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Modulation of TGF-beta signaling during progression of chronic liver diseases

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1. ABSTRACT

A large body of work has established roles for epithelial cells as important mediators of progressive fibrosis and carcinogenesis. Transforming growth factor-beta (TGF-beta) and pro-inflammatory cytokines are important inducers of fibro-carcinogenesis. TGF-beta signaling involves phosphorylation of Smad3 at middle linker and/or C-terminal regions. Reversible shifting of Smad3-dependent signaling between tumor-suppression and oncogenesis in hyperactive Ras-expressing epithelial cells indicates that Smad3 phosphorylated at the C-terminal region (pSmad3C) transmits a tumor-suppressive TGF-beta signal, while oncogenic activities such as cell proliferation and invasion are promoted by Smad3 phosphorylated at the linker region (pSmad3L). Notably, pSmad3L-mediated signaling promotes extracellular matrix deposition by activated mesenchymal cells. During progression of chronic liver diseases, hepatic epithelial hepatocytes undergo transition from the tumor-suppressive pSmad3C pathway to the fibrogenic/oncogenic pSmad3L pathway, accelerating liver fibrosis and increasing risk of hepatocellular carcinoma. c-Jun N-terminal kinase activated by pro-inflammatory cytokines is mediating this perturbed hepatocytic TGF-beta signaling. Thus, TGF-beta signaling of hepatocytes affected by chronic inflammation offers a general framework for understanding the molecular mechanisms of human fibro-carcinogenesis during progression of chronic liver diseases.

2. INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is the third leading cause of cancer deaths worldwide (1). Overall incidence of HCC continues to rise, especially in Western Europe and the US (2). During the past 20 years, striking advances have enhanced our understanding of HCC. More than 85% of HCC cases are related to known hepatitis C virus (HCV) and hepatitis B virus (HBV). Over 170 million people are infected with HCV worldwide, resulting in a large disease burden and significant mortality (3). As HCV is rarely cleared in the acute phase of infection, most patients become chronically infected and develop hepatitis. Some of these patients develop liver fibrosis as a result of chronic liver damage, characterized by extracellular matrix (ECM) protein accumulation in the liver (4). Such an ECM deposition, which is similarly observed in most types of chronic liver disorder, distorts hepatic architecture by forming a fibrous scar. Ultimately, nodules of regenerating hepatocytes become enclosed by scar tissue, which defines cirrhosis. As HCV-infected livers progress from chronic hepatitis to cirrhosis, HCC occurrence increases (5). HCV infection leading to chronic inflammation apparently promotes both fibrosis and carcinogenesis in the liver (Figure 1).

Transforming growth factor-beta (TGF-beta) inhibits growth of mature epithelial cells (6) and induces

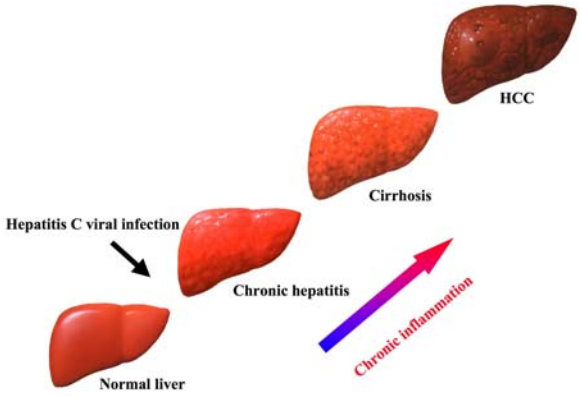

Figure 1. Chronic inflammation causes progressive liver fibrosis and ultimately HCC. As hepatitis C virus (HCV)-infected livers progress from chronic hepatitis to cirrhosis, HCC occurrence increases. Many patients with chronic hepatitis caused by HCV infection develop liver fibrosis with high risk for HCC. Chronic inflammation associated with persistent HCV infection is the primary inducer of both liver fibrosis and HCC.

apoptosis (7), thereby acting as a tumor suppressor. Several components of the TGF-beta signaling pathway are lost or inactivated in a variety of epithelial neoplasms (8-10). However, since only a fraction of liver tumors exhibit inactivating mutations in early stages of cancer formation (11), other mechanisms would appear to play critical roles in human liver carcinogenesis. At present, loss of sensitivity to growth inhibition by TGF-beta in most cancer cells is not synonymous with complete shutdown of the whole TGF-beta signaling (12). Instead, cancer cells gain advantage by selective inactivation of tumor-suppressing activities of TGF-beta accompanied with augmentation of invasive capacity (Figure 2). This process is associated with TGF-beta dependent ECM synthesis by proliferation and invasion of activated mesenchymal cells (13). Cytostatic signaling of TGF-beta in mature epithelial cells has been extensively studied (14), whereas the fibrogenic/oncogenic signaling commonly observed in activated mesenchymal cells and cancer cells has not been elucidated.

Progress over the past 10 years has disclosed important details of how TGF-beta elicits its responses. TGF-beta binds to type II TGF-beta receptor kinase (TbetaRII) and recruits type I receptor (TbetaRI). TbetaRII kinase phosphorylates the GS segments in TbetaRI (15). Smads are central mediators of signals from the receptors for TGF-beta superfamily members to the nucleus (16,17). The catalytically active TbetaRI phosphorylates the C-terminal serine residues of receptor-activated Smads, which are Smad2 and the highly related protein Smad3 (Figure 2). Smad3 phosphorylated at the C-terminal region (pSmad3C)-mediated signaling involves the cytostatic function of TGF-beta in mature epithelial cells (14). Smads are modular proteins with conserved Mad-homology (MH)1, intermediate linker, and MH2 domains. The middle linker domain can undergo regulatory phosphorylation by other kinases including extracellular signal-regulated protein kinase, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and cyclin-dependent kinase 2/4 (18-21). In contrast to the clearly activating role of the C-terminal phosphorylation events, the regulation of Smad activity by phosphorylation of the linker region is complex. Linker phosphorylation of Smad2 during human colorectal carcinogenesis results in cytoplasmic retention of Smad2 and inhibition of tumor-suppressive TGF-beta signaling (14,22). However, Smad3 phosphorylated at the linker region (pSmad3L) is localized predominantly to cell nuclei in actively growing colorectal cancer (22). Phosphorylated Smad2 and Smad3 rapidly oligomerize with Smad4, forming functional trimeric protein complexes (14). While monomeric Smad proteins constantly shuttle in and out of the nucleus, formation of the activated Smad complex favors their nuclear accumulation (23). In the nucleus, the Smad complex binds directly to DNA and associates with a plethora of transcription factors, co-activators, or co-repressors, leading to transcriptional induction or repression of target genes (24).

Although several studies about TGF-beta action in mesenchymal transition/oncogenesis have suggested that the process involves Smad-independent pathways (17), recent studies using Smad3 knockout mice have indicated that signaling through the Smad3-pathway is required for injury-dependent multistage transition of an epithelial cell to a mesenchymal phenotype (25). Lack of antibodies (Abs) able to selectively distinguish phosphorylation sites in Smad3 has impeded determination of phosphorylation sites, and *in vivo* investigation of their distinct phosphorylated domain-mediated signals. We therefore have focused on Smad3 signaling, and have used domain-specific phospho-Smad3 Abs to elucidate how JNK and p38 MAPKs modify the phosphorylation of Smad3 at linker and C-terminal regions (19,20). Moreover, we have reported the JNK/pSmad3L pathway as the signal observed commonly in activated mesenchymal cells and Ras-transformed epithelial cells (26,27). According to these phosphorylation-defined activities, we have sought after the molecular mechanisms by which the two types of phosphorylated Smad3 forms govern progression from chronic hepatitis C through cirrhosis to HCC (28).

3. TGF-BETA SIGNALING OF HEPATOCYTES AFFECTED BY CHRONIC INFLAMMATION DURING PROGRESSION OF CHRONIC LIVER DISEASES

3.1 pSmad3C transmits a tumor-suppressive TGF-beta signal, while oncogenic activities such as cell proliferation and invasion are promoted by the JNK/pSmad3L pathway

Altered TGF-beta responsiveness is observed in Ras-transformed epithelial cells, which typically exhibit a limited growth inhibitory response to TGF-beta, while acquiring the ability to invade tissues and form metastases (14). One therefore needs to distinguish between the tumor-suppressive function of TGF-beta in mature epithelial cells and its tumor-promoting role in Ras-transformed epithelial cells. Originally identified as stress-activated protein kinases that control cell survival and

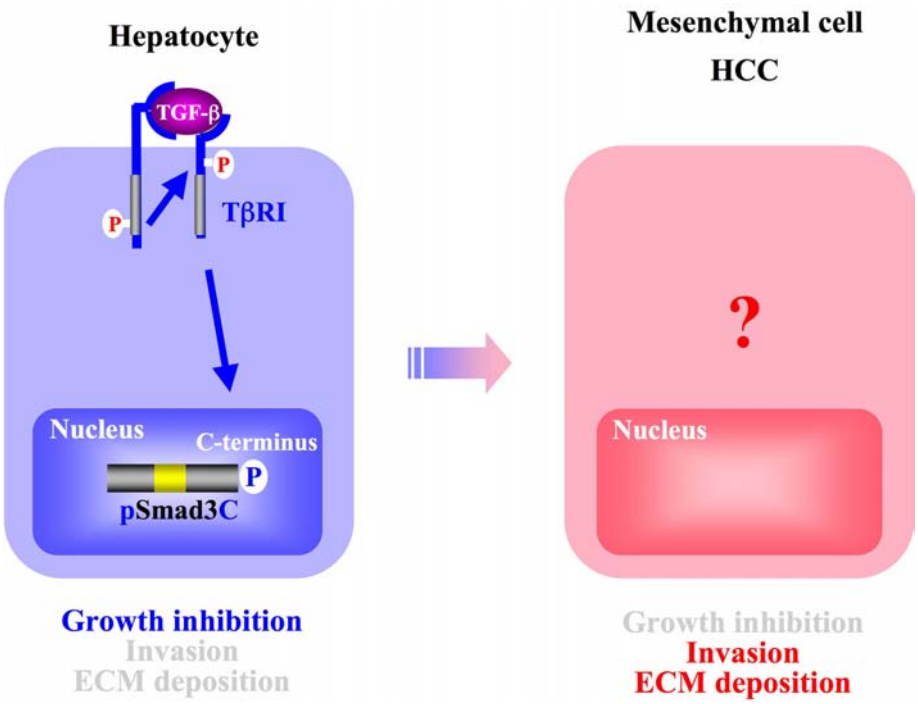

Figure 2. HCC exhibits TGF-beta function related to mesenchymal cells. TGF-beta has a growth inhibitory effect on mature epithelial cells such as hepatocytes. During carcinogenesis, HCC gains advantage by selective reduction of the growth inhibitory activity of TGF-beta together with augmentation of invasive capacity associated with ECM deposition that is observed in activated mesenchymal cells including hepatic stellate cells.

proliferation through transcription factor c-Jun (29), the JNK subgroup of MAPKs has recently emerged as a crucial regulator for the growth and migration of immature epithelial cells (30). When epithelial cells are stimulated by receptor tyrosine kinase growth factors such as hepatocyte growth factor, JNK is activated through the Ras pathway (29), and the activated signal is transmitted to downstream substrates including c-Jun. JNK1 and JNK2 are well known for activation and phosphorylation of c-Jun at Ser^{63} and Ser^{73}. Besides c-Jun, the middle linker region of Smad3 serves as a substrate for JNK1/2 (19,22,26,27). Based on the results from reversible shifting of Smad-dependent signaling between tumor-suppression and oncogenesis in hyperactive Ras-expressing epithelial cells, we have revealed that pSmad3C transmits a tumor-suppressive TGF-beta signal, while oncogenic activities such as cell proliferation and invasion are promoted by the JNK/pSmad3L pathway (27: Figure 3). This notion is supported by the recent finding of Roberts' group that Smad3 is critical for Ras/JNK-mediated transformation (31). We conclude that oncogenic TGF-beta signaling results from functional collaboration of Ras and Smad3 rather than from Ras-mediated inhibition of the Smad3 pathway. Linker phosphorylation of Smad3 indirectly inhibits C-terminal phosphorylation, minimizing tumor-suppressive pSmad3C signaling (27).

3.2. pSmad3C transmits signaling in mature hepatocytes, while pSmad3L takes over in activated mesenchymal cells

To clarify the different roles of pSmad3C and pSmad3L pathways *in vivo*, we have investigated the distribution of pSmad3C and pSmad3L in the injured liver after chemical insult (26). Compensatory growth of the liver to regain mass lost from partial hepatectomy or chemical damage is orchestrated by the interplay of positive and negative polypeptide cytokines and growth factors (32). After CCl_4 intoxication, pSmad3L appears in nuclei of regenerating immature hepatocytes within 8 hr and eventually diminishes by 36 hr, a time when proliferation stops (unpublished observation). Thus, Smad3L is phosphorylated rapidly in immature hepatocytes in response to appropriate mitogenic stimuli. The TGF-beta signal transduction system has been implicated in negative regulation of the growth response in mature hepatocytes. Immunostaining of normal rat liver with an antibody specific to pSmad3C shows slight phosphorylation of Smad3C throughout the liver (Figure 4A, top panel, alfa pSmad3C column). Similarly, the number of nuclei positive for C-terminal phosphorylated Smad3 was increased in mesenchymal cells surrounding centrilobular areas and hepatocytes at 36 hrs after CCl_4 intoxication (Figure 4A, middle and bottom panels, alfa pSmad3C column). C-terminal phosphorylation decreases at 72 hr after CCl4 intoxication. Since TGF-beta secreted from mesenchymal cells including hepatic stellate cells (HSC) is present within hepatocytes, TGF-beta could signal via pSmad3C pathway in mature hepatocytes (Figure 4A, hepatocyte column). In most mature epithelial cell types, TGF-beta arrests cell cycle progression in the G_1 phase by repressing transcriptional activity of the *c-Myc* gene and up-regulating expression of $p21^{WAF1}$ gene (14,34). Both, repression of

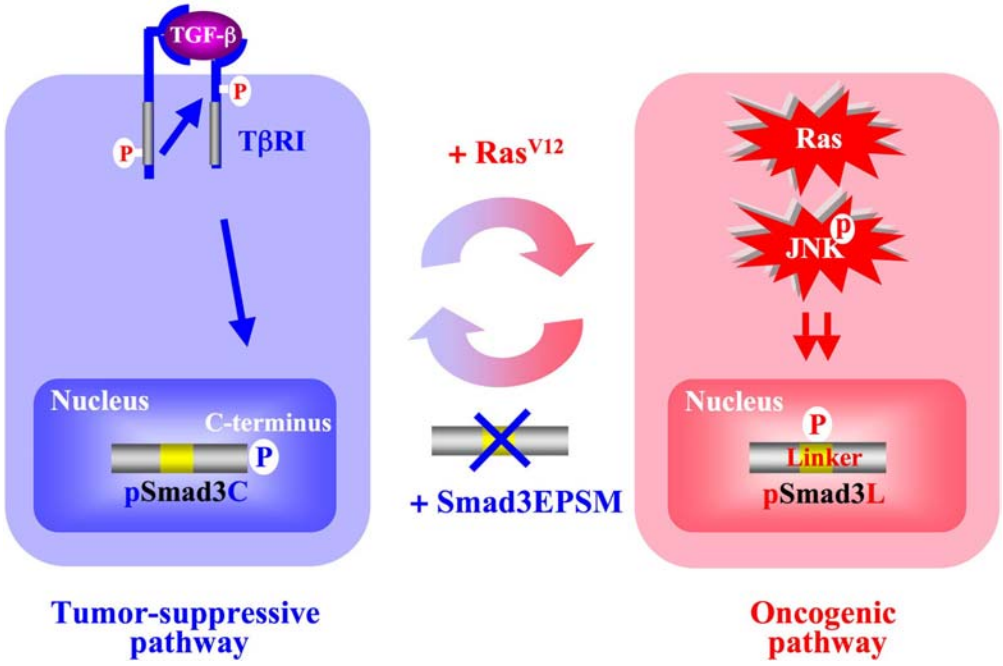

Figure 3. Smad3-dependent signaling shows reversible switching between tumor suppression and oncogenesis. Normal epithelial cells exhibit TGF-beta-mediated pSmad3C, which involves growth inhibition by repression of c-Myc. Hyperactive Ras^{V12} transforms epithelial cells to shut down pSmad3C-mediated signaling, while acquiring constitutively active JNK-mediated pSmad3L signaling that fosters tumor growth and invasion by up-regulating c-Myc and PAI-1. Selective blockade of linker phosphorylation by a mutant Smad3 lacking the JNK-dependent linker phosphorylation sites (Smad3EPSM) abolishes pSmad3L-mediated invasive properties and restores the TGF-beta-dependent tumor-suppressive function involving pSmad3C that is shown by parental epithelial cells. Taken together, domain-specific phosphorylation of Smad3 is a key determinant regulating transcriptional activation of several target genes, ultimately selecting either tumor suppression or oncogenesis.

c-Myc and induction of $p21^{WAF1}$ require C-terminal phosphorylation of Smad3 (35,36). Accordingly, pSmad3C-mediated signaling takes part in the cytostatic response in mature epithelial cells, including hepatocytes (14). The distribution of pSmad3L is different from that of pSmad3C in the injured liver. At 36 hr after CCl_4 intoxication, TGF-$beta_1$ and ECM constituents including alfa 2 (I) procollagen are highly expressed in activated mesenchymal cells immunoreactive for alfa-smooth muscle actin (alfa-SMA)(37). The pSmad3L distribution fits well with the pattern obtained by alfa-SMA immunostaining in mirror image sections. pSmad3L accumulates in nuclei of alfa-SMA-immunoreactive mesenchymal cells (Figure 4B, middle and bottom panels in alfa pSmad3L and alfa alfa-SMA columns), whereas mature hepatocytes are constantly negative for alfa-SMA staining and do not show pSmad3L after 36 hrs CCl4. Activated mesenchymal cells, including HSC, tend to persistently activate the JNK/pSmad3L cascade *in vivo*. Treatment of cultured HSC with pro-inflammatory cytokines results in increased phosphorylation at Smad3L and subsequent ECM deposition after acute liver injury (Figure 4B, mesenchymal cell column)(26).

3.3. Hepatocytes show mesenchymal pSmad3L signaling adjacent to collagen fibers in portal tracts of human chronic hepatitis C specimens

The distribution of pSmad3C and pSmad3L of chronic hepatitis C specimens exactly show characteristics of epithelial and mesenchymal signaling as described above. pSmad3C is predominantly located in hepatocytic nuclei distant from portal tracts (Figure 5A, liver lobule portion in alfa pSmad3C column)(28). pSmad3C is undetectable in the nuclei of mesenchymal cells in the portal portion of chronic hepatitis C specimens. Distribution of pSmad3L presents a sharp contrast to that of pSmad3C in mirror image sections: pSmad3L is observed in nuclei of alfa-SMA immunoreactive myofibroblasts in the portal tracts (Figure 5A, portal tract portion in alfa pSmad3L column; Figure 5B, black arrows in alfa pSmad3L column). HSCs are currently recognized as a key element in developing liver fibrosis (38). As a result of chronic liver damage, HSCs undergo progressive activation to myofibroblast-like cells, which are characterized by synthesis of a large amount of ECM components. During the transdifferentiation process of cultured HSCs, pSmad3C-mediated signal decreases while the pSmad3L pathway predominates and stimulates ECM deposition (20). Besides myofibroblasts, hepatocytes in HCV-infected livers exhibit phosphorylation at Smad3L. pSmad3L is observed particularly in groups of hepatocytes adjacent to collagen fibers in portal tracts (Figure 5B, red arrows in alfa pSmad3L column). Thus, hepatocytes are regulated by the same pSmad3L pathway during chronic inflammation as myofibroblasts. The extent of phosphorylation at Smad3L decreases in hepatocytes distant from portal tracts (Figure 5A, liver lobule portion in alfa pSmad3L column).

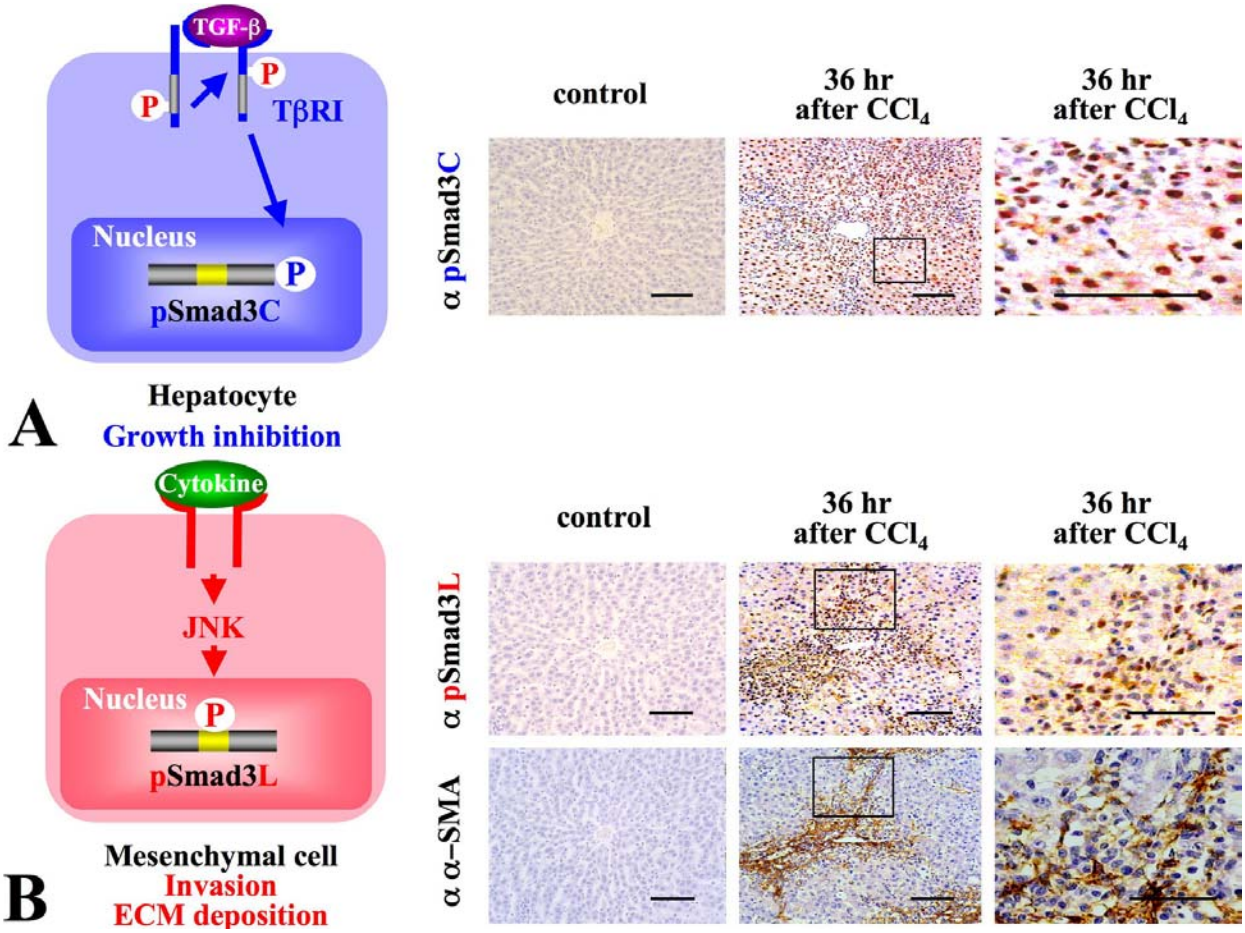

Figure 4. In rat CCl_4 hepatotoxicity, pSmad3C transmits signaling in hepatocytes, while pSmad3L does so in alfa-SMA-immunoreactive mesenchymal cells. A: In normal rat liver, Smad3 is phosphorylated slightly at the C-terminal region (upper panel). Amounts of C-terminal phosphorylation in nuclei of hepatocytes surrounding centrilobular areas increase at 36 hr after CCl_4 intoxication (middle and lower panels). B: In normal rat liver, Smad3 is minimally phosphorylated at the linker region (upper panel). At 36 hr, pSmad3L is localized predominantly in the nuclei of alfa-SMA immunoreactive mesenchymal cells adjacent to necrotic hepatocytes in centrilobular areas (middle and bottom panels). Formalin-fixed, paraffin-embedded sections of normal rat liver (upper panel) and injured liver at 36 hr after CCl_4 intoxication (middle and bottom panels) are stained with anti-pSmad3C Ab (alfą pSmad3C column; A) and anti-pSmad3L Ab (alfą pSmad3L column; B). The pSmad3L section is paired with an adjacent section stained using anti-alfa-SMA Ab (alfa alfa-SMA column; B). Abs then are bound by goat anti-mouse immunoglobulins conjugated with peroxidase-labeled polymer. Peroxidase activity is detected by 3, 3'-diaminobenzidine tetrahydrochloride (DAB). The bottom panels show higher magnification of the boxed areas in middle panels. All sections are counterstained with hemotoxylin (blue). Brown indicates specific Ab reactivity. Bar = 50 micro m.

3.4. Human hepatic fibro-carcinogenesis: reciprocal change in pSmad3L and pSmad3C pathways

Liver fibrosis results from increased deposition of ECM within the hepatic extracellular space, and constitutes a common cardinal signature to all forms of liver injury, regardless of etiology (4,38). End-stage liver fibrosis is recognized clinically as cirrhosis. To discriminate between tumor-suppressive and fibrogenic Smad3 signaling, we have stained serial liver sections using anti-pSmad3L and anti-pSmad3C antibodies, paired with stainings for plasminogen activator inhibitor 1 (PAI-1) and $p21^{WAF1}$. By up-regulating $p21^{WAF1}$ transcription, pSmad3C participates in tumor-suppression (36), while pSmad3L-mediated signaling promotes ECM deposition, partly, by up-regulating PAI-1 transcription (20,39). In specimens from a patient with chronic hepatitis C, the above distribution of pSmad3L fits well with the pattern shown by PAI-1 immunolabeling (Figure 6A, chronic hepatitis panels in alfa pSmad3L and alfa PAI-1 columns), with both strongly apparent in groups of hepatocytes adjacent to collagen fibers in portal tracts (28). Hepatocytes rather than other cells thus appear the cells primarily responsible for PAI-1 expression, associated with chronic inflammation. Amounts of linker phosphorylation and PAI-1 staining increase further in hepatocytes of cirrhotic samples (Figure 6A, cirrhosis panels in alfa pSmad3L and alfa PAI-1 columns). The spatial distribution profiles differ between chronic hepatitis and cirrhosis. In particular, pSmad3L and

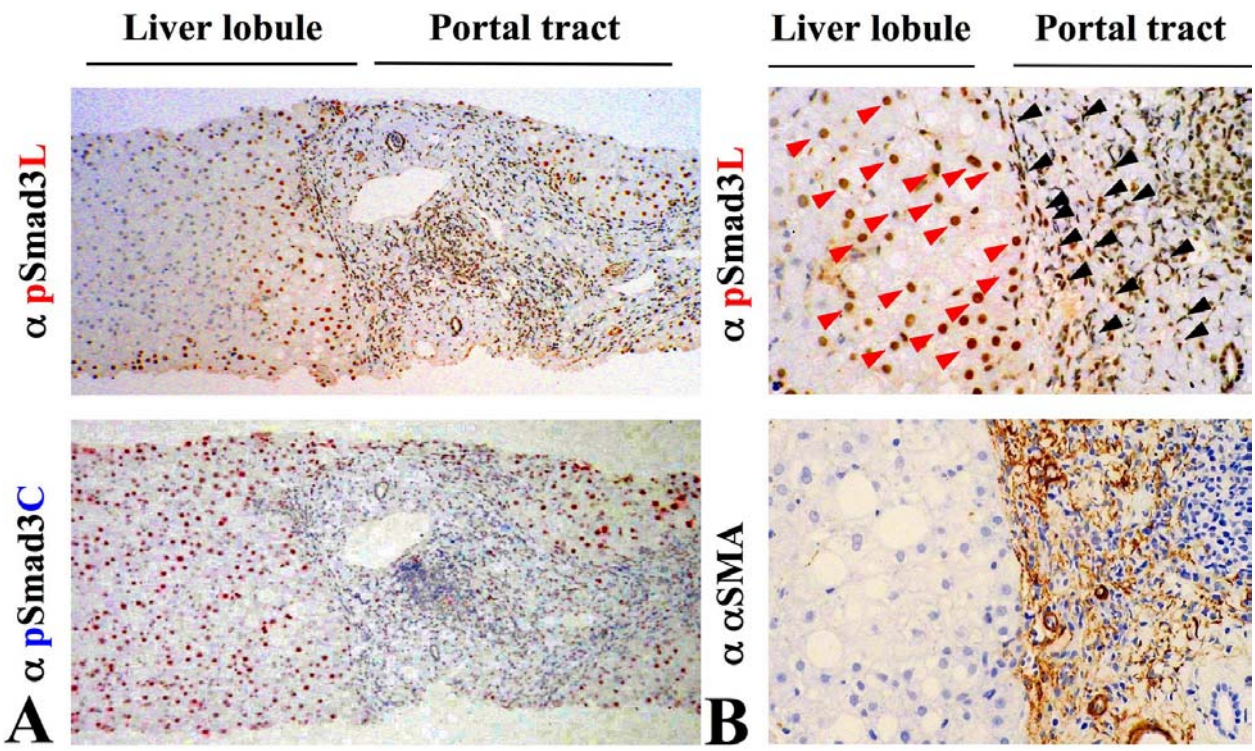

Figure 5. Hepatocytes show the mesenchymal pSmad3L signaling adjacent to collagen fibers in portal tracts. A: While hepatocytes adjacent to collagen fibers of portal tracts in chronic hepatitis C specimens show nuclear localization of pSmad3L, pSmad3C is predominantly located in hepatocytic nuclei distant from the portal tract. Formalin-fixed, paraffin-embedded sections of livers chronically affected by hepatitis C are stained with anti-pSmad3L Ab (alfą pSmad3L panel) and anti-pSmad3C Ab (alfą pSmad3C panel). The pSmad3C section is paired with an adjacent section stained using anti-pSmad3L Ab. Abs are then bound by goat anti-rabbit IgG conjugated with peroxidase-labeled polymer. Peroxidase activity is detected by 3, 3'-diaminobenzidine tetrahydrochloride (DAB). All sections are counterstained with hemotoxylin (blue). Brown color indicates specific Ab reactivity. Bar = 50 micro m. B: In addition to alfa-SMA immunoreactive myofibroblasts, hepatocytes adjacent to collagen fibers in portal tracts carry out pSmad3L-mediated signaling. Formalin-fixed, paraffin-embedded sections of tissues with chronic hepatitis C are stained with anti-pSmad3L Ab (alfa pSmad3L panel) and anti-alfa SMA Ab (alfa alfa SMA panel). The pSmad3L section is paired with an adjacent section stained using anti-alfa SMA Ab. pSmad3L is localized in cell nuclei of hepatocytes (red arrows) as well as alfa-SMA immunoreactive myofibroblasts in the portal tract (black arrows). Brown color indicates specific Ab reactivity. Bar = 50 micro m.

PAI-1 are observed in essentially all hepatocytes in cirrhotic liver. Moreover, linker phophorylation and PAI-1 staining increases further as chronic liver disease progresses to HCC (Figure 6A, HCC panels in alfa pSmad3L and alfa PAI-1 columns). Similarly to the pSmad3C distribution, hepatocytes show increased $p21^{WAF1}$ staining in chronic hepatitis C specimens (Figure 6A, chronic hepatitis panels in alfa pSmad3C and alfa $p21^{WAF1}$ columns). In contrast to intense staining for pSmad3L and PAI-1, pSmad3C and $p21^{WAF1}$ staining decreases in hepatocytic nuclei in cirrhotic liver (Figure 6A, cirrhosis panels in alfa pSmad3C and alfa $p21^{WAF1}$ columns), and it is sparse in HCC (Figure 6A, HCC panels in alfa pSmad3C and alfa $p21^{WAF1}$ columns). Double immunofluorescence studies in chronic hepatitis C and HCC specimens confirm that pSmad3L and pSmad3C co-localize with PAI-1- and $p21^{WAF1}$-, respectively in hepatocytes (Figure 6B). Taken together, the fibrogenic pSmad3L/PAI-1 pathway in hepatocytes becomes predominant while the tumor-suppressive pSmad3C/$p21^{WAF1}$ pathway is shot off as chronic hepatitis progresses to cirrhosis and then HCC.

3.5. Epithelial-to-mesenchymal transition during progression of chronic liver diseases: the shift of hepatocytic TGF-beta signaling from pSmad3C to pSmad3L pathways

While resident tissue fibroblasts are traditionally considered as the principal source of fibrosis, there has been increasing interest in the ability of epithelial cells to assume not only a mesenchymal phenotype (known as epithelial-to-mesenchymal transition: EMT), but to also undertake mesenchymal functions, i.e., contribute to fibrogenesis. TGF-beta can initiate and maintain EMT in a variety of biological systems and pathophysiological context by activating Smad-dependent and Smad-independent pathways (17,40). EMT has been established as a major mechanism for the deposition of ECM in renal and pulmonary fibrosis injury models (41,42). However, possible direct involvement of hepatocytes in liver fibrosis has not been examined. Our recent data

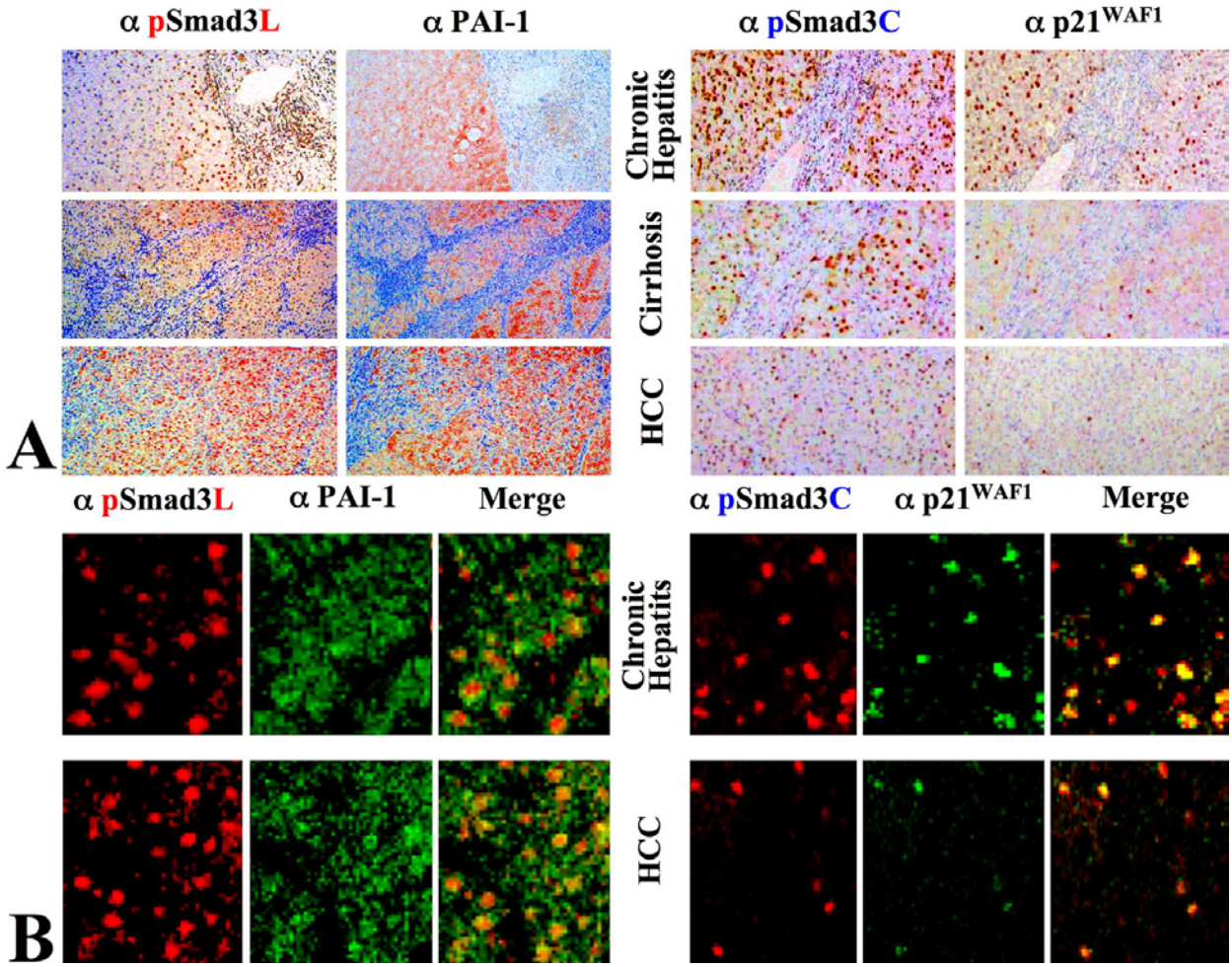

Figure 6. Hepatic fibro-carcinogenesis: reciprocal change in pSmad3L and pSmad3C pathways. A: As the HCV-infected livers progress from chronic hepatitis through cirrhosis to HCC, the pSmad3L/PAI-1 pathway shows increasing prominence, while the pSmad3C/p21^{WAF1} pathway decreases in hepatocytes. Formalin-fixed, paraffin-embedded sections of tissues with chronic hepatitis C, cirrhosis, or HCC are stained with anti-pSmad3L Ab (alfa pSmad3L column), anti-PAI-1 Ab (alfa PAI-1 column), anti-pSmad3C Ab (alfa pSmad3C column), and anti-p21^{WAF1} Ab (alfa p21^{WAF1} column). pSmad3L and pSmad3C sections are paired with an adjacent section stained with anti-PAI-1 Ab and anti-p21^{WAF1} Ab, respectively. Brown color indicates specific Ab reactivity. Bar = 50 micro m. B: pSmad3L and pSmad3C in hepatocytic nuclei of chronic hepatitis C and HCC specimens are co-localized with PAI-1 and p21^{WAF1}, respectively. Sections of chronic hepatitis C (upper panel) and HCC (lower panel) tissues are immunofluorescently stained to simultaneously detect pSmad3L and pSmad3C (red), or PAI-1 and p21^{WAF1} (green). Yellow color indicates overlap of proteins. pSmad3L-immunoreactive hepatocytes show PAI-1 co-localization (left columns), while pSmad3C and p21^{WAF1} are co-localized in hepatocytic nuclei (right columns). Bar = 50 micro m.

indicate that hepatocytes undergo transition from the tumor-suppressive TGF-beta pathway characteristic for mature epithelial cells to the fibrogenic/oncogenic pathway, which appears to favor the state of flux shown by the activated HSCs, accelerating liver fibrosis while increasing risk of cancer (Figure 7)(28). Our finding of hepatocytes involvement in fibrogenesis confirms the reports from other laboratories that mature hepatocytes can acquire a mesencymal phenotype and undertake the activated HSC functions (43,44). In addition, the finding was recently strengthened by the elegant *in vitro* and *in vivo* data by Dooley et al that hepatocytes exert profibrogenic functions of TGF-beta during liver damage (45). Collectively, hepatocytes play a potentially important role in hepatic fibrogenesis and progression to cirrhosis. These findings should open new avenues to understanding the development and progression of hepatic fibrogenesis.

3.6. Chronic inflammation associated with HCV infection shifts hepatocytic TGF-beta signaling from tumor-suppression to fibro-carcinogenesis, accelerating liver fibrosis and increasing the risk for HCC

Several lines of evidence support that TGF-beta and pro-inflammatory cytokines, including interleukin-1beta, are important mediators of fibrosis (46,47). Chronic inflammation associated with persistent HCV infection is clearly the primary inducer of liver fibrosis and cancer. Fibrotic stages and necroinflammatory

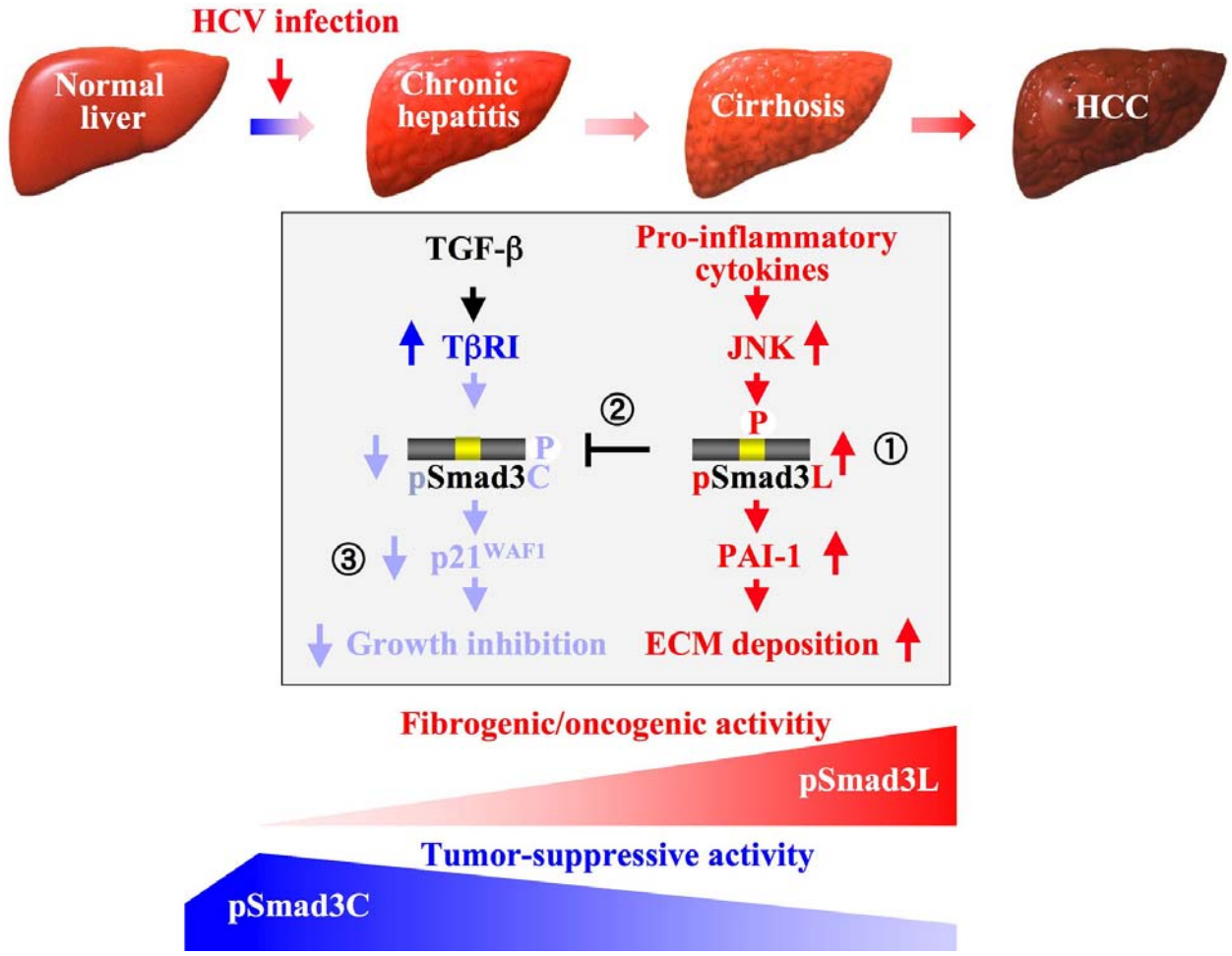

Figure 7. Chronic inflammation associated with HCV infection shifts hepatocytic TGF-beta signaling from tumor-suppression to fibro-carcinogenesis, accelerating liver fibrosis and increasing the risk for HCC. As HCV-infected livers progress from chronic hepatitis through cirrhosis to HCC, pSmad3L/PAI-1 gradually increases while pSmad3C/$p21^{WAF1}$ decreases in hepatocytes, leading to loss of epithelial homeostasis and acquisition of an invasive, mesenchymal phenotype. TGF-beta treatment activates TbetaRI, further leading to direct phosphorylation of Smad3C. The TbetaRI/pSmad3C pathway involves TGF-beta-mediated growth inhibition via cyclin-dependent kinase inhibitors including $p21^{WAF1}$. On the other hand, pro-inflammatory cytokines can transmit the signals through JNK-dependent pSmad3L (①), thus participating in regulation of migratory/invasive capacity and ECM synthesis possibly by stimulating transcriptional activity of the PAI-1 gene. Linker phosphorylation of Smad3 indirectly prevents Smad3C phosphorylation (②), pSmad3C-mediated $p21^{WAF1}$ transcription (③) and consequently the anti-proliferative effect of TGF-beta on hepatocytes.

activities of HCV-related liver diseases therefore correlate closely with the risk of HCC (48). In chronic hepatitis C specimens, Smad3 is phosphorylated at the linker region mainly in nuclei of groups of hepatocytes adjacent to collagen fibers in portal tracts (Figure 5, alfa pSmad3L panel). During inflammation, pro-inflammatory cytokines act as key factors to mediate the immune response (49). In addition, serum levels of pro-inflammatory cytokines are elevated in patients with chronic hepatitis C and cirrhotic livers (50). Pro-inflammatory cytokines and TGF-beta are released from infiltrating macrophages in portal tracts during chronic inflammation (51), indicating that pro-inflammatory cytokines can alter hepatocytic TGF-beta signaling in HCV-related preneoplastic lesions. Thus, TGF-beta signaling of hepatocytes affected by chronic inflammation can act as a key regulatory element that offers a general framework for understanding the origins of HCV-related HCC. However, little information has been available on the molecular mechanisms by which chronic inflammation causes progressive liver fibrosis and ultimately HCC.

3.7. Activated JNK down-regulates the epithelial pSmad3C pathway in favor of up-regulating the mesenchymal pSmad3L pathway in pre-neoplastic hepatocytes

TGF-beta treatment activates TbetaRI, further leading to direct phosphorylation of Smad3C. The TbetaRI/pSmad3C pathway in mature epithelial cells involves TGF-beta-mediated growth inhibition via CDK

inhibitors including $p21^{WAF1}$. On the other hand, phosphorylation at Smad3L is a possible mechanism through which JNK promotes growth and invasion of immature epithelial cells, because JNK directly phosphorylates linker region of Smad3, and a mutant Smad3 lacking JNK phosphorylation sites reduces growth and invasion of the cells (19,27). Resident inflammatory cells (Kupffer cells) as well as newly recruited inflammatory cells (macrophages and neutrophils) are activated to produce pro-inflammatory cytokines, which drive proliferation of hepatocytes and induce liver fibrosis (32,50,51). These pro-inflammatory cytokines can transmit the fibrogenic/oncogenic signals through JNK-dependent pSmad3L (Figure 7①), thus participating in the regulation of migratory/invasive capacity and ECM synthesis possibly by stimulating transcriptional activity of the PAI-1 gene. Treatment with pro-inflammatory cytokines potentiates the JNK/pSmad3L pathway, which in turn antagonizes the TbetaRI/pSmad3C pathway in hepatocytes (19,27). The decrease in pSmad3C is not caused by TbetaRI inactivation, since TGF-beta treatment alone results in an increase of pSmad2C. Parallel linker phosphorylation of Smad3 indirectly prevents Smad3C phosphorylation (Figure 7②)(27), pSmad3C-mediated $p21^{WAF1}$ transcription (Figure 7③) and consequently the anti-proliferative effect of TGF-beta on hepatocytes.

The cellular context is a crucial determinant of the ultimate outcome of TGF-beta signaling in both epithelial and mesenchymal cells (11-14). Our model implies that the JNK pathway directly or indirectly modulates pSmad3C and pSmad3L-mediated signaling to regulate TGF-beta-responsive genes, resulting in antagonistic as well as synergistic biological effects. Differential biological function in response to TGF-beta can depend on the activation state of JNK. The JNK and p38 MAPKs are collectively referred to as stress-activated MAPKs (29). They are activated in response to pro-inflammatory cytokines (30), and also play important roles in hepatic fibro-carcinogenesis (52). JNK activation by pro-inflammatory cytokines shifts hepatocytic TGF-beta signaling from the tumor-suppressive pSmad3C pathway to the fibrogenic/oncogenic pSmad3L pathway. Constitutively phosphorylated Smad3L is observed in pre-malignant lesions including cirrhosis as well as in early HCC (Figure 6). Since JNK is found constitutively phosphorylated and thus activated in an early stage of HCC (53), constitutive Smad3L phosphorylation in cirrhosis and early HCC can be a direct consequence of JNK signaling. JNK acts as an important regulator of TGF-beta signaling by increasing the basal amount of pSmad3L available for fibrogenic/oncogenic action in hepatocytic nuclei, in the meantime shutting down TGF-beta-dependent tumor-suppressive activity by pSmad3C. Loss of an epithelial homeostasis and acquisition of a migratory, mesenchymal phenotype are essential for tumor invasion. In this context, JNK activity is important for pSmad3L-dependent signaling that might interact with other oncogenic pathways, especially activator protein 1, to maintain a mesenchymal phenotype of hepatocytes (53). This mechanism could explain why several studies indicate a correlation between Smad3 and fibrosis as well as metastatic potential (54-56).

4. PERSPECTIVES

HCC is a human neoplasm associated with viral infections (1). The main causative HBV and HCV are responsible for about 80% of all HCC. At present, hepatitis virus-associated carcinogenesis can be seen as a multi-factorial process that includes both direct and indirect mechanisms (57). A major factor in the process of HCC development is the host immune system (58). Chronic inflammation, degeneration and regeneration induced by host cellular immune responses are common to a variety of human diseases, and subsequent cell proliferation increases the risk of cancer (59). Our current data indicate that HCV contributes indirectly to the development of HCC through chronic inflammation in chronic hepatitis C.

Exploring the shift from epithelial to mesenchymal TGF-beta signaling during human hepatic fibro-carcinogenesis appears to offer a new direction for diagnostic and therapeutic approaches. The identification of robust molecular predictive markers for HCC occurrence to supplement conventional pathological staging systems is highly desirable (60). Our approach has identified pSmad3L as a prognostic marker for HCC occurrence that soon should prove clinically useful. Such predictive markers allow us to select a high- or low-risk group of patients with chronic hepatitis C for developing HCC. Although the latter patients can be followed up on a yearly basis, the patients assigned a high risk will require targeted measures to permit early diagnosis of HCC. From the viewpoint of TGF-beta signaling, a key therapeutic aim in chronic liver disorders would be restoration of the lost tumor-suppressive function observed in mature hepatocytes at the expense of effects promoting hepatic fibro-carcinogenesis. Our results strongly suggest that the JNK/pSmad3L pathway is an important target for the development of chemopreventive and therapeutic measures to reduce the emergence of HCC in the context of chronic liver injury and to slow progression of pre-existing tumors. In molecularly targeted therapy for human fibrosis and cancer, pSmad3L and pSmad3C could be assessed as biomarkers to evaluate the benefit from specific inhibition of the JNK/pSmad3L pathway (61).

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Abbreviations: ECM: extracellular matrix, EMT: epithelial-to-mesenchymal transition, HBV: hepatitis B virus, HCC: hepatocellular carcinoma, HCV: hepatitis C virus, HSC: hepatic stellate cell, JNK: c-Jun N-terminal kinase, MAPK: mitogen-activated protein kinase, PAI: plasminogen activator inhibitor, SMA: smooth muscle actin, pSmad3C: C-terminally phosphorylated Smad3, pSmad3L: linker-phosphorylated Smad3, TGF: transforming growth factor, TbetaRI: TGF-beta type I receptor, TbetaRII: TGF-beta type II receptor.

Key Words: TGF-beta, Smad, JNK, Liver fibrosis, Liver carcinogenesis, Review

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