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# Sustained Activation of Rac1 in Hepatic Stellate Cells Promotes Liver Injury and Fibrosis in Mice

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**Rac, a small, GTP-binding protein in the Rho family, regulates several cellular functions, including the activation of NADPH oxidase, a major intracellular producer of reactive oxygen species (ROS). Hepatic stellate cells (HSCs) isolated from mice that are genetically deficient in NADPH oxidase produce less ROS, and their activation during chronic liver injury is abrogated, resulting in decreased liver fibrosis. Therefore, we hypothesized that HSC ROS production and activation would be enhanced, and fibrosis worsened, by increasing Rac expression in HSCs. To achieve this, we used transgenic mice that express constitutively active human *Rac1* under the control of the α-smooth muscle actin (*α-sma*) promoter, because *α-sma* expression is induced spontaneously during HSC activation. Transgene expression was upregulated progressively during culture of primary Rac-transgenic HSCs, and this increased HSC ROS production as well as expression of activation markers and collagen. Similarly, Rac mice treated with carbon tetrachloride ($CCl_4$) accumulated greater numbers of activated HSCs and had more liver damage, hepatocyte apoptosis, and liver fibrosis—as well as higher mortality—than $CCl_4$-treated wild-type mice. In conclusion, sustained activation of Rac in HSCs perpetuates their activation and exacerbates toxin-induced liver injury and fibrosis, prompting speculation that Rac may be a therapeutic target in patients with cirrhosis.** (HEPATOLOGY **2006;44:1267-1277.**)

Chronic liver injury incites fibrosis and, in some instances, this progresses to cirrhosis.[1] During this process, hepatic stellate cells (HSCs), the major profibrogenic cells in the liver, are activated to a myofibroblastic phenotype and produce collagen matrix during injury.[2] Thus, emerging antifibrotic therapies aim to inhibit the accumulation of these fibrogenic cells.[3] Further insight into mechanisms that regulate the activation of HSCs will advance this goal.

Reactive oxygen species (ROS) are important mediators of liver fibrosis.[4] Increased ROS production and resultant oxidative stress are common factors in the progression of chronic liver disease induced by alcohol, hepatitis C virus, chronic cholestasis, and iron overload.[5] Others have recently demonstrated that fibrosis is abrogated by administration of antioxidants during chronic hepatic injury.[6,7]

Several sources of ROS have been identified in the liver. Hepatocyte microsomal enzymes, such as cytochrome P450 2E1, release ROS inducing HSC activation, proliferation, and collagen synthesis.[5] Plasma membrane-associated NADPH oxidase, a major intracellular producer of ROS, has also been identified as an important source of ROS during liver injury. HSCs cultured from mice genetically deficient in NADPH oxidase produce less ROS and are less activated by profibrogenic factors.[8] Consistent with this finding, NADPH oxidase–deficient mice are protected from liver injury and develop less fibrosis following bile duct ligation.[8] Adachi et al.[9] confirmed the critical importance of NADPH oxidase in HSC activation by demonstrating that NADPH oxidase–

*Abbreviations: ROS, reactive oxygen species; HSC, hepatic stellate cell; α-SMA, α-smooth muscle actin; $CCl_4$, carbon tetrachloride; WT, wild-type; DPI, diphenyleneiodonium; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling; PDGF, platelet-derived growth factor.*

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derived ROS are necessary for platelet-derived growth factor to induce proliferation in cultured HSCs. One integral component in the activation of NADPH oxidase is Rac1, a member of the Rho family of small GTPase proteins that regulate cell proliferation and dynamic reorganization of the actin cytoskeleton.[10] Despite growing evidence that induction of NADPH oxidase is critical for HSC activation, it is unknown whether Rac has a role in this process.

Based on evidence that NADPH oxidase–derived ROS are required for HSC activation and hepatic fibrogenesis, we hypothesized that increasing Rac1 activity in HSCs would promote HSC activation. To evaluate this, we studied transgenic mice in which regulatory elements of the α-smooth muscle actin (*α-sma*) promoter control expression of constitutively active mutant human Rac1, thereby increasing NADPH oxidase activity and ROS production in myofibroblastic cells.[11] Because *α-sma* induction occurs spontaneously during HSC activation,[12] these mice serve as a model for HSC-targeted activation of Rac1 and subsequent intrahepatic ROS production.

We report that mutant Rac1 is induced during culture of HSCs from Rac-transgenic mice, and this amplifies their activation *in vitro*. Liver injury, fibrosis, and liver-related mortality caused by carbon tetrachloride ($CCl_4$) are also increased in Rac-transgenic mice. These findings identify Rac as a novel, potential therapeutic target in hepatic fibrosis.

## Materials and Methods

***Animals and Treatments.*** Rac transgenic mice in which *α-sma* promoter elements control expression of a mutant form of human Rac1 ($Rac^{V12}$), produced by a glycine 12-to-valine substitution in the *Rac* coding sequence have been described, as have the construct and procedure for generation of these mice.[11,13] FVB/N transgenic mice were backcrossed with C57BL/6 mice for 10 generations to establish the transgene on a C57BL/6 background. Although the enzymatic activity of mutant Rac protein is constitutive (because GTP remains bound to mutant Rac), mutant Rac retains normal sensitivity to the redox mechanism that controls the cellular content of Rac. Thus, as ROS accumulate, the latter increases the degradation (*i.e.*, shortens the half-life) of active Rac (mutant and endogenous), ultimately limiting further NADPH oxidase generation of ROS despite persistent *α-sma*–driven expression of transgenic *Rac* mRNA.[14] Hence, in Rac-transgenic mice, expression of mutant Rac is controlled at two levels: *α-sma* regulation of mutant *Rac* mRNA expression and ROS-dependent regulation of mutant Rac protein content. These processes constrain the cellular localization of mutant Rac (to cell types that express α-SMA) and the net content of mutant Rac in any given α-SMA–expressing cell. However, all mutant Rac that accumulates exhibits constitutive (*i.e.*, GDP exchange–independent) activity.

Age- and sex-matched wild-type (WT) littermates ($n = 6$) and Rac transgenic mice ($n = 11$) received intraperitoneal injections with $CCl_4$ (0.5 mg/kg; Sigma-Aldrich, St. Louis, MO). $CCl_4$ was prepared as 1 mg/mL in corn oil (Sigma-Aldrich). Mice were sacrificed within 8 weeks of initiating treatment. Serum was collected for biochemical analysis. Livers were either snap frozen in liquid nitrogen or fixed in formalin, paraffin-embedded, serially sectioned at 7 μm thickness, and stained. Histological examination was conducted via light and fluorescence microscopy. Animal experiments fulfilled the National Institutes of Health and Duke University requirements for humane animal care.

***Cell Culture.*** The HSC fraction was isolated via *in situ* perfusion with collagenase and pronase.[15] Viability and purity of HSC preparations were consistently >95% as assessed via Trypan Blue (GIBCO/BRL, Grand Island, NY) exclusion and autofluorescence, respectively.[16] Freshly isolated, primary HSCs were used for RNA analysis or cultured on plastic dishes for ≤10 days in serum-supplemented RPMI 1640 medium (GIBCO/BRL). Culture under these conditions eliminates endothelial cells, vascular smooth muscle cells, and macrophages that contaminate HSC isolates.[17,18]

The human HSC line, LX-2, was obtained from S.L. Friedman (Mount Sinai School of Medicine, New York, NY).[18] LX-2 were cultured in serum-supplemented DMEM (GIBCO/BRL). Inhibition of Rac1 was performed using the small molecule inhibitor of Rac1, NSC23766 (EMD Biosciences, San Diego, CA), at doses of 100 μmol/L and 500 μmol/L.[19] Inhibition of NADPH oxidase was performed using 1 μmol/L diphenyleneiodonium (DPI) (Toronto Research Chemicals, North York, Canada).

Primary hepatocytes were harvested by perfusion with collagenase and further purified by Percoll gradient. Briefly, following perfusion with type I collagenase (0.5 mg/mL, Sigma-Aldrich), isolated hepatocytes were filtered, centrifuged, and resuspended in serum-supplemented DMEM (GIBCO/BRL) containing L-glutamine, insulin, and dexamethasone. An equal volume of Percoll (Amersham, Piscataway, NJ) was added before centrifugation. Cell viability was determined by Trypan Blue exclusion.

***NADPH Oxidase Activity.*** After removing culture medium and washing plates twice to remove nonadherent, dead cells, NADPH oxidase activity was measured via

**Table 1. Reverse-Transcriptase Polymerase Chain Reaction Primers for Analysis**

| Gene | Genbank Accession Number | Direction | Sequence | Amplicon Size (bp) |
|---|---|---|---|---|
| *Gus* | NM_010368 | Forward | GCAGTTGTGTGGGTGAATGG | 142 |
| | | Reverse | GGGTCAGTGTGTTGTTGATGG | |
| *Rac1* | BT007121 | Forward | GAGCAGAAGCTGATCTCCGAGGAG | 650 |
| | | Reverse | TTACAACAGCAGGCATTTTCTCTT | |
| *Gfap* | NM_010277 | Forward | TCCTGGAACAGCAAAACAAG | 224 |
| | | Reverse | CAGCCTCAGGTTGGTTTCAT | |
| *α-sma* | NM_007392 | Forward | GGCTCTGGGCTCTGTAAGG | 149 |
| | | Reverse | CTCTTGCTCTGGGCTTCATC | |
| *Col1α2* | BC007158 | Forward | GAACGGTCCACGATTGCATG | 167 |
| | | Reverse | GGCATGTTGCTAGGCACGAAG | |

lucigenin-enhanced chemiluminescence using previously described methods in which NADPH serves as a substrate for superoxide production.[20,21] The results were standardized to protein concentration in an aliquot of the same homogenates.[22]

***Rac Activation Assay and Western Blotting.*** Rac activation assay was performed as described by Ren et al.[23] Briefly, after preparation of GST-enriched beads, primary HSCs from WT and Rac-transgenic mice were lysed in buffer. Lysates were centrifuged, incubated with prepared beads, and washed in buffer. Equal amounts of protein were resolved by SDS-PAGE and transferred to membranes. Membranes were blotted with anti-Rac1 (Santa Cruz Biotechnology, Santa Cruz, CA), and signals were detected by enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations.

Protein isolated from primary hepatocytes was resolved by SDS-PAGE, transferred to membranes that were probed with anti–Myc-tag (Upstate, Charlottesville, VA) and anti-Rac1 (Santa Cruz Biotechnology). LX-2 transfected with myc-tagged human Rac1 plasmid (gift from R.J. Lefkowitz, Duke University, Durham, NC) were positive controls for Myc-tag, and rat brain microsomal preparation (Upstate) was the positive control for Rac1.

***Two-Step Real-Time Reverse-Transcriptase Polymerase Chain Reaction.*** Total RNA was extracted from primary cells and HSCs with RNeasy kits (Qiagen, Valencia, CA). Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed as previously described.[15] Primers were designed using Genbank sequences or as described (Table 1). For all primer pairs, specificity was confirmed with sequencing PCR products. Target gene levels are presented relative to levels detected in corresponding controls according to the ΔΔCt method.[24] Fold changes were determined using point and interval estimates.

***Immunofluorescent Staining of Cultured Cells and Murine Liver.*** Immunofluorescent staining of culture-activated primary HSCs and murine liver was performed as described.[15]

***Immunohistochemistry and Analysis of Liver Architecture.*** Serial sections were stained with hematoxylin-eosin or Masson's trichrome. Detection of DNA fragments was performed using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) (Roche Diagnostics, Indianapolis, IN). After the reaction was stopped, streptavidin-HRP (Vector Laboratories, Burlingame, CA) and DAB chromagen (DakoCytomation, Carpinteria, CA) were used for detection, with counterstaining in Gill's hematoxylin (Vector Laboratories).

***Quantification of Hepatic Collagen Content.*** Hepatic collagen content was evaluated using morphometric analysis of Sirius red–stained liver sections and by hydroxyproline quantification. Sections were stained with picro-Sirius red (Sigma-Aldrich), counterstained with fast green (Sigma-Aldrich), and subjected to morphometric analysis with MetaMorph software (Molecular Devices Corporation, Downington, PA).[25,26] Collagen staining was quantitated in sections from all mice (magnification ×20; 10 fields from each section). Liver hydroxyproline content was quantified colorimetrically. Freeze-dried samples (30 mg) were hydrolyzed in 6 N HCl at 110°C for 16 hours. Hydrolysates were evaporated under vacuum, dissolved in 1 mL of distilled water, filtered, and then incubated with 0.5 mL of chloramine-T solution, containing chloramine-T (Sigma-Aldrich) dissolved in acetate-citrate buffer and 50% isopropanol, for 20 minutes. Ehrlich's solution (4-dimethylaminobenzaldehyde [Sigma-Aldrich] in 60% perchloric acid [Sigma-Aldrich] with isopropanol) was added. The mixture was incubated at 65°C for 15 minutes, then cooled, and absorbance was read at 561 nm. Hydroxyproline concentration (micrograms of hydroxyproline per grams of liver) was calculated from a standard curve prepared with high-purity hydroxyproline (Sigma-Aldrich).

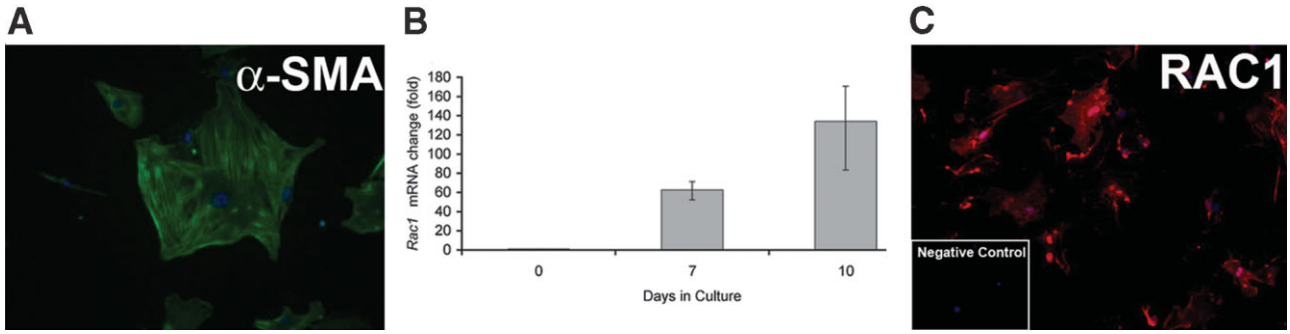


Fig. 1. Characterization of hepatic stellate cells from Rac-transgenic mice. Primary HSCs were isolated and pooled from 6 Rac-transgenic mice and then culture-activated on plastic dishes in serum-containing media for up to 10 days. (A) α-SMA expression in representative HSCs after 7 days in culture. (B) Time-dependent induction of human *Rac1* mRNA was demonstrated by quantitative real-time RT-PCR analysis of RNA from freshly isolated (day 0) HSCs, and RNA from day 7 and day 10 HSC cultures. Results at each time point were normalized to expression of the housekeeping gene, β-glucuronidase, in the same sample. Transgene expression in cultured HSCs was compared with that of freshly isolated cells and displayed as fold-induction of human *Rac1* expression relative to expression on day 0. (C) Immunofluorescent staining for human Rac1 detected with Rho-conjugated secondary antibody in activated primary HSCs. α-SMA, α-smooth muscle actin.

***Serum Biochemical Measurements.*** Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin were measured by Bioreliance (Rockville, MD). Serial dilutions up to 1:4 were performed when measurements exceeded the upper limits of detection for ALT and AST (500 U/l).

***Statistical Analysis.*** Comparisons between groups were made using Intercooled Stata 8.0 (Stata Corporation, College Station, TX). Results are expressed as the mean ± SD or SEM. Comparisons were performed using a Student *t* test. A *P* value of less than .05 was considered statistically significant.

## Results

Earlier studies of α-sma–Rac-transgenic mice could not demonstrate Rac1 transgene expression via RT-PCR analysis of RNA from healthy, uninjured livers.[11,13] This is not surprising, because relatively few cells express α-SMA in uninjured livers.[12] However, because HSC transcription of the *α-sma* gene is dramatically upregulated during liver injury or culture conditions that induce HSC activation, we suspected that constitutively active mutant human *Rac1* would be expressed in activated Rac-transgenic HSCs.[11,13,27,28] To verify this, we isolated primary HSCs from two groups of Rac-transgenic mice (4-6 mice/group) and evaluated them during culture-induced activation. At 2 days, cultures consisted of large polygonally shaped cells with large nuclei surrounded by a ring of lipid droplets, typical of primary HSCs.[29,30] Over the next 7 days, these cells grew to confluence and lost most of their lipid droplets. Because HSCs are known to have a myofibroblastic phenotype when culture-activated, immunofluorescent staining for α-SMA was performed. As expected, the culture-activated Rac-transgenic HSCs strongly expressed α-SMA (Fig. 1A). To determine if activation-related upregulation of *α-sma* expression induced *Rac1* transgene expression, we performed real-time RT-PCR analysis of mRNA extracted from the freshly isolated HSCs (*i.e.*, day 0), as well as from primary HSCs in culture. Transgenic *Rac1* mRNA expression increased by 63-fold at 7 days and >134-fold at 10 days when compared with freshly isolated, quiescent Rac-transgenic HSCs (Fig. 1B). Immunofluorescent staining with monoclonal antibody against human Rac1 demonstrated that the increases in human *Rac1* mRNA were accompanied by increases in Rac1 protein (Fig. 1C).

GTP-bound Rac is the biologically active form that assembles NADPH oxidase. To determine if Rac activity is increased in Rac-transgenic HSCs, primary HSCs were isolated from four additional Rac-transgenic mice and four WT littermates. Assays for GTP-bound Rac1 were performed after cells had been cultured for 10 days. GTP-bound murine Rac1 was demonstrated in cultured HSCs from WT mice, while Rac-transgenic HSCs contained both human and murine GTP-bound Rac1 (Fig. 2A). Although culture-activated WT HSCs have endogenous Rac1 activity, Rac activity was greater in the Rac-transgenic HSCs.

To determine if Rac1 activation increased ROS production by NADPH oxidase, NADPH oxidase activity was evaluated in primary HSCs from additional WT and Rac-transgenic mice (4 mice/group). NADPH oxidase–derived ROS increased with time in culture in WT and Rac-transgenic HSCs. At both 4 and 10 days, Rac-transgenic HSCs produced approximately twofold more ROS than matched WT controls (Fig. 2B). Consistent with published evidence that ROS promote the degradation of both endogenous and mutant Rac proteins,[14] the cellular

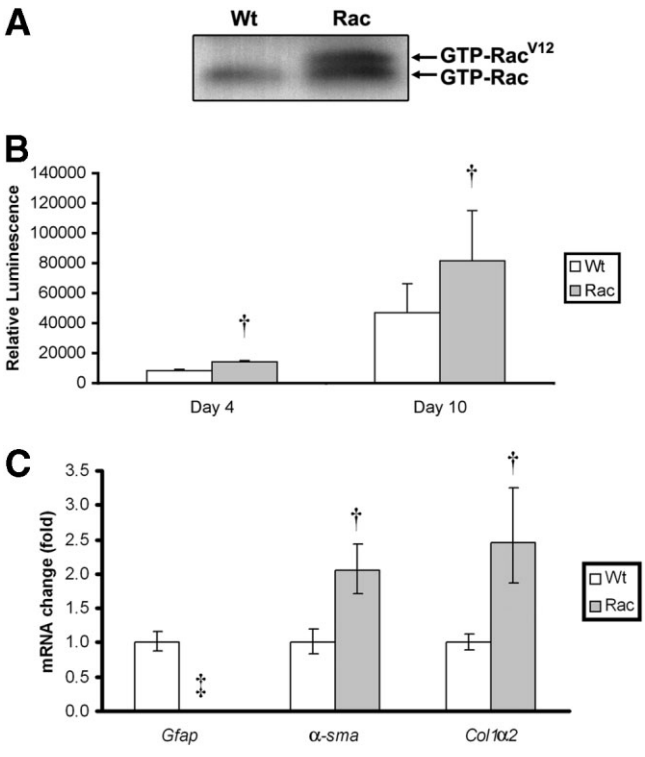


Fig. 2. Rac overexpression amplifies culture-induced activation of HSCs. Primary HSCs were isolated from the livers of 8 healthy WT mice and 8 Rac-transgenic mice using *in situ* liver perfusion and density gradient centrifugation. Cells were placed in culture for up to 10 days. (A) At the end of the culture period, some cultures were processed to assess GTP-bound Rac, which reflects the cellular content of biologically active endogenous Rac (GTP-Rac), as well as constitutively active human Rac1 (GTP-Rac$^{V12}$). Results of a representative assay are displayed. Similar findings were demonstrated in duplicate experiments (data not shown). (B) NADPH oxidase–derived ROS were measured with a luminescence-based assay to detect cellular ROS following 4 and 7 days in culture. Rac-transgenic mice produce greater ROS than their matched WT controls after both 4 and 10 days of culture activation. †$P < .05$. (C) RT-PCR analysis was performed to evaluate markers of stellate cell activation (*α-sma* and *Col1α2*) and quiescence (*Gfap*). For each sample, gene expression was normalized to expression of the housekeeping gene, *Gus*. Gene expression in Rac-transgenic HSCs was then compared with that in WT HSCs; the results are expressed as fold changes in mRNA levels. †$P < .05$. ‡$P < .001$. Wt, wild-type; α-sma, α-smooth muscle actin.

content of Rac protein (Fig. 2A) and NADPH oxidase activity (Fig. 2B) increased only twofold in Rac-transgenic HSCs, although activation-related increases in α-sma promoter activity resulted in 100-fold increases in *Rac* transgene mRNA levels (Fig. 1B). To determine if accumulation of activated Rac was associated with any differences in HSC activation, RNA was isolated from 7-day-old cultures of WT and Rac-transgenic HSCs and analyzed via real-time RT-PCR. As predicted by others,[31,32] the culture-activated WT HSCs strongly expressed *α-sma* and *Col1α2* mRNA, markers of myofibroblastic cells, and low levels of *Gfap* mRNA, a marker of HSC quiescence. Compared with WT HSCs, culture-activated Rac-transgenic HSCs demonstrated approximately twofold higher expression of *α-sma* and *Col1α2* and significantly decreased expression of *Gfap* (Fig. 2C). Together, our findings demonstrate that accumulation of active Rac augments production of ROS by NADPH oxidase and enhances HSC activation during culture.

To examine the general validity of the concept that Rac regulates activation of NADPH oxidase, a major source of intracellular ROS that drive HSC activation,[8] we studied an activated stellate cell line (LX-2) derived from human liver. We treated these cells with NSC23766, a specific, small molecule inhibitor of Rac1[19] and DPI, an NADPH oxidase inhibitor. Changes in NADPH oxidase-dependent endogenous ROS production were then correlated with changes in the expression of plasminogen activator inhibitor (*PAI*)-1 mRNA, a marker of activated HSCs.[33] Treatment with the Rac inhibitor caused a dose-dependent decrease in both ROS production by NADPH oxidase (Fig. 3A) and in *PAI-1* expression (Fig. 3B), supporting a role for Rac in regulating oxidant production by NADPH oxidase during HSC activation. Similarly, treatment with DPI resulted in decreases in both ROS production and *PAI-1* mRNA expression.

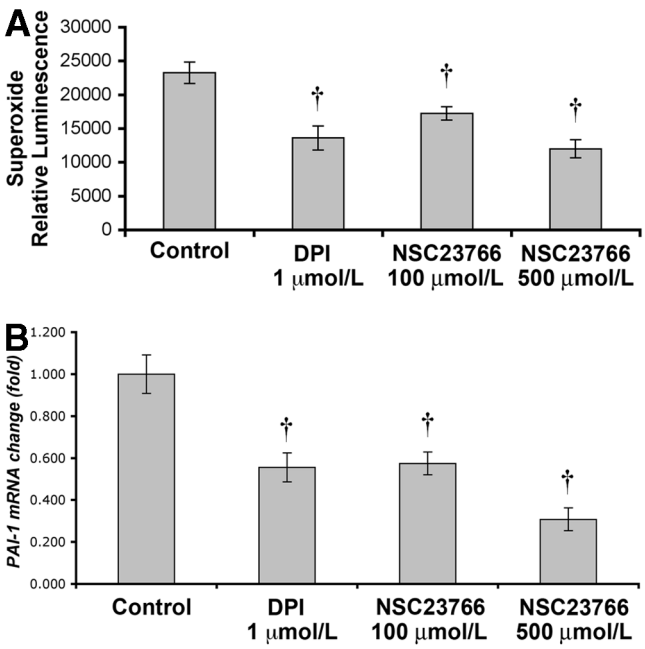


Fig. 3. Inhibition of Rac1 and NADPH oxidase results in decreased ROS production and decreased expression of HSC activation markers. (A) Treatment of the human activated HSC line LX-2 with an inhibitor of NADPH oxidase (DPI) predictably decreased ROS production. Treatment with a specific inhibitor of Rac1 (NSC23766) also decreased ROS production in a dose-dependent manner. †$P < .05$. (B) Rac1 inhibition resulted in decreased mRNA expression of *PAI-1*, an HSC marker of activation, while treatment with DPI similarly decreased *PAI-1* mRNA expression. †$P < .05$.

To assess the pathophysiological consequences of excessive Rac-mediated HSC activation in living animals, we treated WT and Rac-transgenic mice with $CCl_4$. Eleven Rac-transgenic mice and 6 WT controls received injections of $CCl_4$ (0.5 mg/kg) twice a week for 8 weeks to induce chronic liver injury and fibrosis. During treatment, 4 Rac mice but no WT mice died. All remaining mice lost weight. Weight loss in the Rac-transgenic group was greater than in WT controls (Fig. 4A), despite the fact that all Rac-transgenic mice, but none of the controls, had ascites when sacrificed (Fig. 4B). Serum aminotransferases (Fig. 4C) and bilirubin levels (Fig. 4D) were also significantly higher in the Rac-transgenic group. Rac-transgenic mice also exhibited more macroscopic and microscopic liver injury than WT mice after chronic $CCl_4$ exposure. Livers from $CCl_4$-treated Rac-transgenic mice were more grossly nodular (Fig. 4E-F) and demonstrated more acidophilic necrosis (Fig. 4H,K) with greater numbers of TUNEL-positive liver cells (Fig. 4I,L) than $CCl_4$-treated WT controls. Differences in $CCl_4$-induced liver injury between Rac-transgenic mice and WT mice could not be attributed to higher expression of CYP2E1 in the Rac-transgenic animals. Indeed, Western blot analysis of liver extracts from $CCl_4$-treated mice demonstrated that CYP2E1 protein levels were actually lower in Rac-transgenic mice than in controls, making it highly unlikely that their worse liver injury reflected greater exposure to CYP2E1-generated toxic metabolites of $CCl_4$. In addition, we found no evidence that Rac-transgenic mice had hepatic pathology before $CCl_4$ exposure; the livers of age- and sex-matched, untreated WT and Rac-transgenic were normal in appearance both macroscopically (data not shown) and on hematoxylin-eosin–stained sections (Fig. 4G,J).

Interestingly, the injured livers of Rac-transgenic mice accumulated greater numbers of activated, $\alpha$-SMA–positive HSCs than those of WT controls (Fig. 5A,C). Rac-transgenic livers also had denser collagen deposition in these areas (Fig. 5B,D). Morphometric analysis of Sirius red–stained liver sections (Fig. 5E) and quantification of liver hydroxyproline content provided additional evidence for more extensive liver fibrosis in the Rac-transgenic group (Fig. 5F).

Our results show that Rac-transgenic mice developed worse liver injury than WT mice when HSCs were activated by $CCl_4$ exposure. While it has been shown that the Rac1 transgene is not expressed in healthy liver, it is important to verify that hepatocytes are incapable of activating transgene expression. Therefore, we isolated primary hepatocytes from Rac-transgenic mice and performed immunoblot analysis on extracted proteins to identify human Rac1. Constitutively active human Rac1 is myc-tagged, and thus, can be identified by an antibody to myc-tag. Hepatocytes from Rac-transgenic mice expressed endogenous Rac1 (Fig. 5H), but not myc-tagged Rac1 (Fig 5G), confirming that hepatocytes lack factors that activate the $\alpha$-sma regulatory elements that drive expression of the human Rac1 transgene. Hence, differences in $CCl_4$-induced liver injury severity that were noted in Rac-transgenic and WT mice cannot be explained by transgene activation in hepatocytes. Because the turnover of endogenous Rac is generally increased by increased oxidant production,[14] it is also unlikely that endogenous Rac accumulation in hepatocytes explains the increased $CCl_4$ liver injury that occurred in Rac-transgenic mice. Given that ROS production and myofibroblastic activation are enhanced in primary HSCs from Rac-transgenic mice, it seems likely that HSC factors exacerbated liver damage in the Rac-transgenic group. Additional research is necessary to specify these factors.

## Discussion

This study of Rac1-transgenic mice demonstrates that induction of *Rac1* in HSCs enhances their activation during liver injury. This, in turn, exacerbates liver damage, as well as hepatic fibrosis, increasing both acute liver-related mortality and complications of chronic portal hypertension. Although a role for Rac1 in HSC activation had not been tested directly before our study, this seems reasonable based on its actions in other types of myofibroblastic cells.

In fibroblasts and vascular smooth muscle cells, Rac1, a member of the Rho family of small GTP-binding proteins, promotes proliferation and migration by activating the membrane-associated NADPH oxidase complex that generates oxidants for signal transduction.[34-36] Induction of antiapoptotic kinases, including Akt, and reorganization of the actin cytoskeleton ensues, promoting cellular hyperplasia and migration.[10,37] Various studies have proven that both Rac1 and ROS are required for these responses. For example, platelet-derived growth factor (PDGF), a critical mediator of myofibroblastic responses during tissue injury, induces oxidant production in vascular smooth muscle cells.[38] Overexpressing catalase in such cells reduces ROS accumulation after PDGF exposure, and this inhibits proliferation, instead increasing apoptosis.[39] Similarly, inhibition of Rac1 abrogates PDGF-induced migration of vascular smooth muscle cells.[11,40,41] Notably, the biological significance of Rac1-dependent responses in myofibroblastic cells has been demonstrated by studying genetically altered mice. Transgenic mice in which $\alpha$-sma promoter elements control expression of constitutively active human Rac1 exhibit excessive dermal scar formation after skin-wounding,[11] demonstrating that

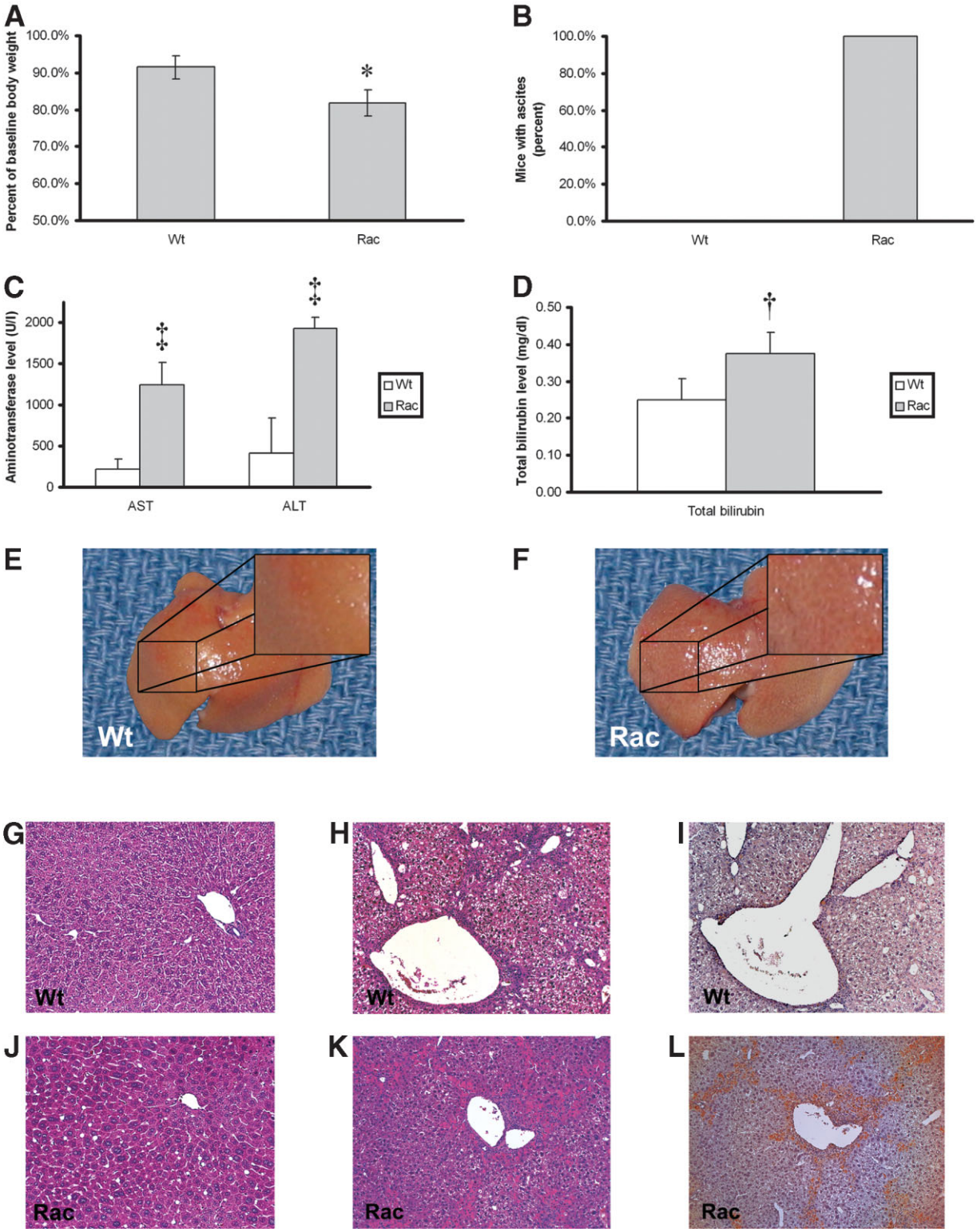


Fig. 4. Rac-transgenic mice exhibit increased liver injury following chronic exposure to $CCl_4$. Six WT and 11 Rac-transgenic mice were given injections of $CCl_4$ (0.5 mg/kg) twice a week for 8 weeks. Four Rac-transgenic mice died during this period, so the results reflect findings in the 7 remaining Rac mice. (A) Body weight was assessed on sacrifice and is expressed as the change from baseline body weight. *$P < .01$ versus WT controls. (B) Despite exhibiting more weight loss during treatment, all Rac-transgenic mice, but no WT mice, had ascites when sacrificed. (C) Serum activities of aspartate aminotransferase and alanine aminotransferase were assessed at sacrifice in both groups. ‡$P < .001$ versus WT controls. (D) Serum bilirubin levels at the end of the treatment period in WT and Rac mice. †$P < .05$ versus WT controls. Livers of WT mice and Rac-transgenic mice were inspected for gross evidence of cirrhosis, as indicated by nodularity and septation. Liver from (E) a representative WT mouse and (F) a Rac-transgenic mouse. (G,J) Hematoxylin-eosin–stained liver sections from untreated, age- and sex-matched WT and Rac mice appear normal. Liver sections from all $CCl_4$-treated mice were stained with hematoxylin-eosin or via TUNEL and then examined to assess the extent of liver injury. Sections from (H-I) representative WT mice and (K-L) Rac-transgenic mice are displayed. These results demonstrate that Rac mice had more extensive areas of acidophilic necrosis and TUNEL-positive cells. Wt, wild-type; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

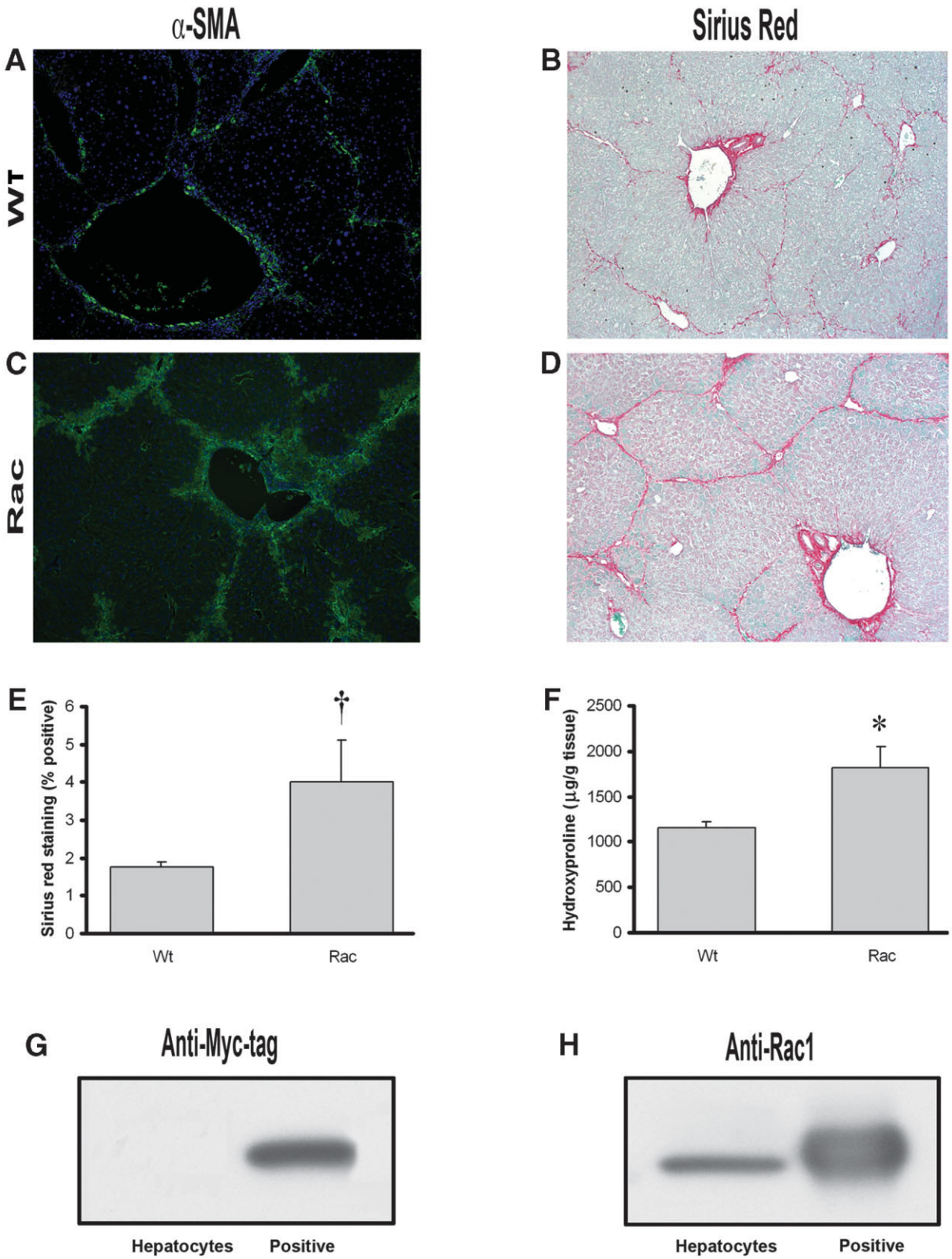


Fig. 5. Rac-transgenic mice accumulate greater numbers of $\alpha$-SMA–positive cells and exhibit more fibrosis after chronic $CCl_4$. (A,C) Immunofluorescent staining for $\alpha$-SMA in the liver of (A) a representative WT mouse and (C) a Rac-transgenic mouse. (B,D) Serial liver sections were also stained with Sirius red to detect collagen deposits. (E) Morphometric analysis of all Sirius red–stained sections from both treatment groups. †$P < .05$ versus WT controls. (F) Quantification of hepatic hydroxyproline content in the two groups. Results are expressed as micrograms of hydroxyproline per grams of liver tissue. *$P < .01$ versus WT controls. (G,H) Primary hepatocytes were isolated from Rac mice and immunoblot analysis was performed to document expression of myc-tagged human Rac1. Immunoblots probed with (G) anti–Myc-tag or (H) anti-Rac1 demonstrate that hepatocytes from Rac-transgenic mice express murine Rac but not human Rac1. The human HSC line LX-2 transfected with myc-tagged human Rac1 was used as the positive control for anti–Myc-tag, and rat brain microsomal preparation was used as the positive control for anti-Rac1. *α-sma*, $\alpha$-smooth muscle actin; WT, wild-type.

activating Rac1 in skin fibroblasts promotes dermal fibrosis when the skin is injured. Our preliminary studies of aortic walls from such Rac-transgenic mice demonstrates that a slight (<20%) but consistent increase in tissue superoxide production accompanies α-sma–induced expression of the Rac transgene by smooth muscle cells in that vessel wall (data not shown). Thus, it is reasonable to assume that the pathological wound-healing responses that others observed in Rac-transgenic mice likely resulted from relatively modest increases in ROS production by α-SMA–expressing cells in injured skin.

Herein, we used a similar approach to evaluate the role of Rac1 in the response to liver injury. Like skin fibroblasts and vascular smooth muscle cells, HSCs upregulate *α-sma* transcription as they become activated during tissue injury.[8,9] As predicted, injury-related activation of α-sma *cis*-acting elements in HSCs induced HSC expression of the human Rac1 transgene. Consistent with abundant evidence that Rac1 activity is required for NADPH oxidase–induced oxidant production in other myofibroblastic cells,[34,35] as well as emerging data that the same enzyme complex is required for HSC activation,[8,9] our work with cultured Rac-transgenic HSCs provides the first direct evidence that activation of HSCs to a myofibroblastic phenotype is enhanced and perpetuated by increasing Rac activity.

The latter point merits emphasis because it refutes recent speculation that HSC activation triggers mechanisms that result in apoptosis of activated cells.[42,43] Rather, evidence that sustained Rac activity in HSCs amplifies their activation and growth in culture, and augments myofibroblastic cell accumulation and fibrosis in injured livers, suggests that oxidants typically generate survival signals in activated HSCs. Similar responses are known to occur in vascular smooth muscle cells.[41] Therefore, injury-related induction of Rac1 appears to initiate a conserved, positive feedback mechanism through which oxidant generation in myofibroblastic cells enhances their survival, thereby perpetuating fibrogenic responses during wound healing.

Our studies did not address which tissue factors might trigger endogenous Rac1 activity in HSCs in injured livers. It is tempting to speculate that PDGF is involved. As mentioned earlier, PDGF is known to induce several Rac-dependent functions in fibroblasts and vascular smooth muscle cells, including oxidant generation, proliferation, and migration.[41,44,45] HSCs express PDGF receptors and, like other myofibroblastic cells, PDGF-treated HSCs exhibit increased oxidant generation, proliferation, and migration.[9,46,47] Rac is required for PDGF to induce HSC migration, suggesting that Rac1 mediates PDGF effects in HSCs, as it does in other myofibroblastic cells.[47] Indeed, the importance of PDGF receptor signaling in the activation of HSCs and evolution of cirrhosis has been the subject of several reviews.[1,5] Consistent with this concept, hepatic expression of PDGF and its receptor are increased in liver injury,[46,48] and PDGF-C transgenic mice with liver-specific overexpression of PDGF spontaneously develop hepatic fibrosis with age.[49]

Although much of the attention on PDGF and the Rac target, NADPH oxidase, has focused on their roles in liver fibrosis, published data suggest that these factors may also promote liver injury. For example, Adachi et al.[9] recently reported that treating HSCs with PDGF to activate NADPH oxidase increases ROS production. Consistent with this observation, mice that are genetically deficient in NADPH oxidase are protected from oxidant-mediated liver injury, including ethanol-induced fatty liver disease.[50] These results raise the intriguing possibility that, in addition to their acknowledged role in hepatic fibrosis, activated HSCs might contribute significantly to progression of liver injury. This concept is supported by recent findings in PDGF-C transgenic mice. In such animals, hepatocyte-specific overexpression of PDGF-C results not only in fibrosis, but also in spontaneous hepatic steatosis and hepatocellular carcinoma.[49]

The present studies complement and extend that work by demonstrating that increasing Rac1 activity in HSCs enhances both HSC acquisition of the myofibroblastic phenotype and susceptibility to toxin-induced liver injury. In Rac-transgenic mice, the transgene for constitutively active Rac1 is expressed exclusively in myofibroblastic cells that transcribe α-SMA. In healthy livers, α-SMA expression is limited to smooth muscle cells in the walls of hepatic arterioles,[51] and RNA analysis of Rac-transgenic livers demonstrated that this was not sufficient to drive appreciable hepatic expression of the transgene.[11] However, cells that express α-SMA are known to accumulate in the parenchyma of injured livers,[12,52] and we found that the severity of $CCl_4$-induced injury, as well as the extent of liver fibrosis, tightly paralleled the accumulation of α-SMA–expressing cells, suggesting that activated myofibroblastic cells (with increased Rac-dependent NADPH oxidase activity and excessive ROS production) play a role in the progression of liver damage in this model. Although we cannot absolutely exclude a role for increased Rac transgene expression in other cells that express α-SMA, this interpretation provides a plausible explanation for earlier observations in WT mice that linked the level of hepatic oxidant stress and the extent and progression of HSC activation during $CCl_4$-induced liver injury.[53]

In conclusion, because sustained activation of Rac1 perpetuates the myofibroblastic phenotype of HSCs and

thereby exacerbates liver injury and fibrosis, we speculate that Rac1 is a potential therapeutic target in various types of chronic liver disease. In this regard, it is intriguing that several clinically useful, statin-type drugs that prevent prenylation of Rho family proteins have been shown to block PDGF-induced migration of vascular smooth muscle cells, a Rac-dependent process.[54,55] Furthermore, simvastatin can reverse angiotensin II–induced increases in intracellular $H_2O_2$ production by inhibiting Rac1 in mesangial cells of the kidney.[56] Further research to explore the efficacy of these and other Rac inhibitors in liver disease is justified and offers the exciting promise of novel, broad-spectrum therapies that might prevent (and reverse) cirrhosis, regardless of etiology.

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