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Reduction in Smad2/3 Signaling Enhances Tumorigenesis but Suppresses Metastasis of Breast Cancer Cell Lines

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ABSTRACT

The role of transforming growth factor β in breast cancer is controversial with tumor suppressor and pro-oncogenic activities having been demonstrated. To address whether the same or different signal transduction pathways mediate these opposing activities, we manipulated the Smad2/3 signaling pathway in cells of common origin but differing degrees of malignancy derived from MCF10A human breast cells. We show that interference with endogenous Smad2/3 signaling enhances the malignancy of xenografted tumors of premalignant and well-differentiated tumor cells but strongly suppresses lung metastases of more aggressive carcinoma cells after tail vein injection. Overexpression of Smad3 in the same cells has opposite effects. The data demonstrate that the Smad2/3 signaling pathway mediates tumor suppressor and prometastatic signals, depending on the cellular context.

INTRODUCTION

The original characterization of TGF-β[1] more than 20 years ago and the properties that led to its naming were based on its ability to induce colony growth of nonneoplastic cells in soft agar, properties that led it to be considered a proximal effector of transformation and a "transforming" growth factor (1). Over the ensuing years, it has become clear that the role of TGF-β in epithelial carcinogenesis is complex with ostensibly different roles in early stages of disease, in which tumor cells retain their sensitivity to inhibition of growth by TGF-β compared with later stages of invasive, metastatic disease, in which tumor cells typically are no longer sensitive to inhibition of growth by TGF-β yet secrete elevated levels of this cytokine (2, 3). Thus tumor suppressor activity and pro-oncogenic activity have been attributed to TGF-β, depending on the particular tumor cell and its stage in malignant progression (2–4).

The progression of cells from normal epithelium to carcinoma is frequently accompanied by a loss of cell surface TGF-β receptors. Most commonly, this involves loss of the ligand-binding TβRII by epigenetic mechanisms involving transcriptional repression (5), although down-regulation of the signal-transducing TβRI by promoter methylation has also been described (6). Together, these receptor changes lead to altered or reduced signaling from TGF-β, typically resulting in diminished sensitivity to inhibition of growth by TGF-β. However, retention of the ability of TGF-β to activate certain gene targets in these cells suggests that all signaling is not lost in most tumor cells, but rather, that certain genes that require only a weak signal flux remain under its control (7). Such genes may, in fact, play an important role in carcinogenesis, especially because late-stage tumor cells additionally secrete relatively high levels of TGF-β, which can act not only on stromal elements in a paracrine fashion but also on the tumor cells themselves (2).

The discovery of the Smad signaling pathway downstream of the TGF-β receptors demonstrated that these molecules were also targets of mutation or epigenetic regulation in carcinogenesis (8, 9). In this pathway, Smad2 and Smad3 are phosphorylated by the TβRI serine/threonine kinase on a COOH-terminal serine motif, enabling them to partner with Smad4 and translocate to the nucleus, where they regulate transcription of target genes. It was shown that the *Smad4* locus (*DPC4*) on chromosome 18q21.1 was either deleted or mutated in 50–80% of pancreatic cancers (10). Loss or mutation of Smad4 has also been described in a variety of other cancers, but the frequency of such mutations in any one cancer is quite low (11). Smad2 is also mutated in some colorectal and lung cancers, yet again with low frequency (12, 13), although its expression is suppressed with relatively high frequency in head and neck squamous carcinomas (14) and some breast cancers. Of note, no mutations have been reported in the *SMAD3* gene, leading us to speculate that there might be some advantage to its retention in fully malignant cells and that it might mediate key oncogenic events in the context of metastasis (8). In addition to the Smad signaling pathway, TGF-β also activates other pathways including the family of MAPKs with downstream kinases ERK1/2, c-Jun NH_2-terminal kinase, and p38. These pathways often act together with Smad signaling to control gene expression and cell phenotype and are likely to contribute to the pro-oncogenic activities of TGF-β (2).

We have used a cell system with defined oncogenic/metastatic potential derived from the parental MCF10A human breast cell line (15–17) to address the role of the Smad signaling pathway in the tumor suppressor and pro-oncogenic activities of TGF-β. Specifically, we have attempted to determine whether the same central Smad2/3 pathway can mediate these two opposing activities or whether there is a change in pathway use as cells progress from premalignant to metastatic during the course of tumorigenesis. To address this, we have examined effects of stable overexpression of either Smad3 or a COOH-terminally truncated dominant negative mutant of Smad3, Smad3ΔC, on responses of the premalignant T-24 mutant Ras-transformed MCF10AT1k cell line (15) and two well-characterized fully malignant variants of that cell line, MCF10CA1 h and MCF10CA1a (17), to TGF-β *in vitro* and on tumorigenesis in nude mice *in vivo*. Although each of these cell lines has a common genetic background, the MCF10AT1k cell line (MII) is the least tumorigenic, forming adenomatous xenografted tumors with a long latency period. One of its derivatives, the MCF10CA1 h cell line (MIII), forms well-differentiated xenografted tumors in about 4–6 weeks, whereas MCF10CA1a (MIV) cells form rapidly growing carcinomas in 7–10 days and are metastatic to lung after i.v. injection [see summary of properties in the paper by Tang *et al.* (18)]. Our results show that signaling through the Smad2/3 pathway can mediate tumor suppressor and oncogenic effects of TGF-β, depending on the stage of progression of the cells and other cooperating contextual changes.

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[1] The abbreviations used are: TGF-β, transforming growth factor β; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; MAPK, mitogen-activated protein kinase; EMT, epithelial to mesenchymal transition; DNTβRII, dominant negative truncated forms of the TGF-β type II receptor; ERK, extracellular signal-regulated kinase; Rb, retinoblastoma; DMEM/F12, DMEM:Ham's F-12.

MATERIALS AND METHODS

Retroviral Constructs. Retroviral constructs expressing wild-type Smad3 or the dominant negative COOH-terminally truncated version of Smad3 (Smad3ΔC) were gifts from Dr. Rik Derynck (UCSF, San Francisco, CA). The mutant was made by deleting the sequence coding for the COOH-terminal SSVS motif of Smad3 (ΔSSVS). Smad3 and Smad3ΔC were expressed from the retroviral construct LPCX, allowing for selection in puromycin (19).

Cell Culture and Generation of Stable Cell Lines. The three human breast cancer cell lines MCF10AT1k.cl2 (MII), MCF10CA1h (MIII), and MCF10CA1a.cl1 (MIV) were obtained from Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI). MII, MIII, and MIV cells were grown in DMEM/F12 (Invitrogen, Carlsbad, CA) and 5% horse serum (Invitrogen) at 37°C with 5% CO_2. MII cells were supplemented additionally with 10 μg/ml insulin (Biofluids, Rockville, MD), 20 ng/ml epidermal growth factor (Biofluids), 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO), and 100 ng/ml cholera toxin (Sigma). The amphotropic retroviral packaging cell line Phoenix A was obtained from Dr. Rik Derynck and maintained in DMEM with high glucose (Invitrogen) and 10% fetal bovine serum (Invitrogen). To generate retroviruses, Phoenix A cells were plated at 4×10^6 cells/100-mm tissue culture dish 24 h before transfection and transfected by the calcium phosphate method (20), using 10 μg DNA/plate. Thirty-six h later, the supernatant containing recombinant retroviruses was collected and filtered through 0.45-μm sterilization filters. Four ml of these supernatants were applied immediately to MII, MIII, or MIV cells preseeded in 100-mm plates at a density of 5×10^5 cells with addition of Polybrene (Sigma) at a final concentration of 4 μg/ml. Selection with 2 μg/ml puromycin (Sigma) was initiated 48 h after infection and continued for 5 days; thereafter, cells were maintained in 0.2 μg/ml puromycin.

Cell Growth Inhibition Assay by TGF-β1 and Immunoblotting. Proliferation of infected MII, MIII, and MIV cells was determined by [^{3}H]thymidine (1 mCi/ml; New England Nuclear, Boston, MA) incorporation for 2 h essentially as described previously (21). For preparation of cell lysates, 60–70% subconfluent cell monolayers were starved to DMEM/F12 and 0.2% horse serum for 18 h and then treated for different time durations with 2 ng/ml TGF-β1 (R&D Systems, Inc., Annapolis, MD). Cells were then solubilized in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 0.05 mg/ml 4-2-aminoethyl-benzenesulfonyl fluoride hydrochloride, and 1 μg/ml leupeptin]. Protein was quantitated by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL), separated by SDS-PAGE gels under reducing conditions, and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Blots were probed using different primary antibodies, detected using horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ), and visualized by chemiluminescence (Pierce Chemical Co.). Antibodies were from the following sources: Smad4, TβRI, and TβRII, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Smad2, Smad3, tubulin, and E-cadherin, Zymed Laboratories, Inc. (South San Francisco, CA); phospho-Smad2 and phospho-Smad3, generous gifts of Dr. Michael Reiss (Cancer Institute of New Jersey, New Brunswick, NJ); phospho-p44/42 and p44/42 protein, Cell Signaling Technology (Beverly, MA); and Rb, BD Transduction Laboratories (San Diego, CA).

Assessment of Tumorigenicity *in Vivo*. MIII and MIV cells from subconfluent monolayers were trypsinized and suspended at a density of 5×10^6 and 1×10^6 cells/ml, respectively, whereas MII cells were suspended in a 1:1 (v/v) mixture of DMEM/F12:Matrigel (BD BioSciences, Bedford, MA) at a density of 5×10^7 cells/ml. Female 4–6-week-old BALB/c-*nu*/*nu* athymic mice were inoculated s.c. on the hind flank with 0.2 ml of the cell suspension. Length and width of tumors (in millimeters) were measured weekly with calipers. Tumor volume was calculated by the formula $(S*S*L) \times 0.52$, where S and L were the short and long dimensions, respectively. Mice were euthanized when tumors reached 2 cm in diameter. Each group had at least five mice with 2 hind flank injections/mouse. Each animal group was repeated at least twice for a minimum total of 10 mice or 20 tumors. Tumor histology was read by a board-certified pathologist (W. T. P.) blinded to the groupings. Paired t test (two-tailed) was used for the comparisons for statistical significance. Protocols for nude mice injections were approved by the National Cancer Institute Animal Care and Use Committee.

Metastasis Assay. Female nude mice, 4–6 weeks old, received i.v. injection of 5×10^5 MIV cells in 0.2 ml of DMEM/F12 via the tail vein. Seven weeks after injection, mice were examined grossly at necropsy for the presence of metastases in internal organs. Microscopic quantification of metastases was performed on representative lung cross-sections of formalin-fixed, paraffin-embedded tissues stained with H&E. The total area of lung metastases and the total area of the lungs were measured by a microscope and its software (QWIN; Leica, Wetzlar, Germany) for each animal.

Indirect Immunofluorescence. Infected MIV cells were plated at $2–3 \times 10^5$ cells onto 22-mm glass coverslips in 6-well plates. At 50–70% confluence, the cells were washed with PBS and switched to DMEM/F12 and 0.2% horse serum overnight. TGF-β1 was then added to treatment groups at a concentration of 8 ng/ml for 72 h. Cells were fixed in cold 3.5% paraformaldehyde for 15 min, permeabilized in cold absolute methanol for 2 min, incubated for 5 min in 50 mM glycine to quench paraformaldehyde autofluorescence, and incubated for 60 min at room temperature with anti-E-cadherin antibody (2 μg/ml; Zymed Laboratories). After washing in PBS, the coverslips were incubated for 30 min at room temperature with FITC-conjugated goat antimouse IgG secondary antibody (Molecular Probes, Inc., Eugene, OR). The coverslips were then mounted in medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Cells were examined using a Leica laser scanning confocal microscope.

Luciferase Reporter Assays. MIII cells infected with different retroviral constructs were plated at a density of 3×10^5 cells/well in 6-well plates 24 h before transfection. Cells were transfected with Fugene6 (Roche, Indianapolis, IN) according to the manufacturer's instructions using the indicated amounts of reporter plasmid [$(SBE)_4$.Luc or FAST1 and ARE.Luc], pSV-β-Gal to normalize transfection efficiency, and pcDNA3 to normalize the amount of transfected DNA. After 48 h, the medium was replaced with low-serum medium (DMEM/F12/0.2% horse serum), and the cells were left untreated or stimulated with 5 ng/ml TGF-β1. Cells were lysed 18 h later, and the luciferase and β-galactosidase activities were determined by VICTOR2 (Perkin-Elmer Life Sciences, Boston, MA).

Immunohistochemical Staining. Staining was performed using the Optimax Plus 2.0 Automated Cell Staining System with research software (BioGenex, San Ramon, CA). For anti-Ki67 antibody (DAKO, Carpinteria, CA), sections were microwaved in 0.01 M citrate buffer (pH 6.0) five times for 4 min at 800 W before blocking nonspecific protein binding. Sections were incubated overnight at 4°C with 1:50 and 1:150 dilutions of monoclonal mouse Ki67 antibody and Smad3 antibody (Zymed Laboratories), respectively. Vectastain Elite mouse or rabbit Avidin: Biotinylated enzyme Complex (ABC) peroxidase kit was used (Vector Laboratories). Other procedures were done according to the manufacturer's protocol. Quantification of Ki67-positive cells was determined for nine randomly chosen high-power fields (magnification, ×400) for each tumor. Similarly, the number of apoptotic cells was quantified after terminal deoxynucleotidyltransferase-mediated nick end labeling staining using the ApoTag kit (Oncor, Gaithersburg, MD).

Biocoat Cell Migration Assay. Cells (5×10^5 cells/0.5 ml) were plated in 24-well inserts (8.0-μm Biocoat Cell Environments plates; Becton Dickinson, Bedford, MA), and 0.5 ng/ml TGF-β1 was added to the bottom wells. Forty-eight h later, cells were fixed and stained by using the Diff-Quik Stain Set (Dade Behring, Deerfield, IL). After wiping off the inside of the chambers using cotton-tipped applicators, migratory cells remained on the bottom of the membranes and were counted under the microscope (magnification, ×320).

Statistical Analysis. All statistical analyses were performed using a two-tailed Student's t test ($P < 0.05$, statistical significance).

RESULTS

Stable Expression of Wild-Type or Dominant Negative Smad3 in MCF10A-Derived Breast Cancer Cell Lines. MCF10At1k (MII), MCF10CA1h (MIII), and MCF10CA1a (MIV) cell lines represent different stages of tumor progression (16, 18). MIII and MIV cell lines were derived from passaging MII cells in mice. Western blot analysis of endogenous TGF-β/Smad signaling components showed that levels of expression of endogenous Smad2, Smad3, Smad4, and TβRI and TβRII did not vary appreciably in the three cell lines, although receptor expression is somewhat higher in MIII compared with MIV cells (Fig. 1*A*). Smad3 was also phosphorylated by TGF-β1

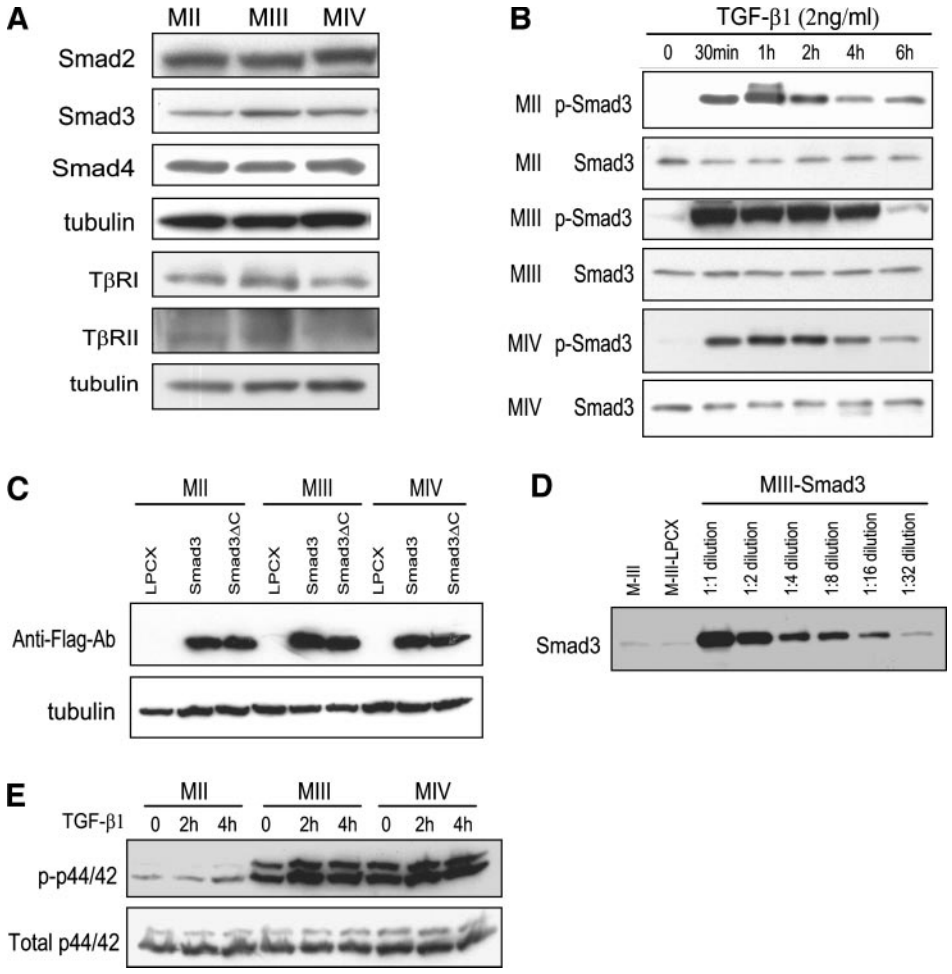

Fig. 1. Expression levels of endogenous Smads, TGF-β receptors, and ectopic Smad3 and Smad3ΔC in MII, MIII, and MIV cells. *A*, expression levels of endogenous components of the TGF-β signaling pathway by Western blotting of cell lysates. Loading control, tubulin. *B*, kinetics of phosphorylation of endogenous Smad3 by TGF-β1 at different times in MII, MIII, and MIV cells. *C*, expression levels of Flag-tagged Smad3 and Smad3ΔC in cells transduced by retroviral LPCX constructs and selected as detected by anti-Flag antibody. Loading control, tubulin. *D*, quantification of the degree of overexpression of ectopic Smad3 by 1:2 serial dilutions of MIII-Smad3 lysates and blotting with an antibody to Smad3. *E*, assessment of MAPK/ERK activity in cells after treatment with TGF-β (2 ng/ml) by Western blotting of cell lysates with antibodies to phospho-p44/42 and total p44/42.

with similar kinetics in all three cell lines, attaining maximal levels at 30 min and sustaining them for 2 h after TGF-β1 treatment (Fig. 1*B*).

To alter the balance of these endogenous signaling pathways, MII, MIII, and MIV cells were transduced with the retroviral expression vector LPCX to create cell lines that stably expressed NH_2-terminally Flag-tagged wild-type Smad3 or its inactive, dominant negative, COOH-terminally truncated mutant (Smad3ΔC; Ref. 19). Detection of the ectopically expressed Smad constructs showed that, on average, expression levels of exogenous wild-type Smad3 and the phosphorylation-defective COOH-terminally truncated mutant, Smad3ΔC, were similar (Fig. 1*C*). The average level of expression of ectopic Smad3 was approximately 32-fold higher than levels of endogenous Smad3 of parental or vector LPCX-infected cells (Fig. 1*D*). Similar expression patterns were found in transduced MII and MIV cell lines (data not shown). Despite the fact that all of these cells were transformed with the T24 mutant of Ha-*ras*, basal levels of activated phospho-p44/42 ERK increased from MII through the most aggressive MIV cells. Whereas TGF-β1 was unable to stimulate MAPK-ERK activity in MII cells, it had moderate effects on activation of phosphorylation of ERK1/2 in MIII and MIV cells, although the basal level of phospho-ERK1/2 activity was higher in MIV cells, suggesting that this pathway becomes more active with the acquisition of more malignant, invasive behavior (Fig. 1*E*).

Dominant Negative Smad3ΔC Blocks Phosphorylation of Endogenous Smad2 and Smad3 and Their Downstream Activities. Binding of TGF-β1 to its receptor triggers the serine/threonine kinase activity of TβRI, resulting in phosphorylation and activation of the Smad2 and Smad3 substrates. To determine whether the levels of endogenous receptor kinase would be sufficient to phosphorylate the overexpressed Smad3, we assessed the levels of phosphorylation of the ectopically expressed protein after treatment of cells with TGF-β1 and found that overexpressed Smad3 was efficiently phosphorylated (Fig. 2, *A* and *B*). Despite being overexpressed to levels approximately 32-fold over background, the presence of ectopic Smad3 in MIII cells had no effect on endogenous levels of either total or phosphorylated Smad2 (Fig. 2*A*), suggesting either that receptors are not limiting or that phosphorylation of Smad2 and Smad3 are noncompetitive. Overexpression of Smad3ΔC, which binds stably to TβRI and acts as a dominant negative mutant blocking phosphorylation of endogenous R-Smads, significantly inhibited phosphorylation of endogenous Smad3 and Smad2 (Fig. 2, *A* and *B*), decreasing levels of phospho-Smad3 and phospho-Smad2 to approximately 50% compared with vector LPCX cells (Fig. 2*B*).

To determine whether these manipulations of the Smad signaling pathway had the expected outcome on gene expression, we examined the response of Smad3-specific reporter genes in MIII cells treated with TGF-β. We used the $(SBE)_4$-luciferase reporter driven by four repeats of the CAGACA sequence identified as a Smad3/4-binding element (22) to show that overexpression of Smad3 or Smad3ΔC had the expected effects of enhancing or inhibiting, respectively, the TGF-β1-induced activation (Fig. 2*C*). Interestingly, there was a small but not significant increase in luciferase activity of $(SBE)_4$.Luc in MIII-Smad3 cells even in the absence of exogenous TGF-β1 (Fig. 2*C*), suggesting that TGF-β might be acting in an autocrine manner in these cells. This was confirmed by the use of neutralizing antibodies to TGF-β1 (data not shown). TGF-β-induced activation of the ARE-

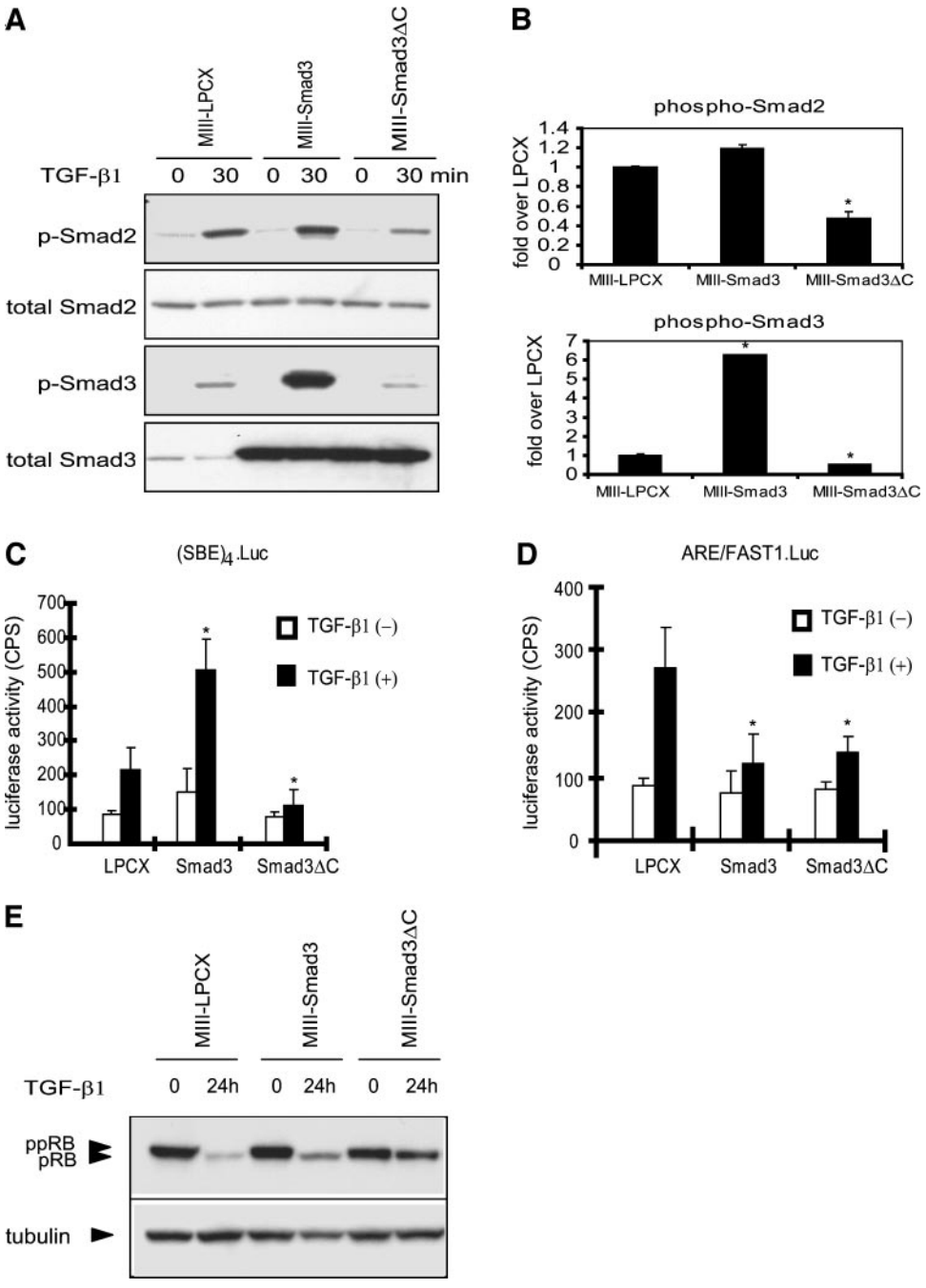

Fig. 2. Functional assessment of the effects of manipulations of the Smad pathway. *A*, Western blots of phospho- and total Smad2 and Smad3 in MIII-LPCX, MIII-Smad3, and MIII-Smad3ΔC without or with TGF-β1 treatment (2 ng/ml) for 30 min. Blots were stripped and reprobed for total Smad2/3. *B*, quantification of the levels of phospho-Smad2 and phospho-Smad3 in LPCX, Smad3, and Smad3ΔC cells by scanning and analysis using ImageQuant software. Data are mean relative pixel ratio ± SD of control LPCX cells of representative blots. Each band was scanned for four times. * indicates statistical significance of $P < 0.05$ relative to vector LPCX cells. *C* and *D*, activity of $(SBE)_4$.Luc and ARE/FAST1.Luc reporter constructs normalized to pSV-β-Gal in response to treatment with TGF-β1 (5 ng/ml) in MIII cell lines as described in "Materials and Methods." Mean counts per second ± SD of a representative experiment performed in triplicate. * indicates statistical significance of $P < 0.05$ relative to LPCX treated with TGF-β1. *E*, regulation of phosphorylation of retinoblastoma protein (pRb) by TGF-β in MIII cell lines. Cells preincubated in DMEM/F12 and 0.2% horse serum for 18 h were treated with TGF-β (2 ng/ml) for 24 h, and cell lysates were analyzed by Western blotting. ppRB, hyperphosphorylated form; pRB, hypophosphorylated form; loading control, tubulin.

luciferase reporter/FAST-1, known to be dependent on Smad2 and to be inhibited by Smad3 (23), also showed the expected outcomes of overexpression of either Smad3 or its dominant negative mutant (Fig. 2*D*). Other signaling pathways examined for basal and TGF-β-stimulated activation, such as MAPK, stress-activated protein kinase/c-Jun NH_2-terminal kinase, and Akt, were not prominently affected by overexpression of either Smad3 or Smad3ΔC (data not shown).

To examine effects of manipulation of the Smad pathway on an endogenous target, we investigated effects on phosphorylation of the Rb protein, known to mediate effects of TGF-β on growth inhibition (24). Rb typically becomes hyperphosphorylated as cells progress through S phase, but treatment with TGF-β blocked this event in LPCX control and Smad3-overexpressing MIII cells (Fig. 2*E*). However, Rb remained predominantly in the hyperphosphorylated state in cells overexpressing Smad3ΔC, consistent with its ability to interfere with the growth inhibitory effects of TGF-β. Together, these effects demonstrated that the overexpressed Smad3 proteins had the expected effects on TGF-β signaling.

Expression of the Dominant Negative Mutant of Smad3 Interferes with the Growth Inhibitory Effects of TGF-β in MII and MIII Cells but not MIV Cells. Because the ability of TGF-β to inhibit the proliferation of epithelial cells has been thought to be the basis of its tumor suppressor activity (25), we examined effects of modulation of the Smad2/3 signaling pathway on the growth response of MII, MIII, and MIV cells to TGF-β1. Using incorporation of [^{3}H]thymidine as a measure of growth, we showed that overexpression of Smad3 had no effect on the response of MII and MIII cells to inhibition of growth by TGF-β1 compared with control cells, suggesting that other components required for growth inhibition were limiting (Fig. 3, *A* and *B*). Cells were exquisitely sensitive to TGF-β;

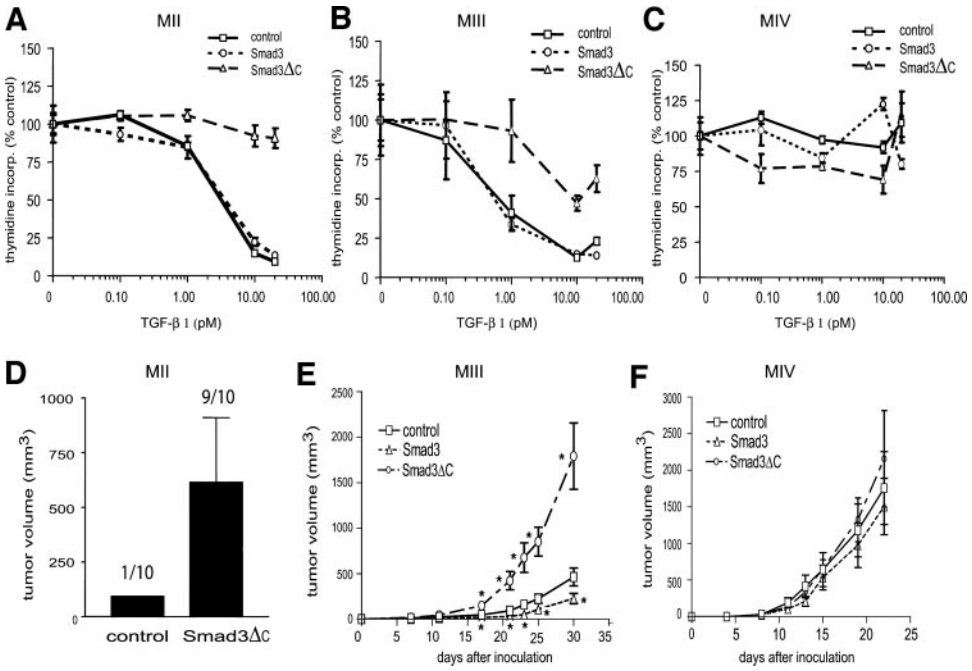

Fig. 3. Manipulation of the Smad2/3 pathway results in altered growth response and ability to form xenografted tumors in MII, MIII, and MIV cell lines. *A–C*, responsiveness to inhibition of growth by TGF-β1 as assessed by incorporation of [^{3}H]thymidine for 2 h. Data are expressed as mean percentage ($\pm$SD) of thymidine incorporation relative to basal counts of each cell line of a representative experiment performed in triplicate. The basal counts for all these cell lines are the following: MII-control, 10,353 cpm; MII-Smad3, 7,616 cpm; MII-Smad3ΔC, 7,799 cpm; MIII-control, 1,270 cpm; MIII-Smad3, 1,308 cpm; MIII-Smad3ΔC, 1,647 cpm; MIV-control, 1,922 cpm; MIV-Smad3, 1,441 cpm; and MIVSmad3ΔC, 1,637 cpm. *D*, mean ($\pm$SD) tumor volume of vector control and Smad3ΔC of MII cells 80 days after inoculation of 1×10^7 cells. *E* and *F*, a representative figure of the growth of xenografted cells of MIII (*E*) and MIV (*F*) cell lines in nude mice. Tumor volumes were calculated as described in "Materials and Methods." Each point represents the mean $\pm$ SD of the tumor volume of a representative experiment with 5 mice and 10 tumors, repeated two or three times. $*$ indicates statistical significance of $P < 0.05$, relative to vector LPCX control (two-tailed paired *t* test).

MIII cells were significantly inhibited (about 60%) by 1 pM TGF-β, and MII and MIII cells were almost completely inhibited by 10 pM TGF-β1. In contrast, overexpression of Smad3ΔC reduced the sensitivity of MII and MIII cells to inhibition by TGF-β. MII-Smad3ΔC cells were insensitive to all of the TGF-β1 concentrations tested (Fig. 3*A*), whereas MIII-Smad3ΔC cells were insensitive to 1 pM TGF-β and only about 50% inhibited by 10–20 pM TGF-β (Fig. 3*B*). The more aggressive MIV cells were relatively insensitive to the growth inhibitory activity of TGF-β, independent of manipulations of the Smad pathway (Fig. 3*C*). Although some sensitivity to inhibition by TGF-β could be seen under modified conditions, MIV cells were consistently less sensitive to inhibition by TGF-β than MIII cells (18).

As correlates of the growth inhibitory activity of TGF-β in these cells, we also examined expression levels of other TGF-β-regulated molecules, such as p21, cyclin D1, and c-Myc. In contrast to the expected outcomes shown for phosphorylation of Rb (Fig. 2*E*), no significant differences in the expression levels of these genes were found in cells overexpressing either Smad3 or Smad3ΔC (data not shown).

Modulation of Smad Signaling Alters the Tumorigenicity of MII, MIII, and MIV Cells *in Vivo*. To address effects of an imbalance in Smad signaling either by overexpression of Smad3 or by inhibition of endogenous Smad2/3 activity, 1×10^7, 1×10^6, and 2×10^5 pooled transduced MII, MIII, and MIV cells, respectively, were injected s.c. into the flanks of nude mice. Representative growth of the xenografts is shown in Fig. 3, *D–F*. Eighty days after injection, 9 of 10 xenograft sites of MII-Smad3ΔC had formed tumors, whereas only a hyperplastic structure was observed at 1 of 10 sites injected with vector control MII-LPCX cells (Fig. 3*D*). In three experiments with 15 mice, tumors derived from MII-Smad3 cells have never been observed for periods as long as 7 months (data not shown). Consistent with this observation, tumors formed from injection of MIII-Smad3 cells grew more slowly than controls ($*$, $P < 0.04$; two-tailed paired *t* test), whereas tumors formed from MIII-Smad3ΔC cells grew substantially faster than controls ($*$, $P < 0.05$; two-tailed paired *t* test) and about four times faster than that of MII-Smad3ΔC tumors (Fig. 3*E*). MIII tumor weights measured 30 days after injection correlated with the growth rate of the xenograft tumors (data not shown). The growth of tumors derived from MIV cells was rapid, and no differences in tumor growth rate dependent on modulation of Smad signaling were seen before the mice had to be sacrificed due to the tumor size (Fig. 3*F*).

Ki67 is a nuclear protein used to assess the proliferation rate of many epithelial cancers (26). Immunohistochemical staining for Ki67 again showed that xenografts of MIII-Smad3ΔC cells displayed about double the rate of proliferation of control tumors, whereas that of cells overexpressing Smad3 had a markedly reduced rate of proliferation with about 1.5-fold fewer positively stained cells (Fig. 4*A*). Use of terminal deoxynucleotidyltransferase-mediated nick end labeling staining to assess the degree of apoptosis in the same tumors showed a higher rate of apoptosis in MIII-Smad3 tumors compared with either controls or tumors of MIII-Smad3ΔC cells (Fig. 4*B*).

All of the xenograft tumors formed by different genotype groups of MIII cells were carcinomas, which showed a mixture of three distinct histologies, similar to that described previously (Ref. 17; Fig. 4*C*). Areas with relatively well-differentiated cribriform structures, clusters of closely apposed round cells with clear cytoplasm ("clear cells"), and poorly differentiated areas characterized by sheets or cords of pleiomorphic cells were observed. The mitotic index of each of these histological subtypes in MIII-LPCX tumors was as follows: cribriform, 0.23 $\pm$ 0.075%; clear cells, 0.46 $\pm$ 0.08%; and sheets or cords, 1.25 $\pm$ 0.23% (means $\pm$ SE). MIII-Smad3 xenograft tumors showed a greater percentage of more differentiated cribriform structures and substantially less necrosis than controls, suggestive of a more differentiated, less aggressive shift in the phenotype (Fig. 4*D*). In contrast, the extent of cribriform structures was substantially reduced in MIII-Smad3ΔC cells (Fig. 4*D*). These tumors were also characterized by a higher mitotic index in the more aggressive sheet structures, closely paralleling the data obtained from Ki67 staining (Fig. 4*A*) and consistent with a more malignant phenotype.

Staining of the MIII-Smad3 tumors for Smad3 showed that the most aggressive-appearing sheets or cords exhibited a diffuse cytoplasmic localization of Smad3, in contrast to areas of clear cells and cribriform structures, in which nuclear localization of Smad3 was prominent (Fig. 4*C*). These data suggest that TGF-β signaling might be compromised selectively in the less differentiated sheet structures,

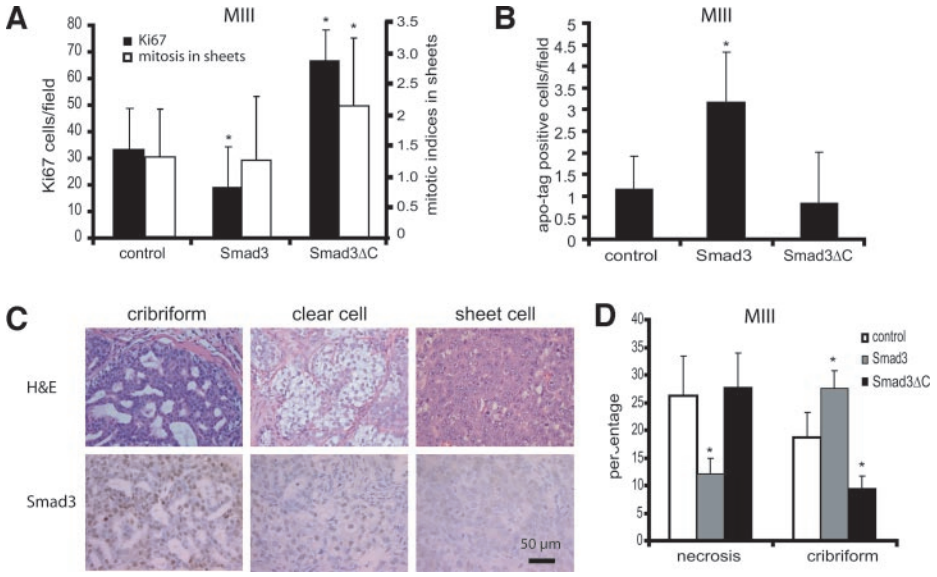

Fig. 4. Altered Smad signaling changes the tumor phenotype of xenografted MIII cell lines. *A*, quantification of proliferation of tumor cells. ■, immunohistochemistry for the proliferation marker Ki67 was assessed in tumor sections. □, the mitotic indices in sheets or cords of xenografted tumors are shown. ∗, significant difference relative to control of $P < 0.05$. Data represent mean positive cell number ± SD of per field (magnification, ×400) of six to nine fields counted. *B*, quantitation of apoptosis by apo-tag staining of the above tumors. Data represent the mean ± SD of positive cells per field (magnification, ×400) of six to nine fields counted. *C*, histology of MIII xenograft tumors. All tumors contained a mixture of three different histologies: cribriform structure, clear cells, and sheets or cords. Smad3 immunohistochemical staining (*brown*) of different histological types from Smad3-overexpressing tumors. *Scale bar* = 50 μm. *D*, the histogram of histology patterns shows mean percentage ± SE of necrotic areas or cribriform structures of the above tumors. ∗, $P < 0.05$ relative to MIII control cells.

even in Smad3-overexpressing MIII cells, which give an overall better outcome in terms of reduced necrosis and increased percentages of the more differentiated cribriform histology.

Down-Regulation of Smad Signaling Suppresses Metastasis in High-Grade MIV Breast Cancer Cells. To examine the role of TGF-β signaling in metastasis, we injected MIV cells, previously shown to form metastases (17), into the tail veins of nude mice. These cells formed rapid-growing, poorly differentiated tumors in the xenograft model even with an inoculum of only 2×10^5 cells (Fig. 3*F*). Effects of manipulation of the Smad pathway on the ability of these cells to form lung metastases after i.v. injection were diametrically opposite to effects of similar manipulations on xenograft tumors of MIII cells at s.c. sites. Overexpression of Smad3 in MIV cells promoted formation of lung metastases, whereas blocking the function of the endogenous Smads by overexpression of Smad3ΔC strongly suppressed formation of metastatic foci in lungs of these mice. The gross histology (Fig. 5*A*) was confirmed by quantitation of the number and area of tumors in representative lung sections (Fig. 5, *B* and *C*), showing that metastatic foci resulting from injection of MIV-Smad3 cells (five of five lungs) were both more numerous and larger and those from MIV-Smad3ΔC cells were less frequent (two of five lungs) and smaller than controls (Fig. 5*C*). As correlates of this observation, assessment of migration *in vitro* with TGF-β1 as the chemotactic stimulus showed that MIV-Smad3 cells migrated faster than controls, whereas MIV-Smad3ΔC showed significantly reduced migration in the same assay (Fig. 5*D*).

Loss of expression of the cell surface adherens junction marker E-cadherin is frequently considered an indication of EMT and has been linked to metastasis of many tumor cells including breast cancer cells (27, 28). MIV control cells showed cell membrane-associated E-cadherin staining was relocalized to the cytoplasm after treatment with TGF-β for 72 h. Smad3-overexpressing MIV cells showed reduced cell surface staining and increased cytoplasmic levels of E-cadherin compared with control cell, and this change was additionally enhanced by treatment with TGF-β. In contrast, overexpression of Smad3ΔC strongly inhibited the TGF-β-induced cytoplasmic re-

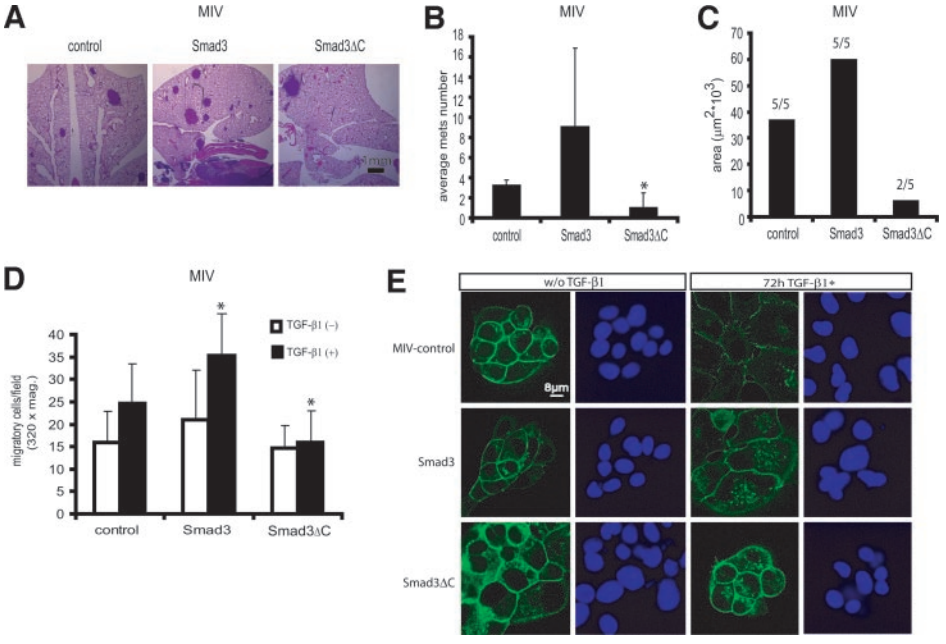

Fig. 5. Effects of altered Smad signaling on metastasis of MIV cell lines to lung. *A*, H&E staining of lung metastases 7 weeks after tail vein injection of 5×10^5 cells/nude mouse. Each slide is a representative of five dissections from each group. *Scale bar* = 1 mm. *B*, quantification of average number of metastatic foci in lungs of mice. Each group represents the mean ± SD of the tumors of a representative experiment of at least two repeats with a total of at least 10 mice. ∗ indicates statistical significance of $P < 0.05$ relative to control MIV-LPCX. *C*, areas of lung metastases (determined in "Materials and Methods"). Only two of five mice that received injection with MIV-Smad3ΔC cells exhibited metastases. *D*, histogram of migratory cells in the Biocoat cell migration assay in the absence or the presence of TGF-β1 (0.5 ng/ml) for 48 h as described in "Materials and Methods." Representative experiment of two independent experiments performed in triplicate. ∗ indicates statistical significance of $P < 0.05$ relative to MIV control treated with TGF-β1. *E*, TGF-β-Smad signaling interferes with E-cadherin localization in MIV cell lines. Representative images of E-cadherin localization detected by fluorescein-conjugated antibody (*green*) from two repeated experiments. Cells were treated with TGF-β1 (8 ng/ml) for 72 h. E-cadherin, *green*; 4′,6-diamidino-2-phenylindole, *blue*. *Scale bar* = 8 μm.

localization of E-cadherin (Fig. 5*E*). These results clearly demonstrate that whereas signaling through the Smad3 pathway is suppressive for formation of tumors by well-differentiated cells still responsive to inhibition of growth by TGF-β (MIII), this same signaling pathway is required for metastasis of more aggressive cells (MIV) because interference with its signaling strongly reduces metastatic foci and the ability of TGF-β to relocalize E-cadherin.

DISCUSSION

Use of a series of cell lines derived from MCF10A cells, which were originated from spontaneous immortalized breast epithelial cells obtained from a patient with fibrocystic disease (16–18), has allowed us to study the effects of perturbations in the Smad signaling pathway in cell lines of a common origin that stably represent defined stages in the progression from premalignant to fully invasive, metastatic breast cancer (17). Although previous conclusions regarding the putative dual tumor suppressor/pro-oncogenic roles of TGF-β in carcinogenesis have been synthesized from a large body of data obtained from different animal models and different cell lines *in vitro* and *in vivo*, this unique model system now allows us to determine whether such a functional switch truly occurs in closely related cells. Based on this system, we can unambiguously state that TGF-β does switch from tumor suppressor to oncogene as cells acquire a greater degree of malignancy associated with metastatic activity. More importantly, we have shown, for the first time, that the same signaling pathway dependent on Smad2 and/or Smad3 mediates both tumor suppressor activities of TGF-β in more differentiated tumor cells (MII and MIII) and its pro-oncogenic activities in cells, which have undergone additional changes conferring the ability to invade and form tumors in lungs (MIV).

Our data confirm previous findings that the endogenous Smad2/3 pathway has tumor suppressor activity (8, 29). We have shown that selective reduction of the signaling flux through this pathway, as might occur naturally by mutation or epigenetic interference with the activity of these proteins, enhances the tumorigenicity of Ha-*ras*-initiated premalignant MII cells and malignant MIII cells. This effect was particularly significant in the MII cells, in which we show a causal role in tumorigenesis for reduced signaling through this pathway in that loss of only about 50% of the capacity for phosphorylation of Smad2 and Smad3 was sufficient to support formation of large tumors at 9 of 10 injected sites, compared with a small tumor found at only 1 of 10 sites injected with control cells. In MII and MIII cells, reduced signaling through the Smad2/3 pathway results in reduced sensitivity to inhibition of growth by TGF-β, and in MIII cells, it results in reduced ability to maintain Rb in its hypophosphorylated, growth inhibitory form. Yet control of growth is likely not the only important tumor suppressor end point of this pathway, as evidenced by the reduced tumorigenicity of MIII cells overexpressing Smad3 in the absence of increased sensitivity to inhibition of growth by TGF-β.

The important question that we have addressed here is whether this same Smad2/3 pathway can also mediate the pro-oncogenic effects of TGF-β on metastasis. The concept that TGF-β signaling might be required for metastasis is not new. Pretreatment of mammary adenocarcinoma cells with TGF-β enhanced their metastatic potential to lung, presumably from increased propensity to extravasate from the vasculature (30). Analysis of immunohistochemical staining of TGF-β1 in breast cancers showed enhanced expression in lymph node metastases with preferential expression at the advancing edges of the tumors (31). More recent studies have shown that TGF-β/Smad and p38 signaling pathways cooperate to promote metastasis of human breast cancer MDA-MB-231 cells to bone via their effects on secretion of the osteolytic factor, parathyroid hormone-related protein (32, 33). Here, we have shown that a reduction in the signaling flux through the Smad2/3 pathway is sufficient to block metastases of MIV cells injected into the tail vein of three of five mice, whereas overexpression of Smad3 in these same cells increased the number and size of lung metastases. These same cells grew so aggressively when injected s.c. that the changes in Smad signaling had no effect on the growth of the xenografts. However, it appears that Smad2/3 signaling is required for cells to either extravasate into the lung or to proliferate in that tissue and induce an angiogenic response. Together, these data provide strong evidence that, at least in the context of the MCF10A series of breast cancer cell lines, the Smad2/3 pathway mediates tumor suppressor activity in premalignant and well-differentiated tumor cells (MIII) and pro-oncogenic signals in cells that have acquired invasive, metastatic behavior (MIV; Fig. 6).

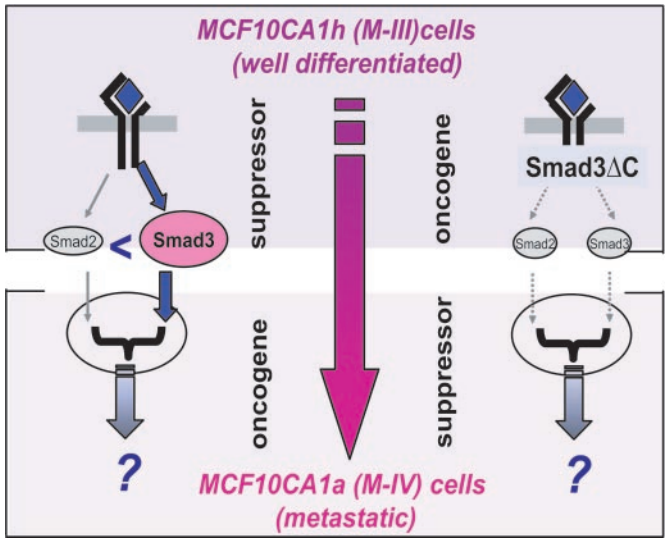

Fig. 6. Model of the opposing effects of modulation of Smad2/3 signaling in MIII cells compared with MIV cells. Suppression of the endogenous Smad2/3 signaling pathway by overexpression of Smad3ΔC enhances tumor growth of the well-differentiated MIII cells but strongly blocks metastasis of the more aggressive MIV cells, whereas overexpression of Smad3 has opposite effects. The data demonstrate that the consequences of the output of the Smad2/3 signaling pathway switch from tumor suppressor to pro-oncogene as cells acquire increasing malignant properties (MIII compared with MIV).

In speculating what might be the basis of the altered outcome of signaling through the Smad2/3 pathway in cells that have acquired the ability to invade and metastasize, insight is provided by the previously described synergism between Ras and TGF-β signaling in regulation of EMT and metastasis. EMT has been implicated in tumor cell invasion and metastasis (34), and one of the hallmarks of EMT, relocalization or loss of expression of an adherens junction protein, E-cadherin, has been demonstrated in many carcinomas, including breast cancer (28, 35). Of note, two highly homologous zinc finger transcriptional repressors, Snail and Slug, master regulators of EMT that bind to E-box elements and suppress transcription of E-cadherin, have each been shown to be transcriptional targets of TGF-β, with the Smad3 pathway directly implicated in induction of expression of the *SNAIL* gene (36).[2] Although studies in breast cancer cell lines suggest that Slug rather than Snail is likely to be the repressor of E-cadherin expression (37), it remains to be determined in MIV cells which of these two proteins might be targeted.

As examples of the putative role of MAPK pathways in this process, treatment of Ha-Ras-transformed mouse mammary epithelial cells, EpRas, with TGF-β results in loss of expression of E-cadherin, and this effect of TGF-β is blocked by inhibition of Ras activity (38).

[2] E. P. Böttinger, personal communication.

Two recent papers describing mutations in the TβRI that selectively disable Smad binding and activation but not signaling through the MAPK pathways demonstrate that Smad signaling and MAPK signaling are required for EMT (39, 40). Moreover, EMT of squamous carcinoma cells *in vitro* was shown to occur only in cells overexpressing oncogenic Ha-*ras* and activated forms of either Smad2 or Smad3, and this correlated with invasive behavior *in vivo* (41). In light of these results, it is important to note that MCF10AT1k cells were transformed with oncogenic Ha-Ras (15), although its expression is not sufficient for tumorigenesis in these cells (42). Although our results show parallel effects of manipulations of the Smad2/3 pathway on loss of E-cadherin expression in MIV cells and formation of lung metastases by these cells (Fig. 5), it remains to be determined whether these effects are dependent on the enhanced signaling through the Ras/ERK1/2 pathway in these cells (Fig. 1) or perhaps on an altered balance between MAPK pathway signaling and Smad pathway signaling as proposed previously (2). However, together with previously published results, our data are highly suggestive that regulation of E-cadherin expression and subsequent EMT are important oncogenic targets of the Smad2/3 pathway in late-stage disease and that these effects likely require cooperation with MAPK pathways.

Several studies have addressed effects of blocking all signaling pathways downstream of the TGF-β receptors by overexpression of DNTβRII to mimic reduced receptor expression seen in late-stage human cancers (2, 5). Although carcinogenesis studies in transgenic mice overexpressing the DNTβRII under control of a mammary gland-specific promoter clearly support a tumor suppressor role for TGF-β receptors (43), expression of a DNTβRII in highly metastatic 4T1 mammary carcinoma cells restricted the ability of the cells to form distant metastases (44), suggesting that receptor signaling also contributes to the oncogenic properties of tumor cells. Most pertinent to the results we have presented here, parallel studies from our laboratory using the series of MCF10A cells have shown a causal role for loss of receptors in breast cancer progression in that expression of DNTβRII enhanced the tumorigenesis of MII and MIII cells but also a requirement of the receptors for metastasis of MIV cells (18). Our results demonstrating that selective manipulation of the Smad pathway, either by suppression of the endogenous pathway or by specific amplification of the Smad3 pathway, can also mediate this "switch" from tumor suppressor to oncogenic behavior now identify the Smad pathway as the key downstream mediator of receptor-dependent effects on carcinogenesis. Activin also transduces signals through Smad2/3, and recent data implicate this TGF-β family member in inhibition of growth of breast cancer cells (45) and possibly also in acquisition of metastatic behavior (46). However, the similarity of our results based on manipulation of the Smad2/3 pathway and those of Tang *et al.* (18) based on overexpression of the DNTβRII would appear to implicate TGF-β as the major ligand upstream of Smad signaling in the MCF10A series of cells.

In light of the data we have presented showing a prometastatic effect of Smad3 overexpression in highly malignant cells, it is interesting to speculate why no mutations have been identified, thus far, in the *Smad3* gene in human cancers. Certainly, a multiplicity of mechanisms exists for epigenetic inactivation of *Smad3* in tumor cells. Thus viral oncoproteins such as the human T-cell lymphotropic virus type I oncoprotein Tax (47) and human papillomavirus oncoprotein E7 (48) as well as other proto-oncogenes including SnoN (49), c-Ski (50), and Evi-1 (51) each inhibit TGF-β signaling by interfering with Smad2/3 signaling. Given that we have shown that the Smad2/3 pathway is required for metastasis in the MCF10A-derived breast cancer cells, it might be advantageous for a tumor cell to use epigenetic mechanisms to block the tumor suppressor activity of Smad3 in earlier stages of carcinogenesis while retaining the ability to reactivate it in metastatic cells (8). Interesting in this regard are recent findings demonstrating that the proto-oncogene c-Ski has tumor suppressor activity in certain contexts, such that its loss enhances tumorigenicity (52).

Although the overexpression approach we have used is admittedly artificial, overexpression of Smad3 may nonetheless model the consequences of an imbalance between Smad2 and Smad3 signaling as might occur in tumor cells harboring inactivating mutations of Smad2 (12, 13). Additional studies in which Smad2 is selectively overexpressed or in which Smad2 or Smad3 is independently eliminated, as with small interfering RNA (siRNA) technology, will be necessary to ascertain whether Smad2 and Smad3 have similar or unique roles in tumorigenic progression. The present studies, in which overexpression of Smad3ΔC was shown to interfere with phosphorylation of Smad2 and Smad3, do not allow independent evaluation of the effects of these two pathways.

Ultimately, approaches such as we have presented here will identify new targets for therapeutic intervention. Recently, two provocative studies have shown that a soluble TβRII can block metastases in certain mouse models of breast cancer in the absence of effects on the primary tumor (53, 54). These studies again show a requirement for TGF-β in metastasis and suggest, in addition, that the form of the ligand secreted by metastatic cells might be selectively amenable to capture by such receptors or, alternatively, that metastatic cells might be particularly susceptible to inhibitors present in the circulation. These findings, together with the recent development of small molecule inhibitors of the TGF-β/activin type I receptor kinases (55), suggest that inhibition of metastatic disease, the usual cause of death from cancer, might be an achievable goal, particularly in combination with other effective therapies.

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