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Restoration of Transforming Growth Factor β Signaling by Functional Expression of Smad4 Induces Anoikis

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ABSTRACT

Smad proteins transduce signals carried by the transforming growth factor β (TGF-β) cytokine superfamily from receptor serine/threonine kinases at the cell surface to the nucleus, thereby affecting cell proliferation, differentiation, as well as pattern formation during early vertebrate development. *Smad4/DPC4*, located at chromosome 18q21, was identified as a candidate tumor suppressor gene that is inactivated in nearly half of all pancreatic carcinomas. For functional characterization of Smad4, a recombinant adenovirus encoding Smad4 (Ad-Smad4) was generated. When Smad4 was expressed in Smad4-null breast carcinoma cell line MDA-MB-468 using the recombinant adenovirus, TGF-β signaling was restored as determined by TGF-β-dependent activity of plasminogen activator inhibitor 1 promoter and p21 expression. Infection with Ad-Smad4 in the presence of TGF-β1 also resulted in an altered cell morphology that coincided with enhanced β1 integrin expression and reduced efficiency of colony formation in soft agar. In agreement with increased p21 expression, Smad4-expressing cells showed modest reduction in S phase. However, Smad4 expression did not lead to induction of apoptosis under normal culture conditions. Interestingly, when Smad4-expressing cells were detached and incubated in suspension, they underwent rapid apoptosis in a TGF-β-dependent manner. Induction of apoptosis caused by loss of anchorage is known as anoikis. Anoikis is believed to prevent colonization elsewhere of detached cells. Additional characterization revealed an increase in the level of focal adhesion kinase 2 (or Pyk2) and activation of caspases 2, 3, 6, and 8 during anoikis because of Smad4 expression and restoration of TGF-β signaling. Because resistance to anoikis in tumor cells is thought to contribute to metastasis, our data suggest a functional basis for the strong correlation between defects in Smad4 and development of malignancy.

INTRODUCTION

The *Smad4/DPC4* (deleted in pancreatic carcinoma, locus 4) gene is a candidate tumor suppressor gene (1, 2) that is frequently inactivated in pancreatic (1–3), biliary (4, 5), and colorectal tumors (6, 7). *Smad4* is homozygously deleted in ~30% of pancreatic carcinomas and inactivated by intragenic mutation in another 20% of the tumors. Smad4 belongs to the evolutionarily conserved family of Smad proteins (8–11) that are essential intracellular effectors of signals from the TGF[2]-β superfamily of cytokines that regulates a number of cellular processes, including ECM formation, cell proliferation, differentiation, and apoptosis (12–14).

During TGF-β and related protein signaling pathways, binding of TGF-β to the type II receptor results in recruitment and phosphorylation of the type I receptor (12–14). The activation of the type I receptor then propagates the signal by phosphorylating receptor-activated Smads. Among the receptor-activated Smads, Smad2 and Smad3 can be phosphorylated by binding of TGF-β and activin, whereas bone morphogenetic proteins phosphorylate Smad1, Smad5, and Smad8. After phosphorylation, receptor-activated Smads dissociate from the receptor complexes and form a heteromeric complex with a common partner Smad such as Smad4. The Smad complex is then translocated to the nucleus, where it either can directly bind to DNA (15) or associate with other transcription factors (16, 17) to induce TGF-β-responsive gene activation. Because Smad4 serves as a common partner of other Smad proteins, it is pivotal in signaling pathways originating from TGF-β and related proteins.

TGF-β exhibits potent tumor suppressor properties at early stages of carcinogenesis but promotes tumor progression at late stages (18). Inhibition of epithelial cell growth by TGF-β is believed to contribute to a large extent to the tumor suppressor functions of TGF-β. However, tumors secrete large amounts of activated TGF-βs late during tumor progression, which is thought to facilitate invasion and metastasis, induce angiogenesis, and suppress antitumor immune response through effects on cells of both tumor and stromal origin (18). Studies have also suggested that TGF-β-overexpressing tumors are a particularly aggressive subset, wherein patients whose cancers express TGF-β have a prognosis worse than those that do not express TGF-β (18). To overcome the growth suppression effects of TGF-β during tumorigenesis, tumor cells often acquire mutations in either type II receptors or in the signal transducers *Smad4* and *Smad2* (18). The extent of TGF-β resistance correlates with metastatic progression, and targeted deletion of an essential component of the TGF-β-signaling cascade, Smad3, promotes metastasis (19).

Results from several studies have suggested that defects in Smad4 play a significant role in the malignant progression of tumors. Pancreatic cancer, which often exhibits defects in Smad4, is highly aggressive, with most of the patients having metastatic disease at the time of diagnosis. In the development of pancreatic adenocarcinoma, inactivation of *Smad4* gene occurs late at the stage of *in situ* or even invasive carcinoma (20). Inactivation of Smad4 in the *APC*-knockout mouse, a model for human familial adenomatous polyposis, results in more rapid development of malignant tumors than in *APC* heterozygotes (21). On the basis of these results, mutations in Smad4 were suggested to play an important role in malignant progression of colorectal tumors (21). Expression of Smad4 in Smad4-defective tumor cell lines has been shown to restore TGF-β signaling and induce cell cycle arrest or apoptosis (22). In addition to the antiproliferative activity, Smad4 expression in tumor cell lines has been shown to inhibit the expression of vascular endothelial growth factor and enhance the levels of angiogenesis inhibitor thrombospondin-1, causing cells to switch from potently angiogenic to antiangiogenic *in vitro* and *in vivo* (23).

To additionally characterize the functional consequences of restoring TGF-β signaling in Smad4-defective cells, we constructed a recombinant Ad encoding human *Smad4*. Infection of Smad4-defective breast carcinoma cell line MDA-MB-468 with this recombinant Ad vector restored TGF-β signaling and growth inhibition, altered cell morphology, and sensitized them to anoikis. Additional characterization of cells expressing Smad4 revealed increased surface expression of β1 integrin and increased levels of FAK2, but not FAK1, and

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[2] The abbreviations used are: TGF, transforming growth factor; ECM, extracellular matrix; Ad, adenovirus; FAK, focal adhesion kinase; PAI-1, plasminogen activator inhibitor 1; β-gal, β-galactosidase; APC, adenomatous polyposis coli; SAPK, stress-activated protein kinase; JNK, c-Jun NH_2-terminal kinase.

activation of caspases, including caspase 8. These findings suggest that the loss of TGF-β signaling mediated by Smad4 plays an important role in matrix-independent survival of tumor cells, which in turn may contribute to the invasiveness of tumor cells.

MATERIALS AND METHODS

Cell Lines and Viruses. All cell lines were obtained from American Type Culture Collection (Manassas, VA) and were propagated as monolayer cultures at 37°C in medium supplemented with antibiotic-antimycotic (Life Technologies, Inc. Grand Island, NY). The breast carcinoma cell line MDA-MB-468 was grown in Kaighn's DME/F12 (1:1) mixture supplemented with 10% FCS and insulin at 10 μg/ml (Clonetics, San Diego, CA). Colon carcinoma cell lines, WiDr, Caco-2, and SW480, pancreatic cell line MiaPaCa-2, and hepatocellular carcinoma cell line Hep3B were propagated in DMEM containing 10% fetal bovine serum. HEK-293, which is transformed with Ad type 5 E1 region, was obtained from Microbix (Toronto, Ontario, Canada) and maintained in Eagle's minimal essential medium with Earle's salts fortified with 10% fetal bovine serum.

Generation of Recombinant Ad Encoding Smad4. Recombinant Ad vectors with deletion in early region 1 rendering them replication deficient were constructed using the standard methods. The coding sequence for Smad4 was cloned downstream of the cytomegalovirus immediate-early promoter/enhancer and the adenoviral tripartite leader into an Ad transfer plasmid, pAN (24). Recombinant Ads were generated by cotransfection of Ad early region 1-complementing HEK-293 cells with linearized transfer plasmid and *Cla*I-digested large fragment of viral DNA as described previously (24). The large fragment of viral DNA is a derivative of Ad serotype 5 with deletion in the early region 1, polypeptide IX, early region 3, and part of early region 4. Viral plaques were isolated, amplified, and purified by column chromatography as described previously (25). The viral preparations were characterized by restriction enzyme digestion and DNA sequencing across the transgene and the promoter sequence.

Immunoblot Analysis. Proteins were separated on SDS-10% polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose. The nitrocellulose blots were blocked in 20% nonfat dry milk in PBS containing 0.05% Tween 20 (PBST) for 30–60 min. The blots were then incubated with the primary antibodies diluted in PBST containing 5% nonfat dry milk and incubated for 2 h at room temperature. Monoclonal anti-Smad4 (Upstate Biotechnology, Lake Placid, NY), anti-p21 (Calbiochem, San Diego, CA), anti-FAK antibody (Transduction Laboratories, Lexington, KY), anti-PYK2/FAK2 (Transduction Laboratories), and anti-β actin antibody (Sigma, St. Louis, MO) were used as recommended by the suppliers. The blots were washed with PBST and further incubated with secondary antibody [horseradish peroxidase-conjugated antimouse (1:4000) or antirabbit IgG (1:10,000; Jackson Immunoresearch Laboratories, West Grove, PA)] in PBST containing 5% nonfat dry milk. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia, Arlington Heights, IL).

Luciferase Assays. The luciferase construct with PAI-1 promoter was a kind gift of Dr. David Luskutoff (Scripps Research Institute, La Jolla, CA; Ref. 26). Cells (6.2×10^4) were seeded in each well of a 24-well tissue culture plate and grown overnight. Transfection was performed using Superfect (Qiagen, Valencia, CA) and 0.375 μg of PAI-1 luciferase plasmid for each well followed by infection with either Ad-Smad4 or Ad-β-gal at indicated concentrations for 1 h and incubated for 48 h with or without the addition of TGF-β1 (Life Technologies, Inc., Grand Rapids, NY) at 2.5 ng/ml. Cell lysates were prepared and luciferase activities determined using a commercially available kit and TopCount (Packard, Meriden, CT).

Analysis of Integrin β1 Expression. Cells were suspended by treatment with Nozyme (JRH Biosciences, Lenexa, KS) and washed twice with PBS. Cells were then resuspended at a concentration of 1×10^6 cells/ml in growth medium containing anti-β1 integrin rabbit polyclonal antibody (Chemicon, Temecula, CA; catalogue no. 21C8) and incubated at 4°C for 1 h. After washing cells with PBS to remove excess primary antibody, cells were incubated with fluorescein isothiocyanate-conjugated rabbit antimouse secondary antibody (Zymed Laboratories, South San Francisco, CA) at 4°C for 1 h. Cells were then washed with PBS and immediately analyzed by flow cytometry.

Soft Agar Colony Formation Assays. Matrix-independent growth of MDA-MB-468 cells infected with either Ad-Smad4 or Ad-β-gal and incubated in the presence or absence of TGF-β1 at 2.5 ng/ml was assessed in soft agar colony formation assays as described previously (27).

Annexin V-FITC Staining. Apotosis was monitored by microscopic observation of blebbed nuclei and by labeling cells with Annexin V-FITC (Boehringer Mannheim, Indianapolis, IN; Ref. 28) followed by flow cytometric analysis according to the instructions from the manufacturer.

Caspase Assays. Caspase assays were performed as described previously (29). Cells (1×10^6) were lysed in 50 μl of cell lysis buffer (Clontech Laboratories, Palo Alto, CA). To the cell lysates, 20 mM appropriate caspase substrate were added and incubated at 37°C for 1 h. Caspase substrates used were Ac-YVAD-AFC for caspase 1, Ac-VDVAD-AFC for caspase 2, Ac-DEVD-AFC for caspase 3, Ac-VEID-AFC for caspase 6, and Ac-IETD-AFC for caspase 8 (all from Enzyme Systems). Fluorescence was then measured on a Cytofluor fluorescence multiwell plate reader (Perspective Biosystems) with 400 nm-excitation and 505-nm emission filters. Activities were expressed in arbitrary fluorescence units.

RESULTS

Functional Expression of Smad4 Using a Recombinant Ad. To transiently express Smad4 for its functional analysis, we constructed a replication-defective Ad encoding Smad4 (Ad-Smad4). To verify Smad4 expression, Smad4-null MDA-MB-468 cells (22) infected with recombinant Ads were fractionated into cytoplasmic and nuclear fractions and subjected to immunoblot analysis. As shown in Fig. 1*A*, Smad4 was detected in cells infected with Ad-Smad4 but not with the control virus Ad-β-gal. Smad4 was detected predominantly in the nuclear fractions, indicating the proper localization of the recombinant protein.

Smad4 Expression Restores TGF-β Signaling. Because the activity of the PAI-1 promoter is dependent upon TGF-β signaling (26, 30), reporter assays using PAI-1 promoter are widely used to determine TGF-β signaling (31). We performed reporter assays using a plasmid construct encoding luciferase under the control of PAI-1 promoter. MDA-MB-468 cells were transfected with the PAI-1 luciferase construct and then infected with Ad-Smad4 or Ad-β-gal. At 48 h after transfection, cells were harvested and assayed for luciferase activity. Consistent with previous studies that showed restoration of TGF-β pathway upon Smad4 expression (22, 32), Ad-Smad4 infection resulted in a dose-dependent increase in the luciferase activity in a TGF-β-dependent manner, indicating that expression of Smad4 restores TGF-β signaling (Fig. 1*B*). Interestingly, when cells were infected with Ad-Smad4, there was an increase in luciferase activity even in the absence of exogenous TGF-β, raising the possibility that overexpression of Smad4 may confer ligand-independent activation of PAI-1 promoter. On the other hand, Ad-Smad4 infection of MiaPaCa-2 cells, which are defective for TGF-β receptor II (33), showed no increase in luciferase activity, suggesting that overexpression of Smad4 does not lead to TGF-β-independent PAI-1 promoter activity (data not shown). Another possibility is that the increase in luciferase activity in the absence of exogenous TGF-β could be because of an autocrine response. Consistent with this notion, when neutralizing anti-TGF-β antibodies were added to the culture, a reduction in luciferase activity was observed (data not shown).

As expected, restoration of TGF-β signaling was not limited to MDA-MB-468 cells. Transient transfection assays showed that in other Smad4-altered cell lines such as WiDr, SW480 (23), and Caco-2 (34), expression of Smad4 using the recombinant Ad resulted in TGF-β-dependent activity of the PAI-1 promoter (Table 1). In both MiaPaCa-2 (33) and Hep3B, cell lines with wild-type Smad4, there was no increase in PAI promoter activity with Smad4 expression, suggesting that restoration of TGF-β signaling because of Smad4 expression is specific to cell lines with defective Smad4.

Cyclin-dependent kinase inhibitor, p21, has been shown to be

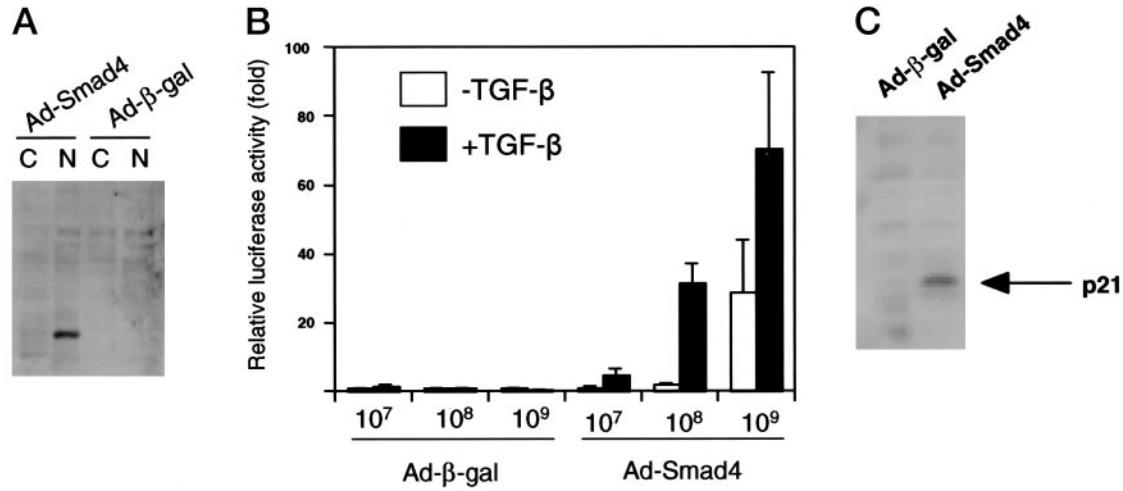

Fig. 1. Smad4 expression using a recombinant Ad restores TGF-β signaling. *A*, MDA-MB-468 cells infected with recombinant Ads encoding either Smad4 (Ad-Smad4) or β-galactosidase (Ad-β-gal) at 1×10^8 particles/ml for 1 h and incubated in the presence of TGF-β1 at 2.5 ng/ml were harvested 48 h after infection. Cells were fractionated into cytoplasmic (*C*) and nuclear (*N*) fractions and subjected to immunoblot analysis for Smad4. *B*, PAI-1 luciferase assays. MDA-MB-468 cells were transiently transfected with a plasmid containing the luciferase gene fused to PAI-1 promoter followed by infection with Ad-Smad4 or Ad-β-gal at indicated concentrations in particles/ml for 1 h. Cells were then incubated for 48 h with or without TGF-β1 addition at 2.5 ng/ml. After measuring the luciferase activity in cell lysates, relative activity was expressed as the fold-increase over the activity obtained with Ad-β-gal infection. The mean was from triplicate transfections. *C*, MDA-MB-468 cells infected with Ad-Smad4 or Ad-β-gal at 1×10^8 particles/ml for 1 h and incubated in the presence of TGF-β1 were harvested 48 h after infection. Cell lysates were subjected to immunoblot analysis for p21.

Table 1 *Ability of Ad-Smad4 to confer TGF-β responsiveness as determined in PAI-luciferase assays*

Cell line[a]	Smad4 status	Fold stimulation[b]
MDA-MB-468	Defective	105.6 ± 33.4
WiDr	Defective	22.4 ± 5.6
Caco-2	Defective	4.1 ± 0.1
SW480	Defective	4.3 ± 0.4
MiaPaCa-2	Functional	1.3 ± 0.3
Hep3B	Functional	0.4 ± 0.1

[a] Among these cell lines, MiaPaCa-2 is known to be defective for TGF-β receptor II.
[b] Cells were transiently transfected with a plasmid containing the luciferase gene fused to PAI-1 promoter followed by infection with Ad-Smad4 or Ad-β-gal at 1×10^9 particles/ml for 1 h. Cells were then incubated with or without TGF-β1 at 2.5 ng/ml for 48 h. After measuring the luciferase activity in cell lysates, fold stimulation was calculated by comparing the activity obtained with TGF-β1-treated Ad-Smad4-infected cells with that in Ad-β-gal-infected cells. In cells infected with Ad-β-gal, induction of luciferase activity upon TGF-β1 addition was 5.8-fold in Hep3B and <1.6-fold in all other cell lines.

transcriptionally regulated after restoration of TGF-β signaling by expression of Smad4 (33, 35). Consistent with these findings, immunoblot analysis of Ad-Smad4-infected cells also showed p21 induction, additionally supporting restoration of TGF-β signaling (Fig. 1*C*).

Smad4 Expression Alters Cell Morphology and Enhances β1 Integrin Expression. Interestingly, expression of Smad4 in MDA-MB-468 cells resulted in subtle but reproducible morphological changes. Smad4-expressing cells showed a flattened and stretched morphology, suggesting alteration in cell-matrix interactions (Fig. 2*A*). Among various receptors for ECM proteins, β1 integrins are known to be transcriptionally regulated by TGF-β (36–38). ECM interactions through β1 integrins play crucial roles in cell cycle progression and cell survival (39). Flow cytometric analysis of Ad-Smad4-infected cells cultured in the presence TGF-β1 indicated dose-dependent increase in β1 integrin levels (Fig. 2*B*).

Smad4 Expression Inhibits Soft Agar Colony Formation. Changes in cell morphology have been shown to be the common feature of malignant transformation in many tumor cells. A hallmark of transformation is that transformed cells can grow in the absence of cell anchorage, as reflected by their ability to grow in soft agar. Restoration of TGF-β signaling by overexpression of TGF-β type II receptor has been shown to result in marked reduction in colony-forming ability in soft agar (40). To determine whether the morpho-

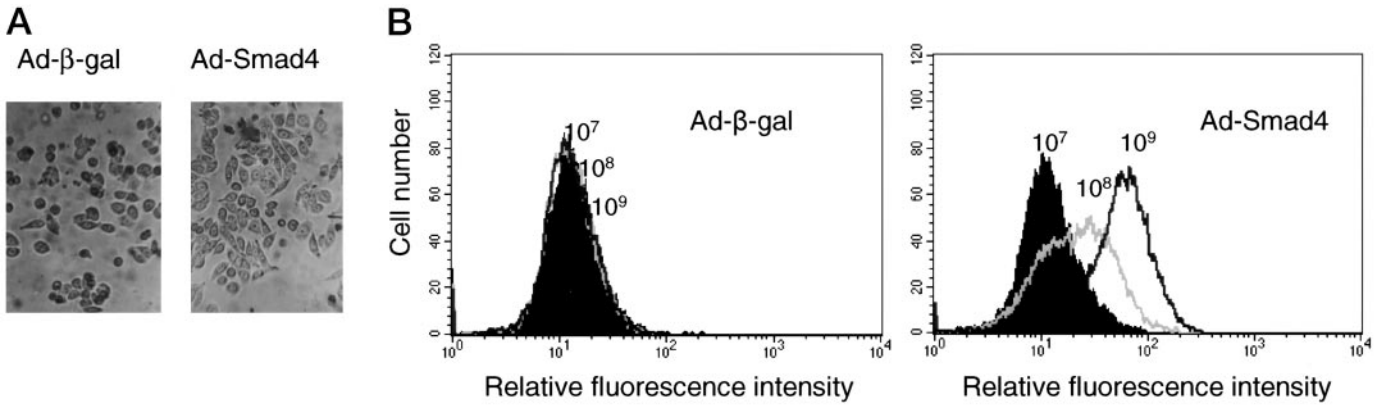

Fig. 2. Smad4 expression leads to a spreading phenotype and increased β1 integrin expression on the cell surface. *A*, MDA-MB-468 cells infected with Ad-Smad4 or Ad-β-gal at 1×10^9 particles/ml for 1 h and incubated in the presence of TGF-β1 at 2.5 ng/ml. Cells were observed under the microscope at 48 h after infection. *B*, MDA-MB-468 cells infected with either Ad-Smad4 or Ad-β-gal at either 1×10^7 (*filled histogram*), 1×10^8 (*gray, unfilled histogram*), or 1×10^9 (*black, unfilled histogram*) particles/ml for 1 h and incubated in the presence of TGF-β1 at 2.5 ng/ml were harvested at 48 h after infection. After staining with FITC-conjugated anti-β1-integrin antibody, samples were assessed by flow cytometric analysis.

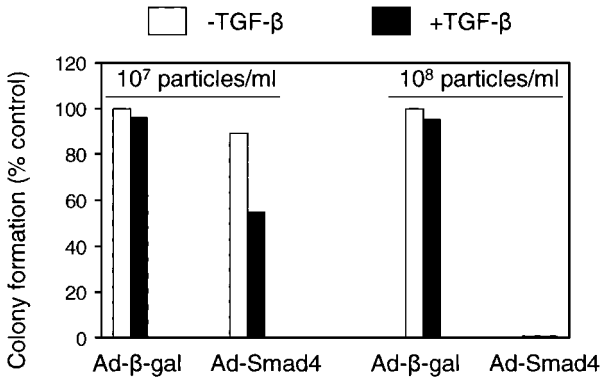

Fig. 3. Smad4 expression leads to inhibition of soft agar colony formation. MDA-MB-468 cells were infected with either Ad-Smad4 or Ad-β-gal at indicated concentrations, and matrix-independent growth in the absence or presence of TGF-β1 at 2.5 ng/ml was assessed in soft agar colony formation assays. Colonies were counted 4 weeks after infection and expressed as the percentage of colony formed in the absence of TGF-β1 after infection with Ad-β-gal at 1×10^7 particles/ml for 1 h. Data from a representative experiment are shown.

logical changes and greater β1 integrin on cell surface induced by Smad4 expression correlate with a reduction in the transformation properties of these cells, we compared the ability of MDA-MB-468 cells infected with Ad-Smad4 or Ad-β-gal to grow in soft agar. Results show that although Ad-β-gal-infected cells readily grew in soft agar, there was a dose-dependent reduction in colony-forming ability of Ad-Smad4-infected cells (Fig. 3). When infected with Ad-Smad4 at a dose of 1×10^8 particles/ml, cells completely lost their ability to grow in soft agar, indicating that Smad4 expression suppressed the anchorage-independent growth of MDA-MB-468 cells. TGF-β-dependent inhibition of soft agar colony formation was also evident when cells were infected at a lower dose of Ad-Smad4 (1×10^7 particles/ml; Fig. 3).

Smad4 Expression Induces Anoikis. Inhibition of anchorage-independent growth could be because of inhibition of cell cycle progression or induction of apoptosis. Smad4 expression and TGF-β pathway restoration have been shown to result in inhibition of cell cycle progression, predominantly by inducing cell cycle arrest at the G_1 phase (22). However, microscopic observation of the cells in the soft agar suggested cell death rather than cell cycle arrest after expression of Smad4. Consistent with previous results, cell cycle analysis of Smad4-expressing cells indicated modest inhibition of cell cycle progression (data not shown). Intriguingly, apoptosis assays such as annexin V staining and caspase activation assays revealed no significant apoptosis in Smad4-expressing cells with or without TGF-β addition. However, because Smad4 expression resulted in increased β1 integrin expression and apparent induction of cell death in soft agar, we sought to evaluate the effect of detachment from the matrix on cell survival. Interestingly, only upon detachment from the matrix and incubation in suspension, the Smad4-expressing cell underwent rapid apoptosis (Fig. 4*A*). Maximum induction of apoptosis was observed within 3–4 h after incubation in suspension. Induction of apoptosis after loss of adhesion, also known as anoikis, was TGF-β-dependent as indicated by >2-fold increase in the percentage of apoptotic cells with the addition of TGF-β1 to the culture (Fig. 4*B*). TGF-β-dependent anoikis upon Ad-Smad4 infection was also observed, albeit at lower levels, in other Smad4-defective cell lines such as WiDr and Caco-2 but not in Smad4 functional but TGF-β receptor II-defective MiaPaCa-2 cell line (Fig. 4*C*).

Increased Levels of FAK2 and Caspase Activity during Anoikis. To obtain additional insight into the mechanism of anoikis induced by Smad4, the levels of FAK, a known transducer of signaling from β1 integrin, and a related protein FAK2 (also known as Pyk2) that shares considerable homology with FAK were examined. Cells were infected at various doses with Ad-Smad4 and the control virus Ad-β-gal. Cell lysates were prepared and subjected to immunoblot analysis using antibodies that specifically recognize either FAK or FAK2. Results indicated that the level of FAK did not increase with Ad-Smad4 infection and detachment from the matrix (Fig. 5), whereas the level of FAK2 was dramatically higher in cells infected with Smad-Smad4 and detached 48 h after infection (Fig. 5). These results suggest the possibility that increased levels of FAK2 might contribute to induction of anoikis by Smad4.

Activation of Caspases. Activation of caspases was determined using specific substrates that become fluorescent upon cleavage by

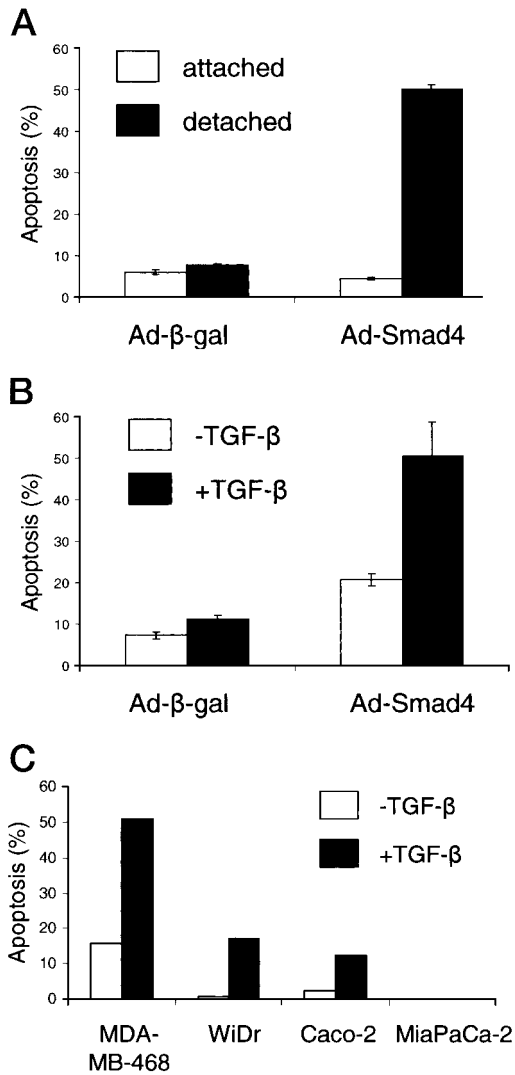

Fig. 4. Restoration of TGF-β signaling sensitizes cells to anoikis. *A*, MDA-MB-468 cells were infected with either Ad-Smad4 or Ad-β-gal at 1×10^9 particles/ml for 1 h and incubated in the presence of TGF-β for 48 h. Forty-eight h after infection, cells were either trypsinized and incubated in suspension in Costar ultra low attachment plates (Corning, Inc., Corning, NY) for 4 h (detached) or trypsinized and analyzed immediately (attached). The extent of apoptosis was determined by annexin V staining and flow cytometric analysis. *B*, MDA-MB-468 cells were infected with either Ad-Smad4 or Ad-β-gal at 1×10^9 particles/ml for 1 h and incubated either in the absence or presence of TGF-β1 at 2.5 ng/ml for 48 h. At 48 h after infection, cells were trypsinized and incubated in suspension for 4 h, and the extent of apoptosis was determined by annexin V staining and flow cytometric analysis. *C*, MDA-MB-468, WiDr, Caco-2, and MiaPaCa-2 cells were infected with either Ad-Smad4 or Ad-β-gal and treated as described in *B*. After determining the extent of apoptosis following incubation in suspension, the percentage of apoptosis was plotted after subtracting the percentage of apoptosis observed with Ad-β-gal infection with that observed with Ad-Smad4 infection. Data from a representative experiment are shown.

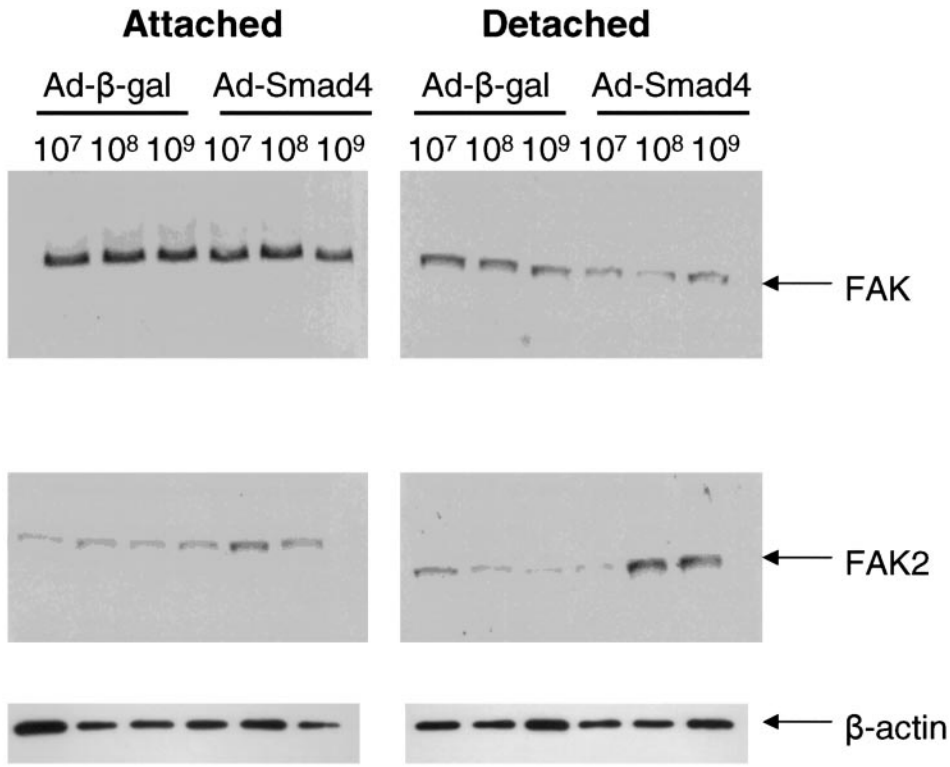

Fig. 5. FAK2/Pyk2 but not FAK levels increased during anoikis sensitized because of Smad4 expression. MDA-MB-468 cells were infected with either Ad-Smad4 or Ad-β-gal at indicated concentrations for 1 h and incubated in the presence of TGF-β1 at 2.5 ng/ml for 48 h. Cells were then either trypsinized and incubated in suspension for 4 h (detached) or trypsinized and analyzed immediately (attached) by immunoblot analysis using anti-FAK (*top*), anti-FAK2 (*middle*), or anti-β actin (*bottom*) antibody.

caspases. Results indicated lack of any significant caspase activation in cells infected with Ad-β-gal either under attached or detached conditions (Fig. 6). However, consistent with the induction of anoikis after detachment from the matrix, detached cells exhibited dramatically activated (4–8-fold) caspases 2, 3, 6, and 8 but not caspase 1 (Fig. 6).

DISCUSSION

Results from this study demonstrate that adenovirus-mediated expression of Smad4 restores TGF-β signaling in Smad4-defective cell lines. Restoration of TGF-β signaling was indicated by TGF-β-dependent activation of PAI-1 promoter, and expression of p21 and integrin β1. In addition to the previously reported growth suppression effects after restoration of TGF-β signaling, we observed sensitization of cells to anoikis. During TGF-β-dependent anoikis, Smad4-expressing cells exhibited increased levels of FAK2 and activation of multiple caspases, including caspase 8.

Integrin receptor-mediated interaction of ECM proteins is known to activate signals that regulate proliferation and survival of epithelial cells. After detachment of cells from ECM, survival signals are down-regulated, and the cells stop proliferating and undergo anoikis (41, 42). Anoikis is believed to suppress expansion of oncogenic transformed cells by preventing proliferation at inappropriate locations. However, many cancer cells are capable of growing at inappropriate locations because of specific defects leading to inhibition of anoikis. Consistent with these findings, suppression of anoikis has been shown to enhance metastasis and dissemination of malignant cells in experimental models (43, 44). Results presented in this study demonstrate that restoration of TGF-β signaling confers sensitivity to anoikis, thus supporting that defects in TGF-β signaling contribute to suppression of anoikis. These findings are consistent with the reports that TGF-β signaling defects result in metastatic phenotypes.

The following findings indicate that sensitization of cells to anoikis is not an experimental artifact or attributable to nonspecific toxicity of Smad4 overexpression. In cells infected with Ad-Smad4, but not with Ad-β-gal, and treated with TGF-β, there was a dose-dependent increase in integrin β1 levels (Fig. 2*B*). Increased level of β1 integrins in Smad4-expressing cells is in agreement with previous findings that TGF-β pathway leads to enhanced transcription of β1 integrins (38). Unligated integrin β1 has been previously shown to be associated with apoptosis and reduced tumor cell growth *in vitro* and *in vivo* (39, 45). It has also been shown that expression of unligated β integrins induces apoptosis by recruiting caspase 8 to the membrane for acti-

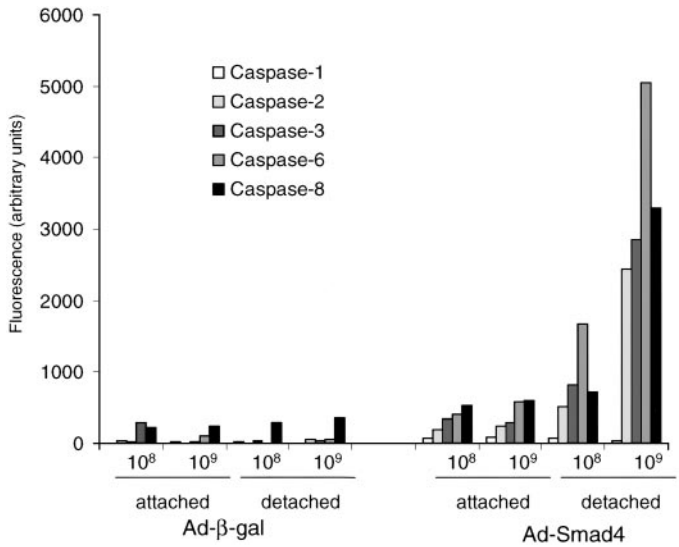

Fig. 6. Multiple caspases, including caspase 8, are activated during Smad4-sensitized anoikis. MDA-MB-468 cells were infected with either Ad-Smad4 or Ad-β-gal at indicated concentrations for 1 h and incubated in the presence of TGF-β1 at 2.5 ng/ml for 48 h. At 48 h after infection, cells were either trypsinized and incubated in suspension for 4 h (detached) or trypsinized and analyzed immediately (attached) for caspase activation using specific caspase substrates that become fluorescent upon cleavage. Data from a representative experiment are shown.

vation (45). Our finding that detachment from the matrix, which results in unligated integrin $\beta 1$, also leads to activation of caspase 8 is consistent with the results of an earlier study (45). We have also observed that anoikis was dependent not only upon Smad4 expression but also upon the addition of TGF-β, indicating that anoikis is not attributable to a nonspecific toxicity associated with Smad4 overexpression. Furthermore, MiaPaCa-2 cells that lack TGF-β receptor II failed to undergo anoikis (Fig. 4*C*) upon Smad4 expression, indicating that in addition to Smad4 expression and TGF-β addition, anoikis requires functional restoration of TGF-β signaling. Additionally, the extent of anoikis in Smad4-defective cell lines (Fig. 4*C*) paralleled with the degree of restoration of TGF-β signaling as determined in the PAI-luciferase assays (Table 1). The differences in the extent of anoikis and PAI-luciferase activities among cell lines are likely because of a different degree of infectivity of these cell lines. A lack of a nonspecific toxicity was also supported by the observation that cells do not undergo apoptosis with Smad4 expression when attached to the matrix either with or without TGF-β.

Signals triggered after integrin receptor-mediated interaction of ECM proteins resulting in regulation of proliferation and survival are known to be mediated mainly through FAK and phosphatidylinositol 3′-kinase/Akt-mediated pathways (46, 47). FAK is a cytoplasmic tyrosine kinase localized in focal contacts, which has been implicated to play a role in regulating cellular morphology and migration in response to cell adhesion to ECM proteins (46, 47). Cell attachment to fibronectin-coated surfaces or integrin clustering by antibodies induces FAK tyrosine phosphorylation. FAK2 is another related focal adhesion tyrosine kinase (also known as RAFTK, Pyk2, and CAKβ) that shows 65% homology with FAK (48, 49). Unlike FAK, FAK2 is activated by an increase in intracellular calcium levels or treatment with tumor necrosis factor α and UV light (48, 49). In this study, we observed no significant changes in FAK levels when Smad4-expressing TGF-β pathway restored cells were undergoing anoikis. However, FAK2 levels were greatly enhanced. Increased FAK2 levels in cells undergoing apoptosis are consistent with the findings that overexpression of FAK2, but not FAK, in rat and mouse fibroblasts results in induction of apoptosis (50). FAK2 is also activated under conditions that lead to apoptosis such as treatment with tumor necrosis factor α, UV irradiation, and changes in osmolarity (48, 49). FAK2 has been shown to be a cell type-specific, stress-sensitive mediator of the SAPK/JNK signaling pathway (49). Overexpression of FAK2 has been shown to result in activation of JNK and a dominant-negative mutant of FAK2 interfered with UV light- or osmotic shock-induced activation of JNK (49). Previously, the SAPK/JNK signaling pathway has been reported to play an important role in anoikis (51, 52). Overexpression of Smad4 in dog kidney epithelial MDCK cells was shown to result in apoptosis that was regulated by SAPK/JNK (53). It remains to be determined if activation of SAPK/JNK occurs in Smad4-expressing TGF-β pathway restored cells after detachment from the matrix. Studies have shown that in both normal and cancer cells, TGF-β responses are tied to Ras function and SAPK signaling (12). Inhibition of TGF-β responses with the use of dominant-negative Ras mutants and upon inhibition of SAPK pathway in Smad4-null cells have also indicated that activation of Ras/SAPK signaling by TGF-β is independent of Smad4 function (12, 54).

Among the caspases studied, caspase 8 was activated during induction of anoikis after restoration of TGF-β signaling. Previously, caspase 8 was suggested to play a central role in the process of anoikis (55). Suspension culture of MDCK cells showed increase in caspase-8 activity, and inhibition of caspase 8 was shown to block anoikis (56). Importantly, loss of anchorage-mediated caspase 8 activation has been shown to be independent of activation of the executioner caspase 3, thereby indicating that caspase 8 activation is an initiating event in anoikis (57). It is worth noting that unligated integrin or β integrin tails have also been shown to recruit caspase 8 to the membrane where it becomes activated in a death receptor-independent manner (45).

Studies have shown a strong association between the loss of TGF-β/Smad4 signaling and tumor malignancy (18). Inactivation of *Smad4* gene occurs late in the development of pancreatic adenocarcinoma at the stage of *in situ* or even invasive carcinoma (20, 58). Studies with mice with mutations in *Smad4* and *APC* have revealed that Smad4 defect also plays an important role in malignant progression of colorectal tumors (21). The finding that restoration of TGF-β/Smad4 signaling confers sensitivity to anoikis suggests a functional basis for the strong correlation between defects in TGF-β/Smad4 signaling and development of malignancy.

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REFERENCES

1. Hahn, S. A., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Schutte, M., Rozenblum, E., Seymour, A. B., Weinstein, C. L., Yeo, C. J., Hruban, R. H., and Kern, S. E. Homozygous deletion map at 18q21.1 in pancreatic cancer. Cancer Res., *56:* 490–494, 1996.
2. Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science (Wash. DC), *271:* 350–353, 1996.
3. Schutte, M., Hruban, R. H., Hedrick, L., Cho, K. R., Nadasdy, G. M., Weinstein, C. L., Bova, G. S., Isaacs, W. B., Cairns, P., Nawroz, H., Sidransky, D., Casero, R. A., Jr., Meltzer, P. S., Hahn, S. A., and Kern, S. E. DPC4 gene in various tumor types. Cancer Res., *56:* 2527–2530, 1996.
4. Hahn, S. A., Bartsch, D., Schroers, A., Galehdari, H., Becker, M., Ramaswamy, A., Schwarte-Waldhoff, I., Maschek, H., and Schmiegel, W. Mutations of the DPC4/Smad4 gene in biliary tract carcinoma. Cancer Res., *58:* 1124–1126, 1998.
5. Goggins, M., Shekher, M., Turnacioglu, K., Yeo, C. J., Hruban, R. H., and Kern, S. E. Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas. Cancer Res., *58:* 5329–5332, 1998.
6. Thiagalingam, S., Lengauer, C., Leach, F. S., Schutte, M., Hahn, S. A., Overhauser, J., Willson, J. K., Markowitz, S., Hamilton, S. R., Kern, S. E., Kinzler, K. W., and Vogelstein, B. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nat. Genet., *13:* 343–346, 1996.
7. Takagi, Y., Kohmura, H., Futamura, M., Kida, H., Tanemura, H., Shimokawa, K., and Saji, S. Somatic alterations of the DPC4 gene in human colorectal cancers *in vivo*. Gastroenterology, *111:* 1369–1372, 1996.
8. Attisano, L., and Wrana, J. L. Smads as transcriptional co-modulators. Curr. Opin. Cell Biol., *12:* 235–243, 2000.
9. Roberts, A. B. TGF-β signaling from receptors to the nucleus. Microbes Infect., *1:* 1265–1273, 1999.
10. Piek, E., Heldin, C. H., and Ten Dijke, P. Specificity, diversity, and regulation in TGF-β superfamily signaling. FASEB J., *13:* 2105–2124, 1999.
11. Zhou, S., Kinzler, K. W., and Vogelstein, B. Going mad with Smads. N. Engl. J. Med., *341:* 1144–1146, 1999.
12. Yue, J., and Mulder, K. M. Transforming growth factor β signal transduction in epithelial cells. Pharmacol. Ther., *91:* 1–34, 2001.
13. Letamendia, A., Labbe, E., and Attisano, L. Transcriptional regulation by Smads: crosstalk between the TGF-β and Wnt pathways. J. Bone Jt. Surg. Am., *83-A* (*Suppl. 1):* S31–S39, 2001.
14. Massague, J. How cells read TGF-β signals. Nat. Rev. Mol. Cell. Biol., *1:* 169–178, 2000.
15. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol. Cell, *1:* 611–617, 1998.
16. Zhang, Y., Feng, X. H., and Derynck, R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription. Nature (Lond.), *394:* 909–913, 1998.
17. Feng, X. H., Zhang, Y., Wu, R. Y., and Derynck, R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-β-induced transcriptional activation. Genes Dev., *12:* 2153–2163, 1998.
18. Reiss, M. TGF-β and cancer. Microbes Infect., *1:* 1327–1347, 1999.
19. Zhu, Y., Richardson, J. A., Parada, L. F., and Graff, J. M. Smad3 mutant mice develop metastatic colorectal cancer. Cell, *94:* 703–714, 1998.
20. Wilentz, R. E., Iacobuzio-Donahue, C. A., Argani, P., McCarthy, D. M., Parsons, J. L., Yeo, C. J., Kern, S. E., and Hruban, R. H. Loss of expression of Dpc4 in

pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res., *60:* 2002–2006, 2000.
21. Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M. F., and Taketo, M. M. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. Cell, *92:* 645–656, 1998.
22. Dai, J. L., Bansal, R. K., and Kern, S. E. G_1 cell cycle arrest and apoptosis induction by nuclear Smad4/Dpc4: phenotypes reversed by a tumorigenic mutation. Proc. Natl. Acad. Sci. USA., *96:* 1427–1432, 1999.
23. Schwarte-Waldhoff, I., Klein, S., Blass-Kampmann, S., Hintelmann, A., Eilert, C., Dreschers, S., Kalthoff, H., Hahn, S. A., and Schmiegel, W. DPC4/SMAD4-mediated tumor suppression of colon carcinoma cells is associated with reduced urokinase expression. Oncogene, *18:* 3152–3158, 1999.
24. Wills, K. N., Huang, W. M., Harris, M. P., Machemer, T., Maneval, D. C., and Gregory, R. J. Gene therapy for hepatocellular carcinoma: chemosensitivity conferred by adenovirus-mediated transfer of the HSV-1 thymidine kinase gene. Cancer Gene Ther., *2:* 191–197, 1995.
25. Huyghe, B. G., Liu, X., Sutjipto, S., Sugarman, B. J., Horn, M. T., Shepard, H. M., Scandella, C. J., and Shabram, P. Purification of a type 5 recombinant adenovirus encoding human p53 by column chromatography. Hum. Gene Ther., *6:* 1403–1416, 1995.
26. Keeton, M. R., Curriden, S. A., van Zonneveld, A. J., and Loskutoff, D. J. Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor β. J. Biol. Chem., *266:* 23048–23052, 1991.
27. Dreijerink, K., Braga, E., Kuzmin, I., Geil, L., Duh, F. M., Angeloni, D., Zbar, B., Lerman, M. I., Stanbridge, E. J., Minna, J. D., Protopopov, A., Li, J., Kashuba, V., Klein, G., and Zabarovsky, E. R. The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc. Natl. Acad. Sci. USA., *98:* 7504–7509, 2001.
28. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods, *184:* 39–51, 1995.
29. Atencio, I. A., Ramachandra, M., Shabram, P., and Demers, G. W. Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. Cell Growth Differ., *11:* 247–253, 2000.
30. Riccio, A., Lund, L. R., Sartorio, R., Lania, A., Andreasen, P. A., Dano, K., and Blasi, F. The regulatory region of the human plasminogen activator inhibitor type-1 (PAI-1) gene. Nucleic Acids Res., *16:* 2805–2824, 1988.
31. Abe, M., Harpel, J. G., Metz, C. N., Nunes, I., Loskutoff, D. J., and Rifkin, D. B. An assay for transforming growth factor β using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal. Biochem., *216:* 276–284, 1994.
32. de Winter, J. P., Roelen, B. A., ten Dijke, P., van der Burg, B., and van den Eijnden-van Raaij, A. J. DPC4 (SMAD4) mediates transforming growth factor β1 (TGF-β1) induced growth inhibition and transcriptional response in breast tumour cells. Oncogene, *14:* 1891–1899, 1997.
33. Grau, A. M., Zhang, L., Wang, W., Ruan, S., Evans, D. B., Abbruzzese, J. L., Zhang, W., and Chiao, P. J. Induction of p21waf1 expression and growth inhibition by transforming growth factor β involve the tumor suppressor gene DPC4 in human pancreatic adenocarcinoma cells. Cancer Res., *57:* 3929–3934, 1997.
34. Barbera, V. M., Martin, M., Marinoso, L., Munne, A., Carrato, A., Real, F. X., and Fabre, M. The 18q21 region in colorectal and pancreatic cancer: independent loss of DCC and DPC4 expression. Biochim. Biophys. Acta, *1502:* 283–296, 2000.
35. Moustakas, A., and Kardassis, D. Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. Proc. Natl. Acad. Sci. USA, *95:* 6733–6738, 1998.
36. Wang, D., Zhou, G. H., Birkenmeier, T. M., Gong, J., Sun, L., and Brattain, M. G. Autocrine transforming growth factor β1 modulates the expression of integrin $\alpha5\beta1$ in human colon carcinoma FET cells. J. Biol. Chem., *270:* 14154–14159, 1995.
37. Weinstein, M., Monga, S. P., Liu, Y., Brodie, S. G., Tang, Y., Li, C., Mishra, L., and Deng, C. X. Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on β1-integrin to promote normal liver development. Mol. Cell. Biol., *21:* 5122–5131, 2001.
38. Kagami, S., Kuhara, T., Yasutomo, K., Okada, K., Loster, K., Reutter, W., and Kuroda, Y. Transforming growth factor β (TGF-β) stimulates the expression of β1 integrins and adhesion by rat mesangial cells. Exp. Cell Res., *229:* 1–6, 1996.
39. Ruoslahti, E. Fibronectin and its integrin receptors in cancer. Adv. Cancer Res., *76:* 1–20, 1999.
40. Guo, Y., and Kyprianou, N. Overexpression of transforming growth factor (TGF) β1 type II receptor restores TGF-β1 sensitivity and signaling in human prostate cancer cells. Cell Growth Differ., *9:* 185–193, 1998.
41. Frisch, S. M., and Ruoslahti, E. Integrins and anoikis. Curr. Opin. Cell Biol., *9:* 701–706, 1997.
42. Frisch, S. M., and Screaton, R. A. Anoikis mechanisms. Curr. Opin. Cell Biol., *13:* 555–562, 2001.
43. Yawata, A., Adachi, M., Okuda, H., Naishiro, Y., Takamura, T., Hareyama, M., Takayama, S., Reed, J. C., and Imai, K. Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. Oncogene, *16:* 2681–2686, 1998.
44. Takaoka, A., Adachi, M., Okuda, H., Sato, S., Yawata, A., Hinoda, Y., Takayama, S., Reed, J. C., and Imai, K. Anti-cell death activity promotes pulmonary metastasis of melanoma cells. Oncogene, *14:* 2971–2977, 1997.
45. Stupack, D. G., Puente, X. S., Boutsaboualoy, S., Storgard, C. M., and Cheresh, D. A. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. J. Cell Biol., *155:* 459–470, 2001.
46. Panetti, T. S. Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration. Front. Biosci., *7:* d143–d150, 2002.
47. Schaller, M. D. Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim. Biophys. Acta, *1540:* 1–21, 2001.
48. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. Nature (Lond.), *376:* 737–745, 1995.
49. Tokiwa, G., Dikic, I., Lev, S., and Schlessinger, J. Activation of Pyk2 by stress signals and coupling with JNK signaling pathway. Science (Wash. DC), *273:* 792–794, 1996.
50. Xiong, W., and Parsons, J. T. Induction of apoptosis after expression of PYK2, a tyrosine kinase structurally related to focal adhesion kinase. J. Cell Biol., *139:* 529–539, 1997.
51. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. Cell, *90:* 315–323, 1997.
52. Frisch, S. M., Vuori, K., Kelaita, D., and Sicks, S. A role for Jun-N-terminal kinase in anoikis; suppression by bcl-2 and crmA. J. Cell Biol., *135:* 1377–1382, 1996.
53. Atfi, A., Buisine, M., Mazars, A., and Gespach, C. Induction of apoptosis by DPC4, a transcriptional factor regulated by transforming growth factor β through stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathway. J. Biol. Chem., *272:* 24731–24734, 1997.
54. Hocevar, B. A., Brown, T. L., and Howe, P. H. TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J., *18:* 1345–1356, 1999.
55. Nishimura, S., Adachi, M., Ishida, T., Matsunaga, T., Uchida, H., Hamada, H., and Imai, K. Adenovirus-mediated transfection of caspase-8 augments anoikis and inhibits peritoneal dissemination of human gastric carcinoma cells. Cancer Res., *61:* 7009–7014, 2001.
56. Frisch, S. M. Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. Curr. Biol., *9:* 1047–1049, 1999.
57. Rytomaa, M., Martins, L. M., and Downward, J. Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. Curr Biol., *9:* 1043–1046, 1999.
58. Iacobuzio-Donahue, C. A., Wilentz, R. E., Argani, P., Yeo, C. J., Cameron, J. L., Kern, S. E., and Hruban, R. H. Dpc4 protein in mucinous cystic neoplasms of the pancreas: frequent loss of expression in invasive carcinomas suggests a role in genetic progression. Am. J. Surg. Pathol., *24:* 1544–1548, 2000.

Restoration of Transforming Growth Factor β Signaling by Functional Expression of Smad4 Induces Anoikis

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