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Early Chronic Inflammation and Subsequent Somatic Mutations Shift Phospho-Smad3 Signaling from Tumor-Suppression to Fibro-Carcinogenesis in Human Chronic Liver Diseases

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide [1]. HCC is strongly associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, which are implicated in about 80% of HCCs in certain geographic area [2]. Risk of HCC is increased 5- to 15-fold in chronic HBV carriers [1] and 11.5- to 17-fold in HCV-infected patients [3]. In addition, epidemiological studies have shown that chronic inflammation of the liver predisposes individuals to HCC. Most HCCs are associated with severe fibrosis or cirrhosis caused by unresolved inflammation. Both HBV and HCV show a wide spectrum of clinical manifestations, ranging from a healthy carrier state to chronic hepatitis, cirrhosis and HCC. Notably, HCC occurs less often in chronic viral hepatitis without cirrhosis. As liver fibrosis progresses from chronic hepatitis to cirrhosis, HCC occurrence increases [4]. Thus, unresolved inflammation with long-term viral infection leads to HCC associated with cirrhosis. Approaches to understanding how human HCC develops in chronic inflammatory liver diseases should therefore focus on molecular mechanisms shared between liver fibrosis and carcinogenesis (fibro-carcinogenesis).

Transforming growth factor (TGF)- β is a key regulator of many important biologic processes. TGF- β can inhibit epithelial cell growth, physiologically acting as a tumor suppressor, but it also can promote neoplasia. TGF- β has been shown to play both tumor-suppressive and tumor promoting roles [5-7]. As disease progresses toward malignancy, cancer cells gain advantage

by selective reduction of the tumor-suppressive activity of TGF- β together with augmentation of TGF- β oncogenic activity [6]. In concert with mitogens, TGF- β induces accumulation of extracellular matrix (ECM), while mitogenic signaling antagonizes cytostatic TGF- β function [8,9]. These results indicate that perturbation of TGF- β signaling by mitogens can promote hepatic fibro-carcinogenesis.

The TGF- β superfamily includes many multifunctional cytokines including TGF- β , activin, and others [6,10]. Progress over the past 10 years has disclosed important details of how the TGF- β family elicits its responses [11-14]. Smads, central mediators conveying signals from receptors for TGF- β superfamily members to the nucleus, are modular proteins with conserved Mad-homology (MH)1, intermediate linker, and MH2 domains [13]. In cell-signaling pathways, various transcription factors are phosphorylated at multiple sites by upstream kinases. Catalytically active TGF- β type I receptor (T β RI) phosphorylates COOH-tail serine residues of receptor-activated Smads (R-Smads), which include Smad2 and the highly similar protein Smad3 [12]. Mitogenic signals alternatively cause phosphorylation of R-Smad at specific sites in their middle linker regions [15-20]. After a phosphorylated R-Smad rapidly oligomerizes with Smad4, this complex translocates to the nucleus, where it regulates transcription of target genes.

Monitoring phosphorylation status of signaling molecules is a key step in dissecting their pathways. In Smad signaling, phosphorylation of not only the COOH-tail but also the linker regions of R-Smads are likely to be important in regulating Smad activity under physiologic and pathologic conditions [21]. Understanding of molecular mechanisms underlying hepatitis virus-induced fibro-carcinogenesis can help to guide early management and improve therapy for patients with chronic liver diseases. This review describes current knowledge of the molecular pathogenesis of human fibro-carcinogenesis, especially concerning Smad3 phosphorylation profiles. We further consider how enhanced understanding of phospho-Smad3 signaling could lead to more effective prevention of human fibro-carcinogenesis.

2. Smad3 phosphoisoforms

The canonical TGF- β pathway involves Smad2 and Smad3 signaling through direct serine phosphorylation of COOH termini by T β RI upon TGF- β binding (Figure 1A), [10,13]. T β RI-mediated phosphorylation of Smad2 and Smad3 induces their association with the shared partner Smad4, followed by translocation into the nucleus where these complexes activate transcription of specific genes [10-14]. Smad2 and Smad3 proteins contain a conserved Mad-homology (MH)1 domain that binds DNA, and a conserved MH2 domain that binds to receptors, Smad4, and transcription co-activators.

More divergent linker regions separate the two domains [13]. The linker domain undergoes regulatory phosphorylation by Ras/mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, and cyclin-dependent kinase (CDK)-2/4, as well as glycogen synthase kinase 3- β , Ca (2+)-calmodulin-dependent protein kinase II, and G protein-coupled receptor kinase-2 (Figure

Figure 1. Reversible phospho-Smad3 signaling between tumor-suppression and fibro-carcinogenesis A) TGF-β treatment activates TβRI, further leading to direct phosphorylation of Smad3C, which inhibits normally hepatocytic growth by up-regulating p21^{WAF1} transcription. B) Mitogens drastically alter phospho-Smad3 signaling via the JNK pathway, increasing basal nuclear fibro-carcinogenic pSmad3L activity while shutting down TGF-β-dependent cyto-static pSmad3C. Although TGF-β signal weakly phosphorylates Smad3L in normal hepatocytes (dotted line), hepatitis viral components including HBx, pro-inflammatory cytokines including TNF-α, and somatic mutations such as Ras additively transmit fibro-carcinogenic signal through the JNK-dependent pSmad3L pathway to participate in hepatocytic growth and ECM deposition, possibly by stimulating transcription of *c-Myc* and *PAI-1* genes. Linker phosphorylation of Smad3 indirectly prevents COOH-tail phosphorylation, pSmad3C-mediated p21^{WAF1} transcriptions and cytostatic function. C) Either various JNK inhibitors or a Smad3 mutation causing lack of JNK phosphorylation sites in the linker region can eliminate fibro-carcinogenic pSmad3L signaling, restoring or maintaining the tumor-suppressive pSmad3C signaling characteristic of mature hepatocytes.

1B), [15-26]. TGF-β alternatively elicits signaling responses through non-Smad pathways representing important effectors for TGF-β activated kinase (TAK) 1 in response to pro-inflammatory cytokines. TAK1 activates JNK and p38 MAPK signaling through mitogen-activated kinase kinase (MKK) 4/7 and MKK3/6 [27,28]. JNK and p38 MAPK have been linked to modification of TGF-β signaling by pro-inflammatory cytokines through their regulation of distinct processes such as cytoskeleton organization, cell growth, survival, migration and invasion [29]. Imbalances between signaling through non-Smad and Smad pathways may occur during fibro-carcinogenesis, with interaction between these pathways mediating pro-fibro-genic and pro-tumorigenic effects of TGF-β [30]

Findings in mice with targeted deletion of Smad3 and JNK1 indicate that both Smad3 and JNK1 pathways promote hepatic fibro-carcinogenesis. When acute liver injury was induced by administration of CCl₄, *Smad3*^{-/-} mice showed approximately half as much of the induction of collagen type I mRNA as seen in wild-type mice [31]. *JNK1*^{-/-} mice resisted not only liver fibrosis

but also HCC development. Remarkable collagen deposition in wild-type and *JNK2*^{-/-} was less evident in *JNK1*^{-/-} mice, suggesting importance of JNK1 in development of liver fibrosis [32]. *JNK1*^{-/-} mice exhibited impaired liver carcinogenesis with reduced tumor mass, size, and number [33]. Importantly, *JNK1*^{-/-} mice displayed decreased HCC proliferation in a carcinogenic model and decreased hepatocytic growth in a model of liver regeneration. In both cases, the impaired proliferation was caused by increased expression of p21^{WAF1}, a cell-cycle inhibitor, and reduced expression of c-Myc, a negative regulator of p21^{WAF1}.

Mitogens simultaneously activate linker-phosphorylated R-Smad and non-Smad signaling, with both usually operating in parallel. Biologic significance of linker-phosphorylated R-Smad pathways is therefore difficult to assess in isolation. Here we will review recent work in this area with a particular focus on how mitogens modulate TGF- β signaling through Smad3 linker phosphorylation, using hepatic fibro-carcinogenesis as an example. Antibodies (Abs) reactive with structurally related phosphorylated peptides are emerging as valuable tools for determining phosphorylation sites *in vivo* and for investigating distinct signals via phosphorylated domains. Domain-specific phospho-Smad3 Abs have allowed us to reveal that T β RI and JNK differentially phosphorylate Smad3 to create 2 phosphorylated forms (phosphoisoforms): COOH-terminally phosphorylated Smad3 (pSmad3C) and linker phosphorylated Smad3 (pSmad3L) [34-37]. Linker phosphorylation can modify COOH-terminally phosphorylated R-Smad signaling [15-17,19-24]. Differential localization of kinases and phosphatases in the cytoplasm or nucleus raises the intriguing possibility of differences in temporal dynamics between cytoplasmic and nuclear R-Smad phosphoisoforms, adding to the repertoire of signaling responses that determine cell-fate decisions [8,9]. Immunohistochemical and immunofluorescence analyses using specific Abs in human tissues can examine the clinical significance of context-dependent and cell type-specific signaling mediated by R-Smad phosphoisoforms by comparison of their tissue and cellular localization in pathologic specimens.

3. Tumor-suppressive (cytostatic) TGF- β signaling: the pSmad3C pathway

TGF- β inhibits proliferation of normal hepatocytes, a crucial function in hepatic homeostasis [38]. In the context of cell cycle control, the most important targets of action by TGF- β are the genes encoding two CDK inhibitors (p21^{WAF1} and p15^{INK4B}) and c-Myc [39]. The pSmad3C signal induces expression of these CDK inhibitors and represses expression of c-Myc, shutting down cell cycle progression in the early to mid G₁ phase of the cell cycle (Figure 1A). Development of HCC is ordinarily blocked through actions of the pSmad3C pathway, which causes normal hepatocytes to cease growth and enter apoptosis after hepatocytic proliferation.

4. Carcinogenic (mitogenic) JNK signaling: the pSmad3L pathway

Mitogens strongly activate the JNK pathway, as TGF- β does more weakly (Figure 1B) [40]. Ras/MAPK signaling has been shown to induce phosphorylation of Smad2 and Smad3 at their

linker regions [15]. Smad2 phosphorylation at the linker region inhibits nuclear accumulation of Smad2 without interfering with TGF- β -induced phosphorylation of its COOH-tail [19,41-50]. In contrast, linker phosphorylation does not retain Smad3 in the cytoplasm, permitting further consequences of Ras/JNK signaling. Mechanisms underlying this difference between the two R-Smads are not known, but phosphorylation sites of Smad3 at clusters of 3 serine residues in its linker region (Ser²⁰⁴, Ser²⁰⁸, and Ser²¹³) somewhat differ in sequence location from the corresponding linker phosphorylation sites of Smad2 (Ser²⁴⁵, Ser²⁵⁰, and Ser²⁵⁵).

Several lines of evidence indicate that JNK transmits carcinogenic (mitogenic) signal via the pSmad3L pathway. First, JNK can directly phosphorylate Smad3 linker sites *in vitro*, while JNK inhibitors block Smad3 linker phosphorylation *in vivo* [16,19]. Second, mitogens translocate pSmad3L into the nucleus [16,19,20]. Third, nuclear pSmad3L forms a hetero-complex with Smad4 [16,23]. Fourth, nuclear pSmad3L binds to the Smad-binding element in the promoter with high affinity and specificity [23,51-53]. Finally, mitogens induce growth of normal epithelial cells by up-regulating c-Myc, and such mitogenic effects are blocked in Smad3 mutants lacking linker phosphorylation sites and by JNK inhibitors [19,54]. These results strongly support the notion that JNK specifically signals via Smad3 [55].

5. Reversible shifts in phospho-Smad3 signaling between tumor-suppression and carcinogenesis

JNK/pSmad3L and T β RI/pSmad3C signals oppose each other; most importantly, the balance between carcinogenesis and tumor-suppression can shift (Figure 1C). Linker phosphorylation of Smad3 blocks COOH-tail phosphorylation induced by T β RI [16,19,24,54,56]. Mitogenic signaling accelerates nuclear transport of pSmad3L from the cytoplasm, while preventing Smad3C phosphorylation, pSmad3C-mediated transcription, and anti-proliferative effects of TGF- β [16,19]. Smad3 mutants lacking linker phosphorylation sites, as well as JNK inhibitors, can restore growth inhibitory and transcriptional responses to TGF- β in Ras-transformed cells and pre-neoplastic hepatocytes, both *in vitro* and *in vivo* [19,54,56]. Our model implies that the JNK pathway directly or indirectly modulates pSmad3C- and pSmad3L-mediated signaling to regulate target genes, resulting in an antagonistic relationship between carcinogenesis and tumor-suppression. Thus, effectiveness of tumor-suppressive TGF- β signaling can depend on extent of Smad3 phosphorylation at the linker region.

6. Homeostatic termination of mitogenic JNK/pSmad3L/c-Myc signaling after liver regeneration by hepatocytic T β RI/pSmad3C/p21^{WAF1} signaling

Carcinogenesis is currently thought to occur as a sequence of steps termed initiation, promotion, and progression. Each step is characterized by disruption of normal cellular control mechanisms. Thus, development of HCC involves sequential alterations of physiological

mechanisms regulating hepatocytic growth. Before consideration of molecular mechanisms of hepatocarcinogenesis, examination of the physiologic role of phospho-Smad3 signaling in liver regeneration is instructive. A unique feature of adult mammalian liver is its ability to accurately regenerate lost mass, which occurs following surgical resection or diffuse liver injury [57]. Although precise identities of cytokines and molecular mechanisms involved in liver regeneration are largely unknown, TGF- β and tumor necrosis factor (TNF)- α apparently act as positive and negative regulators of hepatocytic growth, respectively (Figure 1 A and 1B).

Hepatocytes undergo transition from a resting to a proliferative state after acute liver injury or partial hepatectomy [57]. Loss of parenchyma rapidly induces a wave of hepatocytic proliferation capable of restoring the total mass of the liver to normal. Several converging lines of evidence have established that pro-inflammatory cytokines such as TNF- α and interleukin (IL)-6 are important components of the mitogenic pathways leading to regeneration after acute liver injury [58]. Treatment of hepatocytes with antibodies against TNF- α resulted in decreased DNA synthesis and JNK activity [38]. DNA synthesis during liver regeneration was severely impaired in mice with a TNF- α type I receptor deficiency [59]. After acute liver injury, TGF- β increases in damaged livers within a time frame similar to that of increases in pro-inflammatory cytokines [60-62]. This raises the problem of how hepatocytes manage to proliferate in response to a mitogenic pro-inflammatory cytokine signal despite elevated TGF- β concentration. During liver regeneration, hepatocytes acquire temporary resistance to cytostatic effect of TGF- β , allowing them to proliferate [61-63]. The phosphorylation pattern of Smad3 in regenerative hepatocytes after acute liver injury suggested important participation of phospho-Smad3 in hepatocytic growth regulation. In actively growing hepatocytes, intracellular phosphorylation at Smad3L was found to be high [54,56,64]. Translocated to the nucleus, inflammatory cytokine-induced pSmad3L stimulated c-Myc transcription [54,64,65], which increased proliferation of hepatocytes and opposed the cytostatic action of the pSmad3C/p21^{WAF1} pathway (Figure 1B). Accordingly, pSmad3C/p21^{WAF1} was undetectable in regenerative hepatocytic nuclei; escape from TGF- β -induced cytostasis was crucial in a subset of progenitor cells devoted to ensuring epithelial renewal. Thus, pSmad3L signaling can permit liver regeneration in response to mitogenic pro-inflammatory cytokines even though TGF- β concentration is elevated after acute liver injury.

Liver regeneration is tightly controlled by a delicate balance between hepatocytic growth and inhibition. Anti-mitotic effects of TGF- β contribute to the termination of hepatocyte proliferation observed following the wave of DNA synthesis in the regenerating liver. Post regeneration, return of TGF- β sensitivity thus limits hepatocyte proliferation and terminates liver regeneration [61,63]. After TNF- α and pSmad3L decreased, hepatocytic proliferation ceased, as decreases in pSmad3L allowed increased sensitivity to phosphorylation at Smad3C by T β RI (Figure 1C). TGF- β -dependent pSmad3C appears to limit the proliferative response of regenerating hepatocytes through inhibition of the G1 to S phase transition in the cell-cycle. Such signaling represents a highly effective defense mechanism against development of HCC, since nonproliferating hepatocytes containing pSmad3C that might have sustained any mutations are destined to die [66].

7. Liver fibrosis as the largest single risk factor for HCC occurrence

Liver fibrosis usually precedes the multistage process of HCC development. Liver fibrosis is strongly associated with HCC, with 80 to 90% of HCCs arising in cirrhotic livers [67]. In hepatitis B infection is a risk factor for HCC, along with age, gender, viral DNA load, and viral core promoter mutation [68]. Fibrosis has also been identified as risk factor in hepatitis C infection, where cancer risk is directly related to fibrosis severity [69]. Similarly, HCC development is linked to alcoholic cirrhosis [70], nonalcoholic steatohepatitis (NASH) [70], and hemochromatosis [71], with a yearly HCC incidence of 1.7% in alcoholic cirrhosis [70] and 2.6% in NASH cirrhosis [72].

8. Involvement of both myofibroblasts and hepatocytes in liver fibrosis

Hepatic fibrosis is characterized by accumulation of excess ECM proteins, regardless of underlying etiology. Amount of matrix deposition reflects a balance between matrix synthesis and degradation [73,74]. When synthesis of ECM exceeds degradation, pathologic accumulation of ECM leads to liver fibrosis. Reversibility of experimental hepatic fibrosis and a striking decrease in collagenolytic activity observed in liver fibrosis models suggest crucial importance of impaired matrix degradation in hepatic fibrogenesis [75]. The plasminogen activator/plasmin system, which is situated upstream of the fibrolysis system, can directly degrade matrix components, and indirectly inhibit ECM deposition [76]. Plasminogen activator inhibitor-1 (PAI-1), the major physiologic inhibitor of plasminogen activator, is a potent promoter of fibrosis. Introduction of a PAI-1 small interfering RNA attenuates deposition of ECM and hydroxyproline content in experimental hepatic fibrosis [77].

Liver fibrosis is one of the most common pathologic processes occurring in response to increased inflammatory factors. A complex interplay among different hepatic cell types takes place in injured livers. Hepatocytes are the targets for most hepatotoxic agents, including hepatitis viruses, alcohol metabolites, and chemical toxins [78]. Damaged hepatocytes induce recruitment of white blood cells by local inflammatory cells. Apoptosis of damaged hepatocytes stimulates fibrogenesis by Kupffer cells. Activated Kupffer cells secrete pro-inflammatory cytokines including TNF- α and IL, as well as TGF- β . Intensive studies have shown that hepatic stellate cells (HSC) are the major cell type responsible for matrix production in damaged liver tissues [75]. HSC, characterized by retinoid droplets in the cytoplasm, are present in the space of Disse [79].

Standardized methods of obtaining HSC from livers have been developed [80]. Long-term culture of HSC on plastic substrates is widely accepted as a model of liver fibrosis [79]. HSC spontaneously transdifferentiate to a myofibroblast (MFB) phenotype on plastic dishes, and this response reproduces the features of activation *in vivo*. MFB usually retain fibrogenic TGF- β signaling component, but have lost the capacity to respond to TGF- β with growth arrest [81]. Such a state of altered TGF- β responsiveness is also observed in pre-neoplastic hepatocytes,

which typically exhibit a limited growth inhibitory response to TGF- β , instead responding to TGF- β with pro-fibrogenic behavior [9].

Hepatic fibrosis results from a wound-healing response to repeated injury in chronic liver diseases [82], in which HSC undergo dramatic phenotypic activation, with acquisition of fibrogenic properties. Patients develop liver fibrosis as a result of chronic liver damage, characterized by ECM accumulation that distorts hepatic architecture by forming a fibrous scar [79]. Ultimately, nodules of regenerating hepatocytes become enclosed by scar tissue, an event defining cirrhosis. Excess deposition of ECM of which type I collagen predominates disrupts the normal architecture of the liver, resulting in pathologic damage with pathophysiologic consequences.

A new concept has been proposed that epithelial cells undergo a phenotypical change termed epithelial-mesenchymal transition (EMT), acquiring a fibroblastic phenotype. EMT facilitates metastasis and cancer development [83]. Pioneering studies on EMT in organ fibrosis were carried out in kidney, ocular lens, and lung [84,85]. Involvement of EMT also has been proposed in liver fibrosis. Zeisberg et al. demonstrated that hepatocytes acquire expression of fibroblast-specific protein 1 in response to CCl₄ injury *in vivo* or TGF- β *in vitro* [86].

9. Fibrogenic pSmad3L signaling shared between MFB and pre-neoplastic hepatocytes

As a result of chronic liver damage, HSC undergo progressive activation to become MFB-like cells. During transdifferentiation in culture, pSmad3C-mediated signal decreases while the pSmad3L pathway predominates [23]. These observations complement the finding of pSmad3L rather than pSmad3C in nuclei of α -smooth muscle actin (SMA)-immunoreactive MFB in portal tracts of chronically HCV-infected liver specimens [64]. The presence of α -SMA is associated with transdifferentiation of HSC into scar-forming MFB, an event considered pivotal in the fibrogenic response [75].

Plasma TGF- β , TNF- α , and PAI-1 concentrations are usually elevated in patients with chronic liver diseases [87-89]. Since pSmad3L can transmit a fibrogenic signal by stimulating PAI-1 transcription (Figure 1B) [23], we investigated the pSmad3L pathway in human chronic liver disease. The results indicated nuclear localization of pSmad3L in PAI-1-immunoreactive MFBs and hepatocytes in chronic hepatitis specimens [64]. Thus, hepatocytes are regulated by the same pSmad3L pathway as are MFBs. Hepatocytes in HCV-infected livers, particularly those adjacent to inflamed portal tracts, exhibited phosphorylation at Smad3L [64]. Extent of phosphorylation at Smad3L was less in hepatocytes distant from portal tracts, in sharp contrast to pSmad3C, which was predominantly located in hepatocytic nuclei distant from portal tracts [64]. Extent of hepatocytic pSmad3L/PAI-1 increased in proportion to fibrotic stage in chronic liver diseases [56,74]. TGF- β and pro-inflammatory cytokines are released from infiltrating Kupffer cells in portal tracts to activate JNK [90,91]. Considering these findings together with a previous observation showing transcriptional activation of the *PAI-1* gene by JNK [92], TGF- β and TNF- α can mediate JNK/pSmad3L signaling that in turn induces PAI-1 expression and

promotes ECM deposition in both hepatocytes and MFB. Thus, hepatocytes affected by chronic inflammation undergo transition from the tumor-suppressive pSmad3C pathway, characteristic of mature hepatocytes, to the JNK/pSmad3L/PAI-1 pathway, which favors a state of flux characterized by MFB.

Our findings support many important papers reporting that hepatocytes can promote fibrogenesis via TGF- β /Smad signaling. Dooley et al. reported that overexpression of inhibitory Smad7 in hepatocytes attenuated TGF- β -mediated fibrogenesis by blocking Smad signaling [93]. Since the large latent TGF- β complex consisting of TGF- β , the N-terminal part of its precursor, and the latent TGF- β binding protein exists in not only HSC but also hepatocytes, the complex can transmit a pro-fibrogenic signal [94], although intracellular functions of the TGF- β complex are poorly understood. TGF- β down-stream mediator connective tissue growth factor (CTGF) also involves hepatic fibro-carcinogenesis [95]. CTGF expression increases in fibrotic livers and various tumor tissues [96]. More importantly, *in vivo* knockdown of CTGF by small interfering RNA leads to substantial attenuation of experimental liver fibrosis. Differential regulation of CTGF expression in hepatocytes and HSC by Smad2 signaling may contribute to hepatic fibro-carcinogenesis [97]. Interestingly, a methylxanthine, caffeine, inhibits synthesis of CTGF in hepatocytes and HSC, primarily by inducing degradation of Smad2 [96].

10. Additive promotion of human carcinogenesis by persistent hepatitis viral infection and chronic inflammation

Various experiments support the notion that a single promoting agent is insufficient for development of cancer. Hepatocarcinogenesis is multi-factorial, involving collaboration between 2 or more promoting agents in HCC occurrence [98]. Among tumor-promoting agents, hepatitis viruses and chronic inflammation directly participate in HCC pathogenesis, which frequently occurs during long-standing hepatitis viral infection.

Many clinical observations suggest that persistent hepatitis viral infection and chronic inflammation additively influence development of human HCC. For example, alcohol consumption is a recognized major cause of liver disease, and plays an important role in progression to HCC. However, alcoholic hepatitis progresses less frequently to HCC than HBV- or HCV- related hepatitis. In addition, patients with both viral infection and alcohol consumption have a higher risk of developing HCC than those with alcohol consumption alone [3,99,100]. Autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) are chronic inflammatory disorders that proceed to cirrhosis. However, HCC only rarely arises from AIH or PBC, particularly in the absence of HBV or HCV infection [101,102]. Conversely, asymptomatic HBV or HCV carriers maintaining normal alanine aminotransferase (ALT) levels despite intensive viral replication less frequently develop HCC than patients with chronic hepatitis B. The annual risk of HCC occurrence in HBV healthy carriers is 0.26% to 0.6%, while risk increases to 1% in patients with chronic active hepatitis B [103]. Moreover, HBV can act synergistically with HCV. Patients co-infected with HBV and HCV have a 2- to 6-fold higher

risk of HCC occurrence than those with either infection alone [104,105]. Accordingly, we will consider how the oncogenic JNK/pSmad3L pathway induces development of HCC, with particular attention to potential synergy between hepatitis viruses and inflammation in formation of pre-neoplastic hepatocytes.

11. Hepatitis virus components can activate oncogenic JNK/pSmad3L pathway

One of the earliest evidence linking HBV to development of HCC was obtained in the woodchuck hepatitis virus model, in which 100% of rodents infected with woodchuck hepatitis virus developed HCC [106]. Because HBV contains partially double stranded-DNA, it can directly cause HCC by integrating its DNA into the host genome. HBV genomic integration is present in over 85% to 90% of HBV-related HCC, usually even before development of HCC [107]. Integration of HBV DNA is not restricted to HCC but also is found in non-tumor tissue in patients with chronically HBV infection [108,109]. HBV integration induces a wide range of genetic alterations within the host genome, including chromosomal deletions, translocations, production of fusion transcripts, amplification of cellular DNA, and generalized genomic instability [110,111]. Many integration events occur near or within fragile sites or other cancer-associated regions of the human genome that are prone to instability in tumor development and progression. Genetic instability associated with integration may alter expression of oncogenes, tumor suppressor genes, and microRNAs [111]. A recent large-scale analysis of HBV DNA integration sites in cellular DNA found a preference for sites regulating cell signaling, proliferation, and viability [112]. A large proportion of HCC have integrated HBV sequences encoding HBV X (HBx) and/or truncated envelope pre-S2/S proteins.

The HBx protein encoded by the X gene has been long suspected as a viral oncoprotein participating in hepatocarcinogenesis. This protein is involved in liver cell transformation because of its pleiotropic activities on cell cycle regulation, cell signaling pathways and DNA repair [113-115]. Numerous attempts have been made to examine the oncogenic potential of HBx in cell culture. However, its transforming ability was barely measurable evident only when cells were immortalized by other oncogenes, such as SV40 T-antigen [116,117] or TGF- α [118]. Furthermore, most transgenic mice harboring the HBx gene did not develop serious liver diseases or tumors [119]. Only in a certain transgenic lineage of CD-1 strain, HBx weakly promoted carcinogenesis, where HBx was highly expressed [120]. A second mouse lineage with lower HBx expression developed liver tumors at the same rate as normal CD-1 mice [121]. HBx was shown to potentiate c-Myc-induced liver carcinogenesis in transgenic mice [122]. Thus, HBx does not have strong transforming activity, but HBx overexpression in a certain genetic background might induce tumor formation in a multistage transformation, most likely in collaboration with other cellular oncogenic pathways.

HBx is mainly located in the cytoplasm and exhibits pleiotropic effects that modulate cell responses to oncogenic signaling pathways [114]. HBx protein do not bind directly to DNA, but rather acts on cellular promoters. Such protein-protein interaction can modulate cytoplas-

mic pathways [113,114,123]. For example, HBx protein was found to activate the JNK-dependent pathway and up-regulate oncogenic c-Myc gene expression [124].

To investigate whether HBx alters phospho-Smad3 signaling in hepatocytes, we stably transfected immortalized rat hepatocytes using a construct of HBx with a mammalian expression vector, resulting in high HBx-expressing cells [56]. High expression of HBx protein in hepatocytes tended to shut down pSmad3C-mediated signaling and favored acquisition of constitutively active JNK-mediated pSmad3L signaling, which fostered hepatocytic growth by up-regulating c-Myc (Figure 1B).

In transgenic models, HBx played an important role in hepatocarcinogenesis via the pSmad3L/c-Myc pathway [56]. HBx transgenic mouse livers progressed through hyperplasia to HCC. HBx, pSmad3L, and c-Myc were not detected in normal mouse livers. Beginning at the age of 2 months, HBx transgenic mouse liver showed centrilobular foci of cellular alteration with cytoplasmic vacuolation surrounding central veins where Bromodeoxyuridine (BrdU) was uptaken into the hepatocytes [121]. Smad3L was phosphorylated in hepatocytic nuclei of the centrilobular region, where HBx and c-Myc were expressed. Hepatocytic HBx, pSmad3L, and c-Myc increased as mouse liver progressed through hyperplasia to HCC.

Positivity of hepatocytic nuclei for pSmad3L in early chronic hepatitis B specimens increases with amount of HBV-DNA [56]. Taken together with results of *in vitro* experiments using HBx-expressing hepatocytes and HBx transgenic livers, these human findings indicate that HBx oncoprotein participates directly in hepatocarcinogenesis by shifting hepatocytic phospho-Smad3 signaling from the tumor-suppressive pSmad3C/p21^{WAF1} pathway to the oncogenic JNK/pSmad3L/c-Myc pathway (Figure 1B), [56].

Unlike HBV, HCV is a positive-single-strand RNA virus, apparently incapable of integration into the host's genome. The HCV genome has a long open reading frame, which encodes a polyprotein precursor [125,126]. This polyprotein is cleaved by both host and viral proteases to generate 4 structural proteins (C, E1, E2, and P7) and 6 nonstructural proteins (xlink, NS3, NS4A, NS4B, NS5A, and NS5B) [127,128]. The HCV components modulate a number of cellular regulatory functions by targeting a wide spectrum of cellular signaling pathways [129-136]. HCV core expression has been shown to induce activation of the JNK pathway in regulation of vascular endothelial growth factor [136]. NS5A acts as a positive regulator of the JNK signaling pathway by interacting with tumor necrosis factor receptor-associated factor 2, which may play a key role in HCV pathogenesis [137]. In an HCV infection model, Lin *et al.* demonstrated that HCV directly induced TGF- β release from hepatocytes in reactive oxygen species (ROS)-dependent and JNK-dependent manner [138]. Moreover, recent studies using transgenic mouse models indicate that HCV directly involves hepatocarcinogenesis. Three different HCV core transgenic lines develop liver steatosis and HCC [139-141]. Accordingly, future studies are expected to prove that the HCV components can activate the oncogenic JNK/pSmad3L pathway.

12. Activation of the oncogenic JNK/pSmad3L pathway by chronic inflammation

Inflammatory microenvironments are present in human hepatocarcinogenesis before malignant change occurs. A hepatitis virus infection triggers chronic inflammation, increasing the risk of HCC development. Several studies have discussed how chronic inflammation affects the proliferation and survival of hepatocytes [142,143]. TNF- α , IL-1 β and IL-6 are multifunctional pro-inflammatory cytokines largely responsible for the hepatic response to chronic inflammation [144-146]. Serum concentrations of these cytokines are increased in chronic liver inflammation including hepatitis viral infection and steatohepatitis [147]. JNK is a key signal transducer for inflammatory cytokines and has emerged as an important endogenous tumor promoter [148,149].

TGF- β is also released by infiltrating Kupffer cells, the liver's resident macrophages, in portal tracts during chronic inflammation [150]. These findings suggest that elevated pro-inflammatory cytokines might alter hepatocytic TGF- β signaling in inflammatory microenvironments. We investigated this hypothesis using rat cultured hepatocytes [64]. Pretreatment of hepatocytes with SP600125, a JNK inhibitor, reduced the subsequent increase in pSmad3L, c-Myc transcription, and hepatocytic growth triggered by pro-inflammatory cytokine stimulation (Figure 1C), suggesting a direct role of the JNK/pSmad3L/c-Myc pathway in facilitating hepatocytic growth in response to cytokine stimulation (Figure 1B).

Experimental models of HCC including inflammation can elucidate how chronic inflammation contributes to hepatocarcinogenesis. In a rat model involving diethylnitrosamine (DEN)-induced carcinogenesis, chronic inflammation liver accompanies abnormalities that progress to HCC [151]. This DEN-induced rat HCC is histologically and genetically similar to human HCC, and also is associated with chronic inflammation [152]. In this chemical model, JNK act participates importantly in hepatocarcinogenesis via pSmad3L/c-Myc signaling. In DEN-treated livers, the JNK/pSmad3L/c-Myc pathway was activated in early pre-neoplastic hepatocytes (Figure 1B), [54]. Moreover, a JNK inhibitor SP600125 suppressed HCC development in DEN-treated rat livers by restoring carcinogenic pSmad3L/c-Myc to the basal pSmad3C/p21^{WAF1} pathway in the pre-neoplastic hepatocytes (Figure 1C), [54].

In human chronic hepatitis C specimens, mainly in groups of hepatocytes adjoining inflammatory cells in portal tracts, Smad3 was found to be phosphorylated at the linker region [64]. Furthermore, positivity of hepatocytic nuclei for pSmad3L/c-Myc in chronic hepatitis C specimens showed a significant relationship with necrosis and inflammatory activity [64]. Taken together with the results of *in vitro* experiments and DEN-treated rat livers, the human findings indicate that chronic inflammation directly participates in hepatocarcinogenesis by shifting hepatocytic phospho-Smad3 signaling from the tumor-suppressive pSmad3C/p21^{WAF1} pathway to the oncogenic JNK/pSmad3L/c-Myc pathway [54,64].

Many tumor-enhancing effects of pro-inflammatory cytokines on hepatocytes are exerted at the level of tumor promotion [58]. TNF- α promotes HCC occurrence in mice lacking the P-

glycoprotein Mdr2 [153]. HCC follows cholestatic inflammation in these mice. Incidence of HCC can be enhanced by another member of the TNF family, lymphotoxin β [154]. Tumor-promoting cytokines produced by Kupffer cells activate several transcription factors, including NF- κ B, STAT3, and AP-1, in pre-malignant hepatocytes [155]. The activated transcription factors stimulate transcription of their target genes involved in hepatocytic proliferation and survival, representing a major tumor-promoting mechanism. Similarly to these transcription factors, tumor-promoting actions of hepatocytic Smad3 in human chronic liver disease rarely result from direct mutations [156]. Instead, pSmad3L depends on mitogenic pro-inflammatory cytokine signals produced by neighboring Kupffer cells.

13. Constitutive phosphorylation at Smad3L in pre-neoplastic hepatocytes in cirrhotic human liver

The mechanism regulating regeneration, which avoids accumulation of deleterious mutations in genes that promote cell growth and division, must be disrupted before hepatocytes can throw off normal restraints and behave as an asocial HCC. Constitutive phosphorylation at Smad3L is observed in pre-malignant hepatocytes in cirrhosis [56,64]. Constitutively active pSmad3L stimulates hepatocytes to proliferate continuously in human livers that normally experience little proliferation because hepatocytic regeneration is tightly regulated by cyto-static pSmad3C signaling. Since JNK is constitutively activated in pre-neoplastic hepatocytes in cirrhotic human liver [157], constitutive Smad3L phosphorylation in pre-malignant lesions can be a direct consequence of proto-oncogene-mediated JNK signaling. Somatic mutations in pre-neoplastic hepatocytes include changes in the *Ras* pathway that favor progression from cirrhosis toward HCC [158]. In pre-neoplastic hepatocyte nuclei, pSmad3L/c-Myc can accumulate when somatic mutations constitutively activate the JNK pathway to phosphorylate Smad3 at the linker region (Figure 1B). Then, the proliferative effect mediated via the pSmad3L/c-Myc pathway constitutively keeps on suppresses the growth-inhibitory pSmad3C/p21^{WAF1} pathway in the nuclei of pre-neoplastic hepatocytes.

Pre-neoplastic hepatocytes and HCC show reduction of anti-mitogenic responses to TGF- β [20,37]. 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 [39,159,160]. The TGF- β /pSmad3C pathway is required for maintenance of genomic stability, induction of replicative senescence, and suppression of telomerase [161-163]. 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. In this regard, the TGF- β /pSmad3C pathway contributes to tumor suppression along with its cytostatic effect.

14. Chronic inflammation together with hepatitis virus effects in shifting phospho-Smad3 signaling into oncogene-dependent fibro-carcinogenic signaling

In the pathogenesis of HCC, continuous viral infection and chronic inflammation have a prominent role. On the other hand, detailed analysis of HCC development in experimental animals and correlation of these results with HCC in humans has identified a variety of genomic and molecular alterations in fully developed HCC [164] and to a lesser extent in morphologically defined pre-neoplastic precursor lesions [165]. Thus, a series of mutations may accumulate in individual hepatocytes over time. Finally, hepatocytes come to carry somatic mutations that lead to focal uncontrolled hepatocytic growth and eventual malignant cell transformation, in some cases, HCC [166].

Chronic inflammation associated with hepatitis virus infection may be the primary initial requirement in multistep hepatocarcinogenesis. If pSmad3L-positive and pSmad3C-negative hepatocytes survive in the course of chronic hepatitis, such hepatocytes and their descendants can accumulate, and acquire various mutated alleles. Mutations may involve genes in *Ras* pathway [158] that impel pre-neoplastic hepatocytes with constitutive phosphorylation at Smad3L toward a neoplastic growth [8]. Tumor promotion results in further selective clonal expansion of initiated cells, thereby enhancing the likelihood of additional genetic damage as a consequence of endogenous mutations. During tumor progression, premalignant cells continue to develop progressive phenotypic changes and genomic instability, developing into overt HCC.

15. The JNK/pSmad3L pathway as a therapeutic target to avert HCC development

Clinical analyses of pSmad3L and pSmad3C in human tumor formation have provided substantial mechanistic insights. For example, specimens from patients with chronic hepatitis B who develop HCC show abundant Smad3L but limited Smad3C phosphorylation in hepatocytic nuclei, while other patients with abundant hepatocytic pSmad3C but limited pSmad3L do not develop HCC [56]. The same relationships are observed in human HCV-related hepatocarcinogenesis [64]. These clinical observations support roles for pSmad3C as a tumor-suppressor and pSmad3L as a promoter during human carcinogenesis.

HCC is a highly chemoresistant cancer with no effective systemic cytotoxic chemotherapy [167]. Despite surgical or locoregional therapies, the prognosis remains poor because of high likelihood of tumor recurrence or progression and there are no well-established effective adjuvant therapies [168]. Molecular events that affect carcinogenesis need to be identified and targeted to validate new treatment approaches and expand available therapeutics to include chemoprevention to other therapeutics. Since JNK acts as an important regulator of Smad3 signaling that increases the basal amount of hepatocytic pSmad3L available for cell growth

while inactivating the TGF- β -dependent cytostatic actions of pSmad3C (Figure 1B), pharmacologic interference with JNK/pSmad3L signaling could interrupt carcinogenesis. A key therapeutic aim in chronic liver disorders is restoration of lost tumor-suppressive function observed in normal hepatocytes, at the expense of effects promoting hepatic carcinogenesis [169]. To accomplish this difficult aim, Nagata *et al.* (2009) administered a JNK inhibitor SP600125 to rats and were able to suppress chemical carcinogenesis by shifting hepatocytic Smad3 signaling from the carcinogenic pSmad3L pathway to the tumor-suppressive pSmad3C pathway (Figure 1C), [54]. These studies provide evidence that JNK/pSmad3L is an important target for development of chemopreventive and therapeutic measures to reduce emergence of HCC in the context of chronic liver injury and to slow progression of existing tumors. We must also consider whether long-term use of any drug inhibiting C-terminal phosphorylation of R-Smads might cause cancer development [7].

Figure 2. After hepatitis virus infection, early chronic inflammation and subsequent somatic mutations shift hepatocytic phospho-Smad3 signaling from the tumor-suppressive T β RI/pSmad3C made to the fibro-carcinogenic JNK/pSmad3L mode characteristic of MFB, accelerating liver fibrosis while increasing risk of HCC. Both hepatitis virus infection and chronic inflammation represent early fibro-carcinogenic steps representing non-mutagenic tumor-promoting stimuli. In advanced liver fibrosis, mitogenic genetic or epigenetic alterations can drive multistep fibro-carcinogenesis via the pSmad3L pathway. 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.

16. Conclusion and perspectives

Human fibro-carcinogenesis is a complex multistep process, which involves dysregulation of physiological signal transduction pathways. To maintain hepatic homeostasis, hepatocytic T β RI/pSmad3C/p21^{WAF1} terminates mitogenic JNK/pSmad3L/c-Myc signaling after liver regeneration. During progression of chronic liver diseases, however, early pro-inflammatory cytokines together with hepatitis viruses and subsequent somatic mutations switch hepatocytic phospho-Smad3 signaling from the tumor-suppressive T β RI/pSmad3C to the

fibro-carcinogenic JNK/pSmad3L mode characteristic of MFB, which accelerates liver fibrosis while increasing risk of HCC (Figure 2). Our model is likely to represent a crucial molecular mechanism by which most HCCs arise in from fibrosis or cirrhosis caused by chronic inflammation associated with persistent hepatitis virus infection [164]. Thus, Smad phosphoisoforms function as an important orchestrator of a human chronic inflammation-fibrosis-HCC axis [9,170].

Recent studies in animal models using conditional transgenic expression have suggested an intriguing reversibility of malignant transformation at specific time points if the primary inciting cause of the neoplasia is eliminated [171,172]. However, the fibro-carcinogenic stage in human chronic liver at which the process becomes irreversible. Chronic hepatitis B and C can be cured if patients are treated with antiviral therapy that arrests chronic inflammation by eradicating hepatic HBV and HCV populations. Continued histologic improvement and reversal of fibrosis by antiviral therapy can lead to reduction of HCC development [173,174], but prevention appears most effective when therapy is given before development of cirrhosis. Chronic hepatitis is clearly dependent on continued promoter stimulation - involving in this case the presence of hepatitis viruses and chronic inflammation. However, many patients with cirrhosis have evolved beyond dependence on inflammation because hepatocytes have acquired genetic and epigenetic carcinogenic properties. We are carrying out several trials to determine whether or not antiviral therapy can decrease liver fibrosis and lower HCC incidence. The trials will bear upon important questions regarding relative participation in fibro-carcinogenesis of inflammation-dependent and oncogene-dependent Smad3 phosphoisoform signaling in HBV- and HCV-related chronic liver disorders. In the trials, pathologic analyses using domain-specific phospho-Smad3 Abs, together with clinical data, will be used to evaluate the benefit from antiviral therapy, which decreases stimulation of the inflammation-dependent Smad phosphoisoform pathway. After antiviral therapy, hepatocytic pSmad3L and pSmad3C assessment in liver specimens should prove clinically useful for predicting progression of fibrosis and risk of HCC.

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