

Sp1 Is Required for Transforming Growth Factor- β -Induced Mesenchymal Transition and Migration in Pancreatic Cancer Cells

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Abstract

Transition from a sessile epithelial phenotype to a migrating mesenchymal phenotype is a crucial step in transforming growth factor- β (TGF- β)-induced pancreatic cancer cell migration and invasion. These profound morphologic and functional alterations are associated with characteristic changes in TGF- β -regulated gene expression, defined by rapid repression of epithelial markers and a strong and sustained transcriptional induction of mesenchymal markers such as the intermediate filament vimentin. In this study, we have analyzed the role of the transcription factor Sp1 in TGF- β -induced and Smad-mediated gene regulation during epithelial to mesenchymal transition (EMT) and migration of pancreatic cancer cells. Here, we show that Sp1 is required for TGF- β -induced EMT, and that this function is especially mediated through transcriptional induction of vimentin. Our results emphasize the functional relevance of vimentin in TGF- β -induced EMT because prevention of its induction strongly reduces cell migration. Altogether, this study helps to better understand the role of Sp1 in TGF- β -induced progression of pancreatic cancer. It suggests that Sp1, via transcriptional induction of vimentin, cooperates with activated Smad complexes in mesenchymal transition and migration of pancreatic cancer cells upon TGF- β stimulation.

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Introduction

Transforming growth factor- β (TGF- β) is a pluripotent cytokine that exhibits a dual character during tumorigenesis (1, 2). Although in healthy epithelial cells and early tumor stages, TGF- β acts as a strong inhibitor of cell growth, this ability is lost as the tumor progresses. In late-stage cancer, TGF- β might then contribute to tumor progression through enhanced cellular motility, invasiveness, and metastasis (1–3). In recent years, a growing number of *in vivo* studies have shown that inhibition of TGF- β signaling and transcription reduces the metastatic and/or invasive properties of a variety of experimental cancers, presumably by preventing the induction of an epithelial to mesenchymal transition (EMT) of cancer cells (3, 4). By definition, EMT is an extreme and complex manifestation of epithelial plasticity (5), which has been described in three major physiologic and pathophysiologic contexts:

embryonic development and morphogenesis, chronic fibrotic disorders, and cancer progression.

Oncogenic EMT is well documented *in vivo* and *in vitro*. It is characterized by a reversible conversion of polarized epithelial cells into highly motile fibroblastoid cells (6, 7). On the molecular level, EMT is defined by the loss of cell-cell adhesion molecules (e.g., E-cadherin), down-regulation of epithelial differentiation markers, and transcriptional induction of mesenchymal markers, such as vimentin and fibronectin. We have recently shown that TGF- β -induced EMT in invasive growing pancreatic cancer cells requires an intact Smad-signaling and transcription pathway (8). In fact, mutational inactivation of the Smad-signaling cascade prevents pancreatic cancer cells from TGF- β -induced EMT and tumor cell migration, suggesting that Smads are essential players in the transcriptional regulation of EMT-associated genes. In most cases, however, the Smad proteins alone cannot sufficiently regulate target promoter gene regulation and, thus, need to cooperate with other transcription factor proteins, including members of the AP1 family, Runx1-3, the E2F family, and the Sp1/KLF-like zinc-finger transcription factors (9–12).

Our laboratory investigates the roles of the Sp1/KLF-like proteins in Smad-mediated target gene selection and regulation in normal epithelial cells and in pancreatic cancer cells as well (13, 14). We have recently characterized KLF11, a TGF- β -inducible member of the Sp1/KLF-like family, as a strong tumor suppressor that represses transcription of cell-cycle-promoting genes through both Smad-dependent and Smad-independent mechanisms (15–17). During pancreatic carcinogenesis, however, hyperactive Ras-extracellular signal-regulated kinase mitogen-activated protein kinase signaling prevents KLF11-Smad interactions and subsequently antagonizes the tumor suppressor activities of KLF11 in late-stage pancreatic cancer (15). As a result of KLF11 inactivation, Smad proteins might then partner with other Sp1/KLF-like proteins and together promote pancreatic cancer progression via transcriptional regulation of selected target genes. In this context, Sp1 is of particular interest because recent studies have suggested potential oncogenic functions of Sp1 in several aspects of tumor progression, including those associated with angiogenesis, growth control, and cell motility (18–20). In this study, we investigated whether Sp1 participates in TGF- β -induced gene regulation during EMT and whether this function contributes to increased pancreatic cancer cell migration.

Materials and Methods

Cell culture and transient transfection. Panc-1 and IMIM-PC1 cells were maintained in Dulbecco's modified minimal essential medium (Life Technologies, Invitrogen Corp., Carlsbad, CA) supplemented with 10% FCS. Expression and reporter promoter plasmids were transfected using the

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Transfast reagent (Promega, Madison, WI). Small interfering RNA (siRNA) was transfected into Panc-1 cells with Transmessenger (Qiagen, Hilden, Germany) and into IMIM-PC-1 cells with X-tremeGene (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. The sequences to silence the translation of Sp1 were 5'-GGUAGCUCUAA-GUUUUGAUtt-3' and 5'-GGUCAUUUCUUUGCUUUGtt-3' and, for vimentin, 5'-GGCGAGGAGCAGGAUUtt-3' (Ambion, Austin, TX). The scrambled sequence Silencer Negative Control 2 RNAi (Ambion) was used as a negative control.

Plasmid constructs and luciferase reporter gene assays. The luciferase reporter plasmid vim-wt (-1416 to +73) was obtained from Dr. J.-M. Foidart (Laboratory of Tumor and Developmental Biology, University of Liege, Liege, Belgium). The mutant vimentin reporter plasmids (M1 to M5) were generated from the vim-wt reporter construct by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis primers were as follows: Vim-M1: 5'-GCCCGCGGG-TCTCCCCGTTTGACCGCAGCCCCGAGG-3' and its complementary strand; Vim-M2: 5'-Cctcaccgcgcgaccttgccttttcagcacc-3' and its complementary strand; Vim-M3: 5'-Cgagggtgagccagcttaccattatcatcggaaagccc-3' and its complementary strand; Vim-M4: 5'-Cgagaagcggggaggggtgaacgggtgggggg-3' and its complementary strand; Vim-M5: 5'-Cagcgcctcaacggggtccagtc-3' and its complementary strand.

For reporter gene assays, cells were seeded in 24-well tissue culture dishes. Twenty-four hours later, cells were transfected with the indicated constructs and/or treated with 5 ng/mL TGF- β (PromoCell GmbH, Heidelberg, Germany). Luciferase assays were done with a DLReady Luminometer (Berthold Technologies, Bad Wildbad, Germany) and the Dual-Luciferase-Reporter Assay System (Promega). Firefly luciferase values were normalized to *Renilla* luciferase activity and were either expressed as relative luciferase activity or as mean "fold induction" with respect to empty vector control. Mean \pm SD values are displayed.

Northern blot and Western blot analyses. For Western blotting, the resulting immunoprecipitates or protein extracts from pancreatic cancer cell lines were electrophoresed through a 6% or 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore, Billerica, MA) as described previously (15). Polyvinylidene difluoride membranes were probed with the following antibodies: anti-Smad2/3 (Upstate Biotechnology, Lake Placid, NY, and Transduction Laboratories, San Diego, CA), anti-Smad4 (Upstate Biotechnology), anti-Sp1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-vimentin (Sigma-Aldrich, Saint Louis, MO), anti-phospho-Smad3 (Cell Signaling Technology, Beverly, MA), anti-fibronectin (Biozol, Echingen, Germany), anti-collagen-1 (Biozol), anti-E-cadherin (BD Biosciences, Heidelberg, Germany), and anti- β -actin antibodies (Sigma-Aldrich). Membranes were washed in TBS buffer and then incubated with peroxidase-conjugated secondary antibodies. Lumi-Light Western Blotting Substrate (Roche Applied Science) was used for visualization. For Northern blot hybridization, RNA was extracted using the RNeasy Midi Kit (Qiagen), as indicated by the manufacturer. Northern blots with 20 μ g of total RNA per lane were prepared and hybridized with a 32 P-labeled probe for vimentin, as described previously (8).

Quantitative real-time PCR analysis. RNA was extracted using the RNeasy Midi Kit (Qiagen), and first-strand cDNA was synthesized from 10 μ g total RNA using random primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). The quantitative PCR analysis was done using an ABI PRISM 7700 Sequence Detector System and the SYBR Green PCR Master Mix kit (Applied Biosystems, Wellesley, MA), according to the manufacturer's suggestions. The quantitative reverse transcription-PCR was done with sequence-specific primer pairs determined with the PrimerExpress program (Applied Biosystems). Cyclophilin A was used as a housekeeping gene. The following primer pairs were used: vimentin forward 5'-gccatcaaccaggatggaag-3', vimentin reverse 5'-cgatgtag-tggcgaagcg-3'; fibronectin forward 5'-gccatcaaccaggatggaag-3', fibronectin reverse 5'-caacggcataatgggaaactg-3'; COL1A1 forward 5'-tgtctgattgtcacc-ggg-3', COL1A1 reverse 5'-caccaccatcacatagat-3'; E-cadherin forward 5'-acagacatgagccactgacc-3', E-cadherin reverse 5'-cgatggagcgaactgtctc-tta-3'; cyclophilin A forward 5'-atggtcaaccccacctgt-3' and cyclophilin A reverse 5'-tctgtctctttggacctgtc-3'.

Chromatin immunoprecipitation. The chromatin immunoprecipitation was done with Panc-1 cells treated with TGF- β for 3 h. Cells were cross-linked with 1% formaldehyde for 10 min at 37°C, harvested in SDS lysis buffer (Upstate Biotechnology), and DNA was sheared to fragments of 500 bp by sonication. Antibodies against Smad3 and Sp1 were added to each aliquot of precleared chromatin and incubated overnight. Protein G agarose beads were added and incubated for 1.5 h at 4°C. After reversing the cross-links, DNA was isolated and used for PCR reactions. The primer set 5'-CCCCACCCCGGTTCCAATCT-3' and 5'-GCGCCTCGAGCCT-TCCTG-3' were used to amplify a 357-bp region of the vimentin promoter harboring the Smad binding element and putative GC-rich Sp1 sites.

Fluorescence microscopy. Panc-1 cells were grown on chambered cover slips and were treated with TGF- β for 48 h. Cells were subsequently fixed in 4% paraformaldehyde in PBS at room temperature for 20 min and permeabilized with 0.2% Triton X-100. Cells were incubated with a monoclonal anti-vimentin antibody (Sigma-Aldrich) for 1 h followed by detection with Alexa Fluor 488-labeled secondary antibodies (Molecular Probes, Leiden, Netherlands). Filamentous actin was visualized using Alexa Fluor 488 phalloidin (Molecular Probes). Samples were further analyzed by fluorescence microscopy (100 \times , numerical aperture 1.4) on an Olympus IX71 (Olympus Germany, Hamburg, Germany) equipped with an Orca II-CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were analyzed using ImageJ (1.30v).³

Migration assay. Migration of Panc-1 cells was examined in a two-chamber assay as described previously (8, 21). For this purpose, 1×10^4 cells that were transfected with RNAi were seeded on the upper side of 24-well Falcon cell culture inserts (Becton Dickinson, Heidelberg, Germany). Cells were treated with 5 ng/mL TGF- β . After 48 h, cells on the upper side of the membrane were wiped off, the membranes were fixed, and migrated cells on the lower side of the membrane were either counted or stained with CellTiterGlo (Promega) and measured with a DLReady Luminometer (Berthold Technologies). All migration assays were done in triplicates in at least three independent experiments. Values are expressed as fold induction.

Results

TGF- β stimulates EMT and cell migration in pancreatic cancer. Because the pancreatic cancer cell lines Panc-1 and IMIM-PC1 lack genetic alterations within the TGF- β -Smad signaling pathway and express Sp1, we assumed that these cell lines serve as ideal model systems to explore the role of Sp1 in TGF- β -induced and Smad-mediated tumor progression (22). Figure 1A confirms the expression of Sp1 and Smads in both cell lines and shows rapid and sustained activation of receptor-Smad3 phosphorylation upon TGF- β stimulation. In addition, neither the Smad nor the Sp1 expression levels were affected by TGF- β treatment, even 48 h postapplication. We then examined the potential of TGF- β to stimulate pancreatic cancer cell migration by performing Boyden chamber assays. Figure 1B illustrates the potential of TGF- β to stimulate pancreatic cancer cell migration. It shows a 4-fold induction of tumor cell migration in Panc-1 cells and an approximate 6-fold induction in IMIM-PC1 cells by TGF- β , respectively.

Furthermore, TGF- β treatment for a period of 24 h induced an epithelial mesenchymal transdifferentiation in both cell lines, as illustrated by the acquisition of a fibroblast-like phenotype (Fig. 1C). On the transcriptional level, TGF- β -induced EMT was reflected by the altered expression of the EMT-marker genes E-cadherin, vimentin, and collagen-1 on both the mRNA and protein level (Fig. 1D). In contrast, the expression level of fibronectin remained unaffected by TGF- β . This suggests that TGF- β contributes to increased pancreatic cancer cell migration via transcriptional induction of a gene program that leads to EMT.

³ Available at: <http://rsb.info.nih.gov/ij/>

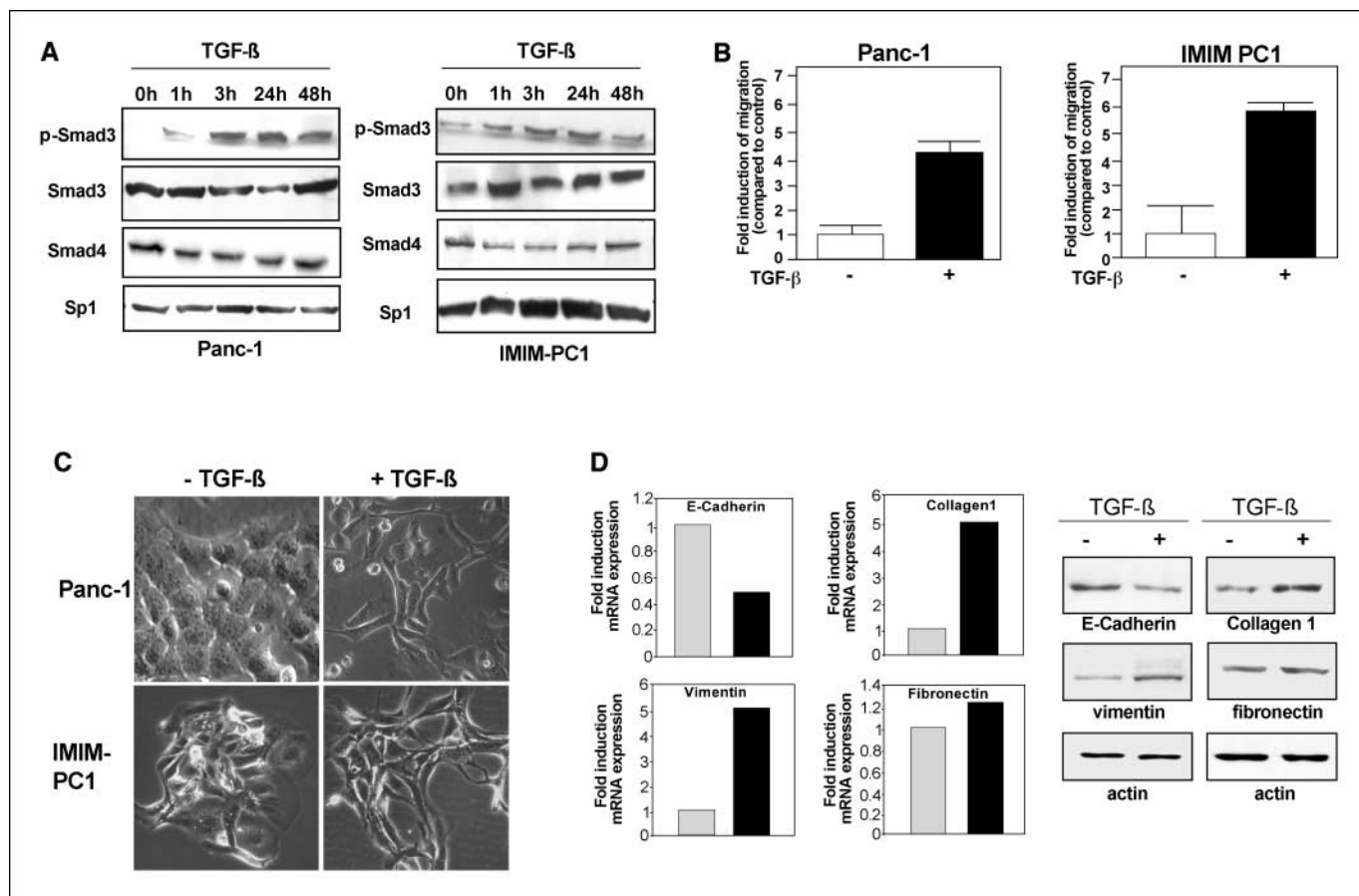


Figure 1. TGF- β stimulates EMT and pancreatic cancer cell migration. *A*, analysis of Sp1, Smad proteins, and phospho-Smad3 expression in Panc-1 and IMIM-PC1 cells following TGF- β treatment over time. *B*, TGF- β -mediated pancreatic cancer cell migration was measured in modified two-chamber assays. Tumor cells were seeded on the upper side of uncoated 24-well plates, serum starved, and finally treated with or without TGF- β (5 ng/mL). Forty-eight hours later, cells were counted, and values were expressed as fold induction as compared with untreated controls. Bars, SD. *C*, phase-contrast photomicrographs documenting the effect of a 48-h TGF- β treatment on cell morphology of responsive Panc-1 and IMIM-PC1 cells. *D*, the effects of TGF- β on EMT marker gene expression levels in Panc-1 cells. Quantitative reverse transcription-PCR was done, and expression levels were calculated relative to basal mRNA expression levels, which were arbitrarily set to 1 for each experiment. TGF- β -mediated changes in mRNA expression levels are shown as fold induction (*left*). Western blot analysis was done 24 h posttreatment (*right*). Cells were harvested, and protein levels of EMT markers were analyzed by using anti-E-cadherin, anti-collagen-1, anti-vimentin, and anti-fibronectin antibodies. Protein loading was controlled using anti-actin antibodies.

Sp1 is required for TGF- β -induced EMT and cancer cell migration. Next, we analyzed the role of Sp1 in TGF- β -induced and Smad-mediated EMT and cancer cell migration and knocked down endogenous Sp1 by RNA interference technology. Figure 2*A* shows successful siRNA-mediated silencing of Sp1 in both cell lines. Figure 2*B* summarizes the results of the migration assays and shows that repression of endogenous Sp1 reduced cancer cell migration under unstimulated conditions and strongly diminished the stimulatory effects of TGF- β in both cell lines (Fig. 2*B*). To control for the effects on cell proliferation, we carried out thymidine assays under conditions identical to those used in the migration assays. To our surprise, these control experiments did not reveal significant effects of Sp1 silencing on TGF- β -mediated cell growth control in pancreatic cancer cells (data not shown). We therefore assumed that the results obtained from Boyden chamber assays cannot be ascribed to a change in cellular proliferation and, instead, reflects altered migration properties of tumor cells upon knockdown of Sp1. Furthermore, reduced cancer cell migration was associated with reduced EMT, as shown on the morphologic (Fig. 2*C*) and the expression level (Fig. 2*D*). More precisely, loss of Sp1 expression lowered the ability of TGF- β to induce a spindlelike

cell shape and prevented the induction of vimentin and, to a lesser extent, collagen I. Interestingly, other TGF- β -regulated EMT marker genes, e.g., E-cadherin, remained unaffected by Sp1 knockdown. These findings were confirmed by Northern blot hybridization experiments and Western blot analysis, which showed lowered vimentin expression following gene silencing of Sp1 (Fig. 3*A* and *B*). Moreover, a similar expression pattern of vimentin was found in immunofluorescence microscopy analysis of Panc-1 cells (Fig. 3*C*). In control cells, the induction of vimentin expression by TGF- β was accompanied by stress fiber formation of actin filaments and loss of cell-cell contacts, indicating the TGF- β -mediated onset of EMT. In contrast, however, vimentin was either absent or only slightly expressed in Sp1 knockdown Panc-1 cells, which also failed to undergo EMT upon TGF- β . Altogether, these results suggest that Sp1 participates in the transcriptional regulation of vimentin and, thus, contributes to the acquisition of a mesenchymal and highly migrating phenotype in pancreatic cancer.

Sp1 participates in vimentin promoter induction by TGF- β . Sequence analysis of the human vimentin promoter revealed the presence of several putative Sp1 binding elements and a neighboring Smad binding element (Fig. 3*D*, *top*). To examine

how TGF- β and Sp1 achieve the concerted expression of vimentin in pancreatic cancer cells, we carried out reporter gene assays using a luciferase reporter gene encoding for the proximal human vimentin promoter (vim-wt; -1416 to +73). As shown in Fig. 3D (bottom), treatment of Panc-1 cells with TGF- β led to a 4-fold induction of the vimentin promoter activity, indicating that TGF- β induces vimentin expression at least in part through increased transcription from its proximal promoter region.

We then studied the role of the TGF- β -responsive Smad proteins in vimentin promoter regulation and cotransfected receptor-Smad proteins (Smad2 and Smad3) either alone or in combination with co-Smad4 into Panc-1 cells. As shown in Fig. 4A, the introduction of Smad3 alone, but not of Smad2, resulted in a significant transcription from the proximal vimentin promoter in Panc-1 cells. Furthermore, an additional increase in vimentin promoter transactivation was observed following a concomitant overexpression of Smad3 and Smad4 in Panc-1 cells. Similarly, in IMIM-PC1 cells, the vim-wt reporter construct was highly induced by Smad3/Smad4 heteromeric complexes (data not shown), but remained unaffected by overexpression of Smad2.

Subsequently, we studied whether Sp1 contributes to Smad3/Smad4-mediated transactivation of the vimentin promoter. For this purpose, we transfected pancreatic cancer cells with hetero-

meric Smad complexes and studied their potential to induce the vimentin promoter depending on the presence or absence of Sp1. Again, the introduction of Smad complexes already induced the vimentin promoter approximately 3.5 times. Moreover, additional transfection of Sp1 greatly increased Smad-mediated transactivation (Fig. 4B), whereas siRNA-mediated knockdown of endogenous Sp1 significantly, although not completely, reduced Smad-mediated induction of the vimentin promoter (Fig. 4C). In line with our reporter gene assays, chromatin immunoprecipitation experiments revealed constitutive binding of Sp1 to the human vimentin promoter and suggested that this interaction was slightly increased upon TGF- β . In addition, Smad3 binding to the vimentin promoter was induced by TGF- β (Fig. 4D). We then went on to analyze the functional significance of the putative Smad binding site and individual GC-rich Sp1 boxes in more detail and did reporter gene assays using vimentin promoter constructs in which either the Smad binding element or the Sp1 binding sites were individually mutated (Fig. 5A and B). These experiments revealed impaired Smad-mediated transactivation of the human vimentin promoter following mutational inactivation of either the Smad binding element (vim-M3) or the neighboring GC-rich binding site (vim-M4). Altogether, our results indicate that Sp1 participates in TGF- β -induced and Smad-mediated transcription from the

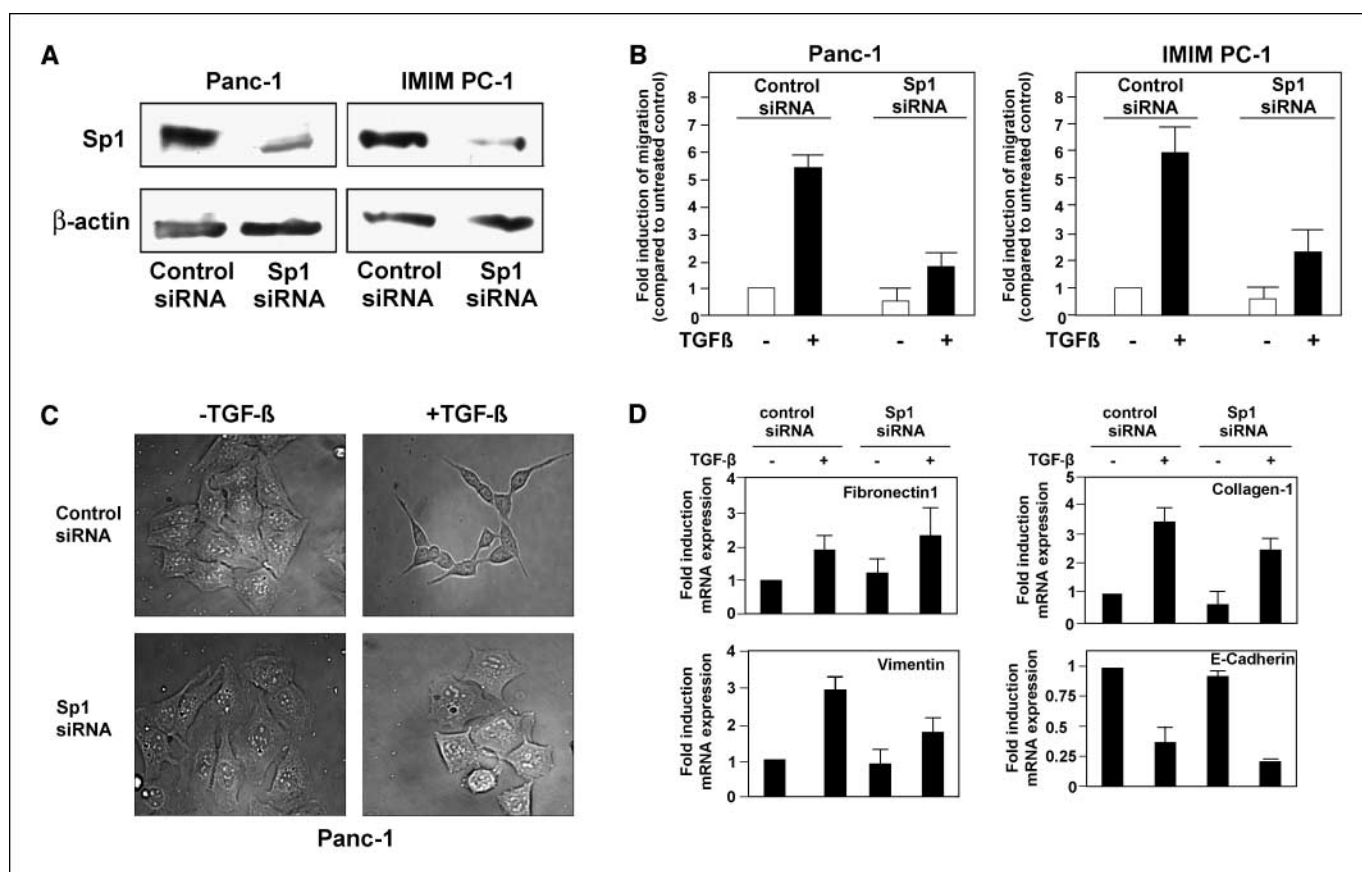


Figure 2. Cells were transfected with either control siRNA or Sp1 siRNA to determine Sp1 dependency of TGF- β -induced EMT and cell migration. *A*, Western blot analysis to show successful Sp1 knockdown in both pancreatic cancer cell lines. Anti- β -actin antibodies served as loading control. *B*, cells were seeded in 24-well chambers before treatment with TGF- β for an additional 24 h. The number of migrated cancer cells was calculated relative to basal migration levels in control siRNA-transfected cells, which were arbitrarily set to 1 for each experiment. Values are expressed as fold induction. *C*, Sp1 (*Sp1 siRNA*)-depleted Panc-1 cells and control (*control siRNA*) cells were allowed to grow as discrete colonies before treatment with or without TGF- β for additional 24 h. The effect of Sp1 knockdown on TGF- β -induced mesenchymal transition was evaluated by phase contrast microscopy. *D*, Panc-1 cells were transfected with either control siRNA or Sp1 siRNA before treatment with TGF- β for 24 h. cDNA was prepared and subjected to quantitative reverse transcription-PCR to determine the effect of Sp1 silencing on mRNA expression of the EMT marker genes.

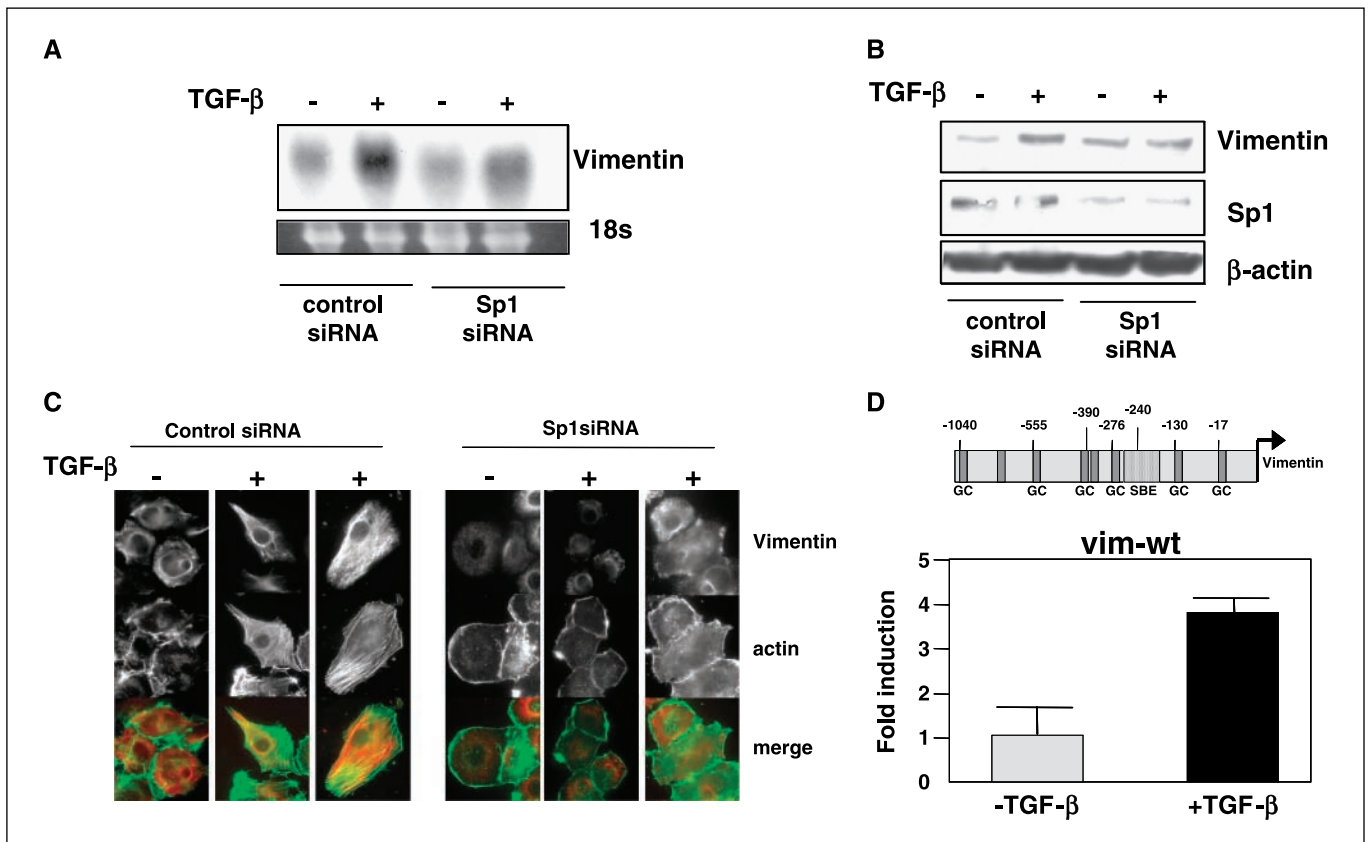


Figure 3. Sp1 participates in TGF- β -induced expression of vimentin. Panc-1 cells were transfected either with Sp1-siRNA or with nonspecific control siRNA. Twenty-four hours later, cells were harvested, and total RNA or proteins were extracted to determine the expression of endogenous vimentin on both the mRNA (A) and the protein (B) level. Northern blot analysis was conducted using a specific 32 P-labeled cDNA probe to detect vimentin. Immunoblotting was done using anti-vimentin antibodies. Anti- β -actin antibodies were used to control for loading. C, confocal microscopy of Sp1 siRNA-transfected Panc-1 cells cultured with (+) and without (-) TGF- β . Note that Sp1 silencing reduces TGF- β -induced acquisition of a mesenchymal phenotype and diminishes induction of vimentin. D, schematic representation of the vimentin (*vim-wt*) reporter gene construct including putative Sp1 sites (GC boxes) and a Smad binding element (SBE; top). Panc-1 cells were transiently transfected with the *vim-wt* reporter gene construct (-1416 to +73) and treated with or without TGF- β for 24 h. Luciferase activity was measured, and induction of *vim-wt* reporter gene activity was expressed as fold induction. Columns, mean values calculated from three independent experiments; bars, SD (bottom).

proximal vimentin promoter. On the other hand, these findings also propose Sp1-independent effects because neither mutation of Sp1 binding sites nor siRNA-mediated knockdown of endogenous Sp1 expression completely prevented Smad-induced activation of the vimentin promoter.

Vimentin plays a central role in TGF- β -induced and Sp1/Smad-mediated cell migration. The intermediate filament vimentin, primarily recognized for its role in cytoskeleton formation, is commonly used as a mesenchymal marker gene to show EMT in cancer cells (23, 24). Recent studies also suggest that vimentin, via modulation of cytoskeleton configuration, plays a direct role in tumor cell migration during EMT (25–27). To assess the relevance of vimentin expression in pancreatic cancer cell migration by TGF- β , we decided to employ two different approaches. First, we knocked down endogenous vimentin in Panc-1 cells by RNA interference and determined the remaining potential of cancer cells to respond to TGF- β with increased migration. Second, vimentin expression was rescued in Sp1 knockdown cells to examine whether the loss of cancer cell migration can be restored through specific induction of vimentin. Figure 6A and B shows successful siRNA-mediated down-regulation of basal expression and TGF- β -induced vimentin expression on both mRNA and protein levels. Transfection of control siRNA into Panc-1 cells did not significantly alter the potential of cells to respond to TGF- β

with increased migration. In contrast, transfection of vimentin siRNA strongly reduced the ability of pancreatic cancer cells to migrate through transwell membranes and almost completely prevented TGF- β -induced cell migration (Fig. 6C). These data clearly supported previous findings that showed a direct link between the level of vimentin expression and the migratory potential of epithelial cancer cells. Moreover, these results further suggested that reduced cell migration in Sp1 knockdown pancreatic cancer cells might particularly be caused by the loss of endogenous vimentin expression. This hypothesis was strongly confirmed by the outcome of our rescue experiments (Fig. 6D), demonstrating that reexpression of vimentin completely restores basal and TGF- β -induced migration of pancreatic cancer cells, even in the absence of Sp1.

Discussion

Cell-type-dependent interaction with partnering transcription factors defines Smad target gene selection and, thus, determines the functional response of a cell to TGF- β (28, 29). In normal epithelial cells, numerous transcriptional binding partners have been discovered that cooperate with the Smads to regulate the transcription of specific target genes involved in cell growth inhibition, apoptosis, and wound healing (28). During

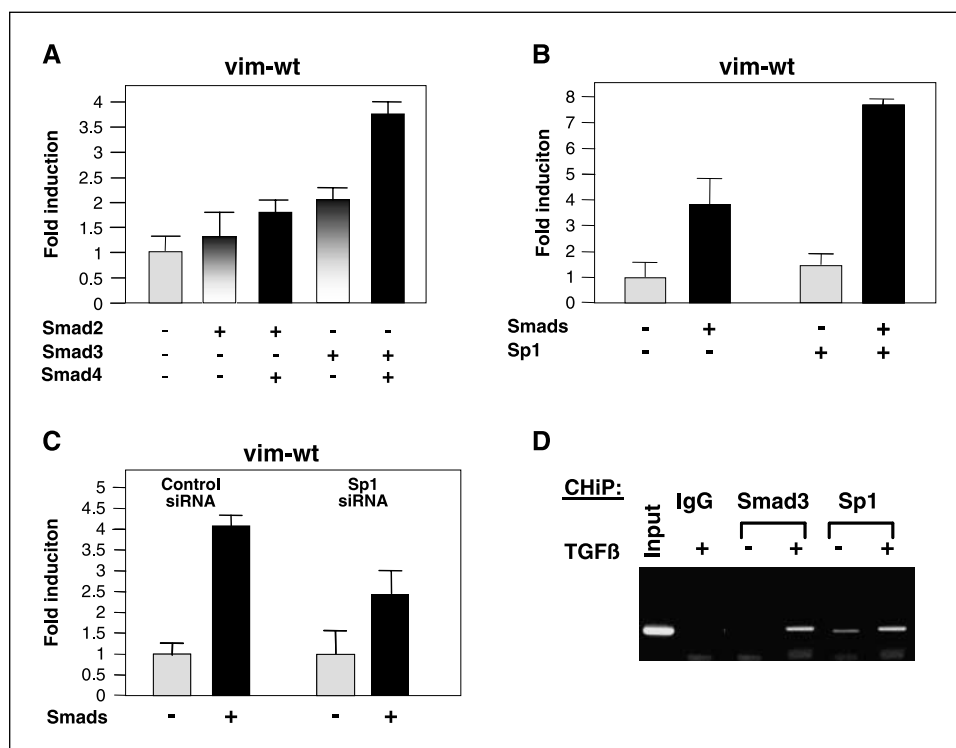


Figure 4. Sp1 and Smads cooperate in TGF- β -induced transcription from the human vimentin promoter. **A**, Panc-1 cells were individually or collectively transfected with Smad2, Smad3, and Smad4 expression constructs together with the vim-wt reporter construct. Luciferase activity was measured, and induction of vim-wt reporter gene activity was expressed as fold induction. **B**, Panc-1 cells were cotransfected with heteromeric Smad3-Smad4 complexes alone or in combination with Sp1 together with the Vim-wt reporter. **C**, Panc-1 cells were transfected with either specific Sp1 siRNA or nonspecific control siRNA. Twenty-four hours later, cells were additionally transfected with Smad3-Smad4 complexes, and the effect of Sp1 depletion on Smad mediated vim-wt promoter induction was determined. **D**, Panc-1 cells were left untreated (-) or treated (+) with TGF- β for 3 h, chromatin immunoprecipitations were done with either anti-immunoglobulin G, anti-Smad3, or anti-Sp1 antibodies, and PCR was done with specific primers to amplify a 357-bp region of the vimentin promoter harboring the Smad binding element and putative GC-rich Sp1 sites.

carcinogenesis, however, the set of active Smad transcriptional partner proteins changes in a way that TGF- β target gene selection is directed towards a promalignant outcome. Sp1, the founding member of the Sp1/KLF-like family of zinc-finger transcription factors, is appreciated as one such oncogenic Smad partner protein involved in TGF- β -mediated gene expression during cancer progression (13, 30–32). It has been shown, for instance, that Sp1 and Smad proteins form transcriptional complexes at specific promoter binding regions and together determine target gene specificity during cell proliferation, survival, and angiogenesis (31–34). This is also true for late-stage pancreatic cancer, where highly expressed Sp1 interacts with activated Smad complexes to regulate the expression levels of distinct tumor-promoting genes (13). The motivation of this study has been to investigate whether Sp1 participates in TGF- β -induced gene regulation during EMT, a hallmark of cancer cell migration (35). We previously showed

that a functional Smad signaling pathway is required for TGF- β -induced EMT, and that genetic inactivation of individual Smads protects pancreatic cancer cells from the acquisition of a migrating mesenchymal phenotype (8). Here, we show that, in addition to the Smads, Sp1 is an essential player in this central aspect of TGF- β tumor progression, and that functional cooperation between both effector transcriptional regulators is required for sufficient induction of EMT and the resulting tumor cell migration. In particular, we show here that siRNA-mediated knockdown of endogenous Sp1 prevents the acquisition of a spindle-shape cell morphology, stabilizes cell-cell contacts, and reduces migration of pancreatic cancer cells upon TGF- β . These impressive effects of Sp1 knockdown on cancer cell architecture and function are accompanied by profound changes in TGF- β -regulated gene expression. Although TGF- β treatment of responsive pancreatic cancer cells induces sustained activation of EMT-defining genes, silencing of Sp1

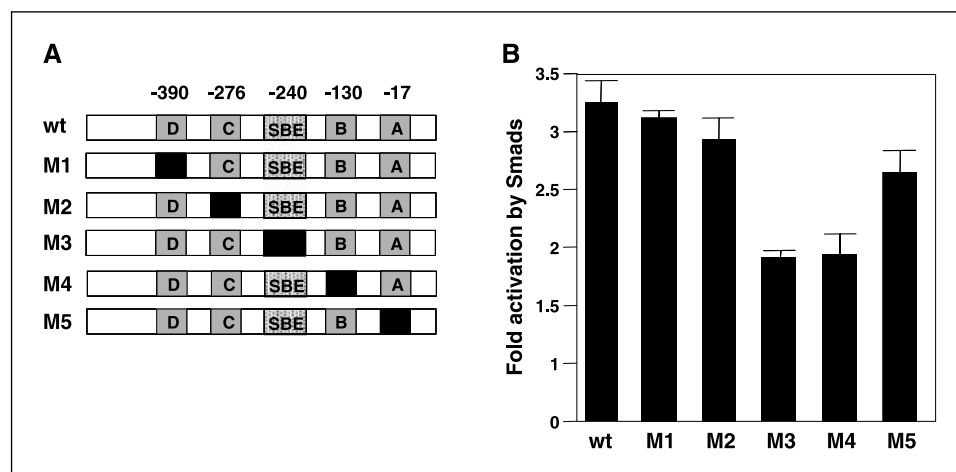


Figure 5. **A**, schematic representation of the wild-type vimentin promoter (vim-wt) and the mutation constructs (M1–M5) used in reporter gene assays. The Smad binding element and the GC-rich Sp1 boxes (A–D) of the proximal vimentin promoter were individually mutated in the context of the vimentin luciferase reporter plasmid. **B**, Panc-1 cells were transiently transfected with either the wild-type vimentin promoter reporter construct (vim-wt) or the mutants (M1–M5) and in the presence or absence of Smads. Firefly luciferase activity was measured 24 h posttransfection and normalized to *Renilla* luciferase controls. Basal levels in the absence of Smad were arbitrarily set to 1 for each experiment. Reporter gene activities are expressed as fold activation by the Smads relative to controls.

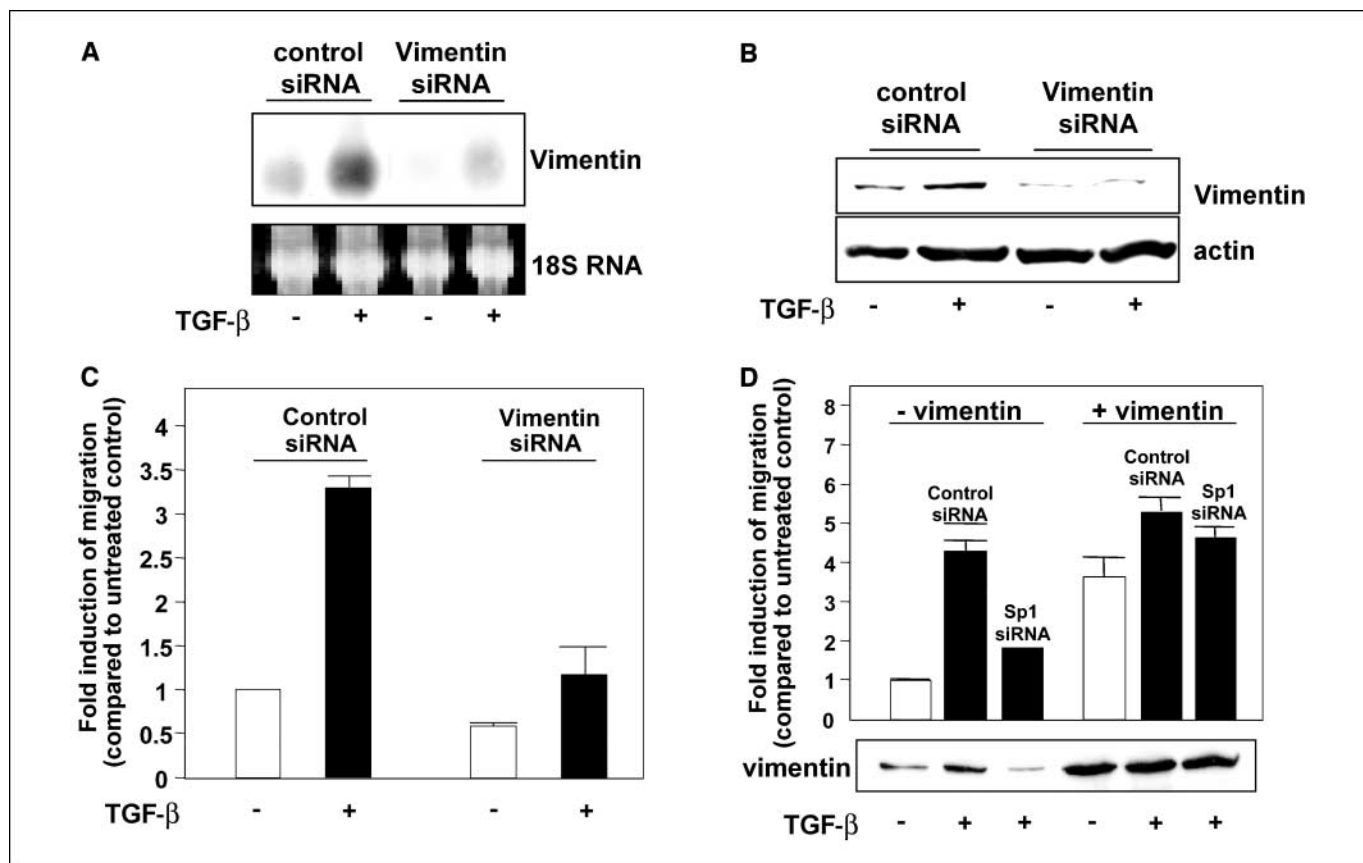


Figure 6. Vimentin plays a central role in TGF- β -induced pancreatic cancer cell migration. Panc-1 cells were transiently transfected with nonspecific control siRNA or a siRNA sequence directed against human vimentin. (A) Northern blot and (B) Western blot hybridizations were done to confirm successful down-regulation of endogenous vimentin in Panc-1 cells. C, controls and vimentin knockdown cells were seeded in 24-well Boyden chambers before treatment with TGF- β for an additional 24 h. The number of migrated cancer cells was calculated relative to basal migration levels in control siRNA-transfected cells, which were arbitrarily set to 1 for each experiment. Note that down-regulation of vimentin significantly lowered the ability of pancreatic cancer cells to respond to TGF- β with increased migration. D, expression of endogenous vimentin was restored in Sp1 knockdown cells to study the relevance of vimentin in Sp1-mediated cancer cell migration upon TGF- β . For this purpose, control cells and Sp1-depleted cells were transfected with a vimentin expression vector before treatment with TGF- β . Twenty-four hours posttreatment, migrated cells were counted, and values were calculated relative to basal migration levels of control siRNA-transfected Panc-1 cells. Vimentin expression levels were monitored by Western blot analysis using anti-vimentin antibodies.

completely abolishes Smad-induced *de novo* expression of the mesenchymal type III intermediate filament vimentin. Our biochemical and functional analyses indicate that TGF- β up-regulates vimentin expression primarily on the level of promoter regulation, and that maximal promoter stimulation by TGF- β requires functional cooperation between Sp1 and activated Smad complexes. Moreover, our studies further revealed that transcriptional induction of vimentin is a key step in TGF- β -induced pancreatic cancer cell migration. In fact, whereas loss of vimentin expression in Sp1 knockdown cells is ultimately associated with reduced cell migration, its reexpression causes complete restoration of pancreatic cancer cell motility, even in the absence of Sp1. Thus, induction of vimentin is required for TGF- β -induced cell migration during pancreatic carcinogenesis. In line with our findings, recent studies also suggested a role for vimentin in tumor cell migration and, in addition, provided first mechanistical insights (27, 36, 37). Most interestingly, vimentin has been identified as a mechanical transducer between cell surface integrins and the nucleus and, thus, potentially controls cell migration through the regulation of cell adhesion stability (38–43). Future work will be required to elucidate whether this mechanism applies to TGF- β -induced cell migration during pancreatic carcinogenesis.

Altogether, our study proposes a model in which Sp1 partners with Smads to induce vimentin expression during EMT and to stimulate pancreatic cancer cell migration upon TGF- β . We were able to show that (a) Sp1 is required for TGF- β -induced and Smad-mediated EMT and the resulting ability of pancreatic cancer cells to migrate; (b) Sp1 cooperates with activated Smad complexes in TGF- β -induced expression of the EMT-associated marker gene product vimentin; and (c) vimentin itself contributes to pancreatic cancer cell migration upon TGF- β . Our study thus provides new insights into the transcriptional regulation of TGF- β -induced EMT and cancer cell migration and might help to better understand the role of Sp1 in this central aspect of pancreatic cancer progression.

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