J Gastroenterol (2014) 49:971–981
DOI 10.1007/s00535-013-0910-2

The Japanese Society of Gastroenterology

REVIEW

TGF-β signal shifting between tumor suppression and fibro-carcinogenesis in human chronic liver diseases

Koichi Matsuzaki · Toshihito Seki · Kazuichi Okazaki

Received: 25 October 2013 / Accepted: 4 November 2013 / Published online: 22 November 2013

Abstract Perturbation of transforming growth factor (TGF)-β signaling in hepatocytes persistently infected with hepatitis viruses promotes both fibrogenesis and carcinogenesis (fibro-carcinogenesis). Insights into hepatocytic fibro-carcinogenesis have emerged from recent detailed analyses of context-dependent and cell type–specific TGF-β signaling processes directed by multiple phosphorylated forms (phospho-isoforms) of Smad mediators. In the course of hepatitis virus–related chronic liver diseases, chronic inflammation, ongoing viral infection, and host genetic/epigenetic alterations additively shift hepatocytic Smad phospho-isoform signaling from tumor suppression to fibro-carcinogenesis, accelerating liver fibrosis and increasing risk of hepatocellular carcinoma (HCC). After successful antiviral therapy, patients with chronic hepatitis can experience less risk of HCC occurrence by reversing Smad phospho-isoform signaling from fibro-carcinogenesis to tumor suppression. However, patients with cirrhosis can still develop HCC owing to sustained, intense fibro-carcinogenic signaling. Recent progress in understanding Smad phospho-isoform signaling should permit use of Smad phosphorylation as a tool predicting the likelihood of liver disease progression, and as a biomarker for assessing the effectiveness of interventions aimed at reducing fibrosis and cancer risk.

Keywords TGF-β · Smad · Liver fibrosis · Hepatic carcinogenesis · Biomarkers

K. Matsuzaki (✉) · T. Seki · K. Okazaki
Department of Gastroenterology and Hepatology, Kansai Medical University, 10-15 Fumizonocho, Moriguchi, Osaka 570-8506, Japan
e-mail: matsuzak@takii.kmu.ac.jp

Abbreviations

CDK	Cyclin-dependent kinase
ECM	Extracellular matrix
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HSC	Hepatic stellate cells
JNK	c-Jun N-terminal kinase
MH	Mad-homology
MFB	Myofibroblast
PAI	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factor
pSmadC	C-terminally phosphorylated Smad
pSmadL	Linker phosphorylated Smad
pSmadL/C	Dually phosphorylated Smad
SMA	Smooth muscle actin
TGF	Transforming growth factor
TβRI	TGF-β type I receptor
TNF	Tumor necrosis factor

Introduction

Hepatocellular carcinomas (HCCs), which are the most frequent primary liver cancers, represent the fifth most common malignant disease in men and the eighth most common in women worldwide [1]. Overall incidence of HCC continues to rise, especially in Western Europe and the US [1]. During the past 20 years, striking advances have enhanced our understanding of HCC. More than 85 % of HCC cases are related to infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) [2]. Lifetime relative risk for HCC is 15–20 among HB surface antigen (sAg)-positive individuals, compared with

the HBsAg-negative population [3]. HCV infection is associated with a 15- to 20-fold increase in risk for HCC compared with HCV-negative subjects in cross-sectional and case–control studies [4].

If hepatitis viruses are not cleared in the acute phase of infection, the liver may develop chronic hepatitis, in which chronic liver damage causes fibrosis, characterized by extracellular matrix (ECM) protein accumulation [5]. Such ECM deposition distorts hepatic architecture by forming a fibrous scar. Ultimately, nodules of regenerating hepatocytes become enclosed by scar tissue, an event defining cirrhosis. As human livers persistently infected by hepatitis viruses progress from chronic hepatitis to cirrhosis, HCC occurrence increases [5]. Chronic inflammation, hepatitis viruses, and host genetic/epigenetic alterations additively promote the fibrogenic process and increase risk of HCC occurrence in individuals with HBV or HCV infection [6]. Approaches to understanding how human HCC develops in chronic liver diseases should therefore focus on hepatitis virus infection–dependent molecular mechanisms shared in common between fibrosis and carcinogenesis (fibro-carcinogenesis) [7].

Conversely, treatment that eliminates hepatitis viruses allows regression of liver fibrosis and decreases risk for HCC occurrence [1]. Patients with mild liver fibrosis have a significant likelihood of histologically evident decreases in fibrosis and inflammation after a sustained virological response (SVR) against HCV infection in response to interferon (IFN) treatment [8]. Furthermore, these patients have marked reductions in HCC occurrence [9]. Such prevention appears to be most effective when therapy is given before development of cirrhosis. In chronic hepatitis, HCC occurrence clearly depends on continued presence of hepatitis viruses and chronic inflammation. In contrast, patients with cirrhosis have relatively low but real risks of HCC occurrence and hepatic decompensation despite SVR [10, 11].

Development of HCC is a complex, multistep process involving alterations of hepatocytes and their physiological control mechanisms. These changes involve both activation of carcinogenic pathways and inactivation of tumor suppressive pathways [12]. Transforming growth factor (TGF)-β inhibits growth of mature hepatocytes and promotes apoptosis, acting as a tumor suppressor [13]. Several components of the TGF-β signaling pathway are lost or inactivated in various epithelial neoplasms [14]. However, only a fraction of liver tumors exhibit inactivating mutations [15, 16], so other mechanisms perturbing hepatocytic TGF-β signaling appear critical in human hepatocarcinogenesis [17]. On the other hand, TGF-β as well as pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin-6 can synergistically promote fibrogenesis by stimulating ECM deposition in activated mesenchymal cells [18]. Although cytostatic signaling of TGF-β in mature hepatocytes has been studied extensively, fibro-carcinogenic signaling shared between activated mesenchymal cells and chronically injured hepatocytes has received less attention [7].

In this review, we provide an overview of current knowledge concerning human hepatocytic TGF-β signal shifting between tumor suppression and fibro-carcinogenesis before and after treatment against hepatitis virus, stressing potential synergy among hepatitis viruses, chronic inflammation, and host genetic/epigenetic alterations in development of pre-neoplastic hepatocytes that eventually develop into HCC.

Context-dependent and cell type-specific TGF-β signaling directed by Smad phospho-isoforms

The canonical TGF-β pathway involves Smad2 and Smad3 signaling through direct serine phosphorylation of COOH termini by TGF-β type I receptors (TβRI) upon TGF-β binding (Fig. 1a, left) [19]. TβRI-mediated phosphorylation of Smad2 and Smad3 induces their association with a shared partner, Smad4, followed by translocation into the nucleus where these complexes activate transcription of specific genes. Smad proteins contain a conserved Mad-homology (MH)1 domain that binds DNA, and a conserved MH2 domain that binds receptors, the partner Smad4, and transcription coactivators [20].

More divergent linker regions separate the two domains. The linker domain undergoes regulatory phosphorylation by mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase, c-Jun N-terminal kinase (JNK), p38 MAPK, and cyclin-dependent kinase (CDK) as well as glycogen synthase kinase 3-β (Fig. 1a, middle) [21–37].

Antibodies (Abs) reactive with structurally related phosphorylated peptides are valuable tools for determining phosphorylation sites in vivo, and for investigating their distinct signals via phosphorylated domains [38, 39]. Domain-specific phospho-Smad2/3 Abs have allowed us to determine that TβRI and JNK/CDK4 differentially phosphorylate Smad2/3 to create 3 phosphorylated forms (phospho-isoforms): COOH-terminally phosphorylated Smad2/3 (pSmad2C and pSmad3C; Fig. 1a, left), linker-phosphorylated Smad2/3 (pSmad2L and pSmad3L; Fig. 1a, middle), and dually phosphorylated Smad2/3 (pSmad2L/C and pSmad3L/C; Fig. 1a, right) [40]. Except for pSmad2L, which shows cytoplasmic localization, the various phospho-isoforms are localized to cell nuclei. Immunohistochemical and immunofluorescence analyses using specific Abs in human tissues can examine clinical significance of context-dependent and cell type–specific signaling directed by Smad phospho-isoforms, by

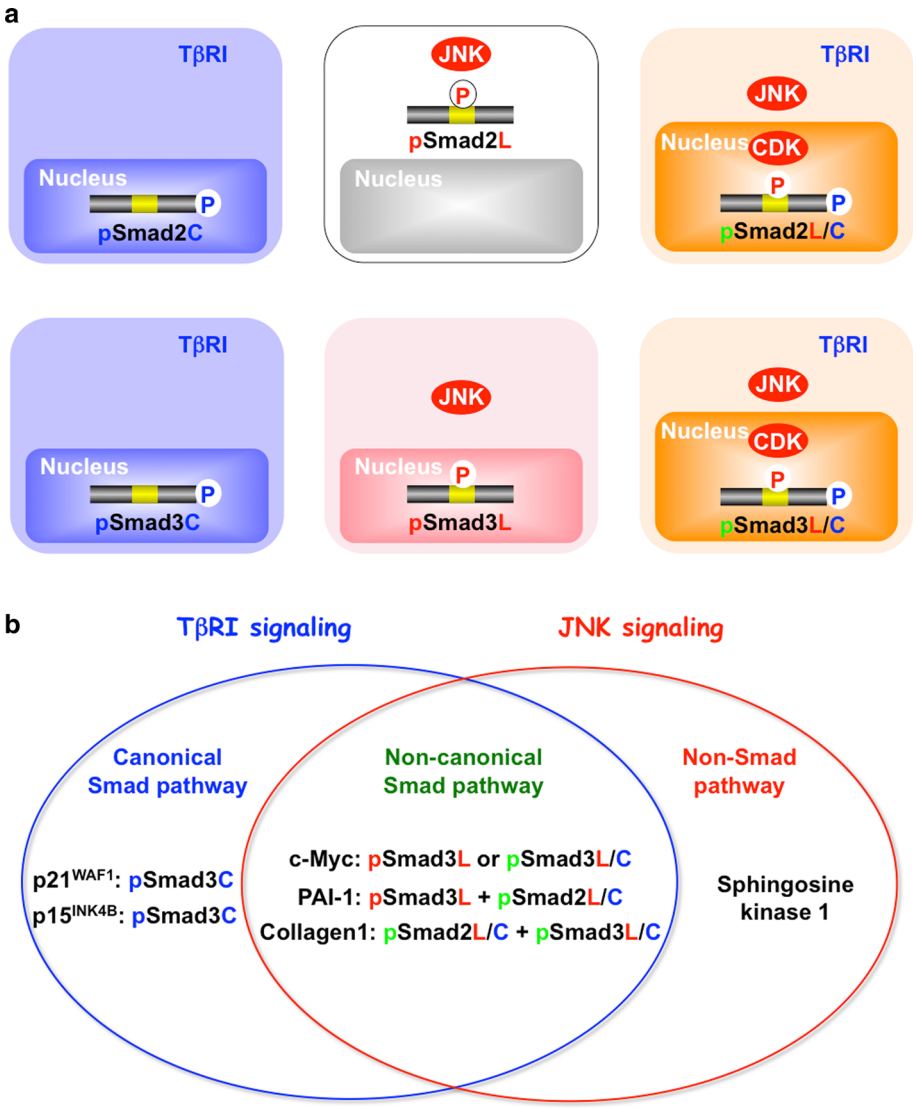


Fig. 1 Multiple Smad phospho-isoform-mediated signaling. **a** Three Smad phospho-isoform types: pSmad2C and pSmad3C; pSmad2L and pSmad3L; and pSmad2L/C and pSmad3L/C. Catalytically active TβRI phosphorylates COOH-tail serine residues of Smad2 and Smad3, while cytoplasmic JNK and nuclear CDK4 alternatively phosphorylate Smad2/3 at specific sites in the middle linker regions. TβRI and JNK/CDK4 differentially phosphorylate Smad2/3 to create three phosphorylated forms (phospho-isoforms): COOH-terminally phosphorylated Smad2/3 (pSmad2C and pSmad3C); linker phosphorylated Smad2/3 (pSmad2L and pSmad3L); and dually phosphorylated Smad2/3 (pSmad2L/C and pSmad3L/C). Except for cytoplasmic localization of pSmad2L, the various phosphoisoforms preferentially localize to cell nuclei. **b** Non-canonical Smad signaling through linker phosphorylation. Canonical Smad signaling activates transcription of cytostatic genes via the TβRI/pSmad3C pathway (i.e., $p21^{WAF1}$ and $p15^{INK4B}$), while JNK activates transcription of other genes via the non-Smad pathway (i.e., sphingosine kinase 1). Despite distinctive signaling pathways, the TβRI pathway shares several target genes (i.e., c-Myc, PAI-1, and collagen 1) with the JNK pathway through Smad linker phosphorylation. Thus, Smad signaling via linker phosphorylation is an important component of the non-canonical Smad pathway

comparing tissue and cellular localization of these phosphoisoforms between various pathologic specimens [37].

Non-canonical Smad signaling through linker phosphorylation

TGF-β also elicits signaling responses through non-Smad pathways that are important effector mechanisms for JNK in response to pro-inflammatory cytokines (Fig. 1b) [41, 42]. Imbalance may occur between signaling through non-Smad and Smad pathways during fibro-carcinogenesis. In particular, JNK activated by TGF-β induces expression of sphingosine kinase 1 in a Smad-independent manner [31, 43].

Numerous reports have suggested that tumorigenic effects of TGF-β involve a pathologic switch of TGF-β signaling from the canonical Smad pathway to a potentially

tumorigenic non-Smad pathway [41, 42]. In cancer cells, however, Smad signaling itself drives pro-tumorigenic gene expression [44, 45] and tumor-initiating stem cell behavior [46]. Linker phosphorylation can explain these long-standing paradoxes concerning Smad signaling, since such phosphorylation occurs apart from the canonical Smad signaling, instead promoting cell growth, invasion, and fibrosis via JNK and CDK pathways [23, 27, 28, 32, 47]. TGF-β can activate the Smad pathway via linker phosphorylation [28], as well as the non-Smad pathway, usually in parallel [7]. Through linker phosphorylation governed by JNK, Smad signaling can be controlled by and functions in conjunction with the alternative non-Smad pathway [41, 42]. Collectively, Smad signaling through linker phosphorylation should be recognized as a major non-canonical Smad pathway [37].

Smad phospho-isoform signaling in hepatocytes after acute liver injury: mitogenic pSmad3L signaling followed by cytostatic pSmad3C signaling

Loss of parenchyma after acute liver injury rapidly induces a wave of hepatocytic proliferation that restores total mass of the liver to normal [48]. Several converging lines of evidence have established that pro-inflammatory cytokines are important components of the mitogenic pathways leading to regeneration [49]. The phosphorylation pattern of Smad3 in regenerative hepatocytes after acute liver injury suggests important participation of Smad3 phospho-isoforms in hepatocytic growth regulation. In actively growing hepatocytes, intracellular phosphorylation at Smad3L is increased (Fig. 2a, upper middle panel) [50–52]. Translocated to the nucleus, TNF-α–induced pSmad3L stimulates c-Myc transcription [53], which increases proliferation of hepatocytes and opposes the cytostatic action of the pSmad3C pathway [7]. Accordingly, pSmad3C is undetectable in regenerative hepatocytic nuclei; escape from TGF-β–induced cytostasis is crucial in a subset of progenitor cells devoted to ensuring epithelial renewal [14]. Thus, mitogenic pSmad3L signaling can permit liver regeneration in response to mitogenic pro-inflammatory cytokines, even though elevated TGF-β concentrations after acute liver injury might otherwise have a cytostatic influence [54, 55].

Liver regeneration is tightly controlled by a delicate balance between hepatocytic growth and inhibition [48]. Anti-mitotic effects of TGF-β contribute to the termination of hepatocyte proliferation observed following the wave of DNA synthesis in the regenerating liver [7, 48]. Return of TGF-β sensitivity at later stages may limit hepatocyte proliferation and terminate liver regeneration [54, 55]. After TNF-α and pSmad3L decrease, hepatocytic proliferation ceases, as decreases in pSmad3L can increase sensitivity to phosphorylation at Smad3C by TβRI (Fig. 2a, upper right panel) [7]. TGF-β-dependent pSmad3C appears to limit the proliferative response of regenerating hepatocytes through inhibition of the G1 to S phase cell-cycle transition [14].

Smad phospho-isoform signaling in hepatic stellate cells after acute liver injury: involvement of pSmad2L/C and pSmad3L/C pathways

After acute liver damage, hepatic stellate cells (HSCs) acquire an activated phenotype associated with gradual loss in retinoid content, which enhances cell proliferation and invasion as well as increases synthesis of ECM components, particularly various collagens [5]. How does cytostatic TGF-β signaling in HSCs take on collagen-producing features within inflammatory microenvironments during acute liver injury? To answer this question, we focused on alternative Smad phospho-isoform pathways—specifically, localization of pSmad2L/C and pSmad3L/C in chemically injured rat livers [47]. These phospho-isoforms are involved in collagen synthesis and transmit a proliferative, invasive TGF-β signal in mesenchymal cells [29]. Nuclear localization of pSmad2L/C and pSmad3L/C is observed in activated HSCs (Fig. 2a, lower right panel) [47]. Because TNF-α and platelet-derived growth factor (PDGF) activate CDK4 via the JNK pathway in HSCs, TNF-α and PDGF can convert cytostatic TGF-β signal to collagen-producing character in activated HSCs under influence of inflammatory microenvironments [7, 47, 56, 57]. Together, pSmad2L/C and pSmad3L/C signaling can mobilize HSCs from the space of Disse to sites of damage, where these activated HSCs contribute to tissue repair by producing large amounts of collagens.

Smad phospho-isoform signaling shared between the chronically injured hepatocytes and myofibroblasts during chronic liver injury: involvement of carcinogenic (mitogenic) pSmad3L and fibrogenic pSmad2L/C pathways

Current evidence suggests that regulation of ECM accumulation in acute and chronic liver diseases involves different mechanisms, even though HSCs are the principal effectors in both cases [5]. As a result of chronic liver damage, HSCs undergo progressive activation to become myofibroblast (MFB)-like cells. During transdifferentiation in culture, pSmad3C-mediated signal decreases, while the pSmad3L pathway predominates [32]. These in vitro observations fully agree with the finding of pSmad3L as opposed to pSmad3C

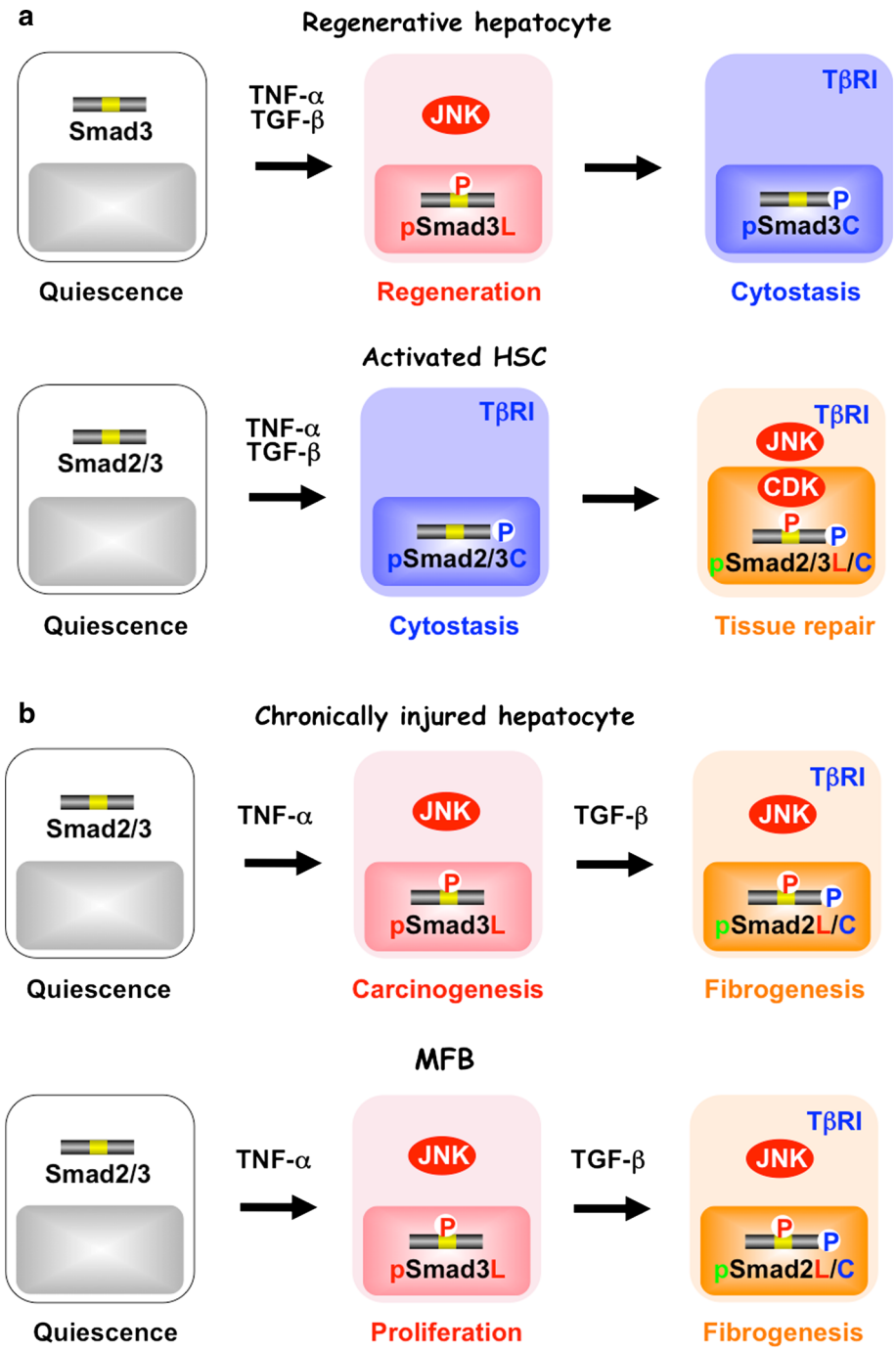
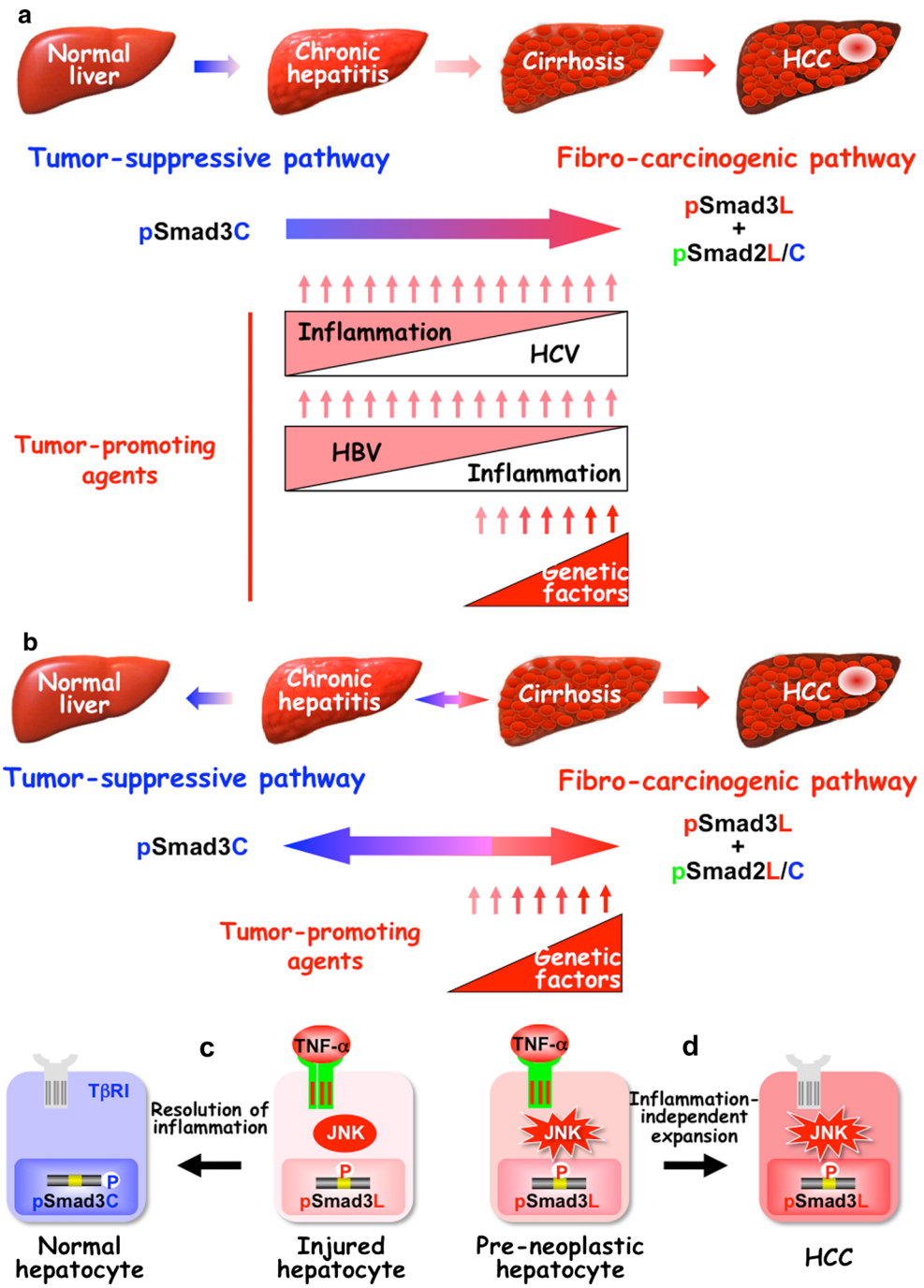


Fig. 2 Fibro-carcinogenic Smad signaling shared between the chronically injured hepatocytes and MFBs in chronic liver diseases. **a** Differential Smad phospho-isoform signaling between hepatocytes and HSCs in acute liver injury. *Upper panel* After acute liver injury, TGF-β-mediated pSmad3C signaling terminates hepatocytic proliferation induced by TNF-α–mediated mitogenic pSmad3L pathway. *Lower panel* After acute liver injury, TGF-β and TNF-α synergistically enhances collagen synthesis by activated HSCs via pSmad2L/C and pSmad3L/C pathways. **b** During progression of chronic liver diseases, chronically injured hepatocytes (*upper panel*) persistently affected by TGF-β together with TNF-α begin to exhibit the same oncogenic (mitogenic) pSmad3L and fibrogenic pSmad2L/C signaling as MFBs (*lower panel*)

in nuclei of α-smooth muscle actin (SMA)-immunoreactive MFBs in portal tracts of chronically HCV-infected liver specimens (Fig. 2b, lower middle panel) [50]. The presence of α-SMA is associated with transdifferentiation of HSC into scar-forming MFB, an event considered pivotal in the fibrogenic response [58].

Similarly to MFBs, hepatocytes in HCV-infected liver exhibit phosphorylation of Smad3L, particularly in cells adjacent to inflamed portal tracts (Fig. 2b, upper middle panel) [50]. Thus, hepatocytes become regulated by the same pSmad3L pathway as MFBs. Extent of phosphorylation at Smad3L is lesser in hepatocytes distant from portal tracts, in sharp contrast to pSmad3C, which is predominantly located in hepatocytic nuclei distant from portal tracts [50]. TNF-α is released from infiltrating Kupffer cells in portal tracts to activate JNK [59]. Together with in vitro data, these findings suggest that TNF-α–dependent JNK can convert Smad3 to pSmad3L in both



chronically injured hepatocytes and MFBs (Fig. 2b, upper and lower middle panels).

A proliferative effect mediated via the JNK/pSmad3L pathway antagonizes TGF-β signaling via the growth-inhibitory pSmad3C pathway in mature hepatocytes (Fig. 2a, upper panels) [7]. Some tumor-promoting effects of pSmad3L involve its ability to indirectly protect hepatocytes from apoptosis [60]. Pre-neoplastic hepatocytes and HCC show reduced anti-mitogenic responses to TGF-β [15, 16, 50]. Escaping the cytostatic action of pSmad3C is a critical step for progression to full malignancy in cancers, which must overcome multiple fail-safe genetic controls [14]. The TGF-β/pSmad3C pathway is required for maintenance of genomic stability, induction of replicative senescence, and suppression of telomerase [61–63]. Selection for genetic instability occurs in clones of aberrant cells able to produce tumors, since genetic instability greatly accelerates accumulation of further genetic and epigenetic changes required for tumor progression [64].

◀ **Fig. 3** Smad phospho-isoform signal shifting between tumor suppression and fibro-carcinogenesis in hepatitis virus–related chronic liver diseases. **a** During hepatitis virus–related chronic liver diseases, chronic inflammation, hepatitis viruses, and host genetic/epigenetic alterations additively shift hepatocytic Smad phospho-isoforms from tumor-suppressive pSmad3C to carcinogenic pSmad3L and fibrogenic pSmad2L/C, accelerating liver fibrosis and increasing risk of HCC. Both chronic inflammation and hepatitis viruses represent an early fibro-carcinogenic step providing nonmutagenic tumor-promoting stimuli. HCV indirectly promotes liver diseases through chronic inflammation in chronic hepatitis C. In contrast, HBV in itself dominantly triggers the pSmad3L pathway in chronic hepatitis B, thus playing a role beyond mere stimulation of the host immune response. In both HBV- and HCV-related liver cirrhosis, mitogenic genetic or epigenetic alterations can drive multistep fibro-carcinogenesis. **b** After successful therapies against hepatitis viruses, patients with chronic hepatitis can experience less risk of HCC occurrence by reversing Smad phospho-isoform signaling from carcinogenic pSmad3L and fibrogenic pSmad2L/C to tumor-suppressive pSmad3C, whereas patients with cirrhosis can still develop HCC owing to maintenance of strong fibro-carcinogenic signaling. If patients with chronic hepatitis C can eliminate HCV before hepatocytes have acquired oncogenic potential, HCV clearance interferes with fibrosis and reduces HCC incidence (*blue arrow*). However, HCC develops in patients with cirrhosis, where an inflammation-independent process of fibro-carcinogenesis, possibly caused by genetic or epigenetic alteration, may have already begun before HCV clearance (*red arrow*). **c** Signaling via pSmad3L initially depends on the presence of chronic inflammation and hepatitis viruses: cessation of chronic inflammation and hepatitis viral infection restores hepatocytic tumor-suppressive pSmad3C signaling, as occurs in normal hepatocytes at the expense of carcinogenic pSmad3L signaling. **d** Patients with cirrhosis are no longer dependent on inflammation and hepatitis viruses for hepatocarcinogenesis. Pre-neoplastic hepatocytes constitutively carry out carcinogenic pSmad3L signaling, possibly as a result of genetic or epigenetic alterations, despite reduced inflammation after elimination of hepatitis viruses

In contrast to the differing distribution of pSmad3L and pSmad3C, Smad2 is phosphorylated at both linker and C-terminal regions in hepatocytic nuclei in hepatitis virus–infected liver specimens [65]. On the other hand, findings in transgenic mice overexpressing plasminogen activator inhibitor (PAI)-1 as well as PAI-1-knockout mice support a critical role in vivo for PAI-1 in experimental fibrotic diseases [66]. Moreover, introduction of PAI-1 small interfering RNA attenuates deposition of ECM and hydroxyproline content in experimental hepatic fibrosis [67]. Plasma TGF-β, TNF-α, and PAI-1 concentrations are usually elevated in patients with chronic liver diseases [68–71]. Since pSmad2L/C in the presence of pSmad3L transmits a fibrogenic signal by stimulating PAI-1 transcription [32], we investigated the pSmad2L/C pathway in human chronic liver disease. The results in chronic hepatitis specimens indicate nuclear localization of pSmad2L/C in PAI-1-immunoreactive MFBs and hepatocytes [65]. Various reports demonstrate transcriptional induction of the *PAI-1* gene by TGF-β and TNF-α [29, 30]. Taken together, TGF-β and TNF-α can mediate pSmad3L and pSmad2L/C signaling that induces PAI-1 expression and promotes ECM deposition in both hepatocytes and MFBs, accelerating liver fibrosis (Fig. 1b; Fig. 2b, upper and lower right panels). As a consequence, the chronically injured hepatocytes exhibit the pro-fibrogenic TGF-β signaling, but have lost the capacity to respond to TGF-β with growth arrest and apoptosis.

During hepatitis virus–related chronic liver diseases, chronic inflammation, hepatitis viruses, and host genetic/epigenetic alterations additively shift hepatocytic Smad phospho-isoform signaling from tumor suppression to fibro-carcinogenesis, thereby accelerating liver fibrosis and increasing risk of HCC

We finally illustrate Smad phospho-isoform signaling during human hepatic fibro-carcinogenesis, which provides substantial mechanistic insight. HBV-and HCV-encoded proteins alter cellular phenotypes that are recognized as hallmarks of cancer [72]. These changes promote mitogen-independent proliferation and resistance to growth inhibition and apoptosis. A strong correlation between chronic HBV infection and HCC occurrence is apparent from epidemiologic evidence [73] and also the finding of integrated HBV-DNA sequences in virtually all HBV-related HCC [74]. The HBV genome carries the *HBx* gene, which has been implicated in HBV-mediated hepatocarcinogenesis [75]. Several studies indicate that the HBx protein alone has no transforming activity, but its overexpression in a certain genetic background induces tumor formation. Persistent expression of HBx oncoprotein in transgenic mice induces HCC by sensitizing them to chemical carcinogenesis [76]. Moreover, HCV core gene transgenic mice develop steatohepatitis and then HCC [77].

Another major factor in HCC development is the host immune system [12]. During inflammation, pro-inflammatory cytokines are key factors mediating immune responses [78]. Chronic inflammation plays a multi-faceted role in carcinogenesis [79]. Possible mechanisms by which inflammation can contribute to carcinogenesis include induction of genomic instability and alterations in epigenetic events causing enhanced proliferation of affected hepatocytes and their resistance to apoptosis. These results suggest that hepatitis viral components together with chronic inflammation can cooperate to create a malignant HCC phenotype.

Two of our papers describe how carcinogenic pSmad3L signaling is similar but yet different between HBV- and HCV-related chronic liver diseases [50, 51]. In early stages of chronic hepatitis, hepatitis viruses and chronic inflammation are independently associated with pSmad3L-mediated signals initiating HCC development (Fig. 3a).

Positivity of hepatocytic nuclei for pSmad3L in chronic hepatitis B specimens increases with amount of HBV-DNA, but not with intensity of inflammation [51]. Thus, HBV directly triggers the pSmad3L carcinogenic pathway in early chronic hepatitis B, playing a role beyond mere stimulation of the host immune response. In chronic hepatitis C, however, positivity of hepatocytic nuclei for pSmad3L in chronic hepatitis C specimens shows a significant relationship with necroinflammatory activity but not with plasma HCV-RNA concentration in chronic hepatitis C patients [50]. Moreover, no consistent evidence indicates that HCV load or quasispecies affects risk of HCC [1]. Taken together, the various lines of evidence suggest that HCV contributes indirectly to development of HCC through chronic inflammation in chronic hepatitis C.

In damaged hepatocytes and MFBs in inflammatory microenvironments, TGF-β can act together with TNF-α to induce fibrogenic signaling via the pSmad2L/C pathway (Fig. 2b, upper and lower right panels). In contrast to typical reciprocal changes in pSmad3L and pSmad3C [50, 51], damaged hepatocytes show a simultaneous increase in both COOH-terminal and linker phosphorylation of Smad2 as liver fibrosis progress [65]. During progression of chronic liver diseases, chronic inflammation and hepatitis viruses additively shift hepatocytic Smad signaling from tumor suppressive pSmad3C to carcinogenic pSmad3L and fibrogenic pSmad2L/C pathways, accelerating liver fibrosis and increasing risk of HCC (Fig. 3a). Thus, Smad phosphoisoforms act as important orchestrators of the chronic inflammation–fibrosis–HCC sequence [7].

After successful therapies against hepatitis viruses, patients with chronic hepatitis are at less risk of HCC because Smad phospho-isoform signaling reverses from fibro-carcinogenesis to tumor suppression, while patients with cirrhosis retain high risk of HCC owing to continued, intense fibro-carcinogenic signaling

Improved understanding of Smad phospho-isoform signaling during human fibro-carcinogenesis suggests better ways to prevent HCC development, exemplifying laboratory-driven translational research. Proving reversibility of human fibro-carcinogenesis is critical, and may point to new therapeutic strategies that attempt mimicry or promotion of this process. A key therapeutic aim in chronic liver disorders is restoration of the lost tumor-suppressive function observed in normal hepatocytes, at the expense of effects promoting hepatic fibro-carcinogenesis.

Patients with mild liver fibrosis are likely to show histologically evident decreases in fibrosis and inflammation after HCV clearance in response to effective antiviral treatment [8]. Such patients experience far less HCC occurrence [9]. Once patients with chronic hepatitis C achieve HCV clearance, Smad phospho-isoform signaling can reverse from fibro-carcinogenesis to tumor suppression (Fig. 3b). Effectiveness of tumor-suppressive TGF-β/pSmad3C signaling appears related to decreases in Smad3 phosphorylation at the linker region, allowing C-tail phosphorylation of Smad3 to approach the normal state after HCV clearance, as pro-inflammatory cytokine-mediated pSmad3L decreases in hepatocytic nuclei (Fig. 3c) [65]. On the other hand, blocking either linker or C-tail phosphorylation of Smad2 abrogates TGF-β– and TNF-α–mediated fibrogenic signaling in hepatocytes [30, 47]. Accordingly, C-tail as well as linker phosphorylation of Smad2 decreases in hepatocytic nuclei after HCV clearance [65]. Thus, our current work offers proof-of-principle that carcinogenic pSmad3L and fibrogenic pSmad2L/C signaling in human chronic liver diseases initially depends on persistent presence of hepatitis viruses and inflammation, gradually shifting to tumor-suppressive pSmad3C signaling when inflammation has abated.

During later stages of the carcinogenic process, livers with either HBV- or HCV-related cirrhosis exhibit constitutively high fibro-carcinogenic signaling [50, 51]. In cirrhosis from either etiology, strong pSmad3L positivity is observed throughout hepatic lobules. Similarly, pSmad2L/C-mediated signaling constitutively induces ECM deposition in pre-neoplastic hepatocytes and HCC by up-regulating PAI-1 transcription [16]. No potentially preventive effects of anti-HCV therapies are able to reduce liver fibrosis and development of HCC once HCV-related cirrhosis is established [10, 11]. Consistent with this observation, hepatocytes maintaining high carcinogenic pSmad3L and fibrogenic pSmad2L/C signaling cannot return to tumor-suppressive pSmad3C signaling, even after HCV clearance (Fig. 3d) [65]. Since genetic or epigenetic alterations of major oncogenic pathway components may lead to sustained linker phosphorylation of Smad2/3 [28, 80, 81], pSmad3L and pSmad2L/C can constitutively transmit fibro-carcinogenic signals even after chronic inflammation resolves.

A key question concerning effectiveness of antiviral therapy for preventing liver fibrosis progression and HCC development is whether it still has value once pre-neoplastic hepatocytes have appeared. Molecular analyses of paired liver biopsy specimens enable us to predict fibrosis progression and HCC risk after HCV clearance. Specimens from HCV-related chronic liver diseases can be divided into 2 subgroups based on phospho-Smad2/3 profiles. One group carries risk of hepatic decompensation and HCC after HCV clearance, while the other avoids fibrosis progression and HCC occurrence. This explains the observation that some patients with HCV-related liver disease respond effectively to antiviral therapy by reversing

TGF-β/Smad signaling from fibro-carcinogenesis to tumor suppression, while others do not. Irrespective of SVR achievement, patients with advanced liver fibrosis who maintain strong pSmad3L and pSmad2L/C signaling in hepatocytic nuclei after HCV clearance require continued close follow-up, since fibrosis progression and HCC risk are not avoided.

Conclusion and perspectives

Risk of HCC development increases in proportion to the degree of liver fibrosis in patients persistently infected with hepatitis virus, especially in those with active viral replication and inflammation. Our model suggests that chronic inflammatory state and hepatitis viruses themselves act in concert with genetic or epigenetic alterations to worsen human liver diseases by promoting hepatic fibro-carcinogenesis. Affected hepatocytes are subject to such interactions until their descendants acquire other genetic or epigenetic alterations. Then, even after withdrawal of promoters such as chronic inflammation and hepatitis viruses, hepatocytes can retain proliferative phenotypes enabling some pre-neoplastic hepatocytes to develop into HCC. Recent progress in understanding Smad phospho-isoform signaling will permit use of Smad phosphorylation as a prognostic indicator concerning progression of human liver diseases, and also as a biomarker for assessing effectiveness of interventions aimed at reducing fibrosis and cancer risk.

Active HBV replication at onset of cirrhosis is an important factor contributing to further liver disease progression. Approval of oral anti-viral agents against HBV has greatly improved prognosis of the patients with liver cirrhosis, as demonstrated in several clinical trials involving therapies with lamivudine, adefovir dipivoxil, or entecavir. These oral antiviral agents are effective in restoring liver function and improving survival in patients with cirrhosis, especially when therapy is initiated early [82–84]. However, convincing effects preventing HCC development have yet to be demonstrated [85–87]. One possible explanation is that genetic events in some hepatocytes in cirrhotic nodules may have occurred before initiation of anti-viral therapy [88]. We are carrying out several trials to determine how anti-HBV therapy for patients with HBV-related cirrhosis affects liver fibrosis and HCC incidence. The trials will answer important questions regarding differential participation of Smad phospho-isoforms in regression of fibrosis and hepatocarcinogenesis after successful anti-viral therapy.

Acknowledgments This study was supported by Ministry of Education, Science and Culture of Japan [22390153 to K.M.].

Conflict of interest The authors declare that they have no conflict of interest.

References

1. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology. 2012;142:1264–73.
2. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol. 2005;5:215–29.
3. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol. 2008;48:335–52.
4. Tanaka Y, Kurbanov F, Mano S, et al. Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinoma mortality. Gastroenterology. 2006;130:703–14.
5. Friedman SL. Evolving challenges in hepatic fibrosis. Nat Rev Gastroenterol Hepatol. 2010;7:425–36.
6. Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. Nat Rev Cancer. 2013;13:123–35.
7. Matsuzaki K. Smad phosphoisoform signals in acute and chronic liver injury: similarities and differences between epithelial and mesenchymal cells. Cell Tissue Res. 2012;347:225–43.
8. Shiratori Y, Imazeki F, Moriyama M, et al. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med. 2000;132:517–24.
9. Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med. 1999;131:174–81.
10. Morgan TR, Ghany MG, Kim HY, HALT-C Trial Group, et al. Outcome of sustained virological responders with histologically advanced chronic hepatitis C. Hepatology. 2010;52:833–44.
11. Kobayashi S, Takeda T, Enomoto M, et al. Development of hepatocellular carcinoma in patients with chronic hepatitis C who had a sustained virological response to interferon therapy: a multicenter, retrospective cohort study of 1124 patients. Liver Int. 2007;27:186–91.
12. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet. 2002;31:339–46.
13. Moses HL, Serra R. Regulation of differentiation by TGF-β. Curr Opin Genet Dev. 1996;6:581–6.
14. Massagué J. TGF-β signalling in context. Nat Rev Mol Cell Biol. 2012;13:616–30.
15. Matsuzaki K, Date M, Furukawa F, et al. Autocrine stimulatory mechanism by transforming growth factor β in human hepatocellular carcinoma. Cancer Res. 2000;60:1394–402.
16. Matsuzaki K, Date M, Furukawa F, et al. Regulatory mechanisms for transforming growth factor β as an autocrine inhibitor in human hepatocellular carcinoma: implications for roles of Smads in its growth. Hepatology. 2000;32:218–27.
17. Meindl-Beinker NM, Matsuzaki K, Dooley S. TGF-β signaling in onset and progression of hepatocellular carcinoma. Dig Dis. 2012;30:514–23.
18. Roberts AB, Sporn MB. The transforming growth factor-βs. In: Sporn MB, Roberts AB, editors. Peptide growth factors and their receptors. Berlin: Springer; 1990. p. 419–72.
19. Heldin CH, Miyazono K, ten Dijke P. TGF-β signaling from cell membrane to nucleus through SMAD proteins. Nature. 1997;390:465–71.

20. Derynck R, Miyazono K. The TGF-β signaling. New York: Cold Spring Harbor Laboratory Press; 2008.
21. Matsuura I, Denissova NG, Wang G, et al. Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature. 2004;430:226–31.
22. Wang G, Matsuura I, He D, et al. Transforming growth factor-β-inducible phosphorylation of Smad3. J Biol Chem. 2009;284:9663–73.
23. Matsuzaki K, Kitano C, Murata M, et al. Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-β signal in later stages of human colorectal cancer. Cancer Res. 2009;69:5321–30.
24. Alarcón C, Zaromytidou AI, Xi Q, et al. Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-β pathways. Cell. 2009;139:757–69.
25. Gao S, Alarcón C, Sapkota G, et al. Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-β signaling. Mol Cell. 2009;36:457–68.
26. Matsuura I, Wang G, He D, et al. Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3. Biochemistry. 2005;44:12546–53.
27. Mori S, Matsuzaki K, Yoshida K, et al. TGF-β and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. Oncogene. 2004;23:7416–29.
28. Sekimoto G, Matsuzaki K, Yoshida K, et al. Reversible Smad-dependent signaling between tumor suppression and oncogenesis. Cancer Res. 2007;67:5090–6.
29. van der Velden JL, Alarcón JF, Guala AS, et al. c-Jun N-terminal kinase 1 promotes TGF-β1-induced epithelial to mesenchymal transition via control of linker phosphorylation and transcriptional activity of Smad3. Am J Respir Cell Mol Biol. 2011;44:571–81.
30. Saito A, Suzuki HI, Horie M, et al. An integrated expression profiling reveals target genes of TGF-β and TNF-α possibly mediated by micro RNAs in lung cancer cells. PLoS ONE. 2013;8:e56587.
31. Yumoto K, Thomas PS, Lane J, et al. TGF-β-activated kinase 1 (Tak1) mediates agonist-induced Smad activation and linker region phosphorylation in embryonic craniofacial neural crest-derived cells. J Biol Chem. 2013;288:13467–80.
32. Furukawa F, Matsuzaki K, Mori S, et al. p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. Hepatology. 2003;38:879–89.
33. Kamaraju AK, Roberts AB. Role of Rho/ROCK and p38 map kinase pathways in transforming growth factor β mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. J Biol Chem. 2005;280:1024–36.
34. Millet C, Yamashita M, Heller M, et al. A negative feedback control of transforming growth factor-β signaling by glycogen synthase kinase 3-mediated Smad3 linker phosphorylation at Ser-204. J Biol Chem. 2009;284:19808–16.
35. Guo X, Ramirez A, Waddell DS, et al. Axin and GSK3-β control Smad3 protein stability and modulate TGF-β signaling. Genes Dev. 2008;22:106–20.
36. Abushahba W, Olabisi OO, Jeong BS, et al. Non-canonical Smads phosphorylation induced by the glutamate release inhibitor, riluzole, through GSK3 activation in melanoma. PLoS ONE. 2012;7:e47312.
37. Matsuzaki K. Smad phospho-isoforms direct context-dependent TGF-β signaling. Cytokine Growth Factor Rev. 2013;24:385–99.
38. Liu F. Smad3 phosphorylation by cyclin-dependent kinases. Cytokine Growth Factor Rev. 2006;17:9–17.
39. Wrighton KH, Lin X, Feng X-H. Phospho-control of TGF-β superfamily signaling. Cell Res. 2009;19:8–20.
40. Matsuzaki K. Smad phosphoisoform signaling specificity: the right place at the right time. Carcinogenesis. 2011;32:1578–88.
41. Zhang YE. Non-Smad pathways in TGF-β signaling. Cell Res. 2009;19:128–39.
42. Moustakas A, Heldin CH. Non-Smad TGF-β signals. J Cell Sci. 2005;118:3573–84.
43. Yamanaka M, Shegogue D, Pei H, et al. Sphingosine kinase 1 (SPHK1) is induced by transforming growth factor-β and mediates TIMP-1 up-regulation. J Biol Chem. 2004;279:53994–4001.
44. Kang Y, He W, Tulley S, et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proc Natl Acad Sci USA. 2005;102:13909–14.
45. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat Cell Biol. 2002;4:487–94.
46. Ikushima H, Todo T, Ino Y, et al. Autocrine TGF-β signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. Cell Stem Cell. 2009;5:504–14.
47. Yoshida K, Matsuzaki K, Mori S, et al. Transforming growth factor-β and platelet-derived growth factor signal via c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation in rat hepatic stellate cells after acute liver injury. Am J Pathol. 2005;166:1029–39.
48. Michalopoulos GK. Liver regeneration. J Cell Physiol. 2007;213:286–300.
49. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140:883–99.
50. Matsuzaki K, Murata M, Yoshida K, et al. Chronic inflammation associated with hepatitis C virus infection perturbs hepatic transforming growth factor β signaling, promoting cirrhosis and hepatocellular carcinoma. Hepatology. 2007;46:48–57.
51. Murata M, Matsuzaki K, Yoshida K, et al. Hepatitis B virus X protein shifts human hepatic TGF-β signaling from tumor-suppression to oncogenesis in early chronic hepatitis B. Hepatology. 2009;49:1203–17.
52. Nagata H, Hatano E, Tada M, et al. Inhibition of c-Jun NH2-terminal kinase switches Smad3 signaling from oncogenesis to tumor-suppression in rat hepatocellular carcinoma. Hepatology. 2009;49:1944–53.
53. Kawamata S, Matsuzaki K, Murata M, et al. Oncogenic Smad3 signaling induced by chronic inflammation is an early event in ulcerative colitis-associated carcinogenesis. Inflamm Bowel Dis. 2011;17:683–95.
54. Date M, Matsuzaki K, Matsushita M, et al. Differential expression of transforming growth factor-β and its receptors in hepatocytes and nonparenchymal cells of rat liver after CCl4 administration. J Hepatol. 1998;28:572–81.
55. Date M, Matsuzaki K, Matsushita M, et al. Modulation of transforming growth factor β function in hepatocytes and hepatic stellate cells in rat liver injury. Gut. 2000;46:719–24.
56. Kaji K, Yoshiji H, Ikenaka Y, et al. Dipeptidyl peptidase-4 inhibitor attenuates hepatic fibrosis via suppression of activated hepatic stellate cell in rats. J Gastroenterol. 2013 (Epub ahead of print).
57. Szuster-Ciesielska A, Mizerska-Dudka M, Daniluk J, et al. Butein inhibits ethanol-induced activation of liver stellate cells through TGF-β, NFκB, p38, and JNK signaling pathways and inhibition of oxidative stress. J Gastroenterol. 2013;48:222–37.
58. Pinzani M, Macias-Barragan J. Update on the pathophysiology of liver fibrosis. Expert Rev Gastroenterol Hepatol. 2010;4:459–72.
59. Kluwe J, Pradere JP, Gwak GY, et al. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. Gastroenterology. 2010;138:347–59.
60. Mu X, Lin S, Yang J, et al. TGF-β signaling is often attenuated during hepatotumorigenesis, but is retained for the malignancy of hepatocellular carcinoma cells. PLoS ONE. 2013;8:e63436.
61. Glick AB, Weinberg WC, Wu IH, et al. Transforming growth factor β 1 suppresses genomic instability independent of a G1 arrest, p53, and Rb. Cancer Res. 1996;56:3645–50.

62. Tremain R, Marko M, Kinnimulki V, et al. Defects in TGF-β signaling overcome senescence of mouse keratinocytes expressing v-Ha-ras. Oncogene. 2000;19:1698–709.
63. Yang H, Kyo S, Takatura M, et al. Autocrine transforming growth factor β suppresses telomerase activity and transcription of human telomerase reverse transcriptase in human cancer cells. Cell Growth Differ. 2001;12:119–27.
64. Weinberg RA. The biology of cancer. New York: Garlang Science; 2007.
65. Yamaguchi T, Matsuzaki K, Inokuchi R, et al. Phosphorylated Smad2 and Smad3 signaling: shifting between tumor suppression and fibro-carcinogenesis in chronic hepatitis C. Hepatol Res. 2013 (Epub ahead of print).
66. Eitzman DT, McCoy RD, Zheng X, et al. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. J Clin Invest. 1996;97:232–7.
67. Hu PF, Chen H, Zhong W, et al. Adenovirus-mediated transfer of siRNA against PAI-1 mRNA ameliorates hepatic fibrosis in rats. J Hepatol. 2009;51:102–13.
68. Shirai Y, Kawata S, Tamura S, et al. Plasma transforming growth factor-β1 in patients with hepatocellular carcinoma: comparison with chronic liver diseases. Cancer. 1994;73:2275–9.
69. Tilg H, Kaser A, Moschen AR. How to modulate inflammatory cytokines in liver diseases. Liver Int. 2006;26:1029–39.
70. Sugano Y, Matsuzaki K, Tahashi Y, et al. Distortion of autocrine transforming growth factor β signal accelerates malignant potential by enhancing cell growth as well as PAI-1 and VEGF production in human hepatocellular carcinoma cells. Oncogene. 2003;22:2309–21.
71. Hidaka H, Nakazawa T, Shibuya A, et al. Effects of 1-year administration of olmesartan on portal pressure and TGF-β1 in selected patients with cirrhosis: a randomized controlled trial. J Gastroenterol. 2011;46:1316–23.
72. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
73. Chen CJ, Yang HI, Su J, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA. 2006;295:65–73.
74. Bréchot C. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. Gastroenterology. 2004;127:S56–61.
75. Kim CM, Koike K, Saito I, et al. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature. 1991;351:317–20.
76. Madden CR, Finegold MJ, Slagle BL. Hepatitis B virus X protein acts as a tumor promoter in development of diethylnitrosamine-induced preneoplastic lesions. J Virol. 2001;75:3851–8.
77. Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med. 1998;4:1065–7.
78. Szabo G, Chang S, Dolganiuc A. Altered innate immunity in chronic hepatitis C infection: cause or effect? Hepatology. 2007;46:1279–90.
79. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860–7.
80. Hamajima H, Ozaki I, Zhang H, et al. Modulation of the transforming growth factor-β1-induced Smad phosphorylation by the extracellular matrix receptor β1-integrin. Int J Oncol. 2009;35:1441–7.
81. Chen D, Lin Q, Box N, et al. SKI knockdown inhibits human melanoma tumor growth in vivo. Pigment Cell Melanoma Res. 2009;22:761–72.
82. Dienstag JL, Goldin RD, Heathcote EJ, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology. 2003;124:105–17.
83. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, et al. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. Gastroenterology. 2006;131:1743–51.
84. Chang TT, Liaw YF, Wu SS, et al. Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B. Hepatology. 2010;52:886–93.
85. Villanueva A, Llovet JM. Targeted therapies for hepatocellular carcinoma. Gastroenterology. 2011;140:1410–26.
86. Eun JR, Lee HJ, Kim TN, et al. Risk assessment for the development of hepatocellular carcinoma: according to on-treatment viral response during long-term lamivudine therapy in hepatitis B virus-related liver disease. J Hepatol. 2010;53:118–25.
87. Papatheodoridis GV, Manolakopoulos S, Touloumi G, et al. Virological suppression does not prevent the development of hepatocellular carcinoma in HBeAg-negative chronic hepatitis B patients with cirrhosis receiving oral antiviral(s) starting with lamivudine monotherapy: results of the nationwide HEPNET. Greece cohort study. Gut. 2011;60:1109–16.
88. Laurent-Puig P, Zucman-Rossi J. Genetics of hepatocellular tumors. Oncogene. 2006;25:3778–86.