Autocrine Transforming Growth Factor-β Signaling Mediates Smad-independent Motility in Human Cancer Cells

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Transforming growth factor-β (TGF-β) is a pleiotropic growth factor that plays a critical role in modulating cell growth, differentiation, and plasticity. There is increasing evidence that after cells lose their sensitivity to TGF-β-mediated growth inhibition, autocrine TGF-β signaling may potentially promote tumor cell motility and invasiveness. To understand the molecular mechanisms by which autocrine TGF-β may selectively contribute to tumor cell motility, we have generated MDA-MB-231 breast cancer cells stably expressing a kinase-inactive type II TGF-β receptor (TβRII-K277R). Our data indicate that TβRII-K277R is expressed, can associate with the type I TGF-β receptor, and block both Smad-dependent and -independent signaling pathways activated by TGF-β. In addition, wound closure and transwell migration assays indicated that the basal migratory potential of TβRII-K277R expressing cells was impaired. The impaired motility of TβRII-K277R cells could be restored by reconstituting TGF-β signaling with a constitutively active TGF-β type I receptor (ALK5TT) but not by reconstituting Smad signaling with Smad2/4 or Smad3/4 expression. In addition, the levels of ALK5 expression sufficient to restore motility in the cells expressing TβRII-K277R were associated with an increase in phosphorylation of Akt and extracellular signal-regulated kinase 1/2 but not Smad2. These data indicate that different signaling pathways have different thresholds for TGF-β activation and suggest that TGF-β promotes motility through mechanisms independent of Smad signaling, possibly involving activation of the phosphatidylinositol 3-kinase/Akt and/or mitogen-activated protein kinase pathways.

Transforming growth factor-β (TGF-β) is a pleiotropic polypeptide growth factor that is part of a superfamily of structurally related ligands that includes the TGF-βs, activins, and bone morphogenetic proteins (BMPs) (1). TGF-β ligands play a critical role in modulating cell growth, differentiation, plasticity, and migration. They elicit their biological effects by binding to a heteromeric complex of transmembrane serine/threonine kinases, the type I and type II receptors. TGF-β ligands can also bind to a transmembrane proteoglycan referred to as the type III receptor, which is thought to present ligand to the signaling type I and type II receptors. Following ligand binding to the type II receptor, the type I receptor is recruited to the complex. This allows the type II receptor, which is a constitutively active kinase, to transphosphorylate and thereby activate the type I receptor (2). Multiple pathways have now been implicated in mediating TGF-β effects downstream of these receptors. These include the extracellular signal-regulated kinase (ERK) (3, 4), c-Jun NH2-terminal kinase (JNK) (5–7), p38 mitogen-activated protein kinase (MAPK) (8, 9), and phosphatidylinositol 3-kinase (PI3K) pathways (10, 11). Several small GTPases can also be activated by TGF-β (12) and are involved in the activation of many of the above-mentioned signaling pathways. However, the Smad pathway was the first signaling pathway identified to mediate TGF-β effects and remains the best characterized (reviewed in Ref. 1).

Signal transduction through the Smad pathway involves phosphorylation of a set of intracellular signaling proteins termed receptor-regulated Smads (R-Smads) by the activated type I receptor. Once phosphorylated, R-Smads can associate with a common mediator Smad, Smad4, to translocate to the nucleus, and regulate gene transcription. In addition to the R-Smads and the common mediator Smad, Smad4, there is a distinct, structurally related class of antagonistic Smads, Smad6 and Smad7, which inhibit TGF-β family signals. Smad6 preferentially inhibits BMP signaling by either competing with Smad4 for binding to R-Smads (13) or interfering with BMP receptor-mediated phosphorylation of Smads (14). Smad7 has been reported to inhibit both TGF-β and BMP signaling by binding to activated type I receptors and interfering with their ability to phosphorylate R-Smads (15, 16).

Although TGF-β1 was originally identified for its ability to cause reversible phenotypic transformation and anchorage-independent growth of fibroblasts (17, 18), TGF-β can act as both a tumor suppressor and a tumor promoter (19, 20). TGF-β elicits most of its tumor suppressor activity by potently inhibiting the proliferation of most epithelial cells. It is thought that escape from the growth inhibitory effects of TGF-β through protein kinase; PI3K, phosphatidylinositol 3-kinase; R-Smad, receptor-regulated Smad; dn, dominant-negative; FCS, fetal calf serum; ALK, activin-like receptor kinase; HA, hemagglutinin; GFP, enhanced green fluorescent protein; m.o.i., multiplicity of infection; MEK, MAPK/ERK kinase; D-PBS, Dulbecco’s phosphate-buffered saline; EMT, epithelial mesenchymal transformation.

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dysregulated expression or mutational inactivation of various components of the TGF-β signaling pathway can contribute to tumorigenesis (21–23). In addition, there is increasing evidence that after cells lose their sensitivity to TGF-β-mediated growth inhibition, autocrine TGF-β signaling may promote tumorigenesis. The importance of autocrine TGF-β signaling in tumor progression has been highlighted by several studies that have shown that expression of a dominant-negative type II TGF-β receptor (dnTβRII) in various tumor cells can prevent the conversion of cells from an epithelial to an invasive mesenchymal phenotype, delay tumor growth, and reduce metastases (24–27). These data suggest that TGF-β can act directly on tumor cells to promote tumor maintenance and progression. In addition to promoting epithelial to mesenchymal transformation of tumor cells, TGF-β can stimulate the motility of many cell types in vitro, suggesting that TGF-β production in vivo may enhance migration of tumor cells and thus contribute to tumor invasiveness and metastases. There is also evidence that TGF-β can increase cellular motility without affecting proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways (28).

To understand the molecular mechanisms by which autocrine TGF-β may selectively contribute to tumor cell motility, we have generated MDA-MB-231 breast cancer cells stably expressing dnTβRII. MDA-MB-231 cells express TGF-β receptor 29, secrete TGF-β 30, and, although they are resistant to the growth inhibitory effects of TGF-β 29, can respond to TGF-β with an increase in spreading 31 and invasiveness 32. In addition, there is evidence that blocking TGF-β signaling by administration of a neutralizing TGF-β antibody can inhibit MDA-MB-231 cell tumorigenicity and metastases in nude mice 33. In this paper we show that expression of dnTβRII in MDA-MB-231 cells impairs their basal migratory potential. This impairment in motility can be restored by expression of a constitutively active type I TGF-β receptor (ALK5) but not by overexpression of Smad4/2 or Smad3/4. In addition, the levels of ALK5 34 expression sufficient to restore motility in the cells expressing dnTβRII are associated with an increase in phosphorylation of Akt and ERK1/2, but not Smad2, suggesting that Smad signaling is dispensable for autocrine TGF-β-mediated motility and that this response depends on alternative signaling pathways activated by TGF-β.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The MDA-MB-231 and MDA-MB-468 breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS). The SW480.7 clone 15.13 (34) was a gift from Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York, NY) and was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 0,3 mg/ml Zeocin, and 0,7 mg/ml G418. TGF-β1 and BMP2 were obtained from R&D Systems (Minneapolis, MN). Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands) graciously provided the rabbit polyclonal sera directed against activin-like receptor kinases (ALKs) (35). Antibodies against the hemagglutinin (HA) epitope (catalog number sc-7392), the type II TGF-β receptor (catalog number sc-220), Smad4 (catalog number sc-7966), and p38 MAPK (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to fibronectin (catalog number F14420) and the phospho-p38 MAPK antibody (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Smad2 (catalog number sc-220), Smad4 (catalog number sc-7966), and p38 MAPK (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to fibronectin (catalog number F14420) and the phospho-p38 MAPK antibody (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Smad2 (catalog number sc-220), Smad4 (catalog number sc-7966), and p38 MAPK (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to fibronectin (catalog number F14420) and the phospho-p38 MAPK antibody (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Smad2 (catalog number sc-220), Smad4 (catalog number sc-7966), and p38 MAPK (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to fibronectin (catalog number F14420) and the phospho-p38 MAPK antibody (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Smad2 (catalog number sc-220), Smad4 (catalog number sc-7966), and p38 MAPK (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA).
Fig. 1. TβRII-K277R is expressed in MDA-MB-231 cells. MDA-MB-231 parental cells, as well as clones and pods stably expressing GFP alone (G15–5, G15–6, and G15 Pool) or GFP and TβRII-K277R (dn15–2, dn15–3, dn15–5, and dn15–11 clones and dn15 Pool), were affinity labeled with 100 pm 125I-TGF-β1 and cross-linked with BS3. Labeled ligand-receptor complexes were resolved by SDS-PAGE using a 3–12% gradient gel and visualized by autoradiography (A) or lysed and incubated with a mouse monoclonal anti-HA antibody for immunoprecipitation of HA-tagged TβRII-K277R (B). Immunoprecipitates were resolved by SDS-PAGE using a 7.5% polyacrylamide gel and visualized by autoradiography. Affinity labeled but non-immunoprecipitated (NIP) G15–5 cells were loaded as a reference (lane 1).

Fig. 2. TβRII-K277R can associate with TβRI. MDA-MB-231 pools expressing GFP alone (GABE 15 Pool; left panel) or GFP and TβRII-K277R (dnTβRII 15 Pool; right panel) were affinity labeled with 100 pm 125I-TGF-β1, cross-linked with BS3, lysed, and incubated with normal rabbit serum (NRS), polyclonal rabbit antiserum directed against various type I TGF-β superfamily receptors (ALK1, ALK2, and ALK5), the type II TGF-β receptor (TβRII), or HA as indicated. Immunoprecipitates were resolved by SDS-PAGE using a 3–12% polyacrylamide gel and visualized by autoradiography. Affinity labeled but non-immunoprecipitated (NIP) cells were loaded as a reference (lane 1).

Transcription Reporter Assays—Cells were transiently transfected with 1 μg per 35-mm dish of the Smad-dependent heterologous promoter reporter construct pCAGA1-Luciferase (38) provided by Dr. Jean-Michel Gauthier (Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) along with 0.01 μg per 35-mm dish of pCMV-Renilla using FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The following day, wells were split into 24-well plates, and ~48 h post-transfection, cells were either left unstimulated or were stimulated with 40 pm TGF-β1 for 16–20 h. All cells were then washed with PBS and lysed. Firefly and Renilla reniformis luciferase activities were measured using Promega’s dual luciferase reporter assay system according to the manufacturer’s protocol. Luciferase activity was normalized utilizing the ratio of Firefly to R. reniformis luciferase activity and presented as -fold induction. All assays were done in triplicate wells, and each experiment was repeated at least twice.

Wound Closure and Transwell Motility Assays—For wound closure assays, confluent cell monolayers were wounded by manually scraping the cells with a pipette tip. Following wounding, wound size was verified with an ocular ruler to ensure that all wounds were the same width. The cell culture medium was then replaced with fresh medium, and wound closure was monitored by microscopy at various times.

Transwell motility assays were performed utilizing 8-mm pore, 6.5-mm polycarbonate transwell filters (Corning Costar Corp., Cambridge, MA). For these assays, single cell suspensions were seeded in serum-free medium containing 0.1% bovine serum albumin on the upper surface of the filters and allowed to migrate toward various concentrations of FCS. After a 16–20 h incubation period, cells on the upper surface of the filter were wiped off with a cotton swab, and the cells that had migrated to the underside of the filter were fixed, stained with 0.5% crystal violet, and counted by brightfield microscopy at ×200 in five random fields.

Adenoviral Expression of ALKs and Smads—The adenoviral construct encoding FLAG-tagged Smad4 (39) was obtained from Dr. Harold Moses (Vanderbilt University, Nashville, TN). All other adenoviral constructs encoding FLAG-tagged Smads or HA-tagged constitutively active mutants of the TGF-β (ALK5Q233D), activin (ALK2Q207D), and BMP (ALK3Q234D) type I receptors (40) were generously provided by Dr. Kohei Miyazono (Japanese Foundation for Cancer Research, Tokyo, Japan). Stocks of recombinant viruses for each of these constructs were generated in 293 cells and titered utilizing the Takara assay (Takara Biomedicals, Tokyo, Japan). Cells were then infected with these or a control β-galactosidase adenovirus at a multiplicity of infection (m.o.i.) that resulted in ~90% cell infection (~15 plaque-forming units/cell or less). The efficiency of infection was evaluated by in situ staining of cells for β-galactosidase activity 48 h following infection.

RESULTS

TβRII-K277R Is Expressed in MDA-MB-231 Cells—To abrogate TGF-β signaling in MDA-MB-231 cells, an expression vector encoding GFP and kinase-inactive TβRII was transfected into cells. Expression of the kinase-inactive TβRII-K277R mutant was verified by affinity labeling cell surface receptors with 125I-TGF-β1 (Fig. 1A). Because TβRII-K277R has an intact extracellular domain, it can still bind TGF-β and should therefore co-migrate with endogenous TβRII. Cell surface labeling of parental cells resulted in the labeling of three proteins corresponding to the endogenous type I, II, and III TGF-β receptors. There was little or no change in the amount of receptor labeling observed in the control cells expressing GFP alone (GABE 15 Pool) compared with parental cells. However, in the pool expressing TβRII-K277R (dnTβRII 15 Pool),
there was a significant increase in the amount of labeled type II receptor, suggesting that the exogenous receptor was expressed. Individual clones obtained from each of these pools expressing either GFP alone (G15–5 and G15–6) or GFP and TgIRI-K277R (dn15–2, dn15–3, and dn15–11) were immunoprecipitated with an HA antibody. As shown in Fig. 1B, the HA antibody immunoprecipitated a labeled type II receptor in the pool and clones expressing TgIRI-K277R but not in the control pool or clones expressing GFP alone, confirming transgene expression.

To confirm that this increase in the labeling of TgIRI was indeed because of expression of the HA-tagged TgIRI-K277R, extracts from affinity labeled cells were immunoprecipitated with an HA antibody. As shown in Fig. 1B, the HA antibody immunoprecipitated a labeled type II receptor in the pool and clones expressing TgIRI-K277R but not in the control pool or clones expressing GFP alone, confirming transgene expression. The type I TGF-β receptor appeared to co-immunoprecipitate with TgIRI-K277R in these experiments. This was confirmed in subsequent co-immunoprecipitation experiments (see Fig. 2).

**TgIRI-K277R Is Functional in MDA-MB-231 Cells**—Having ascertained that TgIRI-K277R was expressed, we then examined whether it was functional. Immunoprecipitation experiments revealed that when affinity labeled cells expressing TgIRI-K277R were precipitated with an HA antibody, a labeled protein of the size of a type I receptor co-precipitated with TgIRI-K277R (see Fig. 1B and Fig. 2, right panel, lane 8). We confirmed that this was TpRI by precipitating similarly labeled cells with various TGF-β superfamily type I receptor antibodies, including ALK1, -2, and -5. Only the ALK5 (TpRI), but not the ALK1 or ALK2 antibodies, precipitated the cross-linked type I receptor (Fig. 2). Although the TgIRI antibody co-precipitated ALK5 efficiently, the ALK5 antibody co-precipitated TgIRI only weakly (see Fig. 2, lane 5, both panels). In the control GABE 15 Pool, the HA antibody failed to precipitate any proteins, as expected. Immunoprecipitations with a TgIRI antibody were carried out in both pools as a positive control, and both resulted in the co-precipitation of TgIRI (Fig. 2, lane 7, both panels). These data indicate that TgIRI-K277R associates with TpRI.

To determine whether TgIRI-K277R prevented TGF-β signaling, we examined its effect on the ability of TgIRI to phosphorylate Smad2. Immunoblot analysis of TGF-β1-treated cell lysates using a phospho-specific Smad2 antibody revealed that although TGF-β1 could induce phosphorylation of Smad2 in both GABE clones (G15–5 and G15–6), its ability to do so in the TgIRI-K277R clones (dn15–2, -3, -5, -11) was impaired (Fig. 3B). In the GABE clone Smad2 staining was relatively diffuse, but upon TGF-β1 treatment for 60 min, Smad2 staining became concentrated in the nucleus. In contrast, in the TgIRI-K277R clones, there was little or no change in Smad2 staining following TGF-β1 treatment, suggesting impaired TGF-β-mediated translocation of Smad2 to the nu-
cleus. We then examined the effect of TβRII-K277R on TGF-β1-induced transcription. A reporter construct containing twelve Smad binding elements repeated in tandem, p(CAGA)₁₂-Luciferase, was transiently transfected into the GABE and TβRII-K277R clones, along with pCMV-Renilla. Normalized luciferase activity indicated that TGF-β1 could induce transcription of both reporter constructs in the GABE clones, but its ability to do so in the TβRII-K277R clones was impaired (Fig. 3C).

To determine whether signaling pathways other than the Smad pathway were also affected by expression of TβRII-K277R, we examined fibronectin expression, which has been reported to be induced by TGF-β in a JNK-dependent but Smad4-independent manner (6). We chose to perform these experiments in our pools as the results obtained in these cells are representative of those obtained in the clones (compare phospho-Smad2 blots in Fig. 3, A and D). Following TGF-β stimulation for 24 h, we observed an increase in fibronectin expression in the GABE pool, but this induction was decreased significantly in the pool expressing TβRII-K277R, as was the basal level of fibronectin expression (Fig. 3D). We were unable to detect any induction of phosphorylation of JNK in response to TGF-β in our GABE pools (data not shown). However, we did observe an increase in phosphorylation of p38 MAPK following TGF-β stimulation for 60 min, and this induction of phosphorylation was slightly attenuated in the pool expressing TβRII-K277R (Fig. 3D).

**TβRII-K277R Impairs the Motility of MDA-MB-231 Cells**—Next we examined the effect of TβRII-K277R expression on the motility of MDA-MB-231 cells in a wound closure assay. In the GABE clones, cells migrated into the wounded area and closed the wound within 24 h, whereas in the TβRII-K277R clones the wound remained open at 24 h (Fig. 4A). This difference in motility did not appear to be because of an effect on proliferation, because when the experiment was performed in the presence of mitomycin C, a compound that inhibits cell division, the same results were obtained (data not shown). Thus, expression.
of TβRII-K277R in MDA-MB-231 cells appears to impair their motility, independent of changes in proliferation. As an alternative measure of cell motility, we also examined the effect of TβRII-K277R on the ability of cells to migrate in a transwell assay system. We observed a 3- to 4-fold reduction in the ability of cells expressing TβRII-K277R to migrate toward FCS, compared with control cells expressing GFP alone (Fig. 4B).

**The Impaired Motility of TβRII-K277R Cells Is TGF-β Type I Receptor-specific**—Because the impaired motility of TβRII-K277R cells was observed in the absence of exogenous TGF-β stimulation, we wished to determine whether this impairment was TGF-β-specific. To do so, we chose to restore TGF-β signaling in TβRII-K277R cells by expressing a constitutively active mutant of TβRII. Mutation of threonine 204 in ALK5 (TβRII) to aspartic acid leads to constitutive activation of the type I receptor kinase, allowing it to induce signals in the absence of ligands or type II receptors (41). Likewise, mutation of corresponding threonine and glutamine residues in the activin (42) and BMP (43) type I receptors to aspartic acid also leads to constitutive activation of these kinases. To test for TGF-β specificity, cells expressing TβRII-K277R were infected with adenoviruses encoding HA-tagged constitutively active mutants of the TGF-β (ALK5TD), activin (ALK2QD), and BMP (ALK3QD) type I receptors (40). Uninfected cells or cells infected with a β-galactosidase adenovirus at the same m.o.i. were used as controls. The ability of Smads to restore TGF-β signaling in dn15–2 was evaluated in transcription reporter assays utilizing the TGF-β responsive transcription reporter p(CAGA)12-Luciferase (B). The effect of Smad expression on wound closure in the control G15 Pool (top panel) and dn15–2 clone (bottom panel) was monitored by microscopy at the times indicated (C).
Smad-dependent signaling was examined utilizing the Smad-body (Fig. 6). Confirmed by immunoblot analysis utilizing an anti-FLAG anti-Smad3 and Smad4, and exogenous Smad expression was confirmed by immunoblot analysis utilizing an anti-FLAG anti-Smad2 and Smad4 or FLAG-tagged Smad expression. Cells were then wounded, washed, and incubated with serum-free medium in the presence of 0, 4, 20, or 100 pm TGF-β1 for 24 h. Wound closure was monitored by microscopy at the times indicated (B). At the conclusion of the wound closure experiment, cells were lysed, and Smad4 expression was examined by immunoblot analysis utilizing a monoclonal antibody directed against Smad4 (A). The blots were also probed with an actin antibody to verify equal loading. Ponasterone was maintained in the culture medium of selected wells throughout the experiment.

Activation of basal transcription was not as marked with Smad3/4, as expected, because Smad2 itself cannot bind DNA. As expected, in the absence of Smad signaling in other cells, the Smad4 defective SW480.7 colorectal cells, conditionally expressing Smad4 via an ecdysone-inducible system (34), were utilized. Cells were infected with adenoviruses encoding FLAG-tagged Smad2 and Smad4 or FLAG-tagged Smad3 and Smad4, and exogenous Smad expression was confirmed by immunoblot analysis utilizing an anti-FLAG antibody (Fig. 6A). The ability of Smad2/4 and Smad3/4 to activate Smad-dependent signaling was examined utilizing the Smad-dependent transcription reporter construct, p(CAGA)$_{12}$-Luciferase. Expression of Smad3/4 resulted in a marked increase in basal transcription (Fig. 6B). Stimulation with TGF-β1 did not cause any further increase in transcription, suggesting that Smad signaling was activated maximally. Despite this, Smad3/4 failed to restore motility in the cells expressing TβRII-K277R (Fig. 6C, bottom panel) and had no effect on the motility of control cells expressing GFP alone (Fig. 6C, top panel). These results indicate that reconstitution of Smad signaling alone is insufficient to restore autocrine TGF-β-mediated motility in cells expressing TβRII-K277R, nor is it sufficient to enhance the motility of control MDA-MB-231 cells.

Re-expression of Smad4 in Smad4-defective Cancer Cells Does Not Enhance Motility—To determine whether Smads are required for cancer cell migration, we examined whether activation of TGF-β signaling could promote motility in the absence of Smad signaling utilizing Smad4 null MDA-MB-468 breast cancer cells (47). Smad4 and ALK5TD were expressed, either alone or in combination, by adenoviral transduction, and their effects on the motility of MDA-MB-468 cells were examined in wound closure assays. Expression of HA-tagged ALK5TD and FLAG-tagged Smad4 was confirmed by immunoblot analysis (Fig. 7A), and their ability to activate Smad-dependent signaling was examined in transcription reporter assays utilizing the Smad-dependent p(CAGA)$_{12}$-Luciferase reporter construct (Fig. 7B). As expected, in the absence of Smad4 (uninfected, β-galactosidase, ALK5TD alone), TGF-β1 was unable to stimulate transcription in these cells. However, upon re-expression of Smad4 a marked increase in both TGF-β1-mediated and ALK5TD-mediated transcription was observed, indicating that both Smad4 and ALK5TD were indeed functional in these cells. Despite this, neither Smad4 nor ALK5TD had any effect on cell motility, whether they were expressed alone or in combination (Fig. 7C). The fact that ALK5TD could not promote motility, even when Smad4 was co-expressed with it, suggests that MDA-MB-468 cells are not responsive to the pro-migratory effects of TGF-β.

To determine whether TGF-β could induce migration in the absence of Smad signaling in other cells, the Smad4 defective SW480.7 colorectal cells, conditionally expressing Smad4 via an ecdysone-inducible system (34), were utilized. Cells were stimulated with increasing concentrations of TGF-β1 in the absence or presence of 3 μM ponasterone to induce Smad4 expression. Smad4 expression in ponasterone-treated cells was confirmed by immunoblot analysis (Fig. 8A), and its effect on TGF-β-mediated motility was examined in wound closure assays. Again, as in the MDA-MB-468 cells, these cells failed to
respond to TGF-β both in the absence and presence of Smad4 (Fig. 8D). Taken together, these data indicate that reconstitution of Smad signaling alone is not sufficient to promote migration of cancer cells.

**Different Signaling Pathways Require Different Thresholds of TGF-β Activation**—To determine whether Smad signaling is actually required in addition to other pathways activated by TGF-β to promote migration, we examined what signaling pathways were activated under conditions where motility was restored following ALK5TD expression in cells expressing TβRII-K277R (see Fig. 5C and Fig. 9B). For these experiments, expression of ALK5TD was confirmed by HA immunoblot (Fig. 9A), and the activation status of candidate signaling pathways was examined utilizing phospho-specific antibodies, as indicated (A). Actin was examined as a loading control. In C, clone dn15–2 was re-infected with ALK5TD at m.o.i. values of 0, 5, 15, 30, or 60, as indicated, and the activation status of candidate signaling pathways was re-examined utilizing phospho-specific antibodies as in A.

![Fig. 9. Different signaling pathways require different thresholds of TGF-β activation.](image)

**DISCUSSION**

In this study, abrogation of autocrine TGF-β signaling in MDA-MB-231 breast cancer cells resulted in an impairment in basal cell migration, which could not be restored by reconstituting Smad signaling, suggesting that Smad signaling alone is not sufficient for autocrine TGF-β-mediated motility. Consistent with this, reconstitution of Smad signaling in the Smad4-defective MDA-MB-468 and SW480.7 cells did not promote migration. In addition, restoration of migration following restoration of TGF-β signaling in cells expressing TβRII-K277R was associated with an increase in phosphorylation of Akt and ERK1/2 but not Smad2. These results indicate that Smad signaling is dispensable for TGF-β-mediated motility and that
pharmacological inhibitors of the PI3K, p38 MAPK, MEK, and JNK pathways activated by TGF-β. This suggests that Smad3 is not required for this response or that residual Smad3 signaling, not blocked by expression of dominant-negative Smad3, is sufficient to mediate motility. This would be consistent with the idea that different biological responses require different thresholds of TGF-β signaling (27). Thus, complete abrogation of Smad3 signaling might be required to observe an impairment in TGF-β-mediated motility whereas partial blockade of Smad function might be sufficient to block the anti-proliferative effects of TGF-β.

Interestingly, expression of either dnSmad4 or antagonistic Smad7 in MDA-MB-231 cells resulted in cell death. Although overexpression of Smad7 has been reported to sensitize various cell types to cell death (56), expression of dnSmad4 has not been associated with such a response. There is, however, increasing evidence that TGF-β can promote the survival of both transformed (7, 57, 58) and non-transformed (59–61) cells. Whether Smad signaling is required for these pro-survival effects of TGF-β is not known. In addition, re-expression of Smad4 in Smad4-defective SW480 has been reported to induce a more adhesive and flat phenotype (62). These results suggest that blockade of Smad signaling could potentially lead to loss of adhesion and result in anoikis. This could explain our inability to express dnSmad4 and Smad7 in MDA-MB-231 cells.

Because we were unable to assess the requirement for Smads in autocrine TGF-β-mediated motility by abrogating Smad signaling, we chose to address this question by activating Smad signaling in cells expressing TβRII-K277R. Having ascertained that the impaired motility of TβRII-K277R cells was indeed TβRII-specific, we overexpressed the TGF-β R-Smads, Smad2 or Smad3, each with Smad4, to determine whether autocrine TGF-β-mediated motility was Smad-dependent. Despite their ability to activate Smad-dependent transcription, neither Smad combination restored the impaired motility of the TβRII-K277R cells. We (data not shown) and others (62) have observed an increase in cell spreading following Smad overexpression. It is tempting to speculate that this increased cell spreading may be associated with increased adhesion, which interferes with cell migration. This could potentially explain why restoration of Smad signaling in TβRII-K277R cells failed to restore migration. These data suggest that in breast cancer cells, autocrine TGF-β signaling mediates motility in a Smad-independent manner or that alternative pathways, in addition to the Smad signaling pathway, are required for these effects.

To address this question, we examined what signaling pathways were activated under conditions where motility was restored in TβRII-K277R cells following expression of a constitutively active type I TGF-β receptor. In these experiments, we observed an increase in the phosphorylation of Akt and ERK1/2 but not Smad2. These data further imply that Smad signaling is not required for TGF-β-mediated motility. Although expression of ALK5(T617) may induce a low level of Smad phosphorylation, which cannot be detected by immunoblot analysis, it is unlikely that Smads are required for autocrine TGF-β-mediated motility as reconstitution of Smad signaling in both MDA-MB-468 and SW480.7 cancer cell lines failed to promote motility, and expression of both low and high levels of either Smad2/4 or Smad3/4 failed to rescue the impaired motility of MDA-MB-231 cells expressing dnTβRII. Thus, alternative signaling pathways activated by TGF-β are more likely to be important for migration. Indeed, blockade of the PI3K, p38 MAPK, MEK, and JNK pathways with pharmacological inhibitors impaired TGF-β-stimulated migration. The fact that inhibitors of p38 MAPK and JNK interfered with TGF-β-induced
migration even though ALK5\textsuperscript{TD} failed to alter their phospho-
rylation status suggests that these signaling pathways, though not activated further by TGF-\(\beta\) in our experimental system, are required for basal cell migration. In agreement with this, we have indeed observed an impairment in the basal migratory potential of these cells in the presence of these inhibitors (data not shown).

The observation that different levels of ALK5\textsuperscript{TD} expression resulted in differential activation of downstream targets (Fig. 9C) indicates that different signaling pathways require different thresholds of TGF-\(\beta\) activation. In agreement with this, others have reported that expression of dnT\(\beta R I I\) in NMuMG mammary cells impairs TGF-\(\beta\)-mediated Smad-dependent in-
hibition of proliferation but not TGF-\(\beta\)-mediated activation of p38 MAPK (9). In addition, there is evidence that different biological responses mediated by TGF-\(\beta\) also require different thresholds of TGF-\(\beta\) signaling. For example, expression of dnT\(\beta R I I\) in squamous carcinoma cells has been reported to block the growth inhibitory effects of TGF-\(\beta\) but not its ability to induce EMT (26). Likewise, expression of dnT\(\beta R I I\) in 4T1 murine mammary cancer cells impairs TGF-\(\beta\)-mediated tran-
scription but fails to block motility (27). Because TGF-\(\beta\) signal-
ing was not completely abrogated in the squamous and mam-
mary cancer cells (26, 27), the molecular mechanisms by which autocrine TGF-\(\beta\) may selectively contribute to tumor progres-
sion could not be fully addressed in those studies. Because we have expressed T\(\beta R I I\)K277R at levels high enough to block both Smad and non-Smad pathways in MDA-MB-231 cells, the model we have generated should prove useful in dissecting the signaling pathways required for the diverse effects elicited by TGF-\(\beta\) in cancer.

Our data indicate that autocrine TGF-\(\beta\)-mediated motility of cancer cells is Smad-independent. This implies that non-trans-
formed cells and transformed cells utilize different mecha-
nisms to promote motility as others have reported that Smad3
null monocytes and keratinocytes exhibit significantly reduced
migration to TGF-\(\beta\) in transwell motility assays (54). More-
ever, Smad3 appears to be required for TGF-\(\beta\)-mediated mono-
cyte chemotaxis in vivo, as mice lacking the Smad3 gene dis-
play a blunted monocyte chemotactic response following
cutaneous wounding (54). Studies in Drosophila also suggest that
Smads may be required for cell migration as mutations in
Mad, the Drosophila receptor-activated Smad, impair migra-
tion of the epidermis during dorsal closure (55). Finally, recent
studies in endothelial cells have indicated that TGF-\(\beta\) acting
through ALK1 stimulates migration in a Smad-independent manner, whereas TGF-\(\beta\) acting through ALK5 inhibits cell
migration in a Smad-dependent manner (50). Taken together,
these studies highlight the importance of Smads in TGF-\(\beta\)-
regulated migration of non-transformed cells.

Despite compelling evidence for the role of Smads in non-
transformed cell migration, a lack of requirement for Smad
signaling in TGF-\(\beta\)-mediated cancer cell migration is consist-
ent with previous studies that have shown that TGF-\(\beta\)
can increase cellular motility of prostate cancer cells without af-
flecting proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways (28). Likewise, expression of Smad7 in pancreatic cancer cells has been shown to abrogate the anti-proliferative effects of TGF-\(\beta\) but enhance matrix-associated transcriptional re-
sponses, highlighting a dissociation between the matrix and
anti-proliferative effects induced by TGF-\(\beta\) (63). If the bio-

gical effects of TGF-\(\beta\) that can contribute to tumor progression
were Smad-independent, it might be possible to selectively
disrupt those pathways, while ensuring that the tumor sup-
pressive, Smad-dependent pathways are maintained. The sig-
naling pathways currently implicated in mediating the various
pro- and anti-tumorigenic effects of TGF-\(\beta\) indicate that this
may in fact be possible. For example, recent studies aimed at
identifying the mechanisms by which TGF-\(\beta\)-1 elicits EMT in
mammary cells have indicated that the PI3K, RhoA, and p38 MAPK
pathways are involved in this process (8, 9, 11, 53). However, whether Smad signaling, which has been implicated in both the anti-proliferative (44, 45) and pro-apoptotic (64, 65) effects of TGF-\(\beta\), is also required for TGF-\(\beta\)-mediated EMT is unclear. In one study, adenoviral expression of low levels of constitutively active ALK5 induced EMT only if Smad2/4 or Smad3/4 were co-expressed (66). In contrast, other investiga-
tors have reported that inhibition of Smad signaling either by
overexpression of Smad7 or dominant-negative Smad3 did not
affect the transdifferentiation, arguing against the involve-
ment of Smads in EMT (53). Because epithelial transdifferen-
tiation to a mesenchymal phenotype is often associated with
acquisition of motile properties, the mechanisms through
which TGF-\(\beta\) mediates EMT may be similar to those required for
TGF-\(\beta\)-mediated motility. Indeed, the PI3K, RhoA, and p38
MAPK signaling pathways, which are required for TGF-\(\beta\)-me-
diated EMT, have also been implicated in TGF-\(\beta\)-mediated
motility (8, 11, 53). Likewise, we have observed that blockade of
these and other pathways interfere with TGF-\(\beta\)-induced motil-
ity (Fig. 10), suggesting that multiple pathways cooperate to
elicit this effect. It will be of interest to determine whether
Smad signaling is required for other effects mediated by
TGF-\(\beta\), as a dissociation between the pathways required for the
tumor suppressive versus the tumor promoting effects of TGF-\(\beta\) could lead to opportunities to selectively inhibit the non-desir-
able effects of TGF-\(\beta\) without compromising its tumor suppres-
sive function.

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