

Silencing of Transforming Growth Factor- β 1 *In situ* by RNA Interference for Breast Cancer: Implications for Proliferation and Migration *In vitro* and Metastasis *In vivo*

Lakisha D. Moore, Tatyana Isayeva, Gene P. Siegal, and Selvarangan Ponnazhagan

Abstract Purpose: Overexpression of transforming growth factor (TGF)- β has been implicated in promoting immune suppression, tumor angiogenesis, tumor cell migration, and invasion in many cancers, including carcinoma of the breast. Thus, targeted down-regulation of TGF- β 1 expression in breast cancer *in situ* and determination of its implications would provide new treatment approaches for disease management.

Experimental Design: Small interfering RNA constructs targeting TGF- β 1 were validated and used to develop clonal derivatives of the metastatic breast cancer cell line MDA-MB-435. The cells were used in several *in vitro* analyses, including migration, invasion, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, apoptosis, and signaling assays. A wound-healing assay was used to determine migration of the cells in culture and a Boyden chamber transwell assay was used for invasion. Further, the clones were used in an *in vivo* mouse model for the kinetics of tumor growth and gene expression in the primary site and in lungs upon metastasis.

Results: Inhibition of TGF- β 1 expression in MDA-MB-435 cells showed a 35% decrease in migration and a 55% decrease in invasion *in vitro*, with a 50% increase in proliferation and no effect on apoptosis. *In vivo* analysis indicated a 90% decrease in the number of mice bearing macroscopic lung metastases; however, the primary tumors did not show any difference in the growth kinetics when compared with the parental MDA-MB-435 cells. Analysis of TGF- β signaling pathways in the clonal derivatives showed a decrease in Smad2 activation and an increase in AKT and extracellular signal-regulated kinase activation. Interestingly, analysis of TGF- β receptor expression showed a decrease in both receptor I and II expression in TGF- β 1 silenced cells. These results suggest that inhibition of TGF- β 1 ligand may act as a negative feedback loop to disrupt the function of all TGF- β isoforms.

Conclusions: Therapies targeting the TGF- β signaling pathway may be more effective in late-stage disease to prevent organ metastasis but not primary tumor formation and may be combined with other tumor-targeted therapies normally limited by increased circulating TGF- β levels.

Transforming growth factor (TGF)- β is involved in physiologic processes, such as wound healing, tissue development, and remodeling. TGF- β has also been implicated in many pathologic conditions, including cancer, and has been shown to regulate a number of events such as angiogenesis, immune suppression, and cell migration (1–5). Most breast cancers express TGF- β , with TGF- β 1 being the most prominent in the

plasma (6–8). Clinically, TGF- β 1 is often elevated in the plasma of breast cancer patients and, preclinically, several models have shown correlations between TGF- β expression and increased tumorigenicity, increased invasion, and increased drug resistance (9–13). In mouse models of breast cancer, TGF- β signaling has been shown to promote lung (14, 15) and bone metastasis (14, 16–18).

TGF- β is present as three different isoforms, which have both overlapping and distinct functions. TGF- β is a homodimer that is secreted as a latent complex composed of the 25 kDa active homodimer, latency-associated peptide, and latent TGF- β -binding protein (19). This latent complex is proteolytically cleaved to its active form and it is the active portion that binds to the TGF- β cell surface receptors to initiate intracellular signaling pathways (20). TGF- β signals through Smad proteins, which translocate into the nucleus to act as transcription factors for several target genes. Smad-mediated signaling of TGF- β has also been shown to interact with other signaling molecules to activate a number of cell signaling pathways (21–24).

Both Smad-dependent and Smad-independent TGF- β signaling pathways have been shown to play a role in normal and pathologic conditions (25–29). TGF- β signaling through

Authors' Affiliation: Department of Pathology, The University of Alabama at Birmingham, Birmingham, Alabama

Received 10/10/07; revised 4/2/08; accepted 4/11/08.

Grant support: U.S. Army Department of Defense grant BC044440 and the Susan G. Komen Breast Cancer Foundation grant BCTR50206.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Requests for reprints: Selvarangan Ponnazhagan, Department of Pathology, University of Alabama at Birmingham, LHRB 513, 701, 19th Street South, Birmingham, AL 35294-0007. Phone: 205-934-6731; Fax: 205-975-9927; E-mail: pons@uab.edu.

© 2008 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-07-4604

mitogen-activated protein kinase, c-Jun-NH₂-kinase, p38, phosphatidylinositol 3-kinase (PI3K), and G-proteins may be responsible for some of the oncogenic effects in late-stage tumorigenesis (30–33). It is believed that these Smad and non-Smad signaling pathways may contribute to the pro-oncogenic effect of TGF- β in late-stage tumors by promoting the epithelial-mesenchymal transition, migration, angiogenesis, immune suppression, and proliferation.

Several drugs are currently being designed to inhibit various stages of TGF- β signaling (34). Both ligand and receptor-targeted therapies have shown promise in mouse models by decreasing the metastatic index, suggesting that TGF- β may be an effective target for anticancer therapies. However, systemic delivery of drugs targeting TGF- β or TGF- β receptors will exert undesired effects on normal cells as here TGF- β modulates growth control. Thus, approaches to target TGF- β expression in tumor cells *in situ* should have specific tumoristic effects without affecting normal cell function.

The present study determined the effects of TGF- β inhibition on cell proliferation, apoptosis, migration, invasion, and cell signaling in the metastatic human breast cancer cell line MDA-MB-435 *in situ* by RNA interference. The results of these studies indicate that small interfering RNA (siRNA)-mediated silencing of TGF- β in the metastatic breast cancer cells decreased the migration and invasion *in vitro* and decreased the number of lung metastasis in an orthotopic model without affecting the growth kinetics of the primary tumors. Increased AKT and extracellular signal-regulated kinase (ERK) signaling in the TGF- β si clone indicates that combination therapies targeting these pathways along with TGF- β silencing may have a synergistic effect on decreasing tumor metastasis.

Materials and Methods

Reagents and cell lines. The metastatic human breast cancer cell line MDA-MB-435 was a generous gift from Dr. Janet Price (M.D. Anderson Cancer Institute, Houston, TX). The MDA-MB-435 cell line was derived from the pleural effusions of a 31-y-old Caucasian female diagnosed with metastatic breast cancer. Cells were maintained in L-15 medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2 \times nonessential amino acids, 2 mmol/L L-glutamine, and penicillin-streptomycin. Cells were grown in a 5% CO₂ incubator at 37°C. Restriction enzymes and other modifying enzymes were purchased from New England Biolabs, Inc. Monoclonal antibody for phospho-Smad2 was obtained from Upstate Biotechnology. Phospho-p85 PI3K, p85 PI3K, TGF- β RI, phospho-ERK 1/2, ERK 1/2, RhoA, phospho-AKT, and AKT antibodies were obtained from Cell Signaling. TGF- β RII antibody was purchased from Santa Cruz Biotechnology. TGF- β 2 and TGF- β 3 antibodies were purchased from Abcam. pRNAT siRNA expression vector was purchased from Genscript Corporation. The TGF- β 1 expression plasmid, pSport, was purchased from American Type Culture Collection.

Establishment of stable siRNA-expressing clones of MDA-MB-435 cells. The pRNAT plasmid expressing TGF- β 1 siRNA was constructed by ligating the short hairpin RNA sequence containing both sense and antisense strands, separated by a 9-bp loop region and containing both *Bam*H1 and *Hind*III restriction enzyme overhangs for directional cloning (5'-GATCCCGTTTAACTTGAGCCTCAGCAGACGCAGCTTCAA-GAGAGCTGCGTCTGCTGAGGCTCAAGTAAATTTTTTCCAAA-3'). MDA-MB-435 cells were transfected at 80% confluency with a pRNAT siRNA plasmid using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Twenty-four hours after transfection, the cells were split into 96-well plates and cultured in medium containing

1 mg/mL geneticin (G418). Single-cell clones and a mixed pool of 10 clones, constitutively expressing TGF- β 1 siRNA, were expanded and screened using reverse transcription-PCR (RT-PCR) as described below to determine transcriptional silencing of TGF- β 1. The mixed pool of clones was used for the remainder of the *in vitro* and *in vivo* studies.

Cell proliferation assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the proliferation rate of the cells. Five thousand cells were plated per well in a 96-well plate and allowed to attach overnight. Cells were serum starved overnight for synchronization and then grown for 72 h before analysis for proliferation. Unmodified MDA-MB-435 cells or clonal derivatives expressing TGF- β 1 siRNA or scrambled siRNA were then incubated with 12 mmol/L MTT stock solution for 4 h at 37°C and then 100 μ L of SDS-HCl solution were added to each well for an overnight incubation. Absorbance was read at 570 nm on a plate reader. A Vybrant MTT Cell Proliferation Assay Kit (V-13154) containing stock solution and SDS was obtained from Molecular Probes.

Apoptosis assay. Cells were plated in a six-well plate and allowed to grow to confluency. The cells were then trypsinized, washed with cold PBS, and then resuspended in 1 \times binding buffer at a concentration of 1 \times 10⁶/mL. One hundred microliters of the cell suspension were incubated with 5 μ L Annexin V and 5 μ L propidium iodine for 15 min, in the dark, at room temperature. After incubation, 400 μ L of 1 \times binding buffer were added to the solution and the buffer was analyzed by flow cytometry. All reagents used were included in the Annexin V FITC Apoptosis Detection Kit from BD Biosciences.

Migration (wound closure) assay. MDA-MB-435 cells and MDA-MB-435 TGF- β si were plated at 10⁵ per well in a six-well plate coated with fibronectin. Once the cells reached 90% confluency, they were serum starved for 12 h. Following serum starvation, 10 μ L/mL mitomycin C were added to the medium for 2 h in complete medium and a "wound" was created using a sterile 200 μ L pipette tip. Photographs of the wounded area were taken at the time of wounding and thereafter every 24 h for 3 d to determine the rate of wound closure. Percent migration was calculated by measuring the length and width of the cell-free area. The width was measured at five points along the scratch area and then averaged to get an accurate representation of the entire scratch. Percent migration was determined by using the following formula: [Δ area/area (day 0)] \times 100.

Invasion assay. To determine invasive ability, MDA-MB-435 and MDA-MB-435 TGF- β si cells were plated on a cell culture insert coated with fibronectin (8- μ m pore size, 24-well format; Becton Dickinson Labware) in serum-free medium and a chemoattractant (5% fetal bovine serum) was added to the lower chamber. To determine the amount of invasion, cells were incubated for 24 h and then removed from the upper chamber using a cotton swab. The cells on the underside of the chamber were fixed to the membrane using methanol for 5 min. Once fixed, the cells were stained with crystal violet for 10 min and rinsed with PBS. The chambers were then photographed to compare the amount of invasive cells on the underside of the membrane. Quantitation of the invasive cells was done using a semiquantitative colorimetric analysis of the crystal violet-stained cells. To remove the crystal violet dye from the cells, the membranes were submerged in 0.01% SDS solution for 20 min. Fifty microliters of the solution were then pipetted into a 96-well plate and absorbance measured at 560 nm. Values were then normalized to the unmodified MDA-MB-435 cells.

Quantitative real-time RT-PCR. Quantitative PCR was done using a SYBR Green detection system from Bio-Rad. Cells were seeded in a six-well plate and allowed to grow to confluency. Cells were transfected using Lipofectamine 2000 (Invitrogen) and then total RNA was isolated after 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen) per the manufacturer's instructions. Total RNA was then reverse transcribed into cDNA using Bio-Rad iScript cDNA synthesis kit. Quantitative PCR was last done using the Bio-Rad SYBR green supermix and iCycler detection system. The primer sequences used for different transcripts are listed below.

TGF- β 1, forward 5'-GAGGGGAAATTGAGGGCTTT-3'; reverse 5'-CGGTAGTGAACCCGTTGATG-3'
 TGF- β 2, forward 5'-AAACAAGAGCAGAAGGCCGAATGGC-3'; reverse 5'-AAAGTGCAGCAGGGACAGTGTAAAG-3'
 TGF- β 3, forward 5'-AAGCGGGCTTTGACACCAATTAC-3'; reverse, 5'-AGCAGAAGTTGGCATAGTAGCCCT-3'
 MMP-9, forward 5'-TGGGCTACGTGACCTATGACAT-3'; reverse 5'-GCCAGCCACCTCCACTCCTC-3'
 MMP-2 forward 5'-GAAGGTGAAGGTCGGAGT-3'; reverse 5'-GAAGATGGTGATGGGATTC-3'
 TGF- β R1, forward 5'-TGGGACCCACTTCCATTTCTTCA-3; reverse 5'-TCCCAAGCCTCATCTGCTCAATCT-3'
 TGF- β R2, forward 5'-TGTTGAGTCTTCAAGCAGACCGA-3; reverse 5'-ACTTCTCCCACTGCATTACAGCGA-3'
 PI3K, forward 5'-AGATCGCTCTGGCCTCATTGAAGT-3; reverse 5'-AGCCAGTTCAGAAGGGCATTTTG-3'
 GAPDH, forward 5'-ATTGCCCTCAACGACCACTT-3; reverse 5'-TTACTCCTTGGAGGCCATGT-3'

The assays were done in triplicate and values were normalized using the GAPDH internal control. PCR was done with a 3-min preincubation at 95°C followed by 45 cycles of a 15-s denaturation at 95°C, and a 30-s annealing and extension at 57°C. PCR products were subjected to melting curve analysis using the light cycler system to exclude amplification of nonspecific products. Quantitation of the PCR data was done using the ΔC_T method as described previously (35).

Immunoblotting. Immunoblotting was done as described (36). Cell extracts from MDA-MB-435 cells and clonal derivatives expressing TGF- β 1 siRNA or scrambled siRNA were prepared using cell lysis buffer (Promega) containing proteinase and phosphatase inhibitors. Lysates were separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. The membranes were blocked with nonfat milk solution for 1 h. Detection of specific proteins was done using antibodies for pSmad2 (Upstate Biotechnology); TGF- β R1, pERK

1/2, ERK 1/2, RhoA, Smad2, pAKT, and AKT (Cell Signaling); TGF- β R2 (Santa Cruz Biotechnology); TGF- β 2 and TGF- β 3 (Abcam). Blots were developed with enhanced chemiluminescence reagent obtained from Amersham Pharmacia Biotech. Densitometric scanning was done to quantify the band intensities.

In vivo xenograft model of breast cancer metastasis. Metastatic breast cancer was developed in athymic nude mice. Four- to 5-wk-old female athymic nude mice were purchased from Frederick Cancer Institute and housed in the Animal Care Facility of the University of Alabama at Birmingham. All animal protocols used for this study were approved by the Institutional Animal Care and Use Committee. A total of four groups consisting of 10 mice each were included in the study. Unmodified or TGF- β 1 siRNA-expressing MDA-MB 435 cells were injected into the second or third mammary fat pad of 4- to 5-wk-old athymic mice. Tumors were allowed to grow for ~6 wk or ~1 cm in diameter and then excised. Six weeks after excision of the primary tumors, mice were sacrificed and lungs were collected to determine the amount of metastases. Incidence of metastasis was determined by the presence of macroscopic lesions on the surface of the lung while sacrificing the mice. The primary tumors were divided into three portions for the isolation of RNA, for cell lysate production, and for making paraffin blocks for immunohistochemistry.

ELISA. Blood samples from mice were allowed to clot for 2 h at room temperature. The samples were centrifuged for 20 min at 2,000 \times g and the serum was removed and stored at -80°C until assayed. Serum samples were diluted 10-fold in PBS. This ELISA kit measures bioactive TGF- β 1. To determine total TGF- β 1 levels, samples were activated using an acid treatment. To activate the samples, 100 μ L of each sample were incubated with 20 μ L of 1 N HCl for 10 min at room temperature. The sample was then neutralized by adding 20 μ L of 1.2 N NaOH/0.5 mol/L HEPES. Active TGF- β 1 levels were measured without treatment of the sample. TGF- β 1 levels were measured as per the instructions of the ELISA kit manufacturer (R&D Systems). The minimum sensitivity of this assay was 4.61 pg/mL.

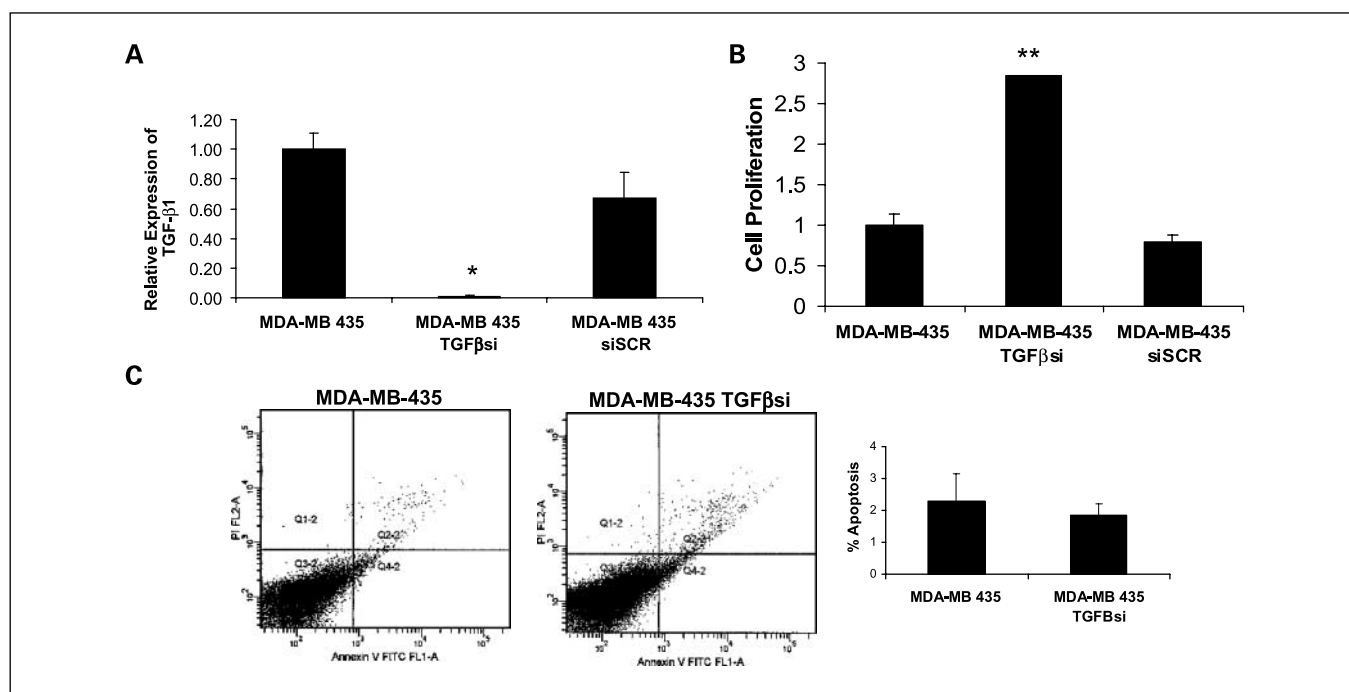


Fig. 1. Characterization of stable clones expressing TGF- β 1 siRNA. The human breast cancer cell line MDA-MB-435 was transfected with TGF- β 1 siRNA plasmid or a plasmid containing a scrambled (SCR) siRNA sequence and stable clones were selected using G418. **A**, RNA isolated from the cells was analyzed for TGF- β 1 expression by real-time quantitative PCR. The values were normalized against GAPDH transcripts from each clone. **B**, a MTT assay was done to determine changes in proliferation of the clones. **C**, Annexin V staining and flow cytometry was used to examine apoptosis in these cells. *, $P < 0.02$; **, $P < 0.0001$. All results are mean values of six independent experiments.

Immunohistochemistry. Immunohistochemical studies were done in 5- μ m sections of paraffin-embedded tumor tissues using antibodies for TGF- β 2 and TGF- β 3 to determine the expression of these isoforms in the primary tumor material. Antigen retrieval was achieved by incubating the slides in citrate buffer for 20 min in a steamer and endogenous peroxidase was blocked by incubation with 3% H₂O₂ for 20 min at room temperature. The anti-TGF- β -2/3 polyclonal antibodies were used in a working dilution of 1:200. Furthermore, the slides were stained with a donkey anti-rabbit and donkey anti-mouse horseradish peroxidase-linked secondary antibody (1:500 dilution). To determine the protein expression, stained slides were examined under high power (\times 40). The antigen-antibody complex was visualized with diaminobenzidine tetrahydrochloride and tissues were counter-stained minimally with hematoxylin.

Statistical analysis. All *in vivo* experiments included at least 10 mice per group. Results consisting of three or more groups were analyzed using single-factor ANOVA. Analysis of results containing two groups was done using the Student's *t* test, assuming unequal variance. Values of *P* < 0.05 were considered statistically significant.

Results

Characterization of MDA-MB-435 stable clones expressing TGF- β siRNA. MDA-MB-435 cells were transfected with the siRNA plasmid and selected for stable clones demonstrating inhibition of TGF- β 1 expression. Silencing of TGF- β 1 transcripts in positive clones was confirmed by RT-PCR. As shown in Fig. 1A, TGF- β 1 mRNA levels were decreased >90% in the MDA-MB 435 TGF- β si clone when compared with unmodified

cells. Next, the effects of TGF- β silencing on both cell proliferation and apoptosis were determined *in vitro*. Analysis of cell proliferation using the MTT assay showed 50% increased proliferation in the TGF- β si clone when compared with the unmodified MDA-MB-435 cells (Fig. 1B). Addition of exogenous TGF- β 1 protein in culture more than doubled the proliferation of MDA-MB 435 cells but had no effect on the proliferation rate of the TGF- β 1-silenced cells (Supplementary Fig. S1). Apoptosis of the cell clone was determined using Annexin V and propidium iodine staining followed by flow cytometry analysis. Unmodified MDA-MB-435 and MDA-MB-435 TGF- β si cells showed 2.1% and 1.8% apoptosis, respectively, indicating no significant difference in the rate of apoptosis between them (Fig. 1C; *P* > 0.05).

Silencing of TGF- β affects the migratory potential of MDA-MB-435 cells *in vitro*. MDA-MB-435 is a highly metastatic breast cancer cell line. *In vitro* studies were done to determine the effects of TGF- β 1 silencing on both migration and invasion of these cell clones. Cell migration was first determined using a wound-healing assay in which cells were scratched and allowed to migrate into the wound area. The amount of migration or wound closure was enumerated 72 hours after disruption. Compared with the normal MDA-MB-435 cells that showed 92% wound closure by 72 hours, clones expressing the TGF- β siRNA showed 56% wound closure in the same period (Fig. 2A). Using a Boyden chamber, coated with fibronectin, we determined changes in cell invasiveness after 24 hours. Cells were

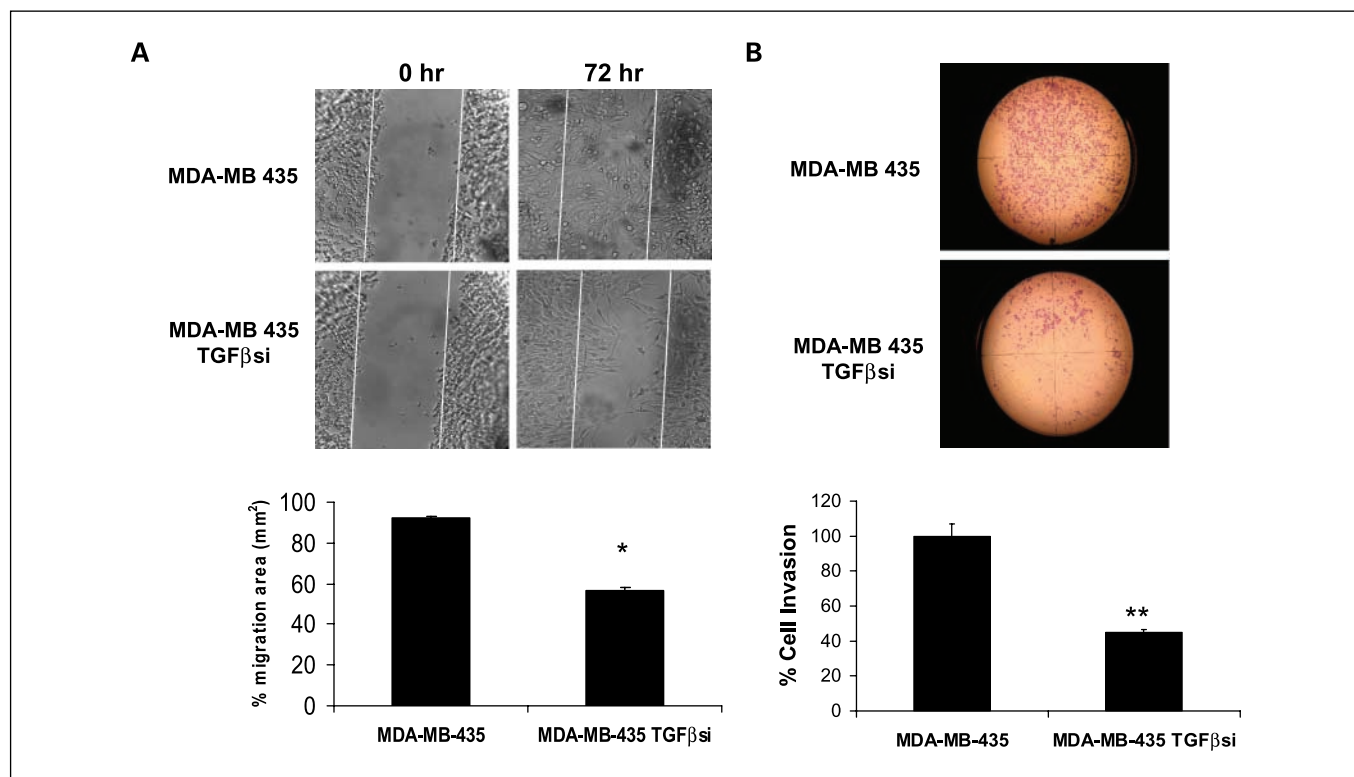


Fig. 2. Silencing of TGF- β mRNA affects migration and invasion of MDA-MB-435 cells *in vitro*. MDA-MB-435 cells and stable clones containing TGF- β siRNA were analyzed for cell migration using the wound-healing scratch assay. *A*, cells were "wounded" and monitored every 24 h for 3 d to determine the rate of migration into the scratched area. Invasiveness of cells was determined using a Boyden chamber assay. Cells were plated in the upper chamber of the apparatus and allowed to grow for 24 h in serum-free medium. A chemoattractant (5% fetal bovine serum) was placed in the lower chamber. *B*, cells were fixed to the membrane and stained after 24 h to determine invasion. *, *P* < 0.0001; **, *P* < 0.001; all assays were done in triplicate.

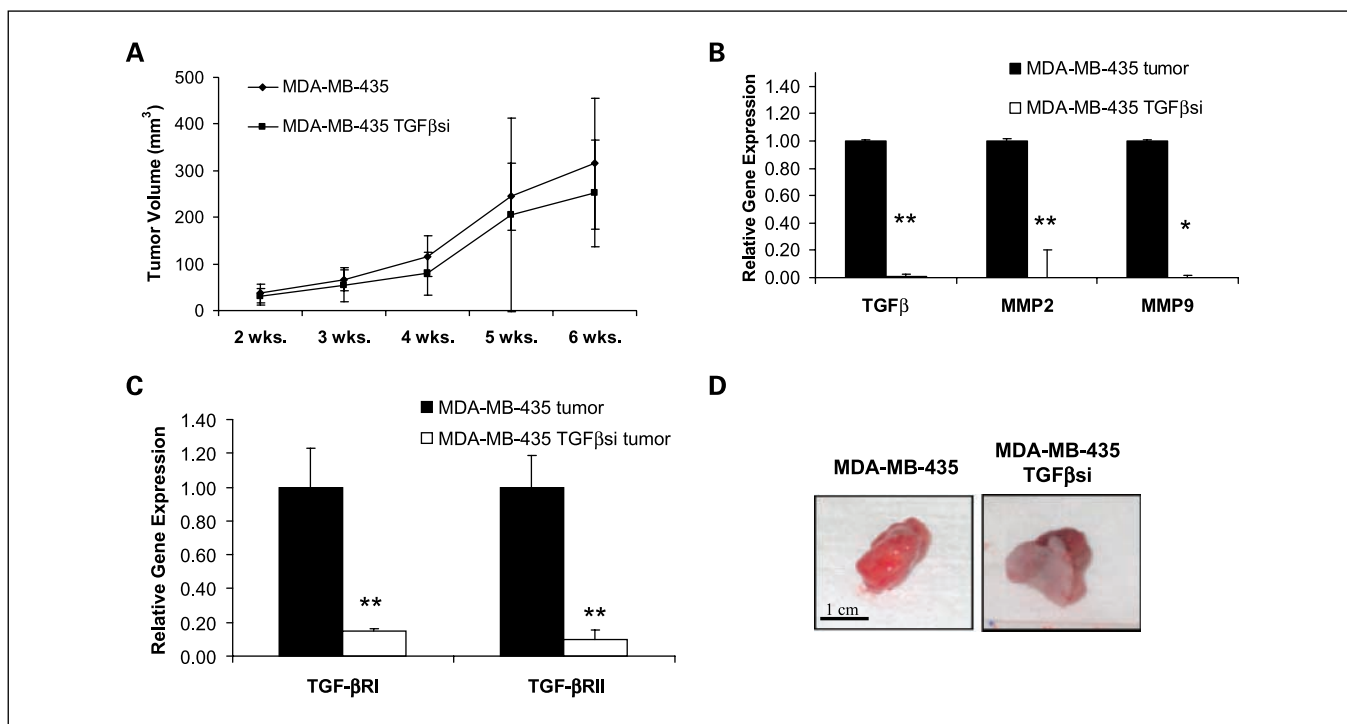


Fig. 3. Analysis of primary tumor growth and gene expression. *A*, by using a xenograft model of breast cancer metastasis in 4- to 5-week-old female athymic nude mice ($n = 10$), both MDA-MB-435 cells and MDA-MB-435 TGF- β si cells were injected into the mammary fat pad and allowed to form primary tumors. The growth kinetics of the primary tumors was measured every week. *B* and *C*, following excision of the tumor, gene expression analysis was done by RT-PCR using RNA isolated from each sample for the expression of TGF- β , MMP-2, MMP-9, TGF- β R1, and TGF- β R2. The values were normalized for GAPDH levels from each sample. *, $P < 0.02$; **, $P < 0.01$. *D*, photographs taken of primary tumors upon excision at 6 wk show no significant difference in primary tumor size.

fixed and stained with crystal violet to determine the number of cells that invaded across the membrane. Compared with unmodified MDA-MB-435 cells, the MDA-MB-435 TGF- β si clone showed a significant decrease in invasion (Fig. 2B). Colorimetric analysis of the crystal violet-stained migratory cells indicated a 55% decrease in the number of invasive MDA-MB-435 TGF- β si cells when compared with parental MDA-MB-435 cells (Fig. 2B).

Analysis of TGF- β 1 silencing in a xenograft model of breast cancer metastasis. MDA-MB-435 has been shown to be a highly metastatic breast cancer cell line when implanted in athymic nude mice. Animals injected orthotopically with this cell line show metastases to the lung, lymph nodes, brain, and bone in as little as 12 weeks postinjection. To determine the growth characteristics of the MDA-MB-435 cells with TGF- β 1 down-regulation, 10^5 cells that were unmodified or modified to constitutively express TGF- β 1 siRNA were implanted in the mammary fat pad of 4- to 5-week-old female athymic nude mice. Cohorts of animals from each group were sacrificed 42 days after removal of the primary tumors to determine lung metastasis. The primary tumors were allowed to grow to 1 cm in diameter before excision. As shown in Fig. 3A, there was no significant difference in the growth kinetics of the primary tumors between the two groups. Analysis of TGF- β 1, MMP-9, and MMP-2 expression in the MDA-MB-435 TGF- β si primary tumors excised after 6 weeks indicated a one-log decrease in gene expression when compared with tumors formed from parental cells (Fig. 3B). Analysis of TGF- β R expression showed an 85% decrease in TGF- β RI and a 90% decrease in TGF- β RII (Fig. 3C). Measurements of primary tumor volume upon

excision showed no difference in the size of tumors (Fig. 3D). Expression of matrix metalloproteinases is important in invasion of the tumor cells through the basement membrane. TGF- β has been shown to modulate expression of the proteases in the tumor microenvironment. Results shown in Fig. 4A indicated a significant decrease in the number of macroscopic metastases present in the MDA-MB-435 TGF- β si clone. Although 100% of mice injected with MDA-MB-435 cells developed macroscopic lung metastasis, only 10% of the mice injected with the MDA-MB-435 TGF- β si clones developed similar lung metastasis. To determine the circulating levels of TGF- β 1, an ELISA was done. Results, as shown in Fig. 4B, indicated no significant difference in levels of total TGF- β 1 in the mice injected with MDA-MB-435 TGF- β si cells when compared with normal MDA-MB-435 cells, suggesting that the TGF- β 1 silencing is limited to the tumor microenvironment. Macroscopic lung lesions were used to determine the incidence of metastasis in each group. Mice injected with unmodified MDA-MB-435 cells revealed several macroscopic lesions in the lungs whereas mice injected with MDA-MB-435 TGF- β si cells show no overt metastatic lesions (Fig. 4C). Histologic analysis revealed the presence of micrometastatic lesions in the lungs of mice injected with the TGF- β 1-silenced clones (not shown). These data suggest that TGF- β 1 does not affect primary tumor growth at the early stages of disease progression but exerts an effect on metastasis, specifically growth of tumor at the secondary site.

Analysis of both Smad and non-Smad TGF- β signaling pathways. Following TGF- β inhibition, to determine the possible influence of signaling pathways affecting metastasis,

both Smad and non-Smad signaling pathways were analyzed using the two cell lines. TGF- β ligand has been known to signal through Smad proteins. In addition, recent studies have shown that several non-Smad signaling pathways may be involved in the prometastatic effects of TGF- β in late-stage disease. Using RT-PCR and Western blot analysis, we examined alterations in gene expression for downstream targets of TGF- β . Results, shown in Fig. 5A, indicated that there was a 95% decrease in gene expression for TGF- β R1 and TGF- β R2 upon silencing of TGF- β 1. These results were supported with protein analysis in which there was a 40% and 20% decrease in TGF- β R1 and TGF- β R2, respectively, in the cell lysate. *PI3K* gene expression was unaltered in the MDA-MB-435 TGF- β si cells using RT-PCR. These results were confirmed at the protein level using antibodies to detect the phosphorylated form of the p85 subunit of PI3K. Analysis of PI3K activation showed no significant difference in the modified MDA-MB 435 TGF- β si cells when compared with unmodified cells. However, *MMP-9* and *MMP-2* gene expression in MDA-MB-435 TGF- β si clone showed a 99% and 90% decrease, respectively (Fig. 5B). Next, we examined the effects of TGF- β , Ras, mitogen-activated protein kinase, and AKT signaling pathways using Western blot analysis. Results of the studies, shown in Fig. 5C, indicated no significant change in RhoA expression but there was a 20% decrease in Smad2 phosphorylation and a 4-fold and 1.7-fold increase in AKT and ERK phosphorylation, respectively, suggesting a possible role of these signaling pathways in both inhibiting and mediating the prometastatic effects of TGF- β

signaling. Quantification of all Western blots has been provided graphically in the Supplementary Fig. S2.

Analysis of TGF- β 2 and TGF- β 3 expression in vitro and in vivo. Although the main focus of the present study is to determine the effects of TGF- β 1 silencing in the highly metastatic MDA-MB-435 breast cancer cell line, all three isoforms of TGF- β have been implicated in various processes involved in tumorigenesis. To determine the effects of TGF- β 1 silencing on the other two isoforms, RT-PCR analysis was done. As shown in Fig. 6A, there was no significant difference in the expression levels of either TGF- β 2 or TGF- β 3 in the MDA-MB-435 TGF- β si cells when compared with parental controls *in vitro*. However, RT-PCR analysis of the primary tumors formed from the TGF- β 1-silenced cells indicated that there was >60% decrease in gene expression for both isoforms *in vivo* (Fig. 6B). These data were confirmed for TGF- β 2 using Western blot analysis (Fig. 6C). Immunohistochemistry was also used to determine the protein expression of TGF- β 2 and TGF- β 3 in the primary tumors. Analysis of TGF- β 2 expression in MDA-MB 435 tumors indicated strong staining throughout the tumor mass, whereas tumors from the MDA-MB 435 TGF- β si clones showed distinct staining only in small areas of the tumor mass (Fig. 6D).

Discussion

TGF- β has emerged as a promising new target for treatment of cancer metastasis. Most of the studies to date have examined

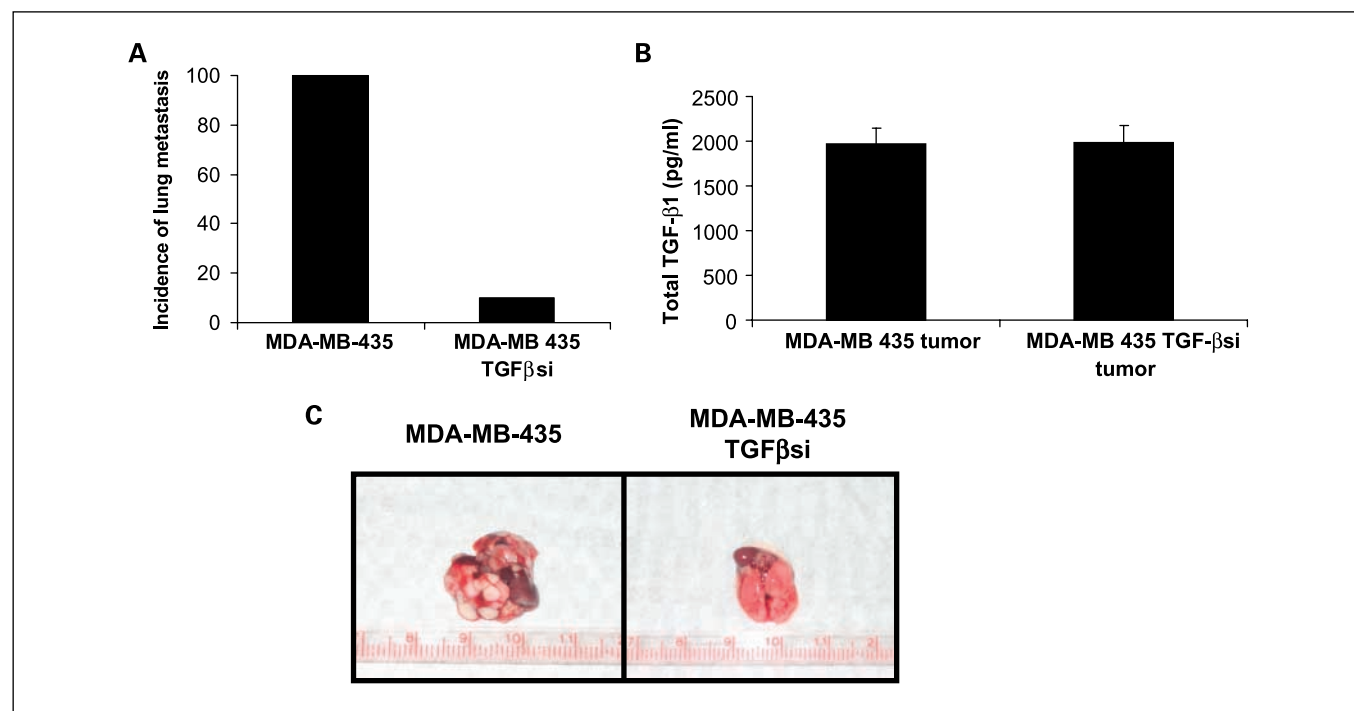


Fig. 4. Analysis of lung metastasis in a xenograft model. MDA-MB-435 cells were injected in the mammary fat pad of 4- to 5-wk-old female athymic nude mice ($n = 10$). **A**, primary tumors were allowed to grow to 1 cm in diameter and then surgically excised. Animals were left for another 6 wk for metastasis to occur and then sacrificed to determine the number of macroscopic lung metastases. When compared with the unmodified MDA-MB-435 cells, which caused macroscopic lesions in 100% of the mice, only 10% of the mice injected with TGF- β si cells developed macroscopic lung metastases. **B**, circulating levels of TGF- β at the time of sacrifice was determined by ELISA using serum samples obtained from mice implanted with the parental MDA-MB-435 cells or its clonal derivative, constitutively expressing TGF- β 1 siRNA. **C**, photographs of the lungs 6 wk after primary tumor excision show macroscopic lesions on the surface of the mice injected with unmodified MDA-MB-435 cells whereas no lesions are seen on the lungs of mice injected with MDA-MB-435 TGF- β si cells.

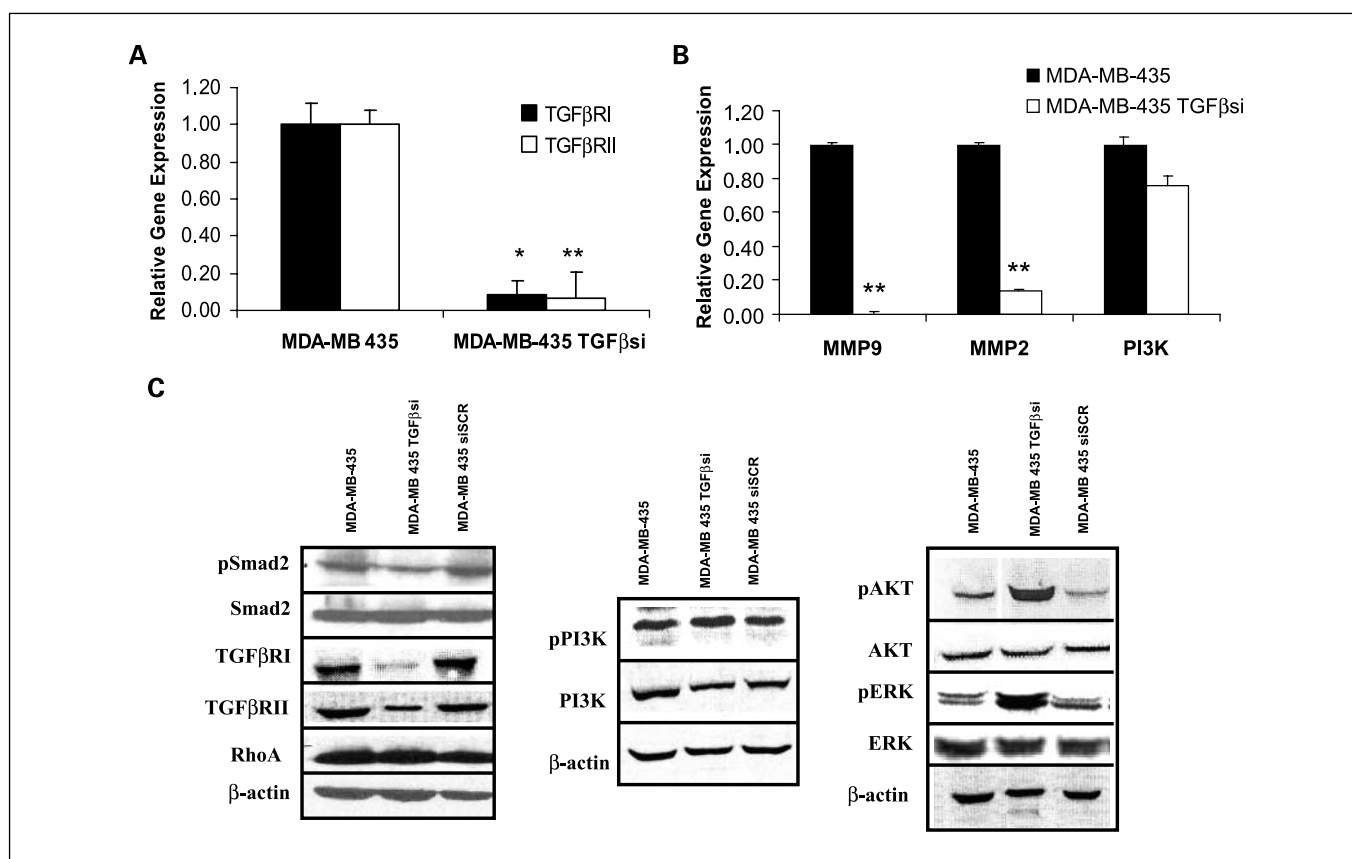


Fig. 5. Effects of TGF- β silencing on Smad-dependent and Smad-independent pathways. RNA isolated from unmodified MDA-MB-435 and TGF- β si containing MDA-MB-435 cell clone was used in a RT-PCR analysis to determine gene expression. Gene-specific primers were used to determine the expression levels of TGF- β receptor I and II (A), and MMP-2, MMP-9, and PI3K (B). Values from individual amplifications were normalized against GAPDH levels. The assays were done in triplicate. *, $P < 0.02$; **, $P < 0.01$. All results indicated are mean values of six independent experiments. Western blot analysis was done using cell lysates from each clone to determine activation of TGF- β receptors, Smad2, and the non-Smad signaling pathways; RhoA, PI3K/AKT, and ERK (C). Western blots were done in duplicates and the mean values were expressed graphically.

the effects of TGF- β receptor inhibition on tumor metastasis (14). Results of the present study indicated that silencing of TGF- β 1 altered the metastatic characteristics of the highly invasive MDA-MB 435 cell line. Furthermore, characterization of the clonal derivatives with TGF- β 1 silencing *in vitro* and *in vivo* indicated a decrease in TGF- β RI and TGF- β RII mRNA expression. Silencing of the TGF- β receptor expression may be through a negative feedback loop in the cells, suggesting that therapies targeting the ligand may have similar effects as indicated by previous studies, which have shown that blocking TGF- β receptors can decrease tumorigenesis. Western blots for TGF- β RII in the present study indicated a 20% decrease in protein expression, which may be significant to decrease signaling of this pathway. Thus, it is possible that a 90% decrease in RNA level, which is relative to the control sample, may not truly reflect the protein level and the remaining 10% RNA may be sufficient to produce the level of protein seen in the Western blots. It is also interesting to note that whereas signaling through TGF- β receptors is the primary method of activation for Smad proteins, several studies have recently shown that mitogen-activated protein kinase and other tyrosine kinases can activate Smads independent of TGF- β receptors (37). This may be the reason for the variation between the levels of Smad activation and the TGF- β receptor levels. Although the present study focused specifically on the role of

TGF- β 1 in tumor metastasis, the decrease in receptor expression would also limit signaling of the other TGF- β isoforms. Both TGF- β 2 and TGF- β 3 have recently been implicated in several processes of tumorigenesis and metastasis. Results from our *in vitro* experiments showed no change in expression for TGF- β 2 and TGF- β 3 isoforms, illustrating the specificity of the siRNA sequence designed for this study. However, analysis of the mRNA levels for all three TGF- β isoforms in the primary tumor material showed a significant decrease when compared with tumors formed from unmodified MDA-MB 435 cells. These data were also confirmed at the protein level by using immunohistochemistry. This decrease in TGF- β 2 and TGF- β 3 gene expression *in vivo* may be partially due to the down-regulation of TGF- β receptors in the tumor cells, which may have abrogated both autocrine and paracrine signaling in the tumor microenvironment. Thus, the decrease in tumor metastasis *in vivo* may be partially attributed to the decrease in signaling from other TGF- β isoforms.

Initial studies to characterize the clonal derivatives of MDA-MB-435 cells constitutively expressing TGF- β siRNA showed no changes in apoptosis, indicating that changes in the number of metastasis are not due to increased apoptosis. Cell proliferation analysis indicated a 50% increase in proliferation *in vitro*. This increase in proliferation in response to TGF- β 1 silencing seems to be due to an increase in AKT and ERK signaling pathways.

When incubated with exogenous TGF- β 1 protein, there was an increased proliferation in unmodified MDA-MB 435 cells; however, no change was seen in the TGF- β 1-silenced clones. This decreased sensitivity to TGF- β 1 may be attributed to a decrease in receptor expression. However, there was no concomitant increase in tumor growth *in vivo*, suggesting a possible role of other inhibitors in the microenvironment of the primary tumor. Silencing of TGF- β 1 by RNA interference specifically inhibited the expression of MMP-9 and MMP-2 *in vitro* and in the primary tumor. Several matrix metalloproteinases have been shown to be overexpressed in the tumor microenvironment and are required for invasion of the tumor cells through the basement membrane. The down-regulation of matrix metalloproteinase expression and the decreased migration and invasion *in vitro* in response to TGF- β 1 silencing supports the role of TGF- β 1 in modulating migration and invasion of the tumor cell and not apoptosis. This was confirmed *in vivo* by analyzing the primary tumor growth, which was not decreased in tumors with TGF- β si clones. Analysis of the primary tumor growth revealed a slight decrease in tumor size after 6 weeks, which was not statistically significant but may suggest the timeframe during which the function of TGF- β switched from a tumor suppressor to a prometastatic phenotype.

The TGF- β /Smad signaling pathway has been well characterized for a number of years (38, 39). TGF- β receptor and Smad proteins have been linked to the activation of mitogen-activated protein kinase, PI3K, and the RhoA signaling pathways in several cancer models (40–44). AKT, a well-known cell survival

and proliferation signaling molecule, has been shown to inhibit TGF- β -induced apoptosis and regulate epithelial-mesenchymal transitions in tumor cells (45, 46). Interestingly, the results of our studies have shown that silencing of the TGF- β 1 ligand *in situ* in breast cancer cells increases both AKT and ERK activation. Studies have shown that ERK signaling can also play a dual role in tumorigenesis. ERK1 up-regulation has been linked to increased cell proliferation, whereas increases in ERK2 seem to mediate apoptosis (47). ERK activation has also been shown to mediate epithelial-mesenchymal transition and is extensively involved in cytoskeletal rearrangement and migration. Our results indicate that the increase in both AKT and ERK could be associated with the increased proliferation found in the MDA-MB-435 TGF- β si cells *in vitro*. Furthermore, an analysis of PI3K activation showed no significant difference in this upstream mediator of AKT. These data suggest that the up-regulation of AKT signaling in TGF- β si clones is likely due to the removal of an inhibitor, TGF- β 1, and not up-regulation through the PI3K pathways. However, *in vivo*, the tumor microenvironment also contains inhibitory signals, which control the growth of the primary tumors. Increased ERK activation may also be involved in the migration of the cells to the secondary site. Whereas we observed a significant decrease in the number of macroscopic lung lesions, there were micrometastatic foci present in the lungs of mice injected with TGF- β 1-silenced cells upon histologic analysis, indicating that silencing of TGF- β 1 does not completely eliminate lung metastasis in this model. Although the study of ERK signaling in tumor progression is somewhat controversial, it is clear that the

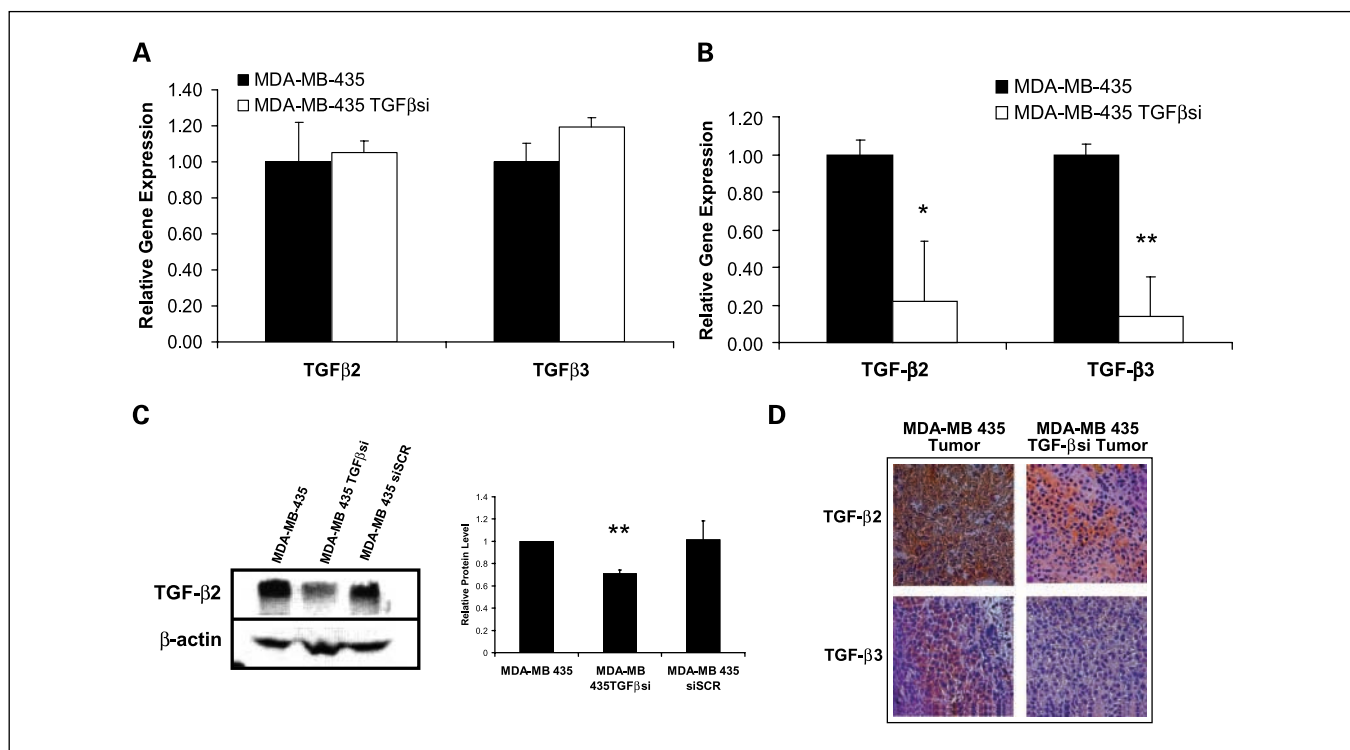


Fig. 6. Analysis of TGF- β 2 and TGF- β 3 isoforms *in vitro* and *in vivo* upon silencing of TGF- β 1. RNA isolated from unmodified MDA-MB-435 and TGF- β si containing MDA-MB-435 clone was used in RT-PCR analysis to determine gene expression. Gene-specific primers were used to determine the expression levels of TGF- β 2 and TGF- β 3 *in vitro* (A) and in the primary tumors *in vivo* (B). Values from individual amplifications were normalized against GAPDH levels. Lysate from the primary tumor tissues were analyzed for the expression of TGF- β 2 and TGF- β 3 isoforms by Western blot and the bands were quantified by densitometry (C). Immunohistochemistry was done with primary tumor tissues following implantation of MDA-MB 435 and MDA-MB 435 TGF- β si cells using antibodies for TGF- β 2 and TGF- β 3 (D). *, $P < 0.05$; **, $P < 0.01$.

overlap between ERK and AKT signaling pathways can mediate several events in tumor progression. Hence, cross-talk between these three pathways may be necessary to induce epithelial-mesenchymal transition and promote metastasis. It is possible that alteration of these two signaling pathways in combination with TGF- β 1 silencing specifically in the tumor microenvironment may have a synergistic effect on the therapy seen in our study.

Although several studies have provided a link between TGF- β inhibition and tumor metastasis, very few have been able to show long-term inhibition that is specific to the tumor cells. Studies have shown significant decrease in tumor metastasis using antisense, small-molecule inhibitors and antibodies that target both the TGF- β ligand and the receptor. Although these studies have shown some success in animal models, the ability to administer these in human patients as a therapy is encountered by limitations (48). Small-molecule inhibitors have also shown promise but have limited biodistribution and lack specificity to both the tumor cells and the tumor microenvironment (49, 50). Constant administration of high doses of these inhibitors is undesirable due to potential toxicity. Thus, alternate modes of delivery based on gene transfer with siRNA targeting TGF- β only in tumor cells and possible combination therapies with non-Smad downstream effectors will make TGF- β -targeted therapies more effective.

The present study suggests that therapies targeting TGF- β 1 in tumor cells may be effective in decreasing metastasis. The ability of this therapy to decrease metastasis may be related to the ability of the cells to grow at the secondary site. This therapy may also result in a decrease in the TGF- β -mediated pro-oncogenic effects such as angiogenesis, immune suppression, and mesenchymal-epithelial transition. Our results also indicate that both the autocrine and paracrine signaling in the tumor microenvironment could be targeted to gain maximum therapeutic effect using this approach due to the variations in results seen between the *in vitro* and *in vivo* studies. Future studies to investigate the effects of growth factors secreted from the stromal cells may give additional insight as to the effectiveness of targeting both the tumor and stromal cells. Although not a focus in these studies, several studies have shown that inhibition of TGF- β signaling modulates the immune response in several cancer models (51). Future studies to understand TGF- β signaling in various stages of tumor progression and metastasis may lead to the further development of more tumor-targeted therapies that may be used in combination with current therapies to decrease the incidence of cancer metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Donovan D, Harme JH, Toomey D, Osborne DH, Redmond HP, Bouchier-Hayes DJ. TGF- β 1 regulation of VEGF production by breast cancer cells. *Ann Surg Oncol* 1997;4:621–7.
- Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005;17:548–58.
- Jeon SH, Chae BC, Kim HA, et al. Mechanisms underlying TGF- β 1-induced expression of VEGF and Flk-1 in mouse macrophages and their implications for angiogenesis. *J Leukoc Biol* 2007;81:557–66.
- Pepper MS. Transforming growth factor- β : vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev* 1997;8:21–43.
- Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF- β inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109:1551–9.
- Kajdaniuk M, Swietochowska, Ostrowska Z, et al. Plasma transforming growth factor β 1 in breast cancer patients treated with CMF chemotherapy. *J Clin Pharm Ther* 2000;25:291–4.
- Sheen-Chen SM, Chen HS, Sheen CW, Eng HL, Chen WJ. Serum levels of transforming growth factor 1 in patients with breast cancer. *Arch Surg* 2001;136:937–40.
- Ivanovic V, Demajo M, Krtolica K, et al. Elevated plasma TGF- β 1 levels correlate with decreased survival of metastatic breast cancer patients. *Clin Chim Acta* 2006;371:191–3.
- Aiping H. Drug resistance and gene amplification potential regulated by transforming growth factor β 1 gene expression. *Cancer Res* 1995;55:1758–62.
- Teicher BA, Maehara Y, Kakeh Y, et al. Reversal of *in vivo* drug resistance by the transforming growth factor- β inhibitor decorin. *Int J Cancer* 1998;71:49–58.
- Bierie B, Moses HL. TGF- β and cancer. *Cytokine Growth Factor Rev* 2006;17:29–40.
- Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. *N Engl J Med* 2000;342:1350–8.
- Elliott RL, Blobel GC. Role of transforming growth factor β in human cancer. *J Clin Oncol* 2005;23:2078–93.
- Bandyopadhyay A, Agyin J, Wang L, et al. Inhibition of pulmonary and skeletal metastasis by a transforming growth factor- β type I receptor kinase inhibitor. *Cancer Res* 2006;66:6714–21.
- Siegel PM, Shue W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor β signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci U S A* 2003;100:8430–5.
- Boyce BF, Yoneda T, Guise TA. Factors regulating the growth of metastatic cancer in bone. *Endocr Relat Cancer* 1999;6:333–47.
- Cicik M, Oursler M. Breast cancer bone metastasis and current small therapeutics. *Cancer Metastasis* 2006;25:635–44.
- Smid M, Wang Y, Klijn JG, et al. Genes associated with breast cancer metastatic to bone. *J Clin Oncol* 2006;24:2261–7.
- Chang CF, Westbrook R, Ma J, Cao D. Transforming growth factor- β signaling in breast cancer. *Front Biosci* 2007;12:4393–401.
- Annes JP, Munger JS, Rifkin DB. Making sense of latent TGF β activation. *J Cell Sci* 2003;116:217–24.
- Dumont N, Arteaga CL. Transforming growth factor- β and breast cancer: Tumor promoting effects of transforming growth factor- β . *Breast Cancer Res* 2000;2:125–32.
- Iyer S, Wang ZG, Akhtari M, Zhao W, Seth P. Targeting TGF β signaling for cancer therapy. *Cancer Biol Ther* 2005;4:261–66.
- Lutz M, Knaus P. Integration of the TGF- β pathway into the cellular signaling network. *Cell Signal* 2002;14:977–88.
- Remy I, Montmarquette A, Michnick SW. PKB/Akt modulates TGF- β signaling through a direct interaction with Smad3. *Nat Cell Biol* 2004;6:358–65.
- Glick AB. TGF β 1, back to the future: revisiting its role as a transforming growth factor. *Cancer Biol Ther* 2004;3:276–83.
- Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor β -mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 2000;275:36803–10.
- Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)- β antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF- β interactions in human breast cancer progression. *J Clin Invest* 1993;92:2569–76.
- Dumont N, Bakin AV, Arteaga CL. Autocrine transforming growth factor- β signaling mediates Smad-independent motility in human cancer cells. *J Biol Chem* 2003;278:3275–85.
- Lamouille S, Derynck R. Cell size and invasion in TGF- β induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J Cell Biol* 2007;178:437–51.
- Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF- β 1-induced EMT *in vitro*. *Neoplasia* 2004;6:603–10.
- Atfi A, Dejjellou S, Chastre E, Davis R, Gespach C. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor β -mediated signaling. *J Biol Chem* 1997;272:1429–32.
- Mulder KM. Role of Ras and Mapks in TGF β signaling. *Cytokine Growth Factor Rev* 2000;11:23–35.
- Wilkes MC, Mitchell H, Penheiter SG, et al. Transforming growth factor- β activation of phosphatidylinositol 3-kinase is independent of Smad2 and Smad3 and regulates fibroblast responses via p21-activated kinase-2. *Cancer Res* 2005;65:10431–40.
- Yingling JM, Blanchard KL, Sawyer JS. Development of TGF- β signaling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011–22.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real time quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* 2001;25:402–8.

36. Mahendra G, Kumar S, Isayeva T, Ponnazhagan S. Antiangiogenic cancer gene therapy by adeno-associated virus 2-mediated stable expression of the soluble FMS-like tyrosine kinase 1 receptor. *Cancer Gene Ther* 2005;12:26–34.
37. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF- β family signaling. *Nature* 2003;425:577–84.
38. Roberts AB, Thompson NL, Heine U, Flanders C, Sporn MB. Transforming growth factor- β : possible roles in carcinogenesis. *Br J Cancer* 1998;57:594–600.
39. Gold L. The role for transforming growth factor- β (TGF- β) in human cancer. *Crit Rev Oncog* 1999;10:303–60.
40. Kamaraju AK, Roberts AB. Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor- β -mediated Smad-dependent growth inhibition of human breast carcinoma cells *in vivo*. *J Biol Chem* 2005;280:1024–36.
41. Mucsi I, Skorecki KL, Goldberg HJ. Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor- β 1 on gene expression. *J Biol Chem* 1996;271:16567–72.
42. Safina A, Vandette E, Bakin AV. ALK5 promotes tumor angiogenesis by upregulating matrix metalloproteinase-9 in tumor cells. *Oncogene* 2007;26:2407–22.
43. Yi JY, Shin I, Arteaga CL. Type I transforming growth factor β receptor binds to and activates phosphatidylinositol 3-kinase. *J Biol Chem* 2005;280:10870–6.
44. Cho HJ, Yoo J. Rho activation is required for transforming growth factor- β -induced epithelial-mesenchymal transition in lens epithelial cells. *Cell Biol Int* 2007;31:1225–30.
45. Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF- β /ALK5-mediated Smad3 activation. *EMBO J* 2006;25:58–69.
46. Involvement of PI3K/Akt pathway in TGF- β 2 mediated epithelial mesenchymal transition in human lens epithelial cells. *Ophthalmic Res* 2008;40:69–76.
47. McCubrey JA, Steelman LS, Abrams SL, Libra M. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* 2006;46:249–79.
48. Arteaga CL. Inhibition of TGF β signaling in cancer therapy. *Curr Opin Genet Dev* 2006;16:30–7.
49. Dong M, How T, Kirkbride KC, et al. The type III TGF- β receptor suppresses breast cancer progression. *J Clin Invest* 2007;117:206–17.
50. Dumont N, Arteaga CL. Targeting the TGF β signaling network in human neoplasia. *Cancer Cell* 2003;3:531–6.
51. Uhl M, Aulwurm S, Wischussen J, Weller M. SD-208, a novel transforming growth factor β receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells *in vitro* and *in vivo*. *Cancer Res* 2004;64:7954–61.