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Asbestos Exposure Induces Alveolar Epithelial Cell Plasticity Through MAPK/Erk Signaling

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ABSTRACT

The inhalation of asbestos fibers is considered to be highly harmful, and lead to fibrotic and/or malignant disease. Epithelial-to-mesenchymal transition (EMT) is a common pathogenic mechanism in asbestos associated fibrotic (asbestosis) and malignant lung diseases. The characterization of molecular pathways contributing to EMT may provide new possibilities for prognostic and therapeutic applications. The role of asbestos as an inducer of EMT has not been previously characterized. We exposed cultured human lung epithelial cells to crocidolite asbestos and analyzed alterations in the expression of epithelial and mesenchymal marker proteins and cell morphology. Asbestos was found to induce downregulation of E-cadherin protein levels in A549 lung carcinoma cells in 2-dimensional (2D) and 3D cultures. Similar findings were made in primary small airway epithelial cells cultured in 3D conditions where the cells retained alveolar type II cell phenotype. A549 cells also exhibited loss of cell–cell contacts, actin reorganization and expression of α-smooth muscle actin (α-SMA) in 2D cultures. These phenotypic changes were not associated with increased transforming growth factor (TGF)-β signaling activity. MAPK/Erk signaling pathway was found to mediate asbestos-induced downregulation of E-cadherin and alterations in cell morphology. Our results suggest that asbestos can induce epithelial plasticity, which can be interfered by blocking the MAPK/Erk kinase activity. J. Cell. Biochem. 113: 2234–2247, 2012.

KEY WORDS: EMT; ASBESTOS EXPOSURE ASSOCIATED DISEASES; FIBROSIS; CANCER

Asbestos is a common name for a group of naturally occurring hydrated silica fibers. Because of their tensile strength and resilient structural and chemical properties asbestos fibers have been used in various construction and insulating purposes. Asbestos exposure raises the risk for non-malignant inflammatory conditions such as pleural effusions, pleural plaques, and asbestosis (asbestos-induced lung fibrosis) [Kamp, 2009]. It is a severe condition in which loss of lung function can lead to death [Frank, 1980]. Malignant conditions associated with asbestos exposure include pleural and peritoneal mesothelioma and lung cancer. Diffuse malignant mesothelioma is a fatal tumor arising from mesothelial cells or underlying mesenchymal cells in the

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pleura, pericardium, and peritoneum [Mossman et al., 1990]. Asbestos-induced lung cancer is indistinguishable from that caused by tobacco smoke [Roggli and Sanders, 2000]. Although asbestos has been banned, the long latency preceding diseases together with the remaining risk for exposure during renovation and demolition works are expected to lead to increased incidence of asbestos exposure-related diseases worldwide [Huuskonen and Rantanen, 2006].

Epithelial-to-mesenchymal transition (EMT), a series of alterations occurring in cell architecture and behavior, is implicated in fibrotic diseases and in cancer progression. In EMT epithelial cells lose their epithelial characteristics and gain properties of mesenchymal cell [Thiery and Sleeman, 2006]. Cells undergoing EMT lose epithelial marker proteins such as the adherens junction protein E-cadherin and tight junction protein zonula occludens (ZO)-1 and begin to express mesenchymal proteins such as smooth muscle actin (SMA), collagen, vimentin, and fibronectin. Loss of epithelial markers and expression of mesenchymal marker proteins correlate with tumor progression and poor prognosis [Thiery, 2002; Peinado et al., 2004; Barrallo-Gimeno and Nieto, 2005; Moody et al., 2005; Thiery and Sleeman, 2006]. Loss of E-cadherin-dependent intercellular adhesion contributes to increased tumor cell motility and invasion into surrounding tissues [Brabletz et al., 2001; Guarino, 2007]. EMT can give rise to metastasizing cells from the primary tumors and contribute to metastasis development [Thiery, 2002; Huber et al., 2005].

After epithelial injury, EMT is likely to have a role in repair and scar formation [Willis and Borok, 2007]. In organ fibrosis including lung, kidney, eye, and liver, EMT may contribute to fibroblast accumulation and parenchyma destruction in advanced fibrosis [Guarino et al., 2009]. Markers of EMT have been identified from epithelial cells of idiopathic pulmonary fibrosis (IPF) patients [Willis and Borok, 2007]. Asbestosis and IPF exhibit many similarities including histopathology of usual interstitial pneumonia (UIP). These diseases may share common pathogenic mechanisms.

Despite active research, understanding of molecular mechanisms underlying the pathogenesis of asbestos-associated malignancies, and of fibrotic lung diseases has remained insufficient for the development of efficient treatments. Increasing evidence has recognized reactive oxygen species (ROS) and reactive nitrogen species (RNS) as important mediators of asbestos-induced toxicity [Mossman and Churg, 1998; Shukla et al., 2003; Kamp, 2009]. These reactive species oxidate and/or nitrolysate proteins and DNA [Shukla et al., 2003] and alter cell signal transduction pathways. Increased levels of ROS as well as endoplasmic reticulum stress have been implicated in alveolar epithelial cell EMT [Zhong et al., 2011]. In addition, asbestos exposure can lead to increased production and activation of various growth factors and cytokines [Robledo and Mossman, 1999; Yin et al., 2007].

Because asbestos exposure can induce signaling pathways previously linked to epithelial cell plasticity, we hypothesized that asbestos may initiate EMT processes which contribute to the pathogenesis of fibrotic and malignant lung diseases. Here, we found that asbestos-induced EMT-like alterations in human A549 lung carcinoma cells. Primary human lung epithelial cells did not show similar alterations, which was likely due to loss of alveolar type II cell phenotype in 2D cultures. However, primary cells cultured in 3D exhibited abundant cell surface E-cadherin expression, surfactant protein production and were sensitive to asbestos-induced loss of E-cadherin protein expression. To identify possible sites of therapeutic interference, we analyzed in detail the mechanism of asbestos-induced phenotypic changes in A549 epithelial cells.

MATERIALS AND METHODS

GROWTH FACTORS, ANTIBODIES, AND INHIBITORS

Transforming growth factor (TGF)-β1 and tumor necrosis factor (TNF)-α were from R&D Systems. Mouse monoclonal anti-E-cadherin, mouse monoclonal Smad2/3, and rabbit polyclonal anti-ZO-1 antibodies were from BD Transduction Laboratories and Invitrogen, respectively. Rabbit polyclonal anti-phospho-42/44 MAP kinase (Thr202/Tyr 204) and mouse monoclonal anti-44/42 MAPK (Erk1/2) (3A7) antibodies were from Cell Signaling Technology, rabbit polyclonal antibody against NF-κB (p65, RelA) from Calbiochem. Rabbit polyclonal anti-β-tubulin and rabbit polyclonal anti-SP-D antibodies were from Santa Cruz Biotechnology. Rabbit polyclonal anti phospho-Smad2 was provided by Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam). Mouse monoclonal α-smooth muscle actin antibody was from NeoMarkers. The inhibitor of TGF-β superfamily type I activin receptor-like kinase (ALK) receptors, SB431542, was from Sigma and used at 6 μM concentration. PD98059 [inhibitor of MAP kinase kinase (MEK), used at 30 μM], SP600125 [inhibitor of c-Jun N-terminal kinase (JNK), used at 10 μM], JSH-23 (inhibitor of the nuclear translocation of NF-kB p65, used at 20 μM) were from Calbiochem.

TWO DIMENSIONAL CELL CULTURES

Primary human bronchial epithelial cells (NHBE) and small airway epithelial cells (SAEC) were from Lonza and cultured according to manufacturer's instructions. One batch of NHBE cells and several independent batches of SAECs were used in the experiments. Cells were used between passages 4 and 8. BEAS-2B cells are non-malignant immortalized human bronchial epithelial cells (ATCC). They were grown in LHC-9 medium according to manufacturer's instructions (Invitrogen). Human A549 lung epithelial cells (ATCC) originate from carcinoma tissue, but have retained some characteristics of alveolar type II epithelial cells. These cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Gibco) and antibiotics.

For experiments the cells were seeded on six-well plates and treated with increasing concentrations of crocidolite asbestos (obtained from the Finnish Institute of Occupational Health) or TGF-β1 for the indicated times. For longer incubations fresh culture medium was changed every 2–3 days. The experiments were performed under standard culture conditions. Only short stimulations for the analysis of Smad2 phosphorylation were performed under serum-free conditions. The inhibitors were added to the medium 1 h prior to stimulation where indicated. Images of cells were captured using Axiovert 200 inverted epifluorescence

microscope and Axio-Cam HR camera (Zeiss) at the Biomedicum Imaging Unit of the University of Helsinki.

THREE DIMENSIONAL CELL CULTURES

Three dimensional (3D) cultures were generated using Matrigel basement membrane matrix (Beckton-Dickinson). Ice cold Matrigel was added to chambered coverglasses (Nunc) on ice and allowed to solidify at 37°C for 2 h. Subconfluent A549 or SAEC cells were trypsinized and seeded on top of Matrigel (5×10^4 cells/chamber). Normal complete culture medium was used to produce the polarized 3D structures. New medium was added every 2–3 days. Asbestos and TGF-β1 treatments were started on 3- to 7-day-old cultures and continued for 1 week.

GENERATION OF IκB 32/36A EXPRESSING STABLE CELL LINES

Retroviral vector pBabePuro was from Addgene and the construct pBabePuro IκB32/36A expressing a dominant-negative mutant of IκB was kindly provided by Juha Klefström (University of Helsinki), dominant negative function described in [Traenckner et al., 1995]. Retroviruses were produced in 293GPG packaging cell line [Ory et al., 1996]. Briefly, the producer cells were transfected overnight using Fugene transfection reagent (Roche) followed by collection of cell-conditioned medium, which was then used to transduce A549 cells. Puromycin selection was started 2 days after transduction.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

Cells to be transfected were seeded in six-well plates. The next day the cells were co-transfected with a total of 2 μg of promoter constructs (CAGA)12-luc (Smad3 responsive) or ARE-luc (Smad2 responsive) and Fast-1 provided by Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam) together with pRL-TK (Renilla Luciferase control, Promega) plasmid using Fugene HD transfection reagent (Roche). The pRL-TK plasmid contains the *Renilla* luciferase gene under the control of a constitutively expressed thymidine kinase promoter, and was used in dual luciferase assays to normalize the transfection efficiency. Dual luciferase based Cignal Finder 10-Pathway Cancer Reporter Array (SABioscience) was used according to manufacturer's instructions. Cells were lysed and subjected to luciferase activity measurements by Dual Luciferase Reporter Assay (Promega) and DCR-1 luminometer (MGM Instruments Digene Diacnostics Inc.). Treatment with TGF-β or asbestos was not found to unspecifically alter luciferase activity of the promoter constructs.

RNA ISOLATION AND QUANTITATIVE RT-PCR

Total cellular RNA was isolated using RNeasy Mini kit (Qiagen) and reverse transcribed to cDNA using Random hexamer primers (Invitrogen) and Superscript III reverse transcriptase (Life Technologies) according to manufacturer's instructions. The cDNAs were amplified using TaqMan Assays-on-Demand gene expression products (Applied Biosystems) and CFX96 Real-time PCR detection system (BioRad). Control amplifications directly from RNA were performed in order to rule out DNA contamination. The levels of gene expression were determined using the C_t method, and the results have been expressed as mRNA expression levels normalized to the levels of a gene with a constant expression (TBP, TATA-binding protein).

SDS–PAGE AND IMMUNOBLOTTING

Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.2% Na-deoxycholate) containing protease inhibitors (Roche Diagnostics) on ice for 15 min. For the analysis of Smad2 phosphorylation cells were lysed in lysis buffer (20 mM Tris–HCl, pH 8, 120 mM NaCl, 0.5% NP-40, 1 mM Na_3VO_4) containing protease inhibitors (Roche Diagnostics) on ice for 15 min. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce). Equal amounts of protein were separated by SDS–PAGE using 10% or 4–20% gradient Tris–glycine gels (Lonza) and transferred to Protran nitrocellulose membranes (Whatman) using a semi-dry blotting system (Bio-Rad). Membranes were first blocked with 5% non-fat milk in TBS/0.05% Tween-20 to prevent non-specific binding of the antibodies and then incubated with the primary or biotin-conjugated secondary antibodies (DAKO) in TBS/5% BSA-0.05% Tween-20 at room temperature for 1 h. After several washing steps, the final detection was performed using HRP-conjugated streptavidin and an enhanced chemiluminescence Western blotting detection system (Amersham). Three independent experiments were included for the analyses of protein band intensities with the Scion Image analysis program (Scion Corporation).

IMMUNOFLUORESCENCE ANALYSIS AND MICROSCOPY

Cells were seeded on glass coverslips and cultured for the indicated times. For the detection of E-cadherin, NF-κB (p65), or F-actin cells were fixed with 4% paraformaldehyde/PBS at room temperature for 10 min. For the detection of ZO-1, the cells were fixed with methanol at −20°C for 15 min. Nonspecific binding of the antibodies was prevented by blocking with 5% BSA/PBS for 30 min. During blocking 0.3% Tx-100 was added when permeabilization of cells was needed. All antibody dilutions and washing steps were made in Dulbecco's PBS containing 0.5% BSA. The cells on coverslips were incubated with the primary antibodies for 1 h at room temperature and washed. The bound primary antibodies were detected using AlexaFluor secondary antibodies (Molecular Probes). TRITC-conjugated phalloidin (Sigma) was used for the detection of F-actin. Finally, the coverslips were washed with water and mounted on glass slides with Vectashield mounting medium containing DAPI (Vector Laboratories). Images were captured with Axioplan 2 epifluorescence microscope and Axio-Cam HR camera using Axiovision 4.5 software (Zeiss) at the Biomedicum Imaging Unit of the University of Helsinki.

3D cultures in Matrigel were fixed with 4% paraformaldehyde/PBS for overnight at room temperature. Fixed gels were embedded in paraffin and cut into 4 μm sections, which were then deparaffinized in xylene and rehydrated in graded alcohol. Antigens were retrieved by heating in 10 mM citrate buffer (pH 6.0). The sections were blocked with 5% non-fat milk/PBS containing normal goat serum (Jackson Immuno Reseach) at room temperature for 30 min followed by incubation with primary antibodies diluted in blocking buffer at +4°C overnight. The sections were then incubated with AlexaFluor secondary antibodies at room temperature for 1 h,

washed several times and mounted using Vectashield Hard Set mounting medium. The nuclei were visualized with DAPI. The images were captured as described above. Digital morphometry was conducted using Image Pro Plus 7.0 (Media Cybernetics) program. A representative picture from the control group was used to generate a filter for the detection of E-cadherin fluorescence intensity relative to unstained area in the 3D structure. Six to 10 independent pictures of each group were analyzed using this filter. Images were captured from H&E and SP-D stained sections with Olympus BX51 and Artray Artcam 300 MI.

STATISTICAL ANALYSIS

Data were analyzed using PASW Statistics 18 program for Windows. Statistical differences in E-cadherin levels in 3D cultures were evaluated using the nonparametric Kruskal–Wallis test (A549 cultures, all groups) or Mann–Whitney *U*-test (SAEC cultures, asbestos-exposed group vs. control group). Differences in mRNA expression levels (asbestos-exposed group vs. control group) and E-cadherin immunoblot band intensities (asbestos-exposed group vs. control group and DMSO treated vs. PD98059 treated group) were analyzed with Mann–Whitney *U*-test. A *P*-value of ≤ 0.05 was considered statistically significant.

RESULTS

ASBESTOS EXPOSURE INDUCES DOWNREGULATION OF EPITHELIAL PROTEINS IN TRANSFORMED LUNG EPITHELIAL CELLS

To test whether asbestos can induce EMT and downregulation of epithelial proteins we exposed cultured lung epithelial cells to crocidolite asbestos (0.5–5 $\mu g/cm^2$) under standard culture conditions for 3 days followed by analyses of E-cadherin protein levels by immunoblotting. TGF-β1, a known inducer of EMT, was used as a control. In primary human bronchial epithelial cells (NHBE) and small airway epithelial cells (SAEC) as well as in immortalized but non-malignant human bronchial epithelial cells (BEAS-2B) neither asbestos nor TGF-β1 treatment was able to alter E-cadherin protein levels (Fig. 1A). In contrast, in human lung carcinoma cells (A549) E-cadherin levels were clearly decreased when treated with asbestos or TGF-β1. Interestingly, TGF-β1 induced E-cadherin downregulation already after 1 day, while asbestos-induced downregulation was not detected until after 3 days (Fig. 1B).

Loss of E-cadherin is considered to be a universal marker for the loss of the epithelial phenotype. In monolayer cultures only in A549 carcinoma derived cells asbestos seemed to induce alterations consistent with the initiation of EMT. We addressed the effects of asbestos on the cell surface localization of epithelial junction proteins in A549 cells next. Cells grown on glass coverslips were exposed to asbestos (5 $\mu g/cm^2$) or TGF-β1 (2 ng/ml) for 3 days after which the cells were fixed and stained with antibodies specific for E-cadherin or the tight junction protein ZO-1. Asbestos exposure induced a significant downregulation of cell surface E-cadherin consistent with the immunoblotting result (Fig. 2). ZO-1 staining was also decreased or lost from cell contacts in asbestos-treated cells. As expected, TGF-β1 induced similar alterations in these epithelial junctional proteins [Kasai et al., 2005].

E-cadherin mRNA levels were examined by quantitative RT-PCR using specific primers (Materials and Methods section). Unexpectedly, A549 cells exposed to asbestos for 3 or 6 days showed no downregulation of E-cadherin mRNA levels (Fig. 1C). In contrast, TGF-β1 treated cells expressed clearly lower levels of E-cadherin mRNA, suggesting that TGF-β1 and asbestos mediate their effects via different mechanisms. Transcription factors Snail and Slug have been implicated in EMT associated downregulation of E-cadherin transcription, and their mRNA levels were analyzed next. As expected, TGF-β1 induced dramatically the expression of Snail and especially of Slug by 3 days (Fig. 1D). The expression was further increased by 6 days. Unlike TGF-β1, asbestos at 3 days had no effect and at 6 days induced only a very modest increase in the expression of these transcription factors, which is consistent with E-cadherin mRNA expression levels. Further analysis of early time points revealed induction of both transcription factors by TGF-β1 already after 1 h stimulation, but no alterations during asbestos exposure (Supplementary Fig. 1).

The BMP inhibitor gremlin is a TGF-β target gene involved in the induction of EMT processes [Koli et al., 2006; Lee et al., 2007]. To analyze whether asbestos exposure can increase gremlin expression in A549 cells we analyzed gremlin mRNA levels by quantitative RT-PCR. Gremlin mRNA levels in asbestos exposed A549 cells were found to be comparable to control levels (Supplementary Fig. 2). In contrast, TGF-β1 increased gremlin mRNA levels nearly fivefold at 3 days and over 10-fold at 6 days.

EPITHELIAL PHENOTYPE IS ALTERED BY ASBESTOS ALSO IN THREE DIMENSIONAL A549 CULTURES

In three-dimensional cultures cells adopt a morphology, which mimics better in vivo situations. The structural organization of cells can be protective against exogenous signals [Schmeichel and Bissell, 2003], which prompted us to study asbestos-induced alterations in A549 cells cultured on Matrigel (Materials and Methods section). Cells cultured for 2 weeks without stimulatory factors formed well-organized structures with polarized cells displaying abundant E-cadherin staining around a central lumen (Fig. 3A). Asbestos or TGF-β1 treatments were initiated on 1-week-old cultures and continued for another week. TGF-β1 was found to alter the appearance of the 3D structures, making them more scattered and less well organized. In control cultures, cells disappeared from the centre of the structure, which was less pronounced in the TGF-β-exposed cultures (Fig. 3A). In addition, the cell surface E-cadherin levels were significantly decreased in asbestos-treated cultures (from ~60% of a total area in the structures to ~35%, Materials and Methods section) and even more in TGF-β1 treated cultures (from ~60% to ~15%; Fig. 3B). These results suggest that the epithelial marker E-cadherin is significantly decreased by asbestos exposure also in three-dimensional cultures consistent with the initiation of EMT.

INDUCTION OF MESENCHYMAL CHARACTERISTICS IN A549 CELLS BY ASBESTOS EXPOSURE

We investigated next whether asbestos exposed cells gained mesenchymal characteristics typical for cells undergoing EMT. Alterations in cell morphology were first analyzed in monolayer

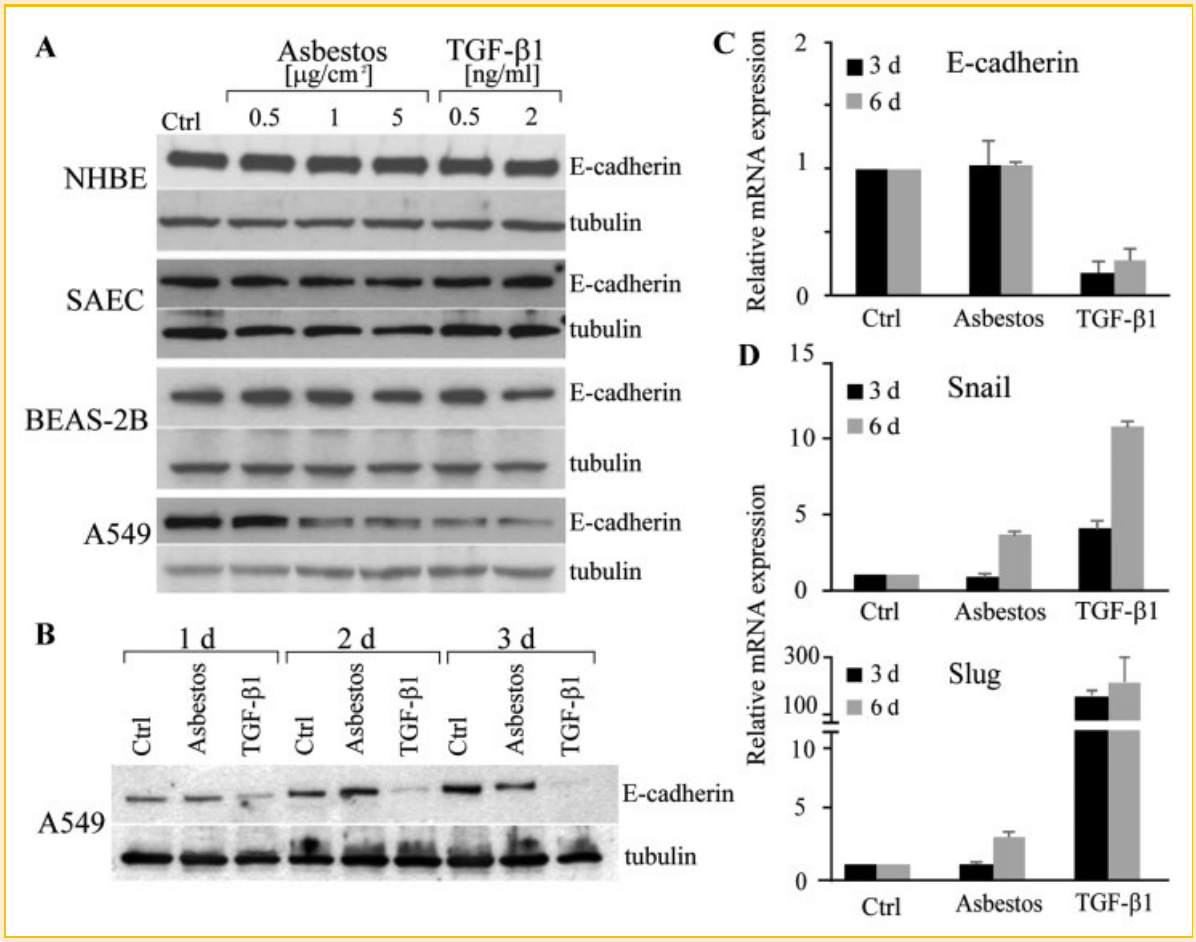

Fig. 1. Asbestos exposure leads to downregulation of E-cadherin protein levels in A549 lung carcinoma cells. A: Primary human bronchial epithelial cells (NHBE), small airway epithelial cells (SAEC), immortalized non-malignant bronchial epithelial cells (BEAS-2B), and lung carcinoma cells (A549) were exposed to asbestos or TGF-β1 for 3 days followed by analyses of E-cadherin protein levels by immunoblotting. Tubulin was used as a loading control. B: A549 cells were exposed to asbestos or TGF-β1 for the indicated times followed by analyses of E-cadherin protein levels. C,D: A549 cells were exposed to asbestos as indicated. Total cellular RNA was isolated followed by quantitative RT-PCR analyses of E-cadherin (C) or Snail and Slug (D) mRNA expression levels. Expression levels were normalized to the expression levels of TBP and are expressed relative to control. Error bars represent standard deviation of the means (n = 2).

cultures exposed to asbestos for 3 days. Asbestos exposure was found to induce the loss of the typical cobblestone-like appearance of A549 cells, which became scattered and more elongated (Fig. 4A). Asbestos exposed cells also frequently exhibited larger intercellular space and less cell–cell contacts. Alterations in actin organization were visualized by phalloidin staining, which showed re-organization of actin into longitudinal stress fibers (Fig. 4A).

The expression of mesenchymal genes associated with EMT was analyzed next. A549 cells were exposed to asbestos for 3 days, total cellular RNA was extracted and the expression levels of vimentin, fibronectin (FN), N-cadherin, collagen 1 (Col1A1), and α-smooth muscle actin (α-SMA) were measured by quantitative RT-PCR. Asbestos exposure-induced fourfold increase in α-SMA and threefold increase in collagen 1 mRNA levels (Fig. 4B). However, only the induction of α-SMA expression reached statistical significance. Vimentin, FN, and N-cadherin mRNA levels were not altered significantly. To analyze alterations in α-SMA protein levels, cells were exposed to asbestos for 3 or 6 days followed by immunoblotting analyses. Consistent with mRNA expression results, asbestos exposure increased α-SMA protein levels (Fig. 4C). The results suggest that in addition to downregulation of epithelial proteins, asbestos is able to alter epithelial cell morphology and induce the expression of certain profibrotic genes as well as proteins associated with the progression of EMT.

ASBESTOS-INDUCED DOWNREGULATION OF E-CADHERIN IN A549 CELLS IS NOT ASSOCIATED WITH INCREASED TGF-β ACTIVATION

Asbestos has been reported to induce gene transcription of TGF-β and other cytokines [Yin et al., 2007]. In addition, ROS are generated during asbestos exposure [Mossman and Marsh, 1989] and asbestos generated ROS may activate latent forms of TGF-β [Pociask et al., 2004]. TGF-β signals mainly through Smad2 and Smad3 transcription factors, which are phosphorylated and transported into the nucleus upon ligand binding [Nakao et al., 1997]. To test whether asbestos increases TGF-β signaling activity in our model, A549 cells were transiently transfected with constructs containing luciferase gene under the control of TGF-β responsive promoters (Smad3

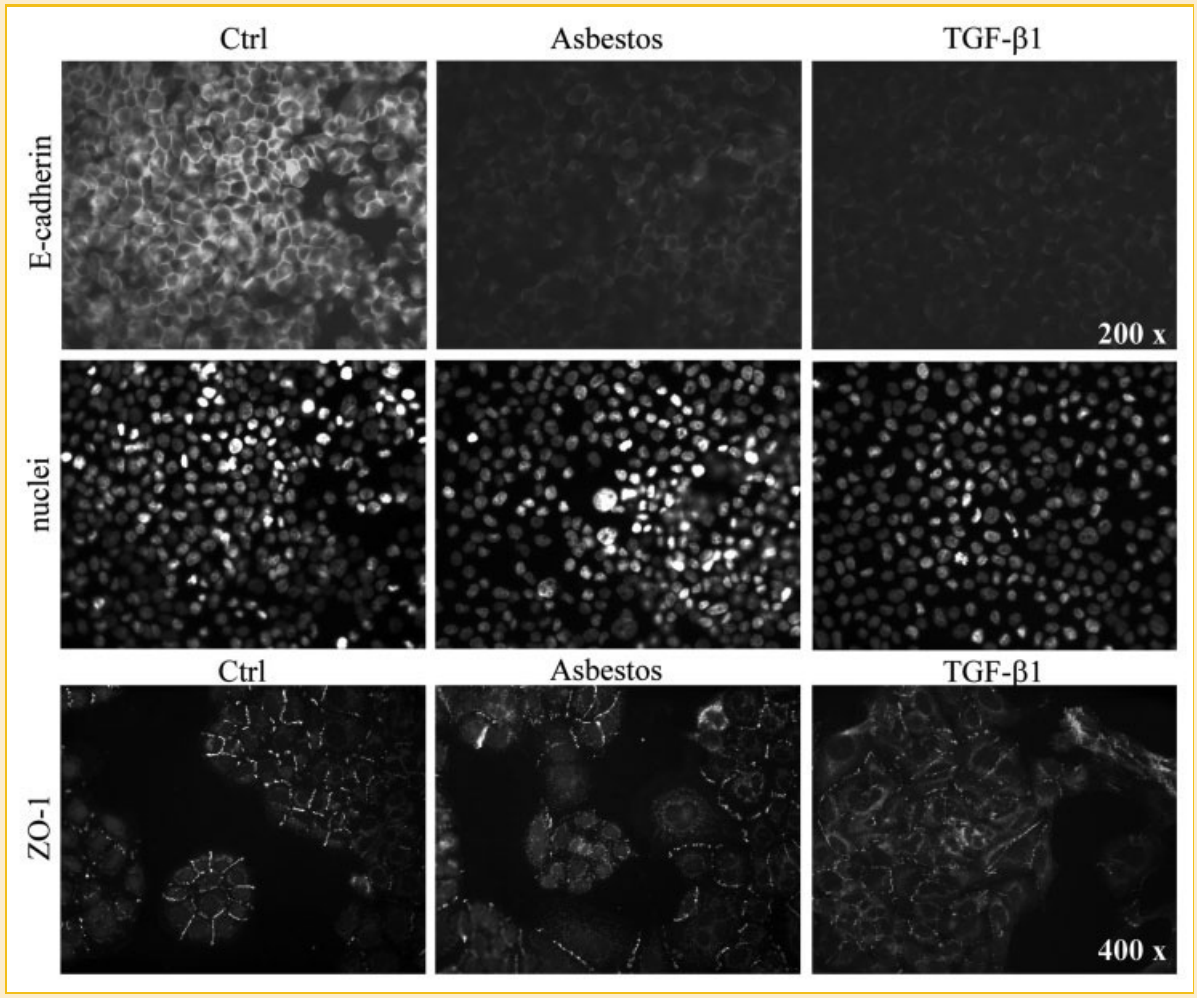

Fig. 2. Epithelial marker proteins decrease from cell junctions of A549 cells after asbestos exposure. A549 cells were seeded on glass coverslips, exposed to asbestos (5 μg/cm^2) or TGF-β1 (2 ng/ml) for 3 days, fixed and stained with antibodies specific for E-cadherin or ZO-1. DAPI staining was used to visualize nuclei. Original magnifications are indicated on the right.

responsive CAGA$_{12}$-luc or Smad2 responsive ARE-luc, Materials and Methods section) followed by exposure to asbestos and measurement of luciferase activity. Exposure to asbestos for 3 days was not able to induce CAGA$_{12}$-luc (Fig. 5A) or ARE-luc (not shown) activity in A549 cells, while TGF-β1 induced signaling activity as expected. To validate these results we analyzed Smad2 phosphorylation, that is, activation at early time points (1, 5, and 24 h) after asbestos or TGF-β1 stimulation. A549 cells were exposed to asbestos or TGF-β1 for the indicated times followed by immunoblotting analyses of P-Smad2. TGF-β1 induced phosphorylation of Smad2 peaked at 1 h and decreased thereafter (Fig. 5B). Asbestos, however, did not induce Smad2 phosphorylation at any time point.

TGF-β can also signal via Smad-independent pathways, which may contribute to cell plasticity. To further assess the possible role of TGF-β in asbestos-induced downregulation of E-cadherin, A549 cells were exposed to asbestos in the presence of SB431542, an inhibitor of TGF-β type I receptors. Interestingly, SB431542 had only a moderate effect on asbestos-induced downregulation of E-cadherin (Fig. 5C). In the presence of SB431542 asbestos was still able to induce significant downregulation of E-cadherin levels, while TGF-β1-induced downregulation was completely blocked as expected (Fig. 5D). The result suggests that asbestos-induced EMT-like phenotype in A549 cells does not depend on Smad2/3 signaling and is not fully explained by induction of TGF-β-mediated pathways.

MAPK/JNK, MAPK/Erk, AND NF-κB SIGNALING ACTIVITY DIFFER BETWEEN NORMAL AND TRANSFORMED CELLS

Cancer cells have typically high activity of cellular signaling pathways, which are silent or active at low levels in non-malignant cells. To screen for differences that make A549 cells more susceptible to asbestos-induced EMT-like alterations than primary cells (Fig. 1A), we compared the basal activity levels of selected cancer-associated signaling pathways in A549 cells and those of normal SAECs using a commercial Cancer Reporter promoter array (Materials and Methods section). Cells were transiently transfected with the promoter constructs and cultured for 2 days under standard culture conditions before the activity measurements. In addition to the p53 pathway, the signaling activities of

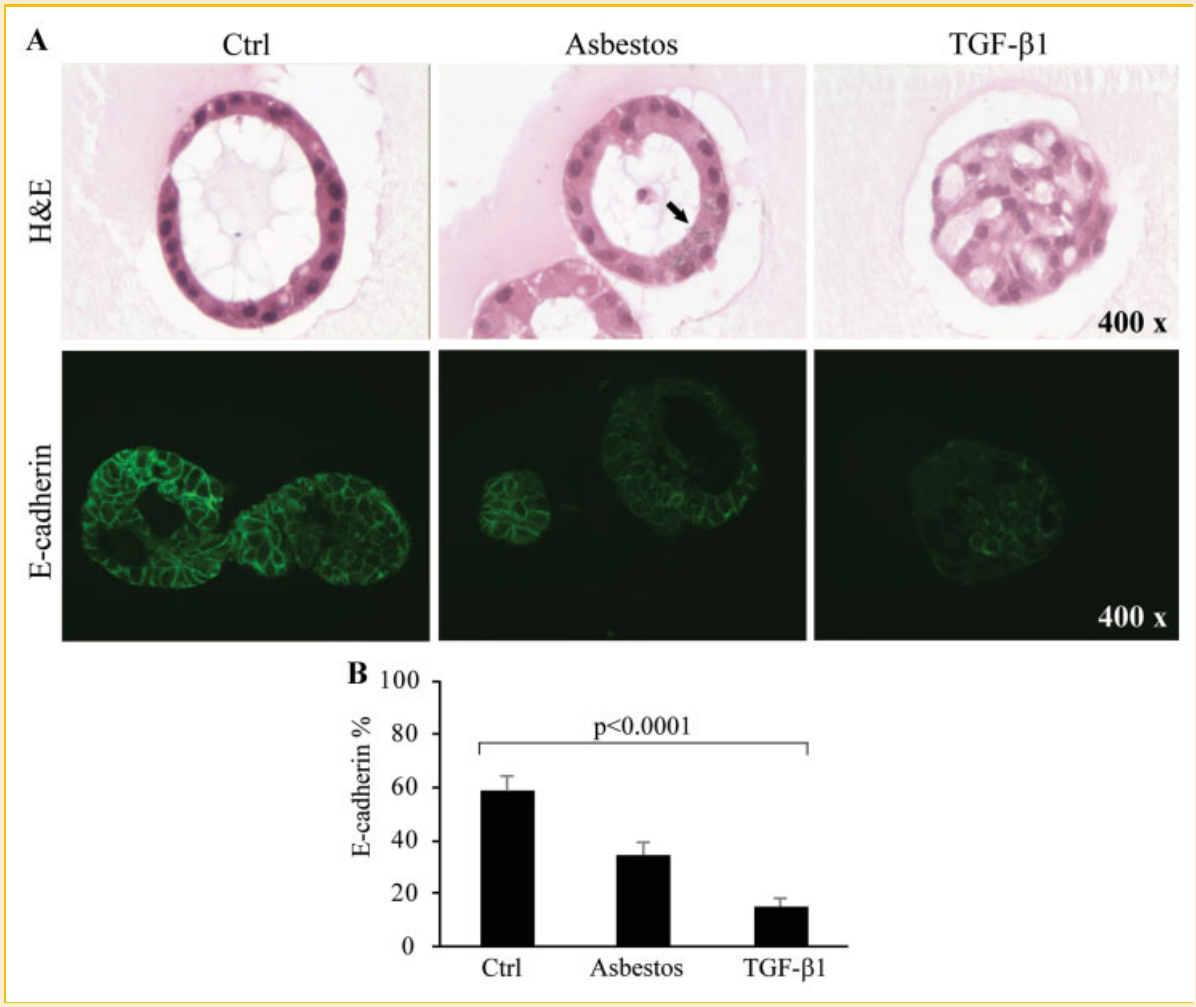

Fig. 3. Epithelial phenotype of A549 cells is altered by asbestos and TGF-β in three dimensional cultures. Cells were grown on Matrigel basement membrane matrix for 1 week followed by exposure to asbestos (5.0 μg/cm^2) or TGF-β1 (2.0 ng/ml) for another week. A: The structures were stained with H&E or antibodies specific for E-cadherin. Asbestos fibers detectable within the cells are indicated with an arrow. Magnifications are indicated on the right. B: Digital image analysis of the staining intensity of E-cadherin relative to non-stained cellular area in the structures. The amount of E-cadherin is expressed as percentage relative to non-stained cellular area. Error bars represent SEM of the samples ($n = 10$). E-cadherin positive area differ significantly between the groups (Ctrl, asbestos, TGF-β), $P < 0.0001$.

mitogen activated protein kinase/c-jun N-terminal kinase (MAPK/JNK), MAPK/extracellular signal-regulated kinase (Erk), and Nuclear factor kappa B (NF-κB) pathways were significantly elevated in A549 cells (Fig. 6A). These three pathways have been previously implicated in EMT and were selected for further analyses.

BLOCKADE OF NF-κB PATHWAY DOES NOT ALTER CELLULAR RESPONSES TO ASBESTOS

The role of NF-κB signaling in asbestos-induced responses in A549 cells was analyzed by blocking this pathway using a dominant negative inhibitor of nuclear factor kappa B (IκB) 32/36A expression construct. Mutated serines in IκB32/36A render it unsusceptible to phosphorylation, and therefore prevent its removal from its inhibitory association with NF-κB [Traenckner et al., 1995]. Stable A549 cell lines expressing IκB32/36A were generated by retroviral transduction (Materials and Methods section). Immunofluorescence analyses of the NF-κB subunit p65 showed that IκB32/36A expression inhibited TNF-α induced nuclear localization of p65 (Fig. 6B) suggesting efficient blockade of NF-κB pathway activity. A549 cells stably transduced with the dominant negative IκB exhibited severely decreased cell proliferation/survival (not shown). The use of JSH-23, a chemical inhibitor of the NF-κB pathway, also resulted in reduced number of cells with altered morphology (not shown) suggesting a role for NF-κB signaling in A549 cell survival. This may have an effect also on cellular responses to asbestos treatment. However, expression of IκB32/36A did not alter asbestos-induced E-cadherin downregulation (Fig. 6C) or α-SMA mRNA induction (Fig. 6D) in A549 cells.

MAPK/Erk PATHWAY CONTRIBUTES TO ASBESTOS-INDUCED EMT-LIKE TRANSITION IN A549 CELLS

The role of MAPK/Erk and MAPK/JNK pathways in asbestos-induced downregulation of E-cadherin was analyzed using chemical inhibitors of MAP kinase kinase MEK (PD98059, 30 μM) and c-Jun

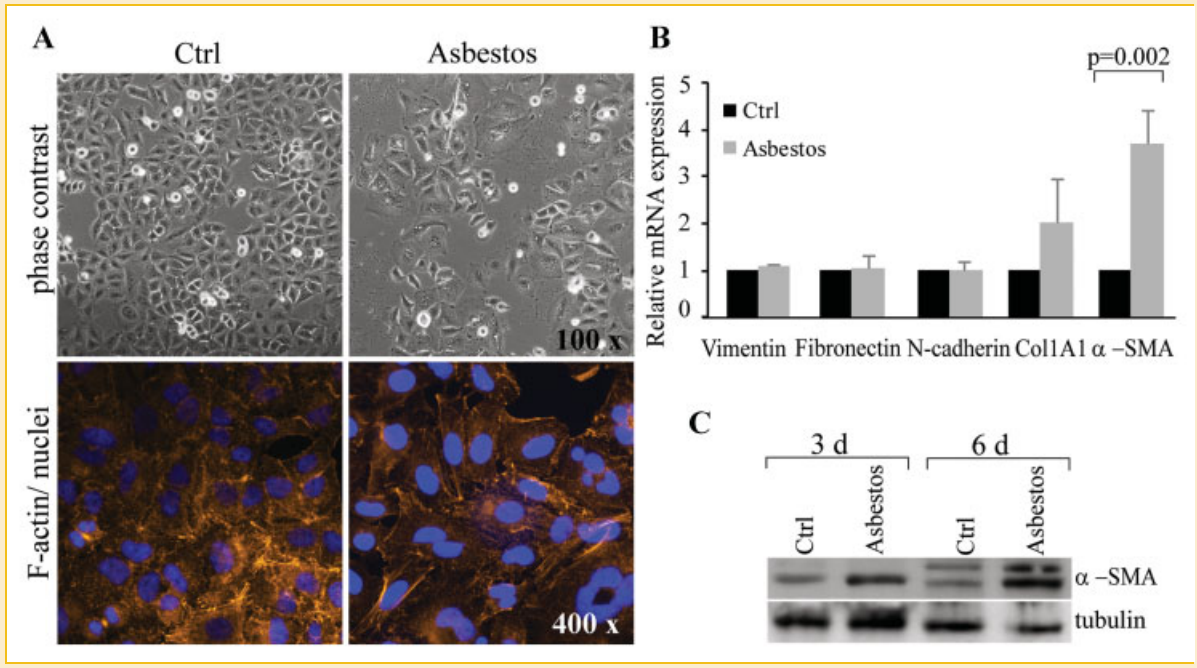

Fig. 4. Asbestos exposed cells gain mesenchymal characteristics. A: A549 cells seeded on culture plates or on glass coverslips, were exposed to asbestos (5 μg/cm^2) for 3 days. Live cells were imaged or the cells were fixed and stained with phalloidin to visualize F-actin. DAPI stain was used to visualize the nuclei. Original magnifications are indicated on the right. B: A549 cells were exposed to asbestos for 3 days. Total cellular RNA was isolated followed by quantitative RT-PCR analyses using specific primers for vimentin, fibronectin, N-cadherin, collagen 1A1 or α-smooth muscle actin (α-SMA). Expression levels were normalized to the expression levels of TBP and are expressed relative to control. Error bars represent standard deviation of the means ($n \geq 2$). C: A549 cells were exposed to asbestos (5 μg/cm^2) as indicated followed by analyses of α-SMA protein levels by immunoblotting. Tubulin was used as a loading control.

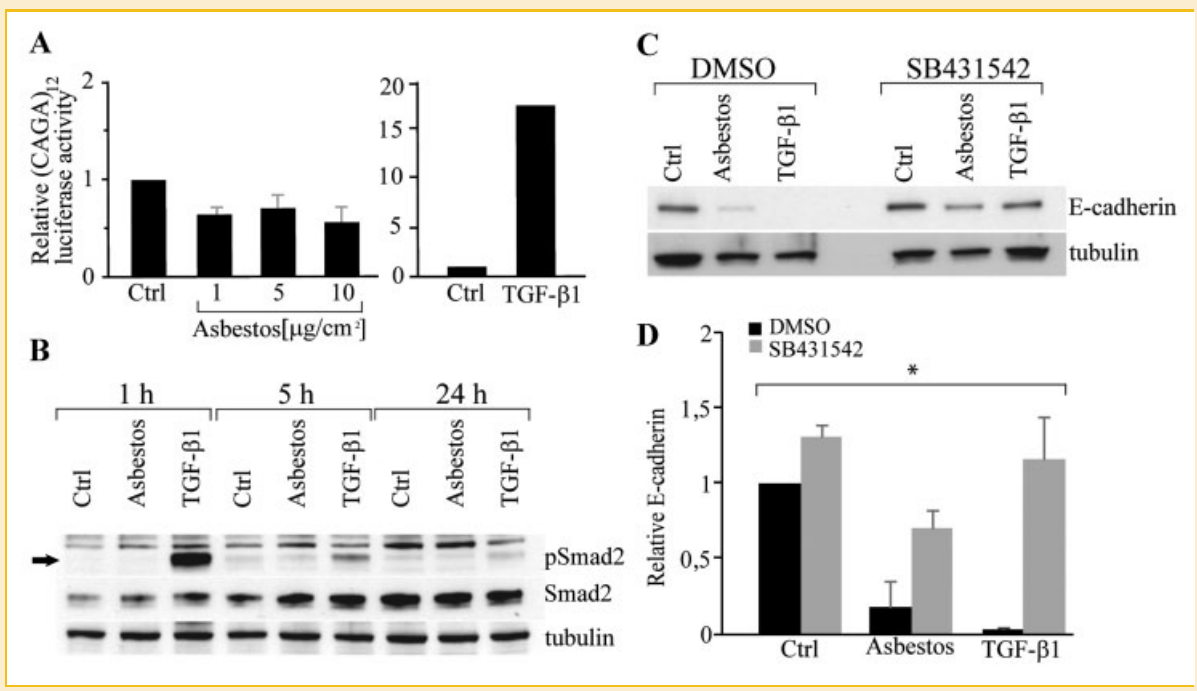

Fig. 5. Asbestos-induced downregulation of E-cadherin is not associated with increased TGF-β activity. A: A549 cells transiently transfected with Smad3 responsive (CAGA)12-luciferase promoter construct were exposed to asbestos (1–10 μg/cm^2) or TGF-β1 (100 pg/ml) for 3 days and subjected to luciferase activity measurement. The luciferase activities were normalized to constitutively expressed *Renilla* luciferase activities, and the results are expressed relative to control. The error bars represent standard deviation of the means ($n = 2$). A representative experiment of TGF-β induced luciferase activity is shown on the right. B: A549 cells were exposed to asbestos (5 μg/cm^2) or TGF-β1 (2 ng/ml) for the indicated times and analyzed for Smad2 phosphorylation (P-Smad2) or total Smad2 by immunoblotting. Tubulin was used as a loading control. C: A549 cells were exposed to asbestos (5 μg/cm^2) or TGF-β1 (2 ng/ml) for 3 days in the presence of an inhibitor of TGF-β type I receptors (SB431542, 6 μM) or DMSO used as a control followed by analyses of E-cadherin protein levels by immunoblotting. Tubulin was used as a loading control. D: E-cadherin band intensities were quantified and normalized to tubulin. The results are expressed relative to control. Error bars represent standard deviation of the means ($n = 3$). Normalized E-cadherin band intensities differ significantly between the groups (Kruskal–Wallis test), $^*P = 0.035$.

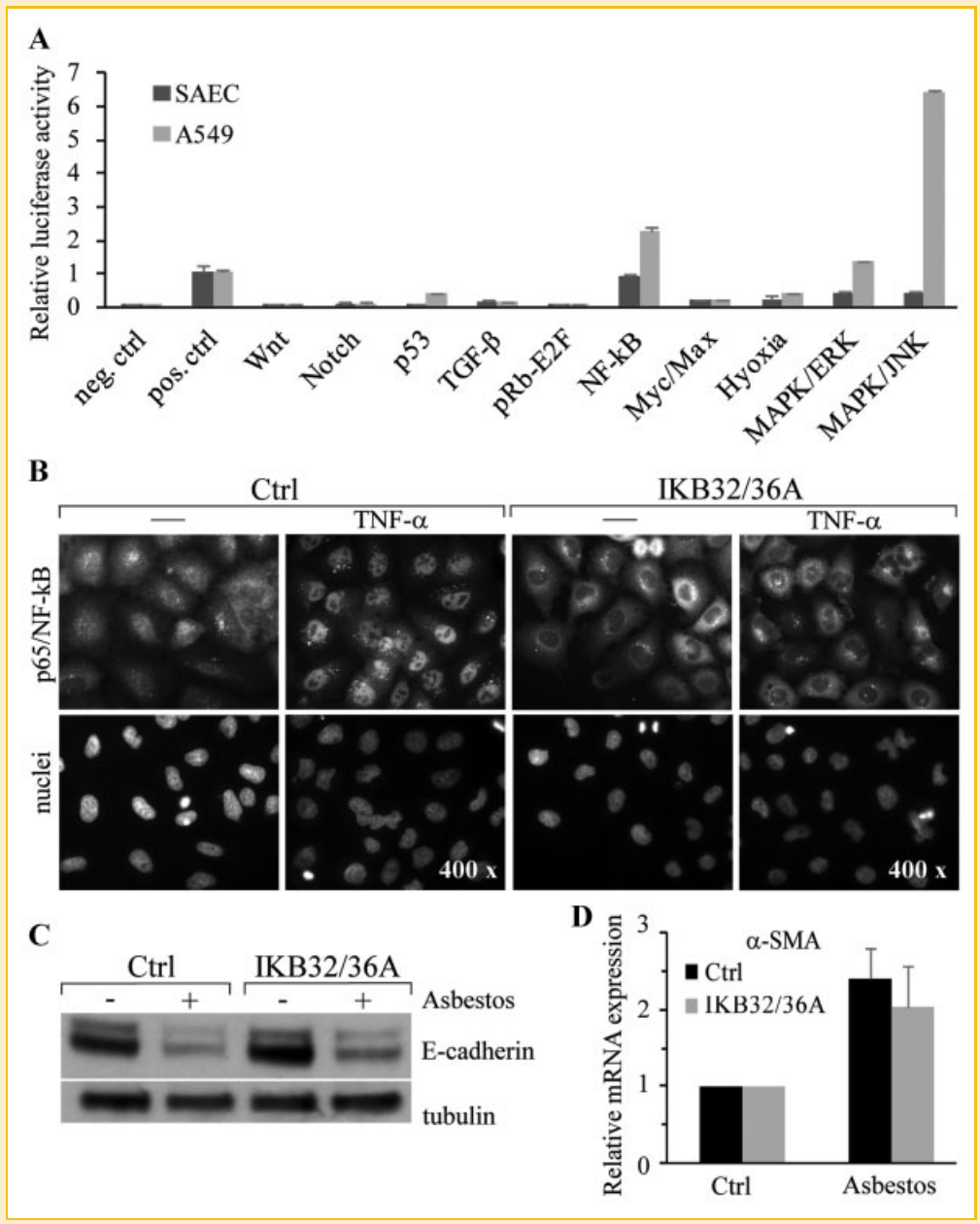

Fig. 6. The activity of MAP-kinases and NF-κB differ between SAECs and A549 cells but blockade of NF-κB pathway in A549 cells does not interfere with E-cadherin downregulation or α-SMA induction by asbestos. A: Basal activities of selected pathways were compared between A549 cells and primary human SAECs using a pathway reporter array. The results are expressed as relative activities and the values are normalized to the positive control. The error bars represent standard deviation of the means ($n = 2$). B: A549 cells transduced with either control virus or virus containing a dominant negative mutant of IκB (IκB32/36A) were seeded on glass coverslips, exposed to TNF-α (5 ng/ml) for 2 h, fixed and stained with p65 specific antibody to analyze NF-κB nuclear localization. Original magnification is indicated on the right. C: Stably virus transduced A549 cells were exposed to asbestos (5 μg/cm^2) for 3 days and analyzed for E-cadherin protein levels. Tubulin was used as a loading control. D: Stably virus transduced A549 cells were exposed to asbestos for 3 days, total cellular RNA was isolated followed by quantitative RT-PCR analyses using specific primer for α-smooth muscle actin (α-SMA). Expression levels were normalized to the expression levels of TBP and are expressed relative to control. Error bars represent standard deviation of the means ($n = 2$).

N-terminal kinase/stress-activated protein kinase JNK (SP600125, 10 μM). A549 cells were pre-treated with the inhibitors for 1 h before exposure to asbestos (5 μg/cm^2) under standard culture conditions for 3 days. Immunoblotting analyses suggested that inhibition of MAPK/Erk pathway during the exposure significantly reversed E-cadherin downregulation, while inhibition of MAPK/JNK pathway had no effect (Fig. 7A). Quantification of band intensities (E-cadherin/tubulin) showed statistically significant E-cadherin downregulation by asbestos in control (DMSO)-treated cells, which was reversed by PD98059 (Fig. 7B). Interestingly, PD98059 also

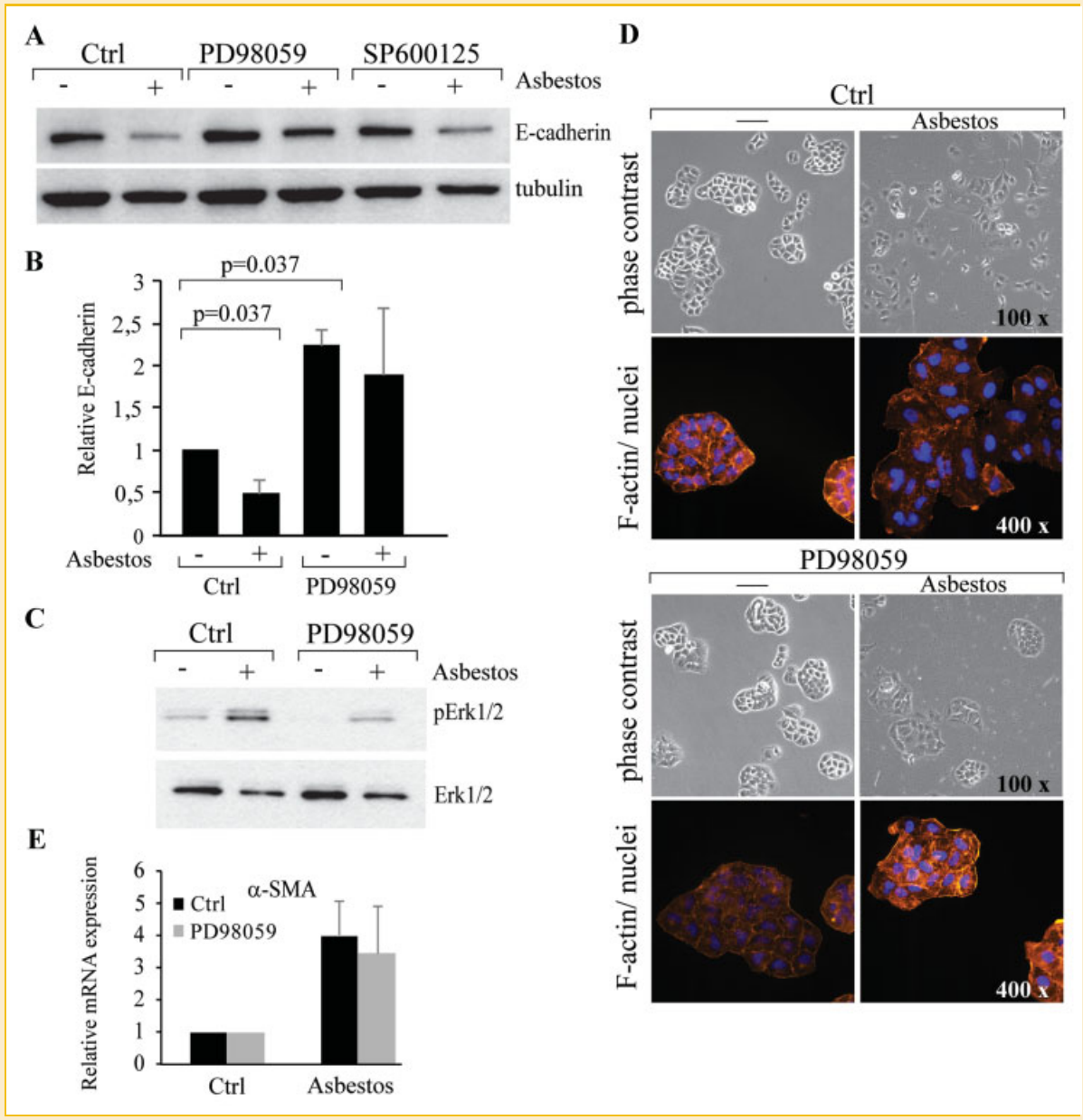

Fig. 7. MAPK/Erk1/2 pathway contributes to asbestos induced loss of epithelial phenotype in A549 cells. A: A549 cells were pre-treated with inhibitors of MAP kinase kinase MEK (PD98059, 30 μM) or JNK (SP600125, 10 μM) or treated with DMSO only (Ctrl) and exposed to asbestos (5 μg/cm^2) for 3 days followed by analyses of E-cadherin protein levels by immunoblotting. Tubulin was used as a loading control. B: E-cadherin band intensities were quantified and normalized to tubulin. The results are expressed relative to control. Error bars represent standard deviation of the means (n = 3). Normalized E-cadherin band intensities differ significantly between the groups (unexposed, asbestos), $P = 0.037$ and (unexposed Ctrl treated, unexposed PD98059 treated), $P = 0.037$. C: A549 cells were pre-treated with PD98059 or DMSO (Ctrl) and exposed to asbestos for 3 days followed by analyses of ERK1/2 phosphorylation, that is, activation or total ERK1/2 levels by immunoblotting. D: A549 cells were seeded on culture plates or on glass coverslips, pre-treated and stimulated as above. Live cells were imaged or the cells were fixed and stained with phalloidin to visualize F-actin. DAPI stain was used to visualize the nuclei. Original magnifications are indicated on the right. E: A549 cells were pre-treated and stimulated as above. Total cellular RNA was isolated followed by quantitative RT-PCR analyses using specific primer for α-smooth muscle actin (α-SMA). Expression levels were normalized to the expression levels of TBP and are expressed relative to control. Error bars represent standard deviation of the means (n = 3).

significantly increased the basal levels of E-cadherin (Fig. 7B) further pointing to a role for MAPK/Erk pathway in the regulation of E-cadherin levels. In agreement, asbestos exposure further enhanced Erk1/2 phosphorylation, which could be reduced to basal levels by the addition of PD98059 (Fig. 7C).

We analyzed next whether the inhibition of MAPK/Erk pathway could also inhibit asbestos-induced morphological alterations in A549 cells. PD98059 pre-treated cells were exposed to asbestos under standard culture conditions for 3 days, fixed and stained with phalloidin to visualize F-actin. Scattering of cells and loss

of cell–cell contacts induced by asbestos were almost completely blocked by the addition of PD98059 (Fig. 7D). However, α-SMA mRNA induction by asbestos exposure was not altered by inhibition of MAPK/Erk activity, suggesting that asbestos-induced mesenchymal gene expression is regulated by other signaling pathways (Fig. 7E).

PRIMARY SMALL AIRWAY EPITHELIAL CELLS RETAIN TYPE II CELL PHENOTYPE IN 3D CULTURES AND DOWNREGULATE E-CADHERIN IN RESPONSE TO ASBESTOS EXPOSURE

Bronchial epithelial cells have been suggested to be less susceptible to EMT than alveolar epithelial cells [Buckley et al., 2010], which may explain our results with monolayer cultures of NHBE and BEAS-2B cells. Furthermore, SAECs in monolayer cultures may spontaneously differentiate and lose alveolar type II cell phenotype, which can influence the sensitivity to EMT. Different interactions between the cells and with the environment in two and in three dimensional cultures are also likely to promote different responses [Schmeichel and Bissell, 2003]. Therefore, we cultured SAEC cells in 3D cultures on Matrigel to investigate the effects of asbestos in 3D environment. Cells seeded on top of Matrigel formed cell aggregates, which in 10 days re-organized into saccular-like structures. Cells facing the lumen were shown to produce surfactant protein D (Fig. 8A) suggesting that in 3D the alveolar type II cell phenotype is retained. These saccular-like structures exhibited abundant E-cadherin staining around the cells (Fig. 8B). Asbestos exposure was initiated on 3-day-old cultures and continued for 1 week. Cell surface E-cadherin levels were significantly decreased in asbestos-treated structures (from ~25% of a total area in saccular structure to ~7,5%; Fig. 8B,C). These results suggest that also normal primary SAEC cells are susceptible to asbestos-induced cell plasticity when cultured in an environment supporting alveolar type II cell phenotype.

DISCUSSION

The development of asbestosis is directly associated with the extent and length of asbestos exposure, but cancer can develop even at low pulmonary concentrations of asbestos fibers [Kamp, 2009]. Asbestos-related or other forms of pulmonary fibrosis, especially IPF, significantly elevate the risk for lung cancer [Kamp, 2009]. Interestingly, Vancheri et al. [2010] have recently suggested that IPF could be considered as a neoproliferative disorder of the lung since there are many similarities between the pathogenic hallmarks of IPF and cancer. Recapitulation of developmental programs such as EMT is one common feature which may play an important role in disease progression [Selman et al., 2008] and the characterization of molecular mechanisms contributing to EMT may provide new

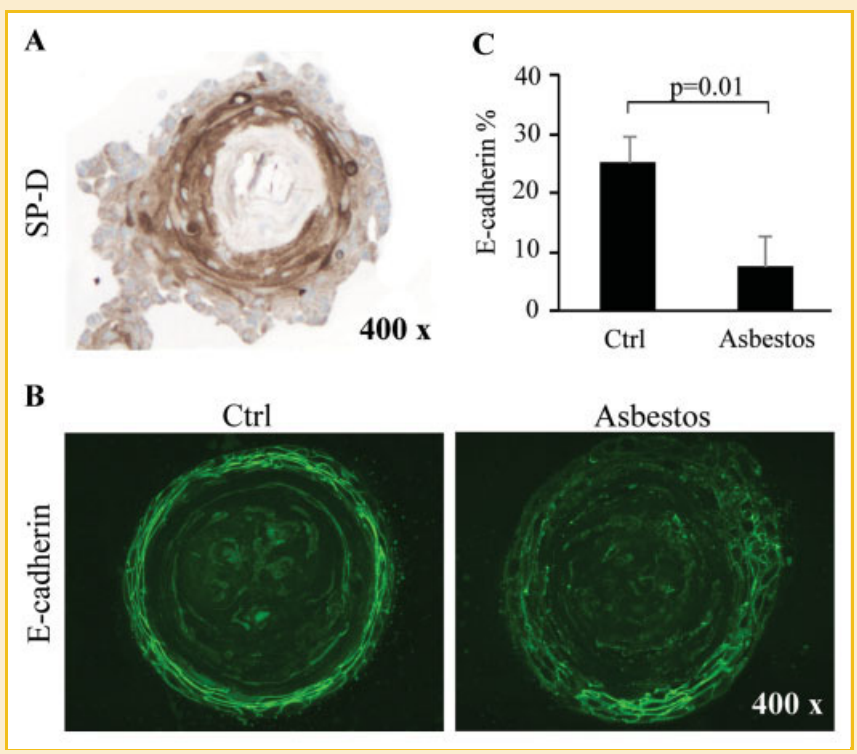

Fig. 8. SAECs retain alveolar type II cell phenotype in three dimensional cultures and downregulate E-cadherin in response to asbestos exposure. Cells were grown on Matrigel basement membrane matrix for 3 days followed by exposure to asbestos (5.0 μg/cm^2) for 1 week. A: Saccular structures were stained with antibodies specific for SP-D. B: Saccular structures were stained with antibodies specific for E-cadherin. Original magnifications are indicated on the right. C: Digital image analysis of the staining intensity of E-cadherin relative to non-stained cellular area in saccular structures. The amount of E-cadherin is expressed as percentage relative to non-stained cellular area. Error bars represent SEM of the samples ($n \geq 6$). E-cadherin positive area differ significantly between the groups (Ctrl, asbestos), $P = 0.01$.

possibilities for prognostic and therapeutic applications. We found here that asbestos exposure can lead to downregulation of epithelial proteins, changes in cell morphology and induction of certain profibrotic genes consistent with the induction of EMT. These molecular alterations may contribute to the progression of asbestos-associated fibrotic and malignant lung diseases.

In the current study, A549 adenocarcinoma cells but not cultured primary small airway or bronchial epithelial cells were induced to undergo EMT by exposure to asbestos or TGF-β under normal 2D culture conditions suggesting that normal cells are not very sensitive to epithelial transitions. This is in agreement with a previous report suggesting that normal mammary epithelial cells are not sensitive to TGF-β mediated EMT unless sensitized by ionizing radiation [Andarawewa et al., 2007]. However, recent studies have described TGF-β induced EMT also in primary human cells [Doerner and Zuraw, 2009; Camara and Jarai, 2010]. Different origin of the cells and differences in culture conditions may explain some of the differences between studies [Buckley et al., 2010]. Intracellular signaling pathways responsible for cell polarity are regulated in significantly different manner in cells depending on whether the cells are cultured in 2D or 3D environment [Schmeichel and Bissell, 2003]. A549 cells cultured on 2D display some features of type II epithelial cells and show cobblestone morphology with cell surface expression of E-cadherin. Primary SAECs, which have not been adapted to monolayer culture, showed abundant levels of E-cadherin but it was mainly localized intracellularly. Furthermore, primary cells rapidly lose the alveolar type II cell phenotype in 2D monolayer cultures. We found that under 3D culture conditions, SAEC cells displayed abundant cell surface E-cadherin, produced surfactant proteins characteristic to type II epithelial cells and were sensitive to asbestos-induced dowregulation of E-cadherin. In conclusion, our results suggest that asbestos can induce epithelial plasticity in both primary cells and A549 cell line displaying type II cell features.

A549 cells provide a tool for the characterization of molecular mechanisms contributing to asbestos pathogenicity in epithelial cells. In the current report, we illustrate a clear asbestos-induced downregulation of epithelial proteins E-cadherin and ZO-1, which is associated with a decrease in cell–cell contacts and a morphological change towards more elongated phenotype characterized by actin reorganization. Asbestos was able to efficiently induce E-cadherin downregulation both in two- and three-dimensional cell culture models. Interestingly, downregulation of E-cadherin protein level was not associated with downregulation of E-cadherin transcription. In agreement with this, only a small and late induction of transcription factors Snail and Slug was detected in asbestos exposed cells. The delayed time and modest nature of Snail and Slug induction by asbestos could be one reason why it was not associated with transcriptional repression of E-cadherin at 6 days. Asbestos-induced late induction of Snail and Slug may, however, become significant during later times and lead to transcriptional repression of E-cadherin. Time points beyond 6 days were, however, not investigated in this study. Transcriptional downregulation of E-cadherin has been suggested to be a late event in EMT [Janda et al., 2002]. However, TGF-β stimulation did result in down-regulation of E-cadherin mRNA levels pointing to different mechanisms of action. Possible mechanisms for asbestos-induced downregulation of E-cadherin are post-translational regulation or proteolytic processing and degradation.

Asbestos containing iron can induce ROS generation, which in turn has been suggested to activate latent forms of TGF-β [Pociask et al., 2004]. Sullivan et al. [2008] propose a model where asbestos elicits TNF-α expression, which leads to increased TGF-β expression by activating MAPK/Erk pathway and that these increased amounts of latent TGF-β are activated by asbestos-induced ROS. We have also previously found that high concentrations of asbestos can induce TGF-β signaling activity in primary human bronchial epithelial cells [Myllärniemi et al., 2008]. Therefore, it was unexpected to find that in A549 cells asbestos did not induce TGF-β signaling activity nor was the downregulation of E-cadherin fully reversed by blocking TGF-β receptor-mediated signaling. Relatively small and late induction of Snail and Slug transcription factors and the profile of mesenchymal gene induction reinforced the notion that asbestos and TGF-β are not likely to share a common mechanism of action at least in A549 lung carcinoma cells. However, it is likely that there exists co-operation between asbestos-induced pathways and TGF-β in in vivo lung.

Analyses of differences in signaling pathway activities between A549 cells and primary SAECs in monolayer cultures highlighted MAPK/JNK, MAPK/Erk, and NF-κB pathway activities. All these pathways have been linked to various EMT-processes, mostly induced by TGF-β [Min et al., 2008; Kalluri and Weinberg, 2009]. Inhibition of MAPK/Erk [Li and Mattingly, 2008] or NF-κB [Huber et al., 2004] prevents EMT in Ras-transformed mammary epithelial cells. A549 cells have homozygous G12S K-Ras, but these cells likely do not depend on Ras for survival [Singh et al., 2009]. As reported earlier [Jiang et al., 2001] blocking NF-κB activity severely impaired the proliferation/survival of A549 cells, but did not appear to interfere with asbestos mediated downregulation of E-cadherin or induction of α-SMA expression. Asbestos can induce NF-κB activity [Nymark et al., 2007], which is likely to play a role in cell survival [Sartore-Bianchi et al., 2007]. However, NF-κB activity did not contribute to asbestos induced EMT-like transition in cell systems used here.

Asbestos is known to induce EGFR activation by direct interaction leading to activation of the MAPK/Erk pathway [Zanella et al., 1996]. In agreement with previous studies, we observed asbestos-mediated induction or Erk1/2 phosphorylation in A549 cells. Inhibition of MAPK/Erk activity clearly prevented asbestos-mediated downregulation of E-cadherin protein levels and restored cell–cell contacts. These results suggest that MAPK/Erk activation plays an important role in asbestos-induced loss of the epithelial phenotype and is in agreement with a previous study showing that MAPK inhibitors were effective in restoring E-cadherin localization to cell–cell junctions in breast epithelial cells with moderate Ras activity without significant effect on E-cadherin expression levels [Li and Mattingly, 2008]. Furthermore, in normal mammary epithelial cells MAPK/Erk activation by ionizing radiation co-operates with TGF-β1 to induce EMT [Andarawewa et al., 2007]. However, a recent article by Ramos et al. [2010] reports reversal of TGF-β1 induced E-cadherin downregulation by co-expression of FGF-1. Particularly, MAPK/Erk activity was shown to contribute

to this FGF-1-mediated inhibitory activity suggesting context-dependent regulation of E-cadherin.

Induction of α-SMA expression by asbestos was not blocked by the inhibition of MAPK/Erk activity. In kidney tubular cells disruption of cell contacts or forced actin polymerization can activate the α-SMA promoter [Fan et al., 2007]. However, in A549 cells restoration of cell–cell contacts by inhibition of MAPK/Erk activity was not able to block α-SMA expression suggesting other mechanism for asbestos-mediated α-SMA expression. We have recently identified GATA-6 transcription factor as an important mediator of TGF-β induced α-SMA expression [Leppäranta et al., 2010]. Although asbestos was not able to induce TGF-β signaling activity in A549 cells, activation of GATA-6 by asbestos through other mechanisms is an interesting possibility.

Induction of EMT is a shared pathogenic mechanism in fibrotic and malignant lung diseases. Here, we reveal the role of asbestos fibers in the initiation of EMT and the importance of the MAPK/Erk pathway in reduced E-cadherin levels and altered cell morphology leading to reduced cell–cell contacts characteristic to a motile phenotype. However, in A549 cells asbestos induced the expression of certain profibrotic genes, but not fibronectin or N-cadherin and induced only slightly the expression of the transcription factors Snail and Slug associated with the transcriptional shift during the progression of EMT. Thus, it is possible that asbestos induces a transitional EMT and/or likely co-operates with other factors activated in diseased lung, such as inflammatory cytokines and growth factors. Our results support the concept of blocking MAPK/Erk signaling as one strategy to develop anti-fibrotic and anti-cancer therapies [Leivonen et al., 2005; Roberts and Der, 2007].

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