

House Dust Mite–Promoted Epithelial-to-Mesenchymal Transition in Human Bronchial Epithelium

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The molecular basis of airway remodeling and loss of epithelial integrity in asthma is still undefined. We aimed to establish if exposure of human bronchial epithelium (16HBE cells) to asthma-related stimuli can induce epithelial-to-mesenchymal transition (EMT), a key process in tissue repair and remodeling associated with loss of intercellular contacts. We studied the effects of fibrogenic cytokine TGF- β and protease-containing aeroallergen house dust mite (HDM) on mesenchymal and epithelial markers, cytoskeleton organization, and activation of β -catenin–driven reporter TopFLASH. TGF- β alone up-regulated vimentin and fibronectin, modestly down-regulated E-cadherin, but did not affect cytokeratin. HDM alone did not affect these markers, but promoted stress fibers. Importantly, when added to TGF- β -primed epithelium, HDM induced E-cadherin internalization, enhanced β -catenin–dependent transcription, and down-regulated cytokeratin. Regarding the underlying mechanisms, the stimuli together induced sustained myosin light chain phosphorylation, which was crucial for E-cadherin internalization and β -catenin–dependent transcription. Previously, we showed that HDM signals through the epidermal growth factor receptor (EGFR). Accordingly, inhibition of EGFR prevented TGF- β /HDM-induced mesenchymalization. TGF- β facilitated uncoupling of EGFR from E-cadherin, its negative regulator, and prolonged EGFR signaling. Thus, we show that HDM promotes EMT in TGF- β -primed epithelium. Analysis of primary epithelium appears consistent with this phenotypic change. We propose that TGF- β secretion and dysregulated EGFR signaling may increase epithelial vulnerability to allergens and trigger the induction of EMT, a hitherto unrecognized contributor to airway remodeling in asthma.

Keywords: TGF- β ; E-cadherin; myosin light chain; EGFR

Allergic asthma is characterized by allergen-induced Th2-mediated airway hyperresponsiveness (AHR), inflammation and, remodeling. Loss of epithelial integrity is thought to play a key role in the development of airway remodeling and AHR. The airway epithelium forms the first barrier against deposited aeroallergens. Barrier function is maintained by intercellular contact formation through homophilic E-cadherin interactions in so-called adherens junctions (1). E-cadherin is linked to the actin cytoskeleton by its association with β -catenin, which stabilizes cell–cell contacts. Integrity of the airway epithelium is often compromised in asthma, with detachment of ciliated cells, increased permeability to allergens, and decreased

CLINICAL RELEVANCE

House dust mite and TGF- β -induced epithelial-to-mesenchymal transition may be a hitherto unrecognized mechanism contributing to asthmatic airway remodeling. The involvement of epidermal growth factor receptor and myosin light chain may offer novel avenues for therapeutic intervention strategies in allergic asthma.

E-cadherin expression at the sites of epithelial detachment (2). Reduction in E-cadherin-mediated cell–cell contacts has been observed during the asthmatic response in animal models of asthma, resulting in increased epithelial permeability and AHR (3). Down-regulation of E-cadherin is an important component of epithelial-to-mesenchymal transition (EMT), a process involved in cell migration, repair, and tissue remodeling. Currently, it is unknown whether this process contributes to the abnormal phenotype of asthmatic epithelium. Furthermore, the molecular basis of airway remodeling and reduced epithelial integrity in allergen-induced asthma is still undefined.

Many allergens contain proteases, which cause epithelial damage. In addition, proteases can activate protease-activated receptor (PAR)s, leading to proinflammatory gene transcription (4–6). Recently, PAR-2 activation has been described to disrupt E-cadherin-mediated cell–cell contacts (7). It is incompletely understood, however, why protease-containing allergens only induce structural changes in asthma, but not healthy epithelium. The asthmatic epithelium may display increased vulnerability and/or an inadequate repair response to noxious stimuli, inducing its migratory/mesenchymal phenotype. The role for inadequate repair is supported by increased expression of the repair markers CD44, epidermal growth factor receptor (EGFR), and TGF- β in asthmatic airways (8–10). Increased expression of TGF- β has also been related to specific TGF- β polymorphisms that are associated with asthma (11). We have recently shown that, in addition to injury-provoked liberation of β -catenin from adherens junctions, the TGF- β -induced stabilization of β -catenin is an important requirement for transcriptional activation of EMT genes in kidney tubular cells (12). Cognizant of this two-hit paradigm, wherein both contact injury (e.g., by a protease-containing allergen) and the presence of a fibrogenic cytokine are required for EMT, we sought to determine the combined effect of TGF- β and house dust mite (HDM), the major allergen in atopic asthma, on bronchial epithelium. Hallmarks of EMT include disassembly of cell–cell contacts, delocalization of E-cadherin from cell junctions, junctional loss of β -catenin and F-actin, cytoplasmic/nuclear redistribution of β -catenin, loss of the epithelial marker cytokeratin, and induction of the mesenchymal markers vimentin and fibronectin. We show that TGF- β and HDM, in combination, induce marked EMT (as defined by the above characteristics) in the bronchial epithelial cell line 16HBE as well as primary bronchial epithelial cells. In a TGF- β -primed epithelium, HDM triggers E-cadherin inter-

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nalization and activation of the E-cadherin/ β -catenin axis, effects that are critically dependent on the phosphorylation of myosin light chain (MLC). Finally, we show that EGFR is a significant mediator in HDM-promoted EMT, and TGF- β facilitates signaling through EGFR.

MATERIALS AND METHODS

Cell Culture and Stimulation

The human bronchial epithelial cell line 16HBE 14o- (16HBE) was kindly provided by Dr. D. C. Gruenert (University of California, San Francisco, CA). 16HBE cells were cultured as described (13). Primary bronchial epithelial cells from different donors were derived from Lonza (Walkersville, MD) as cryopreserved passage 1 normal human bronchial epithelium (NHBE). Primary cultures were grown in 2.5 ml serum-free hormonally supplemented bronchial epithelium growth medium (BEGM; Lonza) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin coated with 30 μ g/ml collagen, 30 μ g/ml fibronectin, and 10 μ g/ml bovine serum albumin (BSA). Cells were passaged using trypsin when 90% confluent and further cultured for use at passage 2. At 80 to 90% confluence, cells were passed or used for experiments. Cells were seeded into coated 6-, 12-, or 24-well plates at a density of $0.5\text{--}2 \times 10^5$ /well until approximately 80 to 90% confluence, serum deprived for 4 hours, and used for experimentation. In case of transient transfection for luciferase assay or transfection with siRNA, cells were transfected on the subsequent day after seeding and grown for 20 hours or 3 additional days, respectively. For preparation of whole cell lysates, medium was replaced with serum-deprived EMEM medium or hormone/growth factor-deprived medium (BEBM) for primary cultures, cells were rendered quiescent for 4 hours to overnight and stimulated with or without TGF- β 1 (5 ng/ml; Sigma, St. Louis, MO) for 1 to 72 hours. Twenty hours (o/n) or 72 hours after pretreatment with TGF- β , cells were treated with or without house dust mite extract (HDM, 50 μ g/ml; Greer Laboratories, Lenoir, NC), PAR-2-activating peptide (1 μ M), or EGF (10 ng/ml) for 5 to 60 minutes or 6 to 24 hours. Myosin ATPase inhibitor blebbistatin (100 μ M), EGFR inhibitor AG1478 (1 μ M; Sigma), cysteine peptidase inhibitor E64 (10 μ M), serine peptidase inhibitor AEBSF (1 mM), and methyl- β -cyclodextrin (m β CD, 500 μ M; Sigma) were added 1 to 4 hours before stimulation.

Air-Liquid Interface

For air-liquid interface (ALI) cultures, primary bronchial epithelial cells were grown to confluence, harvested, and seeded at 50×10^3 cells per insert on semi-permeable Transwell membranes (Transwell, 0.4 μ m pore-size, 12 mm diameter; Corning Inc., Cambridge, MA) coated with 30 μ g/ml collagen, 30 μ g/ml fibronectin, and 10 μ g/ml BSA. Cells were cultured in a 1:1 mixture of DMEM (Lonza) and BEGM supplemented with retinoic acid (RA, 15 ng/ml; Sigma). Cultures were grown submerged until confluence (3–5 d), after which they were exposed to an ALI for another 2 to 4 weeks. Mucociliary differentiation was observed between Days 14 and 26 after exposure to ALI. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Before exposure to HDM and TGF- β , cells were washed twice and placed in growth factor-deprived medium for 4 hours. Twenty hours (o/n) after pretreatment with TGF- β , cells were treated with or without HDM extract for 6 hours (basolateral and apical application). The inserts were fixed with acetone and subjected to immunofluorescence as described below.

Immunodetection

Total cell lysates were obtained by resuspension of the cells in 1 $\times$ Sample buffer (containing 2% SDS, 10% glycerol, 2% β -mercaptoethanol, 60 mM Tris-HCl pH 6.8, and bromophenol blue) and boiling for 5 minutes. Proteins were separated on SDS-polyacrylamide gels. Immunoblotting was performed with anti-fibronectin, anti-vimentin, anti- β -actin, anti-SMA (Sigma), anti-pan-cytokeratin, anti-E-cadherin, anti- β -catenin, anti-EGFR, anti-phospho-EGFR, anti-caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FSP1 (DAKO Diagnostics, Mississauga, ON, Canada), anti-phospho-GSK-3 β , anti-phospho-MLC, and anti-phospho-SMAD2 (Cell Signaling Technology, Hitchin, Herts, UK). Immunoreactive bands were visualized by enhanced chemoluminescence as described (14).

Transient Transfection and Luciferase Assay

The Lef-1/TCF reporter plasmid TopFLASH and its inactive mutant PopFLASH were purchased from Upstate Biotechnology (Charlottesville, VA). The thymidine kinase-driven Renilla luciferase vector (pRL-TK; Promega, Madison, WI) was used as an internal control. Cells were plated on 24-well plates in duplicates and transfected the following day using FuGENE6 (reagent:DNA ratio 2.5 μ l/ μ g; Roche Molecular Biochemicals, Indianapolis, IN). For promoter activity measurements, cells were co-transfected with 0.5 μ g promoter plasmid and 0.05 μ g pRL-TK. After 20 hours, the cells were washed and serum deprived for 4 hours (in the presence and absence of blebbistatin [100 mM] and AG1478 [1 μ M]), followed by stimulation. Next, cells were lysed in 50 μ l Passive Lysate Buffer (Promega), exposed to freezing/thawing, and the samples were clarified by centrifugation. Firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay Kit (Promega) and a Berthold Lumat LB 9507 luminometer according to the manufacturers' instructions. Results were normalized by dividing the Firefly luciferase activity with the Renilla luciferase activity of the same sample.

Immunofluorescent Stainings

Cells were grown on coverslips and fixed in PBS-buffered paraformaldehyde (4%) for 60 minutes, permeabilized in PBS/0.1% Triton X-100 for 20 minutes, and blocked in PBS/5% BSA for 60 minutes. Coverslips were incubated for 60 minutes with primary antibodies (1:100) and subsequently incubated for 60 minutes with Alexa green 488-labeled anti-goat/mouse/rabbit IgG conjugates (1:1,000; Molecular Probes, Eugene, OR), Cy3-labeled anti-mouse/rabbit IgG conjugates (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA) or rhodamine-labeled phalloidin (1:200; Cytoskeleton Inc., Denver, CO) in presence of DAPI (1:500). Coverslips were mounted on slides using Fluorescence Mounting Medium (DAKO). Fluorescence was analyzed by fluorescence microscopy (Olympus microscope, Evolution QEI camera, QED InVivo Imaging software).

Cell Surface Biotinylation

Cells were washed four times with PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ and incubated with 1.5 mg/ml sulfo-succinimidyl 2-(biotinamido) ethyl-dithioproprionate (sulfo-NHS-SS-biotin) (Pierce Chemical Co., Rockford, IL) twice for 20 minutes, followed by washing with 50 mM NH₄Cl in PBS/MgCl₂/CaCl₂ to quench free sulfo-NHS-SS-biotin, followed by four additional washes in PBS/MgCl₂/CaCl₂. Cells were then washed with ice-cold PBS containing 1 mM Sodium orthovanadate (Na₃VO₄) and 1 mM PMSF and subsequently lysed in 200 μ l Triton lysis buffer (30 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.4), 100 mmol/L NaCl, 1 mmol/L ethylene glycol-bis (2-aminoethylether)-N,N'-N'-tetraacetic acid (EGTA), 20 mmol/L NaF, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 20 μ l/ml Protease Inhibitory Cocktail (BD Biosciences, San Diego, CA), and 1 mmol/L Na₃VO₄) for 20 minutes on ice. Cell lysates were clarified at 10,000 $\times$ g for 15 minutes and incubated with streptavidin beads to collect bound biotinylated proteins. Next, immune complexes were washed three times with lysis buffer. The precipitates were subjected to SDS 10% PAGE, immunoblotted on nitrocellulose membrane and E-cadherin and EGFR were detected using ECL, which was performed according to the manufacturer's guidelines. In all cases, membranes were stained with Ponceau S (Sigma) to ensure equal loading and transfer of the proteins.

Immunoprecipitation

Cells were washed with ice-cold PBS containing 1 mM Sodium orthovanadate (Na₃VO₄) and 1 mM PMSF and subsequently lysed in 200 μ l Triton lysis buffer (30 mmol/L HEPES pH 7.4, 100 mmol/L NaCl, 1 mmol/L EGTA, 20 mmol/L NaF, 1% Triton X-100, 1 mmol/L PMSF, 20 μ l/ml Protease Inhibitory Cocktail [BD Biosciences], and 1 mmol/L Na₃VO₄) for 20 minutes on ice. Cell lysates were clarified at 10,000 $\times$ g for 15 minutes and precleared with 30 μ l Protein A Sepharose beads (in a 50% slurry) for 1 hour at 4°C. After 1 minute of centrifugation at 300 $\times$ g, the cell lysates were subjected to immunoprecipitation with a polyclonal antibody specific for EGFR (Santa Cruz Biotechnology) and incubated rotating at 4°C for 1 hour. Protein A sepharose beads were added to

each sample and incubated rotating for 3 hours to bind to the primary antibodies. Next, immune complexes were washed three times with lysis buffer. The precipitates were subjected to SDS 10% PAGE, immunoblotted on nitrocellulose membrane and immunocomplexes were detected using enhanced chemoluminescence (ECL), which was performed according to the manufacturer's guidelines. E-cadherin antibody was purchased from Santa Cruz Biotechnology.

siRNA Down-Regulation of E-Cadherin

E-cadherin gene expression was down-regulated in bronchial epithelial cells by the use of small-interfering RNA (siRNA). Cells were seeded in 24-well plates or 5×10^4 /well and transfected with siRNA with a specific target sequence for human E-cadherin or with nontargeting control oligonucleotides (Eurogentec, Liege, Belgium). The target sequence for E-cadherin was GCAGAAUUGCUCACAUUUC. Cells were transfected with 2 μ g of siRNA purified and annealed synthetic oligonucleotides (Eurogentec) in a final concentration of 20 μ M by the high-efficiency transfection method using Oligofectamine (Invitrogen). Three days after transfection, medium was refreshed and cells were transfected with the TopFLASH plasmid and the Renilla luciferase vector (*see above*).

Transmission Electron Microscopy

After stimulation, cells were fixed for 1 hour at room temperature and then overnight at 4°C in 2% PFA, 2.5% glutaraldehyde, and 0.1 mol/L sodium cacodylate, pH 7.4. Fixed cells were treated with osmium collidine for 1 hour at room temperature, washed in 0.05 mol/L sodium maleate, pH 5.2, and dehydrated in successive incubations of 50, 70, 85, 95, and 100% (2 $\times$) EtOH, for 15 minutes each. Dehydrated cells were incubated in acetone for 3 minutes and infiltrated in a 1:1 araldite/acetone mixture overnight at 4°C, followed by overnight incubation in 100% araldite at 4°C. Cells were then incubated in fresh 100% araldite for 2 hours at room temperature and in fresh araldite overnight at 68°C. Embedded cells were cross-sectioned for electron microscopy (EM) analysis. Transmission electron microscopy (TEM) was performed at the Mount Sinai Hospital core facility (Toronto, ON, Canada).

Statistical Analysis

The Wilcoxon signed rank test was used to test for significant differences. Statistical significance was set at $P < 0.05$. For blots and immunofluorescence pictures, one representative is shown of at least three independent experiments.

RESULTS

TGF- β Induces Mesenchymal Characteristics in the Bronchial Epithelial Cell Line 16HBE

To address whether any features of EMT could be induced by TGF- β , a fibrogenic cytokine that exhibits increased expression in asthmatic airways, we exposed human bronchial epithelial 16HBE cells to TGF- β for 6 to 72 hours. Exogenously added TGF- β modestly down-regulated E-cadherin expression, although this effect was observed only after prolonged periods of TGF- β exposure (72 h, Figure 1A). Upon disruption of E-cadherin-mediated contacts, β -catenin usually undergoes rapid degradation, unless this process is prevented by the concomitant phosphorylation/inactivation of Glycogen synthase kinase (GSK)-3 β , an enzyme critical for the elimination of β -catenin. Indeed, we found that TGF- β increased the phosphorylation of GSK-3 β in 16HBE cells (Figure 1B). Accordingly, β -catenin expression was preserved in presence of TGF- β , despite the reduction in E-cadherin expression (Figure 1A). These changes were accompanied by a slight but significant increase in the activity of the β -catenin-dependent Lef-1/TCF-driven luciferase construct TopFLASH (Figure 1C), indicating that the liberated β -catenin was transcriptionally active. In addition, TGF- β increased vimentin and fibronectin expression; however, it did not induce a clear change in the expression of epithelial marker cytokeratin nor the myofibroblast marker α -smooth muscle actin (SMA, Figure 1D), which was not expressed at all during the course of these experiments.

HDM Enhances TGF- β -Induced Changes in Epithelial Phenotype and the Combination of HDM and TGF- β Induces Internalization of the E-Cadherin/ β -Catenin Complex

HDM itself did not reduce the expression of E-cadherin, nor modify the total levels of E-cadherin or β -catenin after TGF- β treatment (data not shown). Under pathologic conditions, the exposure of the asthmatic epithelium to aeroallergens is thought to occur in the presence of enhanced levels of TGF- β . Therefore, we tested the combined effect of TGF- β and HDM on bronchial epithelial cells. Importantly, the combined addition of TGF- β /HDM had a major impact on the *distribution* of E-cadherin. In the presence of TGF- β , HDM induced robust delocalization of this junctional protein after 60 minutes (Figure 1E). In conjunction with this, the combined treatment with TGF- β and HDM caused marked redistribution of β -catenin. In cells exposed to TGF- β and HDM, E-cadherin and β -catenin adopted a jagged/perpendicular arrangement with increased cytosolic distribution versus a linear distribution parallel to the membrane in untreated cells. Pretreatment with TGF- β also enabled HDM to induce internalization of E-cadherin as detected by the substantial down-regulation of biotinylated E-cadherin from the cell surface, which was most pronounced after 60 minutes (Figure 1F). The disruption of the adherens junctions and the ensuing delocalization of E-cadherin resulted in enhanced β -catenin transcriptional activity (TopFLASH), which was significantly higher than the level achieved by either stimulus alone. In contrast, there was no significant increase in activity of its inactive mutant, FopFLASH (Figure 1C). The observation that E-cadherin redistribution precedes TopFLASH activity suggests that it may be the initiator of β -catenin-dependent signaling and gene expression. In line with the effects on TopFLASH, HDM enhanced the TGF- β -stimulated expression of the mesenchymal marker vimentin (Figure 1G). Moreover, the combined treatment with HDM and TGF- β resulted in a marked down-regulation of the epithelial marker cytokeratin in 16HBE cells (Figure 1G), the level of which was unaffected either by TGF- β (Figure 1D) or HDM (Figure 1G) when applied separately. Taken together, TGF- β and HDM in combination induce mesenchymal transition, causing down-regulation of surface E-cadherin and augmented β -catenin signaling as well as increased vimentin and decreased cytokeratin expression.

In addition to the effects observed in 16HBE, we found that up-regulation of vimentin and down-regulation of cytokeratin are also induced upon combined treatment with TGF- β and HDM in primary bronchial epithelial cells (NHBE, Figure 2A). The mesenchymal phenotype of these cells was further supported by the up-regulation of fibroblast-specific protein-1 (FSP1), a target of β -catenin/TCF signaling (15) that has been extensively used as mesenchymal marker of EMT (Figure 2A). In accordance to the findings in 16HBE, we also observed delocalization of E-cadherin in these cells upon the combined treatment with TGF- β and HDM (Figure 2B). Importantly, this was associated with increased intensity for vimentin (Figure 2B) and FSP1 (data not shown).

Finally, we cultured primary bronchial cells at the ALI to allow for mucociliary differentiation and mimic the phenotype of *in vivo* airway epithelium. Overnight pretreatment of these differentiated cells with TGF- β followed by 6 hours of exposure to HDM also resulted in the delocalization of E-cadherin (Figure 2C) as well as increased intensity of vimentin staining (Figure 2C). These effects were more pronounced when the combination of the stimuli was used than when either stimulus was used alone (data not shown). Thus, we show that stimuli relevant to asthma induce E-cadherin delocalization and push

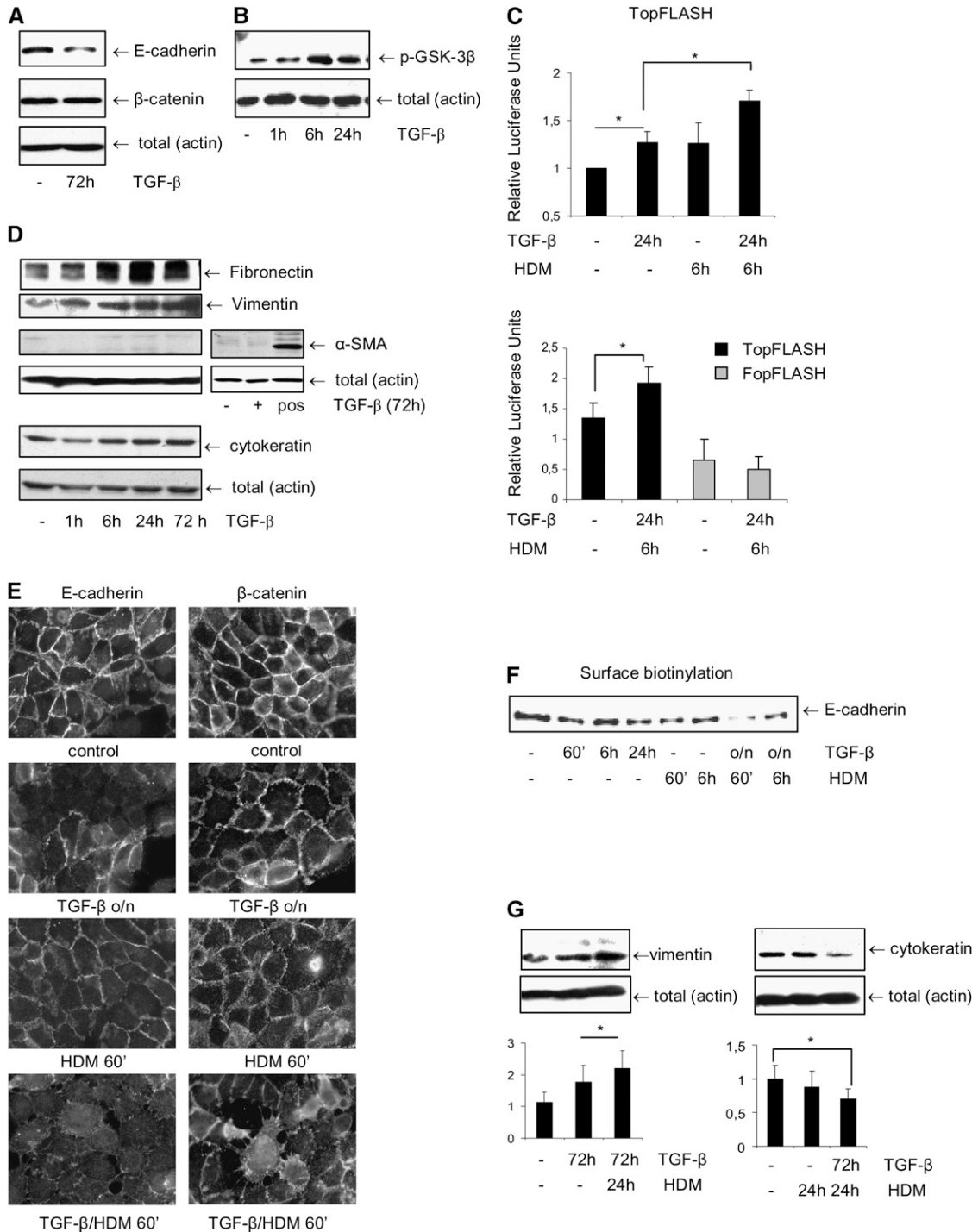
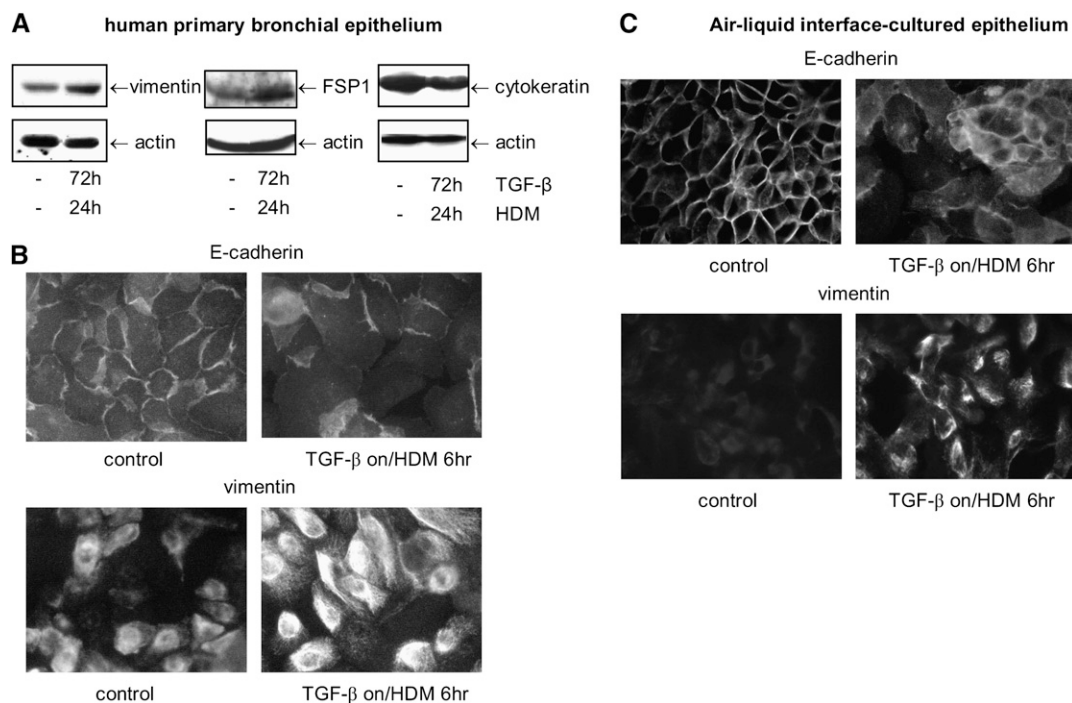


Figure 1. The partial induction of a mesenchymal phenotype in human bronchial epithelial cells by TGF-β is enhanced by house dust mite (HDM), and the combination of HDM and TGF-β induces internalization of the E-cadherin/β-catenin complex. Human bronchial epithelium (16HBE) cells were cultured in 12-well or 24-well plates, grown to approximately 90% confluence, serum deprived, stimulated with or without TGF-β (5 ng/ml) for 1 to 72 hours, and subsequently exposed to HDM (50 μg/ml) as indicated. (A) Total cell lysates were prepared and E-cadherin and β-catenin were detected by Western blotting as indicated by the arrows. (B) Cells were serum deprived for 4 hours, stimulated, total cell lysates were prepared, and phospho-GSK-3β was detected by Western blotting (arrow). (C) Cells were seeded in duplicates in 24-well plates, grown overnight and transfected with TopFLASH or FopFLASH (0.5 μg/ml) and the thymidine kinase-driven Renilla luciferase vector pRL-TK (0.05 μg) as internal control, and stimulated as indicated. Luciferase activity was determined as described in MATERIALS AND METHODS. Values were normalized to the relative luciferase activity

and expressed as means $\pm$ SEM ($n = 6$). $*P < 0.05$ between the treated and control samples. The data were normalized by setting the control value (TopFLASH/Renilla ratio) to 1. Statistical significance was reached when calculated for the absolute TopFLASH/Renilla ratios. (D) Total cell lysates were prepared and fibronectin, vimentin, SMA, and pan-cytokeratin were detected by Western blotting as indicated by the arrows. Positive control: TGF-β-treated LLC-PK1 kidney epithelial cells (72 h). (E) Cells were grown on coverslips. E-cadherin and β-catenin were detected by immunofluorescent staining after fixation and permeabilization of the cells (magnification: $\times 60$). (F) Cells were surface biotinylated and lysed. Biotinylated proteins were recovered using streptavidin beads and analyzed by SDS-PAGE. E-cadherin was detected by Western blotting (arrow). (G) Total cell lysates were prepared and vimentin and cytokeratin were detected by Western blotting (arrows). Protein levels were analyzed using densitometry and normalized for total protein levels (β-actin). Data are expressed as mean ratio $\pm$ SEM. For A, B, D, and G, the β-actin signal in the lower panels is used as control for equal loading. Representatives of at least three independent experiments are shown.



ferent donors were grown on coverslips. E-cadherin and vimentin were detected by immunofluorescent staining after fixation and permeabilization of the cells (*magnification*: $\times 60$). (C) Inserts from ALI cultures from three different donors were fixed, permeabilized, and stained for E-cadherin and vimentin. Representatives are shown.

bronchial epithelial cells toward a mesenchymal phenotype *in vitro*.

The Effects of TGF- β and HDM on E-Cadherin/ β -Catenin Localization Are Dependent on the Activation of MLC

Myosin-based contractility has been proposed to play an important role in destabilization and internalization of the E-cadherin/ β -catenin complex and myosin light chain has been implicated in EMT (16, 17). We therefore conducted experiments to discern whether HDM and TGF- β induce MLC phosphorylation, and whether myosin activation might be causally linked to junction remodeling and its functional consequences. Both TGF- β and/or HDM were able to induce phosphorylation of MLC on Ser19 (Figure 3A; *immunoblot*), an effect known to increase the ATPase activity of myosin and the assembly of F-actin stress fibers. MLC phosphorylation was most pronounced when both TGF- β and HDM were present (Figure 3A). Furthermore, while MLC phosphorylation induced by HDM alone was rapid and transient, it became prolonged when the cells were first pretreated with TGF- β (Figure 3A, *densitometry graph*). There were also marked differences in the localization of phospho-MLC induced by these stimuli. In untreated cells, phospho-MLC labeling was weak and mainly nuclear (Figure 3B). Immunofluorescent staining revealed that TGF- β induced MLC phosphorylation predominantly at the cell periphery and that HDM promoted phospho-MLC accumulation in a stress fiber-like pattern (Figure 3B). Accordingly, exposure to HDM promoted the formation of stress fibers (Figure 3C). TGF- β caused a different pattern of actin reorganization, with the formation of radial F-actin fibers. With the two stimuli combined, stress fiber assembly was most pronounced and accompanied by cortical remodeling. We considered that the myosin-based contractility and sustained peripheral MLC phosphorylation induced by the combined treatment might be required for efficient internalization of E-cadherin. Indeed, the combined effects of

TGF- β /HDM on E-cadherin redistribution and the transcriptional activation of β -catenin were blocked by use of the Myosin ATPase inhibitor blebbistatin (Figures 3D–3F). The latter inhibited TGF- β /HDM-induced internalization of E-cadherin as measured by surface biotinylation, and prevented the increase in TopFLASH activity. These results suggest a crucial role for MLC in the TGF- β /HDM-induced delocalization of E-cadherin, disassembly of the E-cadherin/ β -catenin complex, and transcriptional activation of β -catenin.

HDM Acts in a Protease-Dependent Manner and its Effects Are Mimicked by a PAR-2 Agonist

Since the major allergen of HDM (i.e., Der p) contains proteolytic activity, we tested whether the above described effects of HDM on myosin-based contractility were protease-dependent. Indeed, the HDM-induced MLC phosphorylation (Figure 4A) and stress fiber formation (Figure 4B) were inhibited by the combination of a serine and cysteine protease inhibitor. Cysteine and serine proteases are known to act on PAR-2. Accordingly, we observed that PAR-2 activating peptide (PAR2-AP) exerted effects similar to those of HDM on MLC phosphorylation (Figure 4A) and F-actin reorganization (Figure 4B). This supports a role for PARs in the effect of HDM on myosin-based contractility.

Signaling through the EGFR Is Critical for the HDM-Promoted EMT in a TGF- β -Primed Epithelium

Having shown that the cytoskeletal changes induced by HDM are protease-dependent, we considered that HDM might act primarily through the activation of the EGFR. This assumption was based on previously published data demonstrating that PAR-2 activation induces ADAM17-dependent shedding of heparin-bound (HB)-EGF (18, 19) and our previous findings showing that EGFR activity plays a crucial role in HDM-induced signaling in bronchial epithelial cells, an effect that

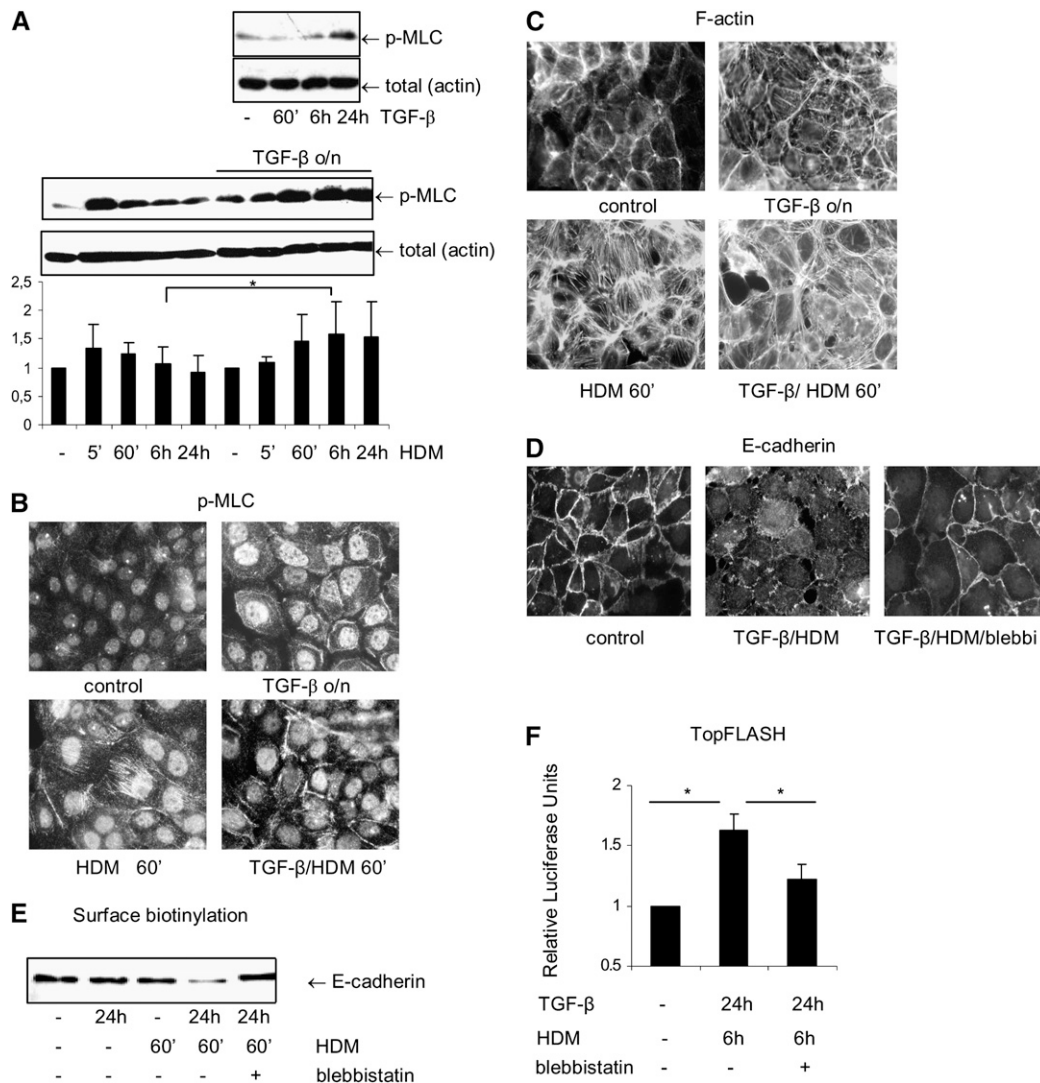


Figure 3. The effects of TGF- β and HDM on E-cadherin/ β -catenin localization are dependent on myosin light chain (MLC) activity and subsequent reorganization of the cytoskeleton. 16HBE cells were cultured in 12-well plates, serum deprived for 4 hours, and incubated with or without blebbistatin (100 μ M) for 4 hours. 16HBE cells were stimulated with HDM (50 μ g/ml) as indicated, with or without overnight preincubation with TGF- β (5 ng/ml). (A) Total cell lysates were prepared and phospho-MLC was detected by Western blotting (arrows). The β -actin signal in the lower panels shows equal loading. Protein levels were analyzed using densitometry and normalized for total protein levels (β -actin) and the condition without HDM. Data are expressed as mean ratio $\pm$ SEM ($n = 3$). (B–D) Cells were grown on coverslips. Phospho-MLC and E-cadherin were detected by immunofluorescent staining. F-actin was visualized by Rhodamine phalloidin staining. (E) Cells were surface biotinylated and lysed. Biotinylated proteins were recovered using streptavidin beads and analyzed by SDS-PAGE. E-cadherin was detected by Western blotting (arrow). (F) Cells were seeded in duplicates in 24-well plates, grown overnight, transfected with TopFLASH and the Renilla

luciferase vector pRL-TK, and stimulated as indicated. Luciferase activity was expressed as means $\pm$ SEM, $n = 4$. * $P < 0.05$ between the indicated values. Representatives of at least three independent experiments are shown.

was dependent on ADAM17 activity (13). In addition, previous publications have proposed a crosstalk between TGF- β and EGF signaling (10, 20, 21). To address the potential involvement of EGFR signaling in the TGF- β plus HDM-induced structural changes and the observed synergy between HDM and TGF- β , we first pretreated the cells with the specific inhibitor of EGFR tyrosine kinase activity AG1478 before exposure to these stimuli. The rapid HDM-induced MLC phosphorylation was prevented by AG1478. Moreover, the prolonged MLC phosphorylation induced by the combination of HDM and TGF- β was also abolished (Figures 5A and 5B). In addition, AG1478 inhibited delocalization of E-cadherin (Figure 5C) and reduced the activation of β -catenin-dependent transcription (as measured by the activation of TopFLASH, Figure 5D). The inhibitory effect of AG1478 was not due to an unspecific action on the TGF- β receptor, as the drug did not interfere with the TGF- β -induced Smad2 phosphorylation (Figure 5E). In agreement with the involvement of EGFR in the HDM-induced responses, the effects of HDM were recapitulated by EGF; similar to HDM, EGF provoked transient MLC phosphorylation (Figures 5F and 5G), which became sustained when the cells were pretreated with TGF- β (Figure 5G). Moreover, EGF induced sustained E-cadherin redistribution in TGF- β -primed

epithelial cells (Figure 5H). We also noted that in the presence of TGF- β , the EGF-induced phosphorylation of EGFR (Tyr 1173) was increased and sustained (Figure 5I), indicating that TGF- β potentiated EGFR signaling. This crosstalk between TGF- β and EGF appeared to be unidirectional: EGF did not induce phosphorylation of TGF- β downstream molecule Smad2, while it did induce robust phosphorylation of EGFR (Figure 5E).

Disruption of Caveolae May Lead to Prolonged EGFR Activation in a TGF- β -Primed Epithelium

In the following experiments we sought to gain insight into the mechanisms whereby TGF- β could enhance EGFR signaling. TGF- β has been reported to reduce expression of caveolin-1 (22), the major component of caveolae. Caveolae are membrane microdomains that are highly immobile and thought to stabilize E-cadherin at the plasma membrane (23–26). Moreover, the scaffolding domain of caveolin-1 has been described as negative regulator of various cellular signaling pathways (27). It also interacts with EGFR and inhibits EGFR autophosphorylation, while disruption of caveolae by cholesterol depletion has been shown to increase EGFR activation and prevent EGFR endocytosis (20). We therefore considered that TGF- β

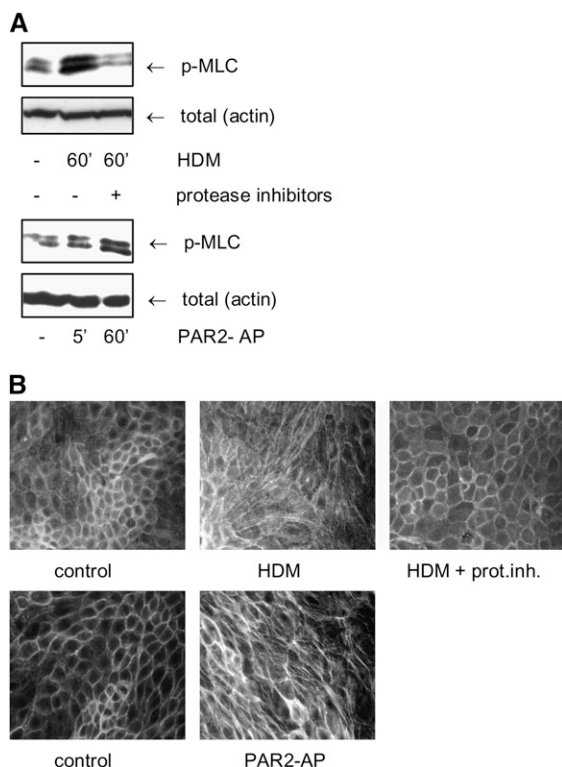


Figure 4. HDM acts in a protease-dependent manner and its effects are mimicked by a PAR-2 agonist. 16HBE cells were cultured in 12-well or 24-well plates, grown to approximately 90% confluence, serum deprived, and stimulated with HDM (50 μ g/ml) or PAR2-AP (1 μ M) for 60 minutes. HDM was pretreated with or without protease inhibitors E64 (10 μ M) and AEBSF (1 mM) for 30 minutes at 37°C. (A) Total cell lysates were prepared and phospho-MLC was detected by Western blotting (arrows). (B) Cells were grown on coverslips. F-actin was visualized by Rhodamine phalloidin staining. Representatives of at least three independent experiments are shown.

might enhance EGFR signaling by down-regulation of caveolin-1. However, in the presence of TGF- β alone we did not observe such an effect in our cells (Figure 6A). The integrity of caveolae and expression of caveolin-1 is maintained not only by its association with cholesterol, but also by the actin cytoskeleton, which regulates the stability of caveolae and internalization of its components. Accordingly, we found that membrane expression of caveolin-1 was dependent on peripheral localization of F-actin (data not shown). With this scenario in mind, we asked whether caveolin-1 distribution is sensitive to exposure to the combination of TGF- β and HDM. Interestingly, TGF- β pretreatment enabled HDM to reduce total caveolin-1 expression (Figure 6A), which could not be observed by HDM alone. Treatment with TGF- β plus EGF had a similar effect (data not shown). Furthermore, the redistribution of E-cadherin that was induced by the combination of TGF- β and HDM was paralleled by delocalization of caveolin-1 from the membrane (Figure 6B). Electron microscopic analysis suggested that this was associated with reduced number of caveolae-like structures (Figure 6C).

To assess whether down-regulation of caveolin-1 and/or disruption of membrane caveolae (as observed in presence of our combined stimuli) could indeed affect the function and expression of EGFR, we destabilized caveolae by the use of methyl- β -cyclodextrin (m β CD) in a relatively low concentration (500 μ M). This concentration depletes cholesterol and thereby specifically disrupts caveolar rafts, but does not interfere with binding of EGF, clathrin-dependent endocytosis of

EGFR, epithelial barrier function, or distribution of tight junction molecules (20, 28). Importantly, the addition of m β CD led to enhanced/sustained EGFR and downstream MLC phosphorylation upon EGF stimulation (Figure 6D). In the same way, HDM-induced MLC phosphorylation was increased in presence of m β CD (data not shown). These data suggest that the combined treatment with TGF- β and EGF or HDM causes liberation of EGFR signaling from negative regulation by caveolae (*see also* Figure 7).

TGF- β Induces Uncoupling between E-Cadherin and EGFR

So far our data indicate that TGF- β plus EGF or HDM are able to induce internalization of E-cadherin, a caveolae-resident protein. Interestingly, E-cadherin has been described as binding partner and negative regulator of EGFR (29, 30). We therefore considered that during the internalization process EGFR and E-cadherin might dissociate from each other, an effect that could contribute to increased EGFR signaling. In support of such a possibility we observed that the EGF-induced endocytosis of EGFR is insensitive to blebbistatin (Figure 6E), in contrast to the blebbistatin-sensitive, myosin-dependent internalization of E-cadherin (Figure 3E). This implies that EGFR and E-cadherin follow two distinct mechanisms of internalization. Indeed, using co-immunoprecipitation we verified that EGFR and E-cadherin interact under resting conditions. While TGF- β and EGF alone did not alter this association, TGF- β preincubation enabled EGF to induce rapid and dramatic uncoupling of EGFR from E-cadherin (Figure 6F). This uncoupling was also observed when TGF- β -primed cells were exposed to HDM (Figure 6F).

To reveal the functional consequences of this uncoupling, we studied the effect of transfection of the 16HBE cells with E-cadherin-specific siRNA, which we have previously shown to cause marked down-regulation of total E-cadherin expression as well as up-regulation of EGFR activation (30). Importantly, down-regulation of E-cadherin significantly increased EGF-induced β -catenin transcriptional activity (Figure 6G). Similarly, costimulation with TGF- β enhanced the activation of TopFLASH by EGF (Figure 6G). Thus, uncoupling of EGFR and E-cadherin may act to further enhance EGFR signaling, MLC-dependent E-cadherin internalization, β -catenin-dependent transcription, and up-regulation of mesenchymal genes in a positive feedback loop (Figure 7).

DISCUSSION

Airway remodeling, a key feature of asthma, is thought to be a consequence of compromised epithelial integrity. The airway epithelium in asthma may be more susceptible to undergo structural changes in response to noxious stimuli. Its barrier function is often compromised, with loss of differentiated columnar cells, reduced intercellular contacts, and lower expression of E-cadherin (2). Down-regulation of E-cadherin is an important component of EMT, a process involved in tissue repair and remodeling. However, it is still unknown whether this process is involved in the abnormal epithelial phenotype in asthma. In the present article we show that bronchial epithelial cells adopt a set mesenchymal features when exposed to TGF- β , a fibrogenic cytokine found in the asthmatic airways (8–10, 31). Importantly, this process is strongly accentuated by HDM, the major allergen in asthma. While HDM alone is not able to trigger major phenotypic changes, it enables redistribution of adherens junctions, transcriptional activation of mesenchymal genes, and loss of epithelial characteristics when it acts on TGF- β -primed epithelium. These findings render EMT as a relevant contributor to the cellular pathology of asthma. This is also

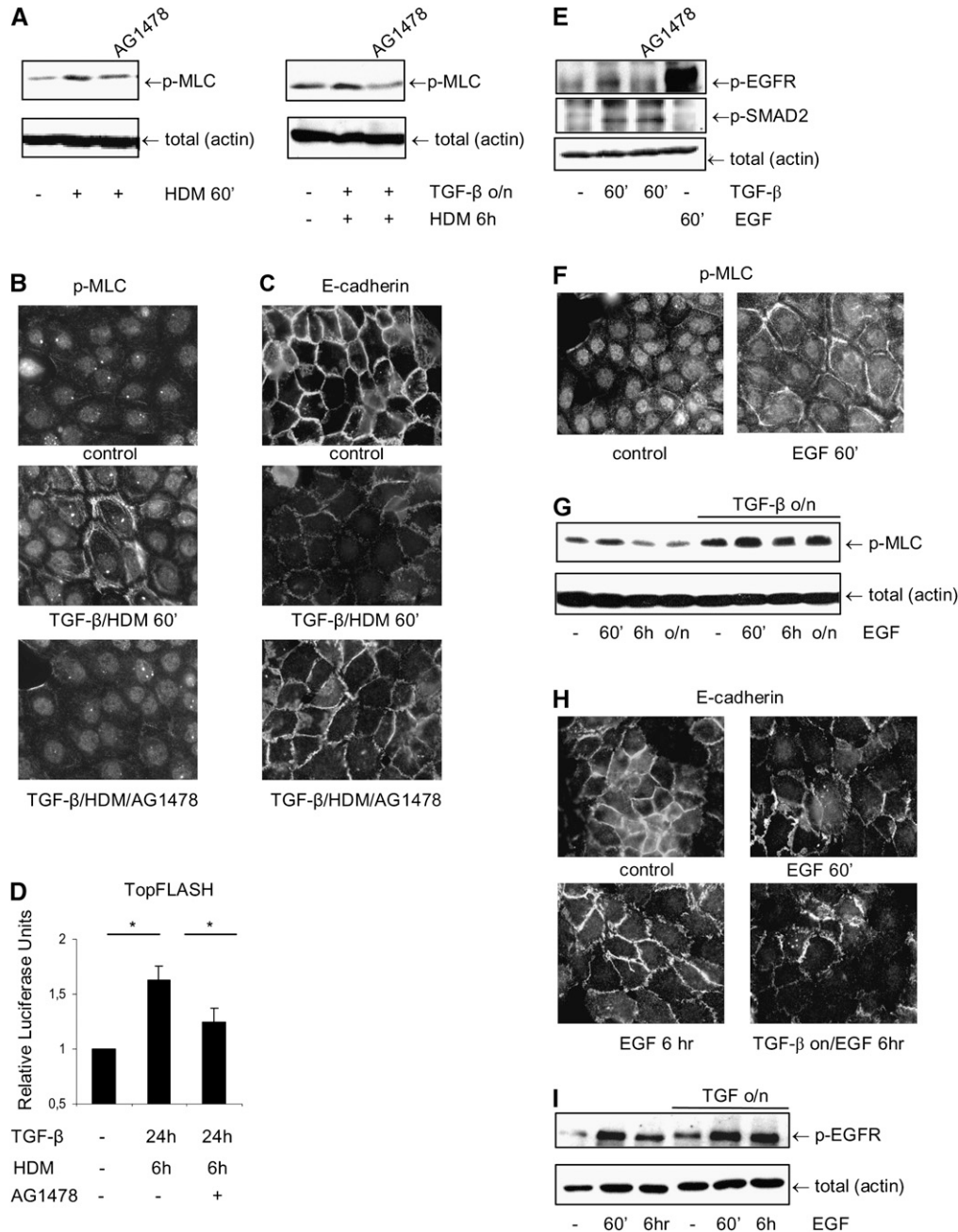


Figure 5. The effects of TGF- β and HDM require EGFR activation. 16HBE cells were cultured in 12-well plates, serum deprived for 4 hours, and incubated with or without EGFR inhibitor AG1478 (1 μ M) for 60 minutes. Cells were stimulated with TGF- β (5 ng/ml) for 60 minutes or 20 to 24 hours or pretreated with TGF- β (5 ng/ml) overnight and subsequently exposed to or HDM (50 μ g/ml) or treated with EGF (10 ng/ml) as indicated. (A) Total cell lysates were prepared and phospho-MLC was detected by Western blotting (arrow). (B, C) Cells were grown on coverslips. Phospho-MLC and E-cadherin were detected by immunofluorescent staining. (D) Cells were seeded in duplicates in 24-well plates, grown overnight, transfected with TopFLASH and the Renilla luciferase vector pRL-TK and stimulated as indicated. Luciferase activity was expressed as means $\pm$ SEM, $n = 3$. * $P < 0.05$ between the indicated values. (E) Total cell lysates were prepared and phospho-Smad2 and EGFR were detected by Western blotting (arrows). For A and E, β -actin was used as a control for equal loading. (F) Cells were grown on coverslips. Phospho-MLC was detected by immunofluorescent staining. (G) Total cell lysates were prepared and phospho-MLC was detected by Western blotting (arrow). (H) Cells were grown on coverslips. E-cadherin was detected by immunofluorescent staining. (I) Total cell lysates were prepared and phospho-EGFR was detected by Western blotting (arrow). Representatives of at least three independent experiments are shown.

supported by our preliminary histologic findings in patients with asthma (data unpublished), where we observed that decreased surface expression of E-cadherin in the bronchial epithelia is accompanied with increased positivity for FSP1, not only in the subepithelial layer, but also within the epithelium. This suggests that delocalization of E-cadherin *in vivo* is also associated with increased expression of this mesenchymal marker, a finding consistent with the possible contribution of EMT in to airway remodeling in asthma. Regarding other lung diseases, EMT has recently emerged to underlie progressive pulmonary fibrosis. Indeed, alveolar epithelium is known to undergo EMT and transform into myofibroblasts upon TGF- β exposure (32). It is noteworthy that bronchial epithelium seems to be less prone to this fulminate form of EMT. We did not observe up-regulation of the myofibroblast marker SMA in 16HBE cells, which might be due to the relatively high endogenous E-cadherin levels or the ectodermic origin of these cells. In any case, this is in

agreement with the scenario that EMT plays a role in the increased numbers of mesenchymal cells in asthma, accompanied by airway remodeling and increased epithelial permeability (10, 33), but does not lead to the development of progressive pulmonary fibrosis. While this manuscript was in preparation, a short report was published showing increased SMA expression in 16HBE cells upon TGF- β exposure (34). While this is in apparent discrepancy with our findings, it should be mentioned that SMA was shown only in a few cells by immunofluorescence, and additional mesenchymal markers were not investigated.

We observed that TGF- β up-regulates the mesenchymal markers fibronectin and vimentin and modestly down-regulates total levels of E-cadherin, without affecting the epithelial marker cytokeratin. We initially hypothesized that the proteolytic activity of HDM would disrupt cell contacts and subsequently induce the liberation of β -catenin from adherens

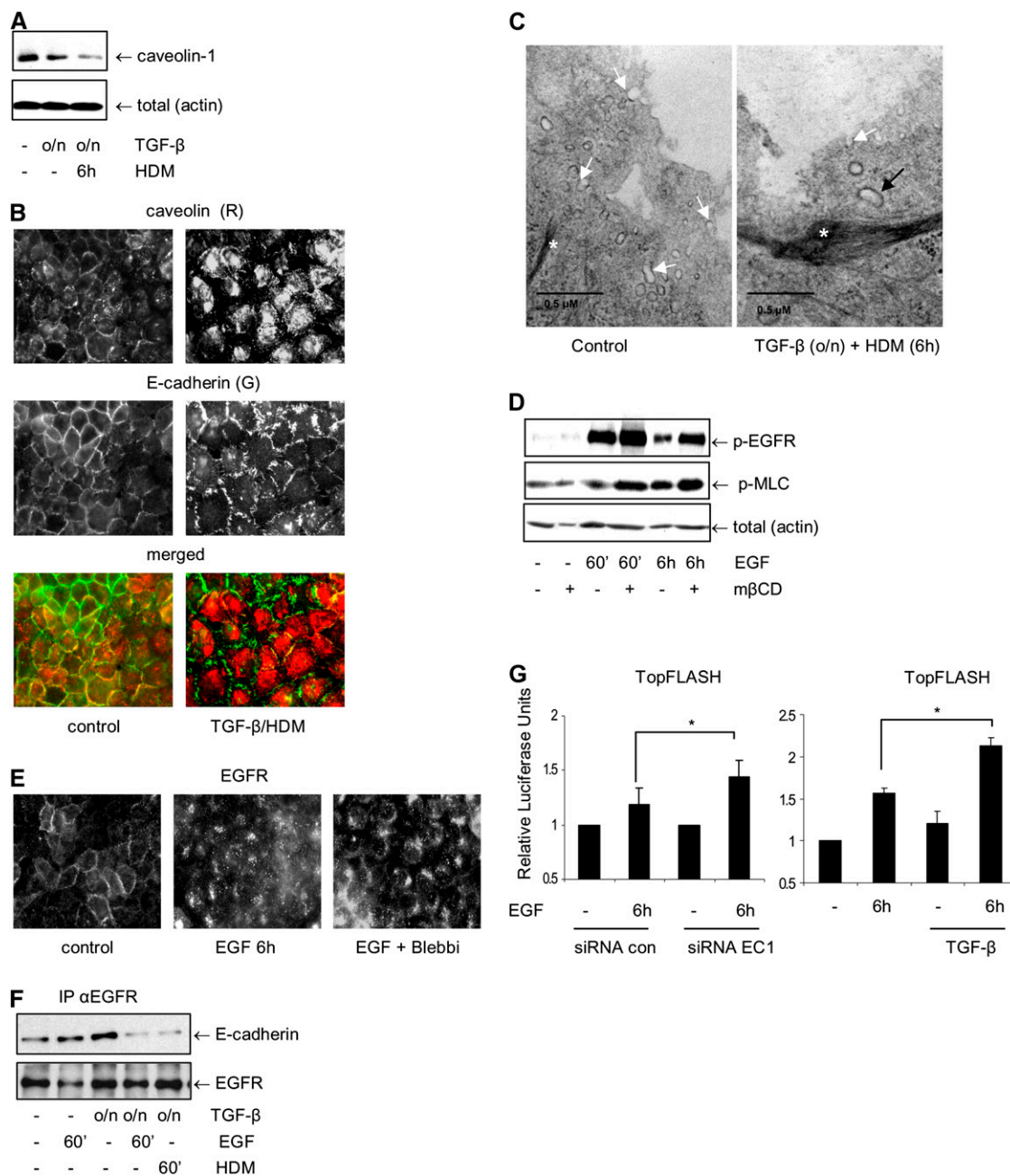


Figure 6. TGF- β preincubation may lead to disruption of caveolar rafts, prolonged EGFR signaling, and uncoupling between E-cadherin and EGFR in HDM- or EGF-stimulated cells. 16HBE cells were cultured in 12-well plates, serum deprived for 4 hours, cultured with TGF- β (5 ng/ml) overnight, and subsequently stimulated with EGF (10 ng/ml) or HDM (50 μ g/ml) as indicated, in the presence and absence of m β CD (500 μ M) or blebbistatin (100 μ M). (A) Total cell lysates were prepared and caveolin-1 was detected by Western blotting (arrow). (B) Cells were grown on coverslips. Caveolin-1 (red) and E-cadherin (green) were detected by immunofluorescent staining. (C) EM analysis of caveolae in 16HBE cells. Representative EM images are shown. The white arrows indicate caveolar structures, the black arrow indicates a coated pit, and the asterisks indicate filamentous actin. (D) Total cell lysates were prepared and EGFR, phospho-EGFR, and phospho-MLC were detected by Western blotting (arrows). (E) Cells were grown on coverslips and EGFR was detected by immunofluorescent staining. (F) Cells were grown in 6-well plates, serum deprived, stimulated as indicated, and subjected to immunoprecipitation with anti-EGFR antibody. Immune complexes were subjected to SDS 10% PAGE and stained with anti-E-cadherin as well as anti-EGFR for control. (G) Cells were seeded in duplicates in 24-well plates and transfected with nontargeting control siRNA (con) or siRNA specific for E-cadherin (EC1). After 3 days, cells were transfected with TopFLASH and pRL-TK and stimulated as indicated. Luciferase activity was expressed as means $\pm$ SEM ($n = 6$). * $P < 0.05$ between the indicated values. For A and D, β -actin was used as a control for equal loading. Representatives of at least three independent experiments are shown.

no fluorescent staining. (F) Cells were grown in 6-well plates, serum deprived, stimulated as indicated, and subjected to immunoprecipitation with anti-EGFR antibody. Immune complexes were subjected to SDS 10% PAGE and stained with anti-E-cadherin as well as anti-EGFR for control. (G) Cells were seeded in duplicates in 24-well plates and transfected with nontargeting control siRNA (con) or siRNA specific for E-cadherin (EC1). After 3 days, cells were transfected with TopFLASH and pRL-TK and stimulated as indicated. Luciferase activity was expressed as means $\pm$ SEM ($n = 6$). * $P < 0.05$ between the indicated values. For A and D, β -actin was used as a control for equal loading. Representatives of at least three independent experiments are shown.

junctions, allowing TGF- β to stabilize and transcriptionally activate β -catenin. However, HDM itself did not reduce membrane expression of E-cadherin. In contrast, when added to a TGF- β -primed epithelium, HDM not only induced robust changes in cellular distribution of E-cadherin and β -catenin, resulting in a significant increase in transcriptional activation of β -catenin, but also further increased vimentin and markedly reduced cytokeratin expression. This suggests that increased levels of TGF- β , as observed in the airways of patients with asthma, may enhance vulnerability of epithelial cells and induce structural changes in response to HDM. Our studies also allow insight in the molecular mechanisms of TGF- β - and HDM-

induced EMT. First, TGF- β and HDM together provoked sustained MLC phosphorylation, which proved to be critical for reorganization of adherens junctions, internalization of E-cadherin, and augmented β -catenin-driven transcription. This finding is in line with recent data showing that MLC plays a role in contact disassembly- and Src-induced redistribution of the E-cadherin/ β -catenin complex (16, 35). Our results imply that myosin-based contractility and reorganization of the actin cytoskeleton are critical for the destabilization of the E-cadherin-catenin complex by asthma-related stimuli. HDM and TGF- β induced differential patterns of MLC phosphorylation (i.e., stress fiber versus peripheral localization), and both of

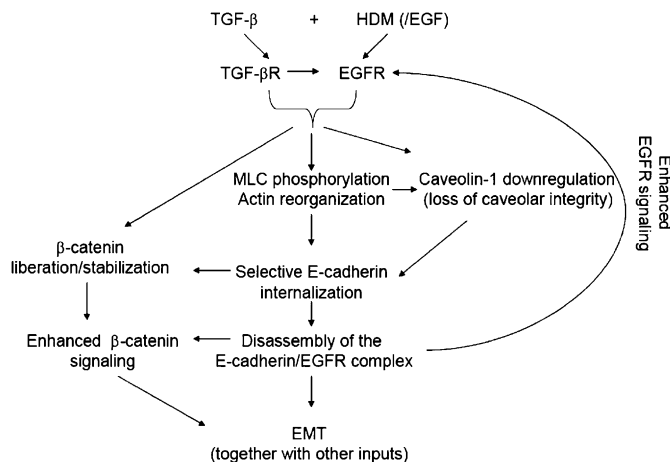


Figure 7. Proposed mechanisms of the interplay between EGFR and E-cadherin during HDM- (or EGF) plus TGF- β -induced mesenchymal transition. Activation of EGFR and TGF- β R induce sustained MLC phosphorylation, which is thought to promote E-cadherin internalization and the dissociation of E-cadherin and β -catenin. Concomitantly, the same stimuli induce F-actin reorganization-dependent loss of caveolar integrity and myosin-independent EGFR endocytosis. These processes facilitate the release of EGFR from E-cadherin (i.e., from its negative regulator), leading to enhancement of EGFR signaling in a positive feedback loop. The liberated and stabilized β -catenin drives the transcription of β -catenin-dependent (mesenchymal) genes, contributing to the ensuing EMT.

these changes seem to be required for the complete loss of surface connection of the E-cadherin/ β -catenin complex. In line with this, peripheral accumulation of phospho-myosin has been linked to Src-induced deregulation of E-cadherin and increased vimentin expression in colon carcinoma cells (36). The MLC-dependent transcriptional activation of β -catenin may target multiple promoters of mesenchymal genes, including fibronectin and vimentin (37, 38). In addition, there is some evidence that β -catenin/Lef-1/TCF signaling regulates Slug, a transcriptional repressor of E-cadherin, which was shown to negatively regulate cytokeratins (39, 40).

We also show that signaling through EGFR contributes to the MLC-dependent induction of a mesenchymal phenotype by HDM and TGF- β and that blocking of EGF signaling prevents crucial steps involved in the loss of epithelial characteristics, including down-regulation of E-cadherin from the cell surface. We previously reported that HDM activates EGFR, which may involve ADAM-dependent shedding of HB-EGF (19). TGF- β has been shown to transactivate EGFR, both through the release of HB-EGF and in a ligand-independent manner involving Src family tyrosine kinases (41, 42). Bidirectional cross-talk between EGF- and TGF- β -dependent signaling has been proposed to promote a migratory phenotype (10, 20, 43–45), while opposing effects of EGF and TGF- β on apoptosis and proliferation have been reported (46). Here, we show that facilitation of EGFR signaling by TGF- β might participate in the induction of a mesenchymal phenotype. The release of EGFR from negative regulation by (E-cadherin-enriched) caveolae and uncoupling between EGFR and E-cadherin may contribute to the prolonged EGFR signaling in presence of TGF- β . Our data suggest that loss of caveolar integrity destabilizes E-cadherin membrane localization and sustains EGFR signaling and downstream MLC phosphorylation (Figure 7). This may allow a further increase in β -catenin-driven transcription of mesenchymal genes in a self-augmenting process. In

future studies, it will be of interest to explore whether prolonged EGFR signaling leads to the maintenance of a mesenchymal phenotype instead of re-differentiation into a functional epithelium, which should normally occur upon tissue injury.

Together, our data indicate that TGF- β and HDM can induce mesenchymal transition in bronchial epithelial cells. TGF- β increases vulnerability to HDM, resulting in internalization of E-cadherin as well as transcriptional activation of β -catenin. TGF- β promotes the uncoupling between E-cadherin and EGFR, creating a positive feedback loop to increase the transcriptional activation of mesenchymal genes. The TGF- β - and allergen-induced changes may be a key component in the dysregulated repair mechanism of the airway epithelium in asthma.

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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