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Activation of Rac1 promotes Hedgehog-mediated acquisition of the myofibroblastic phenotype in rat and human hepatic stellate cells

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Abstract

Hepatic accumulation of myofibroblastic hepatic stellate cells (MF-HSC) is pivotal in the pathogenesis of cirrhosis. Two events are necessary for MF-HSC to accumulate in damaged livers: transition of resident, quiescent HSC (Q-HSC) to MF-HSC, and expansion of MF-HSC numbers via increased proliferation and/or reduced apoptosis. Our group has identified two novel mediators of MF-HSC accumulation: Rac1 and Hedgehog (Hh). It is unclear if Rac1 and Hh interact to regulate the accumulation of MF-HSC. We evaluated the *hypothesis* that Rac1 promotes the activation of the Hh pathway, thereby stimulating signals that promote transition of Q-HSC into MF-HSC, and that enhance the viability of MF-HSC. Using both *in vitro* and *in vivo* model systems, Rac1 activity was manipulated via adenoviral vector-mediated delivery of constitutively active or dominant-negative Rac1. Rac1-transgenic mice with targeted myofibroblast expression of a mutated human rac1 transgene that produces constitutively active Rac1 were also examined. Results in all models demonstrated that activating Rac1 in HSC enhanced Hh signaling, promoted acquisition/maintenance of the MF-HSC phenotype, increased MF-HSC viability, and exacerbated fibrogenesis. Conversely, inhibiting Rac1 with dominant-negative Rac1 reversed these effects in all systems examined. Pharmacologic manipulation of Hh signaling demonstrated that pro-fibrogenic actions of Rac1 were mediated by its ability to activate Hh pathway-dependent mechanisms that stimulated myofibroblastic transition of HSC and enhanced MF-HSC viability. *In conclusion*, these findings demonstrate that interactions between Rac1 and the Hh pathway control the size of MF-HSC populations and have important implications for the pathogenesis of cirrhosis.

Keywords

adenovirus; cyclophosphamide; epithelial-to-mesenchymal transition; fibrosis

Chronic liver injury induces regenerative responses in the liver, including the activation of quiescent hepatic stellate cells (Q-HSC) to an activated, myofibroblastic phenotype that promotes scar formation and liver dysfunction.(1) Hepatic accumulation of myofibroblastic hepatic stellate cells (MF-HSC) is pivotal in the pathogenesis of cirrhosis. Therefore, considerable research has been directed towards delineating mechanisms that promote the transition of Q-HSC to MF-HSC, and that enhance the subsequent growth of MF-HSC populations.(2) Recently, we showed that Rac1 is one factor that modulates hepatic accumulation of MF-HSC.

Rac1 belongs to a sub-family of small GTP-binding proteins that includes Ras-related C3 botulinum toxin substrate 1 (rac1), rac2, rac3, and several rac-related proteins.(3) Rac1 regulates many cellular processes, including the cell cycle, cell-cell adhesion, and motility. (3-6) It also maintains epidermal stem cells that generate epithelial tissues.(7) We demonstrated that Rac1 is activated as Q-HSC become MF-HSC.(8) Using a genetic approach to maintain high levels of Rac1 activity in MF-HSC, we showed that this enhanced MF-HSC growth in culture. We also demonstrated that transgenic mice expressing constitutively active human Rac1 in MF-HSC accumulated more myofibroblastic cells and developed worse liver fibrosis than littermates during liver injury.(8) These data established activated Rac1 as an important mediator of MF-HSC accumulation and fibrosis.

The Hedgehog (Hh) pathway also modulates MF-HSC accumulation and liver fibrosis. (9,10) Hh ligands are lipid-modified morphogens that interact with Patched (Ptc), a membrane-spanning receptor on Hh-responsive cells. This ligand-receptor interaction prevents Ptc from inhibiting its co-receptor, Smoothened (Smo). Smo, in turn, initiates a series of intracellular events that culminate in activation and nuclear localization of Glioblastoma (Gli)-family transcription factors.(11) Hh signaling is antagonized by hedgehog-interacting protein (Hhip), a factor that binds to Hh ligands and blocks Hh ligand-Ptc interactions.(12) We have shown that Hh signaling is involved in adult liver repair. (10,13) Factors produced during liver injury stimulate MF-HSC to produce Sonic hedgehog (Shh) ligand.(9) Shh acts in an autocrine fashion to promote MF-HSC proliferation and viability,(9) and in a paracrine fashion to stimulate the viability of ductular-type progenitor cells (DPC).(13) Genetically-altered mice with an overly active Hh pathway accumulate more MF-HSC and develop worse liver fibrosis after bile duct ligation (BDL) than littermate controls.(13) Whether Hh signaling interacts with, or requires, Rac1 to increase MF-HSC remains unclear. Therefore, the primary objective of the current study was to evaluate the hypothesis that Rac1 and the Hh pathway interact to promote the formation of MF-HSC and/or to enhance accumulation of existent MF-HSC. A secondary aim was to begin to characterize mechanisms involved in these processes.

During development, adult wound healing, and cancer metastasis, the Hh pathway is known to promote formation of mesenchymal cell types by inducing epithelial-to-mesenchymal transitions (EMT).(14,15) EMT is a process that permits tissue remodeling by repressing expression of adherens junction-proteins (*e.g.*, E-cadherin), reducing cell-cell adhesion, and promoting cell motility.(16) EMT may contribute to liver fibrosis.(17) It was recently reported that treating MF-HSC with adenoviral vectors for bone morphogenetic protein (BMP)-7 dramatically down-regulated expression of fibroblast markers, and that injection of BMP-7 adenoviral vectors into rats with advanced thioacetamide-induced cirrhosis abolished liver fibrosis.(18) BMP-7 is a potent inhibitor of EMT, and epithelial cells that are

capable of transitioning to mesenchymal cells typically express BMP-7 to repress mesenchymal gene expression and maintain expression of E-cadherin and other epithelial genes.(19) Overexpressing BMP-7 in hepatocytes was shown to have no effect on hepatocyte E-cadherin expression, suggesting that the beneficial effects of BMP-7 on liver fibrosis were not due to inhibition of hepatocyte EMT.(18) However, the possibility that BMP-7 might be inhibiting EMT in some other type of liver cell was not examined.

Previously, we proved that Shh induces ductular-type progenitor cells to undergo EMT.(20) We also showed that freshly-isolated Q-HSC express biliary epithelial markers, including E-cadherin, and the Hh antagonist, Hhip, and demonstrated that Q-HSC down-regulate Hhip, activate Hh signaling, lose epithelial markers, and express mesenchymal genes as they become MF-HSC. Blocking Hh signaling by pharmacologic inhibition repressed mesenchymal gene expression, while restoring expression of epithelial genes and other Q-HSC markers.(21) In the current study, we show that manipulating Rac1 in rat primary HSC, clonally-derived rat and human MF-HSC lines, and two different animal models of liver fibrosis consistently modulates Hh signaling, EMT, accumulation of MF-HSC, and fibrogenesis. These findings prove that Rac1 and Hh signaling are crucial mediators of HSC activation, confirm our hypothesis that these factors interact to induce and maintain the myofibroblastic HSC phenotype, identify key Hh pathway regulators as targets of activated Rac1, and suggest a novel mechanism for liver fibrosis. The latter involves activated Rac1-dependent induction of Shh and concomitant repression of Hhip, resultant activation of Hh signaling, and consequent transition of Q-HSC to MF-HSC, as well as enhanced MF-HSC viability. Together, these events cause accumulation of MF-HSC and increase fibrogenesis.

Methods

Animal care

Adult, male Sprague-Dawley rats were from Charles River Laboratories (Wilmington, MA). Wild-type (Wt), C57Bl6 mice were from Jackson Labs (Bar Harbor, ME). Rac-transgenic mice that express a constitutively active mutant form of human rac1 (V12rac1) in myofibroblasts were described previously.(22) Animal experiments fulfilled NIH and Duke University-IACUC requirements for humane animal care.

Plasmids

The expression vectors pEXV-V12rac1 and pEXV-N17rac1, encoding the constitutively-active and dominant-negative myc epitope-tagged rac1 cDNAs respectively, were previously described.(6)

Adenoviral vectors

Adenovirus N17rac1 containing the dominant-negative rac1 cDNA was constructed using adenovirus-based plasmid JM17 and the expression vector pEXV-N17rac1 which encodes the dominant-negative myc-epitope-tagged rac1 cDNA.(22) A similar strategy was used to construct an adenovirus encoding a constitutively-active mutant of rac1 (V12rac1) (P. Goldschmidt-Clermont, U. of Miami, FL).(22) The E1-deleted adenovirus dl312,(22) which lacks a cDNA insert, served as a control for all gene transfer studies.

Rac1 manipulation in intact mice

Adult C57Bl6 mice (n=36) received vehicle (100μL) that contained empty adenoviral vectors (dl312) or adenoviral vectors bearing a constitutively active rac1 (V12rac1) or dominant-negative rac1 (N17rac1). Mice then underwent BDL (n=24) (23,24) or sham surgery (n=12). Mice were monitored until sacrifice 10 days post-BDL. Ten-day survival of

the 16 mice that received dl312 or V12rac1 was 75% in each group, whereas none of the 8 mice treated with N17rac1 survived more than 4 days post-BDL.

Rac1-transgenic mice ($n=11$) and age-/gender-matched Wt littermates ($n=6$) received twice-weekly intraperitoneal injections with carbon tetrachloride (CCl_4 , 0.5 mg/kg, Sigma-Aldrich, St. Louis, MO) and were sacrificed after 8 weeks.(8)

Following euthanasia, livers were harvested, snap frozen in liquid nitrogen or fixed in formalin and paraffin-embedded.(8)

Cell isolation and culture

Primary HSC were isolated from Sprague-Dawley rats, assessed for purity and viability, and seeded at a density of 3×10^2 cells/ mm^2 in DMEM supplemented with fetal bovine serum (FBS) and penicillin/streptomycin.(9,21)

The clonally-derived rat HSC line, 8B (M. Rojkind, George Washington University, Washington, DC), human HSC line, LX-2 (S.L. Friedman, Mount Sinai School of Medicine, New York, NY), normal rat cholangiocyte line, NRC (N.F. LaRusso, Mayo Clinic, Rochester, MN), and primary rat hepatocytes were cultured as described.(25-28)

Adenoviral transduction of HSC

HSC (primary, 8B and LX-2) were grown to 70-90% confluency in 6-well plates and serum-starved for 4h prior to infection. Pilot studies demonstrated that maximally efficient transduction occurred at a multiplicity of infection (MOI) of 50. Subsequent experiments were carried out with this MOI of 50 for 24h, then virus-containing media was aspirated and replaced with fresh medium.

Pharmacological manipulation of Hh signaling

MF-HSC (8B) were treated with SAG, a chlorobenzothiophene-containing Smoothed agonist (0.3 μM , Axxora, San Diego, CA),(29) (cyclopamine, 5 μM ; Toronto Research Chemicals, Toronto, ON, Canada), or a catalytically inactive analog (tomatidine, 5 μM) for 24-48h.

Cell migration

MF-HSC (8B) were cultured for 7-days; a standard wound-healing assay was performed after treatment with adenoviral vectors with/without Smo agonist/antagonist.(20,29)

Cell viability assays

Cell viability was measured with the Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD).(30)

mRNA quantification by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA), reverse-transcribed using Superscript reverse transcriptase (Invitrogen). cDNA samples were used for quantitative RT-PCR (qRT-CPR) using iQ-SYBR Green Supermix (Bio-Rad Laboratories).(21)

Rac1 activation assay and Western blotting

Rac1 activation assay was performed.(8,31) Western blots were used to demonstrate changes in relevant proteins; results were normalized to β -actin expression.(21)

Quantification of hepatic collagen content

This was evaluated by morphometry of Sirius red-stained liver sections and hydroxyproline assay.(8)

Statistical analysis

Results are expressed as means $\pm$ SEM. Comparisons between groups were performed using the non-parametric Wilcoxon-Rank-Sums test using SAS version 9.1 software (SAS Institute, Cary, NC). *P*-values are two-tailed; significance was accepted at the 5% level.

Results

Rac1 activity increases during HSC activation in culture and promotes acquisition and retention of the myofibroblastic phenotype

Rat primary HSC were cultured for up to 7 days; affinity purification was performed to identify GTP-bound Rac1, the biologically-active form of Rac1.(6) Although active Rac1 was not identified in freshly-isolated Q-HSC, it increased steadily during culture (Fig. 1A). Rac1 activity was not detectable in normal rat cholangiocytes or hepatocytes, proving that Rac1 activity is a unique characteristic of myofibroblastic liver cells (Fig 1B).

To determine if Rac1 activation played a causal role in myofibroblastic transition, 7-day culture-activated MF-HSC were treated with adenoviral vectors encoding either constitutively-active rac1 (V12rac1), dominant-negative rac1 (N17rac1), or empty cassette (dl312). Preliminary experiments confirmed that V12rac1 increased, and N17rac1 decreased, Rac1 activity (Fig. 1C). V12rac1-treated HSC demonstrated significantly higher expression of the myofibroblastic markers, α SMA and type 1 collagen (Col1 α 1), while treatment with N17rac1 significantly lowered levels of these genes (Fig. 1D-E). Clonally-derived rat (8B) and human (LX-2) HSC lines responded similarly to infection with these adenoviral vectors (Supplemental Figs. 1, 4), assuring that the findings in the primary HSC cultures were not an idiosyncratic response of rodent HSC, or caused by selective out-growth of rare, contaminating cell types. Thus, Rac1 activation not only occurs as Q-HSC transition to MF-HSC, but it actively contributes to acquisition and maintenance of the myofibroblastic phenotype in both rat and human HSC.

Rac1 induces myofibroblastic phenotype by promoting EMT

Transition of Q-HSC to MF-HSC involves an EMT-like process.(21) Therefore, we treated rat primary HSC cultures with adenoviral vectors for V12rac1, N17rac1, or dl312, and determined if modulating Rac1 activity influenced expression of various factors that are involved in EMT. Compared to dl312-treated HSC, V12rac1-treated HSC exhibited greater changes in EMT-related genes. For example, the EMT inhibitor, BMP-7, was significantly down-regulated, as was expression of desmoplakin (epithelial gene). Conversely, expression of snail (an EMT inducer) and S100A4 (a marker of mesenchymal cells of epithelial origin) was enhanced (Fig. 2A). In contrast, treating HSC with N17rac1 to repress Rac1 activity exerted the opposite effects, up-regulating BMP-7 and desmoplakin while down-regulating snail and S100A4 (Fig. 2B). Similar results were noted when 8B and LX-2 were treated with these vectors (Supplemental Figs. 2,4). Because cells that have undergone “complete” EMT acquire a migratory phenotype,(16) we treated clonally-derived rat MF-HSC (8B) with V12rac1, N17rac1, or dl312, and used a standard wound-healing assay to determine if altering Rac1 activity influenced cell migration.(20) Compared to treatment with dl312, V12rac1-treated cells exhibited enhanced migration into the wound, while N17rac1-treated cells showed inhibited cell migration (Fig. 3). Hence, Rac1 activation causes HSC from adult livers to complete an EMT-like process that leads them to acquire a migratory, mesenchymal phenotype.

Rac1 promotes EMT and survival in HSC by enhancing Hh pathway activity

In many cell types, including adult liver HSC, EMT is regulated by the Hh pathway.(20,21) Our data proved that Rac1 activation induced EMT in HSC, suggesting potential interactions between Rac1 and the Hh pathway. To address this more directly, HSC were treated with V12rac1, N17rac1, or dl312, and effects on Hh signaling were examined by qRT-PCR and Western blot. Compared to dl312-treated HSC, HSC treated with V12rac1 demonstrated enhanced Hh signaling, with significant down-regulation of the Hh inhibitor, Hhip, and increased expression of Shh (Hh ligand), Gli2 and sFRP1 (both Hh target genes) (Fig. 4A, C). Conversely, blocking Rac1 activity with N17rac1 increased Hhip expression and decreased expression of Shh and Gli2, reducing Hh signaling and expression of the Gli-regulated gene, sFRP1 (Fig. 4B, C). Similar effects on the Hh pathway were noted when Rac1 activity was manipulated in the clonally-derived 8B and LX-2 lines (Supplemental Figs. 3,5). Thus, Rac1 activity represses HSC expression of Hhip, and increases HSC expression of Shh ligand, leading to activation of Hh signaling that results in increased expression of Hh target genes (Gli2). Gli-family transcription factors promote the transcription of other factors, such as snail, that orchestrate complex, global changes in cellular gene expression to affect EMT.(32) To verify that Hh signaling-regulated events were causally involved in HSC EMT, a wound-healing assay was performed with clonally-derived rat HSC (8B) treated with cyclopamine, a specific antagonist of Hh signaling, or tomatidine, an inactive cyclopamine analog.(30) Cyclopamine (but not tomatidine) decreased HSC migration (Fig. 5A), confirming that Hh signaling is necessary for HSC to undergo complete EMT in culture. Furthermore, adding a Smoothed agonist (SAG) restored the migratory capabilities and changes in gene/protein expression of MF-HSC that had been treated with N17rac1, while blocking Hh signaling inhibited migration and normalized gene/protein expression in V12rac1-treated MF-HSC (Fig. 5B, Supplemental Fig. 6). More protracted treatment of MF-HSC with either N17rac1 or cyclopamine reduced cell survival compared to respective controls (Fig. 5C). Therefore, Rac1 activation initiates a cascade of events, which result in induction of Gli transcription factors, snail, and other factors that cause the cells to acquire a mesenchymal, migratory phenotype and remain viable *in vitro*.

Rac1 promotes fibrosis in a bile duct ligation (BDL) model of fibrosis

To determine if a similar Rac1-regulated process occurs when injury provokes Q-HSC to become MF-HSC in intact animals, C57Bl6 mice were pre-treated with dl312, V12rac1, or N17rac1 by portal venous injection, and then subjected to BDL or sham surgery. Surviving mice were sacrificed after 10 days. Compared to sham surgery, BDL caused liver injury and fibrosis in dl312-treated mice (data not shown). No mice treated with N17rac1 survived beyond 4 days post-BDL, so the effects of inhibiting Rac1 activity could not be analyzed 10-day post-BDL. However, compared to BDL-mice pre-treated with dl312, V12rac1-treated BDL-mice demonstrated significantly greater mesenchymal gene expression (α SMA, Coll α 1 and S100A4), as well as decreased expression of the EMT inhibitor, BMP-7 (Fig. 6A). V12rac1-treated BDL-mice also demonstrated greater Hh pathway activity than dl312-treated BDL-mice (Fig. 6B-C). Consistent with these findings, BDL-mice pre-treated with V12rac1 demonstrated greater liver fibrosis, as evidenced both by Sirius red staining of liver sections (Fig. 7A-C) and quantification of hepatic hydroxyproline content (Fig. 7D).

Overexpression of Rac1 enhances expression of markers of EMT and Hh pathway activity in CCl₄-induced liver injury

Although rac1 transgene expression was verified in primary HSC that were isolated from rats that received portal vein injections of rac1 transgene-bearing adenoviral vectors (Fig. 8A) and results of the *in vivo* study recapitulated our findings in cultured primary HSC and clonal HSC lines, the outcomes observed after BDL might have been unique to this type of

liver injury and/or mediated by other liver cell types that were also transduced during the process. Therefore, responses to another type of chronic liver injury were examined in another animal model in which over-activation of Rac1 was targeted to α -sma-expressing cells (*i.e.*, myofibroblasts). In such rac1-transgenic mice, regulatory elements of the α -sma gene control expression of a human rac1 transgene that produces constitutively-active Rac1 protein, resulting in accumulation of activated Rac1 in α -sma-expressing cells.(8) We reported that rac1-transgenic mice developed greater liver fibrosis after 8 weeks of CCl₄-induced liver injury.(8) Analysis of livers from CCl₄-treated rac1-transgenic mice demonstrated that selectively increasing Rac1 activity in α -sma-expressing cells exacerbated injury-related increases in both Hh signaling and expression of various mesenchymal genes (*e.g.*, α SMA, Col1 α 1 and S100A4), while it decreased expression of BMP-7 (Fig. 8B, D). Thus, results from studies that used two different approaches to activate Rac1 in HSC in intact animals complement data that were generated by gain-/loss- of function studies in cultured primary HSC and clonal HSC lines, providing compelling evidence that Rac1 activation is critically involved in both generation and maintenance of MF-HSC.

Discussion

Hepatic accumulation of MF-HSC is pivotal in the pathogenesis of cirrhosis. Two events are necessary for MF-HSC to accumulate in damaged livers: transition of resident Q-HSC to MF-HSC, and expansion of MF-HSC numbers via increased proliferation and/or reduced apoptosis. Recently, we demonstrated that the first event (transition to MF-HSC) involves a process that has classical features of EMT, and showed that HSC EMT is regulated by Hh signaling.(21) Previously, we demonstrated that Hh ligands enhance the viability of MF-HSC(9) and reported that both Rac1(8) and Hh(9) promote proliferation of MF-HSC. However, it was unclear if Rac1 and Hh interacted to regulate the accumulation of MF-HSC after liver damage. Therefore, in the current study we evaluated the *hypothesis* that Rac1 promotes the activation of the Hh pathway, thereby stimulating signals that promote EMT in Q-HSC, and that enhance the viability of MF-HSC. Using both *in vitro* and *in vivo* systems, Rac1 activity was manipulated via adenoviral vector-mediated delivery of constitutively active or dominant-negative Rac1. Parallel studies were done with adenoviral vectors bearing an empty cassette to control for non-specific effects of the adenovirus. Selected studies were repeated in transgenic mice in which over-activation of Rac1 was restricted to myofibroblastic cells. Results in all models demonstrated that increasing Rac1 activity enhanced Hh signaling, EMT, and fibrogenesis. Conversely, Rac1 inhibition reversed all of these effects in each model system examined. These findings extend existing knowledge about mechanisms that expand populations of MF-HSC in injured livers, and prove that activated Rac1 and Hh are critical mediators of this process.

Many other factors are also known to play important roles in controlling the hepatic content of MF-HSC. These include soluble growth factors, cytokines, chemokines and their respective receptors, as well as pattern recognition receptors and their ligands.(33) It is still unclear, however, whether or not there is a hierarchy of importance among these various mediators, or to what extent different factors might interact to modulate conserved signaling that results in the formation and/or growth of MF-HSC. Previously published data demonstrates interactions between several of these factors and the Hh pathway. For example, TGF- β (10), PDGF(9), and leptin (*unpublished*) have been shown to induce different types of liver cells, including HSC, to produce Hh ligands. TGF- β treatment activates the Hh pathway in cultured human A549 cells (which are used to interrogate EMT regulation)(34) (Supplemental Fig. 7); if/how TGF- β /Rac1/Hh interact to promote EMT has not yet been examined in HSC. On the other hand, it is clear that the mitogenic actions of PDGF on MF-HSC require Hh pathway activity because blocking Hh signaling prevents PDGF from increasing proliferation of MF-HSC.(9) Similarly, leptin-mediated induction of

myofibroblastic genes is blocked by inhibiting Hh signaling in HSC, demonstrating that Hh pathway activity is also necessary for leptin to exert its fibrogenic effects (*unpublished*). Interestingly, although primary HSC from *fa/fa* rats (which have an inherited defect in the long form of the leptin receptor) are unresponsive to leptin-mediated fibrogenesis, they retain Hh pathway activity and remain capable of transitioning into MF-HSC when cultured. This observation suggests that leptin, like PDGF and TGF- β , operates “up-stream” of the Hh pathway and raises the possibility HSC may require a functional Hh pathway in order to respond optimally to other pro-fibrogenic factors. This concept is supported by evidence that Hh pathway inhibition generally abrogates culture-induced transition of Q-HSC into MF-HSC, and causes culture-activated MF-HSC to re-acquire a more quiescent (less myofibroblastic) phenotype.(21) Similarly, treating cultured MF-HSC with antibodies that neutralize endogenously-produced Hh ligands dramatically reduces their viability, demonstrating that Hh ligands are autocrine viability factors for MF-HSC.(9) Hh ligands from MF-HSC also act in a paracrine fashion to stimulate resident liver cells to produce factors that recruit HSC (*e.g.*, MCP-1) and/or exert pro-fibrogenic actions on HSC (*e.g.*, IL-4 and IL-13).(35,36) Thus, the Hh pathway operates at multiple levels to promote the formation of MF-HSC from Q-HSC and to enhance MF-HSC accumulation.

The current study proves that Rac1 controls Hh signaling in HSC and demonstrates that this occurs, in part, because Rac1 activation differentially regulates HSC production of Shh ligand and its inhibitor, Hhip. Rac1 activation induces Shh production while inhibiting expression of Hhip, skewing the ligand/inhibitor balance to favor activation of Hh signaling and consequent induction of Hh-mediated processes, including HSC survival and EMT. Fibrogenic stimuli, such as culture in serum-supplemented medium, are known to activate Rac1 in HSC.(8) The present study proves that blocking Rac1 activity inhibits activation of the Hh pathway, survival, and EMT in HSC that are exposed to fibrogenic stimuli. Thus, it is reasonable to conclude that Rac1 functions as a common intermediate that permits pro-fibrogenic stimuli to promote activation of the Hh pathway in HSC.

Additional research is needed to delineate the mechanisms by which Rac1 activation alters HSC expression of Shh and Hhip. Rac1 regulates diverse cellular processes, including the cell cycle, cell-cell adhesion, and motility.(3) However, to our knowledge, it has not yet been determined if these various outcomes are mediated by conserved Rac1-initiated signals. One function of Rac1 is to direct assembly of the NADPH oxidase enzyme complex, which is an important source of reactive oxygen species (ROS) in many types of cells.(4) NADPH oxidase activity increases as HSC become myofibroblastic and inhibiting this enzyme also inhibits HSC activation, both in culture and in intact animal models of liver fibrosis.(37) The concept that the pro-fibrogenic effects of NADPH oxidase are mediated by ROS is further supported by evidence that antioxidants inhibit accumulation of MF-HSC.(38) Bone marrow chimeric mice were generated to prove that MF-HSC were the most pathophysiologically-relevant cellular source of NADPH oxidase-produced ROS in animal models of liver fibrosis.(39) Given that Rac1 mediates NADPH oxidase assembly, their findings support our conclusion that it is the activation of Rac1 in HSC (rather than some other type of liver cell) that drives MF-HSC accumulation and liver fibrogenesis after BDL or CCl₄-induced liver injury.

Our literature search failed to identify any publications that link NADPH oxidase and the Hh pathway. However, transcription of Shh is known to be regulated by the redox-sensitive transcription factor, NF- κ B.(40) Hence, it is conceivable that Rac1 might increase Shh expression in HSC by activating NADPH oxidase, with resultant increase in ROS, activation of redox-sensitive transcription factors, and increased Shh transcription. Presumably, Hhip expression would be concomitantly repressed in a redox-sensitive manner since Shh and Hhip are reciprocally regulated by Rac1 activation. Preliminary data do not support this

concept, however, because antioxidants failed to attenuate the ability of V12rac1 to induce Shh in cultured HSC (data not shown). Therefore, alternative mechanisms merit consideration since PDGF induces HSC expression of Shh via mechanisms that are dependent upon activation of PI-3-kinase/AKT.(9) Considerable additional work will be required in order to delineate the precise molecular events that link Rac1 activation to altered expression of Hh pathway regulators, and to evaluate whether these mechanisms are important for stellate cell activation in extra-hepatic tissues. Although these efforts extend beyond the scope of this publication, the present studies are important because they identified a previously unsuspected relationship between Rac1 and the Hh pathway in adult mammalian cells, thereby opening entirely novel lines of research that are likely to have general relevance to cellular biology and to liver fibrosis.

In conclusion, targeted manipulation of Rac1 activity in HSC proves that activation of Rac1 is required for Q-HSC to become, and remain, myofibroblastic. Rac1 may promote the myofibroblastic phenotype in HSC via several mechanisms. Our results, however, demonstrate that key pro-fibrogenic actions of Rac1 are mediated by its ability to activate the Hh pathway, with resultant Hh-dependent enhancement in cell viability and transition to a myofibroblastic phenotype via an EMT-like process. The findings also identify two of the early events in this process, namely Rac1 activation-dependent induction of Hh ligand production and coincident repression of the Hh ligand antagonist, Hhip. These discoveries have important implications for the pathogenesis of cirrhosis, because the latter results in large part from hepatic MF-HSC accumulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

αSMA	smooth muscle α -actin
BDL	bile duct ligation
BMP-7	bone morphogenetic protein-7
Col1α1	type I α 1 collagen
CCl₄	carbon tetrachloride
DMEM	Dulbecco's modified Eagle's medium
EMT	epithelial-to-mesenchymal transition
FBS	fetal bovine serum
Hh	hedgehog
Hhip	hedgehog-interacting protein
HSC	hepatic stellate cells
MF-HSC	myofibroblastic hepatic stellate cells

MOI	multiplicity of infection
Q-HSC	quiescent hepatic stellate cells
rac1	Ras-related C3 botulinum toxin substrate 1
RT-PCR	reverse transcription-polymerase chain reaction
Shh	sonic hedgehog

Figure 1. Rac1 activation increases during hepatic stellate cell activation and promotes myofibroblastic gene expression

Primary rat hepatic stellate cells (HSC) were isolated and cultured in serum-containing media. A) Affinity purification was performed to demonstrate endogenous, active (GTP-bound) Rac1. Immunoblot results are representative of data from triplicate experiments. B) Affinity purification of active Rac1 in primary rat hepatocytes, normal rat cholangiocytes (NRC), and HSC. C-D) Primary rat MF-HSC were treated with adenoviral vectors containing constitutively active Rac1 (V12rac1), dominant negative Rac1 (N17rac1), or “empty” adenoviral vector (dl312). Affinity purification was performed (C) and RNA was isolated after 72 h of infection (D). Gene expression was assessed by QRT-PCR. Mean $\pm$ SEM results of triplicate experiments are graphed. * P <0.05, † P <0.005 vs dl312-treated group. E) Representative Western blot analysis of parallel cultures analyzed in (D). β -actin was assessed to control for variations in protein loading.

Figure 2. Increasing Rac1 activity enhances gene expression changes associated with epithelial-to-mesenchymal transitions in primary rat HSC

Primary rat HSC were isolated and cultured in serum-containing media for 7 days to produce a myofibroblastic phenotype. Triplicate plates of day 7 culture-activated MF-HSC were then treated with adenoviral vectors for either A) V12rac1 or B) N17rac1. To control for nonspecific effects of adenoviral infection, triplicate plates were treated in parallel with dl312; RNA was isolated after 72 h of infection; gene expression was assessed by QRT-PCR. Each experiment was done a total of 3 times and mean $\pm$ SEM data are graphed.

* $P < 0.05$, † $P < 0.005$ vs dl312-treated cultures.

Figure 3. Rac1 induces myofibroblastic phenotype by promoting epithelial-to-mesenchymal transitions (EMT)

Triplicate cultures of the clonally-derived rat HSC line, 8B, were treated with adenoviral vectors containing constitutively active Rac1 (V12rac1), dominant-negative Rac1 (N17rac1) or empty adenoviral vector (dl312); a standard wound healing assay was done and 24h later, cell migration into the wound was examined. Representative results are shown.

Figure 4. Increasing Rac1 activity promotes Hedgehog pathway signaling in primary rat HSC
 Primary rat HSC were cultured for 7 days to generate MF-HSC. Triplicate day 7 cultures were treated with adenoviral vectors for either A) V12rac1 or B) N17rac1 as described in Figure 2; RNA was isolated for QRT-PCR. Results are compared to triplicate cultures of MF-HSC that were treated with dl312. Each experiment was done a total of 3 times; mean $\pm$ SEM data are graphed. * $P < 0.05$, † $P < 0.005$ vs. dl312-treated cultures. C) Representative Western blot analysis of cultures described in (A, B). β -actin was used as a protein loading control.

Figure 5. Hh pathway manipulation influences cell migration and inhibition reduces cell survival

A) Triplicate cultures of the clonally-derived rat HSC line, 8B were treated with either the Hh pathway inhibitor, cyclopamine (5 μ M) or its biologically-inert analog, tomatidine (5 μ M), for 24h; cell migration was assessed using a standard wound healing assay. Representative results are shown. B) Triplicate cultures of 8B were treated with adenoviral vectors containing constitutively-active Rac1 (V12rac1) or dominant-negative Rac1 (N17rac1) and then treated with vehicle or SAG (Smo agonist) or tomatidine or cyclopamine respectively; a standard wound healing assay was done and 24h later, cell migration into the wound was examined. Representative results are shown. C) Triplicate cultures of MF-HSC 8B were treated for 48h with either N17rac1 (to inhibit Rac1 activity) or cyclopamine (to block Hh signaling); cell viability was assessed by CCK8 assay. Experiments were done a minimum of 3 times; mean $\pm$ SEM results are graphed. $^{\dagger}P < 0.005$ vs respective dl312-treated cultures or tomatidine-treated control cultures.

Figure 6. Rac1 enhances Hh signaling and myofibroblastic markers in a bile duct ligation (BDL) model of fibrosis

C57Bl6 mice were pre-treated with adenoviral vectors for dl312 (n = 8 mice) or V12rac1 (n = 8 mice) by portal venous injection; subjected to bile duct ligation (BDL) or sham surgery; and sacrificed after 10 days for liver harvest. Liver RNA and protein were analyzed by qRT-PCR and Western blot. mRNA expression of A) fibrosis and EMT markers, and B) Hh pathway. Mean $\pm$ SEM results are graphed. * P <0.05, † P <0.005 vs. dl312-treated group. C) Representative Western blot analysis. β -actin confirms equal protein loading.

Figure 7. Rac1 promotes fibrosis during BDL

Sirius red staining of liver sections from representative A) dl312-treated control mice and B) V12rac1-treated mice at 10 days post-BDL. C) Quantitative morphometry of Sirius red-stained sections from all 16 mice. D) Hepatic hydroxyproline content in both groups. Mean $\pm$ SEM data are graphed. * P <0.05 vs. dl312-treated control group.

Figure 8. Overexpression of Rac1 in myofibroblastic cells increases hepatic expression of mesenchymal genes

Primary HSC were isolated from animals 2 days after intra-portal injection of adenoviral vectors carrying V12rac1, N17rac1 or dl312. Proteins were isolated and Western blots were used to demonstrate the myc-tagged products of the two Rac transgenes. A) Representative membrane that was probed with myc-tag primary antibody. B) Rac1-transgenic mice (n = 11) were treated with CCl₄ or vehicle (n = 6 mice) for 8 weeks; liver RNA and protein were obtained for qRT-PCR and Western blot analysis. B) mRNA levels of fibrosis and EMT markers and C) Hh pathway. Mean ± SEM data are graphed. **P* < 0.05, †*P* < 0.005 vs. vehicle-treated controls. D) Western blot analysis. β-actin is used as a loading control. Results shown are representative of triplicate immunoblot analyses.