Collagen I Promotes Epithelial-to-Mesenchymal Transition in Lung Cancer Cells via Transforming Growth Factor–β Signaling

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Epithelial-to-mesenchymal transition (EMT) is a fundamental biological process whereby epithelial cells lose their polarity and undergo a transition to a mesenchymal phenotype. When cancer cells invade adjacent tissues, they use a mechanism akin to EMT, and understanding the molecular mechanisms that drive this transition will facilitate studies into new targets for prevention of metastasis. Extracellular stimuli, such as growth factors, and their cytosolic effectors cooperate to promote EMT. In highly fibrotic cancers like lung cancer, it is thought that extracellular matrix molecules, including collagen, can initiate signals that promote EMT. Here, we present data showing that collagen I induces EMT in non–small cell lung cancer cell lines, which is prevented by blocking transforming growth factor (TGF)-β3 signaling. In addition, we show that collagen I–induced EMT is prevented by inhibitors of phosphoinositide 3-kinase and extracellular signal-related kinase signaling, which promotes transcription of TGF-β3 mRNA in these cells. Thus, our data are consistent with the hypothesis that collagen I induces EMT in lung cancer cells by activating autocrine TGF-β3 signaling. Epidermal growth factor also seems to initiate EMT via a TGF-dependent mechanism.

Keywords: non–small cell lung cancer; epithelial-to-mesenchymal transition; cadherin switching; collagen I; transforming growth factor–β

CLINICAL RELEVANCE

We show that collagen I induces epithelial-to-mesenchymal transition in lung cancer by autocrine activation of transforming growth factor (TGF)-β and that PI3K and ERK are necessary for TGF-β up-regulation. These studies provide the foundation for the development of new treatments for invasive lung cancers.

Lung cancer is the leading cause of cancer death in the United States, with 80% of patients with lung cancer dying of their disease (1). Treatments such as surgery, chemotherapy, and radiation provide only limited palliation, and there has not been a meaningful improvement in survival of patients with lung cancer in the past 20 years (2). A continuing problem in the management of lung cancer is metastatic disease, pointing out the importance of gaining a better understanding of the biological changes that occur in tumor cells to promote the aggressive neoplastic phenotype. Characterizing the molecular pathways that are activated in metastatic cells may lead to the development of more effective therapeutic modalities (3).

Epithelial-to-mesenchymal transition (EMT) is a fundamental biological process whereby epithelial cells lose their polarity and undergo a transition to a mesenchymal phenotype (4). Hallmarks of EMT include loss of cell–cell adhesion, reorganization of the actin cytoskeleton, and acquisition of increased migratory characteristics (5). E-cadherin is a transmembrane glycoprotein that mediates cell–cell adhesion and plays an important role in maintaining the normal polarized epithelial phenotype (6). E-cadherin is critical in normal development, and alterations in its function have been implicated in tumorigenesis (5). It has been well established that E-cadherin functions as a tumor suppressor, and its loss is associated with poor prognosis in lung cancer (3, 7). Studies from our laboratory and others have shown that expression of an inappropriate mesenchymal cadherin in epithelial cells is another way that tumor cells can alter their adhesive function (8–10). We previously showed that cadherin switching (loss of E-cadherin expression or function and increased expression of other cadherins) is necessary for the increased cell motility that accompanies EMT (11). Furthermore, it has been proposed that, when cancer cells invade adjacent tissues or metastasize, they use a mechanism akin to EMT. Thus, it is important to understand the molecular mechanisms that drive EMT if we are to identify new targets for the prevention of metastasis (12).

EMT can be initiated by external signals, such as transforming growth factor (TGF)-β, hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) (13, 14). Recent studies from our lab and others have shown that interactions with extracellular matrix molecules, such as collagen type I (collagen I), can induce EMT in some cell types (15–17). The mechanisms that govern EMT are being unraveled, and many effectors of EMT are known to modulate adhesion systems, remodel the actin cytoskeleton, and promote the mesenchymal phenotype. For example, during TGF-β–mediated EMT, the TGF-β receptors I and II form a tight complex that leads to phosphorylation of Smad2 and Smad3 (18). Phosphorylated Smads partner with cytosolic Smad4 and translocate to the nucleus where they modulate transcription of target genes. On the other hand, receptor tyrosine kinases, such as HGFR, FGFR, and EGFR, act through Ras and the mitogen-activated protein kinase pathway or the phophoinositide 3- kinase (PI3K) pathway to induce EMT (19). The transcription factors Twist, Snail, and SIP1 play key roles in EMT downstream of growth factor receptor signaling (20). We recently reported that the Jun N–terminal kinase (JNK) pathway plays a key role in collagen I–induced EMT in human pancreatic cancer cells and in mouse and human mammary cancer cells (15, 16).

Prompted by these studies, new approaches to pharmacologic inhibition of EMT have been developed to curb tumor

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progression. For example, the EGF receptor family of receptor tyrosine kinases is a well studied, attractive target for antitumor strategies (21). Expression of high levels of EGF receptor and/or its family members, HER2/erbB2, has been associated with poor prognosis in patients with non–small cell lung cancer (NSCLC), and receptor tyrosine kinase inhibitors, such as gefitinib and erlotinib, are used to treat these patients. Phase II clinical trials demonstrated that gefitinib, as a single agent, produced significant clinical responses in only a small percentage of patients (22). A recent paper by Engelman and colleagues showed that in some of these failed clinical responses, the tumor cells activate the same intracellular pathways that are activated by the EGF receptor via a different route (23). Stimuli from outside the cell and their cytosolic effectors cooperate to induce EMT. The failure of inhibitors of surface receptors to produce a complete clinical response and the ability of the tumor cells to activate signaling pathways through alternative mechanisms highlight the need to further delineate the downstream effectors of EMT signaling.

In the present study, we investigated the signaling pathways that promote EMT in human lung adenocarcinoma cells. We found that extracellular stimuli, such as TGF-β, EGF, and collagen I, induced EMT in the NSCLC cell lines A549, NCI-H358, and NCI-H1299. The onset of EMT was partially or completely prevented by blocking TGF-β3 signaling, indicating that each stimulus induced EMT through a TGF-β–dependent mechanism and suggesting that collagen I promotes autocrine TGF-β3 signaling in these cells.

MATERIALS AND METHODS

Reagents, Antibodies, and Cultured Cells

All reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Chemicals (Fair Lawn, NJ) unless otherwise indicated. A549, NCI-H358, and NCI-H1299 cells were kindly provided by Dr. Stephen Rennard (UNMC) and maintained in Ham's F12K medium or RPMI 1640 with 10% FBS. Mouse mAb against E-cadherin was a kind gift from M. Takeichi (RIKEN). Mouse mAb against N-cadherin (13A9) has been described (11). Antitubulin mouse mAb was from the Developmental Studies Hybridoma Bank (University of Iowa). The 4A6 (anti-birch profilin tag) mAb was kindly provided by Dr. Manfred Rudiger (Zoological Institute, Braunschweig, Germany). Anti-Smad2/3 mouse mAb, rat tail collagen I, and substrate-coated dishes were from BD Biosciences (Bedford, MA). Antiphospho-Smad2 (Ser465/Ser467) rabbit pAb was from Cell Signaling Technology (Beverly, MA). Rabbit antipan TGF-β (catalog number AB-100-NA, lot number E015), goat inhibitory anti–TGF-β3 (catalog number AB-244-NA, lot number CK07), recombinant TGF-β1, and bovine fibronectin were from R&D Systems (Minneapolis, MN). ECM-coated substrates were prepared as described previously (16). DMSO, TGF-β1, HGF, EGF, and FGF-2 were added to the media at the indicated concentrations 1 day after seeding cells onto noncoated dishes with 1% serum containing culture media. The inhibitors SU6656, LY294002, SB203580, PD98059 (Calbiochem), and SP600125 (Biomol) were added at the time of plating the cells at the indicated concentrations.

Detergent Extraction, SDS-PAGE, and Immunoblot

Monolayers of cultured cells were washed with ice-cold PBS and extracted on ice with RIPA buffer (50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin). Cell extracts were resolved by SDS-PAGE and immunoblotted as described (11) and quantified by densitometry using AdobePhotoshop.

Conventional RT-PCR and Quantitative Real-Time RT-PCR

Total RNA was extracted with TRI REAGENT and analyzed by RT-PCR using a TITANIUM One-Step RT-PCR kit (BD Biosciences Clontech) and forward and reverse primers as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGGTATCGTGGAAGGACTCATGAC-3′ and 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′; N-cadherin, TGF-β1, TGF-β2, and TGF-β3 primers were previously reported (24, 25). The conditions for PCR were as follows: 94°C for 45 seconds, 60°C for 30 seconds, and 72°C for 90 seconds for 35 cycles. For quantitative real-time RT-PCR, total RNA was analyzed in an Mx3000P Real-Time PCR System (Stratagene) using the following PCR protocol: 50°C for 30 minutes and 95°C for 10 minutes followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Combinations of primers and probes for TGF-β1, TGF-β3, and GAPDH (control) were from Applied Biosystems, and a reaction mixture was made using Brilliant Probe-Based QRT-PCR Reagents (Stratagene).

Immunofluoresence Microscopy

Cells were fixed with 3.7% formaldehyde for 15 minutes, permeabilized with 0.2% Triton X-100 in PBS for 15 minutes, processed as described (16), and examined on a Zeiss Axiovert 200M microscope (Gottingen, Germany) equipped with an ORCA-ER digital camera (Hamamatsu, Hamamatsu City, Japan). Images were collected and processed using SlideBook software (Intelligent Imaging Innovations, Denver, Co).

Constructs, Transfections, and Infection

cDNA for mouse Smad7 was generated using RT-PCR and total RNA from NMuMG/E9 cells. The PCR product was completely sequenced to ensure there were no mutations. The forward primer (ATGTTCAGGACCAAACGATCTG) and the reverse primer (CTACCGGCTGTTGAAGATGAC) were identical to nucleotides 1592 to 1612 (forward) and 2852 to 2872 (reverse) of mouse Smad7 (GenBank accession number NM_001042660). Smad7 was modified at its N-terminus with two birch profilin epitope tags (26) and subcloned into LZRS-MS-Neo (16). Infected cells were selected with 1 mg/ml G418 (Cellgro; Mediatech, Herndon, VA) as described (11).

RESULTS

A549 Undergo EMT in Response to TGF-β, EGF, and Collagen I

As previously reported (27), A549 human adenocarcinoma cells were converted from an epithelial phenotype to a fibroblastic phenotype in response to TGF-β1 (Figure 1A, compare *panel a* with *panel b*). Mesenchymal markers, including vimentin, smooth muscle actin, fibronectin, and N-cadherin, were up-regulated by TGF-β1 treatment, whereas E-cadherin was down-regulated (Figure 1B, compare *lane 2* with *lane 1*). In agreement with the Hunter lab (28), EGF induced a morphological change in A549 cells (Figure 1A, *panel d*); up-regulation of N-cadherin, fibronectin, and vimentin expression (Figure 1B, *lane 4*); and down-regulation of E-cadherin (Figure 1B, compare *lane 4* with *lane 1*). In contrast, HGF and FGF produced only slight morphological changes and did not produce changes in protein expression that are typical of EMT. Consistent with an epithelial-to-mesenchymal response, A549 cells treated with TGF-β1 or EGF decreased cell surface localization of E-cadherin (Figure 1C, *panels g* and *i*). A549 cell morphology is slightly mesenchymal, so the morphological changes induced by growth factors are subtle. The two characteristics we are most interested in are elongation, which is evident in cells treated with TGF-β, and cell scattering, which is evident in cells treated with EGF. EMT involves the activation of a specific gene expression program, resulting in dramatic changes in differentiation that are critical for normal developmental processes. Typically, tumor cells in culture only partially undergo EMT, which is evidenced by changes in cell morphology, cell behavior, and expression of protein markers (13). Thus, here we are not observing true EMT in the strictest sense, but the changes we see in cultured tumor cells in response to growth factors has been termed "EMT" in the literature, and we refer to it as EMT in this article.

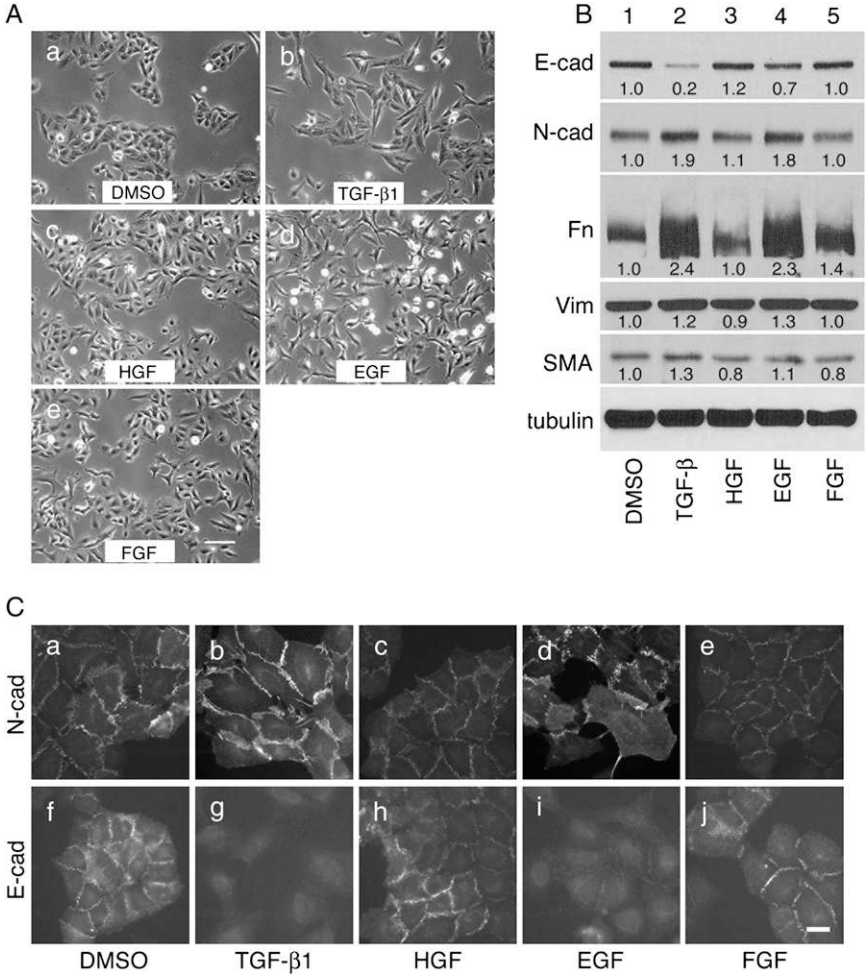

Figure 1. A549 cells undergo epithelial-to-mesenchymal transition (EMT). (*A*) A549 cells were treated with DMSO as a control (*a*), transforming growth factor (TGF)-β1 (2 ng/ml) (*b*), hepatocyte growth factor (HGF) (5 μg/ml) (*c*), epidermal growth factor (EGF) (100 ng/ml) (*d*), and fibroblast growth factor (FGF)-2 (100 ng/ml) (*e*) for 2 days. Phase-contrast pictures were taken using a 10× objective. Bar = 100 μm. (*B*) Cells treated with each growth factor for 2 days were extracted, and 30 μg protein was resolved by SDS-PAGE and immunoblotted for E-cadherin (E-cad), N-cadherin (N-cad), fibronectin (Fn), vimentin (Vim), smooth muscle actin (SMA), and tubulin (as a control). Immunoblots were quantified, and relative expression to control is indicated. (*C*) A549 cells were stained for N-cad (*a–e*) or E-cad (*f–j*) after treatment with DMSO (*a* and *f*), TGF-β1 (2 ng/ml, *b* and *g*), HGF (5 μg/ml, *c* and *h*), EGF (100 ng/ml, *d* and *i*), or FGF-2 (100 ng/ml, *e* and *j*) for 2 days. Photographs were taken using a 40× oil objective. *Scale bar*, 20 μm.

Our lab is interested in the role of collagen I in promoting EMT, particularly in tumors hallmarked by the fibrotic response, such as lung adenocarcinomas. Pancreatic and breast cancers also tend to be highly fibrotic tumors, and we have previously shown that plating pancreatic cancer cells or mammary cancer cells on collagen I induces EMT, whereas plating these same cells on other extracellular matrices does not (15, 16). Therefore, we asked whether plating A549 lung adenocarcinoma cells on collagen I induces EMT. Figure 2A shows that A549 underwent morphological changes typical of EMT when they were plated on collagen I–coated dishes (*panel b*) but not when they were plated on uncoated dishes (*panel a*) or fibronectin-coated dishes (*panel c*). The morphological changes are subtle, but the most prominent feature is increased scattering of the cells. Furthermore, immunoblots showed that mesenchymal markers, including N-cadherin, vimentin, smooth muscle actin, and fibronectin, were up-regulated when the cells were plated on collagen I, whereas E-cadherin was down-regulated (Figure 3C). In agreement with the biochemical data, cell border staining for E-cadherin was barely detectible in cells plated on collagen I (*see* Figure 2B). Thus, like the growth factors TGF-β and EGF, collagen type I also was capable of inducing an EMT-like response in A549 cells.

Collagen I–Induced EMT Is Dependent on TGF-β Signaling

To investigate the mechanism whereby interaction of A549 cells with collagen I might induce EMT, we asked if plating these cells on collagen I activated the TGF-β pathway because exogenous TGF-β1 produced a similar response. When we overexpressed Smad 7, which blocks the TGF-β pathway downstream of the receptor, in A549 cells, phosphorylation of Smads2/3 in response to plating cells on collagen I was decreased (Figure 3A, *panel a*). Furthermore, overexpression of Smad7 prevented collagen I–mediated cadherin switching (Figure 3A, *panel b*). Blocking TGF-β signaling by expressing Smad7 decreased scattering of A549 cells in response to collagen I (Figure 3B, compare *panel d* with *panel b*).

One mechanism whereby inhibiting the TGF-β pathway could prevent collagen I–mediated EMT would be if plating A549 cells on collagen I induced the cells to increase their expression of TGF-β and the endogenous TGF-β then induced EMT. To determine whether the cells up-regulated TGF-β in response to plating on collagen I, we examined mRNA levels for TGF-β1, TGF-β2, and TGF-β3. TGF-β3 was up-regulated when cells were cultured on collagen I, whereas there was no obvious change in the expression levels of TGF-β1 or TGF-β2 (Figure 3C).

To directly examine the role of TGF-β in collagen I–induced cellular changes, we used neutralizing antibodies that inhibit all TGF-β isoforms or neutralizing antibodies that specifically inhibit TGF-β3. Both of these antibodies decreased scattering of A549 cells in response to collagen I, whereas nonspecific rabbit IgG did not (Figure 3D, compare *panel b* with *panels d* or *f*). TGF-β3–specific neutralizing antibody prevented E-cadherin down-regulation in response to collagen I (Figure 3E). These data suggest that TGF-β signaling, especially TGF-β3, is responsible for collagen I–induced EMT.

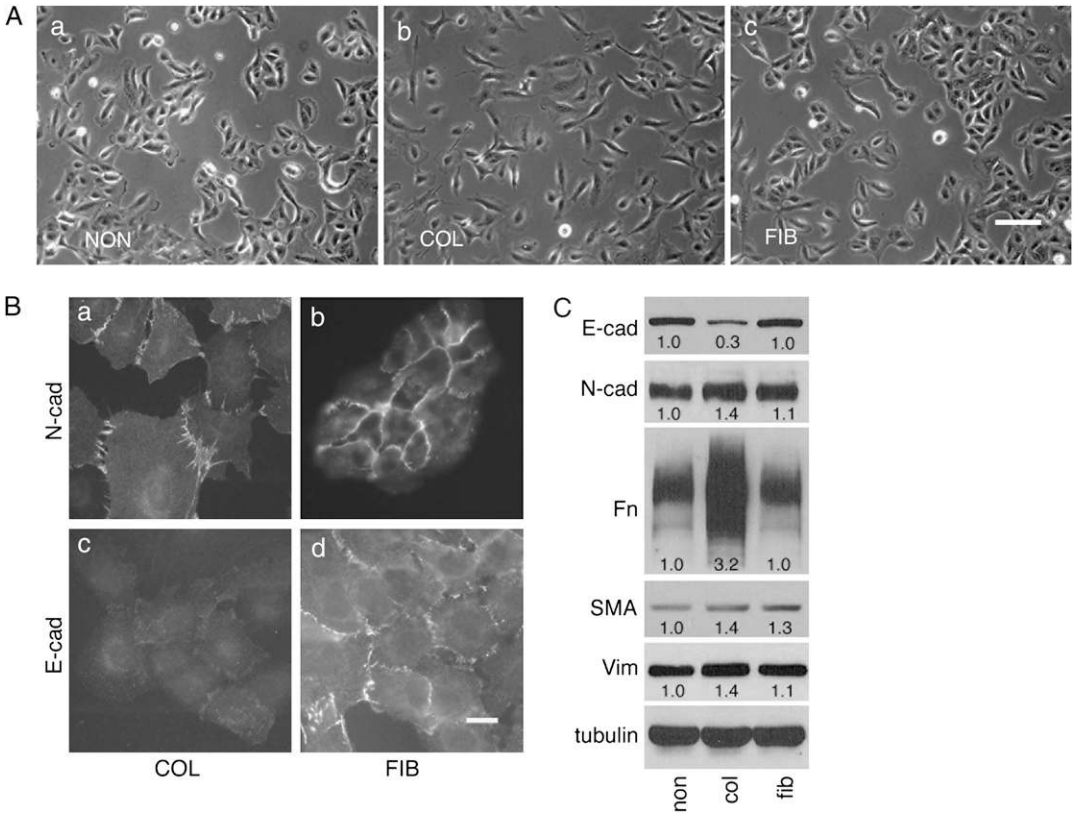

Figure 2. A549 cells undergo EMT in response to collagen type I. (*A*) A549 cells were cultured on noncoated (NON, *a*), collagen I–coated (COL, *b*), or fibronectin-coated (FIB, *c*) dishes. Phase-contrast pictures were taken 2 days after seeding using a 10× objective. Bar, 100 μm. (*B*) Immunofluorescence photographs of A549 cells cultured on substrate for 2 days and stained for N-cadherin (N-cad, *a* and *b*) or E-cadherin (E-cad; c and d) were taken using a 40× oil objective. Bar, 20 μm. (*C*) Cells cultured on each substrate for 2 days were extracted, and 30 μg protein was resolved by SDS-PAGE and immunoblotted for E-cad, N-cad, fibronectin (Fn), smooth muscle actin (SMA), vimentin (Vim), and tubulin (as a control). Immunoblots were quantified, and relative expression to control is indicated.

Biologically Active TGF-β3 Is Secreted by A549 Cells in Response to Collagen I

To determine if biologically active TGF-β3 was secreted by A549 cells in response to collagen I, we examined conditioned media from A549 cells cultured on collagen I for its ability to induce EMT in cells plated on noncoated dishes. We plated A549 cells on collagen I–coated dishes or noncoated dishes for 2 days and collected the medium. We then plated A549 cells on noncoated dishes for 2 days in the conditioned medium. Conditioned medium from cells plated on noncoated dishes did not induce EMT in A549 cells, whereas conditioned medium from cells plated on collagen I induced cadherin switching (Figure 4A, compare *lane 2* with *lane 1*) and cell scattering (Figure 4B, compare *panel c* with *panel b*). These data do not address the possibility that, rather than containing active TGF-β, the conditioned medium contains a factor that induces autocrine production/activation of TGF-β by the target cells. To rule out the possibility that the collagen I used for coating the dishes was contaminated with TGF-β, we collected medium from collagen I–coated dishes that did not have cells plated on them. When we used this conditioned medium to treat cells plated on noncoated dishes, they did not show cadherin switching (Figure 4A, *lane 4*), indicating that the collagen I used to coat the dishes was not contaminated with TGF-β and that TGF-β was produced by cells plated on collagen I. This experiment does not rule out the unlikely possibility that the collagen preparation has insoluble TGF-β that is deposited on the plate. EMT induced by conditioned medium was blocked by pan TGF-β neutralizing antibody and by TGF-β3–specific neutralizing antibody (Figures 4C and 4D). These data indicate that TGF-β3 is secreted by A549 cells into the conditioned media in response to collagen I, and this TGF-β3 is capable of inducing EMT in A549 cells plated on noncoated dishes.

Autocrine TGF-β3 Induced EMT in Other Lung Cancer Cells

To determine if other NSCLC cells undergo EMT in a manner similar to A549 cells, we plated NCI-H358 and NCI-H1299 cells on collagen I or treated them with TGF-β1. NCI-H358 cells were more or less cuboidal when plated on noncoated dishes and became elongated in response to TGF-β1 (Figure 5A, compare *panel c* with *panel a*). The cells also became elongated in response to plating on collagen I, although the difference in cell morphology was slight. Like the A549 cells, NCI-H358 cells are somewhat mesenchymal even without treatment with EMT inducers, so the morphological changes are subtle. However, the biochemical changes, which are easier to quantify, showed that these cells increased N-cadherin and vimentin expression in response to collagen I and TGF-β1 (Figure 5B). NCI-H1299 cells became flat and scattered in response to collagen I and TGF-β1 (Figure 5A, compare *panels e* and *f* with *panel d*). These cells are elongated even in the absence of inducers of EMT, so the biochemical data are more indicative of an EMT response. Figure 5C shows that vimentin and N-cadherin were up-regulated in response to collagen I or TGF-β1.

RT-PCR showed that TGF-β3 mRNA was increased in response to collagen I in both cell lines, whereas TGF-β1 or TGF-β2 mRNA levels were not changed (Figure 5D). Furthermore, overexpression of Smad7 prevented collagen I–mediated N-cadherin up-regulation in NCI-H1299 cells (Figure 5E). Similar to A549 cells, conditioned media from NCI-H1299 cells plated on collagen I–coated dishes induced N-cadherin up-regulation in NCI-H1299 cells, which was blocked by TGF-β or TGF-β3 neutralizing antibodies (Figure 5F). Together, these

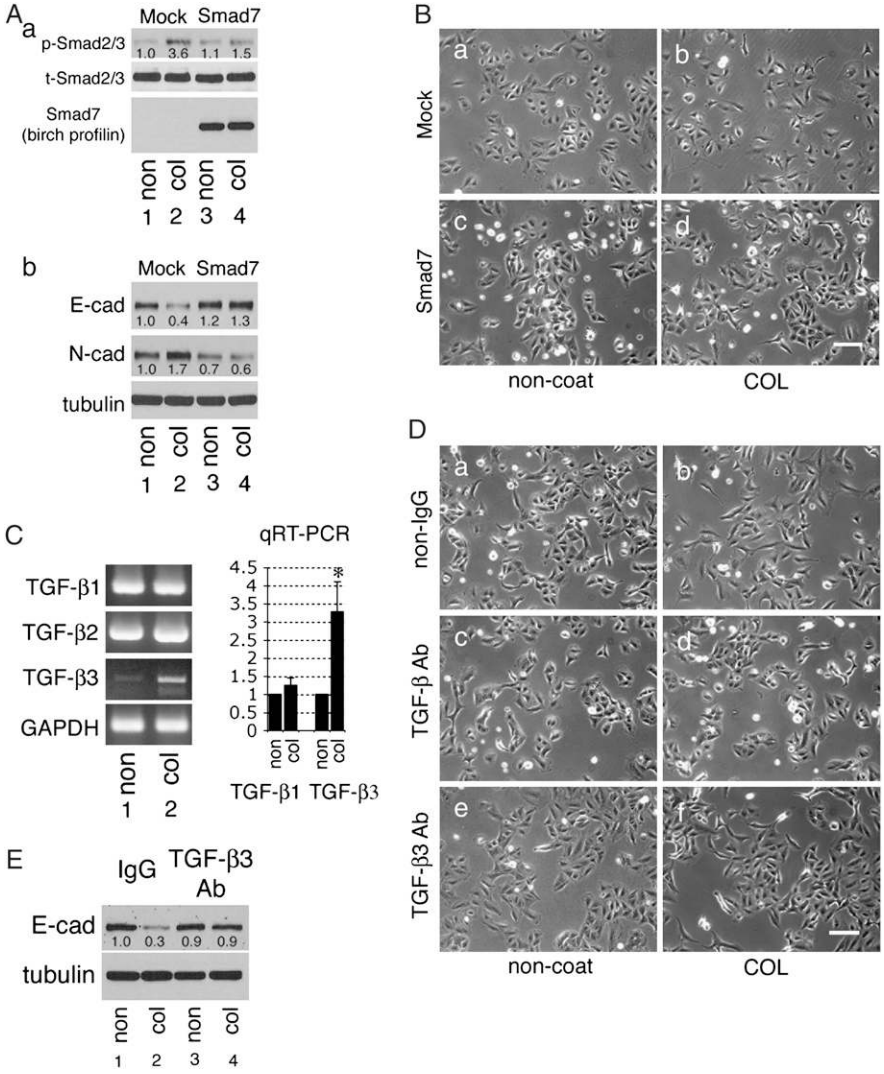

Figure 3. Collagen I–induced EMT is dependent on TGF-β signaling. (*A*) (*a*) Neomycin-resistance gene (Mock) or Smad7-infected A549 cells were plated onto noncoated (non) or collagen I–coated (col) dishes. Four hours after seeding, cells were extracted, and 60 μg protein was resolved by SDS-PAGE and immunoblotted for phospho-Smad2/3 (p-Smad2/3), total Smad2/3 (t-Smad2/3), and Smad7 (anti-tag). (*B*) Cells cultured for 2 days were extracted, and 30 μg protein was resolved by SDS-PAGE and immunoblotted for E-cadherin (E-cad), N-cadherin (N-cad), and tubulin. (*B*) Mock or Smad7 overexpressing A549 cells were plated on noncoated (*a* and *c*) or collagen I–coated (COL, *b* and *d*) dishes for 2 days. Phase-contrast pictures were taken using a 10× objective. Bar = 100 μm. (*C*) One day after seeding onto noncoated or collagen I–coated dishes, total RNA was extracted, and RT-PCR was done for TGF-β1, TGF-β2, TGF-β3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as a control). Quantitative RT-PCR was performed for TGF-β1 and TGF-β3 as described in Materials and Methods. (*D*) A549 cells were plated on noncoated (*a*, *c*, and *e*) or collagen I–coated (COL, *b*, *d*, and *f*) dishes for 2 days in the presence of nonspecific rabbit IgG (non-IgG, 10 μg/ml, as a control; *a* and *b*), pan-neutralizing rabbit Ab against TGF-β (TGF-β Ab, 10 μg/ml; *c* and *d*), or neutralizing rabbit Ab specific for TGF-β3 (TGF-β3 Ab, 10 μg/ml; *e* and *f*). Phase-contrast pictures were taken using a 10× objective. *Bar* = 100 μm. (*E*) A549 cells were plated onto noncoated (non) or collagen I–coated (col) dishes for 2 days in the presence of nonspecific rabbit IgG (10 μg/ml, IgG) or neutralizing rabbit Ab specific for TGF-β3 (TGF-β3 pAb, 10 μg/ml), extracted, and immunobloted for E-cadherin (E-cad) and tubulin.

data suggest that TGF-β3 plays a crucial role in collagen I–induced EMT in NSCLC cells.

PI3K and ERK Are Downstream Mediators of Collagen I–Induced EMT in A549 Cells

To examine the potential pathways downstream of plating A549 cells on collagen I that might be responsible for up-regulating expression of TGF-β3, we treated cells with inhibitors of Src family kinases (SU6656), PI3K (LY294002), extracellular signal–related kinase (ERK; PD98059), JNK (SP600125), or p38MAPK (SB203580) and plated them on noncoated or collagen I–coated dishes. Collagen I–induced morphological changes were prevented by LY294002 (Figure 6A, *panel f*) and by PD98059 (Figure 6A, *panel h*) but not by the other inhibitors. E-cadherin down-regulation in response to collagen I was inhibited by LY294002 (Figure 6B, *lanes 5* and *6*) and by PD98059 (Figure 6B, *lanes 7* and *8*) but not by the other inhibitors. Quantitative RT-PCR showed that up-regulation of TGF-β3 mRNA was prevented by LY294002 and PD98059, whereas TGF-β1 mRNA levels were not changed (Figure 6C). These data suggest that transcription of the TGF-β3 gene is regulated by the PI3K and ERK pathways and that plating cells on collagen I activates these pathways, which promotes transcription of TGF-β3.

Inhibiting PI3K or ERK increased the epithelial characteristics of the cells on noncoated dishes, whereas inhibiting JNK or Src resulted in a scattered morphology even on noncoated dishes, suggesting that each of these pathways plays a role in junction organization in SCLCS cells. To rule out the possibility that any of the pathways mentioned previously were downstream of TGF-β in inducing EMT, we plated A549 cells on noncoated plastic dishes and treated them with or without TGF-β in the presence of the inhibitors mentioned previously. None of the inhibitors, including the PI3K inhibitor (LY294002) and the ERK inhibitor (PD98059), was capable of preventing TGF-β–induced morphological changes in response to TGF-β (data not shown). In addition, none of the inhibitors was capable of preventing the down-regulation of E-cadherin expression in response to TGF-β (*see* Figure E1 in the online supplement). These data are consistent with the idea that plating A549 cells on collagen I induces transcription and autocrine secretion of TGF-β3, which is mediated by PI3K and ERK signaling.

EGF-Induced EMT in A549 Cells Is Mediated by Autocrine TGF-β Signaling

Because EGF induces EMT in A549 cells (*see* Figure 1A, *panel d*), we tested whether EGF-induced EMT in these cells was also due to autocrine TGF-β signaling. Figure E2A in the online

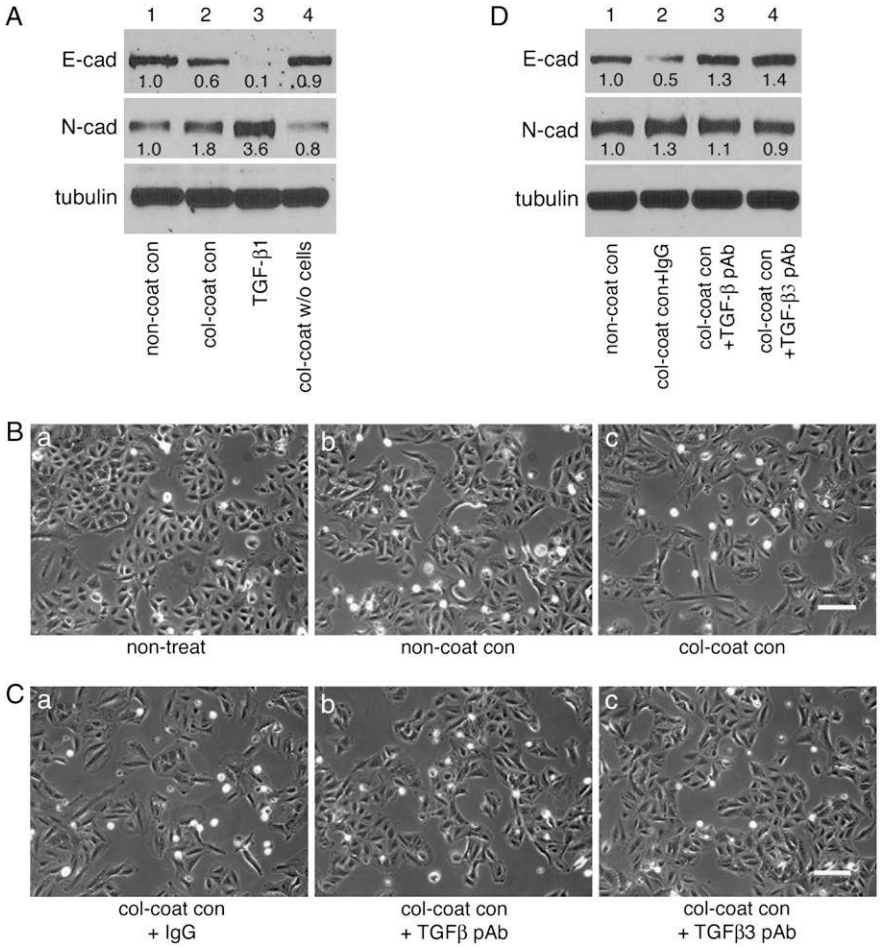

Figure 4. Autocrine TGF-β3 is biologically active and necessary for collagen-mediated changes in A549 cells. (*A*) Conditioned medium was collected from cultures of A549 cells grown for 2 days on noncoated or collagen I–coated dishes. Fresh A549 cells were then plated on noncoated dishes for 2 days in the conditioned media from cells plated onto noncoated (*lane 1*) or collagen I–coated (*lane 2*) dishes, extracted, and immunoblotted for E-cadherin (E-cad), N-cadherin (N-cad), and tubulin (as a control). Extracts from cells treated for 2 days with TGF-β1 (2 ng/ml) were used as a control for EMT (*lane 3*). Extracts from cells treated with conditioned media from cell-free collagen I–coated dishes were immunoblotted to rule out contamination of the collagen I with TGF-β (*lane 4*). (*B*) A549 cells plated on noncoated dishes were treated for 2 days with 1% serum containing media (non-treat, *a*), conditioned media from cells cultured on noncoated (non-coat con, *b*), or collagen I–coated (col-coat con, *c*) dishes for 2 days. (*C*) Cells were treated with conditioned media from cells cultured on collagen I–coated dishes for 2 days in the presence of nonspecific rabbit IgG (col-coat con+IgG, 10 μg/ml; *a*), neutralizing rabbit Ab against TGF-β (col-coat con+TGFβ pAb, 10 μg/ml; *b*), or neutralizing rabbit Ab specific for TGF-β3 (col-coat con+TGFβ3 pAb, 10 μg/ml; *c*). Phase-contrast pictures were taken using a 10× objective. Bar = 100 μm. (*D*) Cells treated for 2 days with conditioned media from cells plated onto noncoated (*lane 1*) or collagen I–coated (*lane 2*) dishes for 2 days were extracted and immunoblotted for E-cadherin (E-cad), N-cadherin (N-cad), and tubulin (as a control). Cells treated with conditioned media from cells cultured on collagen I–coated dishes for 2 days in the presence of neutralizing rabbit Ab against TGF-β (col-coat con+TGFβ pAb, 10 μg/ml; *lane 3*) or neutralizing rabbit Ab specific for TGF-β3 (col-coat con+TGFβ3 pAb, 10 μg/ml; *lane 4*) were extracted and immunoblotted for E-cad, N-cad, and tubulin (as a control).

supplement shows that, like collagen I, EGF treatment of A549 cells resulted in increased transcription of TGF-β3 mRNA. Thus, we asked if expressing Smad7 would prevent EGF-induced EMT in these cells. Figure E2B shows that Smad7 prevented morphological changes in response to EGF, indicating that the Smad pathway plays a role in EGF-induced EMT. To determine if downstream signaling induced by EGF was similar to that induced by collagen I, we treated cells with EGF in the presence or absence of the inhibitors shown in Figure 5. The PI3K inhibitor (LY294002; Figure E2C, *lanes 5* and *6*) and the ERK inhibitor (PD98059; Figure E2C, *lanes 7* and *8*) prevented EGF-induced down-regulation of E-cadherin expression, whereas the other inhibitors did not. The PI3K inhibitor and the ERK inhibitor prevented up-regulation of TGF-β3 mRNA in response to EGF (Figure E2D). These data suggest that EGF-induced EMT is also dependent upon autocrine secretion of TGF-β3, indicating that the pathways that promote transcription TGF-β3 mRNA are similar whether the cells are stimulated by collagen I or EGF.

Together, these data show that multiple extracellular stimuli, including growth factors and extracellular matrix, can induce EMT in NSCLC and that the mechanism for induction of cellular changes is similar in each instance. Interaction of cells with collagen I and with growth factors in the extracellular matrix activates the receptor tyrosine kinase and integrin pathways, respectively. These pathways converge on downstream PI3K and ERK signaling pathways to induce autocrine secretion of TGF-β3, which activates the TGF-β receptors to induce EMT (Figure 7).

DISCUSSION

A549 lung adenocarcinoma is a well-characterized cell line that has been used as a model system to study the mechanisms of carcinogenesis, cancer progression, apoptosis, and drug sensitivity in lung cancer (15, 29, 30). A549 cells retain important characteristics of alveolar type II epithelial cells and have been used to study inflammation and fibrosis in the lung. Because EMT plays a key role in the development of lung fibrosis (31, 32), we chose A549 cells as a model system to investigate signals that induce EMT. In agreement with previous reports (27, 28), we showed that TGF-β1 and EGF induce EMT in A549 cells. In addition, we showed that A549 cells undergo EMT in response to collagen type I, suggesting that A549 cells are highly sensitive to inducers of EMT. In contrast, others have reported that neither TNF-α nor IL-1β induces EMT in A549 cells (27), and here we showed that neither HGF nor FGF induced EMT in these cells. We also showed a similar phenomenon using NCI-H358 and NCI-H1299 cells. NCI-H358 is an adenocarcinoma cell line that expresses E and N-cadherin, whereas NCI-H1299 is large cell carcinoma cell line that expresses only N-cadherin. Both of these cell lines underwent EMT in response to collagen I and TGF-β1, indicating that extracellular stimuli play key roles in lung cancer progression.

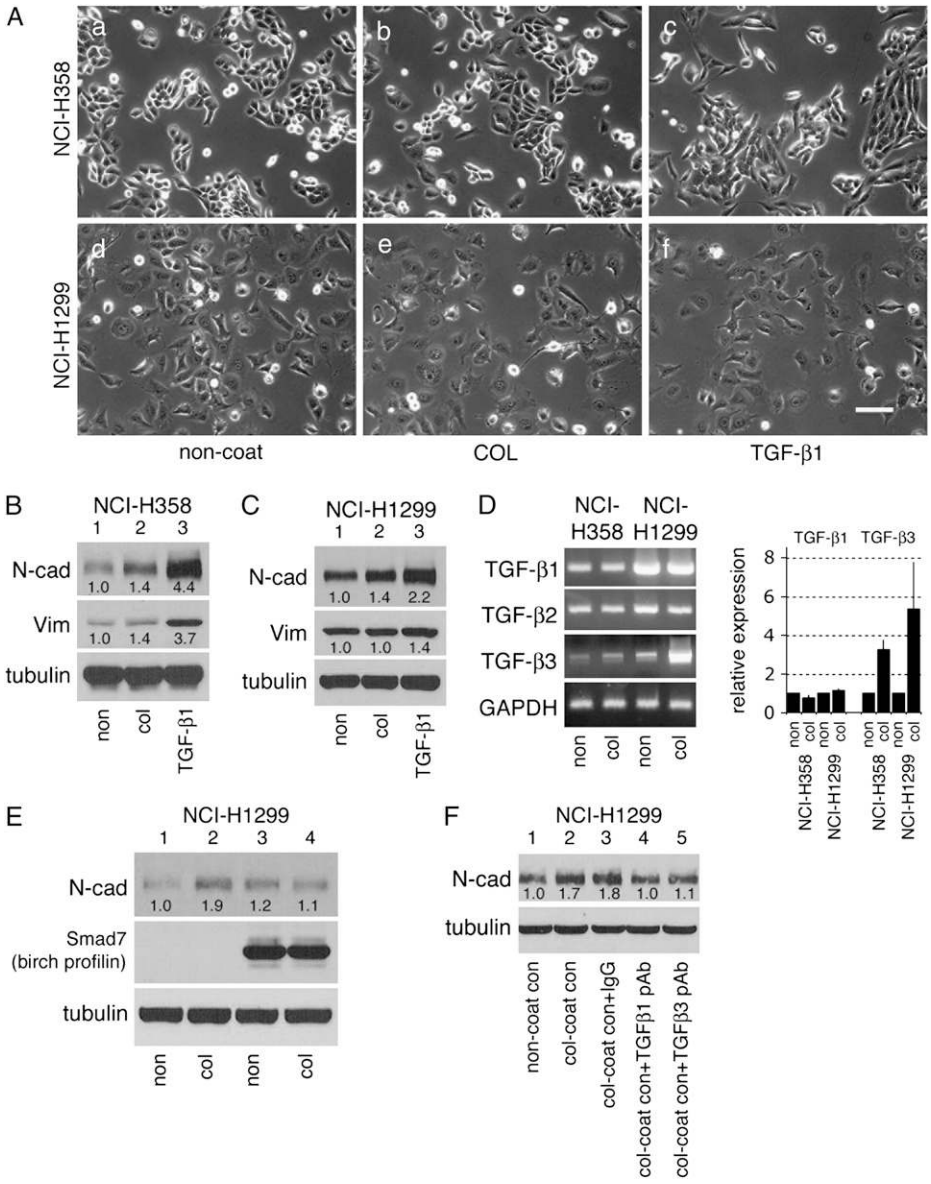

Figure 5. NCI-H358 and NCI-H1299 lung cancer cells undergo EMT via TGF-β3 signaling. (*A*) NCI-H358 and NCI-H1299 cells were cultured on noncoated (*a* and *d*) or collagen I–coated (COL, *b* and *e*) dishes. Cells on noncoated dishes were treated with TGF-β1 (2 ng/ml) for 2 days (*c* and *f*). Phase-contrast pictures were taken using a 10× objective. *Bar*, 100 μm. (*B*). NCI-H358 cells plated on noncoated dishes, on collagen I–coated dishes, or treated with TGF-β1 were extracted, and 30 μg protein was resolved by SDS-PAGE and immunoblotted for N-cadherin (N-cad), vimentin (Vim), and tubulin (as a control). (*C*) NCI-H1299 cells plated on noncoated dishes or on collagen I–coated dishes or were treated with TGF-β1 were extracted, and 30 μg protein was resolved by SDS-PAGE and immunoblotted for N-cad, Vim, and tubulin (as a control). (*D*) One day after seeding onto noncoated or collagen I–coated dishes, total RNA was extracted, and RT-PCR was done for TGF-β1, TGF-β2, TGF-β3, and GAPDH. Quantitative RT-PCR was performed for TGF-β1 and TGF-β3 as described in Materials and Methods. (*E*) Mock or Smad7-infected NCI-H1299 cells were plated onto noncoated (non) or collagen I–coated (col) dishes. Two days after seeding, cells were extracted and immunoblotted for N-cad, Smad7 (anti-tag), and tubulin. (*F*) NCI-H1299 cells were treated for 2 days with conditioned media from cells plated onto noncoated (noncoat con; *lane 1*) or collagen I–coated (col-coat con; *lane 2*) dishes for 2 days. Cells were also treated for 2 days with conditioned media in the presence of nonspecific rabbit IgG (col-coat con+IgG, 10 μg/ml; *lane 3*), neutralizing rabbit Ab against TGF-β (col-coat con+TGFβ pAb, 10 μg/ml; *lane 4*), or neutralizing rabbit Ab specific for TGF-β3 (col-coat con+TGF-β3 pAb, 10 μg/ml; *lane 5*). Cell extracts were immunoblotted for N-cad and tubulin (as a control).

Blocking TGF-β signaling using a pan-neutralizing antibody against TGF-β or overexpressing inhibitory Smad7 completely inhibited collagen I–induced EMT, suggesting that collagen I–induced EMT in these cells occurs through a TGF-β–dependent pathway. Kim and colleagues reported that primary alveolar epithelial cells cultured on fibronectin undergo EMT via integrin dependent activation of endogenous latent TGF-β1 (33). In agreement with their data, we showed that conditioned media from cells cultured on collagen I could induce EMT in A549 and NCI-H1299 cells, indicating that EMT is induced by ECM-dependent activation of TGF-β.

We showed that A549, NCI-H358, and NCI-H1299 cells increased production of TGF-β3 in response to collagen I. Furthermore, neutralizing antibody specific for TGF-β3 prevented collagen I–induced EMT. PI3K and ERK inhibitors prevented collagen I–induced EMT and up-regulation of TGF-β3 mRNA in A549 cells. These data suggest that PI3K and ERK signaling plays a role in the induction of TGF-β3 expression in response to collagen I, which causes EMT in NSCLC cells.

We have previously shown that JNK signaling plays an important role in collagen I–induced EMT in pancreatic cancer (15). Khatlani and colleagues reported that JNK activation promotes oncogenesis in NSCLC (34). In our study, inhibiting JNK activity did not prevent E-cadherin down-regulation but did promote changes in cell morphology and prevented up-regulation of N-cadherin in response to collagen I. Recent studies have shown that expression of N-cadherin in epithelial cells is one way that tumor cells can become more invasive and metastatic, suggesting that investigations of the role of JNK activity in NSCLC are warranted.

Ross and colleagues reported that activating ERK increases Smad3 promoter activity (32). Thus, it is possible that PI3K/ERK signaling might directly activate the Smad pathway. On the other hand, many reports have shown that the interaction of TGF-β, with its receptor on stromal fibroblasts, is a potent inducer of ECM, including production of collagen I, which plays a role not only in cancer progression but also in fibrosis (35, 36). In addition, collagen I can induce EMT in a number of systems (15–17). Thus, EMT-inducing agents, such as growth factors and ECM, may directly induce EMT, or they may promote or enhance EMT by up-regulating the production of other EMT-inducing agents in an autocrine or paracrine manner. For example, IL-1β induces EMT

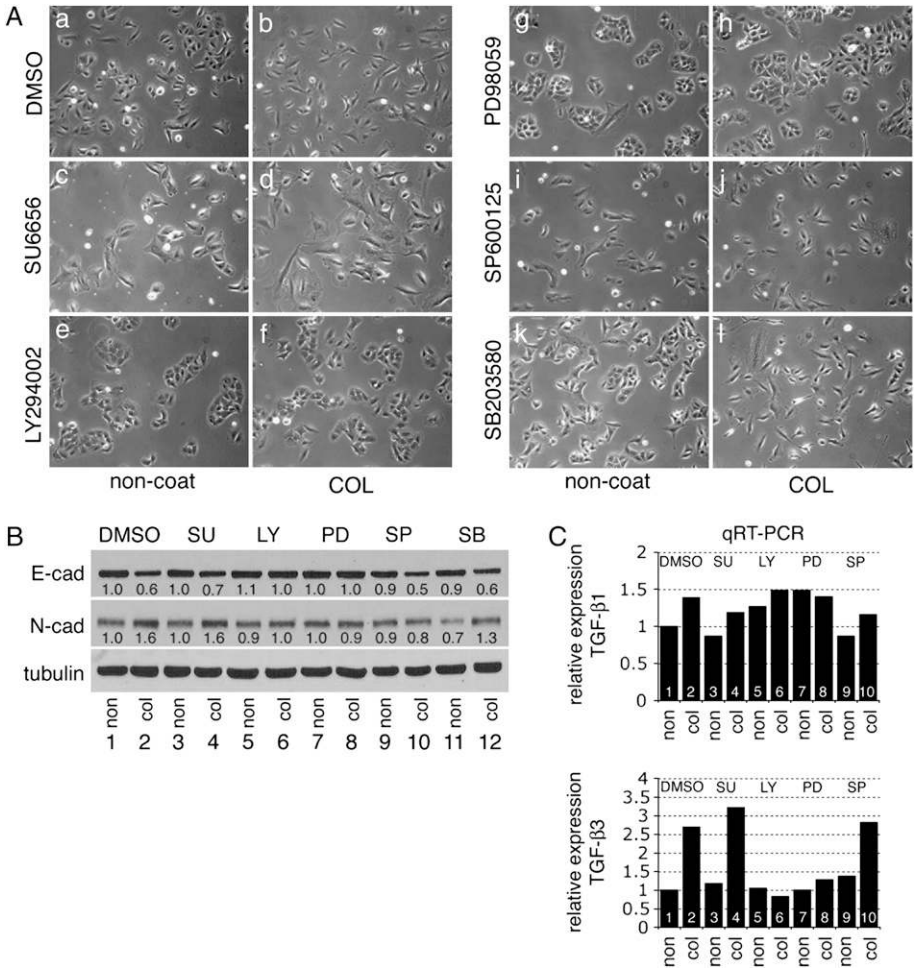

Figure 6. PI3K and ERK signaling are essential for collagen I–induced EMT. (*A*) A549 cells were plated onto noncoated (*a*, *c*, *e*, *g*, *i*, *k*) or collagen I–coated (*b*, *d*, *f*, *h*, *j*, *l*; COL) dishes in the presence of vehicle (DMSO; *a* and *b*), SU6656 (10 μM, *c* and *d*), LY294002 (10 μM, *e* and *f*), PD98059 (10 μM, *g* and *h*), SP600125 (10 μM, *i* and *j*), or SB203580 (10 μM, *k* and *l*). Two days after seeding, phase-contrast pictures were taken using a 10× objective. Bar = 100 μm. (*B*) Cells identical to those in *A* were extracted, and 30 μg protein was resolved by SDS-PAGE and immunoblotted for E-cadherin (E-cad), N-cadherin (N-cad), and tubulin (as a control). (*C*) One day after seeding cells onto noncoated (non) or collagen I–coated (col) dishes in the presence of DMSO, SU6656 (SU), LY294002 (LY), PD98059 (PD), or SP600125 (SP), total RNA was extracted, and qRT-PCR was done for TGF-β1 and TGF-β3.

in renal epithelial cells through a TGF-β1–dependent mechanism (37), and epilysin (MMP-28) has been shown to induce EMT in A549 cells also via activating endogenous TGF-β signaling (38). Thus, many factors can cooperate to induce EMT, and it seems that in lung cancer cells, the mechanisms that induce EMT typically involve the TGF-β pathway.

We showed that mRNA encoding TGF-β3 was up-regulated in response to collagen I and EGF. The TGF-β superfamily

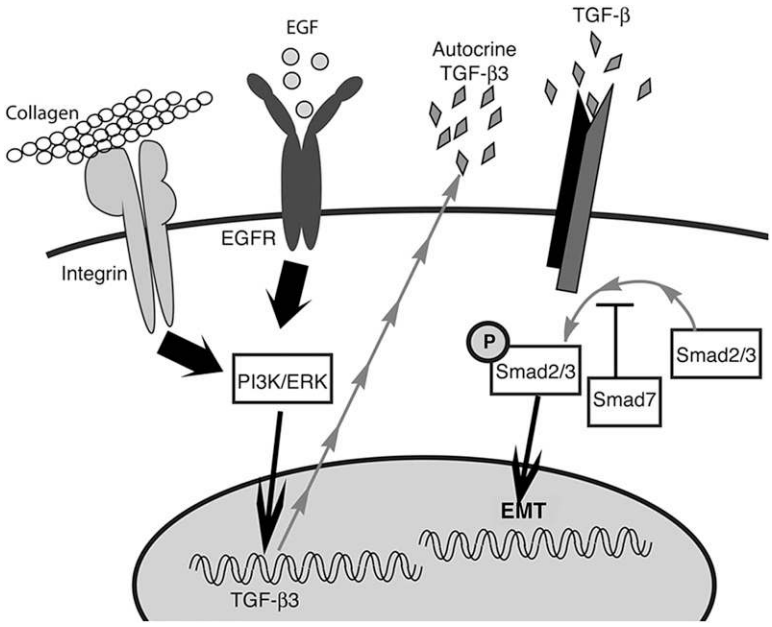

Figure 7. Model for EMT in A549 lung cancer cells. Extracellular stimuli such as collagen I or EGF activate PI3K/ERK signaling, which stimulates transcription of TGF-β3 mRNA and autocrine secretion of TGF-β3 protein, which binds to its receptor on the surface of the cell, activating the Smad pathway and up-regulating genes that promote EMT.

includes three isoforms: TGF-β1, TGF-β2, and TGF-β3. These TGF-β isoforms bind to common transmembrane receptors. A homodimer of TGF-β receptor type II binds to ligand and then interacts with a homodimer of TGF-β receptor type I to initiate signaling. Thus, TGF-β1, TGF-β2, and TGF-β3 are predicted to have similar biological activities (39). However, the localization of each of these isoforms may be different, or the ratios of each isoform may influence the activity of the receptors (40, 41). Bodmer and colleagues reported that secretion of TGF-β2 by tumor cells contributes to decreased immune surveillance for tumor development and neovascularization of the tumor tissue (42). TGF-β1 is the isoform most commonly implicated in regulation of tumorigenesis (43); however, it is possible that other isoforms play a role in cancer progression. In our system, A549 cells make a significant amount of TGF-β1 and TGF-β2 mRNA even when they are not stimulated by collagen I, which is consistent with the hypothesis that the three isoforms of TGF-β do not initiate identical signals even though they bind to the same receptor pair. Themsche and colleagues recently reported a role for TGF-β3 in the invasiveness of endometrial carcinoma cells (44). These authors found that TGF-β3, but not other TGF-β isoforms, induced the expression of MMP-9 in human endometrial cells, which was necessary for cancer invasiveness. Furthermore, retinoic acid, which is used for cancer treatment, did not affect TGF-β1 mRNA but decreased TGF-β3 mRNA in NCI-H1299 cells (45). Further studies are needed to clarify the specific function of the various TGF-β isoforms and their role in cellular signaling.

One characteristic of cells that have undergone EMT is the loss of E-cadherin expression, and loss of E-cadherin has been associated with poor clinical outcome in NSCLC (3, 7). It has also been reported that lung cancer cells that have undergone EMT with reduced expression of E-cadherin are relatively insensitive to the EGF receptor kinase inhibitors erlotinib and gefitinib and that expression of mesenchymal markers could be used as a predictor of the clinical efficacy of treating lung cancer patients with erlotinib (29, 46). Thompson and colleagues also showed that A549 cells originally have an intermediate sensitivity to erlotinib; thus, EMT-induced A549 cells may be even less sensitive to the drug (46). Therefore, we can predict that EMT-inducing pathways are good candidates for intervention in the treatment of cancer. In this article, we show that TGF-β3 signaling is the main pathway promoting EMT independent of the nature of the extracellular stimulus; therefore, blocking TGF-β3 signaling may be the most effective treatment to prevent cancer progression in patients with NSCLC.

Because TGF-β has been reported to be an immunosuppressive cytokine, there are many reports showing that blocking TGF-β signaling in immune cells is an attractive target for anticancer therapy (47, 48). Hojo and colleagues reported that cyclosporine stimulated A549 cells to become invasive by increasing their production of TGF-β and that invasion was prevented by treatment with an inhibitory monoclonal antibody against TGF-β (49). In addition, TGF-β production is frequently up-regulated in human cancers, including lung cancer (50). Together, these studies suggest that TGF-β signaling is a good target for lung cancer treatment. TGF-β has biphasic effects during tumorigenesis. Early in the disease it acts as a tumor suppressor and later promotes cancer progression by its action on the tumor cells and their microenvironment (43). Therefore, therapy targeting TGF-β signaling will necessarily be multifaceted. Here we show that inhibiting the Smad pathway prevented EMT in A549 cells; therefore, Smads and their downstream transcriptional targets may be specific targets for inhibition of TGF-β activation during cancer progression. Furthermore, combination treatment with inhibitors against EGFR and TGF-β signaling may be a promising route for therapy because blocking TGF-β signaling prevents EMT and thus may increase sensitivity to EGFR inhibitors.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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