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ORIGINAL ARTICLE

Identification of Epithelial-Mesenchymal Transition-related Target Genes Induced by the Mutation of Smad3 Linker Phosphorylation

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Background: Smad3 linker phosphorylation plays essential roles in tumor progression and metastasis. We have previously reported that the mutation of Smad3 linker phosphorylation sites (Smad3-Erk/Pro-directed kinase site mutant constructs [EPSM]) markedly reduced the tumor progression while increasing the lung metastasis in breast cancer.
Methods: We performed high-throughput RNA-Sequencing of the human prostate cancer cell lines infected with adenoviral Smad3-EPSM to identify the genes regulated by Smad3-EPSM.
Results: In this study, we identified genes which are differentially regulated in the presence of Smad3-EPSM. We first confirmed that Smad3-EPSM strongly enhanced a capability of cell motility and invasiveness as well as the expression of epithelial-mesenchymal transition marker genes, *CDH2*, *SNAI1*, and *ZEB1* in response to TGF-β1 in human pancreatic and prostate cancer cell lines. We identified *GADD45B*, *CTGF*, and *JUNB* genes in the expression profiles associated with cell motility and invasiveness induced by the Smad3-EPSM.
Conclusions: These results suggested that inhibition of Smad3 linker phosphorylation may enhance cell motility and invasiveness by inducing expression of *GADD45B*, *CTGF*, and *JUNB* genes in various cancers.
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Key Words: Smad3, Epithelial-mesenchymal transition, Pancreatic cancer, Prostate cancer, RNA sequence analysis

INTRODUCTION

TGF-β regulates various biological activities, such as cell proliferation, differentiation, angiogenesis, immune response, apoptosis, adhesion, and migration.[1-5] TGF-β acts as a tumor suppressor as well as a metastasis promoter.[6] In normal cells and early carcinomas, TGF-β inhibits proliferation, induction of apoptosis and plays an essential role in homeostasis. In a later stage of tumorigenesis, TGF-β1 promotes cell migration, invasion, epithelial-mesenchymal transition (EMT), and tumor metastasis .[6]

TGF-β1 ligands interact with two types of serine-threonine kinase receptors. TGF-β directly binds to the type II TGF-β receptor (TβRII) followed by the recruitment of the type I TGF-β receptor (TβRI). Constitutively active the TβRII transphosphorylates type I receptors which lead to transmit signals.[6,7] TβRI recruits and phosphorylates the receptor-associated Smads (R-Smads; Smad2 and Smad3) at the SSXS motif in their C-tails. Phosphorylated R-Smads interact with common mediator (Co-Smad; Smad4) and then translocate to the nucleus where they work as a transcriptional regulator for TGF-β1 target genes.[7,8]

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The Smad proteins consist of conserved N- and C-terminal domains, and a proline-rich linker region. The Smad3 linker region has four phosphorylation sites, Thr179, Ser204, Ser208, and Ser213 which can be phosphorylated by different kinases, such as CDK family and MAP kinase family.[9] TGF-β1 treatment rapidly induces phosphorylation of Thr179, Ser204, and Ser208. The mutation of the Smad3 linker phosphorylation sites to non-phosphorylated form, T179V, S204A, S208A, enhances the Smad3-mediated activation of TGF-β1/Smad3 target genes.[10] In the previous report, we demonstrate that the mutation of the Smad3 linker phosphorylation sites suppresses the tumor growth by apoptosis, growth arrest, and the reduction of cancer cell population and increases the TGF-β-mediated EMT and invasive activity in breast cancer.[11]

In this study, we observed that the mutation of Smad3 linker phosphorylation sites (Smad3-Erk/Pro-directed kinase site mutant constructs [EPSM]) increased the migration and EMT in response to TGF-β1 treatment in human pancreatic and prostate cancer cell lines. Moreover, the RNA-Sequencing analysis showed that the expression of Smad3-EPSM markedly induced the expression of EMT marker genes, such as *GADD45B*, *CTGF*, and *JUNB*. Accordingly, inhibition of Smad3 linker phosphorylation may enhance cell motility and invasiveness by inducing expression of EMT marker genes.

MATERIALS AND METHODS

1. Cell lines and culture conditions

The human prostate cancer cell line PC3M was obtained from Korea Cell Line Bank. DU145 was kindly donated by Prof. Issac Y Kim, Rutgers University, USA. The human pancreatic cancer cell line PANC-1 was kindly donated from Dr. Rhim, University Texas, MD Anderson, USA, and the SNU2543 cell line was kindly provided by Prof. Jin-Young Jang, School of Medicine, Seoul National University, Korea. DU145 and PC3M cells were grown in Roswell Park Memorial Institute (RPMI) 1640, and PANC-1 was cultured in Dulbecco's modified Eagle's medium (DMEM). All culture media contained 10% FBS and 1% penicillin/streptomycin.

2. Reagents

Human TGF-β1 was purchased from R&D Systems (Minneapolis, MN, USA) and treated in the cell at 5 ng/mL concentration. Cell lysates were incubated with antibodies against total anti-E-cadherin, Vimentin and Fibronectin (BD Biosciences, San Jose, CA, USA), Smad3 and Smad3 C-tail phosphorylation (Abcam, Cambridge, MA, USA; Cell Signaling Technology, Beverly, MA, USA) α-tubulin (Sigma Aldrich, Beverly, MA, USA) and HRP-conjugated anti-mouse or rabbit or goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

3. Cell migration and invasion assays

Cells were pretreated with or without TEW-7197 for 2 hours and then cultured with or without TGF-β1 for 24 or 48 hours. Cells were added to the upper well of Matrigel invasion chamber (BD Biosciences) containing 0.1% FBS-DMEM medium. Medium containing 10% FBS was placed in the bottom chamber and incubated for 24 or 48 hours at 37°C. Then, noninvasive cells were removed with a cotton swab. Migrated cells on the lower surface of the membrane were fixed with ethanol and stained with 0.1% crystal violet staining solution. Migrated cells from five fields were counted under a microscope. Migration assays were conducted in the same way as above without Matrigel.

4. RNA extraction and quantitative real-time PCR

Total RNA was isolated from cells using the easy-BLUE Total RNA extraction kit (Promega, Madison, WI, USA) followed by manufacturer's instruction. Reverse transcription was carried out with 2 μg of purified RNA using M-MLV reverse transcriptase (M1705; Promega).

Quantitative real-time PCR (qRT-PCR) was performed by ViiA 7 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Various target gene expression was analyzed using the comparative Ct method. The gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase level.

5. RNA-sequencing and bioinformatics analysis

The quality of total RNA was measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After quantitative PCR validation using KAPA library quantification kit (KAPA Biosystems, Cape Town, South Africa), libraries were subjected to paired-end sequencing with a 100 bp read length using an Illumina HiSeq 2500 platform.

Clean reads that average quality scores for all libraries were more than Q30 were aligned to the human genome using TopHat with a set of gene model annotation (Ensembl 72). Gene expression was quantified using Cufflinks.[12] Differential expression (DE) analysis between sample groups of interests was performed by using Cuffdiff[12] with the cutoff set at $P < 0.05$ and ≥ 1.5-fold change. Also, genes expressed above 1 FPKM in at least one sample were retained for DE analysis due to the possible noise at very low levels of expression. Hierarchical clustering for selected genes was analyzed with MeV (http://mev.tm4.org) using Euclidean

distance and complete linkage method. Functional annotation for differentially expressed genes (DEGs) were performed by Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov),[13] and relevant gene ontology (GO) terms were selected with a cutoff of $P < 0.001$.

RESULTS

1. TGF-β signaling regulates migration, invasion, and epithelial-mesenchymal transition in cancer cells

TGF-β1 is known to stimulate chemotaxis and migration of

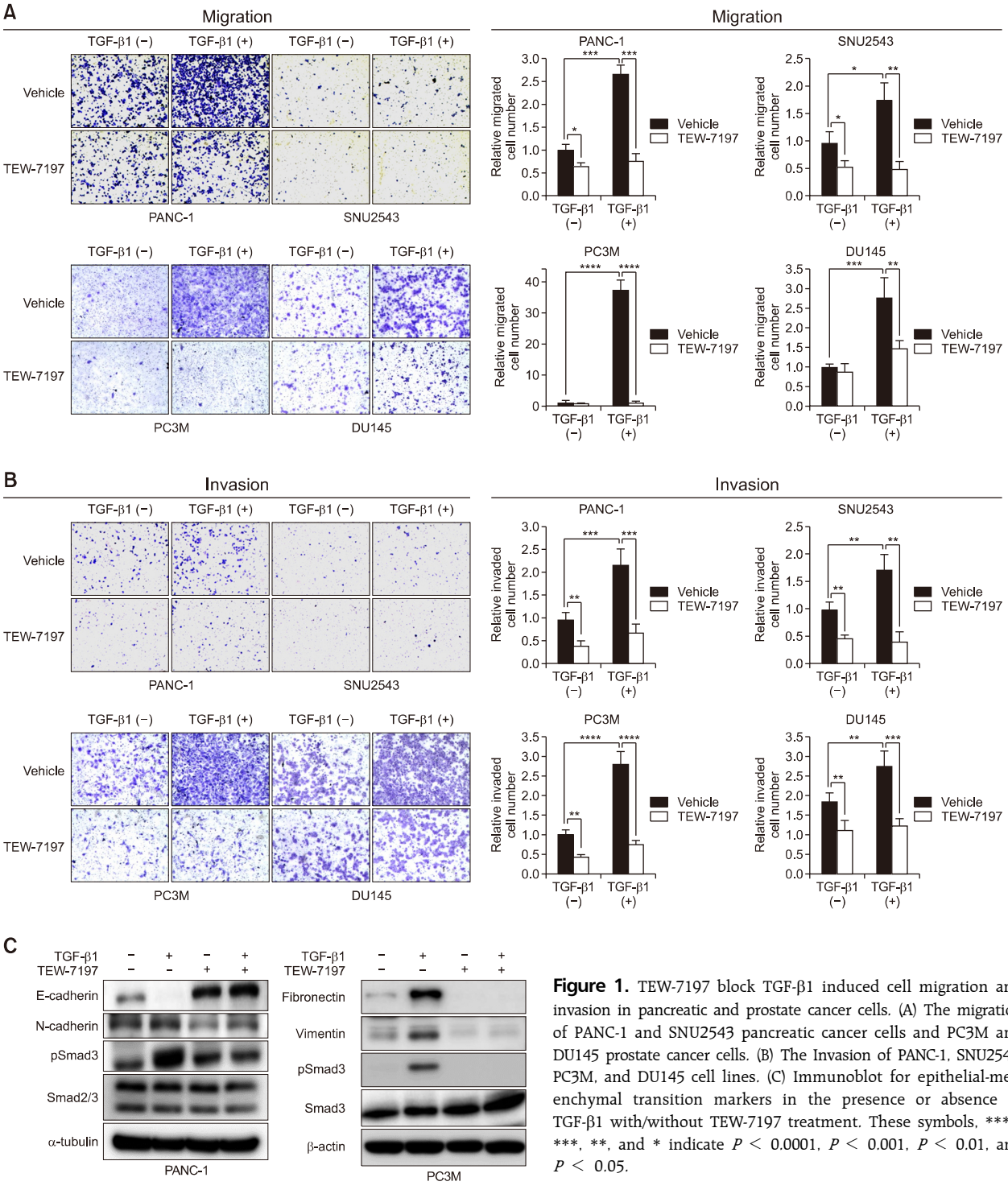

Figure 1. TEW-7197 block TGF-β1 induced cell migration and invasion in pancreatic and prostate cancer cells. (A) The migration of PANC-1 and SNU2543 pancreatic cancer cells and PC3M and DU145 prostate cancer cells. (B) The Invasion of PANC-1, SNU2543, PC3M, and DU145 cell lines. (C) Immunoblot for epithelial-mesenchymal transition markers in the presence or absence of TGF-β1 with/without TEW-7197 treatment. These symbols, ****, ***, **, and * indicate $P < 0.0001$, $P < 0.001$, $P < 0.01$, and $P < 0.05$.

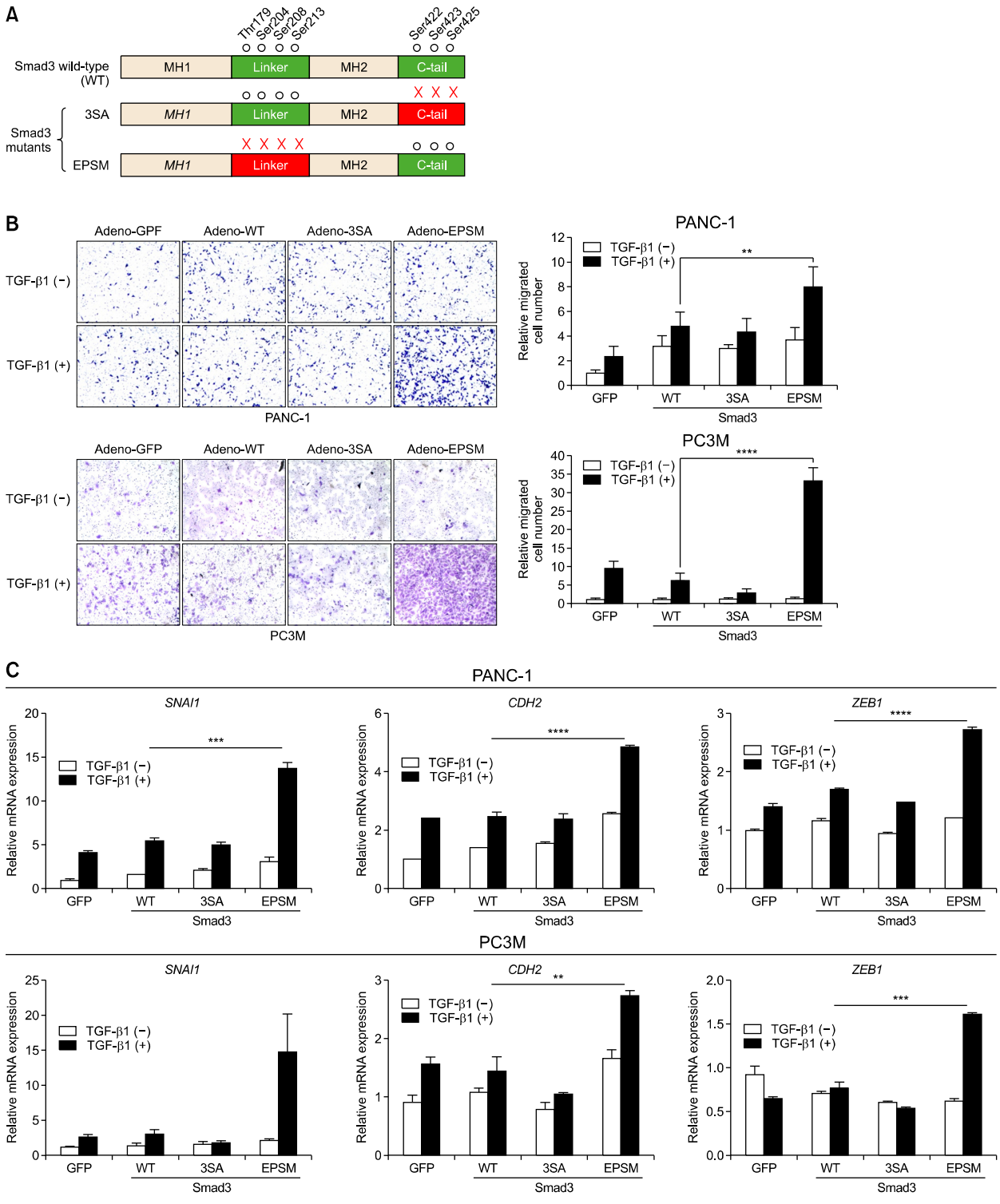

Figure 2. Smad3 linker phosphorylation mutation amplifies TGF-β1-induced epithelial-mesenchymal transition (EMT) in PC3M and PANC-1. (A) Schematic representation of phosphorylation site of linker region mutation and C-tail mutation of Smad3. (B) The migration of PANC-1 and PC3M overexpressing Smad3 either wild-type (WT) or mutants in the presence or absence of TGF-β1. (C) Quantitative PCR analysis to examine the mRNA expression of EMT markers, such as *CDH2*, *SNAI1*, and *ZEB1* in PANC-1 and PC3M cells. These symbols, ****, ***, and **, indicate $P < 0.0001$, $P < 0.001$, and $P < 0.01$. EPSM, Erk/Pro-directed kinase site mutant constructs.

tumor cells. As shown in Figure 1, we observed that TGF-β1 enhanced the migration ability of both PANC-1 and SNU2543 human pancreatic cancer cells, and PC3M and DU145 human prostate cancer cells. Pre-treatment of these cell lines with TEW-7197, a type I TGF-β receptor kinase inhibitor, suppressed TGF-β1-induced migration of the human pancreatic and prostate cancer cells (Fig. 1A). In addition, TGF-β1 significantly increased the invasion ability of the pancreatic and prostate cancer cells through the collagen membrane, while TEW-7197 significantly reduced TGF-β1-mediated cell invasion (Fig. 1B).

Next, we examined the effect of TEW-7197 on the TGF-β1-mediated regulation of EMT marker genes in PANC-1 and PC3M cells. The expression of the epithelial phenotype marker E-cadherin was reduced and the expression of the mesenchymal phenotype marker N-cadherin was increased by TGF-β1 treatment in PANC-1 cells. TEW-7197 reversed the effect of TGF-β1-mediated EMT in PANC-1 cells. Interestingly, TEW-7197 enhanced basal expression of E-cadherin in PANC-1 cells even in the

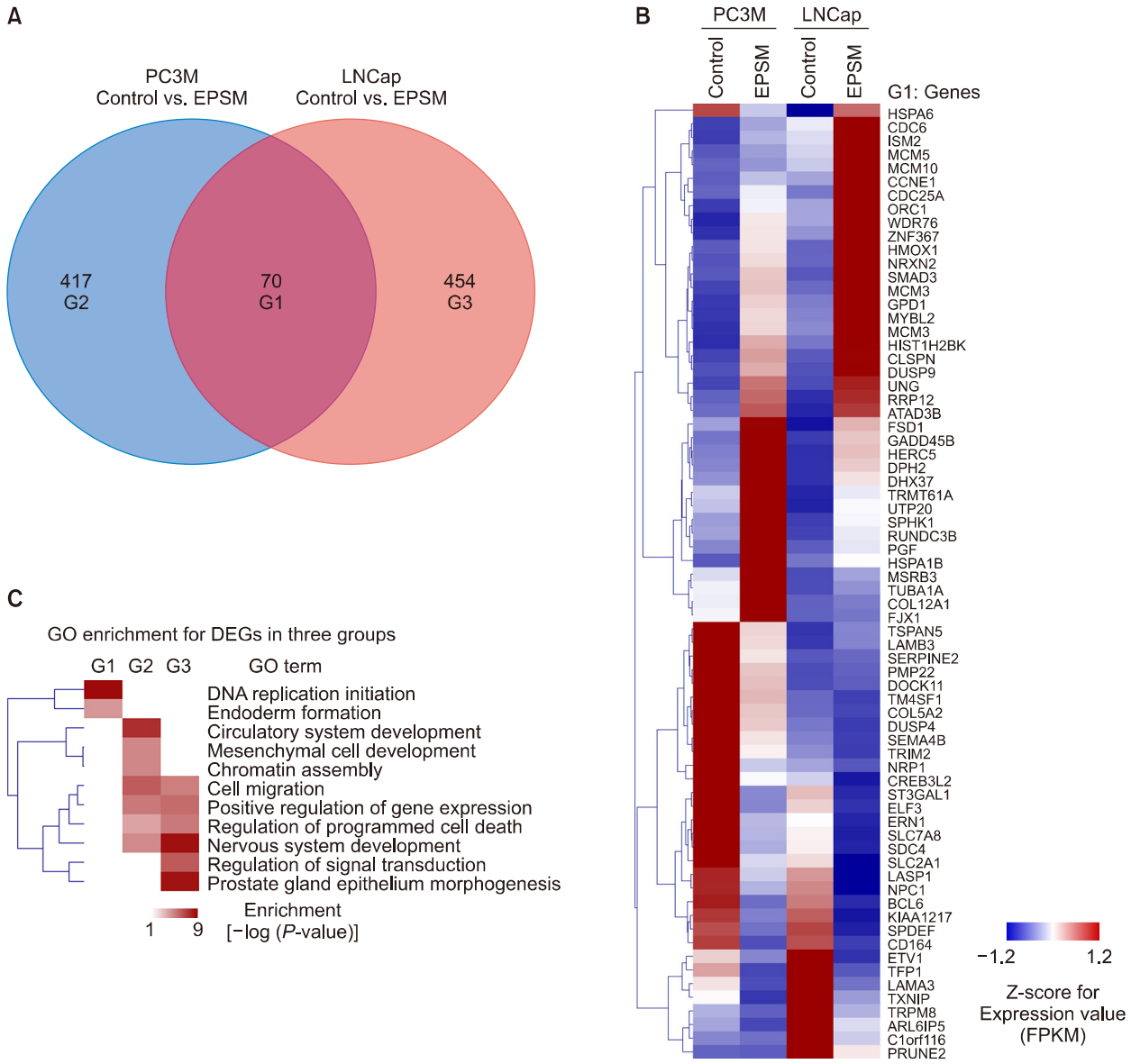

Figure 3. Gene expression profile in PC3M and LNCap after Erk/Pro-directed kinase site mutant constructs (EPSM) treatment. (A) Overlap of differentially expressed genes (DEGs) between control and EPSM in PC3M and LNCap. (B) Hierarchical clustering expression heatmap for 70 overlapping genes. Expression values of genes (i.e., FPKM) were converted to Z-score, and hierarchical clustering for selected genes was performed using Euclidean distance and complete linkage method. (C) Gene ontology (GO) enrichment for DEGs in three groups. Functional categories for DEGs were analyzed by Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov) with a cutoff of $P < 0.001$.

absence of TGF-β1 treatment. We also found that the expression of mesenchymal phenotype markers, fibronectin, and vimentin, was increased upon TGF-β1 treatment and pre-treatment of TEW-7197 effectively suppressed their expressions in PC3M cells (Fig. 1C).

These data suggest that TGF-β1 induces cell migration, invasion and EMT process in pancreatic and prostate cancer cells.

2. Mutations of Smad3 linker phosphorylation sites intensify TGF-β1 mediated epithelial-mesenchymal transition process

Smad3 is an essential mediator in the TGF-β1 signaling pathway. Smad3 consists of MH1 and MH2 domains and a divergent linker region (Fig. 2A). In the previous report, we have shown that expression of Smad-EPSM markedly increased metastasis in breast cancer. To examine whether Smad3-EPSM has similar effects on other cancer cells, we infected control adenovirus (Adeno-green fluorescent protein [GFP]), wild-type Smad3 adenovirus (Adeno-Smad3), C-tail mutant adenovirus (Adeno-3SA), and linker phosphorylation site mutant adenovirus (Adeno-EPSM) in PANC-1 and PC3M cells. Consistent with previous findings, infection of Adeno-EPSM into these cell lines strongly induced cell migration activity compared to the Adeno-GFP upon TGF-β1 treatment. However, Adeno-3SA showed no difference compared to the Adeno-GFP in cell migration upon TGF-β1 treatment in both PANC-1 and PC3M cells (Fig. 2B). Next, we examined the expression of EMT markers such as *SNAI1*, *CHD2*, and *ZEB1* at the transcript level by qRT-PCR. The expression of these genes was increased to a far greater extent in Adeno-EPSM-infected cells as compared to Adeno-Smad3- and Adeno-GFP-infected counterparts (Fig. 2C). These data suggest that the mutation of Smad3 linker phosphorylation sites enhances TGF-β1-induced cell migration and invasiveness in pancreatic and prostate cancer cells.

3. Identification of genes regulated by the mutation of Smad3 phosphorylation sites

To identify genes regulated by the mutation of Smad3 linker phosphorylation sites, we performed RNA sequencing analysis. We used highly metastatic, TGF-β1-responsive PC3M human prostate cancer cell line and the non-metastatic LNCap human prostate cancer cell line which doesn't respond to TGF-β1 due to the deletion of TGF-β1 receptor. We examined the DEGs regulated by Adeno-EPSM compared to the Adeno-GFP (Supplementary Table S1). Based on gene expression quantification with RNA sequencing analysis, we identified 487 and 524 DEGs between control and Smad3-EPSM with the cutoff of $P < 0.05$ and $\geq$ 1.5-fold change of PC3M, and LNCap cells, respectively (Fig. 3A and Supplementary Fig. S1). In the DE analysis, the ratio between up- and down-regulated genes in control versus EPSM

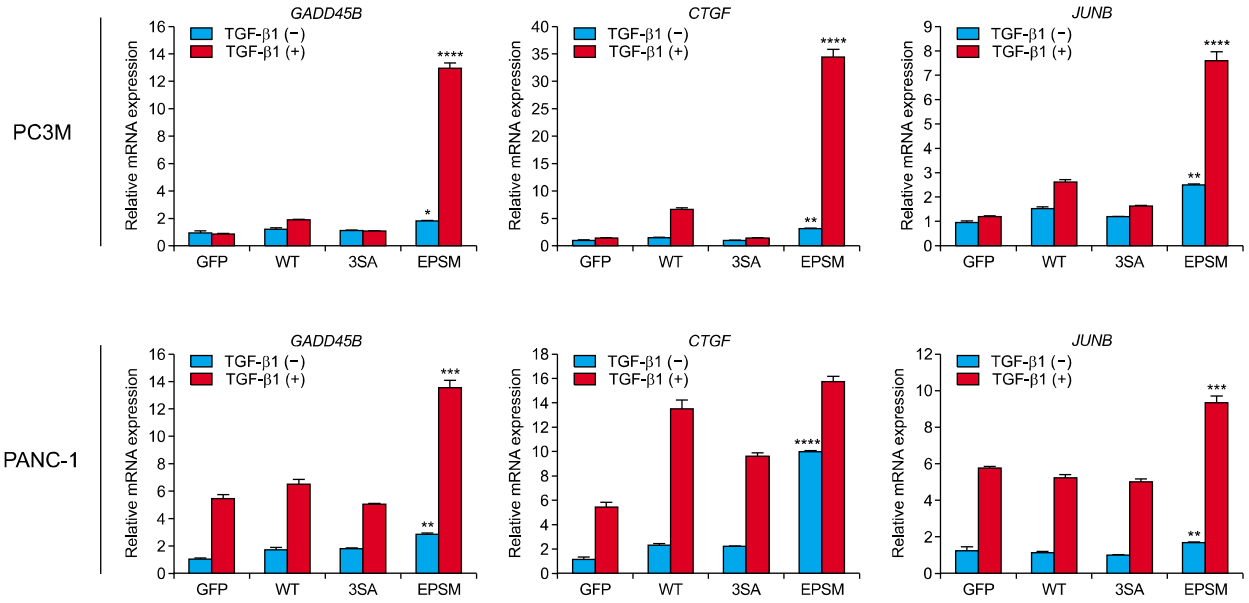

Figure 4. Validation of candidates which is responsible for Smad3-Erk/Pro-directed kinase site mutant constructs (EPSM) induced epithelial-mesenchymal transition. Validation of the gene expressions of *GADD45B, CTGF,* and *JUNB* induced by overexpression of Smad3 either wild-type (WT) or mutant in the presence or absence of TGF-β1. These symbols, ****, ***, **, and *, indicate $P < 0.0001$, $P < 0.001$, $P < 0.01$, and $P < 0.05$. GFP, green fluorescent protein.

was 1.06 and 0.84 for PC3M and LNCap, respectively (Supplementary Fig. S1). About 70 genes were regulated by Smad3-EPSM in both cell lines (Fig. 3A and 3B). In particular, those genes showed high similarity in expression changes. However, the distinction of expression level among samples with four clusters was observed (Fig. 3B). These findings indicate that each cell line operates the distinct TGF-β transcriptional machinery. The GO enrichment analysis revealed unique functions of three groups (Fig. 3C). Common genes between PC3M and LNCap were significantly associated with DNA replication initiation ($P = 2.02 \times 10^{-9}$) and endoderm formation ($P = 5.77 \times 10^{-5}$). Moreover, the molecular event of mesenchymal cell development and cell migration also regulated by Smad3-EPSM (Fig. 3C).

4. Validation of candidates which is responsible for Smad3-Erk/Pro-directed kinase site mutant constructs-induced epithelial-mesenchymal transition

Infection of Adeno-EPSM significantly induced migration in highly metastatic, TGF-β1 responsive PC3M human prostate cancer cell line. We extracted 487 genes (DEGs) regulated by the infection of Adeno-EPSM compared to the Adeno-GFP as the control (Fig. 3 and Supplementary Table S1). Among up-regulated genes, the expression of *GADD45B*, *CTGF*, *JUNB*, *HOXA9*, *HSPA1A*, *HSPA1B*, and *MLLT10* genes were most significantly up-regulated by Adeno-EPSM in PC3M cells (Supplementary Fig. S2). Since most of these genes are known to be involved in cell migration and cancer metastasis, we examined the expression of these genes by qRT-PCR in both metastatic PC3M prostate cancer cells as well as the metastatic PANC-1 pancreatic cancer cell after infection of Adeno-GFP, Adeno-Smad3, Adeno-3SA, or Adeno-EPSM with or without TGF-β1 treatment. Interestingly, the expression of *GADD45B*, *CTGF*, and *JUNB* genes was induced by Adeno-EPSM in both PC3M and PACN-1 cells (Fig. 4). TGF-β1 treatment further induced expression of these genes, suggesting that inhibition of Smad3 linker phosphorylation sites may enhance cell migration and invasion through induction of expression of metastatic genes, such as *GADD45B*, *CTGF*, and *JUNB* in pancreatic and prostate cancer cells.

DISCUSSION

Although it is a potent growth suppressor in cultured epithelial cells, TGF-β is abundantly expressed in cancer cells, and high levels of TGF-β often forecast malignant progression and poor prognosis.[6] A deeper understanding of TGF-β signaling pathway, therefore, becomes critical for controlling aggressive cancers. Signaling of TGF-β is mediated through transcription factors, Smad2 and Smad3. Smad3 has been shown to be a principal mediator of TGF-β-induced transcriptional responses.[14,15]

Smad3 has two Mad-homology domains (MH1 and MH2) and linker region. Phosphorylation of Smad3 on C-tail is essential for downstream signal pathways.[10] Threonine (T) and serine (S), T179, S204, S208, and S213, are phosphorylated by MAP kinases, CDK members, and glycogen synthase kinase 3 (GSK3) and other kinases.[9,10] Smad3 linker and C-tail are differentially activated and exhibit distinct roles in cell context-dependent and cell phenotype-specific manners.[16]

Overexpression of the mutant of the Smad3 linker phosphorylation sites enhances the ability of TGF-β-induced migration, invasion, EMT, growth inhibition, and apoptosis in breast cancer and upregulates the expression of cyclin-dependent kinase inhibitors ($p15^{INK4B}$ and $p21^{WAF1}$) in melanoma cells and $Smad3^{-/-}$ mouse embryonic fibroblasts indicating that abrogation of Smad3 linker phosphorylation intensifies TGF-β-driven transcriptional activities via Smad3.[8,10,11] Here, we demonstrate that overexpression Smad3 linker mutant enhances the migration, invasion, and EMT in pancreatic and prostate cancer cells in concordance with the previous study using breast cancer cell lines.[11]

TGF-β signaling is genetically inactivated in pancreatic cancer.[17] Mutations of TβRI, TβRII, Smad2, and Smad4 genes have the essential role in pancreatic cancer progression. Especially, lost 18q21 chromosome where *Smad4* is located is observed about 60% of pancreatic cancer. Inactivation of Smad4 is specific in pancreatic cancer, relatively.[17,18] PANC-1 pancreatic cancer cells used in this study reportedly carry mutations of KRAS and TP53 together with the homozygous deletion of CDKN2A.[19] A recent report by others demonstrated that inhibition of CDK4/6 with its specific inhibitor substantially suppresses tumorigenic activity in the same pancreatic cancer cell line while intensifying the TGF-β-induced EMT and invasiveness, which is completely suppressed by the treatment with a TβRI kinase inhibitor.[20] These results are in good agreement with the present findings that inhibition of the Smad3 linker phosphorylation by EPSM accelerates the TGF-β-induced EMT and invasion, which is totally blocked by the Smad3 C-tail mutant (3SA).

In the current study, we attempted to identify differentially regulated genes induced by Smad3-EPSM in both highly metastatic TGF-β1-responsive PC3M cells and non-metastatic TGF-β1-low-to-non-responsive LNCap cells. RNA sequencing data in this study disclosed that 70 genes were commonly overlapped in gene arrays both in PC3M and LNCap cells expressing EPSM. However,

levels and patterns of gene expression were quite distinct between two cancer cell lines, probably due to differences in their responsiveness to TGF-β and acquisition of cell type-specific oncogenes.[8,16,21] Analysis of DEGs regulated by Adeno-EPSM in highly metastatic PC3M prostate cancer cell line identified 7 genes (*GADD45B*, *CTGF*, *JUNB*, *HOXA9*, *HSPA1A*, *HSPA1B*, and *MLLT10*) which were most significantly up-regulated by Adeno-EPSM in PC3M cells. Among 7 genes, *GADD45B*, *CTGF*, and *JUNB* were also induced by Adeno-EPSM in PANC-1 pancreatic cancer cells. The significant role of *CTGF* in the induction of EMT in epithelial neoplasms including breast tumor, and head and neck cancers has been well acknowledged.[22-24] Expression of *JUNB* gene induced by TGF-β or other kinases has likewise been known to enhance the EMT in a vast majority of epithelial cells either cancerous or normal. Additionally, induction of *JUNB* by *PDK1* reportedly increased cell proliferation, invasion, and metastasis in gallbladder cancer.[25] Furthermore, the deletion of *JUNB* has been shown to abrogate cell migration, invasion, and distant metastasis of head and neck squamous cell carcinoma.[26] The role of *GADD45B* in EMT is not still unclear. Further study will be performed to understand the molecular mechanism of *GADD45B* in EMT process.

In summary, we have found that *GADD45B, CTGF*, and *JUNB* are commonly induced by Smad3-EPSM in both metastatic PC3M prostate cancer cells and metastatic PANC1 pancreatic cancer cells. It implies that these genes may contribute to the TGF-β1-induced cell migration, invasion, and distant metastasis of various cancer cells and they may have a stronger association with aggressive phenotypes of cancer cells such as the EMT and high invasive activity than those selected from non-EPSM-expressing cancer cells. Accordingly, pathways activated by the *GADD45B*, *CTGF*, and *JUNB* genes may be preventive or therapeutic targets against cancer metastasis.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2018.23.1.11.

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