

Dysregulated PJA1-TGF- β signaling in cancer stem cell-associated liver cancers

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ABSTRACT

The transforming growth factor beta (TGF- β) signaling pathway plays important roles in cell differentiation, stem cell modulation, organ lineage, and immune suppression. TGF- β signaling is negatively regulated by the ubiquitin-proteasome pathway. Although mouse models of cancer arising from a defective TGF- β pathway clearly demonstrate the tumor-suppressive role of TGF- β , the underlying mechanism by which a defective TGF- β pathway triggers liver cancer development is poorly understood. This review summarizes key findings from our recent studies connecting TGF- β to hepatic oncogenesis and highlights the vulnerability of TGF- β signaling to PJA1-mediated ubiquitination. TGF- β , together with the chromatin insulator CCCTC-binding factor (CTCF), epigenetically and transcriptionally regulate tumor promoter genes, including IGF2 and TERT, in TGF- β -defective mice and in human liver cancers. Dysfunction of the TGF- β -regulated SPTBN1/SMAD3/CTCF complex increases stem cell-like properties in hepatocellular carcinoma (HCC) cells and enhances tumorigenesis in tumor-initiating cells in a mouse model. PJA1, a novel E3 ubiquitin ligase, is a key negative regulator of TGF- β signaling. PJA1 overexpression is detected in HCCs and is sufficient to suppress SMAD3- and SPTBN1-mediated TGF- β tumor suppressor signaling, promoting HCC proliferation. Dysregulated PJA1-TGF- β signaling activates oncogenic genes and promotes tumorigenesis in human liver cancers. In addition, inhibition of PJA1 by treatment with E3 ligase inhibitors restores TGF- β tumor-suppressor function and suppresses liver cancer progression. These new findings suggest potential therapeutic avenues for targeting dysregulated PJA1-TGF- β signaling via cancer stem cells in liver cancers.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the solid tumor type with the fastest-rising incidence in the United States as well as the second-leading cause of cancer-related death worldwide [1, 2]. Survival rates for patients affected by HCC remain dismal, despite the development and approval of several targeted chemo- and immunotherapies. Sorafenib, a multi-target tyrosine kinase inhibitor, was the only systemic therapy for advanced HCC with FDA approval prior to 2016 [3].

Recently, three new multi-kinase inhibitors, lenvatinib [4], regorafenib [5], and cabozantinib [6], and an antibody-based VEGFR2 antagonist, ramucirumab, received FDA approval for advanced HCC [7]. Unfortunately, the median overall survival for patients treated with any of the above agents remains less than 15 months [8]. Thus, there is an urgent need for more effective and targeted therapies in this large HCC patient population.

The complexity of tumor initiation, progression, metastasis, and therapeutic resistance in liver cancer is

deeply associated with tumor stem cell development, the tumor microenvironment, tumor immunity, and genomic alterations [9]. The two major causes for failure of liver cancer therapy are cirrhosis and drug resistance, both of which are potentially mediated through cancer stem cells [10]. The TGF- β pathway also plays a complex role in liver diseases including cirrhosis and liver cancer, where it variously exerts fibrogenic/proinflammatory, tumor suppressive, or pro-metastatic effects [11]. Mouse models with a defective TGF- β pathway clearly demonstrate the tumor suppressor role of TGF- β [12-15], although an underlying mechanism by which a defective TGF- β pathway supports liver cancer development has not been established. Clinically, high levels of TGF- β are associated with a favorable prognosis in early-stage cancers, supporting a primarily tumor-suppressive role for this signaling pathway; however, in advanced-stage or metastatic tumors, high levels of TGF- β are associated with tumor invasiveness and dedifferentiation, highlighting the context-dependence of TGF- β signaling effects even in liver cancers [16-19]. How TGF- β alterations initially inhibit liver cancer development but later exacerbate the malignancy's aggressiveness through pro-oncogenic activities remains poorly understood. This review focuses on emerging mechanistic insights into the tumor suppressor role of TGF- β and its primary regulators in liver cancer development, particularly at its early stages.

HCC stem cell signature is characterized by defective TGF- β signaling

We previously showed that disruptions in TGF- β signaling, coupled with loss of the SMAD adaptor SPTBN1, resulted in the spontaneous development of multiple tumors and a high incidence of liver cancer in human fibroblast and mouse models [20]. TGF- β , together with the tumor suppressor CTCF, epigenetically and/or transcriptionally regulate tumor promoter genes, including IGF2, TERT, and Myc in TGF- β -defective mice and in patients with Beckwith-Wiedemann syndrome (BWS), a human stem cell disorder [20]. Dysfunction of the TGF- β -regulated SPTBN1/SMAD3/CTCF complex increases stem cell-like properties in HCC cells and enhances tumorigenesis in tumor-initiating cells in a mouse model (Figure 1) [20].

We analyzed the transcriptome sequencing data from 147 HCCs in The Cancer Genome Atlas (TCGA) for TGF- β pathway genes [21]. TCGA data indicated that genes directly associated with the TGF- β superfamily were consistently dysregulated (i.e., either elevated or suppressed) [21]. Patients demonstrating a defective TGF- β signature (low levels of TGF- β) experienced significantly poorer outcomes than those with an intact TGF- β signature (high and normal levels of TGF- β) (hazard ratio = 3.15, log-rank test p-value = 0.0027) [21].

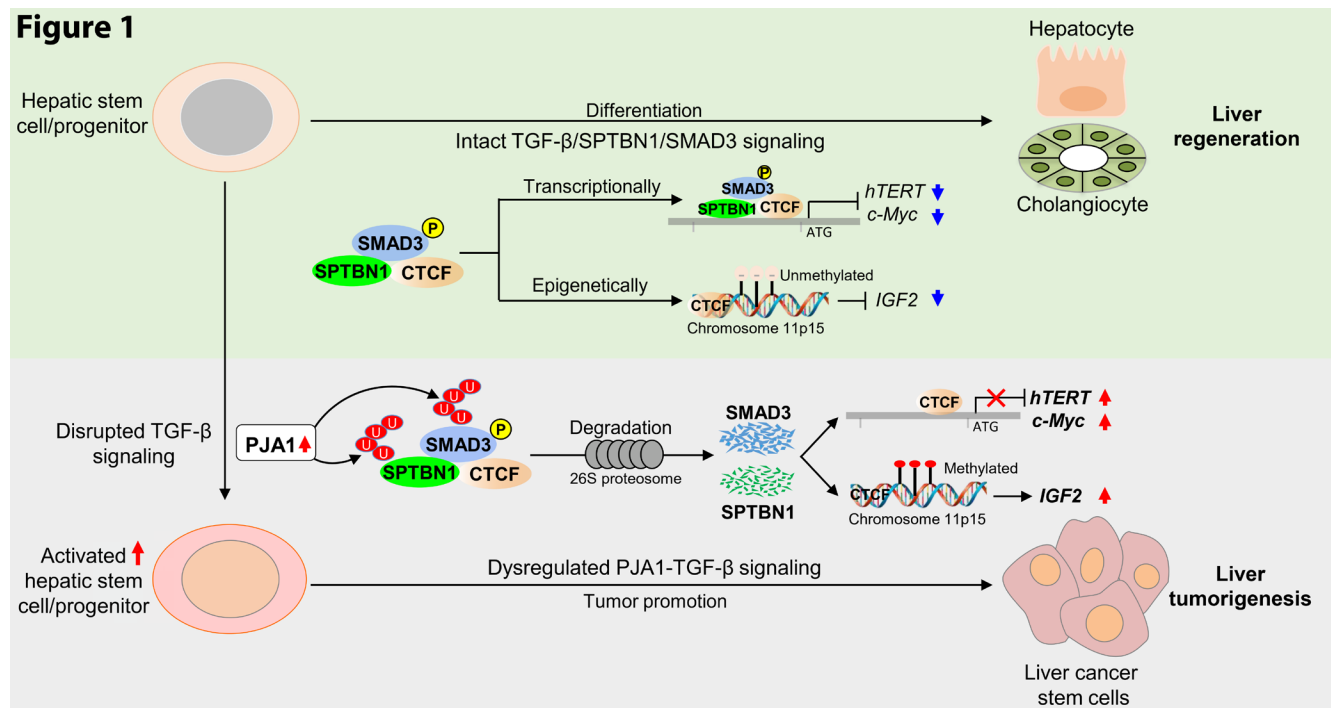


Figure 1: PJA1 suppresses SPTBN1/SMAD3-mediated TGF- β signaling in hepatic stem cells. PJA1 activity disrupts TGF- β signaling at multiple levels, including ubiquitination/degradation of SPTBN1 and p-SMAD3, down-regulation of the SPTBN1/SMAD3/CTCF complex, and promotion of tumor promoter genes IGF2, TERT, and Myc. Collectively, disrupted TGF- β signaling results in development of cancer stem cell-associated tumorigenesis. P, phosphorylated; U, ubiquitinated.

There was variable expression of stem cell signatures across 9,125 samples from 33 tumor types, including 368 HCCs, in the TCGA database [22, 23]. Overall, low TGF- β pathway activity across the 33 tumor types was associated with a higher “stem cell index,” defined by expression of stem cell genes. The HCC samples in this data set were classified into high, intermediate, or low stem cell index tumors. There was a negative correlation between TGF- β pathway activity and stem cell-like character. These results indicate that impairment of TGF- β signaling may contribute to cancer stem cell-associated HCC development [22, 23].

In advanced HCC, high levels of TGF- β are associated with increased inflammation and tumor invasiveness. The oncogenicity of TGF- β signaling is bolstered by results from a recent phase 2 clinical trial, in which inhibition of TGF- β signaling using a TGF- β receptor 1 inhibitor increased survival of patients with advanced HCC whose tumors expressed high levels of TGF- β and alpha-fetoprotein from 9 months to 21 months [24]. The clinical efficacy of TGF- β pathway inhibition and relevance of TGF- β signaling to tumor progression demonstrates that the pathway carries the potential to mediate oncogenic as well as tumor suppressive effects.

Although this treatment modality demonstrated efficacy in patient tumors expressing high TGF- β , there are currently no therapeutic strategies for HCC patients with a defective TGF- β pathway (low levels of TGF- β). Therefore, our recent attention has been turned to identifying oncogenic mechanisms underlying a defective TGF- β pathway, in particular the potential for defective TGF- β signaling to promote liver cancer stem cell growth and survival.

Increased expression of E3 ubiquitin ligase PJA1 in HCC promotes liver tumorigenesis by reducing TGF- β /SMAD3/SPTBN1 activity

We analyzed transcriptome sequencing data of 374 HCC patient samples in TCGA [22]. The amount of PJA1 mRNA was significantly increased in HCC compared with normal liver. An increase in PJA1 transcripts in HCC patients relative to levels in normal liver was also detected in the Roessler liver 2 data from Oncomine and the Wurmbach liver data from the Gene Expression Omnibus [25]. Analysis of PJA1 protein expression revealed increased levels in HCCs compared with normal livers [22]. Furthermore, TCGA data indicated that increased mRNA expression of PJA1 was associated with markedly reduced overall survival of patients with HCC, suggesting that high activity of PJA1 may confer a poor HCC prognosis.

Numerous E3 ubiquitin ligases have been identified to negatively regulate various components of the TGF- β

pathway, and alterations of ubiquitin ligases been recognized in several cancer types [26-34]. TGF- β family members transduce signals through membrane serine/threonine kinase receptors and intracellular effectors from the SMAD family of proteins [35, 36]. Assembly of components for adequate SMAD signal transduction requires adaptor proteins, which tightly regulate SMAD nuclear translocation, receptor phosphorylation and degradation [35].

PJA1, a RING finger E3 ubiquitin ligase of the Praja family, promotes ubiquitination and degradation of multiple targets, including SMAD3, homeodomain protein Dlx5, polycomb repressive complex 2 proteins (PRC2), and Enhancer of zeste homologue 2 (EZH2) [37-39]. Although the expression of PJA1 is increased in some cancers, including glioblastoma and some gastrointestinal tract cancers [40, 41], detailed studies of PJA1 in HCC development and progression have not been conducted.

Recently, we identified the PJA1 as a protein that ubiquitinates SPTBN1 in a TGF- β -dependent manner [22, 40]. Accordingly, PJA1 E3 ligase activity regulates TGF- β signaling by controlling SPTBN1 abundance through ubiquitin-mediated degradation. We also found that PJA1-mediated ubiquitination of phosphorylated (p)-SMAD3 in HCC cells only occurred in cells exposed to TGF- β . These data indicate that PJA1 promotes the ubiquitination and proteasomal degradation of p-SMAD3, resulting in reduced activity of the TGF- β /SMAD3/SPTBN1 tumor-suppressing pathway in HCC cells (Figure 1). Thus, the PJA1 interaction with SMAD3 in HCC is TGF- β -dependent.

Knockdown of PJA1 by short-hairpin RNA (shRNA) significantly reduced colony formation in HCC cells and anchorage-independent growth of SNU475 and HepG2 cells [22]. Moreover, knockdown of PJA1 impaired tumor growth in a xenograft model of subcutaneously injected HepG2 cells in nude mice. Knockdown of PJA1 resulted in reduced numbers of Ki67-positive cells and increased numbers of cells positive for the apoptosis effector Caspase3, suggesting that PJA1 promoted HCC cell proliferation and protected against apoptosis. These data indicate that PJA1 functions as a tumor promoter and that reducing its activity in liver cancer cells impairs malignant phenotypes.

PJA1 may promote liver cancer stem cell proliferation and liver metastasis in the context of defective TGF- β

To elucidate the mechanisms by which PJA1 regulates TGF- β signaling in cancer stem cell-associated liver tumorigenesis, we developed a novel mouse model using a hydrodynamic (rapid injection) delivery approach (PiggyBac Transposon System, System Biosciences,

Inc.). By injecting a PJA1-encoding Sleeping Beauty (SB) transposon through the mouse tail vein, the plasmid primarily accumulates in the liver, allowing for selective overexpression of mouse PJA1 in hepatocytes [22]. This approach built upon our previously established models of IL6-mediated chronic inflammation in both wild-type and TGF- β defective (*Sptbn1*^{+/-}) mice utilizing hydrodynamic injection of the IL6 gene every 2 weeks for 3 months [42].

A mouse PJA1-encoding SB transposon and control plasmid DNA SB transposase were injected hydrodynamically through the mouse tail vein in wild-type Black6 background mice twice per month for 3 months. Stable expression of PJA1 in the liver of wild-type Black6 mice after 3 months of tail vein injection was confirmed by immunohistochemical analysis, while PJA1 expression remained very low in mouse liver infected by control plasmid DNA.

We explored the effects of PJA1 overexpression on stem cell properties in wild-type Black6 and TGF- β defective (*Smad3*^{+/-}) mice. At day 90, single-cell suspensions from the mouse livers were prepared for liver stem cell (LSC) isolation [42]. LSCs (CD133 positive cells) were isolated from each liver preparation by using a magnet-activated cell-sorting column with an antibody recognizing CD133. The cells were assessed for cell proliferation, anchorage-independent growth, and colony formation, and were injected into immune-compromised mice to evaluate for serial tumor formation and liver metastasis [22]. Our data demonstrated that LSCs from the PJA1-injected TGF- β -defective (*Smad3*^{+/-}) mice showed a higher proliferation rate, increased Ki67 staining in cell culture, and exhibited increased cell transformation in soft agar compared with either PJA1-injected wild-type Black6 or the plasmid control-injected TGF- β -defective (*Smad3*^{+/-}) mice. Interestingly, neither the LSCs from the control-injected TGF- β -defective (*Smad3*^{+/-}) (n=6) nor the PJA1-injected wild-type Black6 mice (n=6) formed tumors or liver metastases when LSCs were injected subcutaneously into immune-compromised mice. However, 2 mice from total 6 mice injected with LSCs from the PJA1-injected TGF- β -defective (*Smad3*^{+/-}) mice formed tumors and liver metastases, suggesting that increased PJA1 in the context of defective TGF- β signaling promotes liver stem cell properties and their transformation into cancer stem cells (Figure 1) [22].

Targeting dysregulated PJA1-TGF- β signaling in liver cancers

We recently reported that TGF- β -deficient mice of various genetic backgrounds, including TGF- β signaling adaptor *Sptbn1*^{+/-} mice and double-knockout *Sptbn1*^{+/-}

Smad3^{+/-} mice, develop multiple tumors, including HCC [20, 43]. These mice are characterized by macroglossia, cardiomegaly, renal hypertrophy, and conspicuous cytomegaly of the adrenal cortex [20, 43] and generally resemble the human BWS phenotype. Thus, genetic studies demonstrate that defective TGF- β signaling can phenocopy a human stem cell disorder.

SMAD3 and its adaptor protein SPTBN1 are increasingly recognized as potent regulators of liver cancer stem cell development. Our recent work indicated that PJA1 promotes the ubiquitination of SPTBN1 and p-SMAD3, resulting in reduced activity of the tumor-suppressing TGF- β /SMAD3/SPTBN1-dependent pathway in HCC cells [20, 40]. Although our data strongly support a key role for TGF- β signaling in suppressing liver cancer and highlight how PJA1 E3 ligase inhibits TGF- β signaling, the precise mechanism of dysregulated PJA1-TGF- β signaling and its role in the stages of HCC development remain unclear.

Cirrhotic livers precede HCC in 80%–90% of patients and are considered precancerous lesions [44, 45]. TERT promoter mutations have been reproducibly associated with cirrhosis and cirrhosis-associated HCC [45]. TERT promoter mutations were found in 6% of low grade dysplastic nodules and 19% of high-grade dysplastic nodules in cirrhosis, and were dramatically increased in early HCCs (61%) without additional recurrent HCC driver gene mutations. These tumor genetic studies demonstrate that TERT promoter mutation is one of the earliest recurrent somatic genetic alterations during the transformation sequence from liver cirrhosis to HCC [46-48]. TERT promoter mutations are associated with oncogenic CTNNB1 mutations [46, 47, 49] and epigenetic silencing of the tumor suppressor CDKN2A (p16INK4A) [9], suggesting that activated telomerase may cooperate with TGF- β signaling to initiate liver tumor formation.

Interestingly, our findings revealed that TGF- β , together with CTCF, epigenetically/transcriptionally regulate TERT [20]. Dysfunction of TGF- β -regulated CTCF increases stem cell-like properties in HCC cells and enhances tumorigenesis in tumor-initiating cells in a mouse model, suggesting that defective TGF- β /CTCF might cooperate in liver tumor initiation [14]. Importantly, PJA1 knockdown significantly increases binding of SMAD3 and SPTBN1 to the TERT promoter, thereby transcriptionally increasing TERT gene expression in HCC cells, suggesting that PJA1 may also regulate TERT activity through TGF- β and/or CTCF-mediated epigenetic regulation (Figure 1) [22].

In our ongoing studies, increased levels of PJA1 protein expression were detected in 100% of human liver cirrhosis and cirrhotic HCC patient samples. The strong expression of PJA1 even at the premalignant

cirrhosis stage suggests that PJA1 might drive survival and proliferation of cancer progenitor cells with the potential to develop into HCC. Therefore, we hypothesize that PJA1 plays a central role in chronic fibrosis/inflammation in the liver and cancer stem cell-associated HCC development. These findings also highlight the clinical potential of using PJA1 inhibition to block liver fibrogenesis and possibly oncogenesis (Figure 1).

CONCLUSION

Effective therapeutic strategies targeting HCC are urgently needed and may be achieved by identifying critical signaling pathways relevant in liver cirrhosis and the early stages of liver cancer.

Our genomic analyses of TGF- β in liver and other cancers indicate that genes directly associated with the TGF- β superfamily are recurrently dysregulated (either elevated or suppressed) [21, 50]. However, no therapeutic strategies for HCC patients with a defective TGF- β pathway (low levels of TGF- β) exist. Moreover, patients with tumors harboring a defective TGF- β signature have significantly worse outcomes than those with tumors with an intact TGF- β signature (high and normal levels of TGF- β) [21].

Since TGF- β signaling is recognized to drive the epithelial-mesenchymal transition and liver fibrosis progression toward advanced liver cancer, many drugs targeting TGF- β signaling in liver cancers are currently under investigation in clinical trials [51]. Despite successful outcomes of different anti-TGF- β approaches in cell culture and animal models, cancer clinical trials utilizing strategies to block TGF- β signaling have demonstrated poor or inconsistent results so far [51], suggesting a crucial yet underexplored context-dependent role of TGF- β signaling in human cancers. It is possible that the failure of many of these agents is a consequence of the underappreciated tumor suppressor functions of TGF- β signaling.

Recent mouse model studies have clearly demonstrated the oncogenic potential of disrupting both PJA1 and TGF- β signaling. The consequences of disruption of these genes are likely mediated through liver cancer-initiating cells and/or liver cancer stem cells. Identifying mechanisms to activate TGF- β expression by inhibition of its negative regulators, such as PJA1 E3 ligase, may broaden the usefulness of pathway-based therapies targeting TGF- β . In addition, future therapies may combine E3 ligase inhibition with TGF- β pathway activation to help overcome signaling roadblocks that evolve within tumors. We anticipate significant therapeutic advances to arise from future basic and clinical research exploring the dysregulated PJA1-TGF- β pathway in HCC.

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CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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