Smad-Binding Defective Mutant of Transforming Growth Factor β Type I Receptor Enhances Tumorigenesis but Suppresses Metastasis of Breast Cancer Cell Lines

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ABSTRACT

The role of transforming growth factor β (TGF-β) in carcinogenesis is complex, with tumor suppressor and pro-oncogenic activities depending on the particular tumor cell and its stage in malignant progression. We previously have demonstrated in breast cancer cell lines that Smad2/3 signaling played a dominant role in mediating tumor suppressor effects on well-differentiated breast cancer cell lines grown as xenografts and pro-metastatic effects on a more invasive, metastatic cell line. Our present data based on selective interference with activation of endogenous Smad2 and Smad3 by stable expression of a mutant form of the TGF-β type I receptor (RImL45) unable to bind Smad2/3 but with a functional kinase again show that reduction in Smad2/3 signaling by expression of RImL45 enhanced the malignancy of xenografted tumors of the well-differentiated MCF10A-derived tumor cell line MCF10CA1h, resulting in formation of larger tumors with a higher proliferative index and more malignant histologic features. In contrast, expression of RImL45 in the more aggressive MCF10CA1a cell line strongly suppressed formation of lung metastases following tail vein injection. These results suggest a causal, dominant role for the endogenous Smad2/3 signaling pathway in the tumor suppressor and prometastatic activities of TGF-β in these cells. Using an in vitro assay, we further show that non-Smad signaling pathways, including p38 and c-Jun NH2-terminal kinase, cooperate with TGF-β Smads in enhancing migration of metastatic MCF10CA1a cells, but that, although necessary for migration, these other pathways are not sufficient for metastasis.

INTRODUCTION

Transforming growth factor β (TGF-β) belongs to a family of cytokines that regulate cell proliferation, differentiation, extracellular matrix production, cell motility, and apoptosis of many different cell types (1, 2). The ability of TGF-β to inhibit proliferation of epithelial cells and lymphoid cells from which most human cancers originate suggests a role in tumor suppression. Transgenic mice expressing a dominant-negative TGF-β type II receptor (DNTRII) under control of a mammary gland-specific promoter showed an enhanced incidence of carcinogen-induced mammary, lung, and skin tumors (3). Conversely, in squamous carcinoma cells, TGF-β also can induce an epithelial-to-mesenchymal transition, a process associated with invasiveness (4). Consistent with this, expression of a DNTRII in highly metastatic 4T1 mammary carcinoma cells restricted the ability of the cells to form distant metastases (5). Together, these data suggest that TGF-β acts as a tumor suppressor and a tumor promoter at different stages of tumor progression.

TGF-β signaling is mediated by a heterotrimeric complex of two transmembrane receptor serine/threonine kinases, consisting of a type II ligand-binding receptor (TIIRII) and a type I signaling receptor (TIIRI). Smad2 and Smad3 are direct substrates of TIIRI and, together with the common mediator Smad4, play key roles as cytoplasmic signaling mediators. The specificity of the type I receptor kinase for an R-Smad is determined by the L45 loop of the receptor between kinase subdomain IV and V (6). Replacement of three L45 loop residues (N265, D267, and N268) with alanine (RImL45) abolishes the ability of the receptor to interact with and phosphorylate its Smad substrates without affecting its kinase activity or its ability to activate other signaling pathways such as the mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38. Thus, this mutant receptor provides a useful tool to study the effects of Smad-independent TGF-β receptor signaling (7, 8).

Specific targeting of TGF-β receptors in human cancers has been demonstrated by identification of inactivating mutations in TGFBR2 in colon cancers and head and neck cancers, by homozygous deletion of TGFBR1 in pancreatic and biliary carcinomas and in lymphoma, and by a TGFBR1 tumor-specific mutation in breast cancer (9). However, it has not been possible in these instances to assess the complex cross-talk between the various downstream signaling pathways of TGF-β, including the MAPK pathways known to play a prominent role in metastasis. Nor is it known whether changes in the balance between TGF-β receptor-dependent Smad2/3 signaling and MAPK or other pathways may favor tumor suppression or prometastatic effects on the transcriptome (2).

MCF10CA1h (MIII) and MCF10CA1a (MIV) cell lines stably express features representative of different stages of tumor progression (10, 11). MIII and MIV cell lines were derived from passaging ras-transformed MCF10A1tk (MII) cells in mice. MII cell line forms well-differentiated xenografted tumors in 4–6 weeks, whereas MIV cells form rapidly growing carcinomas in 7–10 days and are metastatic to lung following i.v. injection. We previously showed that levels of expression of endogenous Smad2, Smad3, Smad4, and TIIRI and TIIRII did not vary appreciably in these two cell lines, although receptor expression is somewhat higher in MIII compared with MIV cells (12). Moreover, we showed that signaling through the Smad2/3 pathway could mediate tumor suppressor and prometastatic effects of TGF-β on these cells, depending on the stage of progression of the cells and other cooperating contextual changes. Our previous data demonstrated that interference with endogenous Smad2/3 signaling, by stable expression of a C-terminally truncated form of Smad3 (Smad3ΔC), enhanced the malignancy of xenografted tumors of pre-malignant and well-differentiated tumor cells (MII and MIII) but strongly suppressed lung metastases of more aggressive carcinoma cells (MIV) after tail vein injection. Overexpression of Smad3 in the same cell lines had opposite effects, suggesting a causal effect of the Smad pathway in mediating these effects (12).

Recent data suggest that there is a particularly complicated and intimate inter-relationship between the TGF-β system and Ras/MAPK pathways in tumorigenesis and that TGF-β and activated Ras may cooperate to promote invasive, metastatic disease (13). To change the balance between Smad and alternative signal transduction pathways activated by TGF-β, we stably overexpressed either the wild-type TIIRI or the mutant receptor RImL45 with defective Smad binding in the MCF10A-derived breast cancer cell system. Because the mutant
receptor is competent to activate MAPK pathways, this approach enabled us to investigate changes in the balance of TGF-β signaling through these two pathways on in vitro and in vivo behaviors of well-differentiated MIII and poorly differentiated, metastatic MIV through a strategy similar to that of our Smad3 and Smad3ΔC study (12). Our results provide additional evidence from receptor level interference with endogenous signaling pathway that the TGF-β/Smad signaling pathway is the key suppressor of receptor-dependent effects on carcinogenesis in MIII and the mediator of prometastatic behavior in MIV cells. Interestingly, migration of MIV cells in vitro also showed a dependence on cooperative signals through MAPK pathways, despite the dominant role of the Smad pathway on the prometastatic activity in vivo.

MATERIALS AND METHODS

Retroviral Constructs. Retroviral constructs expressing wild-type TGF-β type I receptor (RI) or the mutant Smad-binding defective type I receptor (RImL45) were constructed by subcloning the cDNA fragments encoding c-terminal Flag-tagged RI or RImL45 (8) into retroviral vector pLPCX (14) to allow for retroviral infection and puromycin selection.

Cell Culture and Generation of Stable Cell Lines. The two human breast cancer cell lines MIII and MIV were obtained from Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI). MIII and MIV cells were grown in DMEM/F12 (Invitrogen, Carlsbad, CA) and 5% horse serum (Invitrogen) at 37°C with 5% CO₂. Retroviral infection of MIII and MIV cells to obtain stable cell lines was done as described previously (12).

Cell Growth Inhibition Assay by TGF-β, Immunoblotting, and Immunohistochemistry. Proliferation of infected MII and MIV cells was determined by [³H]thymidine (1 μCi/ml; NEN, Boston, MA) incorporation for 2 h essentially as described previously (15). Preparation of proteins from cell lysates, Western immunoblot analysis, and immunohistochemistry also were done as described previously (12). Antibodies were from the following sources: Smad2, Smad3, and tubulin, Zymed (San Francisco, CA); TßRI, Santa Cruz Biotechnology (Santa Cruz, CA); phospho-Smad2 and phospho-Smad3, gifts of Dr. Michael Reiss (Cancer Institute of New Jersey, New Brunswick, NJ); phospho-ERK1/2 and total ERK, Cell Signaling Technology (Beverly, MA); Ki67, DAKO (Carpinteria, CA); and CD31, BD Biosciences PharMingen (San Diego, CA).

Assessment of Tumorigenicity and Metastasis in Vivo. Xenograft injection of MII cells into nude mice was done as described previously (12). Each group consisted of at least five mice with two hind flank injections per mouse. Each experiment 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. Parks) blinded to the groupings. The Student t-test (two-tailed) was used for the comparisons of tumor growth rates for statistical significance. Tail vein injections of MIV cells were repeated at least twice for a minimum total of 10 mice in each group as described previously (12). The NCI Animal Care and Use Committee approved protocols for nude mice injections.

Luciferase Reporter Assays. MIII cells injected with different retroviral constructs were plated at a density of 3 × 10⁵ cells/well in six-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 [(CAGA)12-Luc or (ARE)3-Luc/FAST-1or (BRE)2-Luc]. Renilla luciferase reporter plasmid (Promega, Madison, WI) was included in the transfection to normalize transfection efficiency. After 24 h, the medium was replaced with low serum medium (DMEM/F12/0.2% horse serum), and the cells were left untreated or stimulated with 4 ng/ml TGF-β1 or 0.1 μg/ml BMP-2 (R&D, Minneapolis, MN). Cells were lysed 12 h later, and the luciferase activities were determined by VICTOR2 (Perkin-Elmer Life Sciences, Boston, MA).

Biocoat Cell Migration Assay. A total of 2.5–2.8 × 10⁵ cells/0.5 ml were plated in 24-well inserts (8.0-μm Biocoat Cell Environment plates; Becton Dickinson, Franklin Lakes, NJ). Different medium or treatment (without or with TGF-β1 or with TGF-β1 plus different inhibitors) was added to the bottom wells. Inhibitors were added 10 min before addition of TGF-β1. Eighteen or 24 h later, cells were fixed and stained by using the Diff-Quik Stain Set (Dade Behring, Deerfield, IL). After wiping the inside of the chambers using cotton-tipped applicators, migratory cells remaining on the bottom of the membranes were counted under the microscope (magnification, 100×). The inhibitors used in the assay were p38 kinase inhibitor: SB203580 (Calbiochem, La Jolla, CA), 5 μM; ALK4, 5.7 inhibitor: SB431542 (gift from Dr. Caroline S. Hill), 5 μM, JNK inhibitor: SP600125 (Calbiochem), 10 μM; and Rho inhibitor: Y27632 (Calbiochem), 10 μM (16).

Gelatin Zymography. The same amount of MIV cells were plated and cultured to 70% confluency before being changed to low serum medium (0.2% horse serum) either left untreated or treated with TGF-β1 for 48 h. The cell supernatant was collected and concentrated with Amicon filters (Millipore Corporation, Bedford, MA) to 10% of initial volume. The total amount of protein remaining in the cell layer also was measured as quantification reference. Gelatin zymography was performed in 10% polyacrylamide in the presence of 0.1% gelatin (Invitrogen). The gel then was put in renaturing buffer (Invitrogen) for 30 min and developing buffer (Invitrogen) for 48 h. Identification of transparent bands at M₁₂₀,₀₀₀ on the Coomassie blue background of the gel was considered positive for the presence of enzymatic activity.

Statistical Analysis. All statistical analyses were performed using a two-tailed Student’s t-test, with statistical significance at P < 0.05.

RESULTS

Stable Expression of Smad-Binding Defective Type I Receptor Mutant (RImL45) Blocked Phosphorylation of Endogenous Smad2/3 in MCF10A-Derived Breast Cancer Cell Lines. To look at the effect of altering type I receptor expression on TGF-β signaling pathways in MIII and MIV cells, cells were transduced with LPCX retroviral expression vectors to create cell lines that stably expressed C-terminally Flag-tagged wild-type type I receptor (RI) or its Smad-binding defective mutant RI, respectively (16). Identification of transparent bands at M₁₂₀,₀₀₀ confirmed that RImL45 and Smad3 substrates. To determine whether RImL45 blocks downstream phosphorylation, Smad2/3, we assessed the effects of TGF-β on the levels of phosphorylation of Smad2/3 in cells ectopically expressing the RImL45 mutant protein. As expected, overexpression of RImL45 decreased levels of phospho-Smad2 and phospho-Smad3 efficiently (Fig. 1C), whereas overexpression of the wild-type receptor RI increased phosphorylation of endogenous Smad2 (p-Smad2) and slightly increased p-Smad3 (Fig. 1D). Smad4 expression levels showed no changes among the stably infected MIII cells of different constructs with or without TGF-β1 ligand (data not shown). Similar down-regulation of phosphorylation of endogenous Smad2 by RImL45 was found at 30-min and 1-h treatment of TGF-β1 in MIV-RImL45 cells (Fig. 1E).

RImL45 Blocked Smad2/3-Dependent Transcriptional Activities. To determine whether manipulations of the type I receptor had the expected outcome on gene expression, we examined the response of Smad2/3-specific reporter genes in MII cells treated with TGF-β. We used the (CAGA)12-luciferase reporter, driven by 12 repeats of the CAGA sequence identified as a Smad3/4 binding element (17), to show that overexpression of R1 or RImL45 had the expected effects of enhancing or inhibiting, respectively, the TGF-β1-induced activation (Fig. 2A). TGF-β1-induced activation of the (ARE)3-luciferase reporter/FAST-1, known to depend on Smad2 (18), also showed the expected outcomes of overexpression of either R1 or RImL45 mutant (Fig. 2B). To rule out the possibility that the three alanine substitutions in the L45 loop rendered this receptor competent to bind BMP-
dependent Smad proteins (Smad1, Smad5, and Smad8) and to mediate a BMP-like response (9), we tested the ability of RImL45-expressing cells to activate a BMP-specific (BRE)2-Luc reporter (19). In this case, cells expressing either the wild-type RI or mutant RImL45 receptor were unable to activate the BMP-responsive promoter in response to TGF-β1 (Fig. 2C). As a further control, BMP-2 was added to the cells and found to consistently induce (BRE)2-Luc activity to similar levels in MIII control cells, MIII-RI, and MIII-RImL45, suggesting that signaling through the BMP pathway was unaltered by expression of these receptors (Fig. 2C).

MIII and MIV cells are transformed with the T24 mutant of Ha-ras, resulting in high basal levels of activated phospho-ERK1/2, which were slightly higher in MIV cells (12). TGF-β1 activated phospho-ERK1/2 in MIII cells as early as 15 min postaddition (Fig. 3, A and B). In MIV control cells and MIV-RImL45, MAPK activity also was induced significantly at 15 min, whereas the induction time course was possibly slightly slower in MIV-RI. However, the MAPK activity in all cells was nearly equivalent by 45-min TGF-β1 treatment (Fig. 3, D and E). However, by 2 h the levels had returned to basal in the control and the stably infected cells in MIII and MIV (Fig. 3, C and F). Thus, the induction of MAPK in these cells appears to be independent of the manipulation of the TβRI, being strongly induced by TGF-β1 between 15 min and 1 h and then returning to baseline activity. P38 activity also showed a similar pattern by 2-h and 4-h treatment with TGF-β1 (data not shown).

Expression of RImL45 Interfered with the Growth Inhibitory Effects of TGF-β in 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, we examined effects of modulation of the type I receptor on the growth response of MIII and MIV cells to TGF-β1 (20). Using incorporation of [3H]thymidine as a measure of growth, we showed that overexpression of RI had no effect on the response of MIII cells to inhibition of growth by TGF-β1 compared with control cells, suggesting that type I receptors were not limiting for the growth inhibitory response of the cells. In contrast, overexpression of RImL45 reduced the sensitivity of MIII
cells to inhibition by TGF-β (Fig. 4A). The apparent sensitization of RImL45 cells to inhibition by low concentrations of TGF-β was not significant. The more aggressive MIV cells were relatively insensitive to the growth inhibitory activity of TGF-β, although overexpression of RI slightly restored the inhibitory effects of TGF-β (Fig. 4B).

**Modulation of TβRI Altered the Tumorigenicity of MIII in Vivo.** To address effects of modulation of expression of the type I receptors on tumorigenicity of the cells in vivo, 1 × 10⁶ pooled transduced MIII cells were injected s.c. into the flanks of nude mice. Representative growth kinetics and tumor weight of the xenografts are shown in Fig. 5, A and B. Consistent with effects on growth in vitro, tumors formed from injection of MIII-RI cells grew more slowly than controls, whereas tumors formed from MIII-RImL45 cells grew substantially faster than controls (Fig. 5A). MIII tumor weights measured at the end of the experiment correlated with the growth rate of the xenografted tumors (Fig. 5B). Expression levels of Ki67, a proliferation marker of many epithelial cancers, also correlated with the tumor growth rate in vivo (Fig. 5C). Levels of expression of CD31, an endothelial cell marker, also were lower in MIII-RI tumors compared with controls, suggesting reduced levels of angiogenesis in these tumors, consistent with their smaller size (Fig. 5E).

All of the xenografted tumors formed by different genotype groups of MIII cells were carcinomas and showed a mixture of three distinct histologies similar to that described previously (21). MIII-RImL45 tumors showed a significantly reduced percentage of well-differentiated cribriform structures and increased percentage of poorly differentiated sheets of pleomorphic cells (Fig. 5D). Considering that the mitotic index in sheets was more than fivefold higher than in cribriform areas, MIII-RImL45 tumors displayed a substantially more malignant phenotype characterized by a higher mitotic index in more aggressive sheet structures (12).

**RImL45 Suppressed Metastasis in High-Grade MIV Breast Cancer Cells.** To examine the effect of modulation of TβRI on metastasis, we injected stably infected MIV cells, previously shown to form metastases, into the tail veins of nude mice (21). We previously had shown that MIV cells formed rapid growing, poorly differentiated tumors in the xenograft model and that no differences in growth rate could be seen in cells overexpressing Smad3 and Smad3ΔC even with an inoculum of only 2 × 10⁵ cells (12). However, following i.v. injection into the tail vein, MIV-RImL45 cells showed a significantly reduced ability to form lung metastases. This was confirmed by quantitation of tumor areas relative to lung areas, showing that metastatic foci resulting from injection of MIV-RImL45 cells were much smaller than controls (Fig. 6A). As correlates of this observation, assessment of matrix metalloprotease (MMP) 9 activity, an important index in invasiveness of many aggressive cancers (22), and migration in vitro with TGF-β as the chemotactic stimulus showed that MIV-RImL45 cells expressed greatly reduced levels of MMP-9 gelatinase activity in vitro (Fig. 6B) and showed reduced migration mobility in response to TGF-β compared with MIV-LPCX vector control cells (Fig. 6D).

In addition to the Smad signaling pathway, TGF-β also activates other pathways, including the family of MAPKs with downstream kinases ERK1/2, JNK, and p38. These pathways often cooperate with Smad signaling to control gene expression and cell phenotype and are likely to contribute to the prometastatic activities of TGF-β (2). To determine whether these other pathways might contribute to the migration of MIV cells in vitro, we examined the effects of several pathway-specific inhibitors on migration of the cells. Similar to that seen previously for migration of pancreatic cancer cells (23), treatment of the cells with inhibitors alone had little effect on the basal migration of the cells (data not shown). However, when cells were

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**Fig. 4.** Expression of RImL45 resulted in altered growth response in MCF10CA1 h (MIII) but not in MCF10CA1a (MIV) cell lines. A and B, responsiveness to inhibition of growth by transforming growth factor β1 (TGF-β1) as assessed by incorporation of [³H]thymidine for 2 h. Data are expressed as mean percentage (± SD) of thymidine incorporation relative to basal counts of each cell line of a representative experiment performed in triplicate. The basal counts for all of these cell lines are the following (cpm): MIII-control, 16624; MIII-RImL45, 25584; MIII-RI, 15351; MIV-control, 27515; MIV-RImL45, 12157; and MIV-RI, 24719.

![Fig. 3.](image-url) Activation of mitogen-activated protein kinase (MAPK) pathways in stably infected MCF10CA1 h (MIII) and MCF10CA1a (MIV) cells. A–F, MAPK/extracellular signal-regulated kinase (ERK) activity was assessed in stably infected MIII and MIV cells following treatment with transforming growth factor β1 (TGF-β1; 2 ng/ml) at a serial time course by Western blot analysis of cell lysates with antibodies to phospho-ERK1/2 (p-ERK1/2), total ERK.
DISCUSSION

Whereas we previously had proposed that the opposing tumor suppressor and prometastatic activities of TGF-β might result from a shift in pathway utilization, the results of this study based on expression of a type I receptor mutated such that it cannot bind or activate Smad2/3 and those of our previous study based on direct manipulation of the Smad pathway show that the Smad pathway plays a central role in both of these divergent activities (2). Overexpression of Smad3ΔC, as in our previous study, results in binding to the receptor of an ectopic nonphosphorylatable substrate, whereas overexpression of RImL45 used here results in receptor complexes unable to bind endogenous Smad substrates; in both cases the kinase activity of the type I receptor is intact (8). Although the two approaches are different, they have similar functional outcomes in that both result in reduced phosphorylation of endogenous Smad2/3 and interference with downstream transcriptional activities. Similar to our previous finding that selective reduction of the Smad signaling flux by overexpression of Smad3ΔC enhanced the tumorigenicity of Ha-ras-initiated premalignant MII cells and well-differentiated MIII cells, this study shows that overexpression of the Smad-binding defective mutant receptor RImL45 had similar effects (12). RImL45 not only significantly accelerated the growth rate of MIII tumors but also shifted the histology to more malignant patterns with significantly greater areas composed of sheets of pleomorphic cells and reduced areas displaying well-differentiated cribriform structures (Fig. 5D). Although overexpression of the wild-type type I receptor (RI) slowed down the tumor growth in MIII, it did not appreciably alter the histology of MIII expression of the wild-type type I receptor (RI) slowed down the tumor growth in MIII, it did not appreciably alter the histology of MIII consistent with the reports of others (24–26). However, in MIV cells that have acquired metastatic capability, similar manipulations of the Smad signaling pathway by RImL45 reduced the number and size of lung metastases, showing that in this cellular context the same pathway now confers prometastatic activity.

The invasive metastatic phenotype of epithelial tumors often is associated with down-regulation of cellular adhesion molecules, elevated activity of metalloproteases, and increased motility, all of which can be attributed, in part, to TGF-β. Therefore, it is not surprising that TGF-β can enhance cancer progression and metastasis by modulating these critical processes. One of the prometastatic processes regulated treated with TGF-β (0.5 ng/ml) plus different inhibitors, added 10 min after TGF-β, migration was significantly reduced in the presence of the p38 kinase inhibitor SB203580, the JNK inhibitor SP600125, or the ALK4,5,7 inhibitor SB431542. The Rho inhibitor Y27632 was without effect (Fig. 6C). Use of trypan blue to test for cell viability under the same concentrations of inhibitors used in migration assay showed that the inhibitors were not toxic to the MIV cells (data not shown). Correlating with the reduced levels of Smad activation in MIV cells stably expressing RImL45, these cells showed significantly less migration in response to TGF-β compared with MIV-LPCX (P = 0.03; Fig. 6D), suggesting that Smad activation plays an important role in TGF-β-dependent migration. To check whether MAPK pathways still contribute to migration of RImL45-expressing cells, we assessed the effects of the inhibitors. Not unexpectedly, we found that the ALK kinase inhibitor SB431542 resulted in the greatest inhibition of cell migration in agreement with its ability to block not only any residual Smad signaling but also TGF-β-dependent activation of the MAPK pathways (16). However, addition of either the p38 kinase inhibitor or the JNK inhibitor also substantially reduced the migration of MIV-RImL45 cells in the presence of TGF-β, suggesting that TGF-β-dependent activation of these pathways can contribute to migration despite the reduced signaling flux through the Smad pathway (Fig. 6D). Interestingly, the JNK inhibitor SP600125 not only reduced the migration of MIV cells but also changed the cellular morphology and organization (data not shown). Together, we interpret the data to suggest that Smad pathway and other pathways such as p38 and JNK cooperate to effect the migration of MIV cells in vitro.

Fig. 5. Smad-binding defective mutant RImL45 changes the tumor phenotype of xenografted MCF10CA1b (MIII) cell lines. A, a representative figure of the tumor growth kinetics of xenografted cells of stably infected MIII cell lines in nude mice. Tumor volumes were calculated as described in “Materials and Methods.” Each point represents the mean ± SD of the tumor volume of a representative experiment with 5 mice and 10 tumors, repeated two or three times. *Indicates statistical significance, P < 0.05, relative to vector LPCX control (two-tailed t test). B, tumor weights from the aforementioned mice at the end of the experimental observation. Mean weight ± SD in grams from each group was shown. *Indicates statistical significance, P < 0.05, relative to vector LPCX control. C, immunohistochemistry for the proliferation marker Ki67 was assessed in tumor sections. Significantly 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. D, the histogram of histology patterns shows mean percentage ± SD of necrotic areas, cribriform structures, or sheets of the aforementioned tumors. The P values were calculated by two-tailed Student’s t test. E, percentage of CD31-stained areas versus total areas was calculated using Image-Pro Plus imaging analysis software (Media Cybernetics, Silver Spring, MD). Each value represents mean percentage ± SD of per field (magnification, ×400) of at least six fields counted. *Indicates statistical significance, P < 0.05, relative to MIII-control (two-tailed t test).
by TGF-β is control of the turnover of extracellular matrix dependent on regulation of the expression of MMPs and their inhibitors (27, 28). MMPs are zinc-dependent endopeptidases that are activated extracellularly by proteolytic cleavage and play a critical role in the proteolytic degradation of basement membrane that is required for tumor invasion (22). Some of the MMPs, including MMP-1, have been shown to depend on Smad signaling (29). The expression of several MMPs, including MMP-2 (27) and MMP-9 (30–32), can be induced by TGF-β, and expression of these MMPs can be further increased by a positive feedback loop based on their ability to activate latent TGF-β (33). Moreover, TGF-β1 has been shown to selectively induce MMP-9 activity in a subset of metastatic tumors, and the induction of MMP-9 showed strong correlation with the metastatic potential of tumor cells (32). We showed a similar correlation with the different abilities of engineered cells to form metastatic masses in lungs in that TGF-β1 induced MMP-9 activity in MIV-LPCX and MIV-RI but not in MIV-RImL45 mutant cells, in which the number and size of lung metastases were severely reduced (Fig. 6).

Studies on Smad-dependent and Smad-independent effects in epithelial-to-mesenchymal transition, migration, and apoptosis have been given increasing attention during recent years. TGF-β activates a variety of MAPK pathways downstream of its receptors and elicits a wide range of cellular responses, some of which are dependent and some independent of Smad activation (8, 34, 35). Ras MAPK pathways lead to downstream activation of either ERK1/2 or two stress-activated protein kinases (JNK and p38 MAPK). The activation of Ras is an early event in many cancers (36, 37). Study of Ha-Ras-transformed mammary epithelial cells showed that a hyperactive MAPK pathway cooperated with TGF-β signaling, causing epithelial-to-mesenchymal transition and metastasis in vivo, suggesting that Ras and TGF-β together regulated epithelial cell plasticity and metastasis (38). In NMuMG cells, the mutant receptor RImL45 was shown to be sufficient to activate p38 and to promote TGF-β-dependent apoptosis but to be incapable of mediating signals required for more complex endpoints, including TGF-β-mediated growth inhibition and epithelial-to-mesenchymal transition, which presumably require signaling through the Smad pathway (7, 8). Our study based on overexpression of the RImL45 mutant TβRI receptor in the well-differentiated (MIII) and metastatic (MIV) breast cancer cells provides a unique tool to study the effect of these alternative Smad-independent signaling pathways on tumorigenesis and metastasis. By using specific inhibitors of the MAPK pathways and of the ALK5 kinase in either MIV or MIV-RImL45 cells, we have shown that Smad signaling and other non-Smad pathways, including p38 MAPK and JNK, contribute to migration of MIV cells in vitro (Fig. 6). Although the ability of cells to migrate is but one indication of metastatic potential, the observation that the metastatic activity of MIV cells was severely compromised in cells stably expressing RImL45 suggests that the Smad pathway is essential for metastasis, whereas the p38 and JNK pathways also may be necessary but not sufficient. Thus, whereas signaling via the MAPK pathways undoubtedly plays a role in the...
carcinogenicity and metastasis of Ha-Ras-transformed MCF10A cell system, our data clearly demonstrate that TGF-β and Smad play a dominant role in tumorigenesis and metastasis in this cell system.

Others have also investigated effects of alterations in TGF-β receptor expression on the dual tumor suppressor/prometastatic effects of TGF-β in breast cancer. Most pertinent to our own studies using the series of MCF10A cells are studies of Tang et al. (26) demonstrating a causal role for loss of type II receptor in breast cancer progression. Similar to our results, they showed that expression of DNT/BR1II enhanced the tumorigenesis of MII and MIII cells but reduced formation of lung metastases in MIV cells. Using a different model, Siegel et al. (39) showed that expression of an activated TGF-β type I receptor under control of the mouse mammary tumor virus promoter suppressed Neu-induced mammary tumors but increased the subsequent formation of lung metastases. Together, these studies attribute tumor suppressor activities to the TGF-β receptor complex in growth of primary tumors but demonstrate a requirement for receptor function in metastasis. What our study uniquely contributes to this interpretation, based on use of a type I receptor selectively compromised in its ability to initiate signaling through the Smad pathway but competent to activate non-Smad signaling pathways, is the finding that the key downstream pathway involving receptor-activated Smads is essential for both the tumor suppressor and prometastatic effects of TGF-β. This conclusion is strengthened by our previous study in which the Smad pathway was manipulated directly, independent of changes in receptor expression (12).

Further highlighting this bifunctional role of TGF-β in tumorigenesis are recent studies based on bitransgenic mice resulting from a cross of mice expressing an active form of TGF-β driven by the mouse mammary tumor virus promoter with mouse mammary tumor virus-Neu mice. Mammary tumors in the bigenic mice proliferated more slowly but exhibited increased cell survival, local invasion, intravasation, and metastases compared with tumors in mouse mammary tumor virus-Neu mice. These results, involving manipulation of the ligand rather than of the receptor, reinforce the interpretation that TGF-β1 can exert tumor suppressor (anti-proliferative) activities, probably on early progenitors, and tumor-promoting effects (invasion, motility, and survival) on cells as they progress (40).

It is known that growth inhibitory effects of TGF-β are strongly reduced in more advanced cancers and that gene targets involved in growth suppressive effects of TGF-β become insensitive to TGF-β, consistent with the lack of effect of overexpression of either the wild-type or RimL45 receptor in MIV cells on inhibition of growth by TGF-β. Our data suggest that additional investigation of the direct gene targets of the Smad pathway in cells in which it has tumor suppressor activity compared with those in which it elicits prometa-static behavioral changes will deepen our understanding of cancer progression and guide the development of new therapies for management of cancer.

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