Smad4-dependent Regulation of Urokinase Plasminogen Activator Secretion and RNA Stability Associated with Invasiveness by Autocrine and Paracrine Transforming Growth Factor-β*

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Metastasis is a primary cause of mortality due to cancer. Early metastatic growth involves both a remodeling of the extracellular matrix surrounding tumors and invasion of tumors across the basement membrane. Up-regulation of extracellular matrix degrading proteases such as urokinase plasminogen activator (uPA) and matrix metalloproteinases has been reported to facilitate tumor cell invasion. Autocrine transforming growth factor-β (TGF-β) signaling may play an important role in cancer cell invasion and metastasis; however, the underlying mechanisms remain unclear. In the present study, we report that autocrine TGF-β supports cancer cell invasion by maintaining uPA levels through protein secretion. Interestingly, treatment of paracrine/exogenous TGF-β at higher concentrations than autocrine TGF-β further enhanced uPA expression and cell invasion. The enhanced uPA expression by exogenous TGF-β is a result of increased uPA mRNA expression due to RNA stabilization. We observed that both autocrine and paracrine TGF-β-mediated regulation of uPA levels was lost upon depletion of Smad4 protein by RNA interference. Thus, through the Smad pathway, autocrine TGF-β maintains uPA expression through facilitated protein secretion, thereby supporting tumor cell invasiveness, whereas exogenous TGF-β further enhances uPA expression through mRNA stabilization leading to even greater invasiveness of the cancer cells.

Malignant tumors are characterized by their ability to metastasize to distant organs. The initial steps of metastasis involve invasive growth of tumors across the basement membrane and migration through the extracellular matrix (ECM). Because the enzymatic degradation of both the basement membrane and ECM barriers requires a number of ECM-degrading proteases (1, 2) and is a critical early event in metastasis, invasiveness may be modulated by the expression of ECM-degrading proteases in tumor cells in response to autonomous and micro-environmental signals. Among the increasing number of ECM-degrading proteases implicated in metastasis, considerable attention has been focused on the family of matrix metalloproteinases (MMPs) and the plasminogen activator system. One of the regulators of these ECM-degrading proteases is transforming growth factor-β (TGF-β).

TGF-β is a multifunctional cytokine that regulates cell proliferation, differentiation, plasticity, and migration in a context-dependent manner (reviewed in Refs. 3 and 4). TGF-β induces signaling through a transmembrane type II receptor (TβRII), a constitutively active serine/threonine kinase receptor (5). Upon ligand binding, the TβRII recruits and transphosphorylates intracellular TGF-β type I receptor (TβRI), thereby stimulating TβRII serine/threonine kinase activity (6). The TβRI then activates the downstream effectors, Smad2 and Smad3, by phosphorylation. The activated Smad proteins form complexes with the common Smad mediator, Smad4, and then translocate to the nucleus, where the Smad complexes regulate transcription of TGF-β target genes in conjunction with various transcriptional or co-transcriptional regulators. In addition to the Smad pathway, other signaling pathways, including the extracellular signal-regulated kinases (ERK1/2) (7, 8), the mitogen-activated protein kinase (p38) (9, 10), the Src (11), and the phosphatidylinositol 3-kinase (PI3K) (12) pathways can be activated by TGF-β in a context-dependent manner. The precise molecular mechanisms of regulation of these pathways for TGF-β signaling and the physiological and pathological roles of

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1 The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TGF-β, transforming growth factor-β; TβRI, TβRII, transmembrane types I and II; ERK, extracellular signal-regulated kinase; DNIR, dominant-negative type II receptor; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; m.o.i., multiplicity of infection; MT3, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANOVA, analysis of variance.
TGF-β in normal tissues and cancer have not been completely defined.

The importance of autocrine TGF-β in tumor progression and metastatic behavior has been documented previously. For instance, disruption of autocrine TGF-β signaling by a dominant-negative type II receptor (DNIIR) inhibited the invasive and metastatic potential of mammary and colon carcinoma cells (13). This was attributed to prevention of autocrine TGF-β-induced epithelial-to-mesenchymal transition, a process believed to promote tumor cell migration and invasion (12). In a different study, overexpression of a soluble TGF-β type III receptor antagonized autocrine TGF-β activity and resulted in inhibition of tumor cell proliferation and induction of apoptosis (14).

The urokinase plasminogen activator (uPA) is a serine protease capable of initiating cascades of activation of ECM-degrading enzymes (15) and eliciting intracellular signaling through receptor binding. Clinically, elevated uPA expression in tumors is associated with tumor aggressiveness and poor outcome in patients (16, 17) and numerous studies have linked uPA to invasive and metastatic phenotype of tumors in vitro and in animal models (18–21). The metastatic MDA-MB-231 breast cancer cells secrete active TGF-β (22, 23) and are TGF-β-responsive (24). These cells also express both the matrix metalloproteinases-9 (MMP-9) and uPA (20, 25, 26). We hypothesized that autocrine TGF-β may function as a tumor promoter by regulating MMP-9 or uPA activity in MDA-MB-231 cells. The present study provides evidence that autocrine TGF-β regulates both cell invasiveness and uPA secretion. Inhibition of uPA activity is sufficient to suppress tumor cell invasion to the same extent as inhibition of autocrine TGF-β signaling, suggesting that autocrine TGF-β stimulation of invasiveness occurs via its regulation of uPA release. The Smad pathway appears to be required for the regulation of uPA release as silencing of Smad4 protein expression suppressed uPA secretion. Interestingly, although autocrine TGF-β regulates uPA production through protein secretion, exogenous TGF-β further increases uPA expression through RNA stabilization also through a Smad4-dependent fashion. Finally, this work demonstrates that a pharmacological kinase inhibitor of TGF-β receptors inhibits both uPA secretion and tumor cell invasiveness, thereby providing evidence for the potential efficacy of targeting TGF-β signaling for therapeutic intervention in cancer and suggests that uPA expression or secretion may be an important mediator of such effects.

**IMMUNOBLOT ANALYSIS—**To harvest protein lysates, cells were washed with cold phosphate-buffered saline (PBS) and lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml aproamin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovandate, and 1 mM sodium fluoride) for 20 min on ice. Lysates were sonicated and then clarified by centrifugation at 15,000 × g for 15 min at 4 °C. Protein contents of lysates were determined by the Bradford Assay (Bio-Rad). Proteins in the lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% milk PBS-T (0.1% Tween 20 (v/v) in PBS) for 1 h at room temperature and then probed with primary antibodies in 5% milk PBS-T overnight at 4 °C. After several washes with PBS-T, membranes were incubated in PBS-T containing horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and washed again with PBS-T. Immunoreactive bands were visualized by chemiluminescence reaction using ECL reagents (Amersham Biosciences) followed by exposure of the membranes to XAR5 films (Kodak, Rochester, NY). To detect secreted uPA, conditioned media were collected, centrifuged at 15,000 × g for 5 min to remove cell debris, and then subjected to immunoblotting under non-reducing conditions. 2- to 8-h conditioned media were concentrated using Microcon YM-10 centrifugal filter devices from Millipore (Billerica, MA). The volumes of conditioned media loaded on gels were normalized to the protein concentrations of cell lysates. The fibronectin antibody was purchased from BD Transduction Laboratories, Inc. The Smad2 and phospho-Smad2 antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The uPA antibody (cat. no. 394) was obtained from American Diagnostica, Inc. The polyadenosine diphosphate ribose polymerase and Rho GDI (guanine nucleotide dissociation inhibitor) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The actin and FLAG antibodies were purchased from Sigma-Aldrich, Inc.

**TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAY—**Cells at 50–70% confluence on 12-well plates were co-transfected with 0.5 μg of a firefly luciferase promoter-reporter construct and 0.01 μg of the Renilla reniformis luciferase reference reporter construct, phRL-TK (Promega, Madison, WI) using Lipofectamine Plus reagents (Invitrogen). Four hours after transfection, cells were cultured back in regular media. Forty-eight hours after transfection, firefly and R. reniformis luciferase activities were measured using the Dual Luciferase Reporter System kit (Promega) in an Optocomp II Luminometer (MGM Instruments, Inc., Hamden, CT). Normalized firefly luciferase activity was plotted as mean ± S.D. from three independent experiments. The phuPA-Luc reporter containing the nucleotide sequence −2345 to +30 of the human uPA promoter was kindly provided by Dr. Shuji Kojima (29). The p3TP-Lux reporter was a generous gift from Dr. Joan Massague (30).

**MATRIGEL INVASION ASSAY—**A modified Boyden chamber assay was performed using Transwells (12-μm pore size, 12 mm in diameter) from Costar (Cambridge, MA) and Matrigel (BD Biosciences). Each Transwell insert was first coated with 100 μl of 2.5 mg/ml Matrigel diluted in serum-free media for 1 h at 37 °C.
and then 10 µl of Matrigel was added in the center of the Transwell 2 h before use. Cells were trypsinized, washed with serum-free media twice, re-suspended in 0.2% bovine serum albumin serum-free medium, seeded in Transwell inserts (150,000 cells/insert), and grown in the presence of 10% fetal bovine serum media in the lower chamber. After 16 h of incubation, Matrigel and cells remaining inside the inserts were removed with Q-tips, and the cells that had traversed to the reverse side of the insert were rinsed with PBS, fixed in 4% formaldehyde for 30 min at room temperature, and stained with 1% crystal violet for 1 h to overnight at room temperature. Results represent the average from three independent experiments ± S.D. (*, p < 0.05; and **, p < 0.003 are derived from one-way ANOVA with a Bonferroni correction).

FIGURE 1. The TGF-β receptor kinase inhibitor, LY364947, inhibits TGF-β signaling and MDA-MB-231 cell invasion. A, LY364947 inhibition of Smad2 phosphorylation induced by TGF-β. Cells were treated with vehicle, LY364947, TGF-β or in combination as indicated. 45 min later, cell lysates were collected and subjected to immunoblotting for total and phospho-Smad2 expression. Cells were treated with vehicle, LY364947, TGF-β, or in combination as indicated. 24 h later, lysates were collected and subjected to immunoblotting for fibronectin and β-actin. C, LY364947 inhibition of basal and TGF-β-induced promoter activation. Cells were co-transfected with the p3TP-Lux reporter construct and a reference reporter construct for 4 h as described under “Materials and Methods” and then treated with vehicle, LY364947, TGF-β, or in combination as indicated. Luciferase activity was determined 48 h after treatments. D, LY364947 inhibition of MDA-MB-231 cell invasion. Cells were treated with LY364947 at the indicated concentrations overnight and then plated in Matrigel-coated Transwells in the presence of vehicle or increasing concentrations of LY364947 as indicated. 16 h later, cells on the reverse side of the Transwell membrane were stained and counted. Results represent the average from three independent experiments ± S.D. (*, p < 0.05; and **, p < 0.003 are derived from one-way ANOVA with a Bonferroni correction).
Northern Blot Analysis—Total RNA isolated with the TRIzol reagent was resolved on formaldehyde-agarose gels, transferred, and immobilized onto Hybond-N nylon membranes (Amersham Biosciences). Blots were blocked in the ULTRAHyb buffer overnight at 68 °C. After several washes with low stringency (2× SSC/0.1% SDS) and high stringency (0.1× SSC/0.1% SDS) buffers at 68 °C, images were acquired by autoradiography using a PhosphorImager. To prepare riboprobes, cDNA plasmids were linearized, purified, and then sub- jected to in vitro transcription using the MAXIscript kit (Ambion) in the presence of 50 μCi of [α-32P]UTP (800 Ci/mmol) for 1 h at 37 °C. Unincorporated nucleotides were removed using NucAway columns (Ambion).

Nuclear Run-on Assay—Cell were collected, washed twice with PBS, and then re-suspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, and 1 mM dithiothreitol). Nonidet P-40 was then added to a final concentration of 0.2–0.5%, depending on cell types. After a 5-min incubation on ice, nuclei were pelleted at 500 × g for 5 min, washed once with nuclear freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl2, 1 mM dithiothreitol), and re-suspended in fresh nuclear freezing buffer. In vitro run-on transcription was performed using 2 × 107 nuclei in 150 μl of reaction buffer (5 mM Tris-HCl, pH 8.0, 2.5 mM MgCl2, 150 mM KCl, 1 mM of ATP, CTP, or GTP, 150 μCi of [α-32P]UTP (800 Ci/mmol), 80 units of RNasin, and 2.5 mM dithiothreitol) for 30 min at 30 °C. Transcription was terminated by adding 350 μl of deoxyribonuclease I solution (20 mM Tris-HCl, pH 7.4, 10 mM CaCl2, and 300 units of RNase-free DNase I) and a 30-min incubation at 37 °C. Next, proteins were digested by adding 50 μl of proteinase K solution (1% SDS, 5 mM EDTA, 1 mM Tris-HCl, pH 7.4, and 300 μg/ml proteinase K) and a 30-min incubation at 50 °C. The 32P-labeled RNAs were phenol/ chloroform purified and precipitated in 10% trichloroacetic acid plus 20 μg of yeast tRNA. After centrifugation at 15,000 × g for 1 h, the RNAs were re-suspended in RNase-free H2O, denatured for 10 min at 65 °C, and then chilled on ice. The radiolabeled RNAs were hybridized to cDNAs pre-immobilized on membranes in hybridization buffer (50% formamide, 5× SSC, 5 mM EDTA, 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA) for 48 h at 42 °C. Next, the membranes were washed several times in 2× SSC (1× SSC = 0.15 M NaCl and 12.5 mM sodium citrate, pH 7) and then in 2× SSC plus 10 μg/ml RNase A for another 30 min or not depending on intensity of background. Signals were acquired and quantified by a PhosphorImager. To immobilize cDNAs to nitrocellulose membranes, 1 μg of linearized plasmid was denatured in 0.2 M NaOH for 30 min at room temperature and then neutralized with 10 volumes of 6× SSC. The DNAs were applied onto nitrocellulose membranes using a slot blot apparatus and immobilized by UV cross-linking.

RNA Interference—To perform Smad4 silencing, 50% confluent cells were transfected with 50–200 pm of a pool of four S. cerevisiae Smad4 or scrambled siRNAs (Dharmacon, Lafayette, CO) using Oligofectamine (Invitrogen) according to manufacturer’s guideline. Conditioned media (48–72 h post transfection) and protein lysates (72 h post transfection) were harvested and subjected to immunoblotting for Smad4 and uPA. To determine uPA mRNA stability under a Smad4-silencing condition, cells were first transfected with 100 nM Smad4 or scrambled siRNAs and then grown in serum media overnight, and then uPA mRNA stability was determined following treatment of TGFB overnight.

Statistical Analysis—p values for multiple comparison tests were derived by an analysis of variance (ANOVA) with a Bonferroni correction.
RESULTS

Autocrine TGF-β Signaling Contributes to MDA-MB-231 Cell Invasion—The metastatic MDA-MB-231 breast cancer cells secrete active TGF-β (22, 23) and are TGF-β-responsive (24). The importance of autocrine TGF-β signaling in regulation of MDA-MB-231 cell invasiveness was assessed by Matrigel invasion assays following abrogation of autocrine TGF-β signaling by the dominant-negative TGF-β type II receptor (DNIIR). Cells were infected with β-galactosidase (Ad-β-Gal) or DNIIR (Ad-DNIIR) adenoviruses at the indicated m.o.i. values. 48 h later, cells were cultured in fresh medium and allowed to grow for 16 h. Conditioned media, protein lysates, and total RNAs were harvested and subjected to immunoblotting for uPA, polyade

 expression significantly decreased MDA-MB-231 cell invasion in a dose-dependent manner (Fig. 1D). These data suggest that autocrine TGF-β plays a role in basal invasive growth of MDA-MB-231 cells.

A Dominant-negative TGF-β Type II Receptor Suppresses Autocrine TGF-β Signaling and Cell Invasion—To substantiate the results obtained using the TGF-β receptor kinase inhibitor, we assessed invasiveness of MDA-MB231 cells following suppression of autocrine TGF-β signaling with a dominant-negative TGF-β type II receptor (TβRII). TβRII is the receptor responsible for ligand binding and for activation of TGF-β type I receptor through its kinase activity. TβRII devoid of the kinase domain (DNIIR) acts as a dominant-negative mutant by competing with wild-type receptors for TGF-β ligands (32). Expression of FLAG-tagged dominant-negative DNIIR was achieved using an adenoviral vector and was confirmed by immunoblotting for FLAG (Fig. 2A). DNIIR expression was increased with increasing amounts of adenovirus, whereas no DNIIR was detected in parental or the β-galactosidase adenovirus-infected cells. Expression of DNIIR inhibited TGF-β-stimulated Smad4

 tin expression (Fig. 1B). In addition, we evaluated the effect of LY364947 on TGF-β-induced promoter activation by reporter assays using p3TP-Lux, a luciferase reporter construct highly responsive to TGF-β (30) and observed that LY364947 significantly inhibited both basal and exogenous TGF-β-induced promoter activation (Fig. 1C). To investigate whether autocrine TGF-β has a role in regulation of MDA-MB-231 invasiveness, Matrigel invasion assays were performed with or without LY364947 treatment. LY364947 inhibited cell invasion in a dose-dependent manner (Fig. 1D). These data suggest that autocrine TGF-β plays a role in basal invasive growth of MDA-MB-231 cells.

FIGURE 3. Inhibition of TGF-β signaling decreases uPA but not MMP-9 protein levels in MDA-MB-231 conditioned media. A, down-regulation of uPA secretion by LY364947. Cells were treated with LY364947 at the indicated concentrations for 24 h. Conditioned media, cell lysates, and total RNAs were collected and subjected to immunoblotting for uPA and β-actin and Northern blotting for uPA and cyclophilin. B, time course of LY364947 inhibition on uPA secretion. Cells were treated with 5 μM LY364947 for 2, 4, 8, 16, and 24 h. Conditioned media were collected, concentrated (early time point media), and subjected to immunoblotting for uPA and β-actin and Northern blotting for uPA and cyclophilin. Cells were treated with 5 μM LY364947 or 5 ng/ml TGF-β for 24 h. Membrane, cytoplasmic, and nuclear fractions were isolated as described under "Materials and Methods" and subjected to immunoblotting for uPA, polyadenosine diphosphate ribose polymerase (a nuclear marker), Rho guanine nucleotide dissociation inhibitor (GDI) (a cytoplasmic marker), and β-actin. D, down-regulation of uPA secretion by the dominant-negative TGF-β type II receptor (DNIIR). Cells were infected with β-galactosidase (Ad-β-Gal) or DNIIR (Ad-DNIIR) adenoviruses at the indicated m.o.i. values. 48 h later, cells were cultured in fresh medium and allowed to grow for 16 h. Conditioned media, protein lysates, and total RNAs were harvested and subjected to immunoblotting for uPA and β-actin and Northern blotting for uPA and cyclophilin. E, the effect of LY364947 on pro-MMP-9 and active MMP-9 protein levels. Cells were treated with LY364947 at the indicated concentrations for 24 h. Conditioned media were collected and subjected to gelatin zymography.
Disruption of Autocrine TGF-β Signaling Suppresses uPA Secretion—The MDA-MB-231 cells secrete uPA (20). To address whether autocrine TGF-β can modulate uPA expression, uPA levels were determined after blockade of autocrine TGF-β signaling using LY364947. Immunoblotting for uPA was conducted under non-reducing conditions, which detect both uPA and complexes of uPA and its inhibitor, plasminogen activator inhibitor–1 (PAI-1) (33). The low motility bands with molecular masses around 100 kDa represent uPA/PAI-1 complexes (uPA ∼55 kDa and PAI-1 ∼52 kDa). Our results show that LY364947 decreased uPA secretion in a dose-dependent manner (Fig. 3A) and that the decreased level of free uPA in the conditioned medium did not result from increased uPA/PAI-1 association and depletion of uPA. The kinetics of the LY364947 effect was determined by a time-course experiment from 2 to 24 h after treatment with 5 μM LY364947. The result (Fig. 3B) shows that uPA secretion was inhibited starting 8 h after treatment. MDA-MB-231 cells constitutively express uPA receptor mRNA and exhibit detectable membrane-associated uPA (20). To determine whether the decreased level of released uPA after LY364947 treatment was the result of increased uPA receptor expression and increased uPA binding to uPA receptor, levels of membrane-bound uPA were determined after LY364947 treatment. The purity of the membrane fractions was confirmed by the absence of nuclear and cytoplasmic proteins, polyadenosine diphosphate ribose polymerase, and Rho GDI (guanine nucleotide dissociation inhibitor), respectively (Fig. 3C). Our results show that neither exogenous TGF-β treatment nor LY364947 treatment altered membrane uPA levels (Fig. 3C). Inhibition of autocrine TGF-β signaling by expression of DNIIR also suppressed uPA secretion in a dose-dependent fashion (Fig. 3D). In contrast, β-galactosidase adenovirus infection did not alter uPA secretion, further supporting that inhibition of autocrine TGF-β signaling suppresses uPA secretion.

Of note, expression of DNIIR was not detectable at an m.o.i. of 10 by immunoblotting (Fig. 2A); however, this low level of DNIIR expression was sufficient to decrease uPA secretion (Fig. 3D). These data demonstrate that disruption of autocrine TGF-β signaling inhibited uPA secretion.

Steady-state levels of uPA mRNA did not change with either LY364947 treatment or DNIIR expression (Fig. 3, A and D). Further, whereas uPA release into the medium was decreased, the intracellular uPA were increased after blockade of autocrine TGF-β signaling (Fig. 3, A and D), suggesting that autocrine TGF-β stimulates uPA secretion and that inhibition of autocrine TGF-β signaling reduces the level of secretion without impairing uPA production, thus leading to intracellular accumulation of uPA. We also examined uPA production in response to TGF-β in the Panc-1 human pancreatic cancer cell line and found that TGF-β treatment stimulated overall uPA production (free uPA plus uPA in complex with PAI-1), whereas LY364947 decreased overall uPA production with no change in uPA mRNA levels (Fig. 4A). In this cell line, total uPA levels also correlate with invasive activity (Fig. 4B). These data demonstrate that the TGF-β-regulated uPA secretion is not limited to a single cell line, and the Panc-1-produced TGF-β also regulated uPA production and invasion in an autocrine manner.

Inhibition of Autocrine TGF-β Signaling Does Not Affect MMP-9 Protein Levels and Activity—MDA-MB-231 cells express the MMP-9 protein (20) that is particularly important among at least 19 MMP proteins identified to date in tumor invasion and metastasis due to its ability to degrade the basement membrane component, type IV collagen. We determined MMP-9 protein levels and activity following abrogation of autocrine TGF-β signaling with LY364947. Gelatin zymography shows no change in both pro- (92 kDa) and active MMP-9 (84 kDa) protein levels after LY364947 treatment (Fig. 3E). These results indicate that autocrine TGF-β does not regulate MMP-9 activity or protein expression in the MDA-MB-231 cells. Consistent with previous reports (20), we did not detect MMP-2 (72 kDa) expression in MDA-MB-231 cells.

Inhibition of Basal uPA Activity Impairs MDA-MB-231 Cell Invasion—Inhibition of autocrine TGF-β signaling resulted in decreased invasiveness and uPA secretion as described above. We next investigated a relationship between uPA activity and MDA-MB-231 cell invasiveness. Inhibition of uPA activity using an anti-catalytic uPA blocking antibody attenuated cell invasion by ∼70% as compared with no treatment or IgG treatment in Matrigel invasion assays (Fig. 5A). These data suggest a correlation between uPA activity and MDA-MB-231 cell invasiveness. Parallel MTT assays suggest that the decreased cell invasion was not due to inhibition of cell proliferation (Fig. 5B). To test the hypothesis that uPA is the molecule that mediates the autocrine TGF-β pro-invasive effect, we examined whether addition of uPA in the medium can reverse the inhibitory effect
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of LY364947 on cell invasion. Our result shows LY364947 failed to decrease cell invasion in the presence of recombinant uPA (Fig. 5C), suggesting that uPA is the cellular molecule that mediates TGF-β-regulated cell invasion. Interestingly, addition of recombinant human PAI-1, an inhibitor of uPA, did not decrease basal cell invasion. As PAI-1 has been previously shown to have a positive role in cell attachment and invasion (34), and concomitant elevation of uPA and PAI-1 has been observed in cancer and linked to poor outcome (35), our data support the hypothesis that invasive potential can be up-regulated by increased uPA expression in conditions with elevated PAI-1 expression.

**Smad4 RNA Interference Decreases uPA Secretion**—TGF-β receptors transduce signals through both Smad and non-Smad pathways, including the extracellular signal-regulated kinases (ERK1/2) (7, 8), the p38 mitogen-activated protein kinase (9, 10), the phosphatidylinositol 3-kinase (PI3K) (12), and the Src (11) pathways. Whether autocrine TGF-β regulates these Smad-independent pathways was evaluated by immunoblotting. LY364947 treatment (up to 10 μM) did not significantly alter activation status of ERK1/2, p38, Akt, and Src proteins (Fig. 6A). In contrast, as little as 0.1 μM LY364947 suppressed uPA secretion (Fig. 6A), implicating that these pathways are probably not involved in autocrine TGF-β regulation of uPA secretion. We hypothesized that the Smad pathway mediates the autocrine TGF-β effect on uPA secretion. In this case, down-regulation of the common Smad mediator, Smad4, should suppress uPA release in response to autocrine TGF-β signaling. Smad4 silencing was achieved using a mixture of four Smad4 siRNAs. Immunoblotting results confirmed down-regulation of Smad4 protein expression in Smad4 siRNA-transfected cells but not in parental or scrambled control siRNA-transfected cells (Fig. 6B). Smad2 protein expression was unaffected by the Smad4 siRNA transfection, further confirming specific silencing of Smad4 protein expression by the Smad4 siRNAs. Interestingly, uPA secretion was also decreased by inhibition of Smad4 protein expression (Fig. 6B), suggesting that the Smad pathway is required for the regulation of uPA secretion by autocrine TGF-β. To further confirm the Smad4 role in uPA secretion, we tested whether forced expression of Smad4 can rescue the inhibitory effect of Smad4 silencing on uPA secretion. Fig. 6C shows that adenovirus-mediated overexpression of Smad4 prevented the inhibited uPA secretion induced by Smad4 silencing.

**Paracrine TGF-β Increases uPA mRNA Levels through RNA Stabilization**—Paracrine/exogenous TGF-β was previously shown to stimulate uPA expression and increased MDA-MB-231 cell invasiveness (20). We further examined uPA regulation by exogenous TGF-β. Unlike autocrine TGF-β, exogenous TGF-β increased both uPA mRNA and protein levels in a dose-dependent manner (Fig. 7A). A time-course study shows that uPA mRNA was induced by TGF-β in a time-dependent fashion to near maximal levels by 16 h after treatment, and the level was sustained for at least 48 h (Fig. 7B). Increased mRNA
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**FIGURE 7.** Paracrine TGF-β increases uPA mRNA levels and protein secretion in a dose- and time-dependent manner. A and B, cells were treated with increasing concentrations (0.1–10 ng/ml) of TGF-β for 24 h (A) or 5 ng/ml TGF-β for various intervals (B) as indicated. Conditioned media, lysates, and total RNAs were then harvested and subjected to immunoblotting for uPA and Northern blotting for uPA and cyclophilin, respectively.

expression is due to either increased transcription or increased RNA stability. The transcription of uPA in response to exogenous TGF-β was determined by a nuclear run-on assay. Exogenous TGF-β failed to enhance uPA transcription (Fig. 8A) but strongly stimulated transcription of PAI-1, a TGF-β target gene that has been shown to be transcriptionally activated by TGF-β (36). Consistent with the nuclear run-on result, transient expression assays using a uPA-promoter-reporter construct containing the nucleotide −2345 to +30 region of the human uPA promoter (phuPA-Luc) show very little changes in uPA promoter activity after TGF-β treatment (Fig. 8B). In contrast, uPA promoter activity was significantly induced by phorbol 12-myristate 13-acetate, a known inducer of activation of the human uPA promoter (37) (Fig. 8B). Next, the stability of uPA mRNA in the presence and absence of exogenous TGF-β was determined by examining uPA mRNA levels at various time points after blocking transcription with the RNA polymerase II-specific inhibitor DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole). Quantitation of the Northern blotting results (Fig. 8C) shows that by 8 h after DRB treatment, uPA mRNA levels were decreased by 50% in untreated cells. In contrast, uPA mRNA levels were only decreased by 25% in TGF-β-treated cells, suggesting that exogenous TGF-β enhances the stability of uPA mRNA. Thus, exogenous TGF-β increased uPA mRNA levels through mRNA stabilization in MDA-MB-231 cells.

**DISCUSSION**

TGF-β is a potent inhibitor of epithelial cell growth through inhibition of proliferation and induction of apoptosis (38, 39)
and is an important tumor suppressor (40). The tumor suppressor role of TGF-β is evident in that mice heterozygous for deletion of the TGF-β gene with expression of 10–30% of TGF-β levels of wild-type animals, developed an increased number of chemically induced tumors than did wild-type littermates (40). However, escape from the growth inhibitory effects of TGF-β occurs frequently in cancer through numerous mechanisms. Moreover, TGF-β displays tumor promoting effects in late-staged tumors. TGF-β has been shown to facilitate tumor progression by inducing epithelial to mesenchymal transition (41, 42), ECM degradation (20), and cyclooxygenase-2 expression (43) or by inhibiting anti-tumor immune responses (44).

Disruption of tumor autocrine TGF-β signaling has been found to delay tumor growth and inhibit metastases (13, 45). However, the molecular mechanisms underlying the tumorogenic effects of autocrine TGF-β remain unclear. The present study demonstrates that autocrine TGF-β regulates both cell invasiveness and uPA secretion. Inhibition of uPA activity decreases tumor cell invasion to the same extent as does the inhibition of autocrine TGF-β signaling. Interestingly, although autocrine TGF-β regulates uPA availability via effects on protein secretion, paracrine/exogenous TGF-β further increases uPA availability by increasing uPA mRNA through RNA stabilization. Our data suggest two distinct levels of regulation of uPA in response to different magnitudes of stimulation by TGF-β. The amount of active TGF-β in MDA-MB-231 cell-conditioned media has been quantitated at ~0.25 ng/ml (22, 23,
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46), whereas at least 10-fold higher concentrations were required to increase uPA mRNA levels (see Fig. 6A).

In the secretory pathway, proteins are transported between intracellular compartments via membranous vesicles that the process involves vesicle formation, motility, and docking, and membrane remodeling and fusion (47, 48). Numerous proteins have been implicated in regulation of vesicle trafficking during protein secretion. For example, members of Ypt/Rabs proteins of the Ras GTPase superfamily have been characterized to be key regulators of protein transport (49–51). A negative role for Rab3, a member of the Ypt/Rab family, in protein secretion has been previously shown by a number of studies (reviewed in Ref. 52). This function of Rab3 suggests that depletion of Rab3 may lead to enhanced protein secretion. Recently, one of the biological functions of TGF-β has been shown to be facilitating ubiquitin-dependent degradation of protein in a Smad-dependent manner (53). These observations raise the possibility that TGF-β signaling may modulate uPA protein secretion by depleting negative regulators of protein secretion through facilitated protein degradation.

Most of TGF-β activities involve modulation of gene transcription. However, TGF-β has been shown to substantially increase stability of mRNAs such as α1(I) collagen mRNA in human hepatic stellate cells (54) and elastin mRNA in human fetal lung fibroblasts (55). In both cases, activation of the p38 MAPK signaling pathway is required for the TGF-β effect. Interestingly, expression of inhibitory Smad7, an inhibitor of TGF-β signaling but not Smad6, an inhibitor of bone morphogenetic protein action blocked TGF-β signaling and dramatically diminished the TGF-β-stabilizing effect on elastin mRNA (55). These results suggest the involvement of both Smad and p38 MAPK pathways in the TGF-β-mediated stabilization of elastin mRNA. In our study, exogenous TGF-β stabilized uPA mRNA in a Smad4-dependent manner. Exogenous TGF-β also induced activation/phosphorylation of p38 MAPK (data not shown) in the same cell context. Therefore, it is conceivable that the p38 signaling pathway may participate in the process of TGF-β regulation of uPA mRNA stability in conjunction with the Smad pathway.

Stabilization of mRNA involves the binding of RNA proteins to certain cis-elements of mRNAs (56). Adenylate-uridylate-rich elements are important regulatory cis-elements present in the untranslated regions of short-lived mRNAs such as proto-oncogenes, cyclooxygenase-2 (57), and c-fos (58) mRNAs. TGF-β and Ras have been shown to synergistically stabilize the COX-2 mRNA through an adenylate-uridylate-rich element in the proximal 3′-untranslated regions (57). Given that MDA-MB-231 cells possess an activating K-Ras mutation (59) and that adenylate-uridylate-rich elements are present in the 3′-untranslated region of the uPA mRNA (60), it is possible that TGF-β may cooperate with the active Ras to regulate the stability of uPA mRNA through a similar mechanism, but confirmation of this awaits further investigation.

Cells release uPA as a single-chain zymogen. The low level of intrinsic proteolytic activity of pro-uPA (61) can convert the plasminogen in tumor microenvironments or Matrigel (62) to plasmin, which in turn activates pro-uPA. This pro-uPA activation by plasmin and activation of plasminogen by uPA in a cyclic fashion promotes degradation of the ECM or the basement membrane and facilitates cell invasion. In addition, plasmin can potentially activate MMPs (15, 63, 64) thereby promoting ECM degradation and tumor cell invasion (65). However, we did not find evidence for up-regulation of MMP-9 activity or expression by TGF-β in the MDA-MB-231 cells under the conditions studied.

TGF-β is released from cells mostly in a latent, inactive form via a constitutive secretion pathway (66). Despite the predominance of latent TGF-β in conditioned media in general, MDA-MB-231 cells express detectable active TGF-β (46). It is interesting that uPA can proteolytically activate latent TGF-β (67). Therefore, TGF-β regulation of uPA production may be a positive feedback loop for activation of latent TGF-β (Fig. 8), and this relationship can be a cycle in cancer progression because TGF-β is overexpressed in both malignant breast tumors and surrounding stroma (68, 69), and uPA expression is increased in human breast carcinomas and bone metastases (70). It will be of interest to determine whether the basal level of uPA secretion contributes to the availability of active TGF-β and autocrine TGF-β signaling.

The plasminogen activator inhibitor (PAI-1) is a TGF-β target gene and a strong inhibitor of uPA. The present study demonstrates that TGF-β increases levels of both uPA and PAI-1 in the MDA-MB-231 conditioned media. Interestingly, despite the fact that PAI-1 inhibits uPA activity, concomitant elevation of uPA and PAI-1 has been observed in breast cancer and is associated with poor outcome (35), suggesting that tumor progression may occur in the presence of high levels of PAI-1. TGF-β has been shown to stimulate attachment and invasion through up-regulation of PAI-1 (34), and these effects may contribute to cell invasion. Our findings suggest that, although PAI-1 is induced by TGF-β, this induction is not sufficient to prevent the invasion promoting effect of uPA.

In summary, autocrine TGF-β regulates cell invasiveness through maintaining uPA levels by facilitated protein secretion, whereas paracrine/exogenous TGF-β further increases invasiveness through stimulated uPA expression by RNA stabilization. The Smad pathway appears to be required for the distinct levels of regulation of uPA in response to different magnitudes of TGF-β stimulation.

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