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Cell Type-specific and Context-dependent TGF-β Signaling: Dialogues between Clinic and Bench

Koichi Matsuzaki[1*]

[1]*Departments of Gastroenterology and Hepatology, Kansai Medical University, Osaka, Japan*

[*]**Corresponding author:** Koichi Matsuzaki, M.D. and Ph.D., Associate Professor, Department of Gastroenterology and Hepatology, Kansai Medical University, 10-15 Fumizonocho, Moriguchi, Osaka 570-8506, Japan, Tel: 81-6-6992-1001; Fax: 81-6-6996-4874; E-mail: matsuzak@takii.kmu.ac.jp

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Abstract

Initial experiments leading to discovery of TGF-β and its designation as a "transforming" growth factor involved its ability to induce malignant behavior in mesenchymal cells such as fibroblasts. TGF-β, together with growth factors signaling via the receptor tyrosine kinase/Ras pathway, allowed proliferation of fibroblasts under anchorage-deficient conditions, a hallmark of cellular transformation. Several years later, TGF-β proved to have profound growth-suppressive effects in normal epithelial cells after transient Ras activation. As human benign tumors progress to carcinoma in situ, tumors with Ras-activating mutations tend to lose susceptibility to growth arrest by TGF-β. At invasive fronts of human advanced cancers, however, Ras and TGF-β pathways synergistically enable cancer cells to undergo epithelial-to-mesenchymal transition, thereby acquiring invasive and metastatic potential. Insights into the stepwise human carcinogenesis have emerged from recent detailed analyses of cell type-specific and context-dependent TGF-β signaling processes directed by multiple phosphorylated forms (phospho-isoforms) of Smad mediators.This review links advances in basic science with real-world clinical problems concerning Smadphospho-isoform signaling.

Keywords: TGF-β; Ras; Smad; Phospho-isoforms; Epithelial-to-mesenchymal transition; Biomarkers

Introduction

Cellular context is a crucial determinant of Transforming Growth Factor (TGF)-β signaling in both normal epithelial cells and Ras-transformed cells [1-5]. TGF-β inhibits proliferation of normal epithelial cells [6]. TGF-β signaling appears to be important for prevention of early-stage carcinogenesis, acting to maintain normal tissue architecture. As genetic mutations involving Ras and other oncogenic pathways gradually accumulate in benign tumors, the tumors tend to lose susceptibility to growth arrest by TGF-β [7]. Upon further, at invasive fronts of advanced cancers, TGF-β signaling together with Ras and other oncogenic pathways enables cancer cells to undergo epithelial-mesenchymal transition, by which they acquire invasive and metastatic potential [7]. Clearly, no simple rule can provide a general explanation of how TGF-β interacts with the mitogenicRas signaling cascade [8].

Smads are tightly controlled as TGF-β signaling mediators by domain-specific phosphorylation, which regulates subcellular localization, transcriptional response, and stability of their components [5,9,10]. Accordingly, monitoring phosphorylation status of signaling molecules is a key step in dissecting their pathways. In this review, we discuss how phospho-Smads transmit cell type-specific and context-dependent signals. We then describe critical interplay between basic research and real-world clinical problems involving phospho-Smad signaling.

Signaling by Multiple SmadPhospho-isoforms

Although C-terminal SXS phosphorylation by the TGF-β Type I Receptor (TβRI) is the key event in Smad activation, additional phosphorylation by intracellular protein kinases can also positively and negatively regulate Smads. R-Smads contain two conserved polypeptide segments, termed MH1 and MH2 domains, coupled by a less-conserved linker region. The structurally diverse linker regions of Smad2 and Smad3 harbor 1 ThrPro (TP) and 3 SerPro (SP) cluster sites (Figure 1). Although the first TP site (Thr-220) in Smad2 is nearly identical with Thr-179 in Smad3, phosphorylation sites in Smad2 at clusters of 3 serine residues in the linker region (Ser-245, Ser-250, and Ser-255) differ somewhat in sequence from corresponding linker phosphorylation sites in Smad3 (Ser-204, Ser-208, and Ser-213). In particular, flanking sequences around the final SP site of Smad3 (Ser-213) are quite different from those in Smad2 (Ser-255), suggesting that modes of regulation could differ between these 2 phospho-proteins [5]. The linker domain undergoes regulatory phosphorylation by cytoplasmic Mitogen-Activated Protein Kinase (MAPK) pathways, as well as by members of the nuclear Cyclin Dependent Kinase (CDK) family [2,3,5,8-10]. MAPKs including Extracellular Signal Regulated Kinase (ERK)1/2, c-Jun N-terminal Kinase (JNK)1/2/3, and p38/MAPKs, are evolutionarily conserved molecules essential for regulation of a variety of cellular events.

In Smad2/3 linker segments, individual serine/threonine residues can be targets for specific kinases. For example, TGF-β induces phosphorylation more strongly at Thr-220/179 in Smad2/3 than at the 3 SP cluster via the nuclear CDK family [2,5,9]. However, Receptor Tyrosine Kinase (RTK) growth factors such as epidermal growth factor, hepatocyte growth factor, and platelet derived growth factor, as well as pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β, bring about strong JNK-mediated phosphorylation

of the 3 SP cluster but only weak phosphorylation of Thr-220/179 [2,5]. Thus, the Smad linker region is a critical regulatory center in the fine-tuning of TGF-β signaling [8-10].

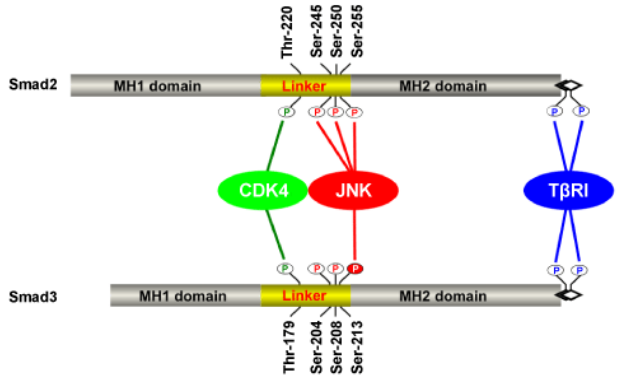

Figure 1: Schematic representation of Smad protein domains and sites of phosphorylation by multiple kinases. Membrane-bound TGF-β type I receptor (TβRI) phosphorylates 2 serine residues in the C-tail. Thr-220 in Smad2L and Thr-179 in Smad3L are preferred phosphorylation sites for nuclear CDK4 in response to TGF-β, while 3 clustered Ser residues are shown as phosphorylation sites for cytoplasmic JNK activated by RTK growth factors and pro-inflammatory cytokines.

Phospho-specific Antibodies (Abs) against individual pSer/pThr residues are critical tools for determining which kinases act at these sites, and how individual phosphorylation events contribute to Smad regulation [9,10]. We therefore developed domain-specific Abs able to distinguish individual pThr/pSer residues in the linker segments of Smad2 and Smad3 [5]. These Abs delineate how membrane-bound kinases (i.e., TβRI), cytoplasmic kinases (i.e., JNK), and nuclear kinases (i.e., CDK) dynamically phosphorylate Smad2 and Smad3 to create 3 types of phospho-isoforms: C-terminally phosphorylated Smad2/3 (pSmad2C and pSmad3C), linker-phosphorylated Smad2/3 (pSmad2L and pSmad3L), and dually phosphorylated Smad2/3 (pSmad2L/C and pSmad3L/C)[5]. Figure 2 depicts representative Smadphospho-isoforms, which differ between Smad2 and Smad3. Differences or apparent contradictions between studies concerning Smad linker phosphorylation and consequences for Smad activity can be related to different genetic and epigenetic backgrounds of the different cellular systems analyzed and fundamental biological differences between normal or immortalized cells and cancer cells (Table 1).

Cytostatic pSmad3C Pathway

Inhibition of cell proliferation is central to the TGF-β response in normal epithelial cells. CDK, cyclins, and CDK inhibitors are important molecules for understanding both TGF-β and Ras signaling [7].Growth arrest by TGF-β occurs via interference with cell cycle progression. Transcriptional activation of $p15^{INK4B}$ and $p21^{CIP1}$ and transcriptional repression of c-Myc genes by TGF-β are pSmad3C-dependent responses that oppose cell-cycle progression beyond the early/mid G_1 phase (Figure 2, left) [2,6]. Development of cancer is impeded by actions of the pSmad3C pathway, which can cause normal epithelial cells to cease growth and enter apoptosis after transient Ras activation, partly, through the ability of pSmad3C to induce or repress expression of a number of apoptosis-associated proteins such as Bcl2 [11].

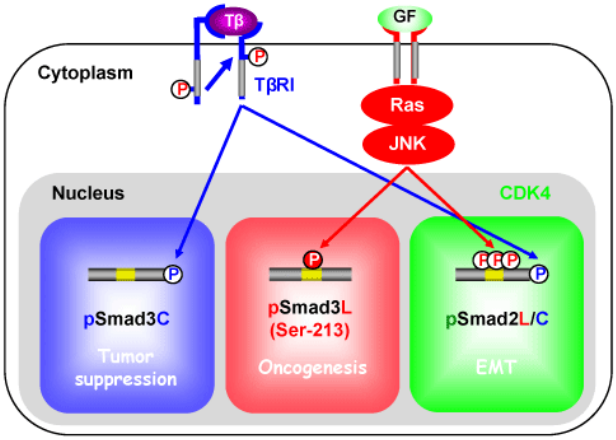

Figure 2: Representative Smadphospho-isoforms in the nucleus. Membrane-bound TβRI, cytoplasmic JNK, and nuclear CDK4 differentially phosphorylate Smad2 and Smad3 to create 3 phosphorylated forms (phospho-isoforms): C-tail phosphorylated Smad2/3 (pSmad2C and pSmad3C), linker-phosphorylated Smad2/3 (pSmad2L and pSmad3L), and dually phosphorylated Smad2/3 (pSmad2L/C and pSmad3L/C). EMT represents a complex biological program that enables cancer cells to acquire attributes of invasiveness and motility.

Samd Phospho-isoform	**pSmad3C**	**pSmad3L (Ser-213)**	**pSmad2L/C pSmad3L/C**
Target Gene	p15INK4B p21WAF1	c-Myc	MMP-2/9
Biological Function	Cytostasis Apoptosis	Cell Growth	Invasion Stemness
Localization Normal Epithelium	Mature Cell	Progenitor Cell	Stem Cell
Cancer	Down-regulation	Up-regulation	Invasion Front

Table 1: Smadphospho-isoforms direct cell type-specific and context-dependent TGF-β signaling. Phenotypes of benign tumors are dictated by genotype and tumorigenic growth is essentially a cell-autonomous phenomenon that involves a shift from tumor suppressive pSmad3C pathway to oncogenic pSmad3L (Ser-213) pathway constitutively induced by alterations in Rasoncogene. At invasive fronts of advanced cancers, TGF-β signaling allows Ras transformed cells to enhance EMT and acquire invasive property via pSmad2L/C pathway in the presence of pSmad3L (Ser-213).

Mitogenic pSmad3L (Ser-213) Pathway

JNK is a serine/threonine kinase affecting proliferation, differentiation, survival, and migration [12]. We have focused on linker phosphorylation of Smad3 at Ser-213 induced by the Ras/JNK

pathway [5]. In contrast to cytoplasmic retention of pSmad2L (Ser-245/250/255) and pSmad3L (Ser-204), pSmad3L (Ser-213) is not retained in the cytoplasm, permitting further consequences of JNK signaling (Figure 2, middle). RTK growth factors, pro-inflammatory cytokines, and to a lesser extent TGF-β, induces phosphorylation of Smad3L at Ser-213. Trimers with two R-Smads and Smad4 are thought to be the principal functional units [1-4]. In the nucleus, R-Smad proteins in activated R-Smad/Smad4 complexes bind other DNA-binding transcription factors as partners for target gene recognition and transcriptional regulation.Both pSmad3C and pSmad3L (Ser-213) can form hetero-complexes with Smad4, and the Smad complex translocates to the nucleus [5]. Because nuclear hetero-oligomerization is essential to assembly of target-specific transcriptional complexes [1-4], Smad3 can utilize 2 different phospho-domains to transmit different signals, consistent with the ability of Smad4 to act as both a tumor suppressor and a tumor promoter [2]. In addition, nuclear pSmad3L (Ser-213) binds multiple 5′-AGAC-3′ sequences, termedSmad binding elements, within the promoters of certain target genes [5]. Ras linking RTK to activation of protein kinase cascades plays a critical role in mitogenic signaling through pSmad3L (Ser-213) [13].

JNK activation is required for transformation induced by Ras, which can overcome TβRI/pSmad3C-mediated growth arrest. Overexpression of c-Myc inhibits Smad3-dependent transcription of $p15^{INK4B}$ and $p21^{WAF1}$, overriding cell-cycle blockade [2]. Resistance to TβRI/pSmad3C/$p21^{WAF1}$-mediated growth arrest has been ascribed to a mitogenic pathway involving JNK/c-Myc [14]. Ser-213 phosphorylation of Smad3L blocks C-tail phosphorylation by TβRI [5]. Mitogenic signaling accelerates nuclear transport of pSmad3L (Ser-213) from the cytoplasm, while preventing Smad3C phosphorylation, pSmad3C-mediated transcription, and anti-proliferative effects of TGF-β. Thus, the TβRI/pSmad3C anti-proliferative pathway and the JNK mitogenic pathway antagonize each other. Because the pSmad3C pathway is also required for maintenance of genomic stability and induction of replicative senescence [15], insensitivity to pSmad3C conveyed by constitutive pSmad3L (Ser-213) results in uncontrolled cell proliferation, contributing to carcinogenesis.

Pro-tumorigenic pSmad2L/C and pSmad3L/C Pathways

In the early 1980s, Roberts and colleagues isolated two fractions from murine sarcoma cell extracts that could synergistically induce remarkable growth of mesenchymal fibroblasts on soft agar, a hallmark of cellular transformation [16]. One, TGF-β, was shown to be a potent inducer of normal fibroblast transformation, but only in the presence of TGF-α, a ligand for RTK that transmits mitogenic signals via the Ras pathway. This was undoubtedly the first classic example of functional interaction between TGF-β and other mitogenic signaling pathways [8].

In addition to the negative impact of JNK on pSmad3C activity in epithelial cells through Smad3L phosphorylation at Ser-213, reports over the past 2 decades have described synergistic effects of JNK on pSmad2C activity in mesenchymal cells [17]. Such differential function between Smad2 and Smad3 pathways can result from variation in amino-acid sequences near the 3 SP cluster in linker segments (Figure 1). Ras-activating mutations drastically alter Smad3 signaling via the JNK pathway, increasing basal mitogenic pSmad3L (Ser-213) activity while shutting down TGF-β-dependent cytostatic pSmad3C function [14]. Ras mutations simultaneously activate the pro-tumorigenic TGF-β signaling to enhance invasive behavior by up-regulation of Epithelial-mesenchymal Transition (EMT)-like proteins via the pSmad2L (Ser 245/250/255)/C pathway (Figure 2, right)[18].

Cyclin overexpression contributes to loss of cell-cycle control and to the potential for cellular transformation and primary tumor growth [7,19]. Notably, the cyclin D-deficient mice are resistant to cancers induced by the Rasoncogene. In addition, Ras-transformed cells exhibit high CDK4 activities because of frequent amplification or overexpression of the cyclin D1 gene. Furthermore, cyclin D1 overexpression has been shown to positively correlate with disease progression and metastasis. Mitogens and hyperactive Ras result in CDK-mediated phosphorylation of Smad3 at Thr-179 [9,20], and of Smad2 at Thr-220 [18]. CDK-dependent phosphorylation of Smad3 inhibits the anti-proliferative action of TGF-β and serves as a novel way by which CDKs promote aberrant cell cycle progression [9,20]. Knock-down experiments also show that Smad linker phosphorylation contributes to cell invasion and migration and is essential for induction of matrix metalloproteinase (MMP) 2/9 and plasminogen activator inhibitor type 1 (PAI-1)[18]. These findings provide another evidence for a switch of phospho-Smad pathways from tumor suppression to pro-metastasis.

Human Carcinogenesis Driven by Ras-activating Mutations via Constitutive pSmad3L (Ser-213) Signaling

In proportion to accumulation of genetic alterations in driver genes including *K-Ras*, a portion of benign tumors are transformed to cancers [7]. On the other hand, a large variety of genetic lesions arising during colonic carcinogenesis, for example, have been assigned to two functional classes: those required to drive proliferation of precursor adenomas, and others that are required for adenomas to block subsequent differentiation and apoptosis.

Differentiation and apoptosis mediated by pSmad3C are blocked by the oncogenic/mitogenicRas/JNK/pSmad3L (Ser-213) pathway. This blockage is a frequent theme in development of gastrointestinal malignancies. Phospho-Smad3 signaling confers a selective advantage upon tumor cells by a shift from tumor-suppressive TβRI/pSmad3C pathway to oncogenic JNK/pSmad3L (Ser-213) pathway during sporadic human colorectal carcinogenesis [21]. This observation has been extended to hepatic carcinogenesis [22,23]. Phenotypes of benign tumors are dictated by genotype, and tumorigenic growth is essentially a cell-autonomous phenomenon that involves the constitutive shift induced by alterations in the Ras oncogene.

At Invasive Fronts of Human Advanced Cancers, Ras-transformed Cells Acquire Properties Resembling those in Epithelial Epithelial-to-mesenchymal Transition (EMT) in Response to TGF-β via the pSmad2L/C Pathway in the Presence of pSmad3L (Ser-213)

During progression of cancer, EMT confers malignant properties, including motility and invasiveness, upon cancer cells [7]. EMT provides cancer cells with capacity to invade surrounding tissues and to repopulate distant sites as metastases. In later stages of human cancer, TGF-β is frequently overexpressed, an occurrence closely associated with poor prognosis [24]. An increasing body of evidence

indicates that late in progression, in co-operation with the Ras pathway, autocrine and/or paracrine TGF-β signaling induces a malignant phenotype through EMT at invasive fronts in advanced cancer [7,24,25]. A variety of intracellular signaling events known as the non-Smad pathway are activated by TGF-β receptors [26,27]. Numerous reports have suggested that pro-tumorigenic effects of TGF-β such as enhancement of invasion and metastasis involve a pathologic switch of TGF-β signaling from the canonical Smad pathway to the pro-tumorigenic non-Smad pathway. However, in cancer cells, Smad signaling indeed drives pro-tumorigenic gene expression [2]. Smads 2 and 3 enhances EMT induction by activated TβRI, because Smad2/3 mutants lacking C-tail phosphorylation site show blockage of TGF-β-induced EMT.Many EMT-promoting transcription factors have the capacity to act as Smad co-factors. These non-Smad proteins interact with Smads to repress epithelial genes and/or activate mesenchymal genes [28].

In one set of influential experiments, EMT of squamous carcinoma cells *in vitro* was shown to occur only in cells overexpressing oncogenic Ras and activated forms of Smad2: this finding correlated with invasive behavior *in vivo* [29]. Insight is provided by the previous finding of synergy between Ras and TGF-β signaling in regulation of EMT and metastasis [30]. A critical role of JNK1, but not of JNK2, in mediating TGF-β-dependent EMT has been elegantly shown for each of these two kinases using tracheal epithelial cells from knockout mice [31]. Further, lack of JNK1 impairs the DNA binding of the Smad complex as well as EMT.

To better appreciate the EMT process, we have examined localization of pSmad2L/C in human advanced colorectal carcinomas carrying the *K-Ras* mutation, since these phosphoisoforms transmit invasive and proliferative TGF-β signals [18]. The results indicate nuclear localization of pSmad2L/C at the boundary between tumor epithelium and reactive stroma in human advanced carcinomas invading adjacent tissues.*In vitro* kinase assay confirms that cytoplasmic JNK obtained from cancerous tissues can phosphorylate Smad2 at the linker region.Ongoing phosphorylation of Smad2 at both linker and C-tail regions is required for tumors to attain a more invasive phenotype because both cell invasion and MMP/PAI-1 expression are completely blocked by Smad2 mutants lacking either linker or C-terminal phosphorylation [18]. MMP-2/9 expression at invasive fronts correlates positively with metastatic activity of primary tumors. Furthermore, PAI-1 is overexpressed at the tumor-stroma interface and is required to produce the TGF-β/Ras-induced invasive phenotype in human cutaneous squamous cell carcinoma. Expression of PAI-1 in cervical cancer strongly correlates with tumor cell invasion, lymph node metastasis, and poor prognosis. All together, our data are highly suggestive that EMT likely involves the pSmad2L/C pathway in the presence of pSmad3L (Ser-213)(32). All told, human advanced carcinomas lose responsiveness to TGF-β in terms of growth inhibition and apoptosis, while at the same time TGF-β enhances EMT in progression to more advanced cancer [15,33].

Prediction of Human Cancer Risk Using pSmad3L (Ser-213) and pSmad3C as Biomarkers

Clinical analyses of pSmad3L (Ser-213) and pSmad3C in human tumor development have provided substantial insight into relevant mechanism. For example, human livers infected by Hepatitis C Virus (HCV) progress from chronic hepatitis C through cirrhosis to Hepatocellular Carcinoma (HCC) several decades later [22]. Specimens from patients with chronic hepatitis C who develop HCC show abundant Smad3L (Ser-213) but limited Smad3C phosphorylation in hepatocytic nuclei, while other patients with abundant hepatocytic pSmad3C but limited pSmad3L (Ser-213) do not develop HCC. The same relationships are observed in human Hepatitis B Virus (HBV)-related hepatocarcinogenesis [23]. These clinical observations points the rolesof pSmad3C as a tumor suppressor and pSmad3L (Ser-213) as a promoter during carcinogenesis.

Useful Biomarkers for Assessing Effectiveness of Interventions Aimed at Reducing Human Cancer Risk Include pSmad3L (Ser-213) and pSmad3C

Improved understanding of Smadphospho-isoform signaling during human carcinogenesis suggests better ways to prevent human cancer development, exemplifying the laboratory-driven translational research. A key question concerning effectiveness of therapy for preventing liver HCC development is whether such therapy still has value once pre-neoplastic hepatocytes have appeared. Molecular analyses of paired liver biopsy specimens enable us to predict HCC risk after HCV clearance. Specimens from HCV-related chronic liver diseases can be divided into 2 subgroups based on phospho-Smad3 profiles [34]. One group carries risk of HCC after HCV clearance, while another carries much less risk of HCC occurrence. This grouping explains the observation that some patients with HCV-related liver disease respond effectively to antiviral therapy in terms of reversal from carcinogenic pSmad3L (Ser-213) to tumor-suppressive pSmad3C signaling, while others do not.Irrespective of HCV clearance, patients with cirrhosis who maintain strong pSmad3L signaling in hepatocytic nuclei require continued close follow-up, since HCC risk likely persists. Deng et al. recently demonstrated reversibility of phospho-Smad3 signaling in stepwise human HBV-related carcinogenesis after anti-HBV therapy [35].

Since JNK acts as an important regulator of Smad3 signaling that increases hepatocytic pSmad3L (Ser-213) favoring cell growth while decreasing TGF-β-dependent cytostatic actions of pSmad3C, pharmacologic interference with the JNK/pSmad3L (Ser-213) pathway might interrupt carcinogenesis. In this respect, Nagata et al. treated rats with a JNK inhibitor to suppress chemically induced hepatocarcinogenesis by reversing phospho-Smad3 signaling from oncogenic pSmad3L (Ser-213) pathway to tumor-suppressive pSmad3C pathway [36]. This study provides proof-of-principle that the JNK/pSmad3L pathway is an important target for therapy devised to reduce emergence of HCC in the context of chronic liver injury.In the future, the phospho-Smad3 profiles should serve as informative biomarkers for assessing effectiveness of interventions that might reduce human cancer risk.

Conclusion and Perspective

Smadphospho-isoform signaling will help both scientists and clinicians to understand complex disease processes. One needs to relate basic science findings to clinical research: a dialogue between bench and bedside. Smad phosphorylation profiles show great promise to allow clinicians to stratify pre-neoplastic epithelia and cancerous tissues into subgroups with distinct biologic properties including oncogenic and pro-tumorigenic potentials. The long-term goal of these analytic techniques is to accurately assess cancer risk, and to supplement existing clinical criteria for judging whether certain drugs should be given to patients with high cancer risk. Such an approach

should offer considerable benefit to precisely defined patient populations.

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