p38α Inhibits Liver Fibrogenesis and Consequent Hepatocarcinogenesis by Curtailing Accumulation of Reactive Oxygen Species

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Abstract

Most hepatocellular carcinomas (HCC) develop in the context of severe liver fibrosis and cirrhosis caused by chronic liver inflammation, which also results in accumulation of reactive oxygen species (ROS). In this study, we examined whether the stress-activated protein kinase p38α (Mapk14) controls ROS metabolism and development of fibrosis and cancer in mice given thioacetamide to induce chronic liver injury. Liver-specific p38α ablation was found to enhance ROS accumulation, which appears to be exerted through the reduced expression of antioxidant protein HSP25 (Hspb1), a mouse homolog of HSP27. Its reexpression in p38α-deficient liver prevents ROS accumulation and thioacetamide-induced fibrosis. p38α deficiency increased expression of SOX2, a marker for cancer stem cells and the liver oncoproteins c-Jun (Jun) and Gankyrin (Psmd10) and led to enhanced thioacetamide-induced hepatocarcinogenesis. The upregulation of SOX2 and c-Jun was prevented by administration of the antioxidant butylated hydroxyanisole. Intriguingly, the risk of human HCC recurrence is positively correlated with ROS accumulation in liver. Thus, p38α and its target HSP25/HSP27 appear to play a conserved and critical hepatoprotective function by curtailing ROS accumulation in liver parenchymal cells engaged in oxidative metabolism of exogenous chemicals. Augmented oxidative stress of liver parenchymal cells may explain the close relationship between liver fibrosis and hepatocarcinogenesis. Cancer Res; 73(1): 1–10. ©2012 AACR.

Introduction

The liver plays an important role in oxidative metabolism and detoxication of endogenous and exogenous chemicals. The most common detoxication mechanism depends on cytochrome p450–mixed function oxidases (1). As a result, extensive and repetitive exposure to toxic chemicals can lead to accumulation of reactive oxygen species (ROS) in hepatocytes that are actively engaged in the detoxication of such chemicals. ROS accumulation can cause liver injury, which often progresses to liver fibrosis, cirrhosis, and cancer.

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and the third leading cause of cancer deaths worldwide and is usually associated with a very poor prognosis (2). In addition to chronic exposure to toxic chemicals, chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) as well as hepatosteatosis are the major risk factors for both liver fibrosis and HCCs (3). In the case of HCV, a virus estimated to infect 4 to 5 million Americans (4), HCC develops only after one or more decades of chronic infection, and elevated risk of HCC progression is restricted largely to patients with cirrhosis or advanced fibrosis (5, 6). Although HCV-infected individuals with mild or nonhepatic fibrosis are unlikely to develop HCCs, once cirrhosis is established, HCC develops at a rate of 1% to 4% per year (6). Thus, the risk of hepatocarcinogenesis depends on background liver factors, of which fibrosis is a major one. Development and progression of liver fibrosis are associated with hepatocyte death and a subsequent inflammatory response (7), both of which involve ROS accumulation in injured hepatocytes (8). Hence, a better understanding of hepatoprotective mechanisms that prevent ROS accumulation and their impact on fibrogenesis and carcinogenesis is of great importance.

The chemical thioacetamide can induce liver cirrhosis and cancer of the bile ducts when given to rats over a period of several months (9). However, as described here, we found that
mice given thioacetamide for 10 months develop HCCs rather than cholangiocellular carcinoma subsequent to appearance of severe liver fibrosis, thus providing a model that closely mimics the natural history of human HCV–related liver disease. In addition, the histology of the thioacetamide-exposed rat liver was reported to resemble human liver cirrhosis (10). Thus, the mouse thioacetamide model may be suitable for studying the relationship between ROS accumulation, liver fibrogenesis, and hepatocarcinogenesis and allows studies to be conducted that are of relevance to human HCV–related liver disease.

Mitogen- and stress-activated protein kinases (MAPK/ SAPK) play a pivotal role in the transduction of extracellular signals to the nucleus, thereby modulating numerous cellular responses, including cell survival, proliferation, differentiation, and metabolism (11, 12). One of the SAPKs, p38α, the major p38/MAPK isoform, is activated in response to inflammation and oxidative stress and, in turn, controls expression of cytokines, inflammatory mediators, survival genes, and antioxidants (13–16). As ubiquitous p38α ablation in all cells results in midgestational lethality, mainly due to placental insufficiency (17–20), we used a conditional p38α "floxed" (p38αfl/fl) strain (21) to generate p38αmice, lacking p38α in liver parenchymal cells, to assess the role of this kinase in development of liver cancer (15). In the course of these studies, we found that p38α prevented the accumulation of ROS in liver parenchymal cells exposed to the hepatic carcinogen diethylnitrosamine. We now describe that p38α also prevents ROS accumulation, liver fibrogenesis, and subsequent hepatocarcinogenesis in mice exposed to thioacetamide. In both models, p38α prevents ROS accumulation by controlling the expression of HSP25, the mouse homolog of human HSP27. Restoration of HSP25 accumulation by controlling the expression of HSP25, the major cytoskeletal kines, in turn, controls expression of inflammatory mediators, survival genes, and antioxidants (13–16).  

Materials and Methods

Animals, tumor induction, and analysis
p38αmice (21) were crossed with Alb-Cre mice (Jackson Lab) to generate p38αmice (15). All mice were maintained in the C57BL/6 background in filter-topped cages on autoclaved or nonautoclaved food at University of California, San Diego (La Jolla, CA) and Kinki University (Osaka, Japan), respectively. Mice were given 0.03% thioacetamide in drinking water. After 10 months on normal chow, mice were sacrificed and analyzed for presence of HCCs. Tumor-occupied areas were measured using ImageJ software.

Biochemical and immunochemical analyses
The e-jun-NH2 kinase (JNK) assays, real-time quantitative PCR (qPCR), immunoblotting, and immunohistochemistry were previously described (15). The primer sequences for TIMP-1, PDGF-B, Sox2 and Gankyrin were: forward primer 5'-CCAGACCCGAGTCGTTTTTC-3', reverse primer 5'-AGAGGACGCTGTAGTCCAG-3', and forward primer 5'-CCTCCGCTGCGAACCGCTAG-3', reverse primer 5'-AGGCTCTGTCACATTGTT-3', forward primer 5'-GACCCCTCATGTA-TGGGT-3', reverse primer 5'-TTGCTGATTCTCGAGGTTGTG-3', respectively. Antibodies used were: anti-HSP27/25 and anti-α-fetoprotein (Santa Cruz Biotechnology); anti-actin (Sigma); anti-α-smooth muscle actin (α-SMA; DukO); anti-p38α, anti-MAPKAPK2, anti-phospho-MAPKAPK2, and anti-Sox2 (Cell Signaling); anti-Pomat1 (CD133, Abnova); anti-c-kit (R&D Systems); and anti-JNK1 (Pharcingen). Immunohistochemistry was conducted using ABC staining kit (Vector Laboratory) according to manufacturer’s recommendations. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done on tissue sections using In Situ Apoptosis Detection Kit (Takara). To examine accumulation of superoxide anions or H2O2, freshly prepared frozen liver sections were incubated with 2 μmol/L dihydroethidine hydrochloride (Invitrogen) or 5 μmol/L 5,5'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen), respectively for 30 minutes at 37°C, after which they were observed by fluorescent microscopy and quantified with Metamorph software. Protein oxidation was assessed by the OxyBlot Protein Oxidation Detection Kit (Millipore). Sirius Red staining was done to quantify the amount of collagen present. To analyze the relative fibrotic area, the Sirius Red–positive areas were measured in 6 random fields (×100) on each slide and quantified using NIH imaging software. Myeloperoxidase (MPO) activity was measured using MPO Activity Assay Kit (Invitrogen). Livers were homogenized in myeloperoxidase buffer (0.5% hexadecyl trimethyl ammonium bromide, 10 mmol/L EDTA, 50 mmol/L Na2HPO4, pH 5.4). Hydroxyproline content was measured as described previously (22).

Adenoviral transduction
Adenovirus-expressing HSP25 was prepared as described previously (15). Adenovirus stocks were injected via the tail vein at 1 x 109 plaque-forming units (PFU) per mouse. Before infection, virus stocks were dialyzed against PBS containing 10% glycerol.

Patients and specimens
HCC tissues and noncancerous liver tissues were obtained from 43 patients, respectively, who had undergone curative hepatectomy for HCC at the Kinki University Hospital between 2004 and 2010. The specimens used were routinely processed, formalin-fixed, and paraffin-embedded. After hematoxylin and eosin (H&E) staining, all samples were diagnosed as HCC. Noncancerous tissue and HCC specimens were frozen and stocked at −80°C. The demographic profiles of the patients are summarized in the Supplementary Table. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Boards. Written informed consents were obtained from all patients for subsequent use of their resected tissues.

Statistical analysis
Data are presented as mean ± SEM. Differences were analyzed by Fisher exact test or Student t test. Recurrence-free survival curves were calculated by the Kaplan–Meier method and analyzed by the log-rank test. P < 0.05 was considered significant.
Results

Enhanced fibrogenesis in p38αΔhep mice

Hepatic stellate cells (HSC) which undergo a transition from a quiescent to an activated state after liver injury play an important part in the pathogenesis of liver fibrosis (23). HSC activation includes increased proliferation rate, a phenotypic transition to a myofibroblast-like α-SMA expression, and a dramatic increase in the synthesis of extracellular matrix proteins. After 8 weeks of thioacetamide treatment, we observed inflammation, HSC activation and formation of fibrotic septa as assessed histologically or by immunohistochemistry with a specific antibody against α-SMA (Fig. 1A). p38αΔhep mice exhibited more thioacetamide-induced liver damage assessed by alanine aminotransferase (ALT) release and hepatocyte apoptosis measured by a TUNEL assay, relative to controls (Fig. 1A and B). Neutrophil infiltration was enhanced, based on measurement of myeloperoxidase activity (Fig. 1C). In addition, there were higher numbers of α-SMA-positive cells, higher levels of hydroxyproline, and larger fibrotic areas in p38αΔhep mice than in control mice (Fig. 1D–F). No significant difference in serum ALT levels or fibrotic areas was found between male and female p38αΔhep mice (data not shown).

We examined the consequences of p38α deletion in liver parenchymal cells on expression of fibrogenic markers. Loss of p38α significantly enhanced expression of the mRNAs for collα1, TIMP1, TGF-β1, and PDGFβ (Fig. 1G). No difference

Figure 1. Enhanced fibrogenesis in p38αΔhep mice. A, histologic and immunohistologic analysis of livers from mice treated with thioacetamide for 8 weeks. Liver sections were examined using H&E and Sirius Red staining, immunohistochemistry with α-SMA–specific antibody, and TUNEL staining (original magnification, ×200). B, ALT levels in serum were determined after 8 weeks of thioacetamide treatment. Results are mean ± SEM (n = 8). *, P < 0.05 versus control (F/F) mice. C, extent of neutrophil infiltration was determined by myeloperoxidase assay. Myeloperoxidase activity in untreated liver was given an arbitrary value of 1.0. Results are mean ± SEM (n = 8). D–F, the surface area stained with Sirius Red or antibody against α-SMA was quantified. Hepatic hydroxyproline content was measured. Results are mean ± SEM (n = 6). G, mice were treated with thioacetamide for 8 weeks and liver RNA was extracted. Relative mRNA amounts of the indicated genes were determined by real-time qPCR and normalized to the amount of actin mRNA. The amount of each mRNA in untreated liver was given an arbitrary value of 1.0. Results are mean ± SEM (n = 8).
in the expression of these fibrogenic markers was found in uninjured livers taken from p38α<sup>−/−</sup> and p38α<sup>Δhep</sup> mice (data not shown).

Enhanced ROS accumulation in p38α<sup>Δhep</sup> mice accounts for increased liver injury and fibrogenesis

A causal link between oxidative stress and liver fibrosis was proposed (24). We assessed the accumulation of hepatocyte superoxides by staining freshly frozen liver sections with dihydroethidine, whose oxidation gives rise to the fluorescent derivative ethidine. More extensive fluorescence was seen in periportal areas (zone 1) after thioacetamide administration in p38α<sup>Δhep</sup> mice than in control mice (Fig. 2A and B). Notably, the histologic location of thioacetamide-induced ROS accumulation differs from that of diethylnitrosamine-induced ROS, which are mainly detected in centrilobular (zone 3) hepatocytes (15, 25). This differential distribution of ROS-positive hepatocytes is likely to be due to a difference in the metabolism of the 2 compounds. Whereas diethylnitrosamine is metabolically activated by Cyp2E1, which is more abundant in zone 3 hepatocytes (26), thioacetamide can be converted to more toxic metabolites including Cyp2B (27), whose spatial distribution in the liver is affected by exposure to different chemicals and growth factors (28). Increased H<sub>2</sub>O<sub>2</sub> accumulation in livers of thioacetamide-treated p38α<sup>Δhep</sup> mice was also detected using the ROS indicator CM-H<sub>2</sub>DCFDA (Fig. 2A). p38α<sup>Δhep</sup> mice were found to have higher levels of oxidized protein than p38α<sup>−/−</sup> mice (Fig. 2B).

To evaluate the contribution of oxidative stress to thioacetamide-induced liver damage and fibrosis, we placed a group of mice on chow diet supplemented with the antioxidant butylated hydroxyanisole (BHA). p38α<sup>Δhep</sup> mice kept on this diet showed a significant reduction in thioacetamide-induced liver injury (Fig. 2C) and fibrosis (Fig. 2D). Thus, loss of p38α enhances thioacetamide-induced cell death and fibrogenesis through mechanisms that may depend on ROS accumulation.

The p38α<sup>−</sup>-induced antioxidant gene HSP25 inhibits thioacetamide-induced fibrosis

As previously described for diethylnitrosamine-treated mice (15), HSP25 expression was also induced by thioacetamide administration, and the extent of induction was much lower in p38α<sup>Δhep</sup> mice relative to p38α<sup>F/F</sup> controls (Fig. 3A and B). HSP25 was reported to inhibit ROS accumulation (29, 30). Adenoviral transduction of HSP25 into p38α<sup>Δhep</sup> mice (Supplementary Fig. S1) prevented thioacetamide-induced ROS accumulation, protein oxidation (Fig. 3C and D), liver damage (Fig. 3E), and fibrogenesis (Fig. 3F and G). These results provide further support for the notion that the enhanced accumulation of ROS in the p38α-deficient liver is responsible for the enhanced fibrogenic response of p38α<sup>Δhep</sup> mice.

![Figure 2. Enhanced ROS accumulation in p38α<sup>Δhep</sup> mice accounts for increased liver injury and fibrogenesis. A. frozen liver sections prepared after 8 weeks of thioacetamide treatment were incubated with 2 μmol/L dihydroethidine hydrochloride (DHE) or 5 μmol/L 5-[and-6]-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) for 30 minutes at 37°C. Cells staining positively for the oxidized dyes were identified by fluorescent microscopy (original magnification, ×100). B, protein oxidation was assessed by immunoblotting (OxyBlot) and quantified using NIH image analysis software. Results are mean ± SEM (n = 6). *P < 0.05 versus control (F/F) mice. C and D, mice were fed either BHA-supplemented (0.7%) or regular chow. After 4 weeks of thioacetamide treatment, serum ALT was measured in p38α<sup>F/F</sup> (F/F) and p38α<sup>Δhep</sup> mice (C) and the surface area stained with Sirius Red was quantified in p38α<sup>Δhep</sup> mice (D). Results are mean ± SEM (n = 6). *P < 0.05.](cancerres.aacrjournals.org)
Decreased expression of MAPKAP kinase-2 and increased expression of SOX2, c-Jun, and Gankyrin in thioacetamide-treated p38α−/− mice

Previous studies have described a critical role for c-Jun and JNKs in mediating HCC development (31, 32). In the thioacetamide model, p38α−/− deficient livers exhibited elevated c-Jun expression and increased JNK activity (Fig. 4A and B). Whereas cytokine-driven compensatory proliferation was suggested to promote diethylnitrosamine-induced hepatocarcinogenesis (15, 25, 33), there was no significant increase in interleukin (IL)-6 and TNF-α and IL-1β expression in thioacetamide-treated p38α−/− mice relative to p38α+/+ controls (Fig. 4B). An important downstream target for p38 is MAPKAP kinase-2 (MAPKAPK2) and MAPKAPK2-deficient cells are more sensitive to DNA damage-induced cell death (34). As shown in Fig. 4C, MAPKAP2 expression and phosphorylation is downregulated in p38α−/− deficient livers. Pluripotency-associated transcription factors such as SOX2 and Nanog are known as regulators of cellular identity in embryonic stem cells. More recently, SOX2 has been shown to participate in reprogramming of adult somatic cells to a pluripotent stem cell state and has been implicated in tumorigenesis in various organs (35). Loss of p38α significantly enhanced the expression of SOX2 mRNA and protein in thioacetamide-treated mice (Fig. 4D and F). Gankyrin, a liver oncoprotein, was reported to mediate dedifferentiation and facilitate the tumorigenicity of rat hepatocytes (36). p38α−/− mice exhibited a significant increase in Gankyrin expression after thioacetamide administration (Fig. 4E and F). No difference in the expression of Gankyrin was found in uninjured livers taken from p38α+/+ and p38α−/− mice (data not shown). To evaluate the contribution of oxidative stress to the increase in expression of these genes, we placed a group of mice on chow diet

Figure 3. The p38α-induced antioxidant gene HSP25 inhibits thioacetamide-induced fibrosis. A, mice were treated with thioacetamide for 8 weeks and their livers isolated and homogenized. Homogenates were gel-separated and immunoblotted with the indicated antibodies. The numbers below the panels indicate relative HSP25 expression levels. B, mice were treated as above, and total liver RNA was extracted at the indicated times. Amounts of mRNA relative to those in untreated p38α+/+ livers were determined by real-time qPCR. Results are mean ± SEM (n = 6). *, P < 0.05 versus control (F/F) mice. C–G, p38α−/− mice were infected with an adenovirus expressing HSP25 or a control adenovirus 20 hours before thioacetamide treatment. C, frozen liver sections prepared after 4 weeks of thioacetamide treatment were incubated with 2 μmol/L DHE for 30 minutes at 37°C and photographed. D, protein oxidation was assessed by immunoblotting (OxyBlot) and quantified using NIH image analysis software. E, ALT levels in serum were determined after 4 weeks of thioacetamide treatment. F, sections of livers prepared after 4 weeks of thioacetamide treatment were examined by Sirius Red staining. The numbers below the panels indicate relative fibrotic areas. G, hepatic hydroxyproline content was measured. Results are mean ± SEM (n = 6). *, P < 0.05.
supplemented with the antioxidant BHA. \textit{p38α}\textsuperscript{-}\textit{Δ} mice kept on this diet showed a significant reduction in SOX2 and c-Jun expression but not in Gankyrin expression (Fig. 4G). Immunohistochemical analysis revealed that putative hematopoietic stem cells, c-kit–positive cells, were recruited to thioacetamide-treated livers (Fig. 4H) but not in nontreated livers (data not shown). We confirmed that c-kit–positive cells expressed p38\textit{α} in \textit{p38α}\textsuperscript{-}\textit{Δ} mice (Fig. 4H). Between \textit{p38α}\textsuperscript{F/F} and \textit{p38α}\textsuperscript{-}\textit{Δ} livers, there was no significant difference in the number of c-kit–positive cells (data not shown), indicating that hepatic p38\textit{α} deficiency does not increase hematopoietic stem cell recruitment.

**Enhanced hepatocarcinogenesis in \textit{p38α}\textsuperscript{-}\textit{Δ} mice**

\textit{p38α}\textsuperscript{F/F} and \textit{p38α}\textsuperscript{-}\textit{Δ} mice were given thioacetamide in their drinking water for 10 months. When sacrificed, all \textit{p38α}\textsuperscript{-}\textit{Δ} mice given thioacetamide developed typical liver cirrhosis and 50% of \textit{p38α}\textsuperscript{-}\textit{Δ} mice had ascites, a common clinical finding indicative of portal hypertension. The liver surface was irregular, closely resembling human cirrhotic liver (Fig. 5A). All of the \textit{p38α}\textsuperscript{F/F} and \textit{p38α}\textsuperscript{-}\textit{Δ} mice given thioacetamide for 10 months developed well-differentiated HCCs (Fig. 5B), whereas only a few and small number of cholangiocellular carcinomas were found. Many tumors were positive for \textit{α}-fetoprotein (AFP) expression, a tumor marker specific for HCCs (Fig. 5C). Tumor sizes and areas were considerably larger in \textit{p38α}\textsuperscript{-}\textit{Δ} mice relative to similarly treated \textit{p38α}\textsuperscript{F/F} controls (Fig. 5D). In contrast to mice, thioacetamide-treated rats develop cholangiocellular carcinoma (9). This may be because CD133-positive stem cells are induced by thioacetamide in mice but not in rats (Fig. 5E).

**Association between risk of HCC recurrence and protein oxidation in human liver**

Forty-two patients with HCCs were recruited in this study. Clinicopathologic profiles of the patients and their HCCs are

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**Figure 4.** Decreased expression of MAPKAP kinase-2 and increased expression of SOX2, c-Jun, and Gankyrin in thioacetamide-treated \textit{p38α}\textsuperscript{-}\textit{Δ} mice. Mice of the indicated genotypes were given thioacetamide for 8 weeks and their livers isolated and homogenized. A, JNK activity was determined by immune complex kinase assay. Protein recovery was determined by immunoblotting with JNK1 antibody. The numbers below the panels indicate relative JNK activities determined by densitometry. B, D, and E, liver RNA was extracted. Relative amounts of cytokine, Nanog, SOX2, and Gankyrin mRNAs were determined by real-time qPCR and normalized to the amount of actin mRNA. The amount of each mRNA in untreated liver was given an arbitrary value of 1.0. Results are mean ± SEM (n = 6). C and F, homogenates of liver tissues were gel-separated and immunoblotted with the indicated antibodies. Representative data are shown. The numbers below the panels indicate relative expression levels determined by densitometry. G, \textit{p38α}\textsuperscript{-}\textit{Δ} mice were fed either BHA-containing (0.7%) or regular chow and treated with thioacetamide for 8 weeks. Relative amounts of mRNAs were determined by real-time qPCR and normalized to the amount of actin mRNA. The amount of each mRNA in untreated liver was given an arbitrary value of 1.0. Results are mean ± SEM (n = 6). H, immunohistochemistry was done on frozen liver sections of thioacetamide-treated \textit{p38α}\textsuperscript{-}\textit{Δ} mice. Cells stained with indicated antibodies were identified by confocal microscopy. Scale bar, 50 μm.
shown in the Supplementary Table. Intrahepatic HCC development after hepatectomy is caused by de novo HCC development and/or metastasis from the resected HCCs. The risk of the former depends on background liver factors such as liver fibrosis, whereas the risk of the latter mainly depends on the characteristics of the resected HCCs (37). In mouse models, HSP25-mediated inhibition of ROS accumulation is involved in control of liver fibrogenesis and can subsequently attenuate de novo HCC development. We examined whether this hypothesis is applicable to humans, focusing on noncancerous liver tissues rather than cancers to assess the potential for de novo HCC development or rapid progression of lesions that were undetectable or pre-neoplastic at the time of resection. In patients exhibiting HCC recurrence after hepatectomy, protein oxidation levels in the nontumor tissues, but not in tumors, were significantly higher than in those without HCC recurrence (Fig. 6A). In addition, patients with low protein oxidation in noncancerous liver had a prolonged recurrence-free survival (Fig. 6B). In conclusion, elevated ROS accumulation in the liver is associated with increased risk of human HCC development or recurrence.

Discussion

Oxidative stress is thought to play a major role in the pathogenesis of hepatic fibrosis (8) and cancer development (38, 39), exerting many effects, including alteration of gene expression (40), enhanced cell death and proliferation as well as genomic instability (39). However, the exact impact of oxidative stress and antioxidant responses on hepatic fibrosis and subsequent HCC development need to be better understood.

Figure 5. Enhanced hepatocarcinogenesis in p38αΔhep mice. A, livers of p38αΔhep and p38α+/F mice after 10 months of thioacetamide treatment. B and C, sections of livers were examined using H&E staining (B) and by immunohistochemistry with AFP-specific antibody (C). Original magnification, ×200. N, noncancerous liver tissues; T, tumors. Distinction between tumor and noncancerous liver tissue was made by H&E staining. D, maximal tumor sizes (diameters) and percentages of liver area occupied by tumors in p38α+/F (F/F, n = 8) and p38αΔhep (n = 8) mice. *, P < 0.05 versus control mice (F/F). E, Sprague-Dawley rats and p38α+/F control mice were given thioacetamide (0.03%) for 5 weeks and their livers isolated. Homogenates of rat and mouse liver tissues were gel-separated and immunobotted with the indicated antibodies. The numbers below the panels indicate relative CD133 expression levels.

Figure 6. Associations between the risk of HCC recurrence and protein oxidation in human liver. A, protein oxidation was assessed in tumors and nontumorous human liver tissues by immunoblotting (OxyBlot) and quantified using NIH image analysis software. *, P < 0.05 versus patients without HCC recurrence. B, recurrence-free survival versus protein oxidation. The Kaplan–Meier method was used to determine recurrence-free survival, and the log-rank test was used to compare recurrence-free survival between patients grouped according to amount of protein oxidation in liver. mo, month.
ROS accumulation in mice exposed to the non-fibrogenic hepatic carcinogen diethylnitrosamine (15). Here, we describe that p38α activity is also important for suppression of ROS accumulation upon thioacetamide administration, which leads to induction of fibrosis, cirrhosis, and HCCs. In the absence of the p38α target HSP25, the thioacetamide-exposed p38α<sup>−/−</sup> liver shows elevated ROS accumulation that correlates with augmented liver damage in these mice. Increased susceptibility to liver damage in p38α<sup>−/−</sup> mice is reversed by administration of the small-molecule antioxidant BHA or restoration of HSP25 expression. These results support the hypothesis that increased ROS accumulation may be the main cause of hepatocyte death in p38α-deficient mice regardless of the hepatotoxic chemical to which they were exposed. Expression levels of MAPKAPK2 and phospho-MAPKAPK2 were decreased in the p38α-depleted livers. Given the protective role of MAPKAPK2 in DNA damage-induced cell death (34), the downregulation of MAPKAPK2 expression and phosphorylation that takes place in the absence of p38α are likely to contribute, at least in part, to increased liver damage in p38α<sup>−/−</sup> mice. Hepatocyte death activates an inflammatory response, which promotes HSC activation via a paracrine mechanism (24), which we and others have suggested to involve IL-1α release (15, 41). This inflammatory response results in excessive synthesis of extracellular matrix proteins and fibrosis development (23). Correspondingly, BHA administration or restoration of HSP25 expression reverses enhanced thioacetamide-induced fibrogenesis caused by the p38α deficiency. A strong correlation between liver fibrogenesis and hepatocarcinogenesis has been reported in patients infected with HCV (6), and HCV infection has been shown to cause ROS accumulation and oxidative stress (42). We find that enhanced protein oxidation in the noncancerous portion of human liver is associated with a high risk of HCC recurrence after hepatectomy. These data support an important role of ROS accumulation within liver parenchymal cells in liver fibrogenesis and subsequent hepatocarcinogenesis.

HSP25/HSP27 has antioxidant properties (29, 30). Mice given thioacetamide showed an inverse correlation between HSP25 expression and ROS accumulation in the liver. In addition, we found that elevated ROS accumulation correlated with the presence of HCC recurrence after hepatectomy. However, we did not observe a statistically significant relationship between HSP27 expression and ROS accumulation in human livers (data not shown). While elevated HSP27 expression reduces ROS accumulation, HSP27 expression itself is upregulated following oxidative stress (43). Most probably, our findings may reflect complex mechanisms regulating ROS accumulation via several molecules in the human liver. The exact relationship between HSP27 and oxidative stress in human parenchymal cells remains to be elucidated. In the mouse liver, however, it is quite clear that p38α negatively regulates ROS accumulation through induction of HSP25, which maintains parenchymal cell viability and suppresses liver fibrogenesis. Enhanced cell death caused by the absence of p38α results in increased inflammation and hepatic fibrogenesis, which eventually augments HCC development, as seen before in diethylnitrosamine-treated mice (15, 32). Thus, the antitumorigenic activity of hepatocyte p38α is not model specific and may also apply to human liver.

Stem cell function is central for the maintenance of normal tissue homeostasis. SOX2 forms the core of the self-renewal transcription network in embryonic stem cells. Selective downregulation of SOX2 induces embryonic stem cell differentiation and exit from the pluripotent stem cell state. In contrast, combinatorial overexpression of SOX2, Nanog, and other transcription factors was shown to reprogram several types of adult somatic cells to a pluripotent stem cell–like state (44–46). In these experiments, cells were reprogrammed fully or only partially (47), possibly because of heterogeneous exposure to reprogramming factors. It is tempting to speculate that acquisition or overexpression of SOX2 can promote tumorigenesis by processes that resemble partial reprogramming (35, 44, 47). In our study, we showed that p38α deletion increased expression of SOX2 through enhanced ROS accumulation. Hepatocyte dedifferentiation has been reviewed as a key cellular event during hepatocarcinogenesis (48). Gankyrin, also named 26S proteasome non-ATPase regulatory subunit 10, is a critical oncoprotein overexpressed in human HCCs. A close association of Gankyrin expression with hepatocyte dedifferentiation was observed, and differentiation induced by Gankyrin interference reduced the population of cancer stem cells in hepatoma cell lines (36), suggesting that Gankyrin promotes HCC development by driving dedifferentiation of hepatocytes and facilitating HCC stem/progenitor cell generation. We found that the p38α deficiency enhances the induction of Gankyrin expression in livers of thioacetamide-treated mice. Recently, JNK activation was reported to be involved in stem cell expansion in human HCCs (49). JNK activity was significantly enhanced in p38α-deficient liver. The inactivation of p38α also leads to an immature and hyperproliferative lung epithelium that is highly sensitized to tumorigenesis (50). It has been shown that the Albumin-Cre driver does not only lead to deletion of genes in hepatocytes but also deletes genes in hepatic precursor cells. These data suggest an important role of p38α in regulating hepatic stem/progenitor cell behavior.

In conclusion, p38α plays a critical role in liver fibrogenesis and hepatocarcinogenesis through the control of HSP27 expression and ROS accumulation in the mouse thioacetamide model, which may be suitable for studying the pathogenesis of HCV-related HCC development. Importantly, the risk of human HCC recurrence after hepatectomy is positively correlated with protein oxidation in liver. Deletion of p38α upregulates expression of SOX2 and Gankyrin, which may be involved in cancer stem cell maintenance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: T. Sakurai, M. Kudo, M. Karin
Development of methodology: T. Sakurai, G. He, A.M. Elsharkawy, M. Karin
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