Summary. Transforming growth factor-β (TGF-β) signaling occurring during human colorectal carcinogenesis involves a shift in TGF-β function, reducing the cytokine’s antiproliferative effect, while increasing actions that promote invasion and metastasis. TGF-β signaling involves phosphorylation of Smad3 at serine residues 208 and 213 in the linker region and serine residues 423 and 425 in the C-terminal region. Exogenous TGF-β activates not only TGF-β type I receptor (TßRI) but also c-Jun N-terminal kinase (JNK), changing unphosphorylated Smad3 to its phosphoisoforms: C-terminally phosphorylated Smad3 (pSmad3C) and linker phosphorylated Smad3 (pSmad3L). Either pSmad3C or pSmad3L oligomerizes with Smad4, and translocates into nuclei. While the TßRI/pSmad3C pathway inhibits growth of normal epithelial cells in vivo, JNK/pSmad3L-mediated signaling promotes tumor cell invasion and extracellular matrix synthesis by activated mesenchymal cells. Furthermore, hepatocyte growth factor signaling interacts with TGF-β to activate the JNK/pSmad3L pathway, accelerating nuclear transport of cytoplasmic pSmad3L. This reduces accessibility of unphosphorylated Smad3 to membrane-anchored TßRI, preventing Smad3C phosphorylation, pSmad3C-mediated transcription, and antiproliferative effects of TGF-β on epithelial cells. As neoplasia progresses from normal colorectal epithelium through adenoma to invasive adenocarcinoma with distant metastasis, nuclear pSmad3L gradually increases while pSmad3C decreases. The shift from TßRI/pSmad3C-mediated to JNK/pSmad3L-mediated signaling is a major mechanism orchestrating a complex transition of TGF-β signaling during sporadic human colorectal carcinogenesis. This review summarizes the recent understanding of Smad3 phosphoisoform-mediated signaling, particularly “cross-talk” between Smad3 and JNK pathways that cooperatively promote oncogenic activities. Understanding of these actions should help to develop more effective therapy against human colorectal cancer, involving inhibition of JNK/pSmad3L pathway.

Key words: TGF-β, Smad, JNK, p38 MAPK, Colorectal cancer

TGF-β function during sporadic human colorectal carcinogenesis

Transforming growth factor-β (TGF-β) can inhibit epithelial cell growth (Moses et al., 1990), thereby acting as a tumor suppressor. Several components of the TGF-β signaling pathway are lost or inactivated in a variety of epithelial neoplasms (Markowitz et al., 1995; Eppert et al., 1996; Hahn et al., 1996). However, since only a fraction of sporadic colorectal tumors exhibit inactivating mutations in early stages of cancer formation (de Caestecker et al., 2000), other mechanisms would appear to play critical roles in human colorectal carcinogenesis. At present, loss of sensitivity to growth inhibition by TGF-β in most cancer cells is not synonymous with complete shutdown of the whole TGF-β signaling (Wakefield and Roberts, 2002). Instead, cancer cells gain advantage by selective inactivation of tumor-suppressing activities of TGF-β together with stimulation of its tumor-promoting activities (Fig. 1) (Piek and Roberts, 2001). On the other hand, TGF-β stimulates growth, invasion, and extracellular matrix synthesis by activated mesenchymal cells. TGF-β is a key regulator of epithelial-to-mesenchymal transition (EMT) in cell phenotypes: a process that is necessary for proper embryonic development, but frequently contributory to pathological conditions in adults. During carcinogenesis, tumors show EMT, thereby becoming refractory to TGF-β-mediated growth inhibition while

Abbreviations: CDKI, cyclin-dependent kinase inhibitor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK1, MAPK/ERK kinase kinase 1; PAI-1, plasminogen activator inhibitor type 1; R-Smads, receptor-regulated Smads; TßRI, TGF-β type I receptor; TßRII, TGF-β type II receptor
showing increased tumor invasion and metastasis (Miettinen et al., 1994; Cui et al., 1996; Oft et al., 1996; Lehmann et al., 2000).

**Nuclear cross-talk between Smad-dependent and Smad-independent pathways in TGF-β signaling**

Progress over the past 10 years has disclosed important details of how TGF-β elicits its responses. The main downstream signaling pathway for TGF-β involves the Smad proteins. TGF-β binds to type II TGF-β receptor kinase (TßRII) and recruits type I receptor (TßRI). TßRII kinase phosphorylates the GS segments in TßRI (Wrana et al., 1994). The activated TßRI then phosphorylates receptor-associated Smad2 and Smad3 at C-terminal serine residues (Macías-Silva et al., 1996; Zhang et al., 1996). These phosphorylated Smads form a complex with Smad4 and translocate into the nucleus (Fig. 2) (Heldin et al., 1997; Wrana, 2000; Shi and Massagué, 2003).

In addition to Smad-mediated signaling, TGF-β can activate other signaling cascades, including phosphatidylinositol 3-kinase, RhoA, and mitogen-activated protein kinase (MAPK) pathways such as ERK, c-Jun N-terminal kinase (JNK), and p38 MAPK (Robinson and Cobb, 1997; Mulder, 2000). In particular, MAPK/ERK kinase 1 (MEKK1) and TGF-β activated kinase 1 (TAK1) can activate JNK through MAPK kinase 4 (MKK4) or MKK7, and p38 MAPK through MKK3 or MKK6, in response to TGF-β.

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**Fig. 1.** TGF-β function during sporadic human colorectal carcinogenesis. Transforming growth factor-β (TGF-β) has an antiproliferative effect on most cells of epithelial origin, but it stimulates growth and migratory/invasive capacity in highly transformed cancer cells. Transition of cells from an epithelial to a mesenchymal phenotype induces loss of sensitivity to growth inhibition by TGF-β, and underlies the pathology of the invasive phenotype associated with metastatic adenocarcinoma.

**Fig. 2.** Nuclear cross-talk between Smad-dependent and Smad-independent pathways in TGF-β signaling. TGF-β binds to type II TGF-β receptor kinase (TßRII) and recruits type I receptor (TßRI). TßRII kinase phosphorylates the GS segments in TßRI. In the Smad-dependent pathway of TGF-β-mediated signaling, activated TßRI then phosphorylates receptor-associated Smad2 and Smad3 at C-terminal serine residues, which in turn form a complex with Smad4 and translocate into the nucleus. In addition to the Smad signaling pathway downstream of TßRI, the activated receptor complex also can activate Smad-independent pathways such as c-Jun N-terminal kinase (JNK) and p38 MAPK pathways, possibly involving mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1) and TGF-β activated kinase 1 (TAK1). JNK and p38 MAPK directly phosphorylate c-Jun and activating transcription factor-2 (ATF-2), respectively. In the nucleus, activated Smad complexes regulate transcription of target genes through physiological interaction and functional cooperation with DNA-binding transcription factors including c-Jun and ATF-2, suggesting intranuclear cross-talk between Smad-dependent and Smad-independent pathways in TGF-β signaling.
Activation of JNK and p38 MAPK pathways by TGF-β affects transcription responses of target genes through direct interactions of Smads with transcription factors, such as the JNK substrate, c-Jun, and the p38 MAPK substrate, activating transcription factor-2 (ATF-2) (Derynck and Zhang, 2003).

Several studies of EMT have suggested that the process involves Smad-independent pathways (Bakin et al., 2000; Bhowmick et al., 2001; Janda et al., 2002; Yu et al., 2002; Dumont et al., 2003). However, recent studies using Smad3 knockout mice have indicated that signaling through the Smad3-dependent pathway is required for the injury-dependent multistage transition of an epithelial cell to a mesenchymal phenotype (Saika et al., 2004). Furthermore, TGF-β-induced activation of JNK and p38 MAPK pathways can result in Smad3 phosphorylation and stimulate Smad3-mediated signaling (Engel et al., 1999; Leivonen et al., 2002).

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. More recently, my coworkers and I have used domain-specific phospho-Smad3 Abs to elucidate how JNK/p38 MAPK signals modify TßRI-mediated Smad3 signaling (Furukawa et al., 2003). Selective antisera (designated “α”) reactive with the phosphorylated linker region of Smad3 (α Smad3 [Ser 208/213]) and with the phosphorylated C-terminal region of Smad3 (α Smad3C [Ser 423/425]) were raised against synthetic peptides including phospho-Ser 208/213 and phospho-Ser 423/425.

To verify that each anti-phospho-Smad3 Ab would react only with its specific phosphorylated domain in Smad3, we created two types of mutants for the phosphorylation sites of Smad3 (Fig. 3B). ERK/Pro-directed kinase site mutant of Smad3 (Smad3EPSM) had mutations at the MAPK consensus sites in the middle linker regions of Smad3 (Kretzschmar et al., 1999), while in another mutant, Smad3(3S-A), three conserved C-terminal serine residues were changed to alanines (Macias-Silva et al., 1996; Zhang et al., 1996). Anti-pSmad3L Ab bound specifically to the phosphorylated linker regions of Smad3, as indicated by failure to react with TGF-β-treated Smad3EPSM protein (Fig. 3C, upper panel). Similarly, anti-pSmad3C Ab

Receptor-activated Smads (R-Smads) such as Smad2 and Smad3 possess two major phosphorylation sites, one at the C-terminal region and the other at the linker region (Kretzschmar et al., 1999). We successfully generated Abs specific for each of the two phosphorylated sites (Fig. 3A) (Furukawa et al., 2003). Selective antisera (designated “α”) reactive with the phosphorylated linker region of Smad3 (α Smad3L [Ser 208/213] and with the phosphorylated C-terminal region of Smad3 (α Smad3C [Ser 423/425]) were raised against synthetic peptides including phospho-Ser 208/213 and phospho-Ser 423/425.

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**Fig. 3. Antibodies selectively distinguish Smad3 phosphorylation at the linker region from that at the C-terminal region.**

A. Phosphorylation sites in Smad3 recognized by the Abs. The anti-pSmad3L (Ser 208/213) Ab recognizes JNK and p38 MAPK phosphorylation sites in Smad3, while anti-pSmad3C (Ser 423/425) Ab recognizes the phosphorylated C-terminal SSVS site in Smad3 that TßRI activates. B. The ERK/Pro-directed kinase site mutant of Smad3 (Smad3EPSM) has mutations at the MAPK consensus sites in the middle linker regions of Smad3. Another mutant is Smad3(3S-A), in which three conserved C-terminal serine residues are changed to alanines (Macias-Silva et al., 1996; Zhang et al., 1996). Anti-pSmad3L Ab bound specifically to the phosphorylated linker regions of Smad3, as indicated by failure to react with TGF-β-treated Smad3EPSM protein (Fig. 3C, upper panel). Similarly, anti-pSmad3C Ab

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selectively recognized the C-terminal region phosphorylated by TBRI, since it did not react with Smad3(3S-A) despite TGF-β treatment (Fig. 3C, middle panel). Collectively, the Abs selectively distinguished pSmad3L from pSmad3C. Notably, in Smad3 mutants for either the linker region or the C-terminal region, TGF-β-dependent phosphorylation was observed at the other site. Moreover, anti-pSmad3C Ab recognized both the upper and the lower bands of wild type (WT) Smad3 phosphorylated in response to TGF-β, while selectively recognizing only the lower band of Smad3EPM. We further examined cross-reactivities of anti-pSmad3L Ab with other Smads proteins (unpublished observation). Anti-pSmad3L Ab did not cross-react with Smad2 upon TGF-β addition. Since the linker regions were not conserved among R-Smads (Massagué, 1998), the anti-pSmad3L Ab was able to distinguish the phosphorylated linker region of Smad2 from that of Smad3.

TGF-β treatment turns nonphosphorylated Smad3 into three phosphoisoforms: pSmad3C, pSmad3L, and pSmad3C/L

Substantial differences in sizes of phosphorylated and nonphosphorylated proteins allow us to know the phosphorylation states of the proteins. Some phosphorylated proteins, including Rb protein, migrate more slowly during electrophoresis than non-phosphorylated proteins. Accordingly, prolonged electrophoresis could separate phosphorylated from nonphosphorylated Smad3. We evaluated pSmad3L and pSmad3C in samples immunoprecipitated with anti-Smad2/3 Ab or anti-pSmad3L Ab by immunoblotting using anti-Smad2/3 Ab, anti-pSmad3L Ab, or anti-pSmad3C Ab. In the absence of exogenous TGF-β, Smad3 was expressed in the immunoblot from clone 9 cells as a 54-kDa protein (Fig. 4A, lane 1) (Weinstein et al., 1975). In response to TGF-β treatment, hyperphosphorylation of Smad3 created a band shift of the 54-kDa protein to an apparent molecular mass near 56 kDa (lane 2 compared with lane 1). Anti-pSmad3L Ab reacted selectively with the 56-kDa band, while anti-pSmad3C recognized both the 54-kDa and the 56-kDa bands (lane 4 compared with lane 6). This indicated that after TGF-β treatment the 54-kDa band contained pSmad3C. Since anti-pSmad3C Ab was able to recognize pSmad3L in the 56-kDa band (lane 8), the proteins in the 56-kDa band included pSmad3L and pSmad3C/L. We further measured the molecular weight of phosphorylated Smad3 in HCC-M cells, in which Smad3L is phosphorylated constitutively (Fig. 4B, lanes 3 and 4) (Watanabe et al., 1983; Matsuzaki et al., 2000). Based on the distinct migration patterns of each phosphorylated Smad3 studied after immunoblotting with each anti-phospho-Smad3 Ab, TGF-β treatment can change nonphosphorylated Smad3 to any of three phosphoisoforms: pSmad3C, pSmad3L, and pSmad3C/L (Fig. 4C).

HGF as well as TGF-β signals induce endogenous pSmad3L, while pSmad3C is not induced by HGF

We next investigated the extent to which Smad3 was phosphorylated in response to TGF-β and HGF (Mori et al., 2004). In contrast to phosphorylation of Smad3L and Smad3C upon TGF-β treatment, HGF-dependent Smad3 phosphorylation occurred in two serine residues within the linker region but not within the C-terminal region.

**Fig. 4.** Three types of Smad3 phosphoisoforms evident upon TGF-β treatment. Clone 9 cells (A) or HCC-M cells (B) were serum-deprived for 15 hr, and then were treated with 20 pM TGF-β, for 30 min. In the samples immunoprecipitated with anti-Smad2/3 Ab or anti-Smad3L Ab, pSmad3C and pSmad3L were detected by immunoblotting (IB) using anti-Smad2/3 Ab, anti-pSmad3C Ab, or anti-pSmad3C Ab. Smad3 was expressed in clone 9 cells as a 54-kDa band on immunoblotting in the presence of exogenous TGF-β1. In response to TGF-β1 treatment, hyperphosphorylation of Smad3 shifted the 54-kDa band to a location indicating a molecular mass near 56 kDa. Anti-pSmad3L Ab selectively reacted with the 56-kDa band, while anti-pSmad3C recognized both the 54-kDa and the 56-kDa bands. Only the 56-kDa band composed of pSmad3C could be identified in HCC-M cells. C. TGF-β treatment turns nonphosphorylated Smad3 into three phosphoisoforms: pSmad3C, pSmad3L, and pSmad3C/L.
We detected pSmad3L within 5 min after HGF treatment; the quantity reached a maximum at 30 min and then decreased. These results suggest that HGF-mediated signaling induces selective phosphorylation of endogenous Smad3L. We further investigated how HGF treatment affected Smad3 phosphorylation in the presence of TGF-β. Smad3L phosphorylation was stimulated additively by co-treatment of TGF-β with HGF. Taken together, these results suggest that shared molecules activated by TGF-β and HGF signals might phosphorylate Smad3L (Fig. 5B). In contrast, treatment with TGF-β plus HGF did not induce as much pSmad3C as TGF-β treatment alone.

**HGF as well as TGF-β signals phosphorylate Smad3L via the JNK pathway**

The JNK pathway has been shown to be activated by TGF-β or HGF treatment (Afti et al., 1997; Robinson and Cobb, 1997; Wang et al., 1997). We therefore investigated whether HGF treatment could affect JNK phosphorylation in response to TGF-β (Fig. 6A) (Mori et al., 2004). At 15 min after simultaneous treatment with TGF-β and HGF, the JNK phosphorylation was additionally stimulated. Because both TGF-β and HGF signals phosphorylated JNK (Fig. 6A) and thereafter Smad3L (Fig. 5A), we next performed an *in vitro* kinase assay. Figure 6B shows that JNK activated by either the TGF-β or HGF signal can directly phosphorylate Smad3L *in vitro*. However, JNK failed to phosphorylate Smad3C upon TGF-β or HGF treatment (unpublished observation). Collectively, these findings indicate that Smad3L serves as a substrate for JNK in *in vitro* after TGF-β and HGF treatment (Fig. 6C). Activation of p38 MAPK at 15 min after HGF treatment also could phosphorylate Smad3L (unpublished observation).

**Complexes of pSmad3L with Smad4 induced by HGF as well as TGF-β signals translocate into the nucleus**

Complexes formed by R-Smads and Smad4 in response to TGF-β are critical for TGF-β/Smad signaling (Lagna et al., 1996). Accordingly, we investigated whether the HGF signal had an effect on the formation of these complexes (Mori et al., 2004). As shown in Figure 7A, interactions between each phosphorylated Smad3 and Smad4 were difficult to detect in the unstimulated culture. TGF-β treatment induced complex formation with Smad4 by either pSmad3L or pSmad3C. Similarly, treatment of the cells with HGF induced complex formation between pSmad3L and Smad4. However, increased association between pSmad3C and Smad4 was not observed in the cells treated only with HGF. These results, together with the findings shown in Figure 6, suggest that HGF-mediated association of Smad3 with Smad4 is JNK-dependent. In light of our observations that each mutant for the phosphorylation sites of Smad3 was able to undergo TGF-β-dependent phosphorylation at the other site (Fig. 3C), we decided to investigate whether or not ligand-dependent signaling occurred at the downstream steps in the TGF-β-induced activation of Smad3 mutants. TGF-β treatment induced interactions between Smad3EPSM or Smad3 (3S-A) and Smad4 (Fig. 7B).

ERK MAPK has been shown to phosphorylate R-Smads at linker regions. Such phosphorylation inhibits R-Smads functions, blocking TGF-β-regulated responses (Kretzschmar et al., 1999). However, we recently found that Smad3 mutants for the linker region or for the C-terminal region did not show shut down of the entire TGF-β signaling pathway. Instead, the predominance of TGF-β signaling was shifted through preferential Smad3 phosphorylation favoring other respective sites (Fig. 6).
Thus, Smad3EPSPM still showed TGF-β-dependent phosphorylation at the C-terminal region, while Smad3 (3S-A) retained TGF-β-dependent phosphorylation at the linker region. This phosphorylation allowed both Smad3 (3S-A) and Smad3EPSPM to associate with Smad4. It is noteworthy that Smad3 (3S-A) showed less phosphorylation at the linker region than Smad3WT (Fig. 3C), possibly because the C-terminal mutation might cause a conformational change in the linker region, which interfered somewhat with phosphorylation by JNK. Accordingly, JNK and TBRI might phosphorylate Smad3 synergistically under physiological conditions.

Translocation of R-Smads into the nucleus upon receptor-mediated phosphorylation is a central event in TGF-β signal transduction (Macías-Silva et al., 1996). To gain additional insight into the significance of Smad3 phosphorylation, we examined intracellular localization of pSmad3L and pSmad3C (Fig. 7C) (Mori et al., 2004). As expected, most pSmad3C was located in the nucleus after TGF-β treatment. In contrast, exposure even to an excess concentration of HGF did not lead to nuclear accumulation of pSmad3C. Either TGF-β or HGF treatment led to nuclear translocation of pSmad3L, and treatment of TGF-β with HGF additively stimulated nuclear translocation. Likewise, Smad3 phosphorylation by JNK facilitated nuclear accumulation (Engel et al., 1999). These data indicate that Smad3 phosphorylation either at the C-terminal region or at the linker region can allow Smad3 to translocate into the nucleus.

### SBE activation by pSmad3L at the PAI-1 promoter

Studies in fibroblasts derived from mouse embryos deficient in Smad3 showed that Smad3 was required for TGF-β-mediated induction of plasminogen activator inhibitor type 1 (PAI-1) transcription (Piek et al., 2001). We therefore used PAI-1 gene expression as an indicator of Smad3-dependent signaling status. Using myofibroblast-like cells in which TGF-β induced pSmad3L but not pSmad3C, we performed electrophoretic mobility shift assays to examine whether or not the element in the PAI-1 promoter was able to interact with the nuclear protein complex including the phosphorylated Smad3 (Fig. 7D) (Furukawa et al., 2003). The AGAC sequence in the probe, known as the Smad binding element (SBE), binds directly to the Smad3-Smad4 complex and is necessary for TGF-β-induced transcription (Yingling et al., 1997; Dennler et al., 1998; Zawel et al., 1998). Basal amounts of Smad binding complex (SBC) contained in the nuclear extracts from cells not treated with TGF-β were detectable (lane 2), while TGF-β treatment increased binding to the SBE (lane 3 compared with lane 2). Binding was eliminated by competition with excess unlabeled wild type oligonucleotide (lane 4), but not oligonucleotide bearing a mutation in the SBE sequence (AGAC→cttg; lane 5). This indicated that the SBE sequence was essential for binding of Smad3 or Smad4 to the promoter. Supershift analyses performed using the two specific Abs raised against phosphorylated Smad3 demonstrated that the primary component present in TGF-β-induced SBC at the PAI-1 promoter was pSmad3L (lanes 6 and 7).

We further investigated the effect of Smad3 phosphorylation on PAI-1 transcription in luciferase assays using PF1-Luc, since the segment in human PAI-1 promoter was sufficient to obtain TGFβ-dependent induction (Hua et al., 1998). In RGM-1 cells co-transfected of PF1-Luc with Smad3WT, TGF-β treatment increased transcriptional activity (Fig. 7E) (Mori et al., 2004). HGF treatment similarly activated the transcription, while treatment of HGF with TGF-β...
led to an additive increase in transcriptional activity. Co-transfection with Smad3EPSM, which lacked phosphorylation sites in the linker region, induced an increase of the transcriptional activity triggered by TGF-β stimulation, but could not significantly modify transcriptional activity in RGM-1 cells treated with HGF alone. Similarly, combined treatment with TGF-β and HGF of the cells expressing Smad3EPSM did not bring about an additive increase in transcriptional activity. Control co-transfection with Smad3(3S-A), which lacked C-terminal serine residues, resulted in an increase in transcriptional activities triggered by either TGF-β or HGF alone. Serum-deprived RGM-1 cells were incubated for 40 min with 20 pM TGF-β1, 400 pM HGF, or a combination of both. After cell lysates were subjected to immunoprecipitation (IP) with each anti-phospho-Smad3 Ab or anti-Smad2/3 Ab, Smad4 in the immunoprecipitates was detected by immunoblotting (IB) using anti-Smad4 Ab. Total Smad4 and Smad2/3 were monitored by immunoblotting (IB) using anti-Smad4 Ab and anti-Smad2/3 Ab, respectively. B. TGF-β signaling stimulates the association of Smad4 with Smad3EPSM. Cells were transfected with Smad4-HA in the presence of Flag-Smad3WT, Smad3EPSM, or Smad3(3S-A), and were treated with 20 pM TGF-β1. Cell lysates were then subjected to immunoprecipitation (IP) with anti-Flag Ab, and Smad4 in the immunoprecipitates was detected by immunoblotting (IB) using anti-HA Ab (upper panel). Expression of Flag-Smad3 and Smad4-HA was monitored by immunoblotting (IB) using anti-Flag Ab (middle panel) and anti-HA Ab (lower panel), respectively. C. HGF as well as TGF-β signals stimulate nuclear translocation of pSmad3L. Serum-deprived cells were incubated for 60 min with 20 pM TGF-β1, 400 pM HGF, or a combination of both. After fixation with 4% paraformaldehyde, the sections were incubated with primary Ab at 4°C for 16 hr. Localization of pSmad3L and pSmad3C was visualized by anti-phospho-Smad3 immunofluorescence. D. The primary component present in TGF-β-induced Smad binding complex (SBC) at the PAI-1 promoter is pSmad3L. Nuclear extracts from the myofibroblasts treated without (-) or with (+) 20 pM TGF-β1 were incubated with the 32P-labeled PAI-1 probe. As competitors, a 100-fold molar excess of unlabeled wild-type (WT) or mutant (MT) PAI-1 oligonucleotide was incubated with a reaction buffer before addition of the probe (lanes 4 and 5). For supershift analysis of the phosphorylated Smad3, nuclear extracts were incubated with anti-pSmad3L Ab or anti-pSmad3C Ab (lanes 6 and 7). E. HGF and TGF-β signals additively stimulate transcriptional activities of PF1-Luc through pSmad3L. RGM-1 cells were transiently co-transfected of PF1-Luc with Smad3WT, Smad3EPSM, or Smad3(3S-A). Under serum-free conditions, cells were incubated for 12 hr with 20 pM TGF-β1, 400 pM HGF, or a combination of both. Luciferase activity was determined and normalized to transfection efficiency. Values of samples from cells transfected with PF1-Luc alone and left untreated were arbitrarily set to 1. F. Schematic of PAI-1 transcriptional activity stimulated by the pSmad3/Smad4 complex.
HGF, as well as an additive increase upon treatment with TGF-β plus HGF. Collectively, the HGF signaling pathway alone increases transcriptional activities of PAI-1. In addition, it enhances TGF-β signal through pSmad3L (Fig. 7F).

**HGF as well as TGF-β signals promote cellular invasion via the JNK pathway**

PAI-1, the main inhibitor of the urokinase-type plasminogen activator system, permits cellular migration and invasion by enhancing cell adhesion (Bajou et al., 1998; Palmieri et al., 2002; Hirashima et al., 2003). We therefore examined the invasive properties of RGM-1 cells using a two-compartment Boyden chamber system that partially mimics in vivo conditions (Fig. 8A) (Mori et al., 2004). Without invasive stimuli from the lower chamber, only a few cells invaded through the pores of the membrane (Fig. 8B). When the medium in the lower compartment was supplemented with either TGF-β or HGF, the activated cells showed an increased invasion. Additional treatment of HGF with TGF-β increased the invasion above that of TGF-β or HGF treatment alone. The addition of a JNK inhibitor SP600125 to the medium in the upper chamber significantly inhibited invasion of the cells triggered by TGF-β and/or HGF stimulation (Bennett et al., 2001), suggesting a direct role of the JNK pathway in facilitating cellular invasion in response to TGF-β and HGF stimulation (Fig. 8C).

**Implication of Smad3 phosphoisoform-mediated signaling for biological function**

TGF-β phosphorylates Smad3 by at least two pathways, one via TβRI and the other via JNK and p38 MAPK. Both of these signaling pathways can be activated by the binding of TGF-β to its cell-surface receptors. The phosphorylation specific Abs used in our recent experiments enables us site-specific analyses of phosphorylation in Smad3. Based on the results, we formulated a model to account for the differential roles of the Smad3 phosphoisoforms: pSmad3C and pSmad3L (Fig. 4). Although Smad3 phosphorylation either at the linker region or at the C-terminal region allows the protein to associate with Smad4 (Fig. 7A, B) and undergo translocation into the nucleus (Fig. 7C), pSmad3C and pSmad3L each transmits its signal independently, and causes distinctive biological effects (Fig. 9).

TGF-β treatment activates the TβRI pathway, further leading to directly phosphorylate Smad3C. However, pSmad3C is not induced by HGF treatment. The TβRI/pSmad3C pathway is likely to be involved in TGF-β-mediated growth inhibition since TGF-β-inducible expression of cyclin-dependent kinase inhibitors (CDKIs) including p21WAF1/CIP1 and p15INK4B (Harper et al., 1993; Hannon and Beach, 1994), which arrest cell cycle progression in the G1 phase, requires C-terminal phosphorylation of Smad3 (Moustakas and Kardassis, 1998; Feng et al., 2000). The role of TGF-β as a potent tumor suppressor has gained widespread acceptance in recent years and has been extensively reviewed (Massagué, 1998; Wakefield and Roberts, 2002).

On the other hand, we recently demonstrated that not only TGF-β but also growth factors acting at tyrosine kinase receptors including HGF and PDGF activate JNK (Fig. 6A) (Mori et al., 2004; Yoshida et al., 2005). Activated JNK can directly phosphorylate serine

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**Fig. 8.** HGF as well as TGF-β signals induce cellular invasion via the JNK pathway. A. Schematic presentation of invasion assay. B. JNK inhibitor SP600125 treatment causes reduction of invasive capacity induced by HGF as well as TGF-β signals. RGM-1 cells were cultured on Matrigel for 48 hr with 20 pM TGF-β1, 400 pM HGF, or a combination of both in the absence or presence of 10 mM SP600125. C. Schematic of invasion promotion via the JNK pathway.
residues 208 and 213 in the linker region of Smad3 in vitro at the same site (Fig. 6B) that is phosphorylated in response to TGF-β or HGF in vivo (Fig. 5). Thus, serine residues 208 and 213 in Smad3L represent a phosphorylation site shared by HGF and TGF-β signaling pathways. Phosphorylation allows Smad3 to associate with Smad4, and thus to enter the nucleus, where the complex binds to the Smad-binding element on the PAI-1 promoter, stimulating its transcriptional activity (Fig. 7). Induction of PAI-1 expression is likely to increase invasion (Fig. 8). Likewise, p38 MAPK-mediated pSmad3L signal stimulates collagen synthesis in activated mesenchymal cells (Furukawa et al., 2003). We therefore concluded that receptor tyrosine kinase growth factors and TGF-β additionally transmit the signals through JNK/p38 MAPK-dependent pSmad3L, thus participating in regulation of migratory/invasive capacity and ECM synthesis.

Signals derived from receptor tyrosine kinase growth factors have been found capable of modulating Smad-dependent effects (Kretzschmar et al., 1997, 1999; de Caestecker et al., 1998). Previous work has also demonstrated the importance of cross-talk between TGF-β receptors and JNK/p38 MAPK pathways for Smad3-mediated signaling. JNK-mediated phosphorylation of Smad3 enhanced its activation and nuclear translocation (Engel et al., 1999). Furthermore, adenoviral expression of Smad3, but not Smad2, augmented the TGF-β-elicited induction of MMP-13 expression (Leivonen et al., 2002). In addition, co-expression of Smad3 with the constitutively active M KK3b and M KK6b, the upstream activators of p38 MAPK, resulted in nuclear translocation of Smad3 and induction of MMP-13 expression. These phosphorylation events were reported to occur at sites distinct from the C-terminal regions in Smad3. Our data are in keeping with such findings, suggesting that JNK/p38 MAPK stimulates phosphorylation of Smad3L.

Cross-talk between Smad3 and JNK/p38 MAPK-mediated signals has been reported to occur in the nucleus (Fig. 2) (Derynck and Zhang, 2003). Several TGF-β-responsive elements in target genes including PAI-1 gene contain AP1 sites. Importantly, TGF-β can activate JNK/p38 MAPK, which in turn regulates the activity of c-Jun and ATF-2 (Atfi et al., 1997; Wang et al., 1997; Hanafusa et al., 1999). Thus, Smads interact in the nucleus with proteins which themselves are targeted by JNK/p38 MAPK. On the other hand, our current model implies that some of the cross-talk begins in the cytoplasm, where JNK/p38 MAPK directly phosphorylates Smad3L to result in subsequent transcriptional activation of the PAI-1 gene in the nucleus. Future work will examine whether pSmad3L and pSmad3C interact differentially with their DNA binding partners or recruit transcriptional co-activators on PAI-1 and CDKI promoters.

**In CCl₄ hepatotoxicity in rats, pSmad3C transmits signaling in hepatocytes, while pSmad3L does so in α-SMA-immunoreactive mesenchymal cells**

We further investigated pSmad3C and pSmad3L-mediated signals in the injured liver after chemical insult (Yoshida et al., 2005). Administration of a single dose of CCl₄ resulted in steatosis and necrosis of centrilobular hepatocytes within 2 days. At 36 hr after CCl₄ intoxication, the centrilobular area in the rat liver contained necrotic hepatocytes, acidophilic bodies, and a dense cellular infiltrate containing macrophages and lymphocytes. At this time point, TGF-β1 and ECM constituents including PAI-1 and α2 (I) procollagen were highly expressed in activated mesenchymal cells immunoreactive for α-smooth muscle actin (α-SMA) (Tahashi et al., 2002). On the other hand, expression of

![Fig. 9. Implication of Smad3 phosphoisoform-mediated signaling for biological function. TGF-β treatment activates TβRI, further leading to direct phosphorylation of Smad3C. The TβRI/pSmad3C pathway involves TGF-β-mediated growth inhibition via cyclin-dependent kinase inhibitors including p21WAF1/CIP1. On the other hand, receptor tyrosine kinase growth factors and TGF-β additionally transmit the signals through JNK/p38 MAPK-dependent pSmad3L, thus participating in regulation of migratory/invasive capacity and ECM synthesis possibly by stimulating transcriptional activity of PAI-1 gene.](image-url)
p21\(^{\text{WAF1/CIP1}}\) was increased in hepatocytes, the hepatic equivalent of epithelial cells (unpublished observation). Necrotic material tended to decrease at 72 hr, when TGF-β1, p21\(^{\text{WAF1/CIP1}}\), and ECM-related transcripts had decreased.

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 (Michalopoulos and DeFrances, 1997). After CCl\(_4\) intoxication, pSmad3L appeared in nuclei of regenerating hepatocytes within 8 hr and eventually diminished by 36 hr, a time when proliferation stopped (unpublished observation). Thus, Smad3L is phosphorylated rapidly in hepatocytes in response to appropriate mitogenic stimuli. The TGF-β signal transduction system has been implicated in negative regulation of the growth response in hepatocytes. Immunostaining of normal rat liver with Ab specific to pSmad3C showed scant phosphorylation of Smad3 throughout the liver (Fig. 10A, top panel in α pSmad3C column). Similar to an increased pSmad3C in the nuclei of mesenchymal cells surrounding centrilobular areas, amounts of the C-terminal phosphorylation were dramatically increased in the nuclei of hepatocytes at 36 hr after CCl\(_4\) intoxication (Fig. 10A, middle and bottom panels in α pSmad3C column). The C-terminal phosphorylation decreased at 72 hr after CCl\(_4\) intoxication (unpublished observation).

Taking the above observations together with a finding that TGF-β secreted by mesenchymal cells including hepatic stellate cells (HSC) is present within hepatocytes (Bissell et al., 2001), TGF-β in hepatocytes would be expected mainly to transmit a growth-inhibitory signal through pSmad3C (Fig. 10A, epithelial cell column). In most normal epithelial cell types, TGF-β arrests cell cycle progression in the G1 phase by up-regulating expression of CDKIs including p21\(^{\text{WAF1/CIP1}}\) and/or p15\(^{\text{INK4B}}\) (Harper et al., 1993; Hannon and Beach, 1994). Induction of CDKI expression requires the C-terminal phosphorylation of Smad3 (Moustakas and Kardassis, 1998; Feng et al., 2000). Accordingly, pSmad3C-mediated signaling could take part in growth inhibition through up-regulation of CDKI expression in epithelial cells such as hepatocytes.

We next investigated the distribution of pSmad3L in normal rat liver and injured liver. Normal rat liver showed scant phosphorylation of Smad3L (Fig. 10B, top panel in α pSmad3L column). Phosphorylation was induced in the injured liver at 36 hr after CCl\(_4\) treatment (Fig. 10B, middle panel in α pSmad3L column). Simultaneously, marked α-SMA immunoreactivity was observed in groups of mesenchymal cells adjacent to necrotic hepatocytes in centrilobular areas of the injured liver (Fig. 10B, middle panel in α-SMA column). Similar to constantly negative α-SMA staining in hepatocytes, pSmad3L was undetectable in most hepatocytes at 36 hr. However, the pSmad3L distribution fitted well with the pattern obtained by α-SMA immunostaining in mirror image sections: pSmad3L accumulated in nuclei of α-SMA-immunoreactive mesenchymal cells (Fig. 10B, bottom panels in α pSmad3L and α α-SMA columns). Mesenchymal cells including HSC tend to persistently activate the JNK/pSmad3L cascade in vivo in contrast to epithelial cells.

After liver injury in vivo, rapid JNK activation occurred in HSC, accompanied by increasing pSmad3L (Yoshida et al., 2005). Co-treatment of primary-cultured HSC with both TGF-β and PDGF activated the JNK pathway, leading to phosphorylate Smad3L. JNK activation induced by TGF-β and PDGF could be involved in invasion of resident HSC within the space of Disse into the area of tissue damage (Yang et al., 2003), since treatment of HSC with a JNK inhibitor SP600125 significantly inhibited HSC invasion triggered by TGF-β and/or PDGF stimulation. Collectively, TGF-β and PDGF could transmit invasive signals of the activated mesenchymal cells through the JNK/pSmad3L pathway (Fig. 10B, mesenchymal cell column).

**Nuclear translocation of pSmad3L by activated JNK may reduce pSmad3C-mediated signaling**

Cellular context is a crucial determinant of ultimate outcome of TGF-β signaling in both normal epithelial cells and cancer cells (Wakefield and Roberts, 2002). Our model implies that the JNK pathway directly or indirectly modulates pSmad3C and pSmad3L-mediated signaling to regulate TGF-β-responsive genes, resulting in antagonistic as well as synergistic biological effects. Thus, differential biological function in response to TGF-β can depend on the activated state of JNK (Fig. 11).

HGF treatment potentiates cellular migration/invasion in response to TGF-β signaling through the JNK/pSmad3L pathway, while HGF signaling antagonizes the antiproliferative effect of TGF-β via the TBR1/pSmad3C pathway in normal epithelial cells (Fig. 11A) (Mori et al., 2004). The decrease in pSmad3C is not caused by inactivated TβRI, since TGF-β treatment results in an increase of pSmad2C by activated TβRI (Fig. 11A.1). HGF signaling cooperates with that mediated by TGF-β to activate the JNK/pSmad3L pathway and accelerate nuclear transport of pSmad3L from the cytoplasm (Fig. 11A.2). This reduces accessibility of unphosphorylated Smad3 to membrane-anchored TβRI, preventing Smad3C phosphorylation (Fig. 11A.3). pSmad3C-mediated transcription, and the antiproliferative effect of TGF-β upon the cells. Although TGF-β signaling also activates the JNK pathway, this activity is relatively weak and transient in normal epithelial cells, as opposed to mesenchymal cells (Fig. 11A.4).

Our present studies have provided important insights into the diversity and complexity of TGF-β-mediated signal transduction. Such mechanisms may have implications concerning the effects of TGF-β on tumor development.
Fig 10. In CCl₄ hepatotoxicity in rats, pSmad3C transmits signaling in hepatocytes, while pSmad3L does so in α-SMA-immunoreactive mesenchymal cells. Formalin-fixed, paraffin-embedded sections of normal rat liver (upper panel) and injured liver at 36 hr after CCl₄ intoxication (middle and bottom panels) were stained with anti-pSmad3C Ab (α pSmad3C column; A) and anti-pSmad3L Ab (α pSmad3L column; B). The pSmad3L section was paired with an adjacent section stained using anti-α-SMA Ab (αα-SMA column; B). Abs then were bound by goat anti-mouse immunoglobulins conjugated with peroxidase-labeled polymer. Peroxidase activity was detected by 3,3′-diaminobenzidine tetrahydrochloride (DAB). The bottom panels show higher magnification of the boxed areas in middle panels. All sections were counterstained with hematoxylin (blue). Brown indicates specific Ab reactivity. Bar: 50 µm.

A. In normal rat liver, Smad3 was phosphorylated slightly at the C-terminal region (upper panel). Amounts of C-terminal phosphorylation in nuclei of hepatocytes surrounding centrilobular areas increased at 36 hr after CCl₄ intoxication (middle and lower panels).

B. In normal rat liver, Smad3 was minimally phosphorylated at the linker region (upper panel). At 36 hr, pSmad3L was localized predominantly in the nuclei of α-SMA immunoreactive mesenchymal cells adjacent to necrotic hepatocytes in centrilobular areas (middle and bottom panels).
formation and progression in vivo (Piek and Roberts, 2001). We previously reported a mechanism of stimulation by tumor-derived TGF-β using highly transformed liver cancer cells (HCC-M cells), that lacked mutations in TGF-β receptors or R-Smads (Watanabe et al., 1983; Matsuzaki et al., 2000). Data obtained from the cells metabolically labeled with [32P] phosphate showed constitutive Smad3 phosphorylation. Accordingly, we investigated whether TGF-β and HGF could phosphorylate Smad3 in HCC-M cells (unpublished observation). Before stimulation, Smad3L was highly phosphorylated (Figs. 4B, 11B.1), and pSmad3L was already located in the nuclei of the cells. Therefore, exogenous TGF-β and HGF were unable to additionally enhance the phosphorylation (Figs. 4B, 11B.2). In comparison, neither basal nor TGF-β-dependent Smad3C phosphorylation was observed in the cells (Fig. 11B.3). The cells underwent TGF-β-dependent, reversible phosphorylation of Smad3C upon treatment with a JNK inhibitor SP600125 (unpublished observation). Impaired phosphorylation of Smad3C was not a result of TßRI inactivation, since TGF-β signaling dramatically stimulated phosphorylation of Smad2C by activated TßRI in the cells (Fig. 11B.4). We therefore conclude that a high degree of Smad3L phosphorylation had suppressed Smad3C phosphorylation. Since constitutively active JNK strongly promotes nuclear accumulation of pSmad3L, membrane-anchored TßRI cannot bind unphosphorylated Smad3. This results in a lack of TGF-β-dependent pSmad3C and loss of sensitivity to growth inhibition by TGF-β in highly transformed cancer cells.

Migration and proliferation of progenitors in normal colonic crypts: Involvement of pSmad3L

During normal tissue homeostasis, escape from TGF-β-induced cytostasis is crucial in a subset of progenitor cells devoted to ensuring epithelial renewal (Siegel and Massague, 2003). pSmad3C was located in the nuclei of normal colonic mucosa (Fig. 12A, top portion in α pSmad3C column). This finding conforms to the concept of the TßRI/pSmad3C pathway representing growth inhibition (Fig. 9). In contrast, pSmad3L immunoreactivity showed a striking distribution, being present in the nuclei of Ki-67 immunoreactive progenitor cells at the bottom of the crypts in the proliferative compartment (Fig. 12B, C, top portions in α pSmad3L and α Ki-67 columns); this staining declined in the upper parts of the crypts which were populated by differentiated cells, becoming undetectable superficially (Yamagata et al., 2005). These findings suggest that normal progenitor cells located at the base of crypts might take advantage of pSmad3L-mediated signaling to proliferate and then migrate upward.

Colonic carcinogenesis: Reciprocal change in pSmad3L and pSmad3C

The biology of intestinal tumors has been proposed to recapitulate developmental and homeostatic features of their progenitor stem cells (van de Wetering et al., 2002; Batlle et al., 2002). To examine this hypothesis in the context of our own, Smad3C and Smad3L

![Fig. 11. Nuclear translocation of pSmad3L by activated JNK reduces pSmad3C-mediated signaling. A. Nuclear translocation of pSmad3L by HGF treatment restricts accessibility of unphosphorylated Smad3 to membrane-anchored TßRI, decreasing Smad3C phosphorylation, pSmad3C-mediated transcription, and TGF-β-mediated growth inhibition in normal epithelial cells. B. Membrane-anchored TßRI cannot bind unphosphorylated Smad3 because of constitutive nuclear transport of pSmad3L by highly active JNK, resulting in loss of Smad3C phosphorylation and loss of sensitivity to growth inhibition by TGF-β in highly transformed cancer cells.](image-url)
phosphorylation was evaluated immunohistochemically in the same specimens (Yamagata et al., 2005). In some part of invasive adenocarcinoma, Smad3 was minimally phosphorylated at the C-terminal region (Fig. 12A, lower portion in α pSmad3C column). Attenuation of the TβRI/pSmad3C pathway would disrupt tumor-suppressive activities, favoring development of colorectal cancers. In contrast, pSmad3L was significantly more abundant throughout the glands formed by the adenocarcinoma than in normal glands (Fig. 12B, C, lower portion in α pSmad3L column). Glands showing highly phosphorylated pSmad3L were evenly distributed in the adenocarcinoma. A significant positive relationship was evident between pSmad3L distribution and Ki-67 expression, while the constitutive distribution of pSmad3L was greater than that of Ki-67 (Fig. 12C, lower portions in α pSmad3L and α Ki-67 columns). Collectively, the JNK/pSmad3L pathway is closely associated with invasive adenocarcinoma, a highly infiltrating tumor with prominent Ki-67 expression. Adenomas showed a degree of linker phosphorylation that was a few times greater than that seen in normal mucosa, i.e. a pSmad3L signal intensity intermediate between those typical of normal epithelial cells and those representative of adenocarcinomas (Fig. 12D).

To address the functional relationship between activated JNK and Smad3 phosphorylation in human colorectal tumors, we next assayed kinase activity in vitro (Yamagata et al., 2005). In proportion to the low activities of JNK1/2 in normal mucosa, JNK showed little ability to phosphorylate Smad3L. In contrast, JNK from human colorectal adenocarcinoma could directly phosphorylate Smad3L. Taken together with the nuclear localization of pSmad3L in human colorectal adenocarcinoma (Fig. 12B, C), the results show that JNK directly phosphorylates Smad3L, leading to translocation of the protein into the nuclei of the adenocarcinoma. The shift from TβRI/pSmad3C-mediated to JNK/pSmad3L-mediated signaling is a major mechanism orchestrating a complex transition of TGF-β signaling as neoplasia progresses from normal colorectal epithelial cells through adenoma to invasive adenocarcinoma with distant metastasis (Fig. 13) (Vogelstein et al., 1988).

Smad3 phosphoisoform-mediated signaling in vivo

Given a family of TGF-β signals that have the potential to control a wide range of biological process in mammalian cells and tissues, multiple layers of tight regulation of their activities are essential to cell survival and function (Siegel and Massagué, 2003). In the colon, epithelial cells are constantly being renewed, with progenitor cells proliferating at the base of colonic crypts and migrating upward to the luminal end while they begin to assume the morphologic characteristics of differentiated cells (Potten and Loeffler, 1990). This process is tightly controlled by a delicate balance between proliferation and differentiation of the migrating cells. The phosphorylation pattern of Smad3 in normal colonic mucosa indicates that it can be a factor important in directing this balancing act (Table 1). Localization of pSmad3L is limited to the base of colonic crypts where progenitor cells are located, suggesting that pSmad3L blocks the growth-suppressive effects of the TβRI/pSmad3C pathway in these cells, allowing them to proliferate and migrate (Moses et al., 1990). On the other hand, pSmad3L is absent from differentiated cells, presumably allowing the cells to become sensitive to the antiproliferative influences mediated by the TβRI/pSmad3C pathway (Barnard et al., 1989).

During neoplastic transformation, the normal balance between proliferation and differentiation in the colonic epithelium is lost (Wakefield and Robert, 2002). Intriguingly, in invasive adenocarcinoma, decreased Smad3C phosphorylation could be demonstrated; this implied ongoing repression of the anti-proliferative effects of TGF-β in the tumor cells. Through a number of experiments, we implicated constitutive phosphorylation of Smad3L in this effect. Similarly, JNK was constitutively phosphorylated and activated in colorectal cancer (Licato et al., 1997; Yamagata et al., 2005). Since the JNK signaling pathway is frequently activated by mutations in Ras during colon tumor development (Dérijard et al., 1994), constitutive phosphorylation of Smad3L can be a direct consequence of Ras/JNK signaling. The activated JNK directly phosphorylates Smad3L, leading to nuclear translocation of the protein in the cancer cells. Constitutively phosphorylated Smad3L is highly infiltrating tumor with prominent Ki-67 expression that was a few times greater than that seen in normal mucosa, i.e. a pSmad3L signal intensity intermediate between those typical of normal epithelial cells and those representative of adenocarcinomas (Fig. 12B, C, lower portion in α pSmad3L column). Attenuation of the TβRI/pSmad3C pathway would disrupt tumor-suppressive activities, favoring development of colorectal cancers. In contrast, pSmad3L was evenly distributed in the adenocarcinoma. A significant positive relationship was evident between pSmad3L distribution and Ki-67 expression, while the constitutive distribution of pSmad3L was greater than that of Ki-67 (Fig. 12C, lower portions in α pSmad3L and α Ki-67 columns). Collectively, the JNK/pSmad3L pathway is closely associated with invasive adenocarcinoma, a highly infiltrating tumor with prominent Ki-67 expression. Adenomas showed a degree of linker phosphorylation that was a few times greater than that seen in normal mucosa, i.e. a pSmad3L signal intensity intermediate between those typical of normal epithelial cells and those representative of adenocarcinomas (Fig. 12D).

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Current evidence suggests that regulation of ECM production in acute and chronic liver disease may involve different mechanisms, although hepatic stellate cells are the principal effector in both cases (Pinzani and Marra, 2001; Tahashi et al., 2002). Hepatic stellate cells undergo progressive activation to myofibroblast-like cells in a state of chronic liver damage (Friedman, 2003). On the other hand, considerable complexity of pSmad3L-mediated signaling can result from temporal variation involving JNK and p38 MAPK (Schnabl et al., 2001). Our in vivo model indicates that JNK activation results in phosphorylation of Smad3L, particularly when JNK is activated transiently in HSC after acute liver injury (Yoshida et al., 2005). When sustained activation of p38 MAPK in myofibroblasts is achieved during
As neoplasia progresses from normal colorectal epithelial cells through adenoma to invasive adenocarcinoma with distant metastasis, pSmad3L gradually increases while pSmad3C declines. Sections of formalin-fixed, paraffin-embedded colonic cancerous tissue and adenoma were exposed to anti-pSmad3C Ab (α pSmad3C column; A and D), anti-pSmad3L Ab (α pSmad3L column; B, C, and D), or anti-Ki-67 Ab (α Ki-67 column; B and C). These Abs in turn were bound by goat anti-rabbit or -mouse immunoglobulins conjugated with peroxidase-labeled polymer. Peroxidase activity was detected with DAB. C shows a higher magnification of the boxed areas in B. All sections were counterstained with hematoxylin (blue). Brown staining indicates specific Ab reactivity.

A. pSmad3C was located in the nuclei of normal colonic mucosa (upper portion in α pSmad3C column). In some part of invasive adenocarcinoma, Smad3 was minimally phosphorylated at the C-terminal region (closed arrow) (lower portion in α pSmad3C column). B and C. In contrast, pSmad3L was localized in nuclei of Ki-67 immunoreactive progenitor cells at the bottoms of crypts in the proliferative compartment (closed arrow) (upper portions in α pSmad3L and α Ki-67 columns). This staining declined in the higher portion of the gland (differentiated compartment), becoming undetectable superficially. Throughout the glands formed by the adenocarcinoma, however, pSmad3L was significantly more abundant than in normal glands (lower portion in α pSmad3L column). Glands showing highly phosphorylated Smad3L were evenly distributed in the adenocarcinoma. pSmad3L was localized exclusively in cell nuclei of actively growing Ki-67 immunoreactive adenocarcinoma (lower portions in α pSmad3L and in α Ki-67 columns). D shows a human colonic adenoma, in which Smad3C and Smad3L were moderately phosphorylated. Bar: 100 µm.
chronic liver injury, TßRI/pSmad3C signaling largely gives way to p38 MAPK/pSmad3L signaling, which could stimulate ECM production persistently (Furukawa et al., 2003).

**Conclusion**

TGF-ß signaling is an important physiological regulator in normal epithelial cells. Further, unraveling the molecular mechanisms of TGF-ß signaling in human carcinogenesis appears promising for the development of new therapies (Siegel and Massagué, 2003). The transition of the cells from an epithelial to a mesenchymal phenotype underlies the pathology of the invasive phenotype associated with metastatic adenocarcinoma. During human colorectal

![Diagram](image_url)

**Fig. 13.** Shift from pSmad3C-mediated tumor-suppressive effects to pSmad3L-mediated oncogenic effects in TGF-ß signal transduction during human colorectal carcinogenesis. As neoplasia progresses from normal colorectal epithelial cells through adenoma to invasive adenocarcinoma with distant metastasis, JNK/pSmad3L-mediated oncogenic activities gradually increase, while TßRI/pSmad3C-mediated tumor suppressor activities decrease.

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Epithelial cell, Cancer cell, Mesenchymal cell, Nucleus, Smad3, pSmad3L, pSmad3C
carcinogenesis, TGF-β signaling might confer a selective advantage upon tumor cells by shifting from the TBRI/psmAd3C pathway characteristic of mature epithelial cells to the JNK/psmAd3L pathway, which is more characteristic of the state of flux shown by activated mesenchymal cells. Loss of epithelial homeostasis and acquisition of a migratory, mesenchymal phenotype are essential for tumor invasion. From the viewpoint of TGF-β signaling, a key therapeutic aim in cancer would be restoration of the lost tumor suppressor function observed in normal colorectal epithelial cells at the expense of effects promoting aggressive behavior in the adenocarcinoma (de Caestecker, 2000). Specific inhibitors of the JNK/psmAd3L pathway might prove useful in this respect. In the case of molecularly targeted therapy for human cancer, psmAd3L and psmAd3C could be assessed as biomarkers to evaluate the likely benefit from specific inhibition of the JNK/psmAd3L pathway.

The use of an Ab against one phosphorylated linker site in Smad3 effectively analyzes the phosphorylation of this domain, but the approach imposes some limitations. Considering the existence of several more phosphorylation sites including ERK and CDK2/4 in the linker segments (Kretzschmar et al., 1999; Matsuura et al., 2004; Kamaraju and Roberts, 2005), our current model does not offer a definitive analysis of linker phosphorylation of Smad3. In fact, Smad3 phosphorylated at Ser204 in the linker segment has been localized to the cytoplasm (unpublished observation). Functional interplay between these kinases and linker segments in R-Smads may be far more complex than previously thought (Kretzschmar et al., 1997, 1999; Javelaud and Mauviel, 2005). Further analyses of each phosphorylation site with specific Abs, particularly against other phosphorylated domains in the linker segments, should enhance our understanding of TGF-β signaling in human colorectal carcinogenesis.

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Smad3 phosphoisoform-mediated signaling

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