A Positive TGF-β/c-KIT Feedback Loop Drives Tumor Progression in Advanced Primary Liver Cancer

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Abstract

Hepatocellular carcinoma (HCC) is globally the second most common cause of cancer mortality. The majority of HCC patients are diagnosed at advanced stage disease for which no curative treatments exist. TGF-β has been identified as a potential therapeutic target. However, the molecular mechanisms mediating its functional switch from a tumor suppressor to tumor promoter in HCC and its interactions with other signaling pathways are poorly understood. Here, we demonstrate an aberrant molecular network between the TGF-β and c-KIT pathway that mediates the functional switch of TGF-β to a driver of tumor progression in HCC. TGF-β/SMAD2 signaling transcriptionally regulates expression of the c-KIT receptor ligand (stem cell factor [SCF]) with subsequent auto- and paracrine activation of c-KIT/JAK1/STAT3 signaling. SCF induces TGF-β1 ligand expression via STAT3, thereby forming a positive feedback loop between TGF-β/SMAD and SCF/c-KIT signaling. This network neutralizes TGF-β-mediated cell cycle inhibition and induces tumor cell proliferation, epithelial-to-mesenchymal-transition, migration, and invasion. Disruption of this feedback loop inhibits TGF-β tumor-promoting effects and restores its antiproliferative functions. Consistent with our in vitro data, we demonstrate SCF overexpression and its correlation to SMAD2 and STAT3 activation in human HCC tumors, advanced tumor-node-metastasis stages, and shorter survival. CONCLUSIONS: Canonical TGF-β and c-KIT signaling forms a positive, tumor-promoting feedback loop. Disruption of this loop restores TGF-β tumor suppressor function and provides the rationale for targeting the TGF-β/SCF axis as a novel therapeutic strategy for HCC.

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Introduction
Hepatocellular carcinoma (HCC) is globally the second most common cause of cancer-related mortality [1]. More than 70% of HCCs are diagnosed at advanced stages not amenable to curative treatments. The multikinase inhibitor sorafenib is the only systemic agent that has demonstrated a survival benefit in advanced-stage HCC that is limited to 2 to 3 months [2]. Novel molecular targeted agents failed to improve outcomes [3]. A better understanding of the molecular signaling networks regulating HCC biology is indispensable for the development of novel therapeutic strategies.

TGF-β signaling through SMAD proteins, known as canonical TGF-β signaling, is a potent tumor suppressor pathway. It is activated through binding of the ligand TGF-β to its cognate receptor resulting in serine phosphorylation and nuclear translocation of transcription factors SMAD2/3. Transcriptional responses to TGF-β are cell type and context specific. Inactivation of TGF-β/SMAD signaling promotes hepatocarcinogenesis [4,5]. Paradoxically, TGF-β1 serum concentrations are elevated in HCC patients and correlate with disease extent and shortened survival [6]. The functional switch of TGF-β to a tumor promoter has been observed in other malignancies; the underlying mechanisms are tumor type specific [7]. TGF-β receptor inhibitors for HCC are being explored in clinical trials, but agent that has demonstrated a survival benefit in advanced-stage HCC that is limited to 2 to 3 months [2]. Novel molecular targeted treatments. The multikinase inhibitor sorafenib is the only systemic agent that has demonstrated a survival benefit in advanced-stage HCC that is limited to 2 to 3 months [2]. Novel molecular targeted agents failed to improve outcomes [3]. A better understanding of the molecular signaling networks regulating HCC biology is indispensable for the development of novel therapeutic strategies.

Materials and Methods

Cell Lines and Culture
Human liver tumor cell lines HepG2 (ATCC, Cat. #HB-8065), SNU398 (ATCC, Cat. #CRL-2233), and SNU449 (ATCC, Cat. #CRL-2234) were cultured under standard conditions in Dulbecco’s modified Eagle’s medium, and Hep3B (ATCC, Cat. # HB-8064) in modified Eagle’s medium (10% FBS, penicillin G [100 U/ml], streptomycin [100 μg/ml]). Prior to cytokine treatment, cells were serum starved for 12 hours followed by cytokine incubation under serum-free conditions.

Stably Transduced Cell Lines
Fourth-generation lentiviral vector systems were used for stable transduction with shRNA. Cells were selected with puromycin followed by GFP-directed fluorescence-activated cell sorting. Knockdown was confirmed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), immunoblot analysis, and/or enzyme-linked immunosorbent assay (ELISA).

Invasion Assay
Invasion assay was performed as previously described [12]. Invasion was assessed after 48 hours using lightmicroscopic (40×) quantification in three high-power fields (invasion [%] = [mean number of cells invading through Matrigel-coated membrane/mean number of cells migrating through uncoated insert membrane] × 100).

Viability Assay
Cell viability was assessed by manual quantification and methyl thiazol phenyl tetrazolium bromide (MTT) assay. Trypan blue–stained cells were quantified using a hemocytometer. For MTT assay, the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Cat. #G4000) was used following the manufacturer’s recommendations.

BrdU Proliferation Assay
The BrdU cell proliferation assay kit (Cell Signaling Technology) was used according to the manufacturer’s recommendations. Absorbance was measured at 450 nM using Synergy H4 hybrid reader (BioTek).

Fluorescence-Activated Cell Sorting
PI-based fluorescence-activated cell sorting was performed as previously described [13]. Cell cycle data analysis was performed using FlowJo software.

Immunofluorescence
Following blocking (1% BSA, TBST), methanol-fixed cells were incubated with primary antibodies overnight at 4°C. Subsequently, cells were incubated with Alexa Fluor 488-/594-conjugated secondary antibodies for 1 hour. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAP) (1 μg/ml) for 20 seconds. Slides were mounted with SlowFade (Cat. #S36939 Invitrogen) and analyzed by fluorescent microscopy.

Immunoblot Analysis
Whole-cell lysates were analyzed by immunoblot analysis as previously described [14].

ELISA
Human SCF Quantikine ELISA kit (R&D Systems, #DCK00) and the Human TGF-β1 Quantikine ELISA kit (R&D Systems, #DB100B) were used for cytokine quantification following the manufacturer’s recommendations.

Chromatin Immunoprecipitation (ChIP)
ChIP-IT Express Enzymatic kit (Active Motif, #53008) was used according to the manufacturer’s recommendations. Three micrograms of anti-SMAD2/3, anti-STAT3, or IgG antibody was used per immunoprecipitation.

Transfection
Transfection was performed according to the manufacturer’s recommendations using Lipofectamine 2000 (Invitrogen, #11668) for Hep3B cells and Lipofectamine 3000 (Invitrogen, #L3000001) for HepG2 cells.

Luciferase Assay
The luciferase expression vector system pRL-TK (Promega, #E2241) was used for promoter analysis. Firefly and Renilla luciferase activities were quantitated using the Dual Luciferase Reporter Assay System (Promega, #E1910).

Microarray Analysis
Microarray data (GEO ID: GSE10186) were obtained from the GEO database. Normalized data were used for detection of genes associated with survival. Genes associated with survival were detected through feature-by-feature Cox PH models in which the gene level was analyzed as the independent variable and the survival status as the
outcome. Genes with a *P* value of < .05 (Suppl. Table 1) were selected for subsequent Ingenuity Pathway Analysis to extract genes involved in the TGF-β signaling pathway. Kaplan-Meier plots were generated for identified genes in the TGF-β signaling pathway. In the Kaplan-Meier plot for each specific gene, samples were divided into two (upregulation and downregulation) groups using the median gene level as the cutoff. A log-rank test was performed to compare the survival rates between two groups.

**Migration Assay**

Cell migration was assessed using the *in vitro* scratch assay as previously described [15]. At 0, 24, and 48 hours after cell layer disruption, wound width was measured using Image J software.

**TUNEL Assay**

Cell apoptosis was evaluated by DeadEnd Colorimetric TUNEL system (Promega) following the manufacturer’s recommendation. Cells were counterstained with hematoxylin.

**Immunohistochemistry**

Tissue microarrays and the corresponding clinical information were obtained from US Biomax (Cat. #LV8013) and Imgenex (Cat. #IMH-318 and Cat. #IMH-360). Immunohistochemistry was performed as previously described [14]. Immunohistochemical staining was performed using VECTASTAIN Elite ABC Kit Universal (#PK-7200) and DAB (#SK-4100) substrate KIT (Vector Laboratories, Burlingame, CA). Counterstain was performed with hematoxylin (Vector Laboratories, #H-3404). Two methods were used for quantification: 1) positivity defined as > 30% of immunohistochemically positive tumor cells and 2) Allred scoring system [16] adapted for intranuclear pTyr

\[705^{\text{STAT3}}, \text{pSer}^{465/467}\text{SMAD2}, \text{and cytosolic SCF}.\]

Proposition of positive tumor cells (PS): 0%, 0 points; <1%, 1 point; 1% to 10%, 2 points; 11% to 33%, 3 points; 34% to 67%, 4 points; and >67%, 5 points. Intensity score of positive tumor cells (IS): none, 0 points; weak, 1 point; intermediate, 2 points; and strong, 3 points. The total score (TS) = PS + IS. *Positivity* was defined as TS of more than 2.

**Quantitative RT-PCR**

Total RNA was extracted using RNAeasy kit (Qiagen, Cat. #74124). cDNA was synthesized using the SuperScript II kit (Invitrogen) and 1 µl added to Power SYBR Green PCR MasterMix (BioRad Cat. #172-5260). Primers (Suppl. Table 1) were added to a concentration of 400 nM. qRT-PCR was performed using the C100 Thermal Cycler (BioRad), and data were analyzed using BioRad CFX Manager.

**RNA extraction from human tumor tissue**

Paraffin-embedded HCC tumor tissue was obtained through the MD Anderson Cancer Center Pathology tissue bank. Studies were approved by the MDACC Institutional Review Board (IRB #PA13-0674). Tissues were reviewed and macrodissected by a hepatobiliary pathologist (W. C. F.). For RNA extraction, the High Pure RNA Paraffin Kit (Roche, #03270289001) was used according to the manufacturer’s instructions.

**Plasmid constructs**

SCF- and TGF-β-promoter fragments were amplified from genomic DNA by PCR (Suppl. Table 1) and cloned into position of the BglII/ NheI-deleted promoter location of the psiCHECK-2 vector (Promega, #C8021). Plasmid DNA was amplified in DH5α competent cells (Invitrogen, #18258-012) and isolated using Pure-Link HiPure MaxiPrep Kit (Invitrogen, #K2100-07).

**Reagents**

TGF-β1 (#101-B1) and SCF (#255-SC-010) were from R&D Systems; TGF-β1 was used at a concentration of 10 ng/ml and SCF at 5 ng/ml, unless otherwise specified. JAK inhibitor I (#420099) and c-Kit inhibitor ISCK03 (#569615) were from EMD Millipore (Billerica, MA); STATTIC (#573099) was from Tocris (Minneapolis, MN). Primers and antibodies are summarized in Suppl. Tables 4 and 5.

**Statistical Analysis**

*In vitro* data represent at least three independent experiments using cells from a minimum of three separate isolations and are expressed as means ± standard deviations unless otherwise specified. Differences between two groups were compared using 2-tailed Student’s *t* tests. For patient data analysis, continuous variables were summarized using descriptive statistics; categorical variables were tabulated with frequency and percentage. Fisher’s exact test and Wilcoxon rank sum test were used to compare categorical and continuous patient characteristics between patients.

**Results**

**SMAD2-activation in HCC and Its Correlation to STAT3-activation and Clinical Parameters**

The status of TGF-β pathway constituents in HCC was analyzed using transcriptomic data of 80 HCC patients (Oncomine database, GEO: GPL5474). Using Cox regression analysis, we identified 342 genes associated with clinical outcomes (Suppl. Table 1). Ingenuity Pathway Analysis of these genes demonstrated a correlation between SMAD2 upregulation and SKI downregulation with reduced patient survival (Suppl. Figure 1A). SKI protein inhibits canonical TGF-β signaling through inhibition of SMAD2 phosphorylation and as a transcriptional repressor [17,18]. Kaplan-Meier survival analysis confirmed significantly decreased survival in patients with SMAD2 upregulation and SKI downregulation (Figure 1A). Subsequently, we analyzed an independent set of matched nonmalignant hepatic and tumor tissues of HCC patients (*n* = 27) for PAI-1, a transcriptional target of TGF-β/SMAD signaling (Suppl. Figure 1B). PAI-1 expression was 3.33-fold (SEM ± 0.77) higher in tumor versus normal tissue (*P* < .0001).

To validate the correlation of TGF-β activation with clinical outcomes, we evaluated nuclear p**Ser**465/467**SMAD2** as a surrogate marker of canonical TGF-β signaling activation in HCC tumor samples of an independent third set of 116 patients (Figure 1B); patient’s characteristics summarized in Suppl. Table 2). SMAD2 activation was significantly higher in advanced HCC (stages III/IV) than early-stage HCC (stages I/II) with a mean Allred score of 4.91 (SEM ± 0.26) versus 3.53 (SEM ± 0.30) (*P* = .0007) (Figure 1C). Survival was significantly shorter in patients with p**Ser**465/467**SMAD2**-positive tumors (Suppl. Figure 1C).

JAK/STAT3 signaling has a key oncogenic role in hepatocarcinogenesis and HCC tumor progression [19]. We evaluated STAT3 activation in the 116 HCC tumors previously evaluated for p**Ser**465/467**SMAD2** (Figure 1B). Intratumoral p**Tyr**705**STAT3** levels were considerably higher (*P* = .02) in advanced than in early-stage HCC with a mean Allred score of 4.72 (SEM ± 0.27) versus 3.74 (SEM ±
To determine a potential association between JAK/STAT and TGF-β/SMAD2 signaling, intratumoral pSer465/467SMAD2/pTyr705STAT3 co-positivity was evaluated (Figure 1D). Only 12% to 18% of pSer465/467SMAD2-negative tumors were positive for pTyr705STAT3. However, 17% to 49% of pSer465/467SMAD2-positive tumors were positive for pTyr705STAT3. Rates of...
**Mechanisms of TGF-β1-induced Tumor Progression in Primary Liver Cancer**

Physiologically, STAT3 is not a target of TGF-β/SMAD signaling. Based upon the above described correlation between activated STAT3 and SMAD2 in human HCC tumors, we evaluated the association between the two signaling cascades in vitro. Importantly, we found that TGF-β1 activated STAT3 only in human liver tumor cells with functional SMAD-mediated TGF-β signaling (Figure 2B). The TGF-β-mediated activation of STAT3 was predominantly observed in liver tumor cells with sustained SMAD2 phosphorylation in response to TGF-β treatment, whereas no Tyr\(^{705}\) phosphorylation of STAT3 was observed in cell lines non- or only transiently responsive to TGF-β treatment in regard to SMAD2 phosphorylation (Suppl. Figure 2B). To confirm canonical TGF-β signaling as the STAT3-activating signaling axis, we evaluated the effect of SMAD2 knockdown on TGF-β-induced STAT3 activation. SMAD2 knockdown abrogated TGF-β1-induced Tyr\(^{705}\) phosphorylation of STAT3 (Figure 2B and Suppl. Figure 2B).

To evaluate the functional relevance of TGF-β/STAT3 signaling, we analyzed prooncogenic TGF-β effects [20,21] in parental versus STAT3-knockdown liver tumor cells (Suppl. Figure 2C). TGF-β1 induced E-cadherin expression and membranous relocalization in HepG2 STAT3-KD and Hep3B STAT3-KD cells but not their parental cell lines (Figures 2, C and D, Suppl. Figure 2D). In parental cells, TGF-β1 induced SNAIL1, ZEB1, TWIST, N-cadherin, and vimentin, but STAT3 knockdown abrogated TGF-β1–induced transcription of these epithelial-mesenchymal transition (EMT) markers (Figure 2C and Suppl. Figure 2E). Unexpectedly, we observed enhanced TGF-β1–induced TWIST transcription in HepG2 STAT3-KD cells, whereas it was abrogated in Hep3B STAT3-KD.

Next, we assessed the effect of TGF-β1–induced STAT3 activation on tumor cell migration and invasion, and the therapeutic efficacy of pharmacologic STAT3 inhibition. Whereas we observed TGF-β1–induced upregulation of p72\(^{WAF1/CIP1}\), this upregulation was maintained in both liver tumor cell lines but considerably enhanced by STAT3 inhibition in Hep3B. Similarly, TGF-β1–induced upregulation of p72\(^{WAF1/CIP1}\) in Hep2 increased with STAT3 inhibition. Interestingly, TGF-β1 induced cyclin D1 expression in HepG2, but this induction was inhibited by STAT3 inhibition. TGF-β1 was recently shown to induce G2/M-phase arrest in Hep3B via upregulation of the negative CDK1 inhibitor Wee1 and downregulation of survivin [25,26]. We did not observe an effect of TGF-β on survivin expression in the presence or absence of STAT3 inhibition in Hep3B cells. Whereas we observed TGF-β1–induced upregulation of Wee1, this upregulation was inhibited by STAT3 inhibition. However, STAT3 inhibition mediated TGF-β1–induced downregulation of cyclin B1 expression.

In summary, TGF-β1 cell cycle inhibitory and antiproliferative functions are neutralized in human liver tumor cells with aberrant TGF-β1–induced STAT3 activation but can be restored by STAT3 inhibition.

**Molecular Interactions between TGF-β and c-KIT Signaling in Primary Liver Cancer**

To evaluate if TGF-β/SMAD2 induces STAT3 phosphorylation through secondary kinases, we analyzed the effect JAK1 inhibition on TGF-β1–induced STAT3 activation. Complete inhibition of TGF-β1–induced Tyr\(^{705}\) phosphorylation and nuclear translocation of STAT3, without inhibition of SMAD2 phosphorylation, was achieved by JAK1 blockade (Suppl. Figure 4, A and B).

In fetal liver cells and human acute megakaryoblastic leukemia cells, the c-KIT ligand SCF activates JAK/STAT3 signaling via its cognate receptor c-KIT [27]. Interestingly, we identified TGF-β1–induced/SMAD2-dependent SCF expression in human liver tumor cells (Figure 4A and Suppl. Figure 4C).

To test whether TGF-β1 activated STAT3 predominantly via SCF/c-KIT signaling, TGF-β1–induced STAT3 activation was evaluated...
in the presence and absence of the c-KIT inhibitor ISCK03. c-KIT receptor inhibition reduced TGF-β-induced Tyr705 phosphorylation of STAT3 (Suppl. Figure 4D) and prevented its nuclear translocation (Figure 4C). Similarly, SCF knockdown (Suppl. Figure 4E) prevented TGF-β1-induced Tyr705 phosphorylation of STAT3 in liver tumor cells (Figure 4C).
Figure 3. TGF-β antiproliferative functions are neutralized in human liver tumor cells but can be restored by STAT3 inhibition. (A) Liver tumor cell viability analysis after 48 hours of TGF-β1 stimulation (10 ng/ml) ± STAT3 inhibitor STATTIC (5 μM) using trypan blue quantification. (B) BrdU assay of parental and polyclonal STAT3-knockdown liver tumor cells treated with TGF-β1 (10 ng/ml) for 12 and 24 hours. Results are shown as percent of untreated cells (mean ± SEM).

Table 1. Cell Cycle Analysis of TGF-β1-Stimulated Parental and STAT3-Knockdown Liver Tumor Cells.

| TGF-β   | PT | STAT3KD |
|---------|----|---------|
| Sub G1  | 1.6 (±2.8) 1.6 (±2.6) | 2.0 (±2.1) 1.7 (±1.6) |
| G0/G1   | 47.8 (±16.3) 49.4 (±10.4) | 45.8 (±14.4) 72.0 (±7.4) |
| S       | 20.9 (±14.1) 25.5 (±15.7) | 15.9 (±6.3) 10.2 (±9.3) |
| G2/M    | 29.6 (±16.8) 23.6 (±3.4) | 36.5 (±10.4) 16.2 (±8.1) |
| Hep3B   | 1.5 (±1.7) 2.8 (±3.9) | 0.4 (±0.7) 0.7 (±1.1) |
| G0/G1   | 51.7 (±6.8) 48.1 (±4.9) | 52.7 (±1.7) 27.0 (±2.6) |
| S       | 15.9 (±7.8) 16.3 (±4.5) | 20.7 (±4.6) 28.3 (±1.7) |
| G2/M    | 30.4 (±5.3) 32.7 (±5.0) | 26.0 (±5.4) 44.0 (±1.9) |

Cell cycle analysis of TGF-β1-stimulated (12 hours) parental versus polyclonal STAT3-knockdown HCC cell lines using Propidium Iodide (PI)-based fluorescence-activated cell sorting. Results represent the mean ± SD from three independent experiments.

Figure 2. TGF-β1 activates STAT3 and drives tumor progression via STAT3. Human liver cancer lines were stimulated for 12 hours with TGF-β1 (10 ng/ml). (A) Immunoblot analysis for TGF-β1 induced activation of SMAD2 and STAT3. (B) Immunoblot analysis for STAT3 activation in parental versus SMAD2-knockdown HepG2 and Hep3B cells. (C) Immunofluorescence analysis for EMT-related proteins vimentin (red) and E-cadherin (green), and nuclear 4',6-diamidino-2-phenylindole (DAPI) stain (blue) in parental versus polyclonal STAT3-knockdown Hep3B cells. (D) qRT-PCR analysis for TGF-β1–induced transcription of EMT markers in parental HepG2 and Hep3B cells, and their polyclonal STAT3-knockdown derivatives. (E) Wound assay for TGF-β1–induced tumor cell migration in the presence or absence of the STAT3 inhibitor STATTIC (5 μM). (F) Invasion assay for TGF-β1–induced tumor cell invasion in the presence or absence of the STAT3 inhibitor STATTIC (5 μM).
In summary, SMAD2-mediated TGF-β signaling transcriptionally regulates c-KIT ligand expression, resulting in STAT3 activation via autocrine and paracrine stimulation of c-KIT/JAK1.

**Molecular Mechanisms of TGF-β-regulated SCF-expression**

TGF-β can regulate gene transcription via SMAD binding to SMAD binding elements (SBE) within the promoter region of its target genes [28]. We analyzed the SCF promoter-containing 2.4-kb 5′-flanking region of the SCF gene for the SBE motif 5′-AGAC-3′ [29] and identified seven putative SBE upstream of the SCF start codon (Suppl. Figure 4F). ChIP assays confirmed TGF-β1-induced SMAD2 binding to the SBE promoter in liver tumor cells (Figure 4D). For validation, we generated luciferase expression vectors regulated by the full-length SCF promoter or SCF promoter deletion mutants (Suppl. Figure 4F). TGF-β1 stimulation induced luciferase expression in liver tumor cells transfected with the full-length SCF promoter; however, sequential deletion of SBE-containing promoter sequences resulted in a gradual decrease in TGF-β1-induced luciferase expression (Suppl. Figure 4G).

To confirm SMAD2 specificity of SCF promoter regulation, parental liver tumor cells and their SMAD2-knockdown derivatives were transfected with the full-length SCF promoter luciferase expression vector followed by TGF-β1 stimulation. Whereas TGF-β1 induced luciferase expression in parental liver tumor cells, this induction was abrogated by SMAD2 knockdown (Figure 4E).

Our data demonstrate that TGF-β regulates SCF expression through direct transcriptional promoter activation via SMAD2 binding to SBE within the SCF promoter.

**SCF-expression in human HCC and its Correlation to Clinical Parameters, SMAD2- and STAT3-activation**

We analyzed SCF expression in the 116 HCC tumors analyzed before for pSer465/467SMAD2 and pTyr705STAT3 (Figure 5A). SCF was overexpressed at significantly higher rates in advanced versus early-stage HCC with a mean Allred score of 5.38 (SEM ± 0.28) versus 3.54 (SEM ± 0.40) (P = .0002) (Figure 5B). SCF expression was correlated to pSer465/467SMAD2 (P = .007) and pTyr705STAT3 (P = .06), and clinically to stages T4 (P = .0002) (Figure 5C). SCF expression correlated to pSer465/467SMAD2 and STAT3 expression. SCF significantly induced STAT3 in primary liver tumor cells, whereas expression was not observed with plasmids containing only STB-2, whereas no activity was observed with plasmids containing only STB-1 (Figure 6D).

To confirm SCF as a key inducer of STAT3-mediated TGF-β1 autoregulation, we performed ChIP analysis for TGF-β1-induced STAT3 binding in parental and SCF-knockdown liver tumor cells. TGF-β1-induced STAT3 binding to STB-2 in parental liver tumor cells, but not in HepG2SCF-KD and Hep3BSCF-KD cells. Whereas TGF-β1 treatment induced TGF-β1 transcription in parental liver tumor cells, SCF knockdown prevented TGF-β1–induced TGF-β1 transcription (Figure 6A). Based on our data on TGF-β1/SCF-induced STAT3 activation and the recently described STAT3 independence of TGF-β1 expression during hepatic fibrogenesis [31], we evaluated STAT3 as a mediator of SCF-induced TGF-β1 expression. SCF significantly induced TGF-β1 ligand expression in parental liver tumor cells, but this induction was abrogated by STAT3 knockdown (Figure 6B and Suppl. Figure 6D).

We analyzed the 5′-flanking region of the TGFβ1 gene for the STAT3 consensus binding motifs 5′-TT(N4)AA-3′ and 5′-TT(N5)AA-3′ [32]. We identified two putative STAT3-binding sites upstream of the TGFβ1 start codon at positions −4384/−4373 (STB-1) and −5365/−5357 (STB-2) (Suppl. Figure 6B). ChIP assays demonstrated TGF-β1–induced STAT3 binding to STB-2 but not STB-1 (Figure 6C). We cloned the STAT3 binding site–containing genomic DNA fragments of the TGFβ1 gene into the enhancer position of a thymidine kinase promoter–regulated luciferase expression plasmid (Suppl. Figure 6B). Following transfection of these constructs in liver tumor cells, TGF-β1-induced luciferase expression only with plasmids containing STB-2, whereas no activity was observed with plasmids containing only STB-1 (Figure 6D).

In summary, SCF as the key mediator of TGF-β1 autoregulation, we performed ChIP analysis for TGF-β1–induced STAT3 binding in parental and SCF-knockdown liver tumor cells. TGF-β1-induced STAT3 binding to STB-2 in parental HCC cells but not in HepG2SCF-KD and Hep3BSCF-KD (Figure 6F). For confirmation, STB-2 STAT3 binding site–containing luciferase constructs were transfected into parental and SCF-knockdown liver tumor cells. TGF-β1 treatment resulted in luciferase expression in parental liver tumor cells but not in HepG2SCF-KD and Hep3BSCF-KD (Suppl. Figure 6C).

In summary, SCF is overexpressed in HCC, and its expression correlates with advanced TNM stages and TGF-β/SMAD2 activation. In conjunction with our *in vitro* data, co-positivity for pSer465/467SMAD2 and SCF is intratumoral and intracellular.

**Formation of a Positive Feedback Loop between SCF and TGF-β1 in Primary Liver Cancer**

Regulation of TGF-β1 expression is cell type specific. TGF-β1 autoregulation has been described, but its mechanisms are incompletely understood [30]. To test if SCF is a key mediator of TGF-β1 autoregulation, we evaluated TGF-β1 autoregulation in HepG2, Hep3B, HepG2SCF-KD, and Hep3BSCF-KD cells. Whereas TGF-β1 treatment induced TGF-β1 transcription in parental liver tumor cells, SCF knockdown prevented TGF-β1–induced TGF-β1 transcription (Figure 6A). Based on our data on TGF-β1/SCF-induced STAT3 activation and the recently described STAT3 independence of TGF-β1 expression during hepatic fibrogenesis [31], we evaluated STAT3 as a mediator of SCF-induced TGF-β1 expression. SCF significantly induced TGF-β1 ligand expression in parental liver tumor cells, but this induction was abrogated by STAT3 knockdown (Figure 6B and Suppl. Figure 6D).

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In summary, SCF is the key mediator of TGF-β1 autoregulation in liver tumor cells. TGF-β1/SCF-activated STAT3 binds to the STB-2 regulatory element upstream of the TGFβ1 gene resulting in its induction. Thereby, TGF-β1 and SCF form a positive feedback loop in HCC (Figure 7A).

**Disruption of the TGF-β/SCF Feedback Loop and Its Therapeutic Potential**

Based upon our previous results, we hypothesized that disruption of the TGF-β/SCF signaling loop will inhibit TGF-β–driven tumor progression and restore TGF-β antiproliferative functions in the presence of functional SMAD signaling.

Tumor cell migration of SMAD2−, STAT3−, and SCF-knockdown cells in the absence of treatment did not differ significantly compared with the parental cell lines. However, TGF-β1 treatment significantly induced tumor cell migration of parental liver tumor cells, whereas
Figure 4. TGF-β transcriptionally regulates SCF expression followed by auto- and paracrine activation of c-KIT/JAK1/STAT3 signaling. HepG2 and Hep3B were stimulated for 12 hours with TGF-β1 (10 ng/ml). (A) ELISA for SCF in the supernatant of TGF-β1–treated parental and SMAD2-knockout liver tumor cells (mean ± SEM). (B) c-KIT dependence of TGF-β–induced STAT3 activation analyzed by immunofluorescence analysis for pTyr705STAT3 following stimulation with TGF-β1 ± c-KIT inhibitor ISCK03 (5 μM). The bar graphs below show the percent of cells in the immunofluorescence analysis with intranuclear pTyr705STAT3 accumulation (mean ± SEM of five high-power fields). (C) Immunoblot analysis for TGF-β1–induced STAT3 activation in parental versus polyclonal SCF-knockdown liver tumor cell lines (protein lysates of Hep3BSCF-KD clone 2 were run on the same gel but noncontiguous). (D) ChIP analysis for SMAD2 binding to the SCF promoter following TGF-β1 stimulation; SMAD2 binding to the PAI-1 gene was used as a positive control. (E) TGF-β1–induced (10 ng/ml, 24-hour stimulation) luciferase activity using SCF full-length promoter-regulated luciferase expression plasmids transfected in parental versus SMAD2-knockdown HCC cells.
SMAD2, STAT3, and SCF knockdown abrogated TGF-β1–induced migration (Figure 7B).

Next, we assessed the effect of SMAD2, SCF, or STAT3 knockdown on TGF-β–induced tumor cell invasion (Figure 7C). Interestingly, even though basal levels of tumor cell invasion were higher in HepG2STAT3-KD and Hep3BSCF-KD cells, TGF-β1 induced tumor cell invasion only in the parental cell lines. Knockdown of SMAD2, STAT3, or SCF resulted in abrogation or inhibition of TGF-β–induced tumor cell invasion.
Lastly, we assessed restoration of TGF-β antiproliferative functions after disruption of SMAD2, STAT3, or SCF (Figure 7D). Consistent with our prior data, no significant antiproliferative effects were observed with TGF-β1 treatment of parental HepG2 and Hep3B cells. As expected, no significant antiproliferative effects were noted after TGF-β1 treatment of SMAD2-knockdown liver tumor cells. However, TGF-β antiproliferative functions were restored in liver tumor cells after STAT3 and SCF knockdown. Interestingly, TGF-β–elicited a strong antiproliferative effect even in genetically modified cells with higher basal proliferation rates than their parental cells (HepG2

Our data show that inhibition of STAT3 and SCF can inhibit the paradoxical tumor-promoting functions of TGF-β and restore its antiproliferative functions. Whereas knockdown of SMAD2 prevents TGF-β–induced tumor cell migration and invasion, it does not restore its antiproliferative effects. This finding is in line with the understanding of canonical TGF-β signaling as the major mediator of TGF-β antiproliferative effects.

Discussion

The multikinase inhibitor sorafenib is the only efficacious systemic therapy, but its survival benefit is limited to 3 months [2]. Novel targeted agents failed to achieve superiority or noninferiority in comparison to sorafenib [33,34]. The TGF-β pathway has been identified as a promising therapeutic target, and TGF-β type 1 receptor inhibition for HCC is currently being evaluated in clinical trials (NCT01246986, [www.clinicaltrials.gov]) [8]. However, the molecular mechanisms mediating the functional switch of TGF-β in HCC are poorly understood, and systemic TGF-β inhibition bears risks such as induction of secondary malignancies [35]. TGF-β–induced tumor suppression is mediated by its canonical, SMAD-mediated signaling axis whose inactivation promotes hepatocarcinogenesis [4,5]. Here, we demonstrate TGF-β/SMAD2 activation in advanced HCC and its correlation with shortened patient survival. Our data are supported by recent immunohistochemical and transcriptomic studies demonstrating intratumoral TGF-β/SMAD2 activation in HCC animal models and in human HCC samples, and its correlation with invasive tumor phenotypes [36–40]. Our in vitro data confirm the tumor-promoting role of TGF-β in our liver cancer model, and we show that the tumor-promoting role of TGF-β1 is mediated by SMAD2-dependent STAT3 activation. In human tumor samples of patients with advanced-stage HCC, we also find an intratumoral and intracellular positive correlation of SMAD2 and STAT3 activation. STAT3 is a key oncogenic transcription factor in hepatocarcinogenesis and a mediator of metastatic spread and chemotherapy-resistance of HCC cells [19,41]. TGF-β inhibits STAT3 phosphorylation in intestinal epithelial cells, prostate epithelial cells, acute myeloid leukemia blasts, and a murine hepatocarcinogenesis model [42–45], indicating that the here-described TGF-β/STAT3 network is specific for advanced-stage HCC. Interestingly, we observed TGF-β–induced Tyr

Several groups reported that malignant hepatocytes that survive TGF-β–mediated cytotoxicity undergo EMT and acquire migratory and invasive properties following the prolonged exposure to TGF-β [8,36,52–55]. In a recent clinical phase 2 trial, it was reported that TGF-β type 1 receptor inhibition reduces E-cadherin serum concentrations [56]. Consistent with these data, we show that TGF-β induces the transcription of EMT regulators and promotes tumor cell migration and invasion in our liver cancer model. Our in vitro data demonstrate that STAT3 inhibition inhibits TGF-β–induced EMT, tumor cell migration, and invasion. Hence, our data suggest that STAT3-targeted therapeutic strategies have the potential to inhibit tumor-promoting effects of TGF-β and restore its tumor suppressor function. STAT3 inhibitors and also agents targeting STAT3-activating kinases (i.e., JAK1/2 and c-KIT) were found to be well tolerated and safe in clinical phase 1 trials [57–59]. This therapeutic approach—curtailing TGF-β’s tumor promoter effects and restoring its tumor suppressor function—has several advantages.
over systemically blocking the TGF-β type I receptor. The potential procarcinogenic effects could be avoided, and elevated TGF-β levels in HCC patients could enhance tumor suppression as an endogenous therapeutic agent. Similarly, Dooley et al. proposed in a recent review article a therapeutic combination strategy aimed at restoration of TGF-β–mediated cytostasis through interference with survival signaling and activation of TGF-β signaling [60].

Mechanisms of STAT3 activation and regulation are cell type and context specific. In preliminary studies using kinase inhibitor approaches, receptor knockdown, and cytokine arrays, we ruled out
other tyrosine kinases (i.e., EGFR, Src, ERK1/2, p38MAPK) and cytokine IL-6 as mediators of TGF-β1-induced Tyr705 phosphorylation of STAT3 (Suppl. Figure 7, A and B) in our liver cancer model. Instead, we identify the c-KIT ligand SCF as the key mediator of TGF-β1–induced STAT3 activation. TGF-β induces SCF expression followed by auto- and paracrine activation of c-KIT/JAK1/STAT3. Interestingly, TGF-β1 is an inhibitor of SCF expression in myelogenous leukemia blasts and hematopoietic progenitor cells [61,62], indicating that TGF-β1–induced SCF expression is specific to liver cancers.

Our data demonstrate that TGF-β regulates SCF expression through direct SMAD2 binding to and subsequent activation of its promoter. Due to the low DNA-binding affinity of SMAD2, SMAD-responsive promoters frequently contain multiple SBE [28,29]. We identified several SBE within the SCF promoter and demonstrate SMAD2 binding to the SCF promoter. Consistent with observations in the PAI-1 binding [63], we find decreasing promoter activity with decreasing numbers of SBE. The SMAD2 specificity of this effect is demonstrated by abrogation of TGF-β–induced SCF promoter activation with SMAD2 knockdown. It is likely that additional factors are involved, as SMAD proteins achieve high affinity and selectivity through interaction with other DNA-binding cofactors [28]. Therefore, a SMAD2-knockdown approach was chosen to validate SMAD2 signaling as the mediator of TGF-β–induced SCF expression, as the lack of such cofactors could result in false-negative results even after restoration of SMAD2 activation (i.e., knock-in model with constitutively activated SMAD2). Studies to identify DNA-binding cofactors aberrantly recruiting SMAD2 to the SCF promoter are currently ongoing in our laboratory.

SCF is a critical mitogen for liver regeneration [10,11], but its expression, regulation, and role in HCC are unknown. Our data demonstrate SCF overexpression in HCC and its correlation with advanced TNM stages and shortened patient survival. Consistent with our in vitro data, we demonstrate the intracellular correlation between pY394/402, SMAD2 and SCF in human HCC tumors. The correlation of SCF overexpression with the T4, N1, and M1 status of our patient population supports the in vitro relevance of our data and the TGF-β/SCF axis as a mediator of TGF-β–mediated tumor progression.

Recently, the need for precision medicine has been recognized to improve outcomes of HCC patients [33,34]. However, the diagnostic benefit of tumor-targeted liver biopsies is limited in HCC by its tumor heterogeneity; noninvasive biomarkers to guide treatment have not been identified [64]. TGF-β1 serum and urine concentrations are elevated in patients with HCC and were found to be predictors of poor outcomes [66,65]. Increased intratumoral SCF expression and SCF serum concentrations were found in patients with ovarian and primary peritoneal carcinoma [66]. Ongoing studies in our laboratory evaluate the diagnostic accuracy of TGF-β/SCF serum analysis to identify subsets of patients whose liver tumors are driven by the TGF-β/SCF/STAT3 axis, thereby providing a noninvasive way to guide targeted therapies.

Recently, it was described that resistance to TGF-β cytotoxicity was mediated by autocrine TGF-β synthesis in fetal rat hepatocytes [67]. Whereas TGF-β autoregulation has been described, its mechanisms are incompletely understood [30]. We characterize the molecular mechanisms of TGF-β1 autoregulation in our liver tumor model and identify SCF/c-KIT as the mediator of its autoregulation. Our data show that TGF-β1/SCF induces STAT3 activation and binding to a regulatory element upstream of the transcription initiation site of TGFBI where it acts as a critical regulatory element for TGF-β1 expression. Based upon the location of the STAT3-binding site in relation to the TGFBI promoter, and the degree of transcriptional induction following stimulation of the STAT3-binding element, it is likely that STAT3 fulfills an enhancer function in TGFBI transcriptional regulation. Our data are supported by recent observations of decreased TGF-β1 expression in IL-6−/− mice and STAT3 dependence of IL-6–induced TGF-β1 expression in hepatocytes [31,68].

In summary, our data show that TGF-β1 and SCF form a positive feedback loop that mediates the functional switch of the TGF-β pathway to a driver of tumor progression in advanced-stage liver cancers. Moreover, we demonstrate that TGF-β–mediated cell cycle inhibition can be restored through disruption of the TGF-β1/SCF/STAT3 axis. Interestingly, some of the parental cell lines and their knockdown derivatives displayed differences in their functional behavior under baseline conditions, and the hepatoblastoma and HCC cell lines HepG2 and Hep3B are known to differ in their p53 and Wnt/β-catenin signaling status—two of the most commonly mutated tumor suppressor genes and oncogenic pathways in human HCC [69–71]. However, disruption of the TGF-β1/SCF/STAT3 axis resulted in inhibition of tumor-promoting effects of TGF-β1 and restoration of its antiproliferative functions independent of these differences, indicating the therapeutic potential of this strategy. Our current study provides the rationale for targeting the SCF/c-KIT/STAT3-axis, thereby aiding the development of precision medicine for HCC.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.04.002.
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assay of parental HCC cells versus their SMAD2-, SCF- and STAT3-knockdown clones.

Figure 7. The TGF-β/SCF signaling loop neutralizes TGF-β tumor suppressor function and drives tumor progression in vitro. (A) TGF-β/SCF positive feedback loop. TGF-β-activated SMAD2 transcriptionally induces SCF expression via direct binding to the SCF promoter. SCF expression and secretion result in autocrine and paracrine stimulation of the c-KIT receptor, followed by activation of JAK1/STAT3 signaling. p185
to STAT3 translocates to the nucleus where it binds directly to the TGF-β ligand gene, positively regulating its expression. Following activation of secreted TGF-β precursor, it can activate the TGF-β receptor. In addition, TGF-β/c-KIT drive tumor progression via STAT3 through neutralization of TGF-β antiproliferative functions and induction of EMT, tumor cell migration, and invasion. Disruption of the TGF-β/c-KIT signaling loop on the level of the SCF/STAT3 axis restores TGF-β tumor suppressor function by inhibition of EMT, tumor cell migration, and invasion, and restoration of its cell cycle inhibitory functions. (B) Migration assay for TGF-β1-induced tumor cell migration in parental liver tumor cells in comparison to their SMAD2-, SCF-, and STAT3-knockdown clones. (C) Invasion assay for TGF-β- induced tumor cell invasion in parental liver tumor cells, and their SMAD2-, SCF-, and STAT3-knockdown clones. (D) MTT cell viability assay of parental HCC cells versus their SMAD2-, SCF- and STAT3-knockdown clones.
