Transcriptional regulation of STAT3 by SPTBN1 and SMAD3 in HCC through cAMP-response element-binding proteins ATF3 and CREB2

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The cytoskeletal protein Spectrin, beta, non-erythrocytic 1 (SPTBN1), an adapter protein to SMAD3 in TGF-β signaling, may prevent hepatocellular carcinoma (HCC) development by downregulating the expression of signal transducer and activator of transcription 3 (STAT3). To elucidate the as yet undefined mechanisms that regulate this process, we present that higher levels of STAT3 transcription are found in livers of heterozygous SPTBN1+/− mice as compared to that of wild type mice. We also found increased levels of STAT3 mRNA, STAT3 protein, and p-STAT3 in human HCC cell-lines after knockdown of SPTBN1 or SMAD3, which promoted cell colony formation. Inhibition of STAT3 overrode the increase in cell colony formation due to knockdown of SPTBN1 or SMAD3. We also found that inhibition of SPTBN1 or SMAD3 upregulated STAT3 promoter activity in HCC cell-lines, which is dependent upon the cAMP-response element (CRE) and STAT-binding element (SBE) sites of the STAT3 promoter. Mechanistically, suppression of SPTBN1 and SMAD3 augmented the transcription of STAT3 by upregulating the CRE-binding proteins ATF3 and CREB2 and augmented the binding of those proteins to the regions within or upstream of the CRE site of the STAT3 promoter. Finally, in human HCC tissues, SPTBN1 expression correlated negatively with expression levels of STAT3, ATF3, and CREB2; SMAD3 expression correlated negatively with STAT3 expression; and the level of phosphorylated SMAD3 (p-SMAD3) correlated negatively with ATF3 and CREB2 protein levels. SPTBN1 and SMAD3 collaborate with CRE-binding transcription factors to inhibit STAT3, thereby preventing HCC development.

Introduction

HCC is the third-most common cause of cancer-related mortality worldwide (1). HCC develops as the result of the progressive accumulation of genetic and epigenetic alterations. These alterations commonly deregulate growth factor-mediated signaling pathways in liver cells, which in turn mediate the development and progression of liver cancer (2).

Transforming growth factor-β (TGF-β) is one of the key growth factors that are deregulated in liver cancer (3). TGF-β has a dual role in HCC development and progression, both as a tumor suppressor that regulates cell proliferation and as a tumor promoter that enhances cancer motility and invasion. Although this dichotomy is well known, the mechanisms by which these dual functions are regulated are not yet understood. It is hypothesized that cancer cells can escape the suppressor function of TGF-β while remaining sensitive to the promoter effect. Studies on the tumor suppressor function of TGF-β have focused on defects in the classical TGF-β signaling pathway in cancer (4,5). The classical pathway of TGF-β signaling transduction occurs through TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII), which function as a heteromeric complex following the phosphorylation of TβRI. Phosphorylated TβRI subsequently phosphorylates SMAD2 and SMAD3, which then translocate to the nucleus, form a complex with SMAD4, and bind to DNA to regulate gene transcription (6). Multiple adaptor proteins, including the Smad anchor for receptor activation (SARA), filamin, and Spectrin, beta, non-erythrocytic 1 [SPTBN1, previously also known as ELF or β5SFP (7,8)], can modulate the activation of SMAD2 and SMAD3 (7). SPTBN1, which was first discovered in embryonic liver development, associates with SMAD3 within the cytoplasmic domain of the TβRI complex. This process is important for the propagation of TGF-β signaling. Disruption of SPTBN1 (SPTBN1−/−) in mice leads to the development of spontaneous liver cancer, suggesting that SPTBN1 functions as a tumor suppressor to prevent liver cancer development (7).

In human HCC tissues with aberrant TGF-β signaling, expression of interleukin 6 (IL-6) and signal transducer and activator of transcription 3 (STAT3) are increased as compared with that in human HCC tissues with normal TGF-β signaling (9). These studies also demonstrated a correlation between downregulated SPTBN1 expression and increased IL-6/STAT3 expression. However, the intrinsic regulatory mechanism by which attenuation of the TGF-β signaling pathway leads to STAT3 upregulation, particularly the roles of SPTBN1 and the related protein SMAD3 in this mechanism, is largely unclear. Research on the role of STAT3 as an oncogene is focused predominantly on post-translational regulation of STAT3. Although STAT3 expression is elevated in many tumors, including HCC, the regulation of STAT3 de novo biosynthesis has received less attention. In this study, we examine the roles of SPTBN1 and SMAD3 in STAT3 regulation in liver tissues from SPTBN1−/− mice, human HCC cell-lines and in a database of human HCC and human HCC tissues. Here, we demonstrate that transcription of STAT3 increases when expression levels of SPTBN1 and SMAD3 are downregulated and that the CRE-binding proteins ATF3 and CREB2 are required for this process.

Materials and methods

Cell culture

All cells were grown in a humidifier in the presence of 5% CO2 at 37°C and cell culture medium was supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Human HCC cell lines, PLC/PRF/5 (CRL-8024), SNU-449 (CRL-2234), and SNU-475 (CRL-2236), were purchased from American Type Culture Collection (ATCC) (Manassas, VA).

Small interfering RNA knockdown and the generation of stable cell-lines expressing SPTBN1-specific short hairpin RNA

RNA interference experiments were performed using either synthesized small interfering RNA (siRNA) (5′-GGAGTTCAGAGGACGTCTAGTATC-3′) that targets the first exon of SPTBN1 (Life Technologies, Grand Island, NY) or siRNAs for SMAD3, ATF3, and CREB2 (Santa Cruz Biotechnology, Santa Cruz, CA) as well as control siRNA (Life Technologies). To generate stable SPTBN1 knockdown cell lines, vectors expressing short hairpin RNA (shRNA) against SPTBN1 exon 1 or non-specific shRNA were generated using a PRS retroviral vector (OriGene Technologies, Rockville, MD). The shRNA constructs were transduced into PLC/PRF/5 cells. Forty-eight hours after transfection, cells were subjected to selection with 2 μg/ml puromycin over 3 weeks.

Quantitative real-time reverse-transcription polymerase chain reaction (QRT-PCR) and reverse transcription PCR (RT-PCR)

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). First strand cDNAs were synthesized using TaqMan reverse transcription reagents

Abbreviations: ATF, activating transcription factor; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; E-box, enhancer box; HCC, hepatocellular carcinoma; IL-6, interleukin 6; SBE, STAT-binding element; shRNA, short hairpin RNA; siRNA, small interfering RNA; SPTBN1, spectrin, beta, non-erythrocytic 1; STAT3, signal transducer and activator of transcription 3; TβR, TGF-β receptor type; TGF-β, transforming growth factor-β.
β mice were developed in Dr Lopa Mishra’s laboratory (Figure 1B). Groups of mouse liver samples had significantly higher 1 was inhibited when SPTBN1 was suppressed in both PLC/PRF/5, right panel) and suppressed cell (Figure 1D). These cell lines from wild-type mice and from wild-type (Materials and methods). All of cDNA was used as a template of gene expression database (Table 1). The statistical significance of mean value differences was assessed using a Student’s t-test. Differences were regarded as statistically significant if P < 0.05 (*), and as highly statistically significant if P < 0.001 (**). The statistic analysis of gene array database is described under Analysis of gene expression database (Materials and methods). U-test is used to compare the percentage differences of subjects in two groups within multiple cohorts during HCC tissue analysis.

Results
Downregulation of SPTBN1 or SMAD3 elevates STAT3 mRNA expression levels in mouse liver tissues and in human HCC cells
To better understand the interplay between SPTBN1 and STAT3, we employed QRT-PCR to initially compare STAT3 mRNA expression levels in liver tissue from SPTBN1−/− mice and from wild-type (WT) mice. SPTBN1−/− mouse liver samples had significantly higher levels of STAT3 mRNA than in WT mouse liver samples (P < 0.01, Figure 1A).

SPTBN1 is a SMAD3 adaptor during TGF-β signal transduction. Thus, we studied SPTBN1 and SMAD3 knockdown in the human HCC cell-lines PLC/PRF/5 and SNU-449 (14, 15). These cell lines were selected for this study because both cell lines have similar levels of endogenous TGF-β1 and p-SMAD3 (Figure 1B and C, lower panel), and responded to TGF-β treatment similarly, as demonstrated by increased p-SMAD3 (Figure 1E, right panel) and suppressed cell viability with TGF-β treatment (Figure 1E, left panel). HCC cells with knockdown of SPTBN1 or SMAD3 had significantly higher mRNA levels of STAT3, resulting in higher STAT3 protein production and greater expression of active phosphorylated STAT3 (p-STAT3). By contrast, no such results were seen in HCC cells treated with control siRNA (Figure 1B and C).

To study the role of SPTBN1 and SMAD3 in the regulation of STAT3 transcription, we analyzed the cellular distribution of SPTBN1 and SMAD3 in PLC/PRF/5 and SNU-449 cells. p-STAT3 and p-SMAD3 found in the nucleus were used as positive controls (Figure 1D). SPTBN1 as well as SMAD3 were localized in both the cytoplasm and the nucleus, suggesting that SPTBN1 serves as both an adaptor protein to SMAD3 in the cytoplasm and as a co-repressor in the regulation of STAT3 gene transcription in the nucleus (Figure 1D). Interestingly, we also found that the endogenous expression of TGF-β1 was inhibited when SPTBN1 was suppressed in both PLC/PRF/5 and SNU-449 cells (Figure 1B and C).
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Fig. 1. Suppression of SPTBN1 or SMAD3 leads to upregulation of STAT3 mRNA expression levels. (A) QRT-PCR analysis of STAT3 mRNA in WT and SPTBN1+/− mouse liver tissues. The two bars represent the mean ± SD fold change in STAT3 mRNA expression levels from five WT mice and five SPTBN1+/− mice, respectively. Statistical significance of the difference in STAT3 mRNA expression levels between WT and SPTBN1+/− mice was evaluated by Student's t-test (**P < 0.01). (B, C) Human PLC/PRF/5 and SNU-449 cells were treated with siRNAs as indicated for 48 h, and the cells were subjected to RT-PCR (upper), QRT-PCR (middle), and western blot (lower) analysis. GAPDH served as an internal control. The intensities of proteins were measured by ImageJ software (National Institutes of Health, Bethesda, MD), and then normalized relative to the control siRNA group. Relative expression units (STAT3/actin) are shown under the lower panels. Significance of the differences of QRT-PCR analysis were evaluated by Student's t-test by comparison with the cells transfected with control siRNA (***P < 0.001, **P < 0.01). (D) Subcellular localization of SPTBN1 and SMAD3 in human HCC cells. PLC/PRF/5 and SNU-449 cells were cultured for 48 h. Cells were then fractionated into cytoplasmic (C) and nuclear (N) fractions, and the subcellular distribution of SPTBN1, SMAD3, p-SMAD3, STAT3, and p-STAT3 was assessed by western blotting. The purities of both the cytosolic and nuclear fractions were verified with anti-α-tubulin and anti-lamin B antibodies, respectively. W, whole-cell lysate. (E) Growth inhibitory response to TGF-β1 treatment in PLC/PRF/5 and SNU-449 cells. Both cells were cultured in the absence or presence of TGF-β1 at the doses as indicated for 2, 4, and 6 days and the cell viability was measured in the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). (Left) The expression of p-SMAD3 in PLC/PRF/5 and SNU-449 cells upon the treatment with TGF-β1 for 2 days was assessed by western blotting (right).
Downregulation of SPTBN1 or SMAD3 enhances cell colony formation, cell viability, and the STAT3 downstream gene BIRC3 in human HCC cells

STAT3 is important for the survival and proliferation of cancer stem-like cells. We tested whether altering the expression of SPTBN1 or SMAD3 influences cell viability in low-serum culture conditions and cell colony formation (i.e., anchorage-independent cell growth) in soft agar using PLC/PRF/5 cells. The viability of PLC/PRF/5 cells was slightly reduced in response to overexpression of SPTBN1 or SMAD3 (Supplementary Figure S1A, available at Carcinogenesis Online). Suppression of SPTBN1 or SMAD3 only slightly increased the rate of cell viability (Supplementary Figure S1B, available at Carcinogenesis Online) but doubled the rate of cell colony formation; cell colony formation increased by an additional 20% when cells were transfected with SPTBN1 and SMAD3 siRNAs simultaneously (Figure 2A, left panel). Moreover, colonies were largest in cells treated with SPTBN1 and SMAD3 siRNAs simultaneously followed by that of cells treated with either SPTBN1 or SMAD3 alone, compared to that of those treated with control siRNA (Figure 2A, right panel). Furthermore, although SPTBN1 or SMAD3 siRNA alone increased cell colony formation and cell viability compared with control siRNA, the inhibition of STAT3 by dominant-negative STAT3 overrode those increases (Figure 2B and Supplementary Figure S1B, available at Carcinogenesis Online). Colony formation was also significantly greater in PLC/PRF/5 cells with stable knockdown of SPTBN1 (generated using SPTBN1 shRNA), as shown in two selected clones (SPTBN1-1 and SPTBN1-2), compared with that in PLC/PRF/5 cells stably transfected with non-specific shRNA (Figure 2C). Thus, the upregulation of STAT3 due to suppression of SPTBN1 or SMAD3 significantly promoted cell colony formation but only slightly increased cell viability.

We then investigated the impact of altering SPTBN1 and SMAD3 expression on the STAT3 downstream genes TWIST1, CCNE1, BIRC3 (CIAP2), and XIAP (BIRC4). We observed that BIRC3 mRNA expression was significantly higher when SPTBN1 was inhibited than when it was expressed in both SNU-449 and PLC/PRF/5 cells. BIRC3 mRNA expression was significantly higher in PLC/PRF/5 cells, but not in SNU-449 cells, when SMAD3 was inhibited, suggesting that STAT3-mediated regulation of its downstream genes varies by cell type and cellular context [Figure 2D (16)]. These increases in BIRC3 expression upon suppression of SPTBN1 or SMAD3 were abolished in cells expressing dominant-negative STAT3 (Figure 2E).

Overexpression of SPTBN1 and SMAD3 inhibits STAT3 promoter activity and protein expression in human HCC cells

We next investigated whether SPTBN1 and/or SMAD3 regulated transcription of STAT3 through the modulation of STAT3 promoter activity. We first tested the promoter activity of six luciferase constructs containing different regions of the human STAT3 promoter by transfecting them into SNU-449 cells (10). Of these transfected STAT3 promoter–luciferase constructs, pHST3-Luc-1, -2, and -3 displayed stronger promoter activity than the basic vector pGV-B2; the rest of the luciferase constructs had little to no promoter activity (Figure 3A). These data indicated that DNA segments from −1807 to −261 of the STAT3 promoter were required for transcriptional regulation of the STAT3 promoter in human HCC cells. We then studied the effects of SPTBN1 and SMAD3 on the promoter activity of two STAT3 promoter–luciferase constructs—pHST3-Luc-1, which possesses a long DNA segment of the STAT3 promoter (−1807/+102), and pHST3-Luc-2, which has a short DNA segment of the STAT3 promoter (−694/+102) but retains all important DNA motifs. Overexpression of SPTBN1 and SMAD3 significantly suppressed the STAT3 promoter–luciferase activity of pHST3-Luc-2 but only weakly inhibited that of pHST3-Luc-1 (Figure 3B), suggesting that the −694 to −261 region of the STAT3 promoter was needed to modulate STAT3 transcription by SPTBN1 and SMAD3. Overexpression of both SPTBN1 and SMAD3 led to stronger suppression of STAT3 promoter activity (pHST3-Luc-2) than SPTBN1 or SMAD3 alone (Figure 3B). Treatment of cells expressing pHST3-Luc-2 with IL-6 markedly increased STAT3 promoter activity but this increase was significantly inhibited by overexpression of SPTBN1 and SMAD3 alone or in combination (Figure 3B). Western blot analysis indicated that overexpression of SPTBN1 or SMAD3 alone or in combination substantially inhibited STAT3 protein expression and the level of active p-STAT3 compared with cells transfected with empty vector (Figure 3C).

The CRE and SBE sites in the STAT3 promoter are responsible for the regulation of STAT3 promoter activity by SPTBN1 and SMAD3 in human HCC cells

The region from −694 to −261 of the STAT3 promoter contains several well-known transcription factor-binding sites, including the E-box, CRE, and SBE sites (Figure 3A). To determine which of these DNA motifs are required for SPTBN1 and/or SMAD3 to suppress STAT3 promoter activity, we generated mutations in the E-box, CRE, or SBE sites of pHST3-Luc-2 (Figure 3D). The responsiveness of these mutated promoters to overexpressed SPTBN1 or SMAD3 was assessed in SNU-449 cells. STAT3 promoters with a mutation at either the CRE or SBE site completely lost their responsiveness to the SPTBN1- and/or SMAD3-mediated suppression of STAT3 promoter activity. Furthermore, mutations at either the CRE or SBE site caused resistance to IL-6 stimulation of STAT3 promoter activation, confirming the finding that CRE and SBE sites are crucial for the induction of STAT3 promoter activity induced by stimulation of IL-6 [Figure 3D (17)]. Conversely, the mutation at the E-box site did not change the STAT3 promoter activity in response to the overexpression of SPTBN1 and/or SMAD3, or to IL-6 stimulation (Figure 3B lower panel and Figure 3D lower panel).

CRE-binding proteins ATF3 and CREB2 bind to regions within or upstream of the CRE site of the STAT3 promoter upon the suppression of SPTBN1 and SMAD3 in human HCC cells

The above data suggest that both the CRE and SBE sites are required for SPTBN1- or SMAD3-mediated inhibition of STAT3 promoter activity. The SBE site is known to be a low-affinity STAT-binding element that, together with the CRE site, is required for full induction of STAT3 promoter activity by IL-6 stimulation; this activation is dependent upon STAT3 protein-mediated positive auto-regulation (17–20). The involvement of the SBE site in the observed inhibition of STAT3 promoter activity by overexpressed SPTBN1 and/or SMAD3 may also result from the effects of STAT3 suppression on the subsequent inhibition of STAT3 promoter activation (17–20). We therefore investigated whether SPTBN1 and/or SMAD3 regulate STAT3 expression through CRE-binding transcription factors.

The activating transcription factor (ATF)/CRE-binding (CREB) family comprises basic-region leucine zipper (bZIP) transcription factors, including ATF1, ATF2, ATF3, ATF4 (also known as CREB2), ATF5, ATF6, ATF7, B-ATF, CREB1, and the CRE modulator (CREM). The basic region of the bZIP element is responsible for binding to the CRE consensus sequence TGACGTCa in various promoters (21). The well-characterized members of the ATF/CRE-binding family include CREB1, CREB2, ATF1, ATF2, and ATF3. We first examined whether SPTBN1 or SMAD3 can regulate the expression of ATF/CRE-binding transcription factors. Of the five well-characterized ATF/CRE-binding proteins, the expression levels of ATF3 and CREB2 only were consistently higher and positively correlated with the expression of STAT3 when SPTBN1 or SMAD3 was suppressed than when SPTBN1 and SMAD3 were not suppressed in SNU-449 and PLC/PRF/5 cells (Figure 4A). Similarly, the expression levels of ATF3 and CREB2 transcripts were consistently higher upon the suppression of SPTBN1 or SMAD3 than when they were not suppressed (Figure 4B). Importantly, the expression levels of ATF3 and CREB2 were higher in human HCC tissues than in normal human liver tissues (Figure 4C).

Because the CRE site is required for the regulation of STAT3 promoter activity, which was involved in the transcriptional activation of STAT3 upon the suppression of SPTBN1 and SMAD3, we performed
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Fig. 2. SPTBN1 and/or SMAD3 inhibit cell colony formation and the STAT3 downstream gene BIRC3. (A) PLC/PRF/5 cells were transfected with siRNAs as indicated for 48 h, and the cells were subjected to a colony formation assay. Significance of differences in colony numbers were compared with numbers of colonies generated by cells treated with negative control siRNA (left) and evaluated using a Student’s t-test (**P < 0.01, *P < 0.05). Colony size was measured by using ImageJ software under the microscope at a magnification of ×20 and shown as area in μM². The percentage of colonies larger than 2000 μM² in each treatment group was calculated (right). (B) PLC/PRF/5 cells were co-transfected with siRNAs and either a plasmid of empty vector or FLAG-tagged dominant-negative STAT3 for 48 h, and the cells were analyzed for colony formation in soft agar. Significance of differences was evaluated using a Student’s t-test (**)P < 0.01). (C) PLC/PRF/5 cells with stably transduced SPTBN1 or nonspecific (ns) shRNA were used for the colony formation assay. Significance of differences was evaluated using a Student’s t-test (versus ns shRNA). (D) SNU-449 and PLC/PRF/5 cells were transfected with siRNAs as indicated for 48 h, and the cells were analyzed by QRT-PCR for expression of BIRC3. Significance of differences were evaluated using a Student’s t-test (versus control siRNA). (E) PLC/PRF/5 cells were co-transfected as shown for 48h, and the cells were subjected to QRT-PCR analysis for BIRC3. The significance of results was evaluated by comparison with the group transfected with control siRNA and empty vector.
Fig. 3. The CRE and SBE sites of the STAT3 promoter are required for inhibition of STAT3 promoter activity by SPTBN1 and SMAD3. (A) Schematic representation of six 5′-deletion human STAT3 promoter–luciferase constructs showing the DNA motifs of E-box, CRE/ATF, SBE, and two GC-rich regions indicated by boxes (left). SNU-449 cells were transfected with basic vector, pGV-B2, one of six pHST3-Luc constructs, or pRL-TK. Following transfection for 48 h, cells were lysed and luciferase activity was measured. Normalized (to the pGV-B2 group) relative luciferase activity is shown (right). (B) Responsiveness of STAT3 promoter activity to overexpressed SPTBN1 and SMAD3. SNU-449 cells were transfected with pCDNA3.1, V5-SPTBN1, or SMAD3 in addition to individual pHST3-Luc constructs and pRL-TK for 36 h. Cells were left untreated or treated with IL-6 (100 ng/ml) for an additional 16 h in 0.1% serum containing medium prior to cell lysis and measurement of luciferase activity. Normalized (to the pCDNA3.1 group without IL-6 treatment) relative luciferase activity is shown. The significance of results was evaluated by comparison with the cells transfected with pCDNA3.1. (C) SNU-449 cells were transfected as indicated. Forty-eight hours after transfection, the cells were analyzed by western blotting. (D) The DNA sequences of the E-box, CRE, and SBE sites of each mutated STAT3 promoter and the unmutated STAT3 promoter sequence are illustrated, with dots representing unchanged nucleic acids (human STAT3 promoter segment −409 to −311) (top). SNU-449 cells were transfected with pCDNA3.1, V5-SPTBN1, or SMAD3 together with each mutant pHST3-Luc−2 (−694/+102 of the STAT3 promoter segment) and pRL-TK for 36 h. Cells were left untreated or treated with IL-6 (100 ng/ml) for an additional 16 h in 0.1% serum containing medium prior to cell lysis and measurement of luciferase activity. Normalized (to the pCDNA3.1 group without treatment) relative luciferase activity is shown. The significance of results was evaluated by comparison with the cells transfected with pCDNA3.1. WT, wild type.
Transcriptional regulation of STAT3 by SPTBN1 and SMAD3

ChIP analysis to examine whether SPTBN1 and SMAD3, in addition to ATF3 and CREB2, bind to the region around the CRE site of the STAT3 promoter in PLC/PRF/5 cells, which were treated with siRNAs targeting SPTBN1 and SMAD3 or control siRNA for 48 h. In cells treated with control siRNA, we observed that SPTBN1 bound to regions within or upstream of the CRE site while SMAD3 bound only to the region within the CRE site (Figure 5A). Very weak binding of ATF3 and CREB2 at the region upstream of the CRE site was observed when cells were treated with control siRNA. The binding of ATF3 and/or CREB2 to the region upstream of the CRE site was increased significantly when SPTBN1 and SMAD3 were inhibited and hence could not bind at the region upstream of the CRE site, and the binding of ATF3 and CREB2 to the region within the CRE site was increased only when SMAD3 was inhibited and hence could not bind at the region within the CRE site (Figure 5A). The increased binding of ATF3 and/or CREB2 to the region within or upstream of the CRE site of the STAT3 promoter was also observed in SNU-449 cells upon the suppression of SPTBN1 and SMAD3 (Supplementary Figure S3, available at Carcinogenesis Online).

**SPTBN1 and SMAD3 downregulate expression of STAT3 via the inhibition of ATF3 and CREB2 expression in human HCC cells**

We next questioned whether ATF3 and CREB2 are required for SPTBN1- and/or SMAD3-mediated regulation of STAT3 transcription. We first

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**Fig. 4.** CRE-binding proteins ATF3 and CREB2 were upregulated in cells upon suppression of SPTBN1 and/or SMAD3. (A, B) SNU-449 cells and PLC/PRF/5 cells were transfected with siRNAs as indicated. Forty-eight hours after transfection, cells were subjected to western blot (A) and QRT-PCR (B) analysis. Significance of differences were evaluated using a Student’s t-test (**P < 0.01, *P < 0.05 versus each control siRNA). (C) Immunohistochemical staining of ATF3 and CREB2 in normal human liver and human HCC tissues.
L.Lin et al. examined whether the overexpression of ATF3 and CREB2 in PLC/PRF/5 and SNU-449 cells can elevate STAT3 transcription. We co-transfected DNA constructs of ATF3 (Supplementary Figure S2A, available at Carcinogenesis Online) or CREB2 (Supplementary Figure S2B, available at Carcinogenesis Online) or an empty vector together with control siRNA, SPTBN1 siRNA, or SMAD3 siRNA. STAT3 mRNA and protein levels were higher in cells transfected with ATF3 or CREB2 than in those transfected with empty vectors upon suppression of SPTBN1 or SMAD3.

We then examined whether ATF3 and CREB2 are required for SPTBN1- or SMAD3-mediated upregulation of STAT3 transcription. In SNU-449 and PLC/PRF/5 cells, although suppression of SPTBN1 or SMAD3 increased STAT3 promoter activity compared with that of controls, the suppression of ATF3 or CREB2 abrogated these increases (Figure 5B), and this phenomenon was also reproducible in another HCC cell-line, SNU-475 (Figure 5B), in which the expression of STAT3, p-STAT3, ATF3, and CREB2 were also elevated upon suppression of SPTBN1 or SMAD3.

Fig. 5. The binding of CREB2 and ATF3 to the regions within or upstream of CRE site of the STAT3 promoter increased when the binding of SPTBN1 and SMAD3 at the regions around the CRE site of the STAT3 promoter was suppressed, and ATF3 and CREB2 were required for the induction of the STAT3 promoter activity caused upon the suppression of SPTBN1 and/or SMAD3. (A) PLC/PRF/5 cells were transfected with siRNAs for 48 h and subjected to ChIP analysis by antibodies as indicated. A schematic representation of the human STAT3 promoter is shown (top). The big arrow indicates the transcription start site. The E-box, CRE, and SBE sites are shown by open boxes from left to right. Small arrows indicate the positions of the qPCR primer pairs. Binding capacities were shown as fold enrichment, which was analyzed by normalizing to each of non-antibody signals (IgG). The significance of differences were evaluated by Student’s t-tests [versus each non-antibody group (IgG)]. (B) SNU-449, PLC/PRF/5, and SNU-475 cells were co-transfected as indicated in addition to pHST3-Luc (−694/+102 promoter segment of STAT3) and pRL-TK. Cells were harvested 48 h post-transfection and analyzed for luciferase activity. Significance of the differences were evaluated by Student’s t-test (**P < 0.01, *P < 0.05) (left).
the suppression of SPTBN1 or SMAD3 (Supplementary Figure S4, available at Carcinogenesis Online). Moreover, although knockdown of SPTBN1 or SMAD3 elevated STAT3 mRNA and subsequently the levels of STAT3 protein, as well as p-STAT3 compared with controls, suppressing ATF3 or CREB2 prevented this elevation in PLC/PRF/5 cells (Figure 6A) and SNU-449 cells (data not shown).

**The expression of SPTBN1 and SMAD3 negatively correlates with the expression of STAT3, ATF3, and CREB2 in human HCC tissues**

To further examine whether SPTBN1 and/or SMAD3 regulate STAT3 in human HCC cases, we analyzed annotated clinical metadata and gene expression data from a completed liver cancer study (11–13). We found that in HCC patients with very early, early, advanced, and very advanced HCC, the expression of STAT3 was negatively correlated with SPTBN1 expression and negatively correlated with SMAD3 expression, whereas SMAD3 expression and SPTBN1 expression were positively correlated and that the expression of both SMAD3 and SPTBN1 were positively correlated with TGF-β1 gene expression (Figure 6B and D). Further analysis of these patients indicated that ATF3 expression and CREB2 expression were both negatively correlated with SPTBN1 expression, and ATF3 expression and STAT3 expression were positively correlated (Figure 6C). These correlations were independent of human HCC stage, suggesting that the downregulation of SPTBN1 or SMAD3 and the upregulation of STAT3, ATF3, and CREB2 occur very early in the development of human HCC.

The correlation of SMAD3 with ATF3 and CREB2 did not reach statistical significance in the analysis of annotated clinical metadata and gene expression data from a completed liver cancer study (data not shown), so we then examined the expression of p-SMAD3, ATF3, and CREB2 in the cell nucleus by immunohistochemistry in 49 human HCC cases. We observed that the majority of human HCC tissues with low levels of p-SMAD3 (p-SMAD3 low) showed high levels of ATF3 and CREB2, while, conversely, the majority of human HCC tissues with medium and high levels of p-SMAD3 (p-SMAD3 middle and p-SMAD3 high), showed low level of ATF3 and CREB2 (Figure 6E).

**Discussion**

In this study, we provide evidence supporting a tumor suppressive role for SPTBN1 and SMAD3 in HCC development. In SPTBN1−/− mouse liver tissue, levels of STAT3 transcripts were elevated by the suppression of SPTBN1. The levels of STAT3 transcripts and subsequently the level of STAT3 protein as well as p-STAT3 were elevated by the suppression of SPTBN1 and SMAD3 in HCC cells. Suppression of SPTBN1 and/or SMAD3 increased HCC cell colony formation although increased cell viability was modest, suggesting increased cancer aggression. This modest increase in cell viability is consistent with the finding that STAT3 activation mainly enhances the potential of cellular transformation induced by the oncoprotein v-Src (22). Moreover, inhibiting SPTBN1 or SMAD3 in HCC cells induced the STAT3 downstream gene BIRC3, a pro-survival factor that increases in response to STAT3 activation and thereby promotes survival of cells against TNF-α–induced apoptosis (16). Further evidence suggests that a genomic region containing BIRC3 is amplified in mouse models of liver cancer and in human liver carcinomas (23), suggesting that BIRC3 drives oncogenesis. The mechanism by which increased BIRC3 gene expression relates to an increase in cell colony formation in our current studies needs to be further investigated, as this might be an essential mechanism contributing to the increased expression of STAT3 upon SPTBN1 or SMAD3 suppression.

Using site-directed mutagenesis, we found that both the CRE and SBE DNA motifs of the STAT3 promoter were important for the inhibitory effect of SPTBN1 and/or SMAD3 on STAT3 transcription. We speculate that increased expression of STAT3 and p-STAT3 induced by suppression of SPTBN1 and/or SMAD3 may act as a positive feedback regulator to enhance the transcription of STAT3 through its SBE and CRE sites. This supports the findings that STAT3 auto-regulates expression of its own gene in a positive feedback manner (17–20).

How this potential feedback mechanism relates to the upregulation of STAT3 by SPTBN1 and/or SMAD3 should be addressed in the future.

The CRE site represents one of the major classes of regulatory cis-acting elements that provide a binding site for several transcription factors (24). Our results indicate that mutations in the CRE site of the STAT3 promoter abolish the inhibitory effect of SPTBN1 and/or SMAD3 on STAT3 promoter activity, which is similar to the previously reported finding that the CRE site, together with the SBE site of the STAT3 promoter, is required for full induction of STAT3 promoter activity by IL-6 (17). In that report, the CRE-binding transcription factors that may be involved in the regulation of STAT3 by IL-6 were not identified (17). We found that out of all of the well-identified CRE-binding transcription factors of the ATF/CRE-binding family, ATF3 and CREB2 showed increased expression and binding to the regions of the CRE site of the STAT3 promoter, and acted as transcriptional activators of STAT3 when the expression of SPTBN1 or SMAD3 was suppressed; hence the binding of SPTBN1 or SMAD3 to the STAT3 promoter was decreased. These results indicate that the binding of ATF3 and CREB2 to the STAT3 promoter is dynamic and that SPTBN1 and/or SMAD3 binds to the region within and upstream of the CRE site of the STAT3 promoter, thus possibly preventing the binding of ATF3 and CREB2 to that region. Therefore, our results suggest that the interplay between transcription factors and SPTBN1 or SMAD3 regulates STAT3.

Our findings were supported by clinical human HCC metadata analysis and by immunohistochemical staining of human HCC tissues. Expression of SPTBN1 and SMAD3 were positively correlated with expression of the TGF-β1 gene. Downregulation of SPTBN1 and SMAD3 expression were significantly correlated with upregulation of STAT3 expression; and downregulation of SPTBN1 expression was significantly correlated with upregulation of ATF3 and CREB2 expression. Moreover, ATF3 expression and STAT3 expression were significantly correlated, indicating that ATF3 may act as a positive transcriptional activator of STAT3. Although SMAD3 gene expression was not significantly correlated (negatively or positively) with ATF3 or CREB2 gene expression in our human HCC metadata analysis, we observed that the expression of functional p-SMAD3 was negatively correlated with nuclear expression of ATF3 and CREB2 in human HCC tissues, which is consistent with our results in human HCC cell lines. We found that STAT3 has an inverse relationship with both SPTBN1 and SMAD3 in human HCC, which is consistent with our previous finding that a STAT3 inhibitor is more effective at inhibition of cell-proliferation in HCC cells with impaired TGF-β signaling than in cells with intact TGF-β signaling (25). It is reasonable to speculate that HCC patients with decreased SPTBN1 or SMAD3 and increased STAT3 are more sensitive to STAT3 inhibitors than those with normal expression of SPTBN1 or SMAD3. Further studies designed to test STAT3 inhibitors in HCC patients are warranted. Hence, we speculate that targeting ATF3 or CREB2 may potentiate the effects of STAT3 inhibitors in the suppression of HCC proliferation.

CREB2 (ATF4) has been observed at high levels in human cancer cells and is a major factor induced in response to tumor hypoxia and anoxia, suggesting that it is involved in cellular processes relevant to cancer progression (26,27). CREB2 (ATF4) has also been reported to be involved in metastasis of several types of tumors (28). ATF3, an adaptive-response gene in participating cellular processes, transduces signals from receptor to nucleus to activate or repress gene expression (29). Increased levels of ATF3 are found in many kinds of cancers, including prostate cancer, breast cancer, colon cancer, and Hodgkin’s lymphoma, as well as in the stromal compartments of several types of cancer. ATF3 stimulates cell proliferation, is anti-apoptotic and promotes metastasis (30,31). Overexpression of ATF3 increases DNA synthesis and cyclin D1 mRNA expression in hepatocytes (32). Additionally, ATF3 and STAT3 have been shown to interact with many growth regulators during liver regeneration (33). These findings suggest that ATF3 has an oncogenic role. Our results provide further evidence that CREB2 and ATF3 have an oncogenic role as transcriptional activators of STAT3 in liver cancer formation when SPTBN1 and/or SMAD3 cease to function.
Fig. 6. The CRE-binding proteins ATF3 and CREB2 are required for upregulation of STAT3 gene expression and increased STAT3 protein, as well as p-STAT3 resulting from suppression of SPTBN1 or SMAD3. Negative correlations between SPTBN1 and/or SMAD3 expression and STAT3, ATF3, and CREB2 expression are observed. There is also a negative correlation between p-SMAD3 with ATF3 and CREB2 in human HCC tissues. (A) PLC/PRF/5 cells were co-transfected as indicated. Forty-eight hours after transfection, cells were analyzed by QRT-PCR (left) and western blotting (right). The significance of results was evaluated by comparison with the group transfected with control siRNA. (B) Correlation analysis of gene expression in human HCC tissues using the Georgetown Database of Cancer platform as described in Materials and methods section. Tumor stages of the analyzed human HCC cases are indicated by different dot colors: blue, very early HCC; green, early HCC; black, advanced HCC; and purple, very advanced HCC. (i) The expression levels of STAT3 and SPTBN1 were negatively correlated ($r = -0.3469$, $P = 0.0479$). (ii) The expression levels of STAT3 and SMAD3 were negatively correlated ($r = -0.6284$, $P = 0.000902$). (iii) The expression levels of SMAD3 and SPTBN1 were positively correlated ($r = 0.5953$, $P = 0.000258$). (C) Correlation analysis as in Fig. 6B. (i) The expression levels of ATF3 and SPTBN1 were negatively correlated ($r = -0.4752$, $P = 0.005201$). (ii) The expression levels of CREB2 and SPTBN1 were negatively correlated ($r = -0.5890$, $P = 0.000311$). (iii) The expression levels of ATF3 and STAT3 were positively correlated ($r = 0.6029$, $P = 0.000205$). (D) Correlation analysis as in Fig. 6B. (i) The expression levels of SPTBN1 and TGF-$\beta$1 were positively correlated ($r = 0.5280$, $P = 0.001584$). (ii) The expression levels of SMAD3 and TGF-$\beta$1 were positively correlated ($r = 0.3714$, $P = 0.033316$). (E) Immunohistochemistry analysis of p-SMAD3, ATF3, and CREB2 in 49 human HCC tissues and the expression scores of each protein in cell nucleus were assessed based on their intensity and distribution. The percentages of ATF3 low (<2) or high (≥2), and CREB low (<2) or high (≥2) in each cohort of p-SMAD low (score: 0–3), middle (score: 4–6), high (score: >6) were compared using $U$-test ($**P < 0.01$, *$P < 0.05$).
In summary, we investigated the regulation of STAT3 by SPTBN1 and/or SMAD3 and propose a molecular mechanism responsible for the tumor-suppressive function of SPTBN1 and SMAD3, which is the central link of TGF-β signaling pathway (Supplementary Figure S5, available at Carcinogenesis Online). A more detailed analysis of the interaction between transcription factors and SPTBN1 and/or SMAD3 in the regulation of STAT3 transcription, and of the connection between this interaction and TGF-β signaling should elucidate mechanisms underlying the pathogenesis of liver cancer and inform a combination treatment strategy for this malignancy.

Supplementary material

Supplementary Figures S1–S5 can be found at http://carcin.oxfordjournals.org/

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References


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