Smad3 reduces susceptibility to hepatocarcinoma by sensitizing hepatocytes to apoptosis through downregulation of Bcl-2

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Summary

In the liver, derangement of TGF-β signaling is associated with an increased incidence of hepatocellular carcinoma (HCC), but the mechanism is not clear. We report here that forced expression of a major TGF-β signaling transducer, Smad3, reduces susceptibility to HCC in a chemically induced murine model. This protection is conferred by Smad3's ability to promote apoptosis by repressing Bcl-2 transcription in vivo through a GC-rich element in the Bcl-2 promoter. We also show that the proapoptotic activity of Smad3 requires both input from TGF-β signaling and activation of p38 MAPK, which occurs selectively in the liver tumor cells. Thus, Smad3 enables the tumor suppression function of TGF-β by serving as a physiological mediator of TGF-β-induced apoptosis.

Introduction

Transforming growth factor β (TGF-β) regulates a diverse array of cellular context-dependent biological processes ranging from growth and differentiation to apoptosis (Massagué, 1998). In the liver, a major function of TGF-β, which is normally produced by nonparenchymal stellate cells, is to limit regenerative growth of hepatocytes in response to injury by inhibiting DNA synthesis and inducing apoptosis (Oberhammer et al., 1992; Romero-Gallo et al., 2005). As in other physiological settings, the proapoptotic activity of TGF-β here is associated with the removal of unwanted or damaged cells, thus playing an indispensable role in maintaining normal cellular homeostasis and organ size.

Hepatocellular carcinoma (HCC) is a common visceral malignancy and among the leading causes of cancer death worldwide (Parkin et al., 2005). It typically arises in a setting of chronic hepatitis or cirrhosis, with infection by hepatitis B and C viruses and chronic exposure to aflatoxin B together responsible for about 80% of all HCC cases in humans (Thorgeirsson and Grisham, 2002; Block et al., 2003). Although the pathogenic causes are well known, no effective treatment is available for most HCC patients except for surgical resection and liver transplantation in the few cases detected at a sufficiently early stage (Varela et al., 2003). TGF-β has a complex role in HCC; it is persistently induced during hepatitis and promotes progression of cirrhosis by accelerating extracellular matrix deposition (Takiya et al., 1995; Wrana, 1999). Persistent upregulation of TGF-β is also a hallmark of HCC in humans, leading to the speculation that TGF-β may accelerate neoplastic growth of liver cancer (Rossmanith and Schulte-Hermann, 2001). However, mice heterozygous for a target-inactivated TGF-β1 allele or a TGF-β type II receptor allele show heightened susceptibility to chemical carcinogens such as *N*-diethylnitrocosamine compared to their wild-type littermates, indicating a haploinsufficiency of tumor suppression (Tang et al., 1998; Im et al., 2001; Kanzler et al., 2001). In rodent HCC models established by transgenic expression of c-*myc*/TGF-α or induced chemically, attenuation of TGF-β signaling was observed as result of downregulation of TGF-β type II receptor (Reisenbichler et al., 1994; Santoni-Rugiu et al., 1999). Apparently, the antiproliferative and proapoptotic roles of TGF-β represent a ''fail-safe'' oversight that has to be evaded by tumor cells to gain malignancy, but the mechanism of this tumor suppression is not fully understood.

Signaling of TGF-β is mediated by a complex of membrane bound type I and type II receptors and Smad proteins that interact with and are phosphorylated at the C-terminal SSXS motif by the serine-threonine kinase domain of the type I receptor (Derynck and Zhang, 2003; Shi and Massagué, 2003). Of the eight Smads identified, Smad2 and Smad3 mediate TGF-β/activin signaling. Once activated, these

SIGNIFICANCE

Hepatocellular carcinoma is a major world health problem for which there is no cure. We report here that forced expression of a TGF-β signaling transducer, Smad3, reduces susceptibility to liver cancer by promoting apoptosis. Our data show that Smad3 represses transcription of an important apoptosis inhibitor, Bcl-2, by directly binding to its promoter. Our data also indicate that, while ectopic expression of Smad3 alone does not ostensibly alter normal function of a resting liver, it nevertheless suppresses tumor formation even well after the onset of carcinogenesis. Thus, Smad3 holds great promise as a gene therapeutic agent for treating liver cancer.

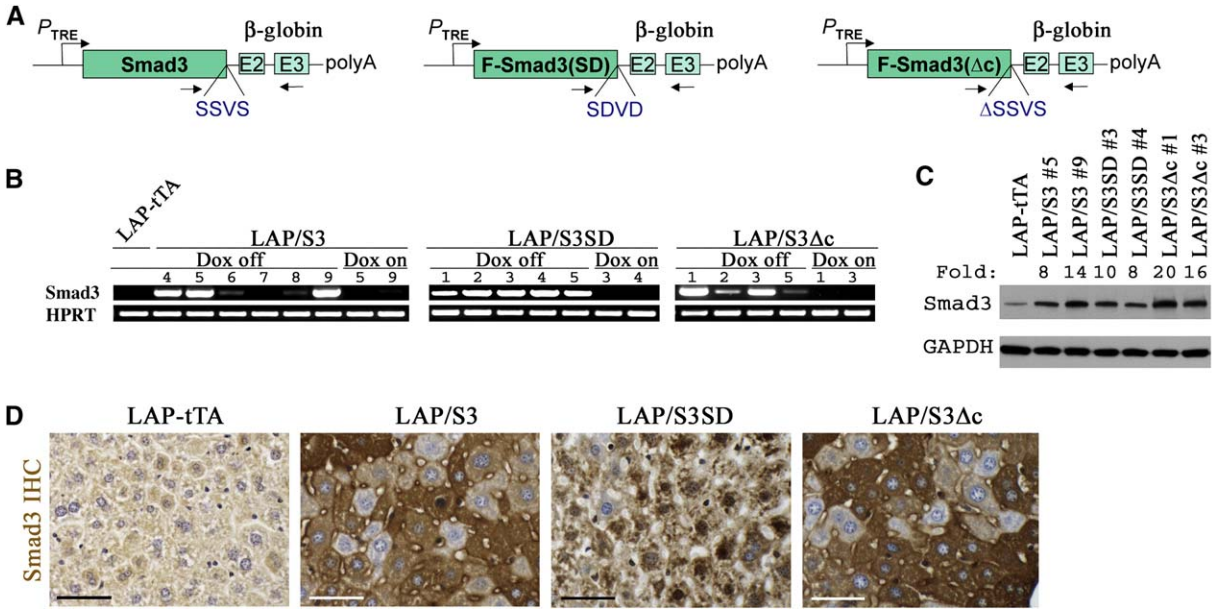

Figure 1. Expression of Smad3 transgenes in hepatocytes

A: Schematic illustration of Smad3, Smad3SD, and Smad3Δc transgenes.
B: Dox-controllable expression of Smad3 transgenes in livers of LAP/S3, LAP/S3SD, and LAP/S3Δc doubly transgenic mice as analyzed by RT-PCR amplification of total liver RNA with primers denoted by arrows in **A**. Transgenic animals were sacrificed 2 weeks after dietary Dox withdrawal.
C: Western analysis of Smad3 transgenes in liver extracts. Fold increase of transgenic relative to endogenous Smad3 level (LAP-tTA control mice) is indicated.
D: Immunohistochemistry staining of Smad3 (brown) in liver sections. Both endogenous and transgenic wild-type Smad3 as well as Smad3Δc showed exclusive cytoplasmic staining in resting hepatocytes, whereas Smad3SD exhibited strong nuclear staining. Scale bar, 60 μm.

receptor-specific Smads associate with Smad4 and traverse into the nucleus to regulate expression of target genes. It is now increasingly appreciated that MAPKs such as Erk, JNK, and p38, as well as cyclin-dependent kinase CDK2/4, also phosphorylate Smads at various sites in the linker region bridging the two conserved MH1 and MH2 domains (Kretzschmar et al., 1999; Engel et al., 1999; Matsura et al., 2004; Mori et al., 2004). These latter phosphorylation events may either inhibit or enhance Smad activity. While it is established that TGF-β exerts growth inhibition via its ability to downregulate the proto-oncogene c-*myc* and to activate transcription of cyclin-dependent kinase inhibitors, such as $p15^{ink4b}$ and $p21^{cip1}$, much less is known regarding the mechanism of TGF-β-induced apoptosis (Derynck et al., 2001). Recent studies in cell culture systems have revealed a number of factors including ARTS, DAXX, and DAP kinase that appear to act through the apoptotic apparatus (Larisch et al., 2000; Perlman et al., 2001; Jang et al., 2002), and that Smad3 is sequestered in a cytoplasmic complex with survival kinase PKB/Akt, which restrains Smad3 from entering the nucleus and activating the apoptotic program independent of the kinase activity (Conery et al., 2004; Remy et al., 2004).

To investigate the role of Smad3 in tumorigenesis, we generated several lines of transgenic mice expressing Smad3 and its mutated variants under the control of the tetracycline repressible system and specifically induced the expression of Smad3 transgenes in the liver. We report here that livers with elevated Smad3 are protected from chemically induced carcinogenesis and demonstrate that Smad3 potentiates apoptosis by repressing Bcl-2 transcription in vivo through a GC-rich element in the *Bcl-2* P2 promoter.

Results

Regulated expression of Smad3 transgenes in hepatocytes

At the outset of this study, we used a plasmid vector that confers controllable cDNA expression via the tetracycline-responsive element (TRE) to create several lines of transgenic mice expressing wild-type Smad3, constitutively active Smad3SD with the last two serines substituted by aspartic residues to mimic phosphorylation, or dominant-negative Smad3Δc lacking the last four amino acid residues, SSVS (Figure 1A). Liver-specific expression of transgenes was achieved by crossing TRE-Smad3, TRE-Smad3SD, or TRE-Smad3Δc mice with LAP-tTA mice, which express tetracycline transactivator (tTA) specifically in hepatocytes under the control of the liver activator protein (LAP) promoter (Kistner et al., 1996). To avoid potential developmental complications, the expression of Smad3 transgenes was held off by feeding each mating pair and their newborn offspring with doxycycline (Dox), an inhibitor of tTA transactivator, until the doubly transgenic offspring were 3 weeks of age or as noted. Two high-expressing strains derived from the intercrosses between separate TRE-Smad founders of each transgenic line and the LAP-tTA mice were selected for further analyses (Figures 1B and 1C). These doubly transgenic mice were designated hereafter as LAP/S3, LAP/S3SD, or LAP/S3Δc, respectively. Western blot analysis confirmed the elevated expression of transgene products in respective liver extracts (Figure 1C), and immunohistochemistry staining indicated that both endogenous and transgenic Smad3 and Smad3Δc were localized exclusively in the cytoplasm of otherwise normal hepatocytes, but Smad3SD was enriched in the nucleus (Figure 1D). Despite the

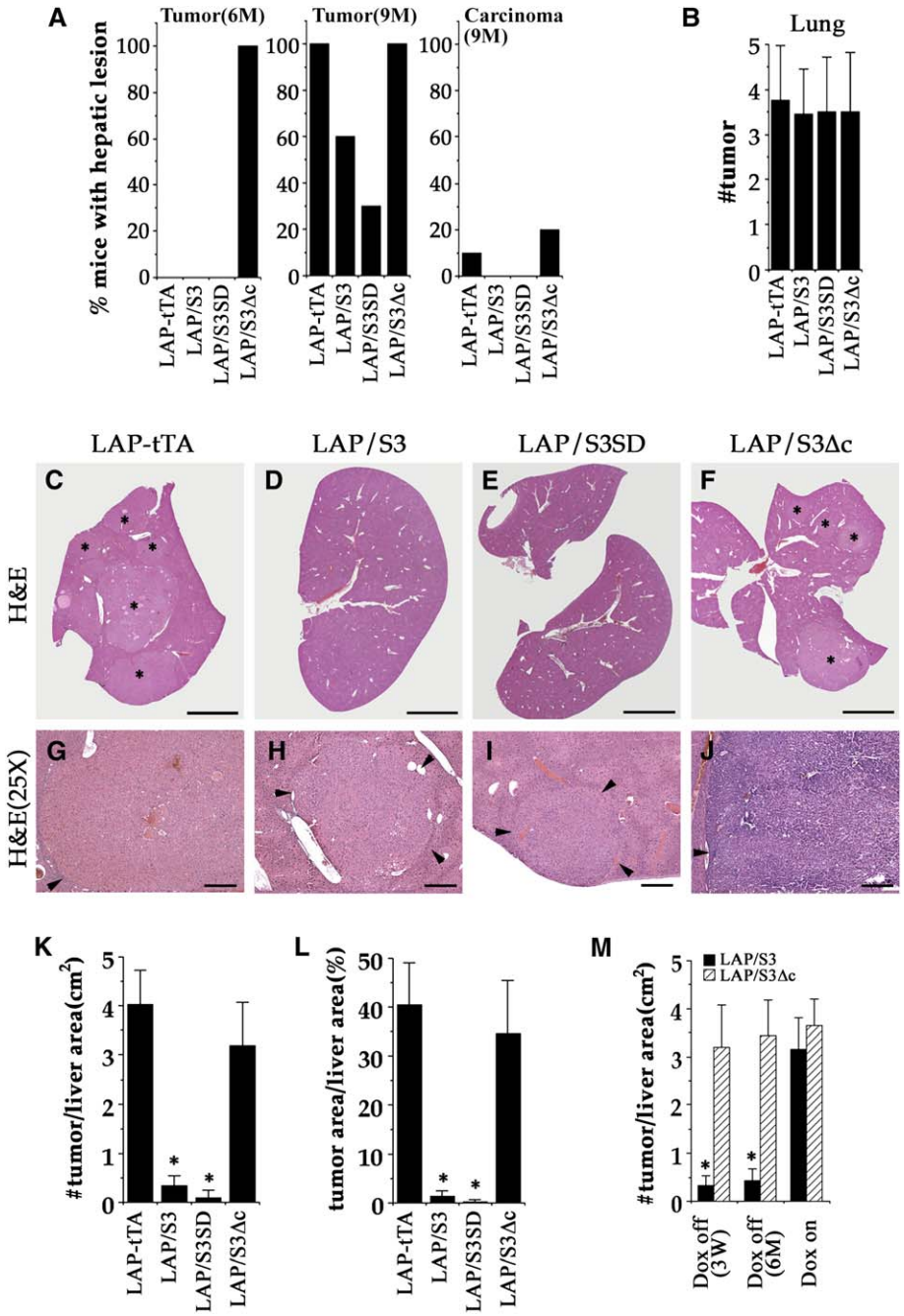

Figure 2. Ectopic expression of Smad3 protects liver from chemically induced carcinogenesis

A: Percentage of mice developed at least one liver tumor at the end of 6th (n = 9) or 9th (n = 10) month after DEN injection. A separate graph shows the percentage of mice with at least one malignant carcinoma at 9th (n = 10) month after DEN injection.

B: Average number of lung tumors per animal at 9th month after DEN injection (n = 10).

C–F: H&E staining of liver sections. No macroscopic tumor was detected in livers from LAP/S3 and LAP/S3SD mice. Scale bar, 5 mm.

G–J: Higher magnification of liver sections in **C**–**F** showing eosinophilic hepatocellular adenomas found in LAP-tTA, LAP/S3, and LAP/S3SD mice and a hepatocellular carcinoma in a LAP/S3Δc mouse. Note the size of adenomas from LAP/S3 and LAP/S3SD livers is much smaller than that from LAP-tTA or LAP/S3Δc livers. Arrowheads denote the tumor margin. Scale bar, 50 μm.

K and L: Density of average number of tumors per cm^2 liver area (**K**) and percentage of tumor in total liver area (**L**) measured in liver sections.

M: Density of average number of tumors per cm^2 liver area. Dox was either taken out of the diet when mice were 3 weeks of age (Dox off, 3W), taken out of the diet 6 months after DEN/Pb treatment (Dox off, 6M), or kept in the diet at all times (Dox on).

All analyses in this figure were carried out 9 months after DEN injection except where otherwise indicated. In **B**, **K**, **L**, and **M**, each cohort consisted of ten mice. *$p < 1 \times 10^{-5}$. Error bars indicate mean ± standard deviation.

overexpression of these Smad3 transgenes, however, none of the doubly transgenic offspring manifested any discernible phenotype in resting livers (Figures S1 and S2 in the Supplemental Data available with this article online; and data not shown).

Ectopic expression of Smad3 protects liver from chemically induced carcinogenesis

To investigate the role of Smad3 in liver tumorigenesis, we subjected the above three lines of doubly transgenic mice as well as the control LAP-tTA mice to a well-established chemical carcinogenesis protocol because the murine tumors developed under this protocol most faithfully recapitulate the human cancer both in tumor morphology and in pathological progression (Pitot, 1990; Tamano et al., 1994; Masui et al., 1997). Thus, liver tumor formation was initiated by a single injection of diethylnitrosamine (DEN) at 5 weeks of age after Smad3 transgenes were turned on, and promoted by continuous administration of phenobarbital (Pb) starting at 9 weeks of age. Nine months into this protocol, all control LAP-tTA and LAP/S3Δc mice developed multiple macroscopic tumor nodules in the liver (Figures 2A, 2C, and 2F), with an average of three to four tumors per square centimeter (Figure 2K) and the neoplasm accounting for about 40% of total liver area (Figure 2L). Although similar in eventual numbers and sizes to the controls, liver tumors of LAP/S3Δc mice had an earlier onset, beginning at the sixth month after DEN injection (Figure 2A), suggesting that

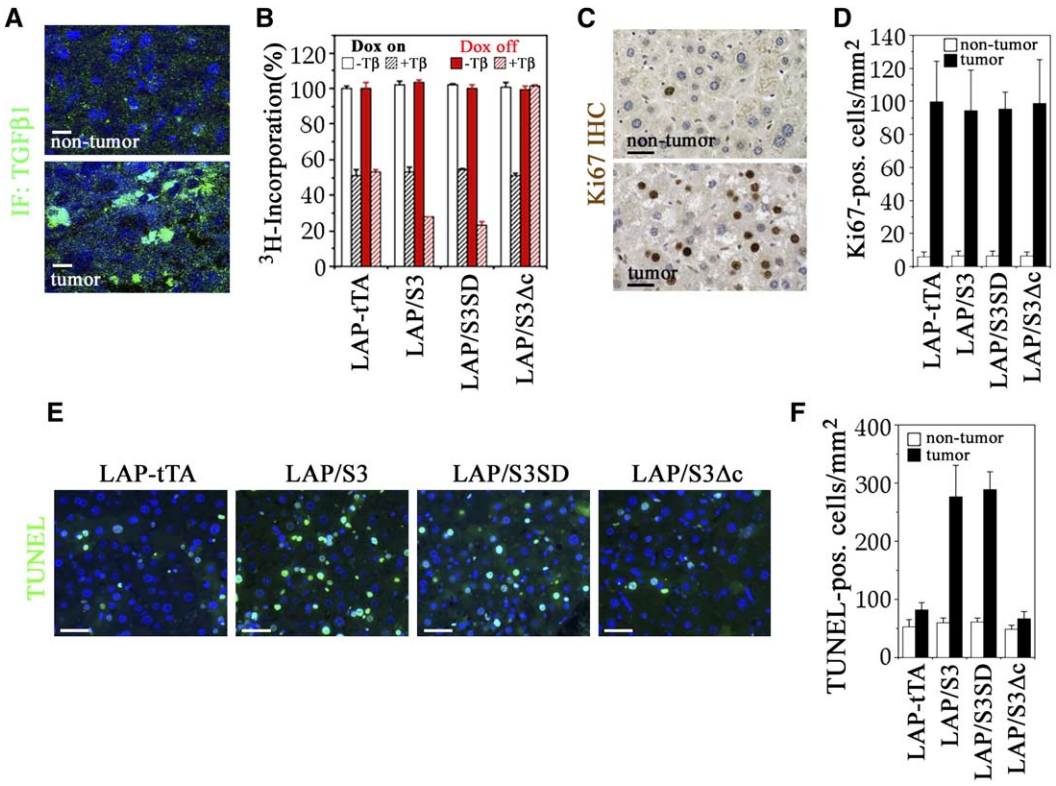

Figure 3. Smad3 promotes apoptosis of liver tumor cells in vivo

A: Immunostaining of active TGF-β1 (green) in normal and neoplastic tissues of LAP-tTA control liver. Cell nuclei were counterstained with DAPI (blue). Scale bar, 10 μm.

B: Thymidine incorporation assay of primary hepatocytes in the presence (Dox on) or absence of Dox (Dox off).

C: Immunohistochemistry staining of the proliferation marker Ki67 (brown) in normal and neoplastic tissues of LAP-tTA liver. Scale bar, 100 μm.

D: Quantification of Ki67-positive cells in normal and neoplastic liver tissues.

E: TUNEL assay of apoptotic cells (green) in tumors. Cell nuclei were counterstained with DAPI (blue). Scale bar, 100 μm.

F: Quantification of TUNEL-positive cells in normal and neoplastic liver tissues.

In **D** and **F**, the results represent mean values of 12 tumors, except in the case of LAP/S3SD, in which the mean value was derived from four tumors. Error bars indicate mean ± standard deviation.

dominant-negative inhibition of Smad3 function accelerates hepatic tumor progression. Pathohistological analysis indicated that, while most tumor nodules present in these mice were eosinophilic hepatocellular adenomas (Figure 2G and data not shown), poorly differentiated trabeculae typical of advanced carcinomas could be occasionally found in the center of large adenomas (Figures 2A and 2J). In contrast, no macroscopic tumor was found in the livers of LAP/S3 or LAP/S3SD mice (Figures 2D and 2E). When scrutinized under a microscope at high magnification, only 60% of LAP/S3 and 30% of LAP/S3SD mice developed liver tumors at the ninth month, respectively (Figure 2A), but the number of tumor nodules was few (Figure 2H-I, 2K) and the size was small (Figures 2H, 2I, and 2L). This reduced tumor formation can be attributed specifically to ectopic expression of Smad3 in the liver because primary lung tumors, frequently observed in this carcinogenesis protocol, appeared at a similar rate in all three transgenic and the control lines after DEN/Pb exposure (Figure 2B). Moreover, when continuously kept on the Dox diet, the extent of liver tumor formation in LAP/S3 mice was comparable to that of LAP/S3Δc mice (Figure 2M). Thus, enhancing Smad3 function through ectopic expression of wild-type Smad3 or Smad3SD protects the liver from chemically induced carcinogenesis, whereas disruption of this function by dominant-negative Smad3Δc has the opposite effect. This protection is likely conferred by suppression of tumor progression rather than prevention of tumor initiation because tumor formation was equally reduced when Smad3 transgenes were induced by dietary Dox withdrawal either before or 6 months after the tumor-initiating DEN injection (Figure 2M).

Smad3 suppresses liver tumorigenesis by promoting apoptosis in tumor cells

In mammals, production and paracrine release of active TGF-β increase substantially accompanying liver regeneration after injury, or during hepatocarcinogenesis (Wrana, 1999; Rossmanith and Schulte-Hermann, 2001). We observed such an increase of the level of active TGF-β1, the predominant variant of three TGF-β isoforms, in the hitherto described DEN/Pb-induced tumors (Figure 3A). Thus, it is possible that the ectopically expressed Smad3 renders hepatocytes hyperresponsive to this endogenous release of TGF-β, effectively amplifying its tumor-suppressive role either through growth inhibition or apoptosis. Conversely, dominant-negative Smad3Δc could accelerate liver carcinogenesis by blocking endogenous TGF-β signaling, and thus the TGF-β-mediated tumor suppression. Indeed, in isolated primary hepatocytes, TGF-β treatment led to a 50% reduction in proliferation of the liver cells expressing normal levels of Smad3

(Figure 3B, LAP-tTA cells or Dox on). Further reduction of proliferation was seen in cells expressing elevated Smad3 or Smad3SD, but this reduction was abolished in cells expressing Smad3Δc (Figure 3B, Dox off), suggesting that the growth of cultured hepatocytes is subject to control by the TGF-β pathway. However, this control appeared to be lost in the DEN/Pb-induced liver tumors because the tumor cell growth, measured by immunohistochemistry staining of the Ki67 proliferation marker in tumor sections (Figure 3C), did not change among various Smad3 transgenic strains (Figure 3D). Outside the tumor tissue, proliferation was extremely low and also did not change in response to transgene expression (Figures 3C and 3D). Instead, the number of apoptotic cells increased substantially in the tumors of LAP/S3 and LAP/S3SD mice as evidenced by TUNEL assay (Figures 3E and 3F). These results suggest that, while liver tumors developed in all transgenic lines may have gained an ability to escape TGF-β-mediated growth inhibition, they succumb to a high rate of apoptosis when the function of Smad3 is enhanced.

Smad3 enhances the responsiveness of hepatocytes to apoptosis

In light of the propensity of LAP/S3 and LAP/S3SD liver tumors to undergo apoptosis, we postulate that the reduced susceptibility of LAP/S3 and LAP/S3SD mice to HCC is due to a heightened sensitivity of "precancerous" liver cells to apoptotic stimuli as result of enhanced Smad3 function. To test this hypothesis, we examined the hepatic response of the above transgenic lines to activation of the death receptor Fas, a potent inducer of liver apoptosis. Fas antigen is ubiquitously expressed in hepatocytes and downregulation of Fas-mediated apoptosis is one of the prominent characteristics of liver tumors (Galle et al., 1995; Ito et al., 1998; Pinkoski et al., 2000). In vivo, mice are known to be sensitive to Fas and become moribund after injection of Jo-2 antibody, a Fas agonist, due to massive liver failure (Ogasawara et al., 1993; Leu et al., 2003). We first detected signs of liver injury and apoptosis in LAP/S3 and LAP/S3SD mice 3 hr after injection of Jo-2 antibody (Figures 4A and 4B); by 5 hr, these signs intensified and death ensued (Figure 4C). In each case, however, the magnitude of cell death and hemorrhagic liver destruction in LAP/S3 and LAP/S3SD mice was much greater than that of the controls (Figures 4B and 4D), and the mortality of LAP/S3 and LAP/S3SD mice also superseded that of the control mice by 1.5 hr (Figure 4C). In contrast, both liver injury and cell death were subdued in LAP/S3Δc mice (Figures 4A and 4B) to such an extent that more than half of the animals in this cohort survived Jo-2 injection (Figure 4C). In line with these physiological changes, the caspase cascade was activated strongly in LAP/S3 and LAP/S3SD, weakly in LAP-tTA, but minimally in LAP/S3Δc livers 3 hr after Jo-2 injection (Figure 4E). Concurrent with the above Fas-induced physiological and molecular changes, Smad3 was C-terminally phosphorylated in the liver (Figure 4F) and translocated into the nucleus (Figure 4G), but both of these two events were blocked by Smad3Δc (Figures 4F and 4G). Thus, although ectopic expression of Smad3 per se causes no phenotypic manifestation in the liver, it nonetheless increases the tendency of hepatocytes to undergo apoptosis.

Proapoptotic activity of Smad3 requires its nuclear translocation and activation of p38 MAPK

As in liver tumors, we detected a release of active TGF-β1 shortly after Jo-2 injection (Figure 5A), most likely stemming from nonparenchymal stellate cells. This suggests that the proapoptotic role of Smad3 in hepatocytes may require activation of the hepatic TGF-β pathway. To test this hypothesis, we isolated primary hepatocytes from the transgenic Smad3, Smad3SD, Smad3Δc, and control mice. When grown in normal medium rich in serum, hepatocytes are refractory to TGF-β but succumb to TGF-β-induced apoptosis when deprived of serum and insulin (Tanaka and Wands, 1996; Chen et al., 1998). In the serum-starved setting, we found that TGF-β-induced apoptosis was augmented in LAP/S3 and LAP/S3SD but suppressed in LAP/S3Δc hepatocytes compared to the controls (Figure 5B). Modulation of the TGF-β-induced apoptosis was specific to Smad3 transgenes, as it was absent in cells that were cultured in Dox-containing medium (Figure 5B). The requirement of Smad3 for triggering cell death in hepatocytes was further corroborated by Smad3-siRNA-mediated RNA interference of the apoptotic response (Figure 5C). It is believed that survival kinase PKB/Akt normally sequesters Smad3 in a cytoplasmic protein complex and, when it is inactivated by serum deprivation, releases Smad3 to translocate into the nucleus (Conery et al., 2004; Remy et al., 2004). However, the fact that ectopic expression of Smad3SD in the liver of LAP/S3SD mice did not cause any phenotype even when Smad3SD was localized to the nucleus argues that C-terminal phosphorylation and nuclear translocation alone are probably not sufficient to render Smad3 fully active in inducing apoptosis. Since both Fas activation and serum deprivation are known to activate JNK and p38 MAPK (Goillot et al., 1997; Juo et al., 1997; Kummer et al., 1997), two important regulators of apoptosis in hepatocytes, we speculated that the activation of such kinases might be an additional requirement for staging full proapoptotic activity of Smad3. Indeed, immunohistochemistry staining showed that p38 MAPK was activated in DEN/Pb-induced liver tumors but not in normal liver tissues, regardless of the expression of Smad3 transgenes (Figure 5D). No such difference in the activation status of JNK was observed (data not shown). To directly test the role of p38 MAPK, we treated normal hepatocytes with various kinase inhibitors and found that only SB203580, a specific p38 MAPK inhibitor, blocked the TGF-β-induced apoptosis (Figure 5E). Treating hepatocytes with JNK, Erk, and Akt kinase inhibitors actually enhanced the apoptotic response, whereas ROCK kinase inhibitor had no effect (Figure 5E). These results demonstrate that the proapoptotic activity of Smad3 in hepatocytes requires nuclear translocation governed by C-terminal phosphorylation as well as activation of p38 MAPK.

Downregulation of Bcl-2 by TGF-β/Smad3

In search of a Smad3 target for its role in hepatocytic apoptosis, we assayed a number of important apoptotic regulators including Bcl-2, Bcl-xL, Bax, Bad, and Bak in Fas-activated liver extracts. Western analyses indicated that the level of Bcl-2 was downregulated in liver extracts prepared after the animals were injected with Jo-2 (Figure 6A), consistent with the role of Bcl-2 as a key antiapoptotic inhibitor (Gross et al., 1999). The suppression of Bcl-2 was more prominent in LAP/S3 and LAP/S3SD extracts but abrogated in LAP/S3Δc extracts (Figure 6A). No detectable fluctuation of other members of the Bcl-2 family was observed (Figure 6A and data not shown). RT-PCR analyses of total liver RNA showed that the level of Bcl-2 mRNA was likewise decreased in response to Fas activation, and this decrease was augmented by the presence of ectopic Smad3

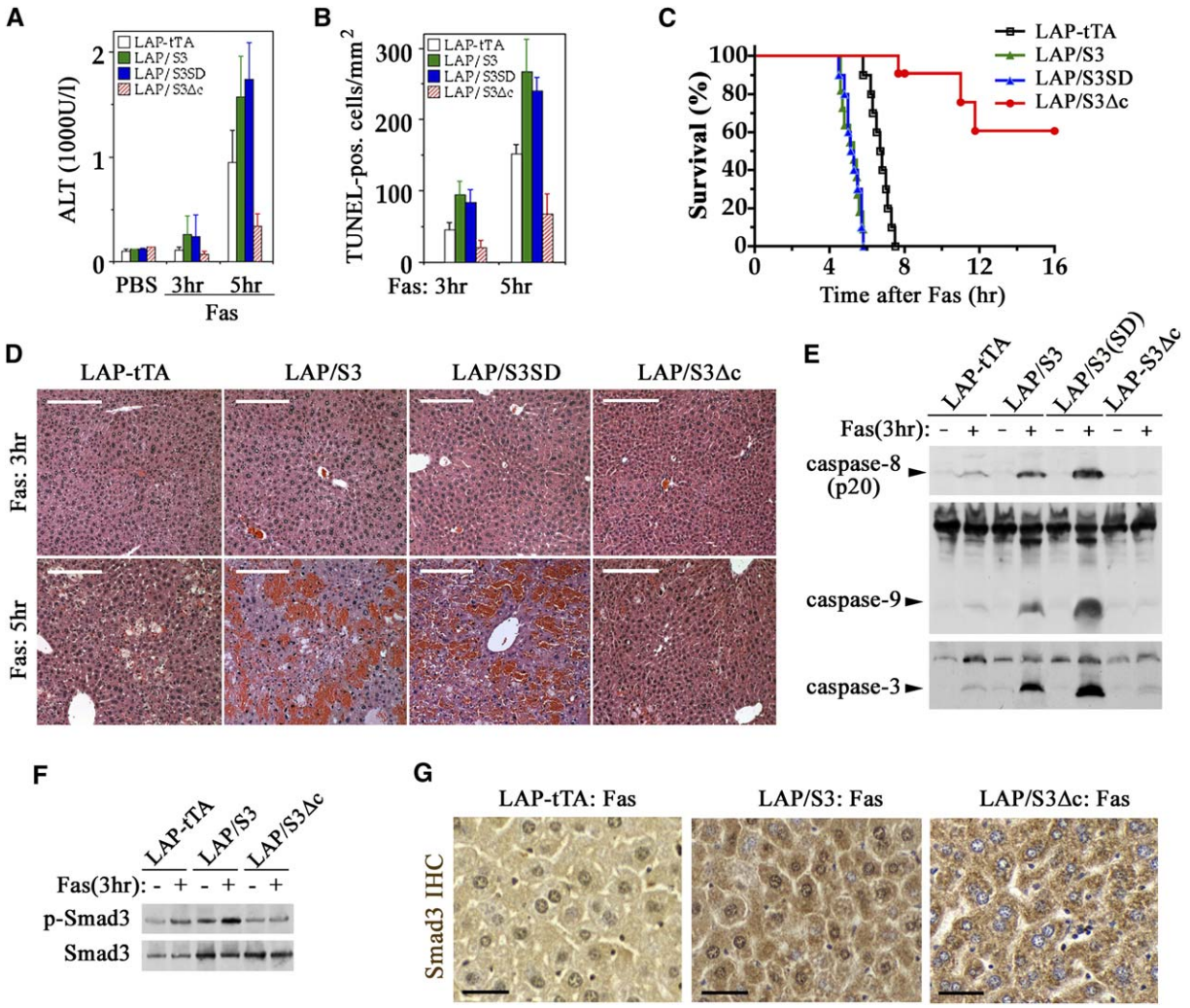

Figure 4. Smad3 enhances the responsiveness of hepatocytes to Fas-mediated apoptosis

A: Liver function of transgenic mice (n = 4) measured as serum alanine aminotransferase (ALT) activity (n = 4).

B: Quantification of TUNEL-positive cells in liver sections of transgenic mice (n = 4) after Fas activation. Mean values compiled after counting five magnification (20×) fields for each liver are shown.

C: Kaplan-Meyer survival curve of each transgenic strain after Fas activation (n = 10).

D: H&E staining of liver sections after Fas activation. Scale bar, 250 μm.

E: Western analysis of caspase cleavage in liver extracts. Arrowheads denote cleaved products of caspases.

F: Western analysis of phospho-Smad3 and total Smad3 in liver extracts.

G: Immunohistochemistry staining of Smad3 (brown) in liver sections 3 hr after Jo-2 injection. Scale bar, 50 μm. Note that wild-type Smad3 (endogenous or transgenic) in LAP-tTA and LAP/S3 accumulated in the nucleus after Fas was activated, while total Smad3 in LAP/S3Δc mice was still retained in the cytoplasm of hepatocytes.

Error bars indicate mean ± standard deviation.

or Smad3SD and diminished by Smad3Δc (Figure 6B), suggesting that Bcl-2 is regulated transcriptionally by Smad3. Similarly, immunohistochemistry staining of Bcl-2 appeared to be decreased in liver tumor sections derived from LAP/S3 and LAP/S3SD mice compared to a persistent high level in tumor sections from LAP-tTA and LAP/S3Δc mice (Figure 6C). This finding is consistent with the apoptotic indexes exhibited by the tumor samples. To investigate whether the same TGF-β/Smad3-mediated repression of Bcl-2 also operates in human liver tumor cells, we expressed N-terminal FLAG-tagged Smad3 and Smad3Δc in human hepatoma SK-Hep-1 cells by retrovirus-mediated transduction and examined the Bcl-2 protein levels in these cells. TGF-β treatment decreased the level of Bcl-2 protein in the SK-Hep-1 cells infected with an empty vector; this reduction was enhanced by forced expression of Smad3 while reversed by Smad3Δc (Figure 6D). To test whether the TGF-β-induced apoptosis can be accounted for by reduction of the Bcl-2 level, we infected SK-Hep-1 cells and Hep3B cells, another line of human hepatoma, with recombinant adenoviruses carrying either a copy of the Bcl-2 cDNA or the control β-galactosidase cDNA. Forced expression of Bcl-2 in both lines of hepatoma cells inhibited the apoptotic response induced by TGF-β (Figure 6E). Taken together, the above results indicate that TGF-β/Smad3 signaling has a direct role in controlling the cellular level of Bcl-2, which is critical for the TGF-β-mediated apoptosis in the liver.

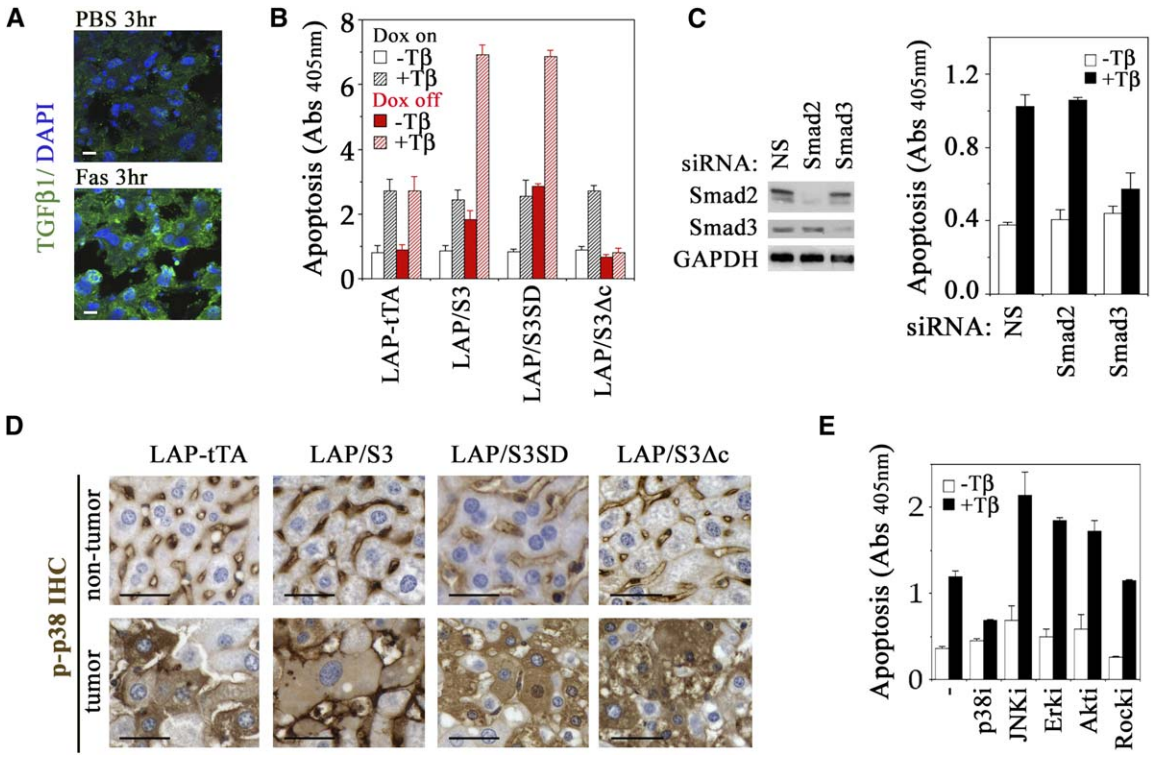

Figure 5. Dual requirements of Smad3 and p38 MAPK in TGF-β-induced apoptosis

A: Immunostaining of active TGF-β1 in mock or Fas-activated liver sections as in Figure 3A. Scale bar, 10 μm.

B: ELISA assay of DNA fragmentation (Cell Death Detection Kit, Roche) as a function of apoptosis in primary hepatocytes isolated from transgenic mice in the presence or absence of Dox, and/or TGF-β.

C: Specific requirement of Smad3 in TGF-β-induced apoptosis in primary hepatocytes. The specificity of Smad siRNAs is shown in the Western analysis of siRNA-transfected hepatocytes in the left panel. NS, nonsilencing control siRNA.

D: Immunohistochemistry staining of phospho-p38 in liver sections. Note that the weak brown staining of phospho-p38 MAPK is concentrated in the interstitial stellate cells in nontumor tissue, whereas it is highly induced in hepatocytes in the tumors. Scale bar, 50 μm.

E: Requirement of p38 MAPK but not JNK, Erk, Akt, or ROCK in TGF-β-induced apoptosis in primary hepatocytes. Cell death was monitored by ELISA assay as in **B**.

Error bars indicate mean ± standard deviation.

Transcriptional repression of Bcl-2 by Smad3

Previously, a decrease of Bcl-2 levels has been observed in TGF-β-mediated apoptosis in several other types of cells (Sánchez-Capelo, 2005). However, the underlying molecular mechanism has not been addressed, nor is it clear whether this reduction is due to direct transcriptional repression by TGF-β signaling. In mammals, transcription of the *Bcl-2* gene is driven by a TATA-less, upstream promoter (P1), and a downstream promoter (P2) (Heckman et al., 2000). When assayed in the Hep3B line of human hepatoma cells, we found that the downstream P2 promoter was repressed by TGF-β (Figure 7A); this repression was enhanced by cotransfected Smad3 but completely blocked by Smad3ΔC (Figure 7B). Similar results were obtained in SK-Hep-1 cells (Figure 7B). This observation was further substantiated in a similar experiment using Smad-specific siRNAs, in which the TGF-β-induced repression of the P2 promoter was specifically blocked by interfering with the function of endogenous Smad3 but not Smad2 (Figure 7C). Consistent with the promoter reporter assays, Smad3- but not Smad2-specific siRNA also diminished the TGF-β-mediated downregulation of endogenous Bcl-2 protein levels (Figure 7D). Thus, Smad3 is essential for the transcriptional repression of Bcl-2 and apoptosis induced by TGF-β.

To delineate the molecular mechanism of Smad3-mediated transcriptional repression of Bcl-2, we conducted promoter mapping studies in transfected Hep3B cells and identified a region between −802 bp and −747 bp upstream from the translational initiation site of the *Bcl-2* gene (Figure 8A). This region encompasses three overlapping copies of a GC-rich repeat, which is conserved between the human and mouse genes and is identical to the *Drosophila* Mad binding element, GCCGnCGc (Kim et al., 1997). Mutation studies showed that altering three nucleotides in the GC-rich region was sufficient to abolish the TGF-β/Smad3-mediated repression (Figure 8B), and the significance of these Smad binding elements was further demonstrated in an experiment employing Smad3 siRNA, which completely ablated the TGF-β-mediated repression of the wild-type *Bcl-2* reporter (−802) while having no effect on the mutant reporter (−802m) (Figure 8C). Incubating a Smad3 DNA binding domain fusion protein, GST-Smad3NL, with a radioactively labeled double-stranded DNA probe containing the GC-rich region, resulted in an electrophoretic mobility shift that could be competed away with unlabeled competitor DNA but not its mutant variants (Figure 8D). Addition of Smad3 antibody caused a supershift of the DNA-protein complex, suggesting a specific binding between Smad3 and the DNA element. To determine

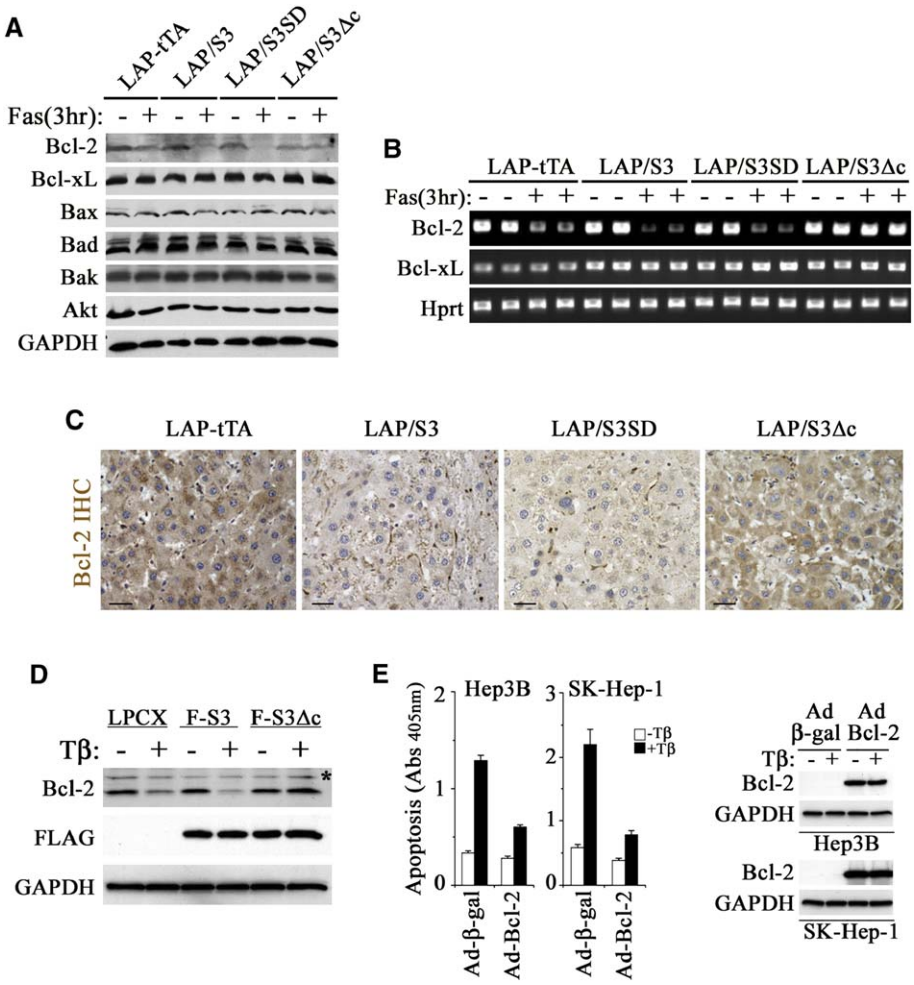

Figure 6. Smad3 downregulates Bcl-2 expression

A: Western analysis of different Bcl-2 family members and Akt in liver extracts from mice treated with or without Jo-2.

B: RT-PCR analysis of Bcl-2 and Bcl-xL mRNA in liver extracts of **A**. RNA from two different livers was used for each group.

C: Immunohistochemistry staining of Bcl-2 in liver tumors after 9 months of DEN/Pb treatment. Representative data of four tumor samples in each cohort are shown. Scale bar, 100 μm.

D: Western analysis of endogenous Bcl-2 protein levels in retrovirus-infected SK-Hep-1 cells expressing LPCX vector, FLAG-tagged Smad3, or Smad3Δc. Cell lysates were collected after TGF-β treatment for 24 hr. Expression of FLAG-tagged Smad3 or Smad3Δc is shown in the middle panel. Endogenous GAPDH was used as the loading control (bottom panel). *Nonspecific band.

E: Suppression of TGF-β-induced apoptosis in Hep3B and SK-Hep-1 cells by adenovirus-mediated expression of Bcl-2. Expression of Bcl-2 in the Hep3B and SK-Hep-1 cell lysates was confirmed by a Western analysis in right panel. Note that TGF-β treatment did not affect the level of exogenous Bcl-2 protein.

Error bars indicate mean ± standard deviation.

whether this interaction occurs in vivo, we performed a chromatin immunoprecipitation (ChIP) assay first in liver tissues isolated from transgenic animals that had been injected with Jo-2 antibody or saline control. After precipitation with Smad3 antibody and amplification by PCR, a DNA fragment containing the GC-rich region was detected in Fas-activated liver extracts obtained from LAP-tTA, LAP/S3, and LAP/S3SD but not LAP-S3ΔC mice (Figure 8E). In a reciprocal experiment, we performed the ChIP assay with an antibody specific for acetylated histone H4, which is generally associated with transcription-active promoter elements (Grunstein, 1997). In this case, the DNA fragment containing the GC-rich region was amplified from all samples except the Fas-activated LAP-tTA, LAP/S3, or LAP/S3SD extracts (Figure 8E). We next repeated this entire set of experiments directly in isolated primary hepatocytes cultured in the absence of Dox. After immunoprecipitation with an anti-Smad3 antibody, the DNA fragment containing the GC-rich region of *Bcl-2* promoter was amplified from lysates of hepatocytes isolated from LAP-tTA, LAP/S3, and LAP/S3SD, but not LAP/S3Δc mice and only when the cells were pretreated with TGF-β (Figure 8F). Clearly, the interaction between Smad3 and the *Bcl-2* P2 promoter correlated with a repressed state of transcription in vivo as demonstrated by ChIP assay using the acetylated histone H4-specific antibody. Taken together, these results demonstrate that Bcl-2 is a bona fide physiological target of TGF-β/Smad3 signaling and suggest that activation of TGF-β/Smad3 signaling amplifies the effect of proapoptotic cues such as death receptor activation or carcinogenesis through transcriptional repression of Bcl-2.

Discussion

TGF-β-induced apoptosis plays an important role in many biological processes such as involution of mammary epithelium, wound healing, elimination of immature lymphocytes, and liver regenerative growth, but the molecular path that leads to its cellular context-dependent activation is not well defined. Unlike death receptor ligands, engagement of the TGF-β signaling pathway does not cause obligatory apoptosis. For this reason, TGF-β-induced apoptosis of cultured cells invariably requires simultaneous removal of serum survival factors. Recent data suggest that the proapoptotic activity of Smad3 is regulated by PKB/Akt in response to cell survival factors via a kinase-independent interaction, which restrains Smad3 from entering the nucleus and activating apoptosis (Conery et al., 2004; Remy et al., 2004). This model is useful in explaining the sensitivity of cultured cells to TGF-β-induced apoptosis but does not specify a mechanism for the TGF-β/Smad3 action. In the current study, we created conditions in which the Smad3 activity in hepatocytes was manipulated by ectopic expression of wild-type

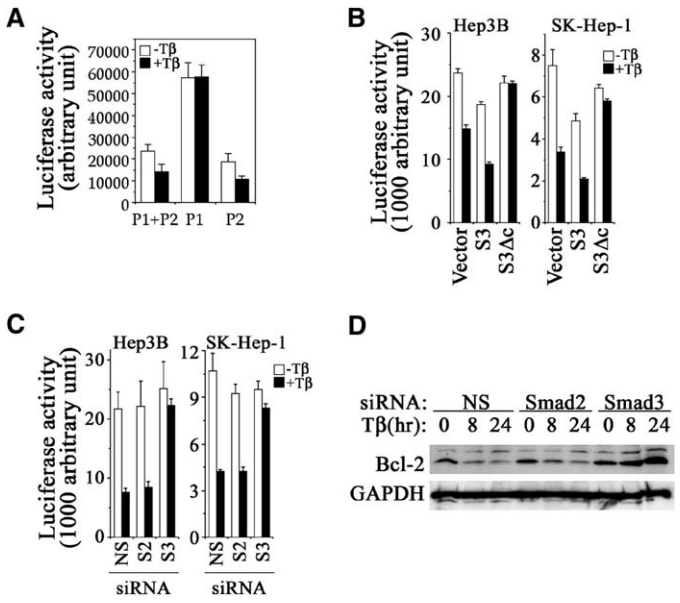

Figure 7. Smad3 represses Bcl-2 transcription

A: Luciferase assays of different *Bcl-2* promoter reporters in transfected Hep3B cells. One day after transfection, the cells were treated with TGF-β for 20 hr.

B: Luciferase assays of the *Bcl-2* P2 (−1278) promoter reporter in transfected Hep3B or SK-Hep-1 cells as in **A**.

C: Luciferase assays of the Bcl-2 P2 (−1278) promoter reporter in the presence of nonsilence control (NS), Smad2, or Smad3 siRNA. The siRNAs were cotransfected with reporter DNAs, and 2 days after transfection, the cells were treated with TGF-β for 20 hr.

D: Western analysis of Bcl-2 expression in SK-Hep-1 cell lysates after 2 days of the siRNA transfection.

Error bars indicate mean ± standard deviation.

Smad3, or its dominant-negative or constitutively active mutant. We show here that the responsiveness of hepatocytes to apoptotic stimuli changes according to the expression of Smad3 transgenes, and we present evidence that identifies Bcl-2 as a direct transcriptional target of the proapoptotic activity of Smad3. These findings are consistent with the previous notion that TGF-β-induced apoptosis is accompanied by reduced mitochondrial transmembrane potential and release of cytochrome c (Freathy et al., 2000). Thus, by attenuating the level of a major apoptosis inhibitor, Bcl-2, TGF-β/Smad3 signaling facilitates a permissive cellular context that is conducive to apoptosis.

In previous cell culture and biochemical studies, Smad3SD with two phosphorylation-mimicking aspartic acid substitutions behaves as a constitutively active mutant and is able to form a homo- or heterotrimeric Smad complex, suggesting that C-terminal phosphorylation is sufficient to activate the transcriptional activity of Smad3 (Feng et al., 1998; Chacko et al., 2001). Our current data indicate, however, that while Smad3SD is nuclear, it does not induce apoptosis by itself in normal hepatocytes. It is possible that either C-terminal phosphorylation alone is not sufficient to fully activate the proapoptotic activity of Smad3, or an independent apoptotic input is required, given the auxiliary nature of TGF-β signaling in apoptosis. Indeed, our data show that p38 MAPK is preferentially activated in liver tumor cells that have the propensity to sustain a high rate of apoptosis as result of ectopic expression of Smad3, and activation of p38 MAPK is required for TGF-β-induced apoptosis in normal hepatocytes. At present, it is not clear whether p38 MAPK is activated in liver tumors by a Smad-independent, noncanonical TGF-β signaling mechanism as we reported previously in mouse mammary epithelial cells (Yu et al., 2002), or it is activated by mitogenic cues emanated from tumor milieu. Nevertheless, p38 MAPK is capable of phosphorylating Smad3 (Mori et al., 2004) or otherwise activating the apoptotic program by other means (Zarubin and Han, 2005).

Although Smad3 was discovered 10 years ago as a major intracellular mediator of TGF-β signaling (Zhang et al., 1996), the role of Smad3 in tumorigenesis still remains elusive. Human genetic studies have revealed both point mutations and deletions of Smad2 and Smad4 in several types of cancers, but the inactivating mutation of Smad3 has yet to be found (Riggins et al., 1997). A recent study of T cell acute lymphoblastic leukemia indicates that Smad3 protein, but not mRNA, is absent, suggesting that changes in the Smad3 protein level rather than mRNA may have an impact on carcinogenesis (Wolfraim et al., 2004). Because target inactivation of Smad3 alleles causes premature death in young adult mice due, at least in part, to compromised immune functions (Yang et al., 1999), we elected to express Smad3 and its mutants specifically in desired tissues to circumvent the obstacle presented by the loss-of-function approach to investigating the role of Smad3 in tumorigenesis. Our data indicate that ectopic expression of Smad3 reduces liver susceptibility to chemically induced carcinogenesis, providing direct in vivo evidence for a tumor suppression role of Smad3.

HCC presents a major world public health problem because of its prevalence in Asia, the Middle East, and Africa, and yet the genetic lesions behind each step along the oncogenic transformation of hepatocytes are not well understood due to the complexity of HCC's etiological origin (Thorgeirsson and Grisham, 2002; Block et al., 2003). Our finding that ectopic expression of Smad3 can reduce liver susceptibility to carcinogenesis in a chemically induced murine HCC model raises a hope of designing a therapeutic strategy for treating HCC in human patients by invoking the TGF-β-induced apoptosis irrespective of the underlying genetic causes. Escaping apoptosis is a critical step in the progression to full malignancy of cancers, which must overcome multiple fail-safe genetic controls. Upregulation of Bcl-2 is one of the traits widely acquired by cancer cells to evade apoptosis, as exemplified in follicular lymphoma, in which a chromosome translocation brings the Bcl-2 structural gene under the control of a constitutively active immunoglobulin gene promoter (Graninger et al., 1987). The significance of Bcl-2 as an anticancer target is reflected by the sheer number of clinical trials designed for various strategies aiming at inactivating Bcl-2 (Reed, 2002; Oltersdorf et al., 2005). However, direct therapeutic use of TGF-β itself may be problematic because it has a complex role in liver physiology. Prolonged exposure to active TGF-β1 increases liver fibrosis and accelerates progression to cirrhosis, and also results in multiple extrahepatic lesions due to elevated serum TGF-β levels (Sanderson et al., 1995; Ueberham et al., 2003). In contrast, our current study indicates that Smad3, even when it is ectopically expressed at high levels, remains dormant in normal adult hepatocytes and causes no obvious health problems during the expected life span of the transgenic animals, making Smad3 a suitable gene therapeutic agent for managing liver tumor growth.

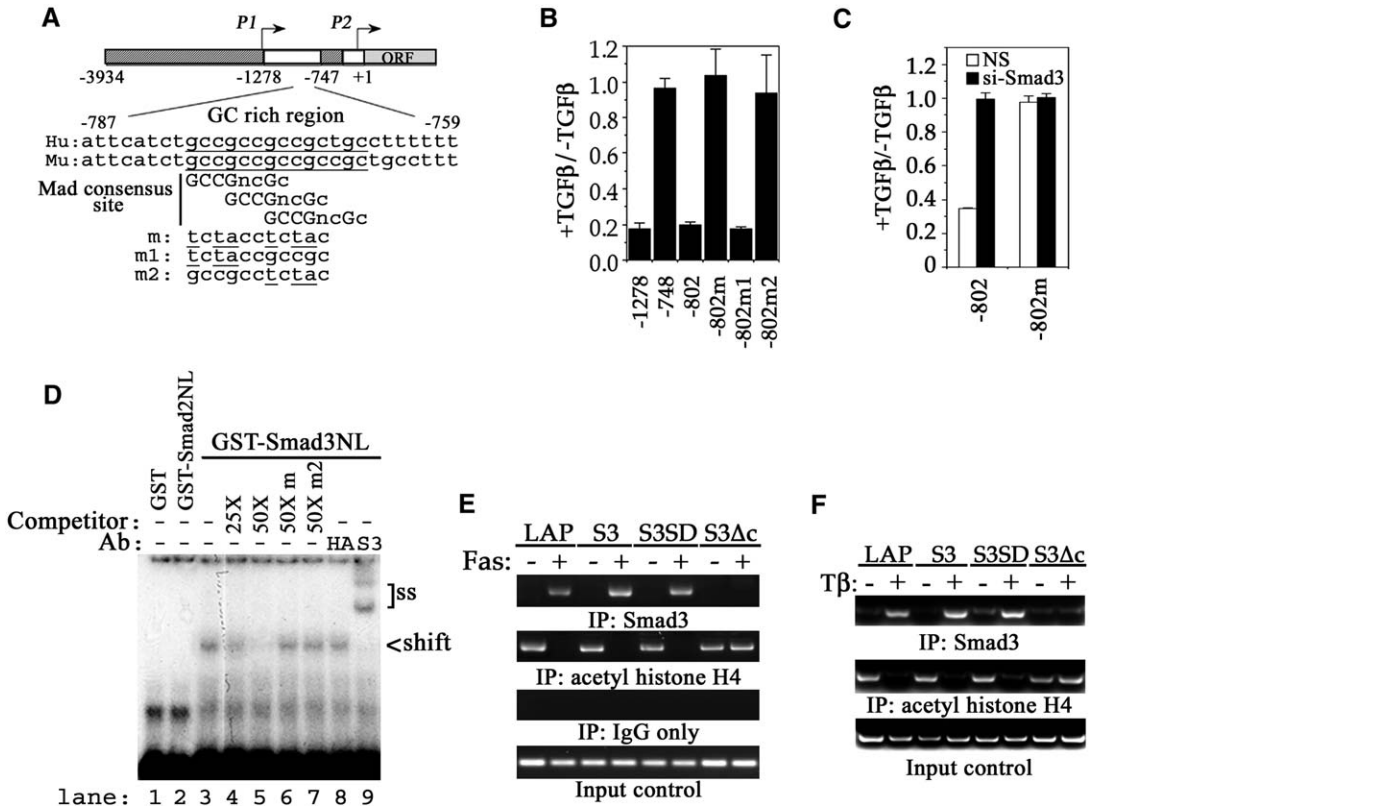

Figure 8. Smad3 specifically represses Bcl-2 transcription via a GC-rich Smad binding element

A: Schematic representation of Bcl-2 promoters. Nucleotide coordinates are anchored to the Bcl-2 translational initiation site. The conserved GC-rich element is underlined, and the *Drosophila* Mad binding consensus and inactivating mutations are shown.

B: Mapping of TGF-β-responsive element in the Bcl-2 promoter region in transfected Hep3B cells. TGF-β treatment lasted for 20 hr, and the promoter activity was presented as the ratio of luciferase activity between TGF-β-treated and nontreated samples.

C: Reversal of the TGF-β-mediated transcription repression of Bcl-2 −802 reporter by Smad3-specific siRNA. Note that neither TGF-β nor Smad3 siRNA had any effect on Bcl-2 *−802m* reporter, in which the GC-rich element was mutated.

D: Electrophoresis mobility shift assay for specific interaction between the purified GST-Smad3 DNA binding domain (Smad3NL) and DNA oligos containing the GC-rich element. GST and GST-Smad2NL were used as controls. Competition by a 50-fold excess of unlabeled wild-type but not mutant (m or m2 as in **A**) DNA probe, and supershift by anti-Smad3 antibody indicate specific binding of Smad3NL to the DNA. DNA-protein (shift) and antibody-induced supershift (ss) complexes are marked.

E: ChIP assay for binding of Smad3 to the Bcl-2 promoter in vivo in the Fas-activated liver tissues. Animals were treated with Jo-2 antibody for 3 hr prior to being sacrificed. PCR primers for amplifying Bcl-2 promoter are specified in the Experimental Procedures.

F: ChIP assay for binding of Smad3 to the Bcl-2 promoter in vivo in primary hepatocytes. Prior to TGF-β treatment for 1 hr, cells were cultured in the absence of Dox for 24 hr to allow for the expression of Smad3 transgenes.

Error bars indicate mean ± standard deviation.

Experimental procedures

Transgenic mice

LAP-tTA mice were originally generated in H. Bujard's laboratory (Kistner et al., 1996) and bred to FVB/N predominant background. Plasmid pTRE-Smad3, pTRE-F-Smad3SD, and pTRE-F-Smad3Δc were constructed by subcloning wild-type Smad3, FLAG-tagged Smad3SD, or FLAG-tagged Smad3ΔSSVS cDNA into pTRE2 (CLONTECH), respectively. Pronuclear injection of linearized pTRE-Smad3, pTRE-F-Smad3SD, and pTRE-F-Smad3Δc into FVB/N oocytes was performed at the NCI transgenic core facility. Founder mice were identified by Southern blot of genomic DNA prepared from tails. PCR genotyping primers for identifying tTA transgenes were described (Kistner et al., 1996) and for Smad3 transgenes are 5′-GTCAGATCGCCTGGA GACGC-3′ and 5′-GTTTCTCCATCTTCACT-3′, which amplify a 698 bp fragment for TRE-Smad3 and a 717 bp fragment for TRE-F-Smad3SD and TRE-F-Smad3Δc, respectively. All mice were maintained and handled according to protocols approved by the NCI Animal Care and Use Committee.

RT-PCR

Total RNA for RT-PCR analysis was prepared from liver tissues using TRIzol (Invitrogen). Total RNA (2.0 μg) was converted into cDNA using the ThermoScript RT-PCR system (Invitrogen), and about one-tenth of the cDNA was used in each PCR reaction. Primers are as follows: Smad3 transgenes, 5′-AGCAGTGGAGCTGACACGGA-3′ and 5′-GGAGTGAATTCTTTG CCAAA-3′; Bcl-2, 5′-TTCTCTCGTCGCTACCGTCG-3′ and 5′-CACTTGTGG CCCAGGTATGC-3′; Bcl-xl, 5′-TTCCGACTGAAGAGTGAGCC-3′ and 5′-TTCTCTCGTCGCTACCGTCG-3′; Hprt, 5′-TATGGACAGGACTGAAAGAC-3′ and 5′-TAATCCAGCAGGTCAGCAAA-3′.

Tumorigenesis and liver function assay

Expression of Smad3 transgenes in mice was activated by dietary withdrawal of doxycycline (Dox) at 3 weeks of age. Diethylnitrosamine (Sigma) was made into a 100 mg/ml solution with 0.9% NaCl, and a single dose was injected at 100 mg/kg body weight intraperitoneally into male mice at 5 weeks of age to initiate tumor formation. Formation of tumors was promoted with 0.07% phenobarbital (Sigma) given in diet when mice were 9 weeks old until mice were sacrificed. Liver function was monitored by measuring serum alanine aminotransferase (ALT) activities.

Histology and immunohistochemistry

Tissues were fixed in 10% buffered formalin, and 5 μm paraffin sections were stained with hematoxylin and eosin (H&E). Tumors were counted in

H&E-stained sections obtained from four different liver lobes for each mouse. For immunohistochemistry, antibodies for Smad3 (Zymed), Bcl-2 (Santa Cruz Biotech), Ki67 (Novocastra), phospho-JNK, and phospho-p38 (Cell Signaling Technology) were used, and enzymatic staining (brown color) was performed using DAB (Vector Lab) with hematoxylin counterstaining for nuclei (blue color). Staining for active TGF-β1 was performed in frozen liver sections as described (Ewan et al., 2002).

Hepatocyte isolation, cell culture, apoptosis, and proliferation assay

Hepatocytes were isolated by a two-step collagenase perfusion of the liver (Kao et al., 1996) from mice fed with a diet containing Dox (Bioserve). Dissociated cells were then plated in Collagen I-coated dishes in DMEM/F12 medium supplemented with 10% FBS, 2 μg/ml Dox, and ITS$^+$ (Collaborative Research). To quantify apoptosis or proliferation, the cells were incubated for 24 hr in the DMEM/F12 medium containing 0.2% FBS in the presence or absence of Dox before harvesting for assay. Hep3B and SK-Hep-1 cells were obtained from ATCC. Apoptosis assay was performed with the Cell Death Detection ELISA assay kit (Roche), which quantitatively determines DNA fragmentation using both anti-histone and peroxidase-conjugated anti-DNA antibodies. Whenever indicated, TGF-β at 4 ng/ml was added to the medium, and chemical inhibitors (Calbiochem) for p38 MAPK (SB 203580), JNK (SP600125), ROCK (Y27632), Erk (U0126), and Akt were used at 10 μM each. Proliferation assays were carried out with ^{3}H-thymidine labeling as described (Danielpour et al., 1989).

ChIP and electrophoretic mobility shift assay

ChIP assays were carried out with a ChIP assay kit (Upstate). A pair of primers (5′-ACAGGACTTCTGCAAATGCT-3′ and 5′-AACCAGAGATCTCAA GAGCA-3′ were used to amplify a 98 bp fragment encompassing the GC-rich repeat of the mouse Bcl-2 P2 promoter. Electrophoretic mobility shift assay was carried out as described (Zhang et al., 1998). Complementary DNA oligos corresponding to the mouse sequence shown in Figure 7A were annealed and labeled with γ^{32}P-ATP. One microliter of antibodies against Smad3 or HA epitope tag was used in the supershift studies.

Transcription reporter assay and knockdown experiments

Luciferase reporter constructs containing −3934/−8, −3934/−1287, and −1278/−8 segments of the Bcl-2 promoter were described by L. Boxer's laboratory (Heckman et al., 2000). The luciferase construct containing the −747/−8 segment was made by digestion of the −1278/−8 construct with SacI followed by ligation. Luciferase constructs containing the −802/−8 segment and the mutants shown in Figure 4D were made by attaching annealed oligos containing respective mutations at the SacI site of the −747/−8 construct. Numbering of these constructs is relative to the translation start site. Transfection of Hep3B or SK-Hep-1 cells was performed with Fugene 6 (Roche) in 12-well plates. Oligofectamine (Invitrogen) was used for siRNA transfection experiments (Elbashir et al., 2001). The target sequences of siRNA (Cellogenetics) against both human and mouse Smad2 and Smad3 are 5′-AACAGGCCTTTACAGCTTCTC-3′ and 5′-AAGGCCATCACCACGCA GAAC-3′, respectively. The nonsilencing siRNA, which does not target any known mammalian gene, was used as the control.

In vivo Jo-2 injection and apoptosis assay

Fas-induced cell death was initiated by intraperitoneal injection of Jo-2 mAb (Pharmingen) at 0.15 mg/kg body weight into mice 3–4 months of age. TUNEL assay on paraffin sections was performed with the In Situ Cell Death Detection Kit (Roche) for demonstrating apoptotic cell death in the liver. Liver lysates were used in Western analyses of caspase cleavage with antibodies specific for caspase-8 (Santa Cruz Biotech), caspase-9, and caspase-3 (Cell Signaling Technology).

Retroviral and adenoviral infections

LPCX-puro derived retroviral vectors were used for expressing FLAG-tagged Smad3 and Smad3Δc as described (Choy et al., 2000). Two days after infection of SK-Hep-1 cells, puromycin (2.5 μg/ml) was added for 3 days, and the selected cells were then plated in 12-well plates for Western blot. Recombinant adenovirus for Bcl-2 was kindly provided by Drs. K. Suh and S. Yuspa (Suh et al., 2004), and infections of Hep3B and SK-Hep-1 cells were conducted as described (Fujii et al., 1999).

Statistical analysis

Student's t test was used for statistical analysis. All bar graphs are displayed as mean ± standard deviation.

Supplemental data

The Supplemental Data include two supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/6/445/DC1/.

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