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Proteomic analysis of the TGF-β signaling pathway in pancreatic carcinoma cells using stable RNA interference to silence Smad4 expression☆

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Abstract

Smad4 is a tumor-suppressor gene that is lost or mutated in 50% of pancreatic carcinomas. Smad4 is also an intracellular transmitter of transforming growth factor-β (TGF-β) signals. Although its tumor-suppressor function is presumed to reside in its capacity to mediate TGF-β-induced growth inhibition, there seems to be a Smad4-independent TGF-β signaling pathway. Here, we succeeded in establishing Smad4 knockdown (S4KD) pancreatic cancer cell lines using stable RNA interference. Smad4 protein expression and TGF-β-Smad4 signaling were impaired in S4KD cells, and we compared the proteomic changes with TGF-β stimulation using two-dimensional gel electrophoresis (2-DE) and mass spectrometry. We identified five proteins that were up-regulated and seven proteins that were down-regulated; 10 of them were novel targets for TGF-β. These proteins function in processes such as cytoskeletal regulation, cell cycle, and oxidative stress. Introducing siRNA-mediated gene silencing into proteomics revealed a novel TGF-β signal pathway that did not involve Smad4.

Keywords: TGF-β; Smad4; Stable RNAi; 2-DE; Proteomics

Smad4, first identified as *deleted in pancreatic carcinoma*, *locus4* (*DPC4*), is a tumor-suppressor gene that is functionally inactivated in one-half of pancreatic carcinomas [1]. *Smad4* belongs to the *Smad* gene family, which encodes intracellular signaling mediators of the transforming growth factor-β (TGF-β) superfamily of cytokines. TGF-β cytokines signal via TGF-β type I and II receptors that phosphorylate receptor-interacting Smad proteins (R-Smads) on activation. Activated R-Smads form heteromeric complexes with Smad4, the common partner Smad (Co-Smad), and translocate into the nucleus, where they function as transcription factors.

Impairment of the Smad pathway results in escape from growth inhibition and promotes cell proliferation, thereby contributing to carcinogenesis. Double knockout mice containing an inactivated allele of the *adenomatous polyposis coli* (*APC*) gene and lacking the wild-type allele of the *Smad4* gene developed invasive adenocarcinoma in multiple intestinal polyps, while single *APC* knockout mice did not [2]. In addition, Smad4 re-expression was recently reported to suppress in vivo tumorigenicity via the restoration of TGF-β growth inhibition [3]; these

☆ *Abbreviations:* TGF-β, transforming growth factor-β; RNAi, RNA interference; KD, knockdown; WT, wild-type; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase.

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mechanisms are thought to support the tumor-suppressor function of Smad4. However, the acquisition of TGF-β resistance and loss of *Smad4* may be independent events [4,5], given that certain TGF-β transcriptional responses are retained in *Smad4*-deficient cells [6] and several *Smad4*-deficient tumor cell lines are still inhibited in growth by TGF-β [7]. Moreover, the TGF-β signal did not recover on rescuing the *Smad* gene in certain TGF-β-response-defective cell lines [8], suggesting that there are other molecules targeted by TGF-β that remain to be investigated.

To identify novel targets of the TGF-β signal, we introduced gene silencing mediated by RNA interference (RNAi) to knockdown *Smad4*. RNAi is an evolutionarily conserved mechanism of gene silencing that is thought to inhibit the replication and expression of selfish DNA elements and viruses [9]. Recently, RNAi has been applied to mammalian cells [10]; it is a powerful tool for analyzing endogenous gene silencing. Although the effect of RNAi introduced transiently into cells is limited because of low transfection efficiency, we discovered that the expression of RNAi from a plasmid vector enables long-term persistence of the silencing effect [10]. Here, we demonstrate the differential expression of proteomes of the human pancreatic carcinoma cell line PANC-1 using stable RNAi targeted to *Smad4* under the stimulation of TGF-β.

Materials and methods

Cell cultures. The human pancreatic ductal cancer cell line PANC-1 (CRL1468), in which the TGF-β-Smad4 signal is preserved [8], was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD). To prepare cell lysate treated with MAPK inhibitor, PANC-1 cells were pre-treated with 50 μM PD98059 (Calbiochem, San Diego, CA) for 1 h before TGF-β treatment.

Establishing Smad4-knocked down cell lines. Plasmids carrying RNAi targeted to *Smad4* were constructed as previously described [10]. Briefly, a 21-nucleotide sequence of the *Smad4* gene, which had no homology to genomic sequences in a BLAST search, was inserted in the sense and antisense directions into the pcPUR + U6i cassette vector containing the human U6 promoter. The targeting and control vectors were transfected into PANC-1 cells using Effectene Transfection Reagent (Qiagen, Hilden, Germany), and the puromycin-resistant clones were selected as stable transfectants. We called PANC-1 cells stably transfected with the plasmid expressing siRNA that targeted Smad4 or with the empty plasmid "PANC-1-S4KD" or "PANC-1-WT," respectively.

Antibodies. Anti-phosphorylated-ERK antibody, anti-ERK antibody, anti-phosphorylated-JNK antibody, anti-JNK antibody, anti-phosphorylated-p38 antibody, anti-p38 antibody, and anti-HSP27 antibody were obtained from Cell Signaling (Beverly, MA). Anti-phosphorylated-Smad2 antibody was obtained from Upstate (Charlottesville, VA). Anti-Smad4 antibody and anti-p21 antibody were from BD Biosciences (San Diego, CA). Anti-vimentin antibody and anti-14-3-3ζ antibody were obtained from Santa Cruz (Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma–Aldrich (St. Louis, MO).

Western blotting. Western blotting was performed as described [11]. Briefly, 35 μg of total cell lysate was electrophoressed and transferred to a PVDF membrane (Hybond-P, Amersham Biosciences, Uppsala, Sweden). The blot was blocked in 5% TBST and incubated with primary antibodies. After washing, the blot was incubated with horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescence detection system (ECL Advance, Amersham Biosciences).

Proteome analysis. S4KD cells were seeded at 8×10^5 cells/10-cm dish and, after 48 h, were treated with 10 ng/ml TGF-β 1 (R&D Systems, Minneapolis, MN) or control buffer (4 mM HCl and 1 mg/ml BSA) for 12 h. The soluble proteins were extracted using lysis buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris–HCl, pH 8.0. Then, 120 μg of the lysate was mixed with immobilized pH gradient (IPG) rehydration buffer containing 7 M urea, 2 M thiourea, 4% Chaps, 2% DTT, and 1% IPG buffer (Amersham Biosciences). The IPG strips (pH 3–10 NL or pH 4–7, 18 cm; Amersham Biosciences) were allowed to rehydrate overnight, and isoelectric focusing (IEF) was performed using IPG phor (Amersham Biosciences), as previously described [12]. After IEF, the first equilibration was performed in a buffer containing 50 mM Tris–HCl, pH 8.8, 6 M urea, 2.0% SDS, 30% glycerol, and 1% DTT for 15 min; the second equilibration was performed in the same buffer without DTT but with 4% iodoacetamide for 15 min. Equilibrated strips were placed on top of 18 × 20-cm, 12.5% acrylamide gels containing SDS. SDS–PAGE was performed for 12 h at 15 mA/gel. After electrophoresis, silver staining was performed according to a modified protocol [13].

Image analysis and data processing. The images of protein spots were scanned, and spot intensities were obtained using the software PDQuest (BioRad, Hercules, CA). After global normalization, we selected reproducible spots from among four replicate experiments using the spot intensity-dependent standard deviation (SD). Common spot numbers (SSP numbers) were assigned to identify unique protein spots over the four gels, and we calculated the mean and coefficient of variation (CV) of the spot intensities in four replicate experiments. The range of mean spot intensity was divided into 10 classes. For protein spots included in each class, the SD of the CV values was calculated. From these SD values, corrected SD (cSD) values were calculated by locally weighted regression using the LOWESS algorithm (www.rproject.org). In each class, protein spots varying by over 2× cSD were considered irreproducible and were eliminated. The mean intensities of the remaining spots were used in the following fold-change calculation. All analyses except LOWESS were performed using our custom Perl codes.

Protein identification. The protein spots were excised by hand and in-gel digestion was performed. Briefly, the spots were destained, trypsin (sequencing grade; Promega, Madison, WI) was added, and the gel pieces were incubated overnight. The tryptic peptides were extracted (50 μl of 50% acetonitrile/5% TFA), the supernatant was taken to dryness in a vacuum centrifuge, and the residue was redissolved in 0.1% TFA. The resulting peptide mixture was desalted using ZipTips C18 (Millipore, Bedford, MA) [14], eluted on a spot of a sample plate with matrix solution (10 mg/ml α-cyano-carboxycinnamic acid in 50% acetonitrile/0.1% TFA), and then analyzed using MALDI-TOF/MS (Voyager-DE STR, Applied Biosystems, Foster City, CA) or MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems). The spectra were initially calibrated using auto-proteolysis products of trypsin, and a search of the NCBInr sequence database using Mascot (www.matrixscience.com) was performed with a tolerance of 100 ppm. One miscut and oxidation of methionine or carbamidomethylation were allowed. The search results were evaluated by considering the probability score, peptide coverage, and correspondence of the estimated and experimental p*I* and molecular mass.

Quantitative real-time RT-PCR analysis. S4KD or WT cells were seeded and treated with TGF-β, as described above. After 2 h of stimulation with TGF-β, mRNA was extracted using ISOGEN

(Nippon Gene, Tokyo, Japan). One microgram of total RNA was converted into cDNA using the ImProm-II Reverse Transcription System (Promega). Real-time PCR primers (Qiagen, Valencia, CA) targeting human HSP27, 14-3-3ζ, vimentin, DPC1, and GAPDH were designed using Primer Express software (Applied Biosystems). The ABI Prism 7000 Sequence Detection System and SYBR Green Master Mix kit (both from Applied Biosystems) were used to detect real-time PCR products from reverse-transcribed cDNA samples.

Results

Establishing Smad4-knocked down pancreatic carcinoma cell lines

We established PANC-1 cells stably transfected with the plasmid expressing siRNA that targeted Smad4 or with the empty plasmid. Fig. 1A shows that introducing siRNA resulted in the loss of Smad4 protein in the PANC-1-S4KD clone and S4KD "cell pools," which were mixtures of polyclonal cells. To avoid deriving biased data due to the selection of a specific clone, we performed further analysis using "cell pools."

To exclude the possibility that the siRNA induced off-target effects, the expression of *deleted in pancreatic carcinoma, locus1* (*DPC1*) gene, which has a sequence similar to that of the targeted *Smad4* gene sequence, was examined using quantitative RT-PCR. S4KD and WT cell pools showed the same amount of DPC1 mRNA expression (data not shown), which revealed the Smad4-specific knockdown in S4KD cells. To confirm that the TGF-β-Smad4 signaling pathway was preserved in WT cells and impaired in S4KD cells, we performed Western blotting of phosphorylated Smad2 and p21/WAF1. p21/WAF1, an inducer of G1 cell cycle arrest, is a major downstream molecule of the TGF-β-Smad4 signaling pathway [15]. Fig. 1B shows TGF-β-induced phosphorylation of Smad2 in both S4KD and WT cells, whereas up-regulation of p21/WAF1 was markedly inhibited in S4KD cells. We also performed a luciferase assay using the Smad-dependent reporter $(CAGA)_9$-luc. In this assay, WT cells showed significant TGF-β-induced transcriptional activation, whereas the induction was dramatically reduced in S4KD cells (Amarsanaa et al., manuscript in preparation). These results indicate that TGF-β-Smad4 signaling was preserved in Smad4-intact cells and that inactivation of Smad4 impaired this signaling.

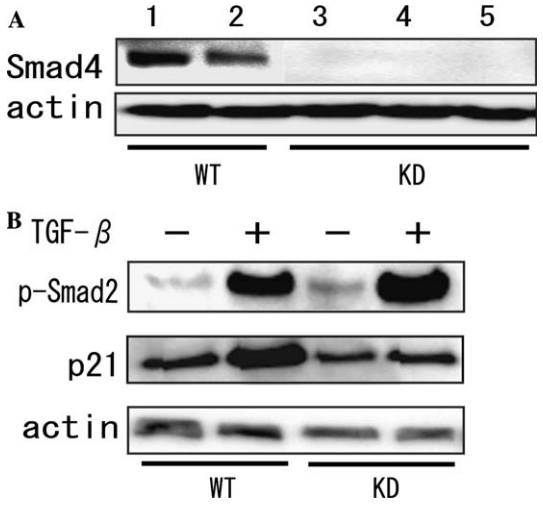

Fig. 1. (A) siRNA inhibits Smad4 protein expression in S4KD cells. Western blots from whole cell extracts (35 μg/lane) probed with anti-Smad4 antibody. Lane 1, WT pool; lane 2, WT clone No.1; lane 3, S4KD pool; lane 4, S4KD clone No.1; and lane 5, S4KD clone No.2. (B) Protein expression of phosphorylated-Smad2 (p-Smad2) and p21/WAF1 in S4KD and WT cells confirmed by Western blotting.

2-DE of PANC-1 cells

To investigate the changes in protein expression quantitatively, we investigated proteomes of S4KD cells. Cells were treated with TGF-β for 12 h and the cell lysate was resolved by 2-DE (Fig. 2A). Initially, 2-DE was performed using pH 3–10, IPG strips for the first dimension. After several repeated runs, we found that most of the differentially expressed proteins were included in the pH range from 4 to 7. To isolate these proteins more efficiently, we performed further experiments with pH 4–7, IPG strips for the first dimension. We analyzed more than 10 gels per experimental condition, and the four gels with the fewest artifacts were selected for statistical analysis. We detected 789 spots on silver-stained, 2-D gels reproducibly. Of these, 216 spots with variations greater that 2× cSD were considered irreproducible and were eliminated; consequently, 573 spots were selected for further analysis. Spots showing more than a 1.3-fold increase or 0.7-fold decrease after TGF-β stimulation were used for protein identification according to a previous study [16].

Of the spots investigated, five had increases of more than 1.3-fold, and 17 showed more than 0.7-fold decreases after TGF-β stimulation. These spots were first subjected to MALDI-TOF/MS. For the spots for which peptide mass fingerprinting (PMF) using MALDI-TOF/MS was unsuccessful, peptide sequence tagging was performed using MALDI-TOF/TOF. We identified all five spots that increased and 8 out of 17 spots that decreased after TGF-β stimulation. Table 1 lists the proteins identified, and Figs. 2B and C show the corresponding spots on the 2-D gel. The proteins identified are involved in cytoskeletal regulation (HSP27, vimentin, ARP3, and profilin-II), cell cycle regulation (14-3-3ζ, CSN8, and stathmin), cell proliferation (DJ-1 protein), nuclear transport (NTF2), and oxidative stress (peroxiredoxin3). Vimentin was identified from two

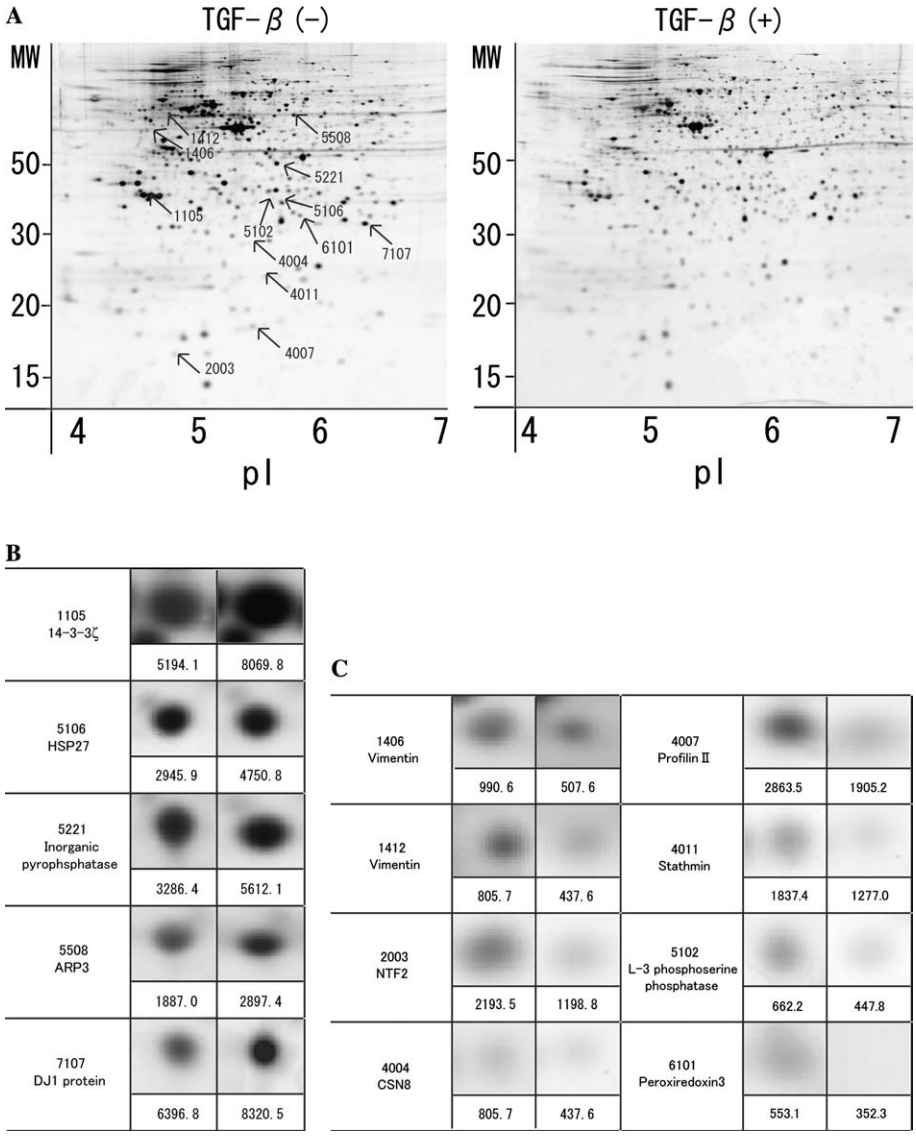

Fig. 2. (A) 2-D gel of S4KD cells. Aliquots (120 μg) of total lysate obtained from cells were resolved with a 2-D gel. The arrows indicate protein spots that showed TGF-β-induced alteration and were identified by MS. The pH gradient of the first dimension obtained using a marker for isoelectric points (p*I*) is shown at the bottom of the gel, and the migration of molecular weight (MW) markers for SDS–PAGE is shown to the left of the gel. The spots that increased or decreased after TGF-β stimulation are shown in (B and C), respectively.

spots, which suggests posttranslational modification. To confirm the expression of these proteins, Western blotting was performed to detect HSP27, vimentin, and 14-3-3ζ, for which antibodies are available (Fig. 3). The expression of each protein in WT cells was also confirmed using Western blots, and most of the changes were identical to those in S4KD cells (Fig. 3).

The quantitative changes in the transcripts of HSP27, 14-3-3ζ, and vimentin after TGF-β stimulation were investigated using real-time RT-PCR. In S4KD cells, the levels of HSP27, 14-3-3ζ, and vimentin mRNA increased 1.1-, 1.6-, and 1.5-fold, respectively, whereas in WT cells, they increased slightly by 1.0-, 1.1-, and 1.1-fold (data not shown). As previously described [17,18], our results show that changes in mRNA levels are not always correlated with changes in protein expression.

Quantitative changes of Smad4-independent targets treated with an ERK inhibitor

In addition to Smad-mediated transcription, TGF-β is reported to activate MAPKs in certain cells [4]. Therefore, we examined these pathways in S4KD and WT cells. As shown in Fig. 4A, TGF-β stimulation induced phosphorylation of extracellular signal-related kinase (ERK), p38 MAPK was slightly phosphorylated, and c-JUN *N*-terminal kinase (JNK) signaling was not detected in either cell. To investigate whether ERK signaling regulates the amount of protein, the cells were

Table 1
Proteins that showed altered experssion after TGF-β stimulation

Spot No.[a]	TGF-β (−)[b]	TGF-β (+)[b]	Fold change	Protein identified	NCBI ID No./ Accession No.	Instrument used[d]	Score[e]	Sequence coverage (%)	Estimated[e]		Experimental[a]	
									p*I*	M_r (kDa)	p*I*	M_r (kDa)
1406	990.6	507.8	0.511	Vimentin[c]	A25074	MS/MS	48	3	5.06	53.7	4.6	49
4004	805.7	437.6	0.543	CSN8	gi|5729979	MS/MS	63	10	5.25	23.2	5.4	23
1412	1045.0	568.7	0.544	Vimentin[c]	A25074	MS/MS	71	7	5.06	53.7	4.8	52
2003	2193.5	1198.8	0.547	NTF2	gi|5031985	MS/MS	39	11	5.10	14.5	4.9	13
6101	553.1	352.3	0.637	Peroxiredoxin3	AAH08435	MS/MS	64	14	7.11	28.1	5.9	25
4007	2863.5	1905.2	0.665	Profilin II	1D1JA	MS/MS	39	17	5.84	15.3	5.5	15
4011	1837.4	1277.0	0.695	Stathmin	P16949	MS/MS	56	8	5.76	17.3	5.5	19
5102	662.2	447.8	0.676	L-3-phosphoserine phosphatase	gi|4758972	MS/MS	64	11	5.53	25.0	5.6	26
7107	6396.8	8320.5	1.301	DJ1 protein	JC5394	MS/MS	70	13	6.33	20.1	6.3	25
5508	1887.0	2897.4	1.535	ARP3	gi|5031573	MS/MS	77	10	5.61	47.3	5.7	55
1105	5194.1	8069.8	1.55	14-3-3ζ	1QJBA	MS	199	71%	4.99	26.1	4.6	26
5106	2945.9	4750.8	1.61	HSP27	E980237	MS/MS	57	13	5.98	22.9	5.7	26
5221	3286.4	5612.1	1.71	Inorganic pyrophosphatase	AAF17222	MS/MS	57	11	5.54	33.2	5.6	36

[a] Spot No., experimental p*I*, and experimental M_r were obtained using PDQuest software.
[b] Quantitative changes in protein expression were expressed as ppm.
[c] Vimentin was identified from two spots (No. 1406 and No. 1412) on the 2-D gel.
[d] MS indicates MALDI-TOF/MS (Voyager-DE STR) and MS/MS indicates MALDI-TOF/TOF (4700 Proteomics Analyzer).
[e] Score, estimated p*I*, and estimated M_r were obtained from the Mascot search.

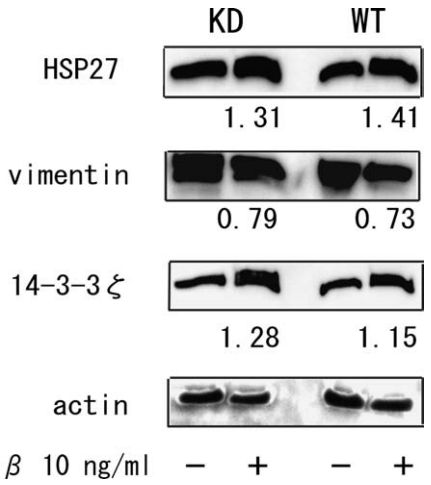

Fig. 3. Quantitative changes in protein expression confirmed by Western blots. S4KD or WT cells were incubated with or without 10 ng/ml TGF-β for 12 h. The numbers under the blots indicate the relative changes in protein levels based on densitometric analysis.

treated with TGF-β in the presence or absence of the selective ERK inhibitor PD98059. Pretreatment with PD98059 did not alter the amounts of the proteins investigated (Fig. 4B), suggesting that these molecules are not stimulated via either the Smad4 or MAPK pathways.

Discussion

This study combined a proteomic approach with RNAi technology to identify novel targets of TGF-β. RNAi shows tremendous promise as a tool for targeted gene silencing. Although recent reports suggest that RNAi induces off-target effects [19], the equal expression of the *DPC1* gene in S4KD and WT cells showed the specificity of the RNAi used. Recently, RNAi has been used to obtain a genome-wide view of the influence of specific genes using cDNA microarrays [20]. Indeed, cDNA microarrays are useful for screening mRNA expression, although there is conflicting evidence regarding the correlation between mRNA and protein abundance [17]. By introducing RNAi-mediated gene silencing into the analysis of protein expression, we can understand the global changes in protein expression associated with the loss of a specific gene function. Although a similar study was carried out using gene silencing mediated by antisense DNA [21], there might be a problem with its specificity and reproducibility.

Our study identified 12 molecules by MS as proteins involved in TGF-β signals. Interestingly, some of the identified proteins have known roles in TGF-β-signaling. In osteoblasts, HSP27 and vimentin are stimulated by TGF-β via a non-Smad pathway [22,23]. HSP27 is a

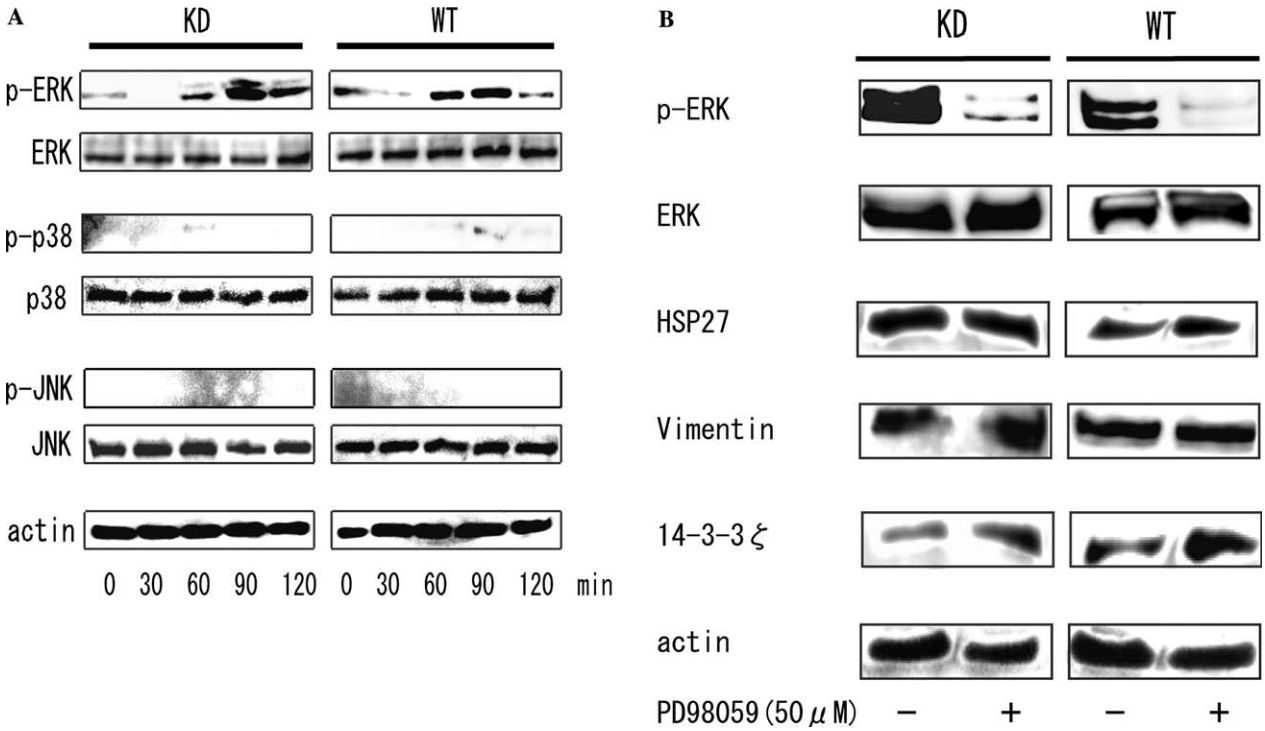

Fig. 4. (A) The effects of TGF-β on MAPKs. S4KD and WT cells were treated for the indicated time with 10 ng/ml TGF-β. The cell extracts were then immunoblotted to detect total and phosphorylated-ERK (p-ERK), JNK, and p38 MAPK. (B) The effects of an MEK inhibitor on TGF-β-induced changes of protein expression. S4KD and WT cells were treated with or without PD98059 (50 μM) for 1 h and were then stimulated with 10 ng/ml TGF-β for 90 min (p-ERK, ERK) or 12 h (HSP27, vimentin, and 14-3-3ζ). Thirty-five micrograms of total cell lysate was probed with anti-p-ERK antibody, anti-ERK antibody, anti-HSP27 antibody, anti-vimentin antibody, and anti-14-3-3ζ antibody.

molecular chaperone that belongs to the heat shock family of stress proteins [24], and it plays a major role in actin filament dynamics and prevents apoptosis [25]. Vimentin is a major structural component of intermediate filaments in many cell types, and it plays an important role in vital mechanical and biological functions, such as cell contractility, migration, stiffness, and proliferation [26]. In our study, TGF-β increased the expression of vimentin mRNA, but decreased its protein level. The molecular mechanisms that account for the discrepancy remain to be clarified.

There are no previous reports on the involvement of the other 10 proteins identified as TGF-β signals, which suggests that TGF-β signaling acts in cytoskeletal regulation, cell cycle regulation, cell proliferation, nuclear transport, and oxidative stress. Actin-related protein 3 (ARP3) and profilin-II are actin regulatory proteins [27]. *ARP3* was first cloned from *Schizosaccharomyces pombe*, where it encodes a protein that is essential for the germination of haploid spores [28]. Profilin-II is a multifunctional actin regulatory protein that binds to actin monomers, and it is widely thought to promote actin filament assembly.

Interestingly, some of the proteins identified act in cell cycle regulation. 14-3-3 proteins are broadly expressed in a wide range of eukaryotes, and at least seven different isoforms have been identified in mammalian cells [29]. 14-3-3 proteins have been implicated in cell cycle control and interact with many signaling molecules. In this study, 14-3-3ζ protein was up-regulated by TGF-β in the presence of PD98059. Because mitogen-activated protein kinase kinase (MEK) phosphorylates Smad2/3 and inhibits its nuclear translocation [30], PD98059 might promote Smad2/3 nuclear translocation, resulting in an increase in 14-3-3ζ protein. The inhibition of stathmin expression results in growth inhibition and the accumulation of cells in mitosis [31]. The down-regulation of stathmin that we observed might account for the growth inhibition mediated by TGF-β.

Recently, TGF-β signaling was also reported to regulate protein stability. One of the identified proteins, CSN8, is a component of the subunit of COP9 signalosome (CSN) and was previously reported to accelerate p27 degradation [32]. Because p27 induces growth inhibition by causing G1 cell cycle arrest [33], our results suggest that TGF-β induces down-regulation of CSN8, which in turn causes growth inhibition.

DJ-1 was first reported to be a novel oncogene showing cooperative transforming activity with H-ras [34]; it is a ubiquitously expressed, highly conserved protein that plays important roles in several biological processes. Recently, mutations in DJ-1 were identified as the cause of an autosomal recessive, early-onset form of familial Parkinson's disease [35]. Human L-3 phosphoserine phosphatase is the enzyme responsible for the third and last step in L-serine formation [36]. Fairchild et al. [37] first reported human inorganic pyrophosphatase (PPase), which catalyzes the hydrolysis of pyrophosphate to form orthophosphate. Nuclear transport factor 2 (NTF2) mediates the nuclear import of RanGDP. Although NTF2 is reportedly essential for mediating the active nuclear import of phosphorylated Smad3, the relationship between NTF2 and TGF-β signals is not clear [38]. Peroxiredoxin3 is an antioxidant enzyme that is involved in the protection of protein and lipid against oxidative injury and in cellular signaling pathways regulating apoptosis [39].

In order to elucidate the phenotypic change due to the knockdown of *Smad4*, we performed cell proliferation and cell invasion assays in vitro. However, there were no significant differences in the results with S4KD and WT cells. We are analyzing whether the loss of *Smad4* changes the cellular phenotype, and further study is necessary to clarify the molecular mechanism by which TGF-β regulates these identified targets.

In conclusion, we established Smad4 knockdown PANC-1 pancreatic carcinoma cells using a stable RNAi method. Proteomic analyses with RNAi could be a useful method for examining novel target molecules in unknown pathways, and these molecules might play significant roles in the pathogenesis of pancreatic carcinoma. Further investigation of the signaling pathway of the identified molecules is needed.

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