Phosphorylation status at Smad3 linker region modulates transforming growth factor-β-induced epithelial-mesenchymal transition and cancer progression

Akira Ooshima1 | Jinah Park1 | Seong-Jin Kim1,2

1 Precision Medicine Research Center, Advanced Institutes of Convergence Technology, Suwon, Korea
2 Graduate School of Convergence Science and Technology, Seoul National University, Suwon, Korea

Correspondence
Akira Ooshima, Precision Medicine Research Center, Advanced Institutes of Convergence Technology, Suwon, Korea.
Email: aooshima@snu.ac.kr
Seong-Jin Kim, Graduate School of Convergence Science and Technology, Seoul National University, Suwon, Korea.
Email: jasonsjkim@snu.ac.kr

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Smad3, a major transcription factor in transforming growth factor-β (TGF-β) signaling, plays critical roles in both tumor-suppressive and pro-oncogenic functions. Upon TGF-β stimulation, the C-terminal tail of Smad3 undergoes phosphorylation that is essential for canonical TGF-β signaling. The Smad3 linker region contains serine/threonine phosphorylation sites and can be phosphorylated by intracellular kinases, such as the MAPK family, cyclin-dependent kinase (CDK) family and glycogen synthase kinase-3β (GSK-3β). Previous reports based on cell culture studies by us and others showed that mutation of Smad3 linker phosphorylation sites dramatically intensifies TGF-β responses as well as growth-inhibitory function and epithelial-mesenchymal transition (EMT), suggesting that Smad3 linker phosphorylation suppresses TGF-β transcriptional activities. However, recent discoveries of Smad3-interacting molecules that preferentially bind phosphorylated Smad3 linker serine/threonine residues have shown a multitude of signal transductions that either enhance or suppress TGF-β responses associated with Smad3 turnover or cancer progression. This review aims at providing new insight into the perplexing mechanisms of TGF-β signaling affected by Smad3 linker phosphorylation and further attempts to gain insight into elimination and protection of TGF-β-mediated oncogenic and growth-suppressive signals, respectively.

KEYWORDS
breast cancer, EMT, metastasis, Smad3, TGF-β

1 | INTRODUCTION

Transforming growth factor-β (TGF-β)/Smad3 signaling plays critical roles in a wide variety of biological processes and carries out multiple functions such as growth arrest, apoptosis, differentiation, and epithelial-mesenchymal transition (EMT).1-4 Studies using mouse embryonic fibroblasts with targeted disruption of either Smad2 or Smad3 showed that Smad3 plays significant roles in TGF-β-mediated transcriptional responses.5,6 A critical part of Smad3 in TGF-β-induced EMT also stems from observations that Smad3 knockout mice abrogate EMT in response to epithelial injuries in vivo or by exposure to TGF-β in cell culture.7,8 TGF-β has been reported to be suppressive in the early development of cancer, while acquiring cancer-promoting propensities in the later stages. TGF-β is abundantly expressed in most cancer tissues, and high levels of TGF-β often bode for adverse clinical outcomes.1,2 Biological activities of TGF-β initiate its binding to a heteromeric complex of two types of transmembrane receptors consisting of type I and type II
S204, it can provide a priming site (pS208) for phosphorylation by GSK-3. GSK-3 is responsible for phosphorylation at S204. Flavopiridol, therefore, can reduce the level of GSK-3 including CDK family, ERK, JNK, p38 MAPK, and glycogen synthase kinase-3 transcriptional activity. A, Serine/threonine residues at Smad3 C-terminal and linker region are depicted together with responsible kinases, β.

**FIGURE 1** Smad3 linker phosphorylation by intracellular kinases attenuates transforming growth factor-β (TGF-β)-induced Smad3 transcriptional activity. A, Serine/threonine residues at Smad3 C-terminal and linker region are depicted together with responsible kinases, including CDK family, ERK, JNK, p38 MAPK, and glycogen synthase kinase-3β (GSK-3β). TGF-β induces Smad3 phosphorylation at three sites in the linker region, threonine 179 (T179), serine 204 (S204), and serine 208 (S208), along with the two C-terminal residues, serine 423 (S423) and serine 425 (S425). Even in the absence of TGF-β, the linker region in cancer cells often becomes phosphorylated at a certain level (pink) by intracellular kinases that are constitutively activated, but less weakly as compared to that in the presence of TGF-β (red). In the breast cancer cell line (MCF-10CA1a.c1, hereafter known as CA1a) in culture was first treated with a pan-CDK inhibitor, flavopiridol. TGF-β transcriptional activity was significantly upregulated by treatment with flavopiridol concomitant with reduction of Smad3 linker phosphorylation at T179, S204, and S208, suggesting that Smad3...
linker phosphorylation negatively regulates Smad3 transcriptional activity as determined by (CAGA)$_{12}$-luciferase ([CAGA]$_{12}$-Luc) reporter assay (Figure 1B). Likewise, inhibition of Smad3 linker phosphorylation by a specific phosphatase (SCP1) acting on the Smad2/3 linker region has resulted in upregulation of TGF-β signaling.

3 | MUTATION AT SMAD3 LINKER PHOSPHORYLATION SITES GREATLY ENHANCES TGF-β SIGNALING

It was initially reported that Ras-induced Smad2/3 linker phosphorylation inhibits TGF-β-induced responses by interrupting nuclear translocation of Smad2/3 and subsequent Smad-dependent transcription. Direct evidence that Smad3 linker phosphorylation suppresses TGF-β responses comes from studies using a Smad3 mutant at linker phosphorylation sites. We have adopted recombinant adenoviruses constitutively expressing wild-type Smad3 (Ad-Smad3) or its mutant at the linker region (Ad-EPSP), the C-terminal (Ad-3SA) or both the linker and the C-terminal (Ad-EPSP/3SA), in which all serine and threonine phosphorylation sites are replaced with alanine (A) and valine (V), respectively (Figure 2A). When Ad-EPSP was infected with the breast cancer cell line (CA1a), TGF-β-induced Smad3 transcriptional activity as evaluated by (CAGA)$_{12}$-Luc reporter assay was greatly enhanced in Ad-EPSP-infected cells as compared to Ad-Smad3-infected counterparts. Infection with either Ad-3SA or Ad-EPSP/3SA, in contrast, completely abolished Smad3 transcriptional activity, confirming that Smad3 linker phosphorylation downregulates TGF-β-induced Smad3 transcriptional responses (Figure 2B).

Although both Ad-Smad3- and Ad-EPSP-infected cells undergo TGF-β-induced EMT as characterized by spindle-shaped phenotype and loss of polarity, Ad-EPSP-infected cells show a far more prominent EMT as compared to Ad-Smad3-infected counterparts. Even in the absence of TGF-β, Ad-EPSP-infected cells showed phenotypic changes of EMT at the edge of a cell colony, suggesting that cells become highly sensitive to TGF-β so that they are capable of responding to even a trace amount of endogenous TGF-β (Figure 2C). Use of Ad-EPSP, therefore, showed that phosphorylation of the Smad3 linker region suppresses TGF-β signaling leading to EMT (Figure 3).

Remarkable changes induced by TGF-β other than EMT in Ad-EPSP-infected cells are noted by prominent apoptosis and growth arrest along with the reduction of stem cell population in breast cancer cell lines (CA1a and 4T1). The magnitude of these changes is much greater in Ad-EPSP-infected cells as compared to Ad-Smad3-infected counterparts. Even in the absence of TGF-β1, Ad-EPSP-infected cells show mild features of EMT. Both Ad-3SA and Ad-EPSP/3SA infection completely abrogated TGF-β-induced EMT. Scale bar, 50 μm.
higher in Ad-EPSM-infected cells than in Ad-Smad3-infected controls. We have reported in a mouse orthotopic xenograft model with breast cancer cell lines (CA1a and 4T1) that Ad-EPSM-infected cells generate the smallest primary tumor while showing the highest frequency of lung metastasis. Conversely, Ad-3SA-infected cells create the largest primary tumor without metastatic invasion into the lung, suggesting that as a result of enhanced TGF-β signaling induced by blockade of Smad3 linker phosphorylation, Ad-EPSM-infected cells promote TGF-β-induced EMT along with apoptosis, growth arrest and even a loss of stem cell population. Augmentation of TGF-β responses by Ad-EPSM was also confirmed in our separate studies. All of these observations indicate that phosphorylation of Smad3 linker sites causes inhibitory effects on TGF-β responses, including EMT and cytostatic functions, creating part of a negative feedback loop in Smad3 transcription (Figure 3).

4 | LINKER REGION-PHOSPHORYLATED SMAD3 RECRUITS FUNCTIONAL MOLECULES THAT EITHER SUPPRESS TGF-β SIGNALING OR PROMOTE TGF-β-INDUCED CANCER PROGRESSION

Smad3 linker phosphorylation has critical functions, such as temporary augmentation of TGF-β function, and promotion of cancer metastasis in addition to inhibition of TGF-β responses. It was first reported that functional molecules, such as peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) and neural precursor cell expressed developmentally downregulated gene 4-like (Nedd4L), preferentially bind to the phosphorylated Smad3 linker region and elicit either positive or negative signals in response to TGF-β in a cell context-dependent way (Figure 4). Both TGF-β and bone morphogenetic protein (BMP) receptor kinases in principle activate Smads by C-terminal phosphorylation to form active transcriptional complexes. The linker region of Smads in the complexes become phosphorylated first by nuclear CDK8/9 and later by GSK-3β. In the case of BMP signaling, Yes-associated protein (YAP), an effector of Hippo organ size control pathway, is recruited to the phosphorylated Smad1 linker sites and supports BMP/Smad1-dependent transcription as a linker coactivator. Smad3 linker phosphorylated sites are likewise recognized by Pin1, resulting in activation of TGF-β/Smad3-induced responses. It has now become evident that following activation of Smad3 signaling by Pin1, a ubiquitin ligase Nedd4L recognizes phosphorylated linker sites through its WW domains, resulting in poly-ubiquitination and proteasome-mediated degradation. Smad1 is similarly degraded but specifically by Smurf1. Interaction of Pin1 and Nedd4L with phosphorylated linker sites acts as a sequential on/off switch for activation first and degradation later in the TGF-β/Smad3 pathway, respectively (Figure 4A).
Pin1 has also been shown to promote cell migration and invasion in a human PC3 prostate cancer cell line with concurrent expression of N-cadherin. Knockdown of Pin1 suppresses TGF-β-induced cell migration and invasion while causing no effect on growth arrest (Figure 4B). Although EGF phosphorylates Smad3 linker serine/threonine residues, Pin1 is unable to recognize the phosphorylated linker sites of Smad3, indicating that C-terminal phosphorylation by TGF-β is essential for interaction with Pin1. In a separate report, Pin1 has been shown to promote interaction of linker-phosphorylated Smad3 with a cofactor Olig1 and accelerate TGF-β-induced cell motility but neither growth arrest nor EMT.

Transforming growth factor-β signaling is known to be regulated by the ubiquitin-proteasome system. It has previously been reported that Pin1 binds preferentially to phosphorylated Smad2 and 3 at the linker region and enhances their interaction with Smad ubiquitination regulatory factor 2 (Smurf2), leading to proteasomal degradation of Smad2 and 3 by poly-ubiquitination (Figure 4C). Generation of mice harboring targeted disruption of a Smurf2 allele has now shown that Smurf2 selectively binds to the phosphorylated Smad3 linker region and induces mono-ubiquitination instead of poly-ubiquitination in vivo under physiological conditions. Mono-ubiquitination by Smurf2 has been confirmed to inhibit the formation of homo- or hetero-trimeric Smad3 complexes required for TGF-β/Smad3 transcriptional activation. It became further apparent that mono-ubiquitination by Smurf2 interrupts access of Smad3 to the TGF-β type I receptor for C-terminal phosphorylation. Smurf2 seems highly likely to play a significant role in the suppression of TGF-β signaling by Smad3 linker phosphorylation (Figure 4C).

Recently, an additional mechanism has been reported that Smad3 linker phosphorylation promotes TGF-β-mediated cancer progression by inducing alternative splicing of a stem cell marker CD44. Phosphorylated linker sites of Smad3 are first recognized by an RNA-binding protein, poly(rC)-binding protein 1 (PCBP1), and both molecules form an active complex. On the cue from TGF-β and EGF stimulation, two proteins in the complex start to interact with the variable exon region of CD44 pre-mRNA and interrupt splicing assembly, favorably generating the mesenchymal isoform CD44s over the epithelial isoform CD44E (Figure 4D). Alternative splicing induced by Smad3 linker phosphorylation is now extended to occur in a large number of genes required for TGF-β-induced EMT and metastasis. Upon binding of either Pin1, Nedd4L, Smurf2, or PCBP1 to phosphorylated linker sites of Smad3, phosphorylation at the T179 residue has been determined to play a pivotal role in eliciting biological activities.

5 | MECHANISTIC INSIGHT INTO EMT AND CANCER METASTASIS

Cancer progression and acquisition of malignant properties are often associated with accumulation of genetic mutations that often lead to...
high activities of intracellular kinases such as oncogenic Ras, CDK family, and MAPK family.\textsuperscript{1,2,36} Growing evidence has shown that both canonical and non-Smad pathways collaborate and participate in TGF-β signaling to regulate a wide array of downstream cellular responses. Inhibition of any one of EGF/Ras/ERK or p38 signaling branches has been shown to abrogate TGF-β-induced EMT, suggesting the involvement of multiple signaling pathways and their cross-talks (Figure 5).\textsuperscript{37-39}

It has previously been reported that active Ras is required for induction of TGF-β-induced EMT in keratinocytes.\textsuperscript{50} In pancreatic cancer cells, transfection of oncogenic Ras has markedly enhanced expression of Snail1, a critical mediator for EMT. However, the synergism between TGF-β and active Ras is confined to the expression of Snail1 but not any of other TGF-β target genes such as Smad7.\textsuperscript{41} From our previous work, it can be speculated that oncogenic Ras might raise the level of Smad3 linker phosphorylation by activating the MEK/ERK pathway, probably resulting in suppression of TGF-β-induced EMT. Activation of Ras, however, actually enhances TGF-β-induced EMT by upregulating Snail1 expression as stated above. It is, therefore, reasonable to assume that cancer cells might be well equipped with prometastatic machinery that counteracts or overrides the inhibitory effect of Smad3 linker phosphorylation. Supporting part of this notion, Pin1 and PCBP1 are now known to mediate the malignant conversion of cancer by accelerating motility and conferring tumor-promoting properties, respectively.\textsuperscript{31,34}

Signals from the Wnt pathway promote activation of Snail in addition to Zeb, β-catenin and other EMT-promoting transcription factors. It is of note that GSK-3β works as a nodal protein in non-Smad and Wnt pathways. Activation of Wnt and Ras/ERK-P13K/AKT pathways leads to inhibition of GSK-3β, thereby stabilizing Snail and β-catenin.\textsuperscript{39,42} Moreover, inhibition of GSK-3β most likely reduces the phosphorylation level of the Smad3 linker region, leading to sensitization of TGF-β responses, including EMT.

6 | CONCLUDING REMARKS AND PERSPECTIVES

Smad3 signaling is mediated through multiple cross-talks between the canonical- and non-canonical pathways in a cell context-dependent way. A previous report by others showed that inhibition of CDK4/6 by a highly specific inhibitor induces TGF-β-induced EMT and enhances invasiveness of pancreatic cell lines, while significantly suppressing cell growth.\textsuperscript{43} In melanoma cells, suppression of Smurf2 by RNA interference was reported to increase the cytotoxic effects.
of mitogen-activated protein kinase (MAPK) inhibition by sensitizing the TGF-β signaling. These findings underscore the significance of suppressive impacts on TGF-β responses by Smad3 linker phosphorylation.

Abrogation of Smad3 linker phosphorylation, therefore, acts as a double-edged sword, either enhancing TGF-β-induced EMT or increasing cytotoxicity of anticancer reagents. Although Smad3 linker phosphorylation adds another layer of complexity to TGF-β responses, it may help find a particular target to block EMT or promote cell death. A deeper understanding and clearer insight into EMT and cell growth mediated through TGF-β signaling may provide better combinatorial cancer chemotherapies in the future.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

ORCID

Akira Ooshima https://orcid.org/0000-0002-2637-1219

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