SCF ^{β -TrCP1} Controls Smad4 Protein Stability in Pancreatic Cancer Cells

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Smad4, also known as deleted in pancreatic carcinoma locus 4 (DPC4), is a critical co-factor in signal transduction pathways activated by transforming growth factor (TGF)- β -related ligands that regulate cell growth and differentiation. Mutations in Smad4/DPC4 have been identified in ~50% of pancreatic adenocarcinomas. Here we report that SCF ^{β -TrCP1}, a ubiquitin (E3) ligase, is a critical determinant for Smad4 protein degradation in pancreatic cancer cells. We found that F-box protein β -TrCP1 in this E3 ligase interacted with Smad4 and that SCF ^{β -TrCP1} inhibited TGF- β biological activity in pancreatic cancer cells by decreasing Smad4 stability. Very low Smad4 protein levels in human pancreatic ductal adenocarcinoma cells were observed by immunohistochemistry. By analyzing pancreatic tumor-derived Smad4 mutants, we found that most point-mutated Smad4 proteins, except those within or very close to a mutation cluster region, exhibited higher interaction affinity with β -TrCP1 and significantly elevated protein ubiquitination by SCF ^{β -TrCP1}. Furthermore, AsPC-1 and Caco-2, two cancer cell lines harboring Smad4 point mutations, exhibited rapid Smad4 protein degradation due to the effect of SCF ^{β -TrCP1}. Both Smad4 levels and TGF- β signaling were elevated by retrovirus-delivered β -TrCP1 siRNA in pancreatic cancer cells. Therefore, inhibition of Smad4-specific E3 ligase might be a target for therapeutic intervention in pancreatic cancer. (Am J Pathol 2005; 166:1379–1392)

Human pancreatic cancer is one of the most deadly of all malignancies,¹ with a death:incidence ratio of ~0.99.² The mechanisms responsible for the loss of growth regulation in pancreatic adenocarcinoma cells remain primarily unknown. It is known, however, that pancreatic

cancers exhibit numerous alterations such as overexpression of mitogenic growth factors and their receptors,^{3,4} as well as gene mutations such as those of the proto-oncogene k-ras⁵ and the tumor suppressor genes p53⁶ and p16.⁷

Loss of sensitivity to transforming growth factor (TGF)- β -dependent signaling, one of the major signaling pathways regulating proliferation, differentiation, and death of various cell types, is thought to be an important contributing factor in tumor development.^{8,9} Smad4, also known as DPC4 (deleted in pancreatic carcinoma locus 4), is a key downstream determinant in TGF- β signaling.¹⁰ Smad4/DPC4 was originally isolated from human chromosome 18q21.1 as a tumor suppressor gene for pancreatic cancer.¹¹ Mutations in Smad4/DPC4 have been identified in ~50% of pancreatic adenocarcinomas but only in ~10% or less of other cancers,^{11,12} suggesting a possible pivotal role of Smad4 in TGF- β functional loss in pancreatic tumorigenesis. In fact, defects in Smad4 play a significant role in the malignant progression of tumors. Tumors lacking functional Smad4 tend to be more invasive and angiogenic, and consequently, are more likely to form metastatic lesions.¹³ Among patients undergoing surgical resection of pancreatic adenocarcinoma, survival is significantly longer for those patients whose tumors expressed Smad4 protein.¹⁴

The mechanisms underlying the Smad4 inactivation caused by mutations in human pancreatic ductal adenocarcinomas are not fully understood. Although some missense mutations may result in loss of a specific function of the Smad4 protein,^{15,16} it is possible that some missense point mutations represent only chance variants that do not affect protein function. Recently, some mutations in Smad4 have been shown to target the protein for rapid degradation via the ubiquitin-proteasome pathway,^{17–19} indicating that protein instability of Smad4 may contribute to the loss in cellular responsiveness to TGF- β in tumors. Studies of the mechanisms by which Smad4 is

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degraded in pancreatic carcinoma should be instructive for further understanding of the role of Smad4 in human neoplasia, and may also help in development of effective therapeutic intervention strategies for patients with pancreatic cancer.

The ubiquitin proteasome pathway controls degradation of the majority of regulatory proteins in mammalian cells,^{20,21} and E3 ubiquitin protein ligases (E3 ligases) determine substrate specificity and the timing of ubiquitination for given substrates. SCF (Skp1-Cullin1-F-box protein) E3 ligases recognize specific protein substrates through their variable F-box proteins and confer their ubiquitination.²² F-box proteins classified as Fbw, Fbl, and Fbx,^{23,24} contain two essential modular domains: the F-box that is required for binding to Skp1 and a protein-protein interaction domain for binding distinct substrates. The β -transducin repeat-containing protein (β -TrCP), members of the Fbw subfamily of F-box proteins, are known to recognize the phosphorylated DSG(X)_{2+n}S motif within their substrates, which include the I κ B,^{25–36} β -catenin,^{26,30,31–33} ATF4,³⁴ Emi1,^{35,36} p100 nuclear factor (NF)- κ B B2,³⁷ NF- κ B p105,³⁸ Dlg,³⁹ IFNAR1,⁴⁰ Cdc25A,^{41,42} Wee1,⁴³ prolactin receptor,⁴⁴ and Snail⁴⁵ proteins. Therefore, SCF $^{\beta$ -TrCP E3 ligases ubiquitinate and regulate the stability of specific protein substrates, and play a pivotal role in the regulation of cell division and various signal transduction pathways that are essential for many aspects of tumorigenesis.

Previously, we have identified Smad4 as a novel substrate for the SCF $^{\beta$ -TrCP1 E3 ligase. We have demonstrated that F-box protein β -TrCP1 in this E3 ligase interacts with Smad4 and SCF $^{\beta$ -TrCP1 contributes to the protein ubiquitination and degradation of Smad4 protein.⁴⁶ In this study, we sought to investigate whether very low Smad4 protein stability, due to rapid proteasome degradation, is a common phenomenon in pancreatic ductal adenocarcinomas, and whether the SCF $^{\beta$ -TrCP E3 ligase is a critical determinant for both Smad4 stability and TGF- β activity control in pancreatic cancers. Here, we report that F-box protein β -TrCP1 in SCF E3 ligase interacts with Smad4 in pancreatic cancer cells and SCF $^{\beta$ -TrCP1 inhibits TGF- β biological activity in pancreatic cancer cells by decreasing Smad4 stability. Smad4 protein level is significantly lower in human pancreatic ductal adenocarcinoma cells compared to adjacent ductal cells with a chronic pancreatitis morphology. By systematically examining the protein stability of Smad4 mutants found in pancreatic tumors, we found most of the Smad4 mutants exhibited significant decreased protein stability and had higher interaction affinity with β -TrCP1. Using siRNA-induced F-box protein β -TrCP1 gene silencing, the protein steady-state level of Smad4 was elevated, and TGF- β signaling was rescued in pancreatic cancer cells.

Materials and Methods

siRNA Constructs and Antibodies

GFP RNAi and β -TrCP1 RNAi plasmids were generated by using BS/U6 vector.⁴⁷ Briefly, a 22-nucleotide oligo

(oligo 1) corresponding to nucleotides 106 to 127 of GFP or nucleotides 453 to 474 of the human β -TrCP1 coding region was first inserted into the BS/U6 vector digested with *Apal* (blunted) and *HindIII*. The inverted motif that contains the six-nucleotide spacer and five Ts (oligo 2) was then subcloned into the *HindIII* and *EcoRI* sites of the intermediate plasmid to generate BS/U6/GFP and BS/U6/ β -TrCP1. For cloning into retrovectors, the U6 promoter region plus the siRNA cassette was digested with *XbaI* and cloned into the *XbaI* site of a retrovirus vector Δ U3. The wild-type (WT) Smad4 expression plasmid was amplified by polymerase chain reaction (PCR) using pRK5-Smad4 as the template and subcloned into the *SalI* and *BamHI* sites of the pCMV5B vector with a Flag tag at the amino terminus. Tumor-derived mutation sequences were introduced within the Smad4 coding region of Flag-tagged WT Smad4 by site-directed mutagenesis. Monoclonal antibody recognizing human Smad4 and rabbit antisera against β -TrCP1, Skp2, and ubiquitin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against Flag and Myc were from Sigma (St. Louis, MO). Monoclonal antibody to HA was from CRP Inc. (Denver, PA).

Real-Time Reverse Transcriptase-PCR

Total RNA was isolated from cultured cells and tissues using the RNeasy mini kit (Qiagen Inc., Chatsworth, CA). One μ g of total RNA was used for the synthesis of first strand cDNA using the Superscript preamplification system (Life Technologies, Rockville, MD). Quantitation of Smad4 and GAPDH with specific primers was performed using a DNA Engine Opticon continuous fluorescence detection system (MJ Research, Logan, UT) with SYBR Green I as the method of detection. Details of the method are as previously described.⁴⁸ Briefly, quantitative PCR was performed in a total reaction volume of 20 μ l per capillary for the LightCycler format. This reaction mix contained 10 μ l of a SYBR Green mix, 0.5 to 10 pmol of each forward and reverse primer, 2 μ l of cDNA, and nuclease-free water to make up the reaction volume. Runs were performed in duplicate and mean values were subsequently used for analysis. To ensure unbiased analysis, real-time quantitative PCR was performed blindly and the identity of the samples was only revealed after mRNA measurements had been made. Primers used were as follows: Smad4: forward, 5'-TGGCCTGATCTTCACAAAAA-3' and reverse, 5'-TCACAGTGTATCCTGAGAGAT-3'; GAPDH: forward, 5'-TAAAGGGC-ATCCTGGGCTACACT-3' and reverse, 5'-TTACTCCTTGAGGCCATGTAGG-3'.

Immunoprecipitation and Western Blotting Analysis

Immunoprecipitation and Western blotting analysis of cell lysates were performed as described previously.⁴⁶ All blots were developed by the enhanced chemiluminescence technique (Amersham, Little Chalfont, UK). The density of the bands was quantitated using Amersham

Pharmacia Biotech Storm System and image analysis software.

Protein Stability Assays

To measure the rate of degradation of Smad4 and Smad4 mutants, Flag-tagged versions were transfected into 293T cells that were treated with 80 $\mu\text{g/ml}$ of cycloheximide 48 hours after transfection to prevent further protein synthesis. Whole cell extracts were prepared from samples taken at different time points, and the amounts of the Smad4s were determined by Western blotting using the anti-Flag antibody.

In Vivo Ubiquitination Assays

In vivo ubiquitination assays using cell lysates were conducted as described previously.⁴⁶

Luciferase Assays and Statistical Analysis

Luciferase activities were assayed using the Dual-Luciferase assay kit (Promega, Madison, WI) according to the manufacturer's directions. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments. Statistical significance was determined by two-tailed Student's *t*-test ($P < 0.05$) or analysis of variance ($P < 0.05$) when indicated.

Cell Proliferation and Cell-Cycle Analysis

Cell proliferation was assayed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO] method.⁴⁹ Briefly, after transfection, 50 μl of MTT solution (5 mg/ml) was added to the culture medium. After 4 hours at 37°C the medium was removed and 50 μl of acidified isopropanol was added to each well. The color was allowed to develop for 5 minutes and optical density at 570 nm was determined with a microplate reader (Bio-Rad, Richmond, CA). Cell cycle was analyzed by flow cytometry. Briefly, cells were harvested, fixed with 100% cold ethanol, stained with 50 $\mu\text{g/ml}$ of propidium iodide (Sigma), and subjected to flow cytometric analysis by using a FACScan instrument (Becton Dickinson, Mansfield, MA). Approximately 10,000 events (cells) were evaluated for each sample. Two independent experiments were performed, and one is presented.

Clinicopathological Parameters of Patients

Thirty-five randomly selected pancreatic adenocarcinoma tissue samples from resected samples were retrieved from the surgical pathology files of the Department of Pathology at the University of Alabama at Birmingham. Tissue samples were obtained with informed consent and institutional review board approval. Patient identifiers were removed from the samples and reports in compliance with Health Insurance Portability

and Accountability Act (HIPAA) legislation. The group included 21 male and 14 female patients. The age range of the patients was 60 to 81 years. All resected samples also had adjacent chronic pancreatitis, which served for comparing the frequency and pattern of SMAD4 distribution with pancreatic adenocarcinoma.

Immunohistochemistry of Pancreatic Tumor Tissue

Formalin-fixed tissue sections of 5 μm thickness were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was achieved by incubating tissue sections in boiling 10 mmol/L citrate buffer (pH 6.0) for 5 minutes in a microwave oven. All sections were then incubated with hydrogen peroxide for 5 minutes. Subsequently, sections were incubated with the primary monoclonal antibody against Smad4 (catalog no. sc-7966, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. After rinsing the primary antibody in T-PBS, antibody detection was accomplished using the Super Sensitive biotin-streptavidin horseradish peroxidase detection kit (Biogenex, San Ramon, CA). The diaminobenzidine tetrachloride Super Sensitive substrate kit (Biogenex, San Ramon, CA) was used to visualize the antibody-antigen complex. The sections were then counterstained with hematoxylin. Appropriate negative controls, consisting of tissue sections of each case processed without the addition of primary antibody, were prepared along with positive multitissue control sections.

Evaluation of the Immunohistochemical Results and Statistical Analysis

Only those cases with greater than 10% immunohistochemical stains were considered as positive. Assessment of the immunohistochemical staining was performed independently by two pathologists. Contrasting results were discussed until an agreement was reached. Statistical analysis to assess the differences of Smad4 expression level between pancreatic ductal adenocarcinoma and adjacent chronic pancreatitis tissue was performed using the χ^2 test.

Results

SCF ^{β -TrCP1} Inhibited TGF- β Function in Pancreatic Cancer Cells because of Decreasing Smad4 Stability

Previously, we found that an E3 ligase complex SCF ^{β -TrCP1} is responsible for Smad4 degradation. F-box protein β -TrCP1 in this complex associates with Smad4 both in yeast and in mammalian cells, and SCF ^{β -TrCP1}-overexpressing cells display increased ubiquitination and degradation of Smad4.⁴⁶ To further determine the biological role of SCF ^{β -TrCP1} in controlling Smad4 protein

stability in pancreatic cancer cells, we examined the interaction of endogenous Smad4 with β -TrCP1 in PANC-1 cells using immunoprecipitation assays. Figure 1A shows that endogenous Smad4 in PANC-1 cells co-immunoprecipitated with β -TrCP1 by using antibody specific against β -TrCP1 (lane 2), but not co-immunoprecipi-

tated with Skp2 (lane 3), another well-characterized F-box protein of SCF E3 ligase. Fbw subfamily consists of many F-box proteins besides β -TrCP1.^{23,24} To investigate whether the interaction of β -TrCP1 and Smad4 is specific, we detected the association of other F-box proteins with Smad4. Unlike β -TrCP1 (Figure 1B, lane 2; Figure 1C, lane 2), ectopically expressed Flag- β -TrCP2 (Figure 1B, lane 3), Flag-Fbw2 (Figure 1B, lane 4), HA-Fbw3 (Figure 1C, lane 3), or HA-Fbw5 (Figure 1C, lane 4) has either undetectable or very weak interaction with Smad4. This indicates that β -TrCP1 is the specific F-box protein that recognizes Smad4.

To determine whether SCF $^{\beta$ -TrCP1-induced Smad4 degradation affects TGF- β -mediated transcriptional activity, we co-transfected a TGF- β -responsive luciferase reporter (SBE), 4-luc (four repeats of Smad-binding element) with SCF components (Cul1, Roc1, and β -TrCP1) in PANC-1 cells. TGF- β induces approximately threefold of luciferase activity, and SCF complex down-regulates the transcription activity (Figure 2A). To clarify the effect of SCF $^{\beta$ -TrCP1 on TGF- β biological functional activity in pancreatic cancer cells, cell proliferation was investigated. SCF components, Cul1, Roc1, β -TrCP1, were transfected in PANC-1 cells with or without TGF- β treatment. TGF- β inhibited cell proliferation, and SCF restored such TGF- β -induced cell proliferation (Figure 2B). The distribution of the cell population in the G₁ and S phases of the cell cycle was analyzed by flow cytometry after expression of SCF $^{\beta$ -TrCP1 in PANC-1 cells. As shown in Figure 2C, left, cell growth was arrested by TGF- β treatment, as judged by the decreased number of cells in S phase and the concomitant increase of cells in the G₁ phase. SCF inhibited the TGF- β -induced cell growth arrest. Smad4 protein levels were significantly reduced in these SCF component transfected cells (Figure 2C, right), suggesting that SCF $^{\beta$ -TrCP1 inhibits TGF- β biological activity by decreasing Smad4 protein level.

Low Smad4 Protein Level in Human Pancreatic Ductal Adenocarcinoma

To investigate whether Smad4 protein instability is a common phenomenon in human pancreatic tumor cells, immunohistochemistry was conducted in 35 cases of pan-

Figure 1. Specific interaction of Smad4 with β -TrCP1 in pancreatic cancer cells. **A:** Cell lysates of PANC-1 cells were immunoprecipitated using preimmune (lane 1), anti- β -TrCP1 (lane 2), or anti-Skp2 (lane 3) antibody, and the immunocomplex was detected by Western blotting using anti-Smad4 (top), anti-Skp2 (middle), and anti- β -TrCP1 (middle) antibodies. The expression level of Smad4 in cell lysates was also detected as shown at the bottom. **B:** PANC-1 cells were transfected with the indicated plasmids. F-box proteins were immunoprecipitated from the cell extracts using anti-Flag antibody, and the immunocomplex was detected by Western blotting with antibodies specific for HA (top) and Flag (middle) antibody to detect Smad4 and F-box proteins, respectively. The expression level of HA-Smad4 in cell extracts was also detected by Western blotting with antibody specific for HA. **C:** PANC-1 cells were transfected with the indicated plasmids. F-box proteins were immunoprecipitated from the cell extracts using anti-HA antibody, and the immunocomplex was detected by Western blotting with antibodies specific for Flag (top) and HA (middle) antibody to detect Smad4 and F-box proteins, respectively. The expression level of Flag-tagged Smad4 were also detected by Western blotting with antibody specific for Flag (bottom) antibody.

Figure 2. SCF β -TrCP1 inhibited TGF- β function in pancreatic cancer cells due to decreasing Smad4 stability. **A:** SCF β -TrCP1 inhibits TGF- β -induced gene transactivation in pancreatic cancer cells. SBE-lux luciferase reporters were co-transfected with the indicated plasmids into PANC-1 cells in 24-well plates. TGF- β (2 ng/ml) was added in some experiments. Thirty-six hours after transfection, the cells were lysed and the luciferase signal was detected. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments. **B:** SCF β -TrCP1 prevents cell proliferation inhibited by TGF- β . PANC-1 cells were transfected with indicated doses of SCF components in 96-well plates, and treated with 5 ng/ml of TGF- β 1 for 48 hours. Cell proliferation was measured by the MTT assays as described in the Materials and Methods. **C:** SCF β -TrCP1 releases cells from TGF- β -mediated growth arrest. PANC-1 cells were transfected with 5 μ g of β -TrCP1, 1 μ g of Cul1, and 1 μ g of Roc1 in 60-mm plates, and treated with 5 ng/ml of TGF- β 1 for 48 hours. Cells were stained with propidium iodide and cell-cycle arrest was analyzed by flow cytometry (**left**). The expressions of Smad4 and transfected protein were also detected by Western blotting (**right**).

creatic ductal adenocarcinoma. In all sections, chronic pancreatic ductal cells could be seen adjacent to carcinoma cells. The percentage of cells positive of Smad4

Table 1. The Distribution of Smad4 Protein Expression in Pancreatic Carcinoma and Chronic Pancreatitis Ductal Cells from 35 Pancreatic Adenocarcinoma Patients

	Pancreatic carcinoma	Chronic pancreatitis	Significance (χ^2)
Smad4+	5	31	$P < 0.001$
Smad4-	30	4	
Total	35	35	

protein staining was evaluated. Only those cases with greater than 10% immunohistochemical stains were considered as positive. The case distribution positive of Smad4 protein expression in the 35 sections with either chronic pancreatitis or pancreatic adenocarcinoma is demonstrated in Table 1. Of 35 patients, 30 (85.7%) were evaluated as Smad4-negative and the rest 5 (14.3%) were positive in carcinoma cells. In contrast, 31 (88.6%) were evaluated as Smad4-positive and the rest 4 (11.4%) were negative in adjacent chronic pancreatitis ductal cells. Cytoplasmic and occasional nuclear immunoreactivity was noted in all of the cases with positive staining of Smad4 (Figure 3). Smad4 was strongly expressed in relative normal or chronic pancreatitis ductal cells (Figure 3C), however, Smad4 was either undetectable or very weak in most of the pancreatic ductal adenocarcinomas (Figure 3D).

Pancreatic Tumor-Derived Point Mutations of Smad4 Cause Protein Instability through Ubiquitin-Proteasome Pathway

It has been demonstrated that some mutations in Smad4 have been shown to target the proteins for rapid degradation via the ubiquitin-proteasome pathway,¹⁷⁻¹⁹ indicating that protein instability of Smad4 may contribute to its protein loss in pancreatic cancers. To investigate this possibility, seven Flag-tagged Smad4 expression constructs harboring Smad4 mutations previously identified from pancreatic tumor were generated (Table 2). These mutated (MT) and wild-type (WT) Flag-tagged Smad4 expression plasmids were transfected individually into 293T cells and cycloheximide was added after transfection. Both point mutations (m100 and m130) that lie on MH1 domain cause much quicker Smad4 protein degradation than the WT (Figure 4A, top). The degradation rate of the three mutants (m351, m355, and m493), whose mutations reside on MH2 domain, also exhibit much quicker degradation than the WT (Figure 4A, middle). The results indicate that rapid protein degradation is the main contributor leading to protein loss of most Smad4-harboring point mutations. However, the degradation rate of Smad4 m370 and m383, whose mutations are also within MH2 domain, has no obvious differences compared with the WT (Figure 4A, bottom), which imply that rapid protein degradation may not be the primary defect for these two mutations.

We next determined whether rapid protein degradation of human pancreatic tumor-derived Smad4 mutations is

Figure 3. Immunohistochemistry of human pancreatic adenocarcinoma sections. **A:** H&E staining of a section from pancreatic tissue adjacent to pancreatic ductal carcinoma demonstrates lack of acinar cells, increased fibrosis, and proliferation of reactive ductal epithelial cells. **B:** H&E staining of section from moderately differentiated pancreatic adenocarcinoma shows neoplastic glands with filtrating pattern and marked cytological atypia including increased cell pleomorphism, irregular nuclear membrane, prominent nucleoli, and occasional mitoses. **C:** Immunohistochemical staining of the reactive ductal epithelial cells in the same area of **A** shows positive cytoplasmic immunoreactivity for Smad4 protein. **D:** Immunohistochemical staining of the carcinoma cells in the same area of **B** shows no immunohistochemical expression of Smad4. Original magnifications: $\times 200$ (**A**, **B**); $\times 400$ (**C**, **D**).

via the proteasome pathway. MG-132, a proteasome inhibitor,⁵⁰ was added to the cells. MG-132 significantly elevated the level of Smad4 protein in most point-mutated Smad4-transfected cells, including m100, m130, m351, m355, and m493 (Figure 4B), but not in m370, m383, and WT Smad4 transfected cells, indicating that the changes of Smad4 protein steady-state levels in most point-mutated Smad4-transfected cells were due to protein degradation via the 26S proteasome. We also examined whether the enhanced turnover observed in Smad4 mutants is mediated through ubiquitination. Individual MT or WT Smad4 expression plasmids were co-transfected with or without ubiquitin expression plasmid in 293T cells. As shown in Figure 4C, WT Smad4 exhibits very little ubiquitination with or without ubiquitin. Stronger ladders of high molecular weight, ubiquitin-conjugated Smad4

products were observed in most of the MT Smad4-transfected cells. Consistent with this, ubiquitination of Smad4 m370 and m383, although much higher than WT Smad4, has no comparable difference between with or without ubiquitin addition. Taken together, these results suggest that most of Smad4-harboring tumor-derived point mutations, except m370 and m383, are degraded more rapidly through ubiquitin-proteasome pathway when compared to their WT counterparts.

Instability of Point-Mutated Smad4 Proteins Mediated by SCF ^{β -TrCP1}

The low protein stability of point-mutated Smad4 raises the question of whether point-mutated Smad4 proteins

Table 2. Seven Pancreatic Tumor-Derived Smad4 Point Mutations

Sample	Domain	Exon	Codon	Mutation	Predicted effect	Reference
Cell line (ASPC1)	MH1	2	100	AGG $\rightarrow$ ACG	R $\rightarrow$ T	Cancer Res 1996, 56:2527–2530
Xenograft	MH1	2	130	CCA $\rightarrow$ TCA	P $\rightarrow$ S	Cancer Res 1998, 58:4592–4597
Cell line (MZPC-2)	MH2	8	351	GAT $\rightarrow$ CAT	N $\rightarrow$ H	Biochem Biophys Acta 2000, 283–296
Cell line (PC)	MH2	8	355	GAC $\rightarrow$ GGC	N $\rightarrow$ G	Virchows Arch 2001, 439:798–802
Xenograft	MH2	8	370	GTC $\rightarrow$ CAC	V $\rightarrow$ H	Cancer Res 1998, 58:4592–4597
Cell line (SK-PC-3)	MH2	9	383	ATA $\rightarrow$ AAA	I $\rightarrow$ K	Biochem Biophys Acta 2000, 283–296
Xenograft	MH2	11	493	GAT $\rightarrow$ CAT	N $\rightarrow$ H	Science 1996, 271:350–353

Figure 4. Pancreatic tumor-derived point mutations of Smad4 cause protein instability through ubiquitin-proteasome pathway. **A:** Protein degradation rate of point-mutated Smad4 increased compared with WT Smad4. 293T cells were co-transfected with Flag-tagged Smad4 or individual Smad4 mutants. After 48 hours, cycloheximide (80 μ g/ml) was added to the culture to prevent any further Smad synthesis. Whole cell extracts were prepared at 0, 4, 8, 12, and 16 hours and assayed by Western blotting with an anti-Flag antibody to detect Smad4 expression level. The intensity of the bands was quantitated by phosphorimaging and plotted relative to the amount present at time 0. **B:** Proteasome inhibitor MG132 enhances the expression levels of Smad4 mutants. 293T cells were transfected with the indicated expression plasmids and incubated with or without the proteasome inhibitor, MG132 (20 μ mol/L), for 4 hours. Extracts were assayed by Western blotting with Flag antibody to detect Smad4 (top) or β -actin antibody (bottom). **C:** Higher ubiquitination of MT Smad4 than WT Smad4 protein. 293T cells were transfected with the indicated plasmids and MG132 (20 μ mol/L) was added 4 hours before cell lysis. Smad4 protein was immunoprecipitated from the cell lysates with Flag antiserum, and then reprecipitated before immunoblotting with anti-ubiquitin to detect the polyubiquitinated Smad4.

Figure 5. Instability of point-mutated Smad4 proteins mediated by SCF^{β-TrCP1}. **A:** Interaction of point-mutated Smad4 proteins with β-TrCP1. 293T cells were transfected with the indicated plasmids. Immunoprecipitation assays were performed using anti-β-TrCP1 antibody and the immunocomplex was detected by Western blotting using anti-Flag antibody to detect Smad4. The expression levels of Flag and β-TrCP1 proteins in cells were also detected, as indicated at the bottom. **B:** The interaction affinity was normalized by quantitation of the band density and calculation of the density ratio between the band in first panel of **A** with the corresponding band in the second panel of **A**. The intensity of the bands was quantitated by phosphorimaging and plotted relative to the amount present at time 0. **C:** Enhanced protein ubiquitination of Smad4 mutants by SCF^{β-TrCP1}. 293T cells were transfected with the indicated plasmids. Smad4 were immunoprecipitated from the cell lysates with Flag antiserum, and then reprecipitated before immunoblotting with anti-ubiquitin to detect the polyubiquitinated Smad4. **D:** β-TrCP1 siRNA elevated protein level of pancreatic cancer-derived Smad4 mutants. BS/U6 GFP (siGFP, irrelevant siRNA control) or BS/U6/β-TrCP1 (siβ-TrCP1) plasmid as indicated were co-transfected into 293T cells with individual Flag-tagged Smad4 mutants. Cells were harvested 3 days later and the extracts were assayed by Western blotting with antibodies specific for Flag (top), β-TrCP1 (middle), or β-actin (bottom).

Figure 6. Instability of endogenous Smad4 protein in pancreatic cancer cells harboring Smad4 point mutation. **A:** Smad4 protein expression in different human pancreatic cancer cell lines. Western blotting of cell lysates from PANC-1, ASPC-1, and Caco-2 cells with antibodies specific for Smad4 (top) or β -actin (bottom). **B:** Smad4 mRNA expression in different human pancreatic cancer cell lines. reverse transcriptase-PCR of total RNA isolated from BxPC-3, PANC-1, ASPC-1, and Caco-2 using primers specific for Smad4 (top) and GAPDH (bottom). **C:** Degradation rate of Smad4 protein increased in ASPC-1 and Caco-2 cells. Cycloheximide (80 μ g/ml) was added to the culture of PANC-1 (top), ASPC-1 (bottom), or Caco-2 cells. Whole cell extracts were prepared at 0, 2, 4, 8, and 16 hours and assayed by Western blotting with an anti-Smad4 antibody. **D:** Proteasome inhibitor enhances the Smad4 expression level in human pancreatic cancer cell lines harboring Smad4 point mutation. PANC-1, ASPC-1, and Caco-2 cells were incubated with or without the proteasome inhibitor, MG132 (20 μ mol/L), for 4 hours as indicated. Extracts were assayed by Western blotting with antibodies against Smad4 (top) or β -actin (bottom).

interact with β -TrCP1. The interactions of mutated Smad4 with β -TrCP1 were detected by immunoprecipitation assays with Flag-tagged WT or MT Smad4 individually transfected 293T cells. The results indicated that β -TrCP1 interacted with WT and almost all of the Smad4 mutants (Figure 5A). Because the expression level of Smad4 in WT and MT are quite different, the density of the immunoprecipitated bands was quantitated. The interaction affinity of β -TrCP1 with most of Smad4 mutants, except m370 (Figure 5B, lane 7), was much enhanced in comparison with the WT Smad4 (lane 2). These results indicate that the interaction of β -TrCP1 with these Smad4 mutants was enhanced. Consistent with the above observation, ubiquitination of most of the MT Smad4, except m370, by the SCF complex is stronger than the WT Smad4 in *in vivo* ubiquitination assays (Figure 5C), suggesting that tumor-derived Smad4 mutants exhibit a stronger interaction with its F-box protein β -TrCP1, thereby leading to their rapid degradation.

Since we demonstrated that the use of RNAi was successfully inhibiting the expression of β -TrCP1 and enhancing the expression of endogenous Smad4,⁴⁶ we asked whether this RNAi functioned to increase the stability of tumor-derived Smad4 mutants. si β -TrCP1 decreased the expression of β -TrCP1 (Figure 5D, middle), and the expression of most of the Smad4 mutants is elevated (Figure 5D, top). We did not demonstrate obvious enhancement of Smad4 expression level in lanes 10 (m370) and 12 (m383), which is consistent with the above results and indicate that the protein stability of Smad4 m370 and m383 is relatively high and may not degrade through SCF $^{\beta$ -TrCP1 pathway. These results suggest that β -TrCP1 is a key factor in mediating the instability of most tumor-derived Smad4 mutants.

Figure 7. SCF $^{\beta$ -TrCP1 controls Smad4 protein stability in pancreatic cancer cell line harboring Smad4 point mutation. **A:** Interaction of endogenous Smad4 with β -TrCP1 in pancreatic cancer cell lines. Cell lysates of PANC-1, ASPC-1, and Caco-2 cells were immunoprecipitated using preimmune or anti- β -TrCP1 antibody and the immunocomplex was detected by Western blotting using anti-Smad4 antibody. The expression levels of Smad4 and β -TrCP1 proteins in cells were also detected, as indicated at the bottom. **B:** Suppression of β -TrCP1 by retroviral delivery of siRNA into pancreatic cancer cells increases endogenous Smad4 expression. PANC-1 (lanes 1 and 2), ASPC-1 (lanes 3 and 4), and Caco-2 (lanes 5 to 7) cells were infected with virus containing siGFP (irrelevant siRNA control), si β -TrCP1, or Smad4 (positive Smad4 expression control) as indicated. Three days after infection, cells were harvested and expression levels of β -TrCP1, Smad4, and β -actin were measured by Western blot analysis.

SCF^{β-TrCP1} Controls Smad4 Protein Stability in Cancer Cell Line Harboring Smad4 Point Mutation

We then examined whether SCF^{β-TrCP1} is required for endogenous Smad4 protein rapid degradation in pancreatic cancer cells harboring Smad4 point mutation. PANC-1 cells has WT Smad4, whereas, AsPC-1 (m100) and Caco-2 (m351) cells harbor Smad4 point mutation.^{12,51} Figure 6A shows that PANC-1 expresses high level of Smad4 protein (Figure 6A, left and right panels, lane 1), and both AsPC-1 (Figure 6A, left, lane 2), and Caco-2 (Figure 6A, right, lane 2) expresses very low level of Smad4 protein. To clarify whether the low Smad4 protein expression in pancreatic cancer cell lines harboring Smad4 point mutation was the result of low Smad4 mRNA level, we evaluated the mRNA expression of Smad4 in cells by real-time reverse transcriptase-PCR. No significant differences in Smad4 mRNA expression were noted among PANC-1, AsPC-1, and Caco-2 cells (Figure 6B). A Smad4-deficient cell line, BxPC-3, exhibits no Smad4 mRNA expression as a negative control.

Smad4 protein degradation rate was then determined in pancreatic cancer cell lines. Cycloheximide was added to PANC-1, AsPC-1, and Caco-2 cultures to prevent further Smad protein synthesis. The level of endogenous Smad4 m100 rapidly decreased after the addition of cycloheximide and almost disappeared at 8 hours (Figure 6C, middle). In contrast, WT Smad4 was considerably more stable and was still present 16 hours after cycloheximide addition (Figure 6C, top). The level of Smad4 m351 has a relatively lower degradation rate, however, degrades faster in comparison with the WT (Figure 6C, bottom versus top). We next determined whether rapid protein degradation of this mutated Smad4 protein is via the proteasome pathway. MG-132 was added in PANC-1, AsPC-1, and Caco-2 cells. Consistent with Figure 6A, the expression level of Smad4 in AsPC-1 and Caco-2 was much lower than that in PANC-1 (Figure 6D, left and right sides, lane 1 versus lane 3). MG-132 increased the level of Smad4 protein in both AsPC-1 (Figure 6D, left and right sides, lane 2 versus lane 1). These results suggested that endogenous Smad4 m100 and m351 degrades rapidly in AsPC-1 and Caco-2 cells via proteasome pathway.

To determine whether SCF^{β-TrCP1} is the key determinant for Smad4 protein rapid degradation in AsPC-1 and Caco-2 cells, we examined the interaction of endogenous Smad4 with β-TrCP1 in PANC-1, AsPC-1, and Caco-2

Figure 8. Suppression of β-TrCP1 by retroviral delivery of siRNA into pancreatic cancer cells elevated TGF-β-mediated gene transcription. SBE-luc, MBE-luc, p15P114-luc, and p15Smad mutation-luc luciferase reporters were transfected into PANC-1 (A), AsPC-1 (B), and Caco-2 (C) cells that were infected with virus containing siGFP or siβ-TrCP1 as indicated in 24-well plates. TGF-β (2 ng/ml) was added in some experiments. Thirty-six hours after transfection, the cells were lysed and the luciferase signal was detected. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments. Relative increased fold of the luciferase activity stimulated by TGF-β in each cell line was calculated. Statistical significance of the fold from siβ-TrCP1 group versus siGFP group was determined by two-tailed Student's *t*-test. * *P* < 0.05.

cells. Interaction was observed in all three cell lines. The middle panel in both sides of Figure 7A shows that the expression level of Smad4 in PANC-1 cells is much higher than that in AsPC-1 and Caco-2 cells, whereas the interaction of Smad4 with β -TrCP1 in AsPC-1 (Figure 7A, left side, top) and Caco-2 (Figure 7A, right side, top) cells is stronger than that in PANC-1 cells.

We attempted to investigate whether β -TrCP1 siRNA could elevate Smad4 level in pancreatic cancer cells. Because the efficiency of transient transfection of siRNA into pancreatic cancer cells is very low, we generated retrovirus constructs that contain Δ U/U6 empty vector, Δ U/U6/GFP (siGFP) and Δ U/U6/ β -TrCP1 (si β -TrCP1). High infection efficiencies of the siRNA in PANC-1, AsPC-1, and Caco-2 cells were yielded (data not shown). Consistent with the results in 293T cells, β -TrCP1 siRNA efficiently reduced the expression of β -TrCP1 (Figure 7B, top), and the expression of Smad4 is elevated in all of the three cell lines (Figure 7B, middle).

We then examined whether β -TrCP1 silencing affects Smad4-mediated transcriptional activity in pancreatic cancer cells. TGF- β response reporter plasmids, (SBE)4-luc⁵² and p15P114luc⁵³ were transfected in PANC-1, AsPC-1, and Caco-2 cells infected by retrovirus containing GFP siRNA or β -TrCP1 siRNA. TGF- β significantly stimulates luciferase activity in PANC-1 cells (Figure 8A) and has a mild effect in AsPC-1 (Figure 8B) and Caco-2 (Figure 8C). β -TrCP1 siRNA significantly increased TGF- β -induced transcriptional activity in both PANC-1 cells (Figure 8A, $P < 0.05$) and Caco-2 cells (Figure 8C, $P < 0.05$). However, the elevation of TGF- β -induced transcriptional activity mediated by β -TrCP1 siRNA has no statistical significance in AsPC-1 cells, indicating that Smad4 m100 has some functional defect in transducing TGF- β activity. To determine whether the function of β -TrCP1 siRNA on TGF- β response is through elevating Smad4 amount and DNA binding, two additional luciferase reporters containing Smad-binding site mutations, MBE6-luc⁵² and p15SBE mutant-luc,⁵³ were transfected in PANC-1, AsPC-1, and Caco-2 cells infected by retrovirus containing GFP siRNA or β -TrCP1 siRNA. β -TrCP1 siRNA no longer increased TGF- β -induced transcriptional activity in these cells (Figure 8; A to C). Taken together, the results indicated that β -TrCP1 is a key molecule mediating Smad4 degradation and TGF- β functional loss in pancreatic cancer cells.

Discussion

Loss of Smad4 is associated with poor prognosis of pancreatic cancer. Smad4 is also a critical intercellular transducer of TGF- β signals. In the present work, we provide evidence that the instability of Smad4 protein may play a role in Smad4 functional loss and TGF- β resistance in pancreatic cancer cells. TGF- β causes a G₁ arrest of cell-cycle progression by inducing expression of the cyclin-dependent kinase inhibitors p15^{INK4b} and p21^{CIP/WAF1} in pancreatic cancer cells.^{54,55} Our data demonstrate that SCF ^{β -TrCP1} impairs TGF- β -induced gene transcription and cell-cycle arrest in pancreatic

cancer cells. Accordingly, Smad4 protein level decreased in the cells. In addition, SCF ^{β -TrCP1} mainly regulates the cell cycle in TGF- β -treated cells, it has little effect on the cell-cycle progression in those cells without TGF- β treatment, indicating that the effects of SCF ^{β -TrCP1} are Smad-dependent. On the other hand, our data also shown that SCF ^{β -TrCP1} does not completely restore TGF- β -inhibited cell proliferation and cell-cycle progression, suggesting that Smad4-independent effect may also be involved in TGF- β -mediated cell responses. Recent Smad4 RNA interference⁵⁶ and gene microarray⁵⁷ studies demonstrated that some cell-cycle regulation signals, including p21/WAF1, were activated by TGF- β in a Smad4-independent manner in pancreatic cancer cells, providing evidence for our observation.

Our data give us new insight into the mechanism by which point-mutated Smad4 proteins undergo rapid degradation in tumor cells. We found strong association between SCF ^{β -TrCP1} and most mutated Smad4 proteins, and the ubiquitination and degradation of these mutated Smad4 proteins are significantly higher than that of WT Smad4. Consequently, TGF- β -mediated biological function in pancreatic cancer cells has been disrupted. This observation provides a partial explanation for why and how human tumors frequently lose TGF- β responsiveness. Further studies will be needed to define the detailed mechanisms by which the interaction of β -TrCP1 with Smad4 is enhanced by the point mutations. It has been demonstrated that SCF complex containing β -TrCP as the F-box protein (SCF ^{β -TrCP}) recognizes phosphorylated substrates and mediates their protein degradation.²⁵⁻⁴⁵ One possibility is that some point-mutated Smad4 might be phosphorylated easily by certain protein-associated kinases, thereby making it much easier for β -TrCP1 to recognize Smad4.

Our study shows that most, but not all, of the Smad4 point mutations affect their protein stability. The protein stability of Smad4 m370 and m383 is relatively high and rapid protein degradation may not be the primary defect for these two mutations. Smad4 is a multifunctional protein that contains a DNA-binding domain (mad homology domain 1, MH1), a protein-interaction domain (mad homology domain 2, MH2), and a linker region.¹⁰ A mutational hot spot within the MH2 domain corresponding to codons 330 to 370 was termed the mutation cluster region (MCR) by a recent work.⁵⁸ The immunohistochemical studies indicated that the majority of missense mutations inactivate Smad4 by protein degradation, whereas carcinomas with missense mutations within the MCR retain Smad4 protein stability. The MCR, corresponding to the loop/helix structure of the MH2 domain, possesses its functional significance proposed from structural studies. The fact that the mutations in Smad4 m370 and m383 reside either right within or very close to MCR might be the reason why the proteins cannot be degraded by the proteasome. Another possibility is that some mutations, such as m370, do not affect the interactions with β -TrCP1, their protein stability may not be degraded very fast. It is interesting to examine whether other mutations within MCR would affect their recognition by F-box protein β -TrCP1. Further characterization of the mutations

whose levels of expression in human cancers are already known will also help to better understand the protein degradation mechanisms of Smad4 mutants.

Functional inactivation caused by some Smad4 mutations cannot be excluded in tumorigenesis in some circumstances. Especially, some mutations in the MH2 domain, independent of its DNA-binding activity, have been shown to prevent homo- and hetero-oligomerization with other Smads.^{59,60} It has also reported that C-terminal deletion of Smad4 was reported to have dominant-negative activity.⁶¹ Our luciferase assays from AsPC-1 cells demonstrated that TGF- β -induced gene transcription was not significantly elevated by si β -TrCP1 even though the protein level of Smad4 m100 increased. These imply that Smad4 m100 must have some functional defect in transducing TGF- β signal besides its protein instability. Previously studies demonstrated that this mutation does not affect the function of its oligomerization with Smad3 and keeps its ability to translocate into nuclear translocation,¹⁷ but loses its DNA-binding ability.⁶² Therefore, the mild effect of si β -TrCP1 on this cell line is possibly because of the elevation of Smad3 protein nuclease translocation by Smad4 m100 stabilization. However, one should realize that the role of Smad4 in cells does not only reside in its capability to directly bind to DNA and mediate TGF- β growth inhibitory responses. Smad4 was reported to serve as a transcriptional co-factor of other DNA-binding proteins such as Hox proteins^{63,64} and estrogen receptor α .⁶⁵ In these cases, Smad4 instability could also be involved in other types of tumors. The future understanding of Smad4 instability caused by Smad4 mutations may require detailed analysis of these function of Smad4 other than its DNA-binding and TGF- β signal-transducing activity.

Cellular responses are highly sensitive to the level of Smad protein.⁶⁶ Taken together of our series studies on protein degradation of point-mutated Smad4 and the luciferase results from Caco-2 cells, our data indicate that protein instability, not loss of a specific function, is the primary consequence of the majority of the point mutations in pancreatic cancers. Consistent with other studies on MCR,⁵⁸ our data also imply that the missense mutations within or very close to a distinct MCR may affect some of their functions but permit continued Smad4 stable expression. More importantly, previous studies suggest that ~50% of pancreatic cancers have either homozygous deletion (30%) or intra-genic Smad4 mutation (20%).^{11,12} Our human pancreatic adenocarcinoma tissue immunohistochemistry data demonstrated that of 35 patients, 30 (85.7%) were evaluated as Smad4-negative and the rest ($n = 5$; 14.3%) were positive in carcinoma cells. These results indicate that Smad4 protein instability is a common phenomenon in pancreatic carcinoma cells even though Smad4 has no mutations. Therefore, inhibiting the Smad4/SCF β -TrCP1 association might be a promising approach for increasing Smad4 stability in pancreatic tumors. Our data also demonstrate that using siRNA triggered β -TrCP1 gene-specific silencing significantly increases the stability of Smad4 protein and restores TGF- β signaling in human pancreatic cancer

cell lines. Further characterization of the structure and function of Smad4/SCF β -TrCP1 interaction might lead to the discovery of new targets for anti-cancer drug discovery.

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