells, and the percentage of YFP-labeled bile ducts increased to 46% ± 7% in the BDL K19CreERT mice. Similar numbers of YFP+ cholangiocytes were detected in mice treated with CCl4 (41% ± 5%) or corn oil (27% ± 6%). The number of genetically labeled cells was quantified in FSP-1Cre, GFAPCre+, and Col2(I)Cre mice expressing constitutive Cre, before and after induction of liver injury. The number of YFP+ cells was significantly increased in the livers of the BDL FSP-1Cre mice in comparison with the sham-operated controls (from 31% ± 3% to 3% ± 1%) of total liver cells (100%). Similar results were obtained in CCl4-treated FSP-1Cre mice (24% ± 2% versus 2% ± 0.6% in control mice), indicating that induction of FSP-1 is a general feature of hepatic fibrosis.

CCl4 injury also induced activation of HSCs, as indicated by the increased numbers of YFP+ cells detected in preparations of myofibroblasts (100%) isolated from Col2(I)Cre mice (Figure 3C). In response to CCl4-induced injury, the number of genetically labeled HSCs ranged from 21% ± 4% to 95% ± 3% in Col2(I)Cre mice and from 82% ± 7% to 84% ± 4% in GFAPCre+ mice. Taken together, Cre-loxP recombination was achieved in all transgenic mice; resulted in specific labeling of K19+ cholangiocytes, FSP-1+ cells, and quiescent GFAP+; and activated Col2(I)+ cells.
HSCs. The number of genetically labeled K19+ cholangiocytes, FSP-1+ cells, and GFAP+ HSCs increased in response to injury (Figure 3D) in comparison with control mice.

**EMT of Cholangiocytes Does Not Contribute to the Myofibroblast Population in Fibrotic Livers**

The role of EMT in the pathogenesis of liver fibrosis was studied in K19YFP mice, in which K19+ cholangiocytes and their progeny were permanently labeled by YFP expression. In addition to existing cholangiocytes, repeated tamoxifen administration induced expression of YFP in new cholangiocytes in response to injury. YFP+ cholangiocytes were located in the portal areas (Figure 4A). EMT in the YFP+ cholangiocytes was evaluated in K19YFP mice with liver fibrosis and was determined by coexpression of mesenchymal markers (α-SMA, desmin) in cells with a history of K19 expression.
Expression of GFAP, a marker of HSCs, was not detected in YFP+ cholangiocytes. Immunostaining with anti-α-SMA and antidesmin Abs, which detect myofibroblasts, showed no colocalization of α-SMA or desmin in YFP+ cells in the livers of BDL-operated K19YFP mice. Similarly, double-positive YFP+α-SMA+ or YFP+desmin+ cells were not observed in CCl4-treated K19YFP mice, suggesting that EMT of cholangiocytes does not produce myofibroblasts in response to experimental liver fibrosis. To corroborate these results, the myofibroblast population was purified with the gradient centrifugation method from fibrotic livers of BDL-injured K19YFP or Col2/IYFP mice. No YFP+ cells were detected in these cultures from K19YFP mice, whereas in control experiments 97% ± 3% of myofibroblasts, derived from Col2/IYFP mice, expressed YFP. Thus, our results show that EMT of genetically labeled cholangiocytes does not contribute to HSCs, or myofibroblasts, as shown with the use of the 2 models of experimental hepatic fibrosis.

**Cholangiocytes Do Not Up-Regulate FSP-1 in Response to Fibrogenic Liver Injury**

We hypothesized that cholangiocytes may undergo an incomplete EMT and, therefore, would express FSP-1 without being transformed into myofibroblasts. Livers from BDL- or CCl4-injured K19YFP mice were ana-
lyzed for the EMT marker FSP-1 expression. Although FSP-1⁺ and K19⁺ cells were located in the portal areas in close proximity to each other, there was no colocalization (Figure 4C, panel a).

We next examined the expression of FSP-1 on epithelial cells. We used FSP-1/GFP reporter mice, which express GFP under the control of the FSP-1 promoter, and mark cells currently expressing FSP-1. We analyzed for the EMT marker FSP-1 expression. Although FSP-1⁺ and K19⁺ cells were located in the portal areas in close proximity to each other, there was no colocalization (Figure 4C, panel a).

Figure 3. Induction of liver injury in K19⁺ mice. (A) Tamoxifen-induced Cre-loxP recombination in K19⁺ mice, as shown by immunohistochemistry for YFP (upper panel) and immunofluorescence for Pan-CK and YFP (lower panel). Bile ducts (bd), hepatic artery (ha), and portal vein (pv) are indicated. (B) Cre-loxP recombination was quantified in K19⁺ mice. The bars display the number of YFP-labeled cholangiocytes in comparison with Pan-CK⁺ cholangiocytes (100%) (P < .05; n = 10). (C) Efficiency of Cre-loxP recombination was estimated in tamoxifen-treated K19 YFP mice in comparison with total Pan-CK⁺ cholangiocytes (100%), in GFAP⁺ or Col2⁺ mice was in isolated myofibroblast fractions (100%) by the number of genetically labeled GFP⁺ or YFP⁺ cells, respectively. (D) Liver injury increases the number of K19⁺, FSP-1, and GFAP⁺ cells. The number of labeled cells is compared in BDL and sham-operated mice, or in CCl4- and corn oil-treated mice and expressed as the ratio calculated for each group.
were subjected to BDL, and the livers were stained with anti–Pan-CK Ab to visualize cholangiocytes. Pan-CK+ cholangiocytes were located in the portal areas of FSP-1/GFP mice, but they never overlapped with FSP-1/GFP cells, indicating that cholangiocytes did not express FSP-1 (Figure 4C, panel b). Similar results were obtained for CCl4-treated mice. Because expression of FSP-1 by cells of epithelial origin may be transient during EMT, we next examined the fate of FSP-1+ expressing cells. To address this question, FSP-1^{YFP} mice, generated by crossing FSP-1^{Cre} mice with Rosa26^{f/f-YFP} mice, were used to identify the progeny of FSP-1^{+} cells, even if these cells completed EMT and down-regulated FSP-1 expression. Liver from BDL- or CCl4-treated FSP-1^{YFP} mice was stained with anti–Pan-CK Ab. Liver sections from FSP-1/GFP reporter mice (n = 7) were stained with anti-GFP and anti–Pan-CK Abs. Representative images are shown at 40× and 400× magnifications.

**Figure 4.** EMT in cholangiocytes does not contribute to the myofibroblast population in response to liver injury. (A) In BDL- or CCl4-injured K19^{YFP} mice, genetically labeled YFP+ cholangiocytes did not coexpress myofibroblast markers (α-SMA, desmin, or GFAP), shown at 400× magnification. (B) Myofibroblast fraction isolated from BDL-operated K19^{YFP} mice lacked EMT-derived YFP+ cells, as detected by immunostaining with anti-GFP Ab. As a control, expression of YFP was detected in 97% of plated cell myofibroblasts isolated from CCl4-treated Col2(I)^{YFP} mice (P < .05). (C) Liver tissues from K19^{YFP} mice were stained with anti–FSP-1 Ab. Liver sections from FSP-1/GFP reporter mice (n = 7) were stained with anti-GFP and anti–Pan-CK Abs. Similarly, FSP-1^{YFP} mice (n = 8) were stained with anti-GFP and anti–Pan-CK Abs. Representative images are shown at 40× and 400× magnifications.
costained with anti-GFP and anti–Pan-CK Abs (Figure 4C panel c). Examination of YFP+ cells showed no coexpression of Pan-CK, suggesting that cholangiocytes did not express FSP-1 before or during liver injury. Consistent with this, cholangiocytes immunostained with anti–Pan-CK Ab did not express α-SMA in response to BDL or CCl4 (Supplementary Figure 4).

EMT is best characterized during embryonic development and usually occurs between embryonic day (E) 6.5 (gastrulation) and E9.5.19 Because K19 is strongly upregulated in tissues during gastrulation (>90% cells),14 Cre-loxP recombination was induced in embryos at E9.5 by tamoxifen administration to pregnant K19YFP mice. Embryos (labeled at E9.5) contained K19-YFP+ cells. Most YFP+ cells were located in the skin, epithelial lining, lungs, and liver (Supplementary Figure 5). FSP-1+ cells colocalized with K19-YFP+ cells, mainly in the lungs, indicating that K19-expressing cells undergo EMT during embryogenesis. Furthermore, immunostaining with anti–α-SMA or antidesmin Abs also identified double-positive cells. In particular, desmin+ K19YFP+ cells were identified in the developing gastrointestinal tract. Hence, K19YFP mice are well suitable to study EMT during embryogenesis.

**Cell Fate Mapping of HSCs to Study MET in Liver Fibrosis**

MET is a process opposite to EMT, in which myofibroblasts undergo differentiation into epithelial-like cells.4 To follow the fate of HSCs in adult mice in response to liver fibrosis, GFAPGFP mice were crossed with Rosa26f/f-mT/GFP mice to generate GFAPGFP mice (Figures 5A and 3C). These mice showed successful Cre-loxP recombination by expression of GFP in the GFAP+ cells but not in the Tomato-red+ hepatocytes. To induce liver injury, GFAPGFP mice were treated with CCl4, and the ability of HSCs to undergo MET in response to acute injury or during recovery was assessed by immunostaining with anti–α-SMA, antidesmin, anti–Pan-CK, and E-cadherin Abs. As expected, activated HSCs proliferated, migrated into fibrotic septa, and expressed myofibroblastic markers α-SMA and desmin (Figure 5B). Double-positive α-SMA+GFP+ and desmin+GFP+ cells were visualized by pseudo-red cytosolic staining surrounded by membrane-tagged GFP signal. Although the epithelial marker E-cadherin was detected in fibrotic areas of GFAPGFP livers, it did not colocalize with the GFAPGFP+ HSCs (Figure 5B). Similar results were obtained for Pan-CK+ cholangiocytes, although located in close proximity to HSCs, they showed no colocalization with GFAPGFP+ cells (Figure 5B and C).

As an alternative approach to assess MET, Col2(I)−Cre mice were crossed with Rosa26f/f-YFP mice, and collagen-expressing activated HSCs (as well as any other myofibroblasts) were analyzed for expression of MET markers in response to CCl4-induced liver injury (Figure 5D).

However, similarly to GFAPGFP mice, Col2(I)YFP+ cells showed no colocalization with Pan-CK+ cholangiocytes.

To further analyze the concept of stellate cell plasticity, livers from CCl4-treated GFAPGFP mice were stained for markers specific for murine oval cells.17 Several Abs have recently been generated that recognize progenitors in Diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate–induced mice. Although these Abs recognized a small number of progenitors in CCl4-treated mice, we did not find any GFAPGFP+–labeled HSCs staining for these markers (Figure 6).

**Discussion**

Chronic inflammation and persistent liver injury may induce deregulation of normal physiology with inherent cellular plasticity, causing EMT, MET, or both. Although both processes may enhance regeneration, they may also accelerate the progression of liver fibrosis. Using 2 models of liver fibrosis, BDL and CCl4, we have determined that genetically labeled K19+ cholangiocytes do not undergo MET. Although FSP-1+ cells were widely present in fibrotic livers, this marker was not up-regulated in Pan-CK+ cholangiocytes. Moreover, fibrogenic liver injury did not prompt HSCs to express epithelial cell markers, such as Pan-CK or E-cadherin. HSCs also lacked markers of hepatic progenitors. Taken together, the current study provides evidence that EMT in cholangiocytes and MET in HSCs do not contribute to experimental hepatic fibrosis or liver regeneration (Table 1).

Recent studies have reported that EMT plays a role in biliary fibrosis in human beings, rats, and mice.12 With the use of mostly immunohistochemical analysis, EMT in cholangiocytes was identified by colocalization of the EMT-specific marker FSP-1 in CK-7+ cells.12,13,20 Although these studies have shown costaining in CK-7+ cells, the contribution of EMT to the deposition of ECM and fibrosis was not documented. Moreover, EMT was solely based on coexpression of FSP-1 and CK-7, or examination of EMT-activated signal transduction pathways in response to injury,12 without following cholangiocyte differentiation into cells with mesenchymal phenotype. Taken together, detection of EMT in human beings and rodents provides several difficulties. (1) Specificity of Abs used for immunohistochemistry, such as CK-7, may recognize a population slightly distinct from K19+ or Pan-CK+ cholangiocytes. (2) Coexpression of FSP-1 and epithelial markers (CK-7, Pan-CK, or K19), typical for early EMT, may be transient. Therefore, finding no coexpression of these markers does not rule out the occurrence of EMT. (3) Colocalization studies may generate false positives by bleed-through of fluorescent probes or by overlapping cells expressing different markers being misinterpreted as single cells. To overcome these obstacles, genetic in vivo labeling of the cells of interest, K19+ cholangiocytes, and monitoring their changing phenotype throughout dura-
Figure 5. Genetically labeled quiescent or activated HSCs do not undergo MET in response to CCl₄ injury or during recovery. (A) Morphology of liver tissues from GFAPGFP mice is shown before injury (upper panel) and after CCl₄ administration (lower panel). Quiescent and activated HSCs are labeled by membrane-bound GFP expression (green arrows), and hepatocytes retain expression of mTRed (white arrows; 200× magnification). (B) Genetically labeled GFAP⁺ quiescent HSCs do not express MET markers in response to CCl₄ or during recovery in GFAPCre mice (n = 10). Images show morphology (upper panel) and costaining (lower panel) of the same tissue section with anti-α-SMA, antidesmin, E-cadherin (E-cad), or Pan-CK Abs and were visualized with Alexa Fluor 633–conjugated secondary Abs for α-SMA, desmin (shown in pseudo-red color), and E-cadherin and Pan-CK (pseudo-blue color; 600× magnification). (C) Serial liver sections from GFAPGFP mice show differential localization of genetically labeled GFAP⁺ HSCs and Pan-CK⁺ cholangiocytes in response to CCl₄. (D) Genetically labeled activated HSCs do not undergo MET in CCl₄-treated Col2(1)YFP mice (n = 10). Col2(1)YFP mice up-regulate YFP in all activated HSCs and coexpress α-SMA and desmin but lack Pan-CK expression (200× magnification).
tion of the liver injury, was considered as the method of choice in the current study.

We genetically labeled K19\textsuperscript{CreERT} cholangiocytes in adult mice to investigate EMT in response to fibrogenic liver injury. Tamoxifen-inducible K19\textsuperscript{CreERT} mice were chosen for a number of reasons. (1) To avoid genetic labeling of K19\textsuperscript{CreERT} cells occurring during embryonic development, the tamoxifen-inducible Cre-ERT gene was knocked into the genetic locus of the K19 promoter.\textsuperscript{14} (2) The K19 promoter is highly specific for cholangiocytes in the liver, as confirmed by immunostaining with anti-K19 and Pan-CK Abs. (3) Up-regulation of mesenchymal markers (FSP-1, \(\alpha\)-SMA, and desmin) is monitored in K19-labeled cholangiocytes throughout liver injury, tracing initial (epithelial) and final (mesenchymal) phenotypes in single cells. (4) BDL causes maximal damage to the biliary epithelium with proliferation of the bile ducts, a condition that has been proposed to induce EMT. Thus, using K19\textsuperscript{YFP} mice (K19\textsuperscript{CreERT} mice \(\times\) Rosa26\textsuperscript{YFP} mice), we have shown that cells with a history of K19 expression did not express \(\alpha\)-SMA or desmin, mesenchymal markers typically expressed by myofibroblasts. Furthermore, K19-labeled cholangiocytes did not co-express FSP-1, a marker associated with EMT, suggesting that fibrogenic liver injury does not induce EMT in cholangiocytes.

Our results differ from the findings reported by Omedetti et al\textsuperscript{12} and Roderfeld et al,\textsuperscript{20} who demonstrated coexpression of FSP-1 in CK-7\textsuperscript{+} biliary cells. Several factors may explain this discrepancy. First, inducible K19\textsuperscript{Cre} mice label a highly specific population of mature cholangiocytes in the liver, as confirmed by immunostaining with anti-K19 and Pan-CK Abs. (3) Up-regulation of mesenchymal markers (FSP-1, \(\alpha\)-SMA, and desmin) is monitored in K19-labeled cholangiocytes throughout liver injury, tracing initial (epithelial) and final (mesenchymal) phenotypes in single cells. (4) BDL causes maximal damage to the biliary epithelium with proliferation of the bile ducts, a condition that has been proposed to induce EMT. Thus, using K19\textsuperscript{YFP} mice (K19\textsuperscript{CreERT} mice \(\times\) Rosa26\textsuperscript{YFP} mice), we have shown that cells with a history of K19 expression did not express \(\alpha\)-SMA or desmin, mesenchymal markers typically expressed by myofibroblasts. Furthermore, K19-labeled cholangiocytes did not co-express FSP-1, a marker associated with EMT, suggesting that fibrogenic liver injury does not induce EMT in cholangiocytes.

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giocytes is heterogenous. This fact is supported not only by the identification of K19+/CK-7- cholangiocytes but also by the presence of oval cells and other liver progenitors in the canal of Hering.17 Third, development of liver fibrosis often depends on the model of injury and the choice of transgenic/knockout mice. Thus, for example, Omenetti et al12 studied Ptc-deficient mice, whereas Roderfeld et al20 used Abcb4−/− mice. Our current study focused on EMT in K19+ cholangiocytes in wild-type mice undergoing experimental fibrosis. To strengthen our findings, we analyzed cells that might have undergone EMT and at some point up-regulated FSP-1 in response to injury. However, genetically labeled FSP-1+ cells did not express cholangiocyte markers (Pan-CK), proving that cholangiocytes did not express FSP-1 and do not undergo EMT in response to experimental liver fibrosis. Of course, all of these genetic mouse experiments are limited by the short duration of the studies (usually 2–8 weeks) in comparison to the years in which liver fibrosis progresses in patients. Therefore, EMT of cholangiocytes in patients, originally defined by the coexpression of K19+ and the presence (Pan-CK label) or absence (E-cadherin) of coexpression of specific markers is indicated.

Unfortunately, the genetic studies of EMT in fibrosis with the use of the Cre-LoxP system has its own limitations. Genetic labeling of a specific cellular population is achieved by crossing mice expressing Cre under control of a cell-specific promoter with reporter mice, ubiquitously expressing the β-gal or yfp genes in which transcription is blocked by a floxed Stop cassette.25 The choice of the reporter mice is critically significant, and Rosa26YFP mice are superior to Rosa26β-gal mice, which increase nonspecific B-gal accumulation in tissues with age. Two problems are associated with cell fate mapping in mice: specificity of Cre and efficiency of Cre-lox recombination. Because specificity of Cre expression can be confirmed by immunostaining, efficiency of Cre-lox recombination depends solely on Cre concentration. Therefore, 100% of Cre-lox recombination can be rarely achieved in mice. It is especially difficult in ER-Cre mice, in which Cre expression is regulated by tamoxifen administration and can be strongly affected by the dose, route, and frequency of administration.26 A number of well-established Cre or ER-Cre mice have incomplete Cre-Lox recombination, which creates problems with conditional gene ablation in mice.27 However, the Cre-lox system remains the most reliable tool to monitor specific cellular populations and their progeny in mice. Thus, using Albumin-Cre mice complete Cre-LoxP recombination was not achieved in hepatocytes, but it still showed the lack of EMT in response to injury.11 Low efficiency of Cre-LoxP recombination can be overcome in cell fate mapping studies by examining a large number of mice per experiment and cells per mouse. This way a statistically significant result can be obtained. Because we were not able to determine any genetically labeled cells coexpressing markers of EMT in >1000 K19YFP+ cells, (eg, α-SMA and K19YFP, or desmin and K19YFP), this number was not increased when calculated for 100% of cholangiocytes.

Our present study also investigated the role of MET of HSCs in response to liver injury. Again, using a genetic approach, we showed that neither quiescent GFAP−/−labeled nor activated Col2(I)−/−labeled HSCs express epithelial cell or cholangiocyte markers after CCl4 activation or recovery. Our findings differ from Yang et al,28 who suggested that quiescent HSCs may differentiate into hepatocytes and, therefore, belong to the oval cell family. Because of the expression of neural markers, HSCs are believed to originate during embryonic development from the same common precursor as brain astrocytes. In addition, it has been suggested that adult bone marrow can serve as a source of HSC replenishment in response to injury.29 Recent studies in rats suggested that, in addition to epithelial cells, Thy-1.1+ oval cells may also give rise to cells with myofibroblastic phenotype.30 Moreover, expression of progenitor markers CD133 raised a possibility that HSCs may arise from a common liver precursor (or liver stem cell) in the adult liver.28 Using immunostaining with recently generated monoclonal Abs for the oval cell response in ductal and periductal areas,17 we demonstrated that GFP-labeled HSCs from CCl4-treated GFAPGFP mice lack markers of oval cells. Although our study does not provide insight into

### Table 1. Genetic Cell Fate Mapping of Transgenic Mice Used to Study EMT and MET

<table>
<thead>
<tr>
<th>Coexpression</th>
<th>Costaining</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>Pan-CK</td>
</tr>
<tr>
<td>FSP-1</td>
<td>Desmin</td>
</tr>
<tr>
<td>Oval cell</td>
<td>E-cadherin</td>
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**NOTE.** The genetically labeled cell type is stated for each transgenic mouse; coexpression of the cell of interest with other markers is shown, and the presence (+ +; +) or absence (−) of coexpression of specific markers is indicated.
whether there is a common hepatic precursor cell for HSCs, hepatocytes, and cholangiocytes, it does show that HSCs and myofibroblasts did not undergo MET to become epithelial cells.

**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.2010.05.005.

**References**


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**Reprint requests**

Address requests for reprints to: Tatiana Kisseleva, Department of Medicine, University of California, 9500 Gilman Drive, La Jolla, California 92093-0702. e-mail: tkkisseleva@ucsd.edu.

**Conflicts of interest**

The authors disclose no conflicts.

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