Letter to the Editor

Transcriptional Factor Snail Mediates Epithelial-Mesenchymal Transition in Human Bronchial Epithelial Cells Induced by Silica*

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Epithelial-mesenchymal transition (EMT) plays an important role in fibrotic diseases. We have previously showed that silica induces EMT in human bronchial epithelial cells (BECs); however, the underlying mechanism of silica-induced EMT is poorly understood. In the present study, we investigated the role of Snail in silica-induced EMT in human BECs *in vitro*. Human BECs were treated with silica at various concentrations and incubation times. Then MTT assay, western blot, electrophoretic mobility shift assay (EMSA), and small interfering RNA (siRNA) transfection were performed. We found that silica increased the expression and DNA binding activity of Snail in human BECs. SNAI siRNA inhibited the silica-induced expression of Snail. Moreover, SNAI siRNA upregulated the expression of epithelial marker E-cadherin, but attenuated the expression of mesenchymal marker α-smooth muscle actin and vimentin in silica-stimulated cells. These results suggest that Snail mediates the silica-induced EMT in human BECs.

Inhalation of silica particles can induce an inflammatory response and fibrosis in the lung, and bronchial epithelial cells are especially sensitive to silica exposure.

In our previous study, we showed that silica induced epithelial-mesenchymal transition (EMT) in human bronchial epithelial cells (BECs)[1]. The hallmark of silica-induced lung disease is the aggregation and lesion of fibroblast-like cells. EMT, a process whereby fully differentiated epithelial cells are transformed into mesenchymal cells such as fibroblasts and myofibroblasts, is increasingly recognized as the source of fibrotic extracellular matrix[2]. Silica-induced EMT may be involved in the development of silicotic fibrosis. However, the underlying mechanism of silica-induced EMT remains to be revealed.

During EMT, there is a downregulation of several adhesion molecules, such as E-cadherin, and an upregulation of genes typically found in myofibroblastic or fibroblastic cells, such as α-smooth muscle actin (α-SMA) and vimentin[3]. Although we demonstrated that the Rho/Rock signaling pathway regulates the silica-induced EMT in human BECs, Rho kinase is not responsible for the silica-induced EMT exclusively[1]. In addition, the activation of zinc-finger transcription factors and downregulation of E-cadherin in the epithelial cells are necessary for EMT[4]. The transcription factor Snail is a member of the zinc finger transcription factor family. In adults, Snail is mainly expressed in the heart, lung, brain, and skeletal muscle, and regulates the TGF-β-induced EMT during embryonic development and tumor progression[5].

In the present study, we confirmed the role of Snail in silica-induced EMT in human BECs and tested whether the inhibition of Snail expression can block the silica-induced EMT.

Crystalline silica (Sigma, 0.1-10 μm in diameter) was boiled in 1 mol/L HCl to remove contaminating Fe_2O_3, washed with water, and dried in an oven at 110 °C for 90 min. Then the particles were sterilized by heating at 180 °C for 6 h. Before use, the particles were suspended in 1 mL sterile saline and were sonicated for 10 min.

The immortalized human BEC line was kindly provided by Professor Gruenernt (San Francisco Branch Campus, University of California). Human BECs were cultured in 100 mL cell culture bottle in RPMI medium 1640, containing 10% fetal bovine serum and 2.5 μg/mL insulin, at 37 °C in a humidified atmosphere of 5% CO_2. At 80% confluence, the

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medium was changed to serum-free medium 24 h before the addition of silica.

Cell viability was assayed with the MTT test. Cells were exposed to silica (100, 200, 300, 400 μg/mL) for 72 h. At the end of the experiment, MTT (5 mg/mL) was added to the wells (20 μL/well). After 4 h incubation at 37 °C, the medium was removed, and DMSO was added (150 μL/well). Then the plates were agitated at room temperature for 10 min. Absorbance was read at a wavelength of 570 nm using an assay reader.

Cells were incubated with silica (200 μg/mL) for various incubation times (0, 1, 6, 18, and 24 h). After the treatment, cells were harvested and total proteins were extracted. Nuclear extracts were harvested as follows. Cell pellets were resuspended in 5 pellet volumes of buffer A (10 mmol/L KCl, 20 mmol/L HEPES, 1 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethanesulfonyl fluoride) and then in 5 pellet volumes of buffer B (10 mmol/L HEPES, 400 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 15% glycerol). Protein extracts were cleared by centrifugation at 4 °C for 15 min. The supernatants containing the nuclear proteins were collected. Equal amounts of proteins, quantified by the BCA protein assay kit (Pierce), were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 2 h and then incubated with various primary antibodies for 18 h at room temperature. After an incubation with a suitable secondary antibody at room temperature for 2 h, immunoreaction was visualized using enhanced chemiluminescence reaction. Anti-E-cadherin(sc-7870), anti-vimentin (sc-5565), and anti-Snail(sc-28199) antibodies were obtained from Santa Cruz; anti-α-SMA antibody (SMA-CE) was provided by Novocastra.

Electrophoretic mobility shift assays (EMSAs) were performed according to the protocol of the Light Shift Chemiluminescence EMSA Kit (Pierce). Biotin-labeled oligonucleotides that bind Snail (5′-CTGTGGCCGG CAGGTGAACCCTCAGCCA-3′) were prepared with 5′ end labeling. In brief, nuclear proteins (10 μg) were incubated with the reaction mixture (10×binding buffer, 1 μg/L poly(dI-dC), 1% NP-40, 2 μL labeled probe) for 20 min at room temperature. Each sample was electrophoresed at 100 V for 1.5 h using 6% non-denaturing polyacrylamide gels. Then, the biotin labeled DNA was transferred to a nylon membrane electrophoretically. Finally, biotin was detected using chemiluminescence. In competitive experiments, the unlabeled probe was added at a 100-fold excess to the reaction mixture.

The knockdown of Snail expression in BECs was achieved using the SNAI small intervening RNA (siRNA) (Santa Cruz). The cells were transiently transfected with 100 nmol/L SNAI siRNA according to the manufacturer's protocol. Scrambled siRNA used at the same concentration served as control. Post-transfection (6-8 h), the cells were treated with silica (200 μg/mL) and then lysed 24 h after the treatment; the efficiency of the knockdown was verified by Western blot.

Statistical analysis was performed using analysis of variance (ANOVA); $P<0.05$ was considered significant. All data are expressed as mean±standard deviation (SD).

Firstly, MTT assay was conducted to ensure that doses used in the next experiments had no effect on cell viability. After treatment with 0, 100, 200, 300, and 400 μg/mL ilica, the absorbance values were 0.39±0.01, 0.38±0.02, 0.38±0.01, 0.36±0.01, and 0.18±0.01, respectively. Therefore, exposure to 100-300 μg/mL silica did not reduce cell viability.

E-cadherin is an epithelial transmembrane protein, and its loss is strongly associated with EMT. Similarly, α-SMA and vimentin are markers of EMT[6]. Snail is an inducible zinc finger transcription factor with a molecular weight of 29 kDa and downregulates the expression of E-cadherin and upregulates the expression of vimentin[7]. Therefore, we considered Snail as a candidate protein that might mediate silica-induced EMT, and therefore, examined the total and nuclear protein level of Snail in silica-treated cells. The total protein level of Snail started to increase after 1 h of treatment with silica, peaked at 18 h, and decreased at 24 h of treatment (Figure 1A, 1B). In addition, silica exposure noticeably increased the nuclear level of Snail protein. This effect was visible after 1 h of treatment, peaked around 18 h, and decreased at 24 h of treatment with silica (Figure 1C, 1D). These findings suggest that silica induces Snail in human BECs.

Next, we examined the DNA binding activity of Snail in silica-treated human BECs. Silica treatment significantly increased the DNA binding activity of Snail in human BECs (Figure 2), which suggests that silica induces Snail activation in human BECs. Furthermore, this effect preceded EMT[1]. Similarly, a previous study demonstrated that TGF-β activated the Snail pathway in human BECs[8]. These findings

suggest that Snail regulates the silica-induced EMT in human BECs.

To assess the role of Snail in silica-induced EMT in human BECs, we first inhibited Snail protein expression in silica-treated cells using Snail siRNA. Silica-treated cells were transfected with SNAI1 siRNA or with non-silencing control siRNA constructs. Silica-induced Snail protein expression was inhibited by SNAI1 siRNA (data not shown).

Next, we examined whether the inhibition of Snail expression by SNAI1 siRNA affects the expression of EMT markers (E-cadherin, α-SMA, and vimentin).

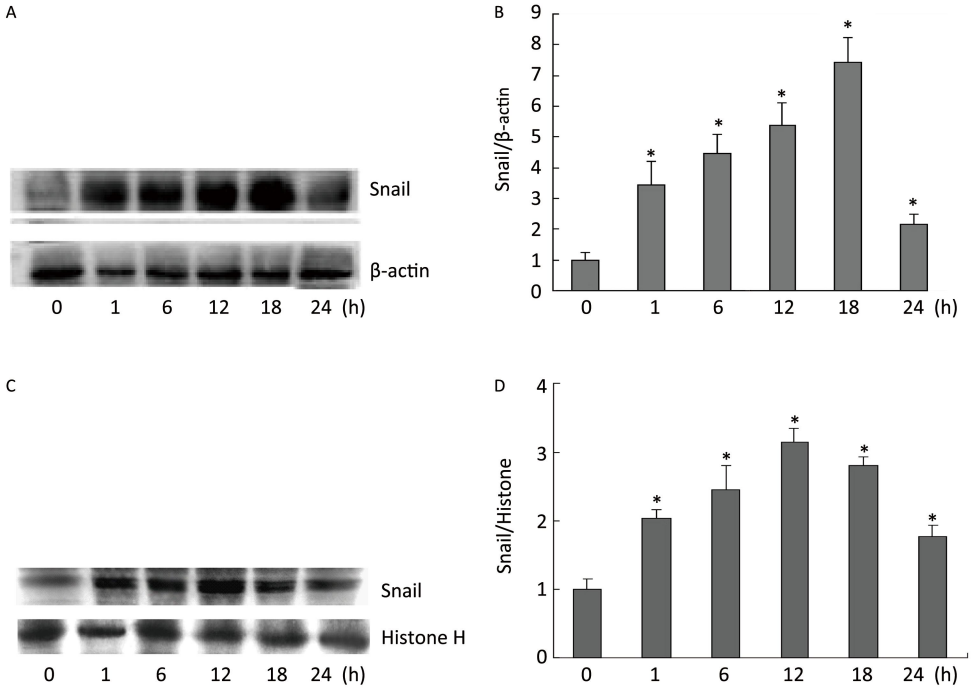

Figure 1. Silica treatment induced the expression of Snail in human BECs. Cells were treated with 200 μg/mL silica for 0, 1, 6, 12, 18, and 24 h. Using Western blot for the detection of total (A) and nuclear (C) Snail, we determined its relative expression level (B, D). Mean±SD values were calculated based on the densitometric analysis of three individual experiments. $^{*}P<0.05$ compared to the unexposed group.

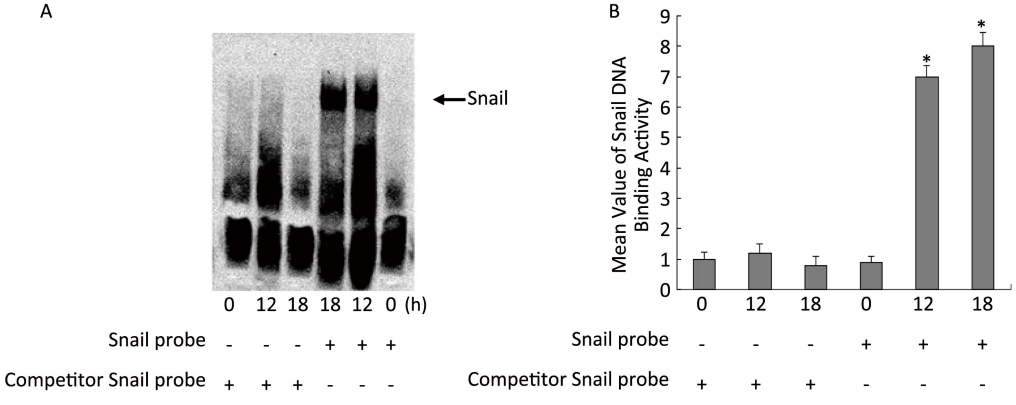

Figure 2. Silica increased the DNA binding activity of Snail in Human BECs. Cells were stimulated with 200 g/mL silica for the indicated periods (0, 12, and 18 h). The nuclear proteins were extracted, and electrophoretic mobility shift assay was performed (A). Mean±SD values were obtained from the densitometric analysis of three individual experiments (B). $^{*}P<0.05$ compared to the unexposed group.

We found that SNAI1 siRNA treatment attenuated the silica-induced loss of E-cadherin in human BECs (Figure 3). Furthermore, SNAI1 siRNA treatment decreased the silica-induced expression of α-SMA and vimentin (Figure 3). These data provide direct evidences that Snail might regulate silica-induced EMT in human BECs. Xi and colleagues found that methacycline attenuated the bleomycin-induced pulmonary fibrosis, which was accompanied by the inhibition of Snail[9]. In addition, a HSP27 inhibitor blocked the development of pulmonary fibrosis by promoting Snail degradation[10]. These antifibrotic factors might be associated with the prevention of EMT through the inhibition of Snail activity.

To our knowledge, this study provides the first evidence that the Snail signaling pathway plays an important role in the development of silicainduced EMT in human pulmonary epithelial cells. These novel findings might help to reveal the molecular mechanisms involved in silica-induced fibrosis.

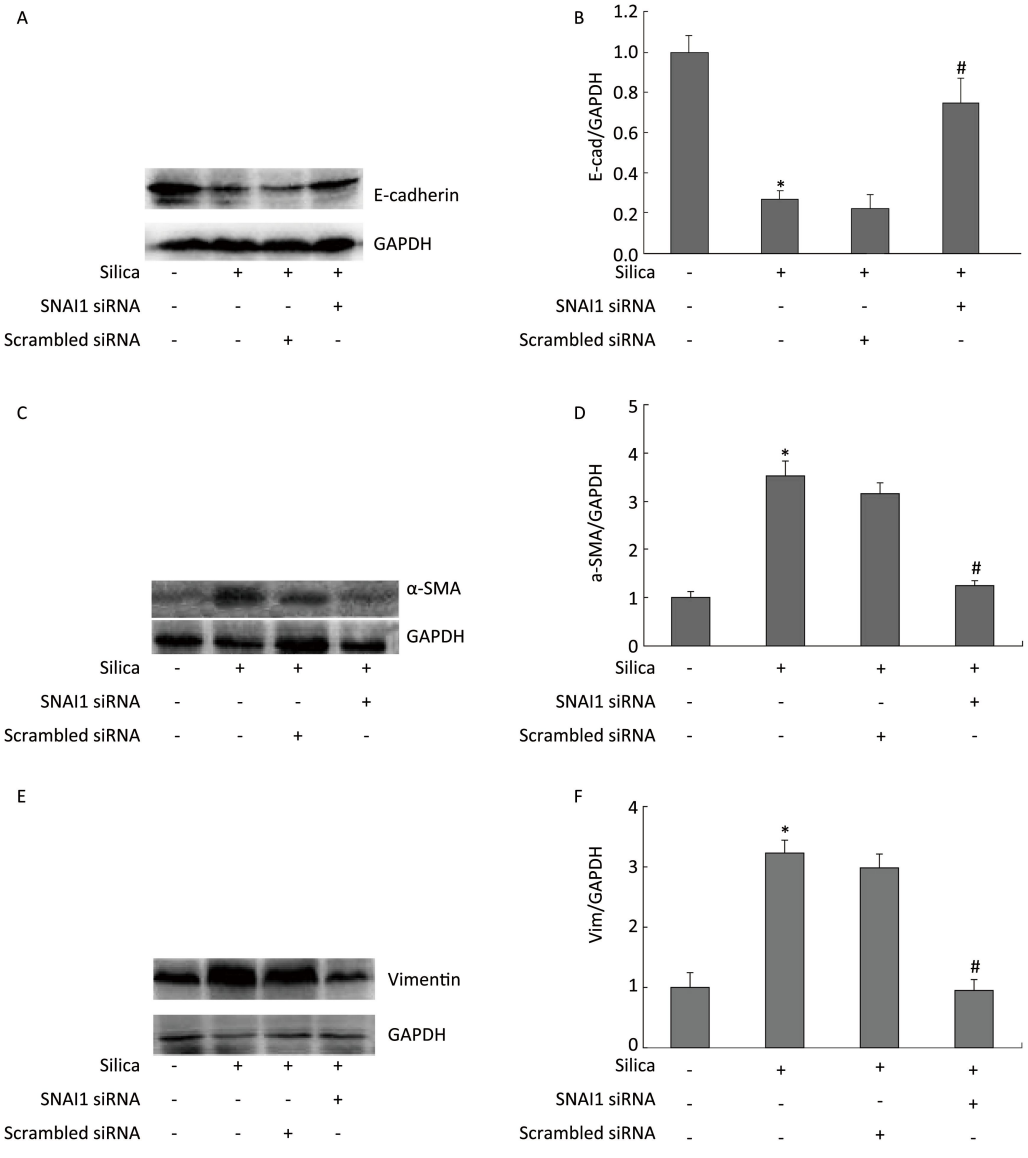

Figure 3. Snail siRNA inhibited the silica-induced EMT in human BECs. Cells were pre-incubated with SNAI1 siRNA or scrambled siRNA, and then they were stimulated with silica (200 μg/mL) for 24 h or were left untreated (A, C, E). Mean±SD values were quantified by densitometric analysis of three individual experiments (B, D, F). $^{*}P<0.05$, compared to the untreated control group. $^{\#}P<0.05$, compared to the silica-treated group.

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