TGFBR1*6A Enhances the Migration and Invasion of MCF-7 Breast Cancer Cells through RhoA Activation

Diana S. Rosman,1,2 Sharbani Phukan,1,2 Chiang-Ching Huang,2,3 and Boris Pasche1,2

1Cancer Genetics Program, Division of Hematology/Oncology, Department of Medicine, 2Robert H. Lurie Comprehensive Cancer Center; and 3Department of Preventive Medicine, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois

Abstract

TGFBR1*6A is a common hypomorphic variant of the type 1 transforming growth factor β receptor (TGFBR1), which has been associated with increased cancer risk in some studies. Although TGFBR1*6A is capable of switching TGF-β growth-inhibitory signals into growth-stimulatory signals when stably transfected into MCF-7 breast cancer cells, the biological effects of TGFBR1*6A are largely unknown. To broadly explore the potential oncogenic properties of TGFBR1*6A, we assessed its effects on NIH-3T3 cells as well as its effect on the migration and invasion of MCF-7 cells. We found that TGFBR1*6A has decreased oncogenic properties compared with TGFBR1. However, TGFBR1*6A significantly enhances MCF-7 cell migration and invasion in a TGF-β signaling-independent manner. Gene expression profiling studies identified two down-regulated genes involved in cell migration and invasion: ARHGAP5, encoding ARHGAP5, and FN1, encoding fibronectin-1 (FN1). ARHGAP5 and FN1 expression was similarly down-regulated in MCF-7 cells stably transfected with a kinase-inactivated TGFBR1*6A construct. Functional assays show that TGFBR1*6A-mediated decreased ARHGAP5 expression is associated with higher RhoA activation, a crucial mediator of cell migration. Extracellular signal-regulated kinase (ERK) activation is also higher in cells that harbor the TGFBR1*6A allele. We conclude that TGFBR1*6A is not an oncogene but enhances MCF-7 cell migration and invasion through RhoA and ERK pathway activation and down-regulates two crucial mediators of this phenotype. These results provide the first evidence that TGFBR1*6A may contribute to cancer progression in a TGF-β signaling-independent manner. [Cancer Res 2008;68(5):1319–28]

Introduction

TGF-β plays dual roles during cancer development and progression. TGF-β acts as a tumor suppressor by inhibiting the growth of most cell types, including epithelial, endothelial, hematopoietic, and neuronal cells. However, once tumors form, most cells become resistant to TGF-β growth inhibition and TGF-β becomes pro-oncogenic (1).

As a tumor suppressor, TGF-β inhibits cell growth predominantly by signaling via the SMAD pathway. Secreted TGF-β is normally kept inactive by a latency-associated peptide and latent TGF-β binding protein (LTBP1; refs. 2–4). Thrombospondin-1 and 6 integrin bind LTBP1, thereby activating TGF-β (5, 6). Upon binding to the type 2 TGF-β receptor (TGFBR2), TGF-β causes TGFBR2 and TGFBR1 to heterodimerize with two TGFBR1/TGFBR2 complexes. The ensuing TGFBR1 autophosphorylation induces phosphorylation of SMAD2/3, thereby allowing SMAD2/3 to bind to SMAD4. Although SMAD4 is not required for translocation into the nucleus, it is required for the SMAD complex to act as a transcription factor (7).

TGF-β signaling is both enhanced by, and runs in parallel to, the mitogen-activated protein kinase (MAPK) signaling. TGF-β induces migration by activating AKT and extracellular signal-regulated kinase (ERK) 1/2 (8, 9). The role of SMADs in migration is controversial. There is evidence that SMAD4 is required for TGF-β-induced migration in human immortalized keratinocytes (HaCaT) and in pancreatic tumor cells (Colo-357; ref. 10). The fact that the metastatic ability of RAS-transformed MCF10A1k cells signaling through TGFBR1 requires the SMAD2/3 binding domain (11) is in agreement with these findings. Other groups have suggested that TGF-β-induced migration requires TGFBR1 but is independent of SMADs (8). In a severe combined immunodeficient mouse model, invasion and metastasis of MDA-MB-231 cells is mediated by MAP/ERK kinase–ERK signaling, which results in the activation of matrix metalloproteinase-9 (MMP-9), a SMAD4-independent event (12). ERK, JNK, and RhoA regulate TGF-β-induced migration in MCF-7 cells as well as in the SMAD-deficient breast cancer cell line, MDA-MB-468. TGF-β-induced activation of ERK has also been shown to be independent of TGFBR1 (13).

A common variant of TGFBR1, TGFBR1*6A (or *6A for short), is a low penetrance, tumor susceptibility allele that is found in 14.2% of the general population. Carriers of this mutation have a 24% increased risk of cancer. Breast cancer risk seems to be increased by 31% for heterozygotes and 169% for homozygotes, respectively (14). TGFBR1*6A results from a 9-bp in-frame deletion, which truncates 3 alanines from a 9-alanine tract located within TGFBR1 signal sequence. The wild-type allele will therefore be called TGFBR1*9A or *9A. Previous studies have shown that transient and stable transfection of mink lung epithelial cells with *6A results in a small but significant decrease in TGF-β-induced growth inhibition (15, 16). We have also shown that TGFBR1*6A and TGFBR1 share the same signal sequence cleavage site and that TGFBR1*6A mature receptor is identical to and processed similarly to its wild-type counterpart (17). This suggests that the biological effects of TGFBR1*6A are mediated by its signal sequence and not by its mature receptor. This report aims to determine whether TGFBR1*6A is an oncogene and whether it affects the migration and invasion of MCF-7 breast cancer cells.

Materials and Methods

Cell lines and conditions. MCF-7 cells were cultured in RPMI 1640 (Invitrogen Corp.) supplemented with 10% heat inactivated fetal bovine serum (Hyclone), 2 mmol/L L-glutamine (Invitrogen), nonessential amino acids, 100 μg/mL streptomycin, and 100 U/mL penicillin. The cells were grown in a humidified atmosphere of 5% CO2 at 37°C. For transfections, the cells were plated in 6-well plates in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin. The cells were allowed to grow to 60% confluence before transfection. The cells were transfected using Lipofectamine 2000 for 48 hours. The transfected cells were then trypsinized and replated in 24-well plates in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin. The cells were allowed to grow for 24 hours before immunofluorescence experiments.
acids, 1,000 units/mL penicillin, 10,000 μg/mL streptomycin, 0.006 mg/mL human recombinant insulin (Sigma), and 0.5 mg/mL amphotericin B (Biologos, Inc.).

Starvation medium was identical except that 0.5 μg/mL bovine serum albumin was substituted for 10% FBS. MCF-7 cells were stably transfected with pires-TGFBR1*9A-HA-FLAG, pires-TGFBR1*9A-HA/FLAG, or vector alone and selected for with 0.5 μg/mL G418 (17). NIH-3T3 cells were cultured in DMEM (Invitrogen) supplemented with 10% heat inactivated FBS, 1,000 units penicillin/streptomycin, amphotericin B, 2 mM L-glutamine, and 1 mg/mL G418 for pires selection.

Phoenix cells (American Type Culture Collection) were cultured according to ATCC recommendations.

**Plasmid constructs.** TGFBR1-HA-Flag (TGFBR1*9A-HA-Flag) and TGFBR1*6A-HA-Flag were constructed in the pires vector (BD Biosciences, Clontech) as described previously (17). TGFBR1-HA-Flag and TGFBR1*6A-HA-Flag were excised from pCMV5-TGFBR1-HA-Flag or pCMV5-TGFBR1*6A-HA-Flag, respectively, using EcoRI and BamHI restriction enzymes and inserted into the pBABE vector, linearized with EcoRI, and blunt ended with Klenow. pBABE and pBABE-RASV12 were gifts from Dr. Vince Cryns (Northwestern University, Chicago, IL). The pBSE4-lux vector was a gift from Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD). The 3TP-Lux vector was a gift from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY).

**Transfections.** pires-TGFBR1-HA-Flag, pires-TGFBR1*6A-HA-Flag, or pires-TGFBR1*6A-HA-Flag were stably transfected into MCF-7 cells as previously described. Stable clones from each *6A and *9A lines were chosen based on similar levels of TGFBR1 and HA expression. We refer to *9A-5, *9A-9, *6A-5, *6A-1, and *6A-15 as *9A-low, *9A-high, *6A-low, *6A-intermediate (*6A-int), and *6A-intermediate (*6A-int), respectively (17). pBABE, pBABE-TGFBR1-HA-Flag, or pBABE-TGFBR1*6A-HA-Flag was transfected into MCF-7 cells using retroviral transfection. The pBABE vectors were transfected into Phoenix cells using FuGENE6 (Roche Applied Science). Viral-containing medium was collected 48 h after transfection, filter sterilized, and treated with 4 ng/mL polybrene (Sigma). The clones were selected for in 1 μg/mL puromycin. We selected two low-expressing and high-expressing clones for each experiment. We refer to these cells in the text as pBABE, pBABE*9A-low, pBABE*9A-high, pBABE*6A-low, and pBABE*6A-high.

NIH-3T3 cells were stably transfected with pires-TGFBR1-HA-Flag or pires-TGFBR1*6A-HA-Flag using FuGENE6 (Roche Applied Science) and selected for with 1 μg/mL G418. pBABE*6A-HA-Flag or pBABE*6A-HA/Flag was transiently transfected into the NIH-3T3*6A*- or *6A stably transfected cells by viral transfection. The pBABE vector was mixed with 125 mM/L CaCl2 and HBS and transfected into Phoenix cells. Two days after transfection, the viral supernatant was collected, filter sterilized, and treated with 4 ng/mL polybrene (Sigma). NIH-3T3 cells were incubated with the viral supernatant for 24 h then refeed with complete medium.

**Assessment of TGF-β signaling.** MCF-7 cells stably transfected with pires, pires-TGFBR1-HA-Flag, and pires-TGFBR1*6A-HA-Flag were plated in triplicate at a density of 1.5 × 104 per well in a six-well plate (BD Falcon) a day before being transfected with either the pBSE4-lux vector (18) or 3TP-lux vector (19) using FuGENE6 (Roche Applied Science) according to the manufacturer’s recommendations. Starting 24 h later, cells were treated with 100 μmol/L TGF-β1 (R&D Systems) for 18 h, then harvested for the luciferase assay using the protocol from the Luciferase Assay Systems kit (Promega) using the Flash ‘n Glow system (Berthold Technologies).

**Migration assay.** MCF-7 cells were grown to 80% confluence then serum starved overnight before setting up the experiment. Cells were washed twice in Dulbecco’s PBS and harvested from the plate using 0.5 mL EDTA (pH 6.8). The cells were collected and resuspended in starvation medium. We used 24-well transwell chambers (BD BioCoat Control Inserts from BD Biosciences) with 8.0-μm pore size polycarbonate membrane for this experiment. The cells were plated at a density of 5 × 104 per well in 0.5 mL in the upper well, which was placed into a lower well containing one of the following conditions: complete medium + 5 ng/mL TGF-β or complete growth medium (10% FBS). After 24 h at 37°C, 5% CO2 incubator for 24 h, the experiment was stopped by wiping the cells from the well with a cotton swab and fixed and stained using the Diff-Quik kit (Dade-Behring). Migration was quantitated by counting 12 fields at a magnification of ×400. Each experiment was repeated in triplicate and the results were averaged. Statistical analysis was done using the Student’s t test.

**Invasion assay.** The invasion assay was identical to the above migration assay except that inserts were coated with 100 μL Matrigel (BD Biosciences) diluted to 1 mg/mL. The experiment was stopped after 72 h using the same method as above.

**Scratch wound.** MCF-7 cells were plated in 12-well plates. At 100% confluence, cells were scratched with a 10 μL pipette tip in the shape of a cross. The cells were fed with complete medium (10% FBS) or 1 μg/mL anti–TGF-β (clone: 1D11, R&D Systems) in complete medium. Pictures were taken with a Nikon camera fitted to a microscope eyepiece either above the intersection or to the left of the intersection. The gaps were measured and calculated into % wound closure. Results represent the average of four experiments.

**Growth inhibition assay.** Briefly, a ×104 NIH-3T3 cells were plated per well in a six-well plate, and allowed to adhere overnight. Cells were treated with 100 pmol/L TGF-β1 for 18 h, before addition of 3[H]thymidine (Amersham) for an additional 4 h. After the 4 h incubation, cells were washed with ice-cold PBS, fixed for 1 h with 95% methanol, rewashed in PBS, and lysed with 0.2 N NaOH. [3H]thymidine incorporation was measured using the Beckman Coulter scintillation counter.

**Oncogenic assessment.** To test foci formation, NIH-3T3 cells and stable clones were plated in 60-mm plates at a density of 1 × 105 per plate. The medium was replaced every 2 to 3 days and grown for 21 days. The cells were fixed with methanol and stained with methylene blue. To determine whether *6A enhances H-RasV12 transformation of NIH-3T3 cells, a transient transfection using pBABE-H-RasV12 was done 24 h after plating the NIH-3T3 clones.

To assay colony formation, NIH-3T3 cells and stable clones were plated at a density of 1 × 104 in 0.3% agar and laid overtop 0.6% agar. The plates were incubated at 37°C in 5% CO2 and monitored every 2 to 3 days for colony formation up to 28 days.

**Gene array.** MCF-7-TGFBR1-HA-Flag and MCF-7-TGFBR1*6A-HA-Flag cells expressing similar amounts of transgene were grown in complete medium. RNA was collected from each cell line in triplicate using the RNeasy protect Mini kit (Qiagen). RNA quality was confirmed using the 2100 Bioanalyzer from Agilent. The microarray was done using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array and carried out according to the protocols from Affymetrix, Inc. The array was read on the Affymetrix GeneChip Scanner 3000. The microarray data consist of 54,675 probe sets and were normalized using BMA algorithm (20). Once normalized, genes that were both up- or down-regulated 1.5-fold in *6A cells over *9A cells, and had a P value of <0.01 using t test, were uploaded into the Ingenuity Pathway Analysis software (Ingenuity Systems). The Ingenuity Pathway Analysis software sorts the genes into their appropriate signaling pathways, from which we were able to search through genes specifically involved in migration and invasion pathways. This search led to the identification of two genes that were down-regulated in *6A cells compared with *9A cells that would directly lead to an increase in migration. Results of the gene array were then confirmed using real-time PCR.

**Real-time PCR.** RNA was collected from cells using the RNeasy protect Mini kit (Qiagen). Reverse transcription was carried out using 2 μg RNA in a 100 μL reaction volume using the TaqMan Reverse Transcription Kit (Applied Biosystems). Two microliters of cDNA were combined with 10 μmol/L of each forward and reverse primer, 50 μmol/L of the TaqMan probe, and TaqMan Fast Universal PCR Master Mix 2× (Applied Biosystems). We used the following primers and TaqMan probes for ARHBGAP5, fibronectin 1 (FN1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): ARHBGAP5 sense primer (5′-AGGCGCATTCTTGGCACA-TAG-3′), TaqMan probe (5′-FAM TACTTGGAGATTCTTACTGTGCGGC CAT QSY7-3′), FN1 sense primer (5′-TGGCCATCATGAGAAGTGAC-3′), and TaqMan probe (5′-FAM TTCTTGATTTTCCTTACACAGTA-3′), TaqMan probe (5′-FAM...
TCAACCTTCCTGAAACTGCAAACTCCGTC QSY7-3

GAPDH sense primer (5'-GAAGGTGAAGGTCGGAGTC-3'), antisense primer (5'-GAAGATGGGATGGGATTC-3'), and TaqMan probe (5'-FAM CAAGCTTCCCGTTCT-CAGCC QSY7-3').

PCR amplification and detection was done on the Applied Biosystems 7500/7500 Fast PCR system (Applied Biosystems). The ARHGAP5 and FN1 transcripts were quantitated relative to GAPDH by comparative C_T method following the Applied Biosystems protocol.

Western blots. Cells were grown to 80% confluence, serum starved overnight, treated with 100 pmol/L TGF-β1 for 18 h, and lysates were collected. Cells were lysed, boiled, and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked in 5% milk in TBST, and immunoblotted with the appropriate antibodies. Fibronectin (EP5), RhoA (26C4), JNK1 (C-17), and p38 (N-20) were obtained from Santa Cruz Biotechnology; phospho-Smad2 (Ser465/467) was from Cell Signaling Technology; Akt/PKB; phospho-Akt1/PKBα (Ser463), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-p44/42 MAPK (Thr202/Tyr204), and p44/42 MAPK were obtained from Upstate Cell Signaling Solutions; anti–p190-B RhoGAP (EP489Y) was from Novus Biologicals; SMAD3 and pSMAD3 were a gift of Dr. Koichi Matsuzaki (Osaka, Japan); and α-tubulin was from Sigma-Aldrich (Sigma-Aldrich). Secondary antibodies were peroxidase-conjugated affinity-purified anti rabbit or mouse IgG (Rockland), and detection was done with ECLPlus Western Blotting Detection System (Amersham Applied Biosystems). Densitometry analysis was done using UN-SCAN-IT software (Silk Scientific, Inc.).

RhoA-GTPase activity assays. Pull-down assays to detect GTP-bound RhoA were done as described previously (21, 22). Briefly, a fusion protein containing the Rho binding domain (RBD) for rhotekin (23) and a glutathione S-transferase (GST) were used (a kind gift from Dr. Martin A. Schwartz, Scripps Institute, La Jolla, CA). Cells were serum starved overnight before being fed with complete medium (10% FBS). After 10 min, cells were washed twice in ice-cold TBS and lysed in lysis buffer [50 mmol/L Tris (pH 7.2), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mmol/L NaCl, 10 mmol/L MgCl2, 1 mmol/L DTT, 10 μg/mL each of leupeptin, aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride]. Cell lysates were incubated at 4°C for 60 min with the RBD-GST precoated to agarose-glutathione beads (Sigma) to precipitate the GST-bound RhoA. The product was separated on a 13% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with a RhoA antibody (Santa Cruz Biotechnology). The membrane was stripped and reprobed with α-tubulin (Sigma-Aldrich).

The amount of GTP-bound RhoA was also assessed using the G-LISA RhoA Activation Assay Biochem Kit (Absorbance Based; Cytoskeleton, Inc.) according to the manufacturer’s protocol, which came with all the reagents used in the assay. Briefly, 2 days after plating 1 × 10^6 cells in a 100-mm dish, the cells were serum starved overnight and refed the following day with fresh complete medium (10% FBS) for various early time points. Cells were washed once with ice-cold PBS and lysed in the lysis buffer supplied in the kit. Protein concentration was evaluated using the Precision Red Advanced Protein Assay Reagent, and 1.75 μg of protein were used for the assay. The absorbance was measured at 490 nm using a microplate spectrophotometer.

Results

Oncogenic assessment. To test the hypothesis that TGFBR1*6A may act as an oncogene, we stably transected NIH-3T3 cells with
*6A, *9A, or the empty vector. Transfected cells were plated and foci formation was assessed after 21 days. Neither the *9A- nor the *6A-expressing plates exhibited foci, indicating that *6A alone cannot act as an oncogene (data not shown).

**H-Ras** is a bona fide oncogene, which transforms NIH-3T3 cells via the phosphatidylinositol 3-kinase pathway and leads to uncontrollable cell proliferation. A constitutively active mutant H-Ras known as H-RasV12 maintains the gene in its activated GTP state (24). The high-expressing *9A clone cooperates with H-RasV12 to increase transformation (data not shown). This is not surprising given the fact that transforming growth factor-β (TGF-β) was first identified as a fibroblast TGF (25). In contrast, *6A has no effect on H-RasV12-induced transformation, thus indicating that the known hypomorphic properties of *6A with respect to TGF-β-mediated signaling result in decreased oncogenesis when compared with its wild-type counterpart.

In the colony formation assay in soft agar, neither *9A nor *6A alone was able to transform NIH-3T3 cells. *9A and *6A were also unable to synergize with H-RasV12 to induce more colony growth than just the H-RasV12 alone (data not shown).

There was no evidence of a difference between TGFBRI*6A-transfected and TGFBRI*9A-transfected NIH-3T3 cells with respect to TGF-β-mediated growth inhibition (data not shown). Various *6A clones are growth inhibited upon TGF-β treatment. Hence, transfection of NIH-3T3 cells with the *6A allele does not significantly alter TGF-β-mediated growth inhibition.

**TGF-β signaling.** To assess TGF-β signaling, MCF-7 *9A-low and *6A-low cells were transiently transfected with the luciferase reporter constructs SBE4-lux or 3TP-lux. The SBE4 construct measures the ability for SMAD2 and SMAD3 to induce transcription of the reporter construct (18). The 3TP-Lux expresses luciferase under the control of three 12-O-tetradeca-

---

Figure 2. Assessment of MCF-7 cell migration. A and B, MCF-7 cells stably transfected with the empty vector (pIRES), *9A, or *6A were first plated in transwell chambers in starvation medium overnight. Then, either complete medium containing 5 ng/mL TGF-β (A) or complete medium (B) was added to the bottom wells. After 24 h, cells were scraped off the top of the insert and the cells on the bottom of the insert were counted. The experiment was done five times in TGF-β and seven times in FBS; bars, SE. Migration of *6A clones was significantly higher than that of *9A clones (A). In the absence of exogenously added TGF-β, the difference was greater (B). C, MCF-7 cells stably transfected with the empty vector (pBABE), *9A, or *6A were first plated in transwell chambers in starvation medium overnight. Then, either complete medium alone or complete medium containing 5 ng/mL TGF-β was added to the bottom wells. After 24 h, cells were scraped off the top of the insert and the cells on the bottom of the insert were counted. The experiment was done thrice. Bars, SE. Migration of *6A clones was significantly higher than that of *9A clones. D, a confluent monolayer of MCF-7-*9A and MCF-7-*6A cells was scratched with a pipette tip and the gap was measured after 24 h. *6A-low cells closed the wound completely in FBS, whereas *9A-low cells closed the wound 66%. Addition of a TGF-β-neutralizing antibody resulted in 89% and 47% wound closure in *6A-low and *9A-low cells, respectively. *6A-int cells closed the wound 85% and 93% in FBS and TGF-β, respectively, whereas *9A-high cells closed the wound 60% and 80% in FBS and TGF-β, respectively. The pictures are representative of three assays.
nophorbol-13-acetate response elements and a segment of the plasminogen inhibitor promoter (19). After TGF-β treatment, the amount of induction in the SBE4-lux reporter is the same in pIRES cells and *9A cells: 1.3- and 1.2-fold induction, respectively. SMAD4 signaling in *6A cells is decreased after TGF-β treatment; however, the difference is not statistically significant (P = 0.067; Fig. 1A).

TGF-β signaling downstream of SMADs was assessed by using the 3TP-lux reporter assay (19). TGF-β treatment resulted in 1.7-, 1.3-, and 1.1-fold induction of reporter gene expression for pIRES, *9A, and *6A cells, respectively. The differences between the different cell lines are again not statistically significant (P < 0.175; Fig. 1B). Thus, transfection of MCF-7 cells with *6A results in a small but not statistically significant decrease in TGF-β signaling when compared with *9A.

Lastly, we assessed SMAD-mediated TGF-β signaling by measuring the levels of phosphorylated SMAD2 (pSMAD2) and SMAD3 (pSMAD3). Exposure to TGF-β leads to similar levels of pSMAD2 for MCF-7*/6A cells and MCF-7*/9A cells (both for low and high expressers), which is minimal and consistent with the minimal increase we saw in the SMAD luciferase assays (Fig. 1C). There is no difference in SMAD3 phosphorylation between *6A and *9A cells.

Migration and invasion. To comprehensively explore possible roles for *6A in tumor development and progression, we investigated its ability to modify cell migration and invasion of MCF-7 cells. Transwell chambers were used to assess the migratory potential of MCF-7 cells. The upper chambers contained the cells seeded in starvation medium, and the bottom wells held the complete medium with 5 ng/mL TGF-β (gray columns). Columns, average of seven experiments (A) or three experiments (B); bars, SE. The number of *6A cells invading the Matrigel was significantly higher than that of *9A cells (P < 0.005). C, basal growth rate of MCF-7 cells was determined by thymidine incorporation over 4 h after growth for 18 h in culture. For *9A-low and *6A-low, n = 10 assays were done in triplicate. For MCF-7, pIRES, *9A-high, and *6A-int, n = 3 assays were done in triplicate.

Figure 3. Assessment of MCF-7 cell invasion. A and B, MCF-7 cells stably transfected with the empty vector (pIRES), *9A or *6A (A) or pBABE empty vector, or with pBABE*9A or pBABE*6A (B) were first plated in transwell chambers in starvation media overnight. Cells were seeded on top of Matrigel-coated inserts and allowed to invade toward complete medium (black columns) or complete medium with 5 ng/mL TGF-β (gray columns). Columns, average of seven experiments (A) or three experiments (B); bars, SE. The number of *6A cells invading the Matrigel was significantly higher than that of *9A cells (P < 0.005). C, basal growth rate of MCF-7 cells was determined by thymidine incorporation over 4 h after growth for 18 h in culture. For *9A-low and *6A-low, n = 10 assays were done in triplicate. For MCF-7, pIRES, *9A-high, and *6A-int, n = 3 assays were done in triplicate.
to the physiologic levels (17). First, we assessed migration in the absence of exogenously added TGF-β. Parental MCF-7 and pIRES control cells had an average of 37 and 33 migrating cells, respectively. Although the migration of *9A cells was essentially identical to that of the control cells, the number of migrated *6A cells was 3.2-fold higher than the *9A cells (P < 0.005; Fig. 2B).

Migration in MCF-7 cells was further assessed with several clones from cells transfected with TGFBR1*6A or TGFBR1*9A inserted into the pBABE vector. Compared with vector-only controls, low-expressing *6A clones migrated 2.6-fold more in the presence of FBS (P < 0.001; Fig. 2C). Low-expressing *6A cells on the other hand migrated 3.5-fold more than pBABE cells in FBS (P < 0.001). This 1.3-fold difference between the *9A-low and *6A-low cells is also highly significant (P < 0.001). TGF-β does not increase the amount of migration in pBABE, *9A-low, or *6A-low cells (P < 0.1 for each cell line). Although the high-expressing *9A clones migrated 0.4-fold less than vector cells, the high-expressing *6A cells migrated 1.8 times more than the vector cells in FBS (P < 0.001). Again, addition of TGF-β did not alter the amount of migrating cells in the *6A-high cells (P < 0.1), demonstrating that *6A expression results in an increase in migration that is not enhanced by TGF-β.

Migration of MCF-7 cells was also determined by performing a scratch wound assay (Fig. 2D). At 24 h, in the presence of 10% FBS, *6A-low cells closed the entire wound, whereas *9A-low cells only closed 66%. The *6A-int cells closed 85% whereas the *9A-high cells closed 60% after 24 h. These results mirror the results from the transwell assays. These data indicate that *6A enhances migration in MCF-7 cells, both in the presence and in the absence of exogenously added TGF-β.

To examine the possible role played by traces of TGF-β in FBS, the differential effect of *6A and *9A on cell migration was further characterized by the addition of a pan–TGF-β neutralizing antibody to the medium. As seen in Fig. 2D, TGF-β blockade resulted in slightly less wound closure when compared with closure in FBS alone. The percent closure for *9A-low, *6A-low, *9A-high, and *6A-int was 47%, 89%, 80%, and 93%, respectively, after TGF-β neutralization. This further shows that the increase in migration that *6A cells have over *9A cells is independent of TGF-β signaling. The high percent of wound closure seen after blocking TGF-β also shows that TGF-β is not required for migration or wound closure, and although migration is higher in complete medium (with possible traces of TGF-β present in serum), TGF-β is not the driving force for cell migration.

Having shown that *6A enhances the migration of the MCF-7 breast cancer cell line, we sought to assess its effect on invasion. Cell invasion of MCF-7 cells was determined by the ability of the cell to invade through a Matrigel barrier. Cells were plated in starvation medium on top of the Matrigel-coated inserts, whereas the bottom wells contained either complete medium with 10% FBS or complete medium with 10% FBS and 5 ng/mL TGF-β. As with cell migration, we observed that *6A enhances cell invasion independent of TGF-β. In the presence of FBS alone, MCF-7 parental cells, pIRES control cells, and *9A cells have the same number of invading cells (Fig. 3A). However, *6A cells show a 1.8-fold greater amount of invasion than the *9A cells (P < 0.005).

Addition of TGF-β results in similar proportions of invading cells in each of the cell lines tested. The pRES and *9A cells have a slight decrease in invading cells compared with the MCF-7 cells; however, *6A cells invade the same as the parental cells, indicating that *6A has no effect on TGF-β–induced cell invasion.

Invasion was also tested in the MCF-7 pBABE clones. The *9A-low cells invade 1.3 times more than the vector controls (P < 0.001), whereas the *6A-low cells invade 2.2 times more than the vector cells (P < 0.001; Fig. 3B). This 1.7-fold increase in *6A-low cell invasion over *9A-low cells is also significant (P < 0.001). Similar to what was seen with the pIRES clones, TGF-β does not alter the amount of invasion, compared with the basal invasion (P = 0.1 for *9A-low and P = 0.1 for *6A-low cells). *9A-high clone invasion was decreased compared with pBABE controls; however, the *6A-high clones invaded 1.9-fold more than the pBABE cells (P < 0.001), demonstrating that *6A increases invasion. TGF-β caused a 2.1-fold induction in invasion in the *6A-high cells compared with pBABE cells (P < 0.001); however, TGF-β did not change the amount of invading cells compared with nontreated cells (P < 0.2).

To determine whether the increase in cell migration, wound closure, and invasion seen in *6A cells is partially or entirely due to increased cell growth compared with *9A cells, basal growth rate was assessed by thymidine incorporation. As seen in Fig. 3C, there was no difference between the growth rate of *6A compared with *9A or to parental MCF-7 or vector control cells. These data strongly suggest that the differences in actual cell migration are not due to differential cell growth.

These results establish the fact that both migration and invasion of MCF-7 cells are significantly enhanced by *6A in a TGF-β–independent manner.

**Gene array.** To dissect the molecular mechanisms underlying the differences in migration and invasion of *6A and *9A cells, we analyzed the differential gene expression of the two low-expressing cell lines. The Affymetrix GeneChip Human Genome U133 Plus 2.0 Array was used to identify differentially expressed genes in MCF-7 pIRES*6A cells compared with MCF-7 pIRES*9A cells. The low-expressing *6A and *9A cells were used for the array. The results were analyzed using Ingenuity Pathway Analysis focusing on genes involved in cell migration and invasion. Using a cutoff of 1.5-fold difference in gene regulation and a P value of <0.01, two genes involved in cell migration were identified that were down-regulated in *6A cells compared with *9A cells, ARHGAP5 and FNL.

**ARHGAP5** encodes the Rho GTPase–activating protein 5. Affymetrix gene array analysis shows that ARHGAP5 expression is 3.4-fold lower in *6A cells than in *9A cells in the presence of normal growth medium (P = 0.00017). Real-time PCR confirmed these findings and showed a 3.8-fold reduction in the expression of ARHGAP5 in *6A cells compared with *9A cells (Fig. 4A), as well as a 4.3-fold reduced expression in the *6A-int cells compared with the *9A-high cells, indicating that this response is not due to clonal variation. Furthermore, we observed a 2.4-fold reduction in ARHGAP5 in a TGFBR1*6A kinase–inactivated MCF-7 cell line compared with the low-expressing *9A cells (data not shown), suggesting that ARHGAP5 down-regulation is independent of TGFBR1 kinase signaling. Western blotting for ARHGAP5 also shows that ARHGAP5 is down-regulated in *6A compared with *9A for cells transfected with both the pRES and pBABE vectors (Fig. 4B).

RhoGAP5s down-regulate GTP-bound RhoA by hydrolyzing GTP into GDP. By down-regulating ARHGAP5, RhoA remains bound to GTP and remain active. We used two different methods to determine whether the differential regulation of ARHGAP5 alters ARHGAP5 activity: a commercially available G-LISA RhoA Activation Assay kit and the pull-down method described by Ren et al. (21). It has been shown that the serum component LPA rapidly
induces RhoA activation before declining (21). As shown in Fig. 4C, RhoA activation was consistently higher in *6A cells compared with *9A cells. The greatest differences were observed at 10 and 20 min and 60 min after serum induction.

Because GTP-bound RhoA is rapidly hydrolyzed into the GDP form, a pull-down assay was used that utilizes the ability of the Rhotekin protein to specifically bind GTP-bound RhoA (23). At 10 min post–FBS induction, we were able to confirm our finding that GTP-RhoA is higher in *6A-low cells than in *9A-low cells, indicating that RhoGAP activity is decreased in *6A cells (Fig. 4D).

FN1 encodes for fibronectin. FN1 was down-regulated 2.5-fold in *6A cells when compared with *9A cells (P = 0.0046). Real-time PCR confirmed this finding and showed a 6.4-fold reduction of FN1 in the low-expressing *6A cells compared with *9A. The difference among high-expressing clones was also significant with a 3.1-fold lower level in the *6A cells compared with *9A cells (Fig. 5A). FN1 was down-regulated 2-fold in the kinase-dead *6A MCF-7 cells compared with the *9A-low cells (data not shown), indicating that *6A-mediated down-regulation of FN1 is independent of TGFBR1 kinase signaling.

Western blotting analysis further confirmed FN1 down-regulation, as seen by the almost complete disappearance of FN1 in all *6A clones tested compared with its *9A control (Fig. 5B).

**Analysis of MAPK signaling.** Signaling through the MAPK pathway contributes to migration and invasion (26). To determine whether TGFBR1*6A increases migration in our model via activation of the MAPK pathway, we examined the various components involved in MAPK signaling. After growth in complete growth medium (10% FBS) for 24 h following overnight serum starvation, we observed 44% higher levels of ERK1/2 phosphorylation in the *6A-low over the *9A-low clones, and 43% higher levels of ERK1/2 phosphorylation in the *6A-int over the *9A-high clones (Fig. 5C). Considering that the *6A-int clones express TGFBR1 to a lesser extent than the *9A-high cells, the enhancing effect of *6A on ERK1/2 phosphorylation may be underestimated.

There was no consistent difference in signaling between p38 and JNK activation in *9A cells compared with *6A cells grown in complete medium.
Discussion

One of the most essential homeostatic functions of TGF-β is inhibition of cell proliferation, thereby functioning as a tumor suppressor gene. TGF-β signaling components are often mutated during tumor formation.

In this report, we show that transfection of TGFBRI*6A into MCF-7 breast cancer cells results in a significant increase in both cellular migration and invasion. The difference in migration and invasion were observed with multiple clones both in the presence and in the absence of exogenously added TGF-β. However, the difference between *6A cells and *9A cells was more pronounced in the absence of TGF-β, which indicates that this phenomenon is independent of TGF-β signaling and that TGF-β may blunt the effect of other serum components.

Gene expression profiling analysis identified two differentially expressed genes involved in migration, ARHGAP5 and FN1. Differential expression of these genes was confirmed in high- and low-expressing *6A and *9A clones. Furthermore, our functional assays established that down-regulation of ARHGAP5 is associated with increased RhoA activation. RhoGTPases (Rho, Rac, and Cdc42) regulate cell migration by mediating distinct cytoskeletal changes. Rac induces lamellipodia extensions and membrane ruffling; Cdc42 regulates filopodia formation; and Rho induces stress fiber formation (27). ARHGAP5 encodes the RhoA GTPase–activating protein 5, which causes the inactivation of GTP-bound RhoA. We found that down-regulation of ARHGAP5 is associated with increased levels of activated RhoA as early as 10 min postinduction. Although mutations in RhoA are rare, RhoA is often overexpressed or functionally hyperactive in breast cancer tissue, and overexpression correlates with more advanced breast disease (28, 29). Furthermore, knockdown of RhoA using small interfering RNA inhibited migration and invasion of aggressive MDA-MD-231 and Hs578T breast cancer cell lines (30, 31). Expression of constitutively activated RhoA in MCF-7 breast cancer cells resulted in an increase in serum-mediated motility, which was directly blocked by ROCK inhibition. In addition, RhoA activation signaling via ROCK led to a direct increase in MMP-9 activity and ERK1/2 activation, leading to the increase in motility (32). These findings lead us to...
hypothesize that the observed increase in ERK activation is a direct effect of RhoA hyperactivation induced by TGFBR1*6A expression, resulting in the observed increased migratory phenotype of MCF-7*6A cells.

Loss of FN1 expression has been correlated with a poorer prognosis in breast cancer. Patients with FN1-negative tumors have decreased relapse-free survival (33). Furthermore, women with invasive breast carcinoma who died without metastases (27 of 31 patients, 87%) had a higher frequency of FN1-positive stromal cells compared with women who died with disseminated invasive breast carcinoma (3 of 26 patients, 12%; \( P < 0.0005 \); ref. 34).

Loss of FN1 expression has been shown in cell lines derived from metastatic carcinomas while still present in cell lines from nonmalignant tissues or from primary carcinomas (35). It has also been shown that FN1 production in MCF-7 cells is inhibited by 17\( \beta \)-estradiol; however, treatment with tamoxifen, an anti-estrogen, restores FN1 expression, which is partially mediated by TGF-\( \beta \) induction (36). Decreased FN1 expression due to *6A most likely causes a loss of adhesion, thus promoting early events that lead to migration.

Because the mature *9A and *6A receptors are identical (17), it is expected that TGFBR1 kinase–mediated TGF-\( \beta \) signaling is identical in *9A and *6A cells. Indeed, MCF-7 cells expressing either *9A or *6A do not show a difference in TGF-\( \beta \) signaling as assessed by levels of pSMAD2 or pSMAD3. Similarly to our previous findings with respect to TGF-\( \beta \)-mediated growth inhibition (17), the observed effects are likely due to secondary signaling events triggered by *6A signal sequence. This explanation is supported by the findings that MCF-7 cells transfected with a kinase-deficient TGFBR1*6A construct also show decreased expression of ARHGAP5 and FN1 when compared with cells transfected with *9A.

TGFBR1*6A causes TGF-\( \beta \)-mediated growth stimulation in both MCF-7 breast cancer cells and DLD-1 colon cancer cells and transduces TGF-\( \beta \) growth-inhibitory signals less effectively than TGFBR1 in mink lung epithelial cells (15–17). These previous findings led us to test the hypothesis that TGFBR1*6A may act as an oncogene. Using foci and colony formation assays in NIH-3T3 cells, we were unable to uncover any oncogenic properties for *6A, either alone or collectively with H-RasV12. Overexpression of the wild-type receptor increased H-RasV12 oncogenic transformation in NIH-3T3 cells to a greater degree than overexpression of the *6A receptor. Furthermore, transfection of *6A did not provide NIH-3T3 cells with a growth advantage after TGF-\( \beta \) treatment.

In summary, we show that *6A enhances MCF-7 cell migration and invasion and results in the activation of the RhoA and ERK pathways as well as the down-regulation of FN1. This is the first report of a TGFBR1*6A phenotype that is independent of TGF-\( \beta \) signaling. Given the fact that \( \sim 16\% \) of patients with breast cancer harbor the *6A allele (37), our findings may have important implications for the relatively large proportion of patients suffering from this disease.

Acknowledgments

Received 9/17/2008; revised 12/17/2007; accepted 1/8/2008.

Grant support: Walter S. Mander Foundation, Chicago, IL; the Jeannik M. Littlefield-AACR Grant in Metastatic Colon Cancer Research; grants CA112520 and CA108741 from the National Cancer Institute; the Katten Muchin Rosenman Travel Scholarship Award from the Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL; and the Malkin Scholar Program, Chicago, IL.

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.

We thank Dr. Hiroaki Kiyokawa for advice and Dr. Neureh Jalari for the gene array experiments.

Figure 6. TGFBR1*6A, ERK signaling, and cell motility. TGFBR1 and TGFBR1*6A encode for identical mature receptors but the signal sequence of TGFBR1*6A is three amino acids shorter than the signal sequence of TGFBR1. Transfection of MCF-7 cells with TGFBR1*6A results in the down-regulation of FN1 and ARHGAP5, and enhances RhoA and ERK activation. RhoA activates ROCK, which activates myosin light chain kinase (MLCK), then myosin regulatory light chain 2 (MRLC), causing myosin II to induce stress fiber formation and enhancing motility.
References

TGFB\textsuperscript{1*6A} Enhances the Migration and Invasion of MCF-7 Breast Cancer Cells through RhoA Activation

Diana S. Rosman, Sharbani Phukan, Chiang-Ching Huang, et al.