Nicotine-induced epithelial-mesenchymal transition via Wnt/β-catenin signaling in human airway epithelial cells

Weifeng Zou,1 Yimin Zou,1 Zhuxiang Zhao,1 Bing Li,2 and Pixin Ran1

1The State Key Laboratory of Respiratory Diseases, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, Guangdong, China; 2The Research Center of Experiment Medicine, Guangzhou Medical University, Guangzhou, Guangdong, China

Submitted 12 March 2012; accepted in final form 30 November 2012

Zou W, Zou Y, Zhao Z, Li B, Ran P. Nicotine-induced epithelial-mesenchymal transition via Wnt/β-catenin signaling in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 304: L199–L209, 2013. First published November 30, 2012; doi:10.1152/ajplung.00094.2012.—Epithelial-mesenchymal transition (EMT) has been proposed to be a mechanism in airway remodeling, which is a characteristic of chronic obstructive pulmonary disease (COPD). Studies have shown that cigarette smoke and nicotine are factors that induce Wnt/β-catenin activation, which is a pathway that has also been implicated in EMT. The main aim of this study was to test whether human bronchial epithelial cells are able to undergo EMT in vitro following nicotine stimulation via the Wnt3a/β-catenin signaling pathway. We show that nicotine activates the Wnt3a signal pathway, which leads to the translocation of β-catenin into the nucleus and activation of β-catenin/Tcf-dependent transcription in the human bronchial epithelial cell (HBEC) line. This accumulation was accompanied by an increase in smooth muscle actin, vimentin, matrix metalloproteinases-9, and type I collagen expression as well as downregulation of E-cadherin, which are typical characteristics of EMT. We also noted that the release of TGF-β1 from these cells was stimulated by nicotine. Knockdown of Wnt3a with small interfering RNA (siRNA) prevented these effects, implying that β-catenin activation in these responses is Wnt3a-dependent. Furthermore, specific knockdown of TGF-β1 with TGF-β1 siRNA partially prevented nicotine-induced EMT, suggesting that TGF-β1 has a role in nicotine-mediated EMT in HBECs. These results suggest that HBECs are able to undergo EMT in vitro upon nicotine stimulation via the Wnt3a/β-catenin signaling pathway.

chronic obstructive pulmonary disease (COPD) involves partially reversible air flow obstruction, which is often ascribed to airway remodeling (28). The link between airway remodeling and cigarette smoking, the most commonly encountered risk factor for COPD, has been established in both human and animal models (2, 6, 28), and smoking contributes to ongoing airway inflammation and remodeling.

Recently, epithelial-mesenchymal transition (EMT) has frequently been mentioned with regard to its involvement in airway remodeling. EMT is the transdifferentiation of epithelial cells into mesenchymal cells. During this process, epithelial cells no longer have cellular polarity, and both epithelial cell-cell and cell-matrix adhesion contacts are remodeled. Markers of polarized epithelial cells, such as E-cadherin and some cytokeratins, are lost. Furthermore, myofibroblast or mesenchymal cell markers, such as vimentin and α-smooth muscle actin (α-SMA), are acquired (44). EMT has only recently been recognized in the human lung or airway (41, 42). The transformation of airway epithelial cells via EMT is one of the potential sources for fibrogenic cells within the airway wall (11, 42). EMT is also likely to be active in the airways of smokers, especially COPD patients who currently smoke (35, 36).

Endobronchial Wnt signaling (19, 29, 31). Furthermore, nicotine enhances the expression of fibronectin, an EMT marker (41). However, whether nicotine can induce bronchial EMT in HBECs is not clear, and the cellular mechanisms of nicotine-induced EMT in HBECs are not known.

Wnt signaling is a key regulator of multiple aspects of tissue development. In the canonical Wnt pathway, Wnt ligands bind to the frizzled/IRP coreceptor complex, which then leads to the stabilization and nuclear translocation of β-catenin. β-Catenin acts as a powerful transactivator of LEF/TCF transcription factors, which regulate important downstream target genes that promote cell proliferation, differentiation, and tissue development. Recently, the importance of Wnt signaling as a regulator of EMT was demonstrated through the direct participation of β-catenin in this process (13, 14) and through the induction of EMT by Wnt ligands in vitro (7). Wnt signaling can interact with other profibrotic growth factors, such as TGF-β1.

In dermal fibroblasts, Wnt3a increased TGF-β1 expression, suggesting that Wnt/β-catenin signaling lies upstream of TGF-β1 (5). Cigarette smoke stimulates TGF-β1 to induce small airway extracellular matrix deposition (9, 38). Recently, it was demonstrated that TGF-β1 could induce EMT in alveolar epithelial cells in vitro and in vivo (12, 15, 43), and in human bronchial epithelial cells (8, 45) β-catenin is involved in alveolar epithelial cell EMT (16).
To our knowledge, no study has systematically examined the in vitro effects of nicotine on HBECs and the possible molecular mechanisms that may be involved in EMT. Because epithelial cells play a key role in the airway remodeling process (10), we aimed to determine whether nicotine can induce EMT via Wnt/β-catenin signaling in HBECs. Understanding the specific molecular mechanism(s) that underlie EMT should allow the identification of specific molecular intermediate targets that can prevent nicotine-induced EMT and, hence, nicotine’s detrimental effects on airway remodeling in COPD.

MATERIALS AND METHODS

Materials. DMEM and fetal bovine serum (FBS) were purchased from Sigma Chemical (St. Louis, MO). Mouse anti-E-cadherin, mouse anti-β-catenin, and mouse anti-COL1A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-matrix metalloproteinase-9 (MMP9) was purchased from Abcam Biotechnology (Abcam), mouse anti-α-SMA was purchased from Sigma Chemical (St. Louis, MO), rabbit anti-Wnt3a was purchased from Millipore (Bedford, MA), and rabbit anti-β-catenin was purchased from Cell Signaling Technology (Beverly, MA). Nicotine was purchased from Calbiochem (San Diego, CA). Wnt3a siRNA (h), TGF-β1 siRNA (h), nonspecific control siRNA, and siRNA transfection reagent were purchased from Santa Cruz Biotechnology.

Cell culture. Human bronchial epithelial cells (HBECs; ATCC) were cultured in 2 ml of DMEM containing 10% FBS and penicillin (100 U/ml) at a density of 5 × 10^5 cells in each well of a six-well plate. The cells were grown at 37°C in a humidified 5% CO2 atmosphere. Prior to the experiments, the cells were serum starved for 24 h and then stimulated with 6 × 10^{-8} to 6 × 10^{-4} mol/l nicotine in the growth medium for 24–72 h. The cells were then harvested for further analysis.

Small interfering RNA preparation and transfection. One day before transfection, cells were plated in growth medium without antibiotics so that they were 60–80% confluent at the time of transfection. The cells were transfected with 80 pmol/l siRNA duplexes (control, Wnt3a, or TGF-β1) by using transfection reagent and transfection condition according to the manufacturer’s recommendations. The siRNA concentrations were chosen on the basis of dose-response studies.

Transient transfection and luciferase activity assays. The cells (3 × 10^5 cells/well in 24-well plates) were transiently transfected with 0.1 μg of TOPFlash or FOPFlash (Upstate, Lake Placid, NY) along with 5 ng of pRL-SV40 (Promega, Madison, WI) by using Lipofectamine LIXV reagent (Invitrogen). After 24 h, the cells were stimulated with nicotine. The cells were lysed, and the luciferase activity was determined according to the manufacturer’s recommendations. The luciferase activity of each sample was normalized against Renilla luciferase activity for monitoring the transfection efficiency.

Real-time quantitative PCR. Total RNA was prepared from cells by using the RNeasy plus mini kit (Qiagen) according to the manufacturer’s instructions, and RNA was reverse transcribed into cDNA (TaKaRa). The cDNA was then amplified by real-time quantitative TaqMan PCR in a reaction containing 1× Taqman Universal PCR master mix, 1 μM primers, and 0.3 μM probe and analyzed by use of an ABI Prism 7900 sequence detector (Applied Biosystems). The housekeeping gene GAPDH was used as an internal control. The data were normalized to GAPDH and expressed as the fold change over control.

Cytoplasmic and nuclear protein extraction and Western blotting. The cells were lysed and incubated in a cytoplasmic extract (CE) buffer composed of 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris pH 8.0, 1 mM sodium orthovanadate, 5 mM NaF, and a protease inhibitor cocktail. The CE was then placed in a clean tube. The remaining nuclei were washed and resuspended in nuclear extract (NE) buffer composed of 20 mM Tris-CI, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM PMSF, and 25% (vol/vol) glycerol and adjusted to pH 8.0. After incubation on ice, the CE and NE were centrifuged for 5 min. Protein lysates (30 μg) were loaded on and resolved by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes, which were then blocked in 5% BSA for 1 h at room temperature and probed with an antibody to mouse anti-E-cadherin, mouse anti-MMP9, mouse anti-α-SMA, mouse anti-COL1A, rabbit anti-Wnt3a, or rabbit anti-β-catenin. Antibody binding was detected by using chemiluminescence according to the manufacturer’s instructions with either a peroxidase-conjugated anti-mouse or anti-rabbit antibody. We ran the gels with a prestained marker in all experiments. The housekeeping gene GAPDH was used as an internal control. The data were normalized to GAPDH and expressed as the fold change over the control.

Immunocytochemistry and immunofluorescence. The cells were seeded onto sterile round coverslips that had been placed inside 12-well plates. At 70% confluency, the medium was changed to serum-free DMEM, and nicotine was added to a subset of wells for a final concentration of 6 × 10^{-6} mol/l. Seventy-two hours later, all of the wells were washed twice with cold PBS, and a subset of the wells were fixed with cold methanol-acetone (1:1) at room temperature for 20 min. The cells were then treated with 0.2% Triton X-100 (Sigma-Aldrich) at room temperature for 10 min. The cells on the coverslips were then incubated with mouse anti-α-SMA Mob (1:200), mouse anti-COL1A (1:50), mouse anti-MMP9 (1:50), mouse anti-β-catenin (1:50), or mouse anti-E-cadherin (1:50) for 1 h at room temperature. Antibody binding was detected with peroxidase-conjugated anti-mouse and 3,3-diaminobenzidine according to the manufacturer’s instructions; the slides were treated with fluorescein isothiocyanate donkey anti-mouse IgG (1:250) or donkey rabbit anti-IgG (1:250) for 40 min in the dark. The slides were again rinsed with PBS three times, mounted with 50% glycerol and stored in the dark. Immunofluorescence was examined using a Leica confocal microscope.

ELISA. The cell supernatants were collected, and TGF-β1 and Wnt3a were measured by using a Wnt3a or TGF-β1 ELISA kit (Ray Biotech) according to the manufacturer’s instructions. Samples with a concentration exceeding the standard curve limits were diluted until an accurate reading could be obtained. Four replicate wells were used to obtain all of the data points, and all of the samples were processed in duplicate and averaged.

Statistical analysis. The data for analysis were obtained from at least 3 independent sets of experiments and expressed as means ± SD. The data were analyzed by use of the SPSS 17.0 statistical package.

![Fig. 1](http://ajplung.physiology.org/)
Statistical evaluation of the continuous data was performed by ANOVA or the independent-samples t-test for between-group comparisons. The level of significance was considered to be $P < 0.05$.

RESULTS

Nicotine induces the expression of Wnt3a and activates β-catenin signaling in HBECs. Initially, we found that the cells exposed to nicotine for 24 h significantly increased Wnt3a secretion as determined by ELISA (Fig. 1A). Real-time PCR revealed a dramatic upregulation of Wnt3a mRNA expression after 24 h of nicotine stimulation (Fig. 1B), which suggested that Wnt3a upregulation occurred at the transcriptional level. Western blotting analysis showed that an increase in the level of Wnt3a protein occurred as early as 30 min, and it was sustained at all of the time points examined (Fig. 1C). A 24-h exposure of cells to nicotine ($6 \times 10^{-6}$ mol/l–$6 \times 10^{-6}$ mol/l) led to an increase in Wnt3a production in a concentration-dependent manner (Fig. 1D). Because Wnt3a has been repeatedly reported to potently stimulate Wnt/β-catenin signaling in vitro (23), we examined whether nicotine could induce β-catenin accumulation. Indeed, a 24-h exposure of cells to nicotine also led to an accumulation of the total β-catenin, which was concentration dependent (Fig. 1D). Cytoplasmic accumulation and nuclear translocation of β-catenin were observed after 24 h of exposure of the cells to nicotine ($6 \times 10^{-6}$ mol/l, Fig. 1, E and G). This finding raised the question of whether nicotine is able to activate β-catenin/Tcf-dependent

![Fig. 2. Nicotine induces epithelial-mesenchymal transition (EMT)-related changes. HBECs were incubated with nicotine ($6 \times 10^{-6}$ mol/l) for 72 h to induce the EMT. A: Western blot analysis shows that nicotine induces a downregulation of the epithelial marker E-cadherin (E-cad) and an upregulation of the mesenchymal proteins type I collagen (Col I), matrix metalloproteinase-9 (MMP9), and α-smooth muscle actin (α-SMA). The ratios of EMT/GAPDH in control cells are designated as 1. B: nicotine induces a mesenchymal morphology. *$P < 0.05$ compared with the control group, $N = 3$ (t-test).](http://ajplung.physiology.org/)

A: Nic (6 × 10^{-6} mol/L)

- E-cad
- α-SMA
- MMP9
- Col I
- GAPDH

<table>
<thead>
<tr>
<th>EMT marker/GAPDH protein</th>
<th>Control</th>
<th>Nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cad</td>
<td><img src="control.png" alt="image" /></td>
<td><img src="nicotine.png" alt="image" /></td>
</tr>
<tr>
<td>α-SMA</td>
<td><img src="control.png" alt="image" /></td>
<td><img src="nicotine.png" alt="image" /></td>
</tr>
<tr>
<td>MMP9</td>
<td><img src="control.png" alt="image" /></td>
<td><img src="nicotine.png" alt="image" /></td>
</tr>
<tr>
<td>Col I</td>
<td><img src="control.png" alt="image" /></td>
<td><img src="nicotine.png" alt="image" /></td>
</tr>
</tbody>
</table>

B: Control Nicotine 72 h
transcription in HBECs. To address this question, we transfected TOPflash and FOPflash luciferase reporter constructs into HBECs. The TOPflash and FOPflash constructs contain the wild-type and mutated β-catenin/Tcf binding sites, respectively. As shown in Fig. 1F, nicotine induced β-catenin/Tcf-dependent transcription.

Nicotine induces EMT-related changes in HBECs. Because canonical Wnt signaling is a key determinant of EMT, we examined whether nicotine could induce biochemical and morphological changes indicative of EMT in HBECs. Western blots and immunofluorescence and immunocytochemistry analysis showed that the epithelial marker E-cadherin was downregulated in these cells after a 72-h exposure to nicotine (Figs. 2A, 7, and 8). In contrast, a dramatic upregulation in the expression of mesenchymal marker type I collagen, MMP9, vimentin, and α-SMA was observed in the presence of nicotine (Figs. 2A, 7, and 8). Thus nicotine has the ability to induce biochemical changes consistent with EMT. In addition, HBECs in culture have a cobblestone morphology. However, HBECs in the presence of nicotine had a morphological phenotype characteristic of EMT, with a loss of cell-cell contact and an elongated shape (Fig. 2B). These data suggest that nicotine induces EMT characteristics in HBECs. A concomitant upregulation of Wnt3a/β-catenin with the induction of EMT in HBECs prompted us to investigate which signaling pathways are involved in this phenomenon.

The role of Wnt/β-catenin signaling in nicotine-induced EMT of HBECs. To test whether Wnt3a/β-catenin is involved in the nicotine-mediated EMT, siRNAs were used to deplete Wnt3a. Transfection of Wnt3a siRNA into the cells significantly decreased the Wnt3a protein level in both nicotine-treated and untreated cells. Compared with the negative control siRNA, which had no inhibitory effect on enhanced Wnt3a expression.

Fig. 3. Effects of nicotine and Wnt3a siRNA on β-catenin level in HBECs. Cells were transfected with Wnt3a siRNA or control siRNA for 48 h and then stimulated with nicotine (6×10⁻⁶ mol/l) for 72 h. A: Western blotting shows that knockdown of Wnt3a reduces Wnt3a expression in the presence of nicotine. The ratios of Wnt3a/GAPDH in control cells are designated as 1. B: ELISA shows that knockdown of Wnt3a reduces Wnt3a secretion in the presence of nicotine. C: knockdown of Wnt3a decreases cytoplasmic accumulation and nuclear translocation of β-catenin in the presence of nicotine. The ratios of β-catenin/tubulin and β-catenin/lamin B in the control cells are designated as 1. D: knockdown of Wnt3a inhibits nicotine-induced β-catenin/Tcf-dependent transcription. The cells were transfected with Wnt3a siRNA or control siRNA, and then after 48 h, they were transfected with 1 μg of TOPflash or FOPflash along with 5 ng of pRL-SV40. The luciferase activity was normalized against the Renilla activity. The activity of control cells transfected with TOPflash and FOPflash is designated as 100%. *P < 0.05 compared with the control group; #P < 0.05 compared with the control siRNA group, N = 3 (ANOVA).
after nicotine treatment, Wnt3a siRNA transfection suppressed this increase in Wnt3a expression; however, Wnt3a expression was still higher than that in the untreated control (Fig. 3, A and B). Wnt3a lies upstream of β-catenin, and translocation of β-catenin into the nucleus acts as a powerful transactivator of LEF/TCF transcription factors. We next examined whether transfection of Wnt3a siRNA would block nicotine-induced activation of β-catenin. Cells transfected with a Wnt3a-specific siRNA showed decreased cytoplasmic accumulation and nuclear translocation of β-catenin compared with cells that were stimulated with nicotine (Fig. 3C). Similarly, cells transfected with a Wnt3a-specific siRNA also inhibited nicotine-induced β-catenin/Tcf-dependent transcription (Fig. 3D). We tested whether Wnt3a/β-catenin activation elicited by nicotine is associated with EMT markers. As expected, cell transfection with our Wnt3a-specific siRNA inhibited nicotine-induced EMT, whereas no inhibitory effect was observed in cells transfected with a control siRNA (Figs. 4A, 7, and 8). Taken together, these results show that HBECs can undergo EMT in vitro in response to nicotine via a Wnt3a/β-catenin mechanism.

The role of TGF-β1 in nicotine-mediated Wnt3a/β-catenin signaling and EMT. It is well known that TGF-β1 can induce EMT in HBECs. In other systems, Wnt3a increases TGF-β1 expression. Thus we hypothesized that nicotine-induced upregulation of TGF-β1 would also be mediated by Wnt3a activation during the process of EMT. To determine the involvement of TGF-β1 in nicotine-induced cell responses, the total amounts of TGF-β1 were measured in HBECs. The total TGF-β1 was increased by nicotine at the 12-, 24-, and 72-h time points (Fig. 5, A and B), and this increase was prevented by knocking down Wnt3a (Fig. 5B). To better understand the role of TGF-β1 in the process of EMT, we specifically knocked down TGF-β1 expression with TGF-β1 siRNA. Cells transfected with TGF-β1 siRNA showed a decrease in TGF-β1 production in the presence of nicotine, whereas no inhibitory effect was observed in cells transfected with a control siRNA (Fig. 5B). Because β-catenin is a key mediator in the canonical Wnt signaling pathway, we checked whether its expression might be regulated by TGF-β1 in HBECs. Knockdown of TGF-β1 expression did not significantly attenuate the effects of nicotine on cytoplasmic accumulation or the nuclear translocation of β-catenin (Fig. 5C). Similarly, cells transfected with a TGF-β1-specific siRNA also did not significantly inhibit nicotine-induced β-catenin/Tcf-dependent transcription (Fig. 5D). Furthermore, knockdown of TGF-β1 expression partially prevented the nicotine-induced increase in EMT markers (Figs. 6, 7, and 8). These results suggest that in HBECs, the TGF-β1 and Wnt pathways interact during nicotine-induced EMT.

DISCUSSION

Our work reveals that, in HBECs, exposure to nicotine at a concentration that is similar to the plasma concentration in active and passive smokers (1) was able to induce EMT, which was characterized by the expression of mesenchymal phenotypes α-SMA, vimentin, and MMP9 and the production of collagen I as well as the loss of the adherens junction protein E-cadherin and cell morphological changes. For the first time, our data provide evidence for nicotine-induced EMT in HBECs. Our data also demonstrate an upregulation of the Wnt3a/β-catenin pathway in this process, which provides a possible mechanism for nicotine-induced EMT in HBECs. The Wnt/β-catenin system is largely expressed in the lung bronchial and alveolar epithelium and is known to regulate epithelial and mesenchymal cell biology in an autocrine and paracrine fashion. Interestingly, bronchial epithelial expression of Wnt signaling is augmented in smokers with or without COPD (21, 37). Recent studies have revealed that the Wnt target Wnt1-inducible signaling protein-1 (WISP1) induces EMT in vitro, highlighting a role for Wnt signaling in the process of EMT and lung fibrosis (18). Nicotine induces alveolar interstitial fibroblast-to-myofibroblast transdifferentiation by upregulating Wnt signaling. Together, these data suggest that Wnt/β-catenin pathway may be an important determinant of epithelial responses to nicotine and closely correlate with nicotine-mediated EMT marker expression. Our findings demonstrate that HBECs stimulated by nicotine produce excessive amounts of Wnt3a, accompanied by cytoplasmic accumulation and nuclear translocation of β-catenin and EMT.

There are several Wnt proteins that exert different overall effects in cells (27). In this study, we examined Wnt3a for two reasons. First, Wnt3a has been repeatedly reported to potently stimulate β-catenin-dependent Wnt signaling in vitro (23), and it has been recognized as the prototypic Wnt ligand for in vitro stimulation (26). Königshoff et al. (17) reported that Wnt3a induces lung epithelial cell proliferation, fibroblast activation and collagen synthesis. Regulation of Wnt/β-catenin signaling...
by Wnt and Wnt inhibitors is one of the emerging therapeutic approaches for controlling tissue remodeling and regeneration (3, 25). In our preliminary study, we found that exposure to cigarette smoke increased the levels of Wnt3a/β-catenin protein in rat lung tissue. To better understand the role of Wnt3a in nicotine-mediated β-catenin activation and EMT production, we transfected HBECs with Wnt3a siRNA. The Wnt3a siRNA used in this study has been reported to efficiently inhibit Wnt3a expression (20). The results showed that cells with repressed Wnt3a expression had lower cytoplasmic accumula-

by 10.220.33.4 on November 6, 2017 http://ajplung.physiology.org/ Downloaded from

Fig. 6. Role of TGF-β1 in nicotine-mediated EMT. The cells were transfected with TGF-β1 siRNA or control siRNA for 48 h and then stimulated with nicotine (6×10^{-6} mol/l) for 72 h. Western blotting shows that knockdown of TGF-β1 affects nicotine-induced protein expression of EMT markers. The ratios of the EMT marker/GAPDH in the control cells are designated as 1. {*}P < 0.05 compared with the control group; {#}P < 0.05 compared with the control siRNA group, N = 3 (ANOVA).

Fig. 5. Role of TGF-β1 in nicotine-mediated Wnt3a/β-catenin signaling. Cells were transfected with Wnt3a siRNA, TGF-β1 siRNA, or control siRNA for 48 h and then stimulated with nicotine (6×10^{-6} mol/l) for different times. A: nicotine induces the production of TGF-β1 in HBECs. B: knockdown of TGF-β1 and Wnt3a affects 72-h nicotine-induced production of TGF-β1. The relative production of TGF-β1 was evaluated by ELISA. C: knockdown of TGF-β1 does not significantly decrease the cytoplasmic accumulation and nuclear translocation of β-catenin upon 72-h nicotine treatment. The ratios of β-catenin/tubulin and β-catenin/lamin B in control cells are designated as 1. D: knockdown of TGF-β1 does not significantly inhibit 72-h nicotine-induced β-catenin/Tcf-dependent transcription. Cells were transfected with TGF-β1 siRNA or control siRNA, and then after 48 h they were transfected with 0.1 μg of TOPflash or FOPflash along with 5 ng of pRL-SV40. The luciferase activity was normalized against Renilla activity. The activity of the control cells transfected with TOPflash and FOPflash is designated as 100%. {*}P < 0.05 compared with the control group; {#}P < 0.05 compared with the control siRNA group, N = 3 (ANOVA).
Fig. 7. Role of Wnt3a/β-catenin and TGF-β1 in nicotine-mediated EMT. Cells were transfected with Wnt3a siRNA, TGF-β1 siRNA or control siRNA for 48 h and then stimulated with nicotine (6×10^{-6} mol/l) for 72 h. Immunoreactivity for E-cad (A to E), α-SMA (F to J), and Col I (K to O) was evaluated by immunocytochemistry. A, F and K: Phase images of the control group. B, G and L: Phase images of the nicotine group. C, H and M: nicotine + control siRNA. D, I and N: nicotine + Wnt3a siRNA. E, J and O: nicotine + TGF-β1 siRNA. Original magnification ×400. Immunocytochemistry shows that knockdown of TGF-β1 and Wnt3a affects nicotine-induced protein expression of the EMT markers.
tion and nuclear translocation of β-catenin as well as lower EMT marker synthesis compared with cells that had been transfected with a negative siRNA and pretreated with nicotine. On the basis of these data, we propose that nicotine activates Wnt3a, which in turn upregulates the cytoplasmic accumulation and nuclear translocation of β-catenin that then binds to LEF-1/TCF and results in EMT. The TOPflash luciferase assay using the LEF-1/TCF element reporter demonstrated that β-catenin target genes were upregulated upon nicotine stimulation and that this process is Wnt3a dependent. These results are consistent with a previous study that demonstrated that the Wnt/β-catenin signaling pathway is activated in the epithelium during the process of EMT in lung fibrosis (18).

Wnt3a increased TGF-β1 expression in both fetal and postnatal fibroblasts (5). Nicotine upregulates TGF-β1 protein expression in fibroblasts both in vitro (33) and in vivo (30). In accordance with these findings, our results show that nicotine increases the total amount of TGF-β1, and, intriguingly, cells transfected with Wnt3a siRNA downregulated TGF-β1 expression in HBECs upon nicotine stimulation. It has been reported that the epithelial cells from smokers and COPD patients released more TGF-β1 than those from nonsmokers (38). Wnt/β-catenin signaling is a critical upstream regulator of proximal-distal patterning in the lung, partly through the regulation of BMP4 (34), a member of the TGF superfamily. Both of these studies and our present work provide supportive evidence that nicotine-induced secretion of TGF-β1 is partly involved in nicotine-induced Wnt3a activation.

It is well known that TGF-β1 can induce EMT in HBECs. We also investigated the role of TGF-β1 in nicotine-induced EMT in HBECs. Our data show that knockdown of TGF-β1 expression also partially inhibits EMT marker expression induced by nicotine. Some reports have mentioned that the TGF-β1 pathway may interact with the Wnt/β-catenin pathway and induce β-catenin nuclear translocation, involving in hypertrophic scars in fibrosis (32), in the fibroblast-to-myofibroblast transition in human lungs (4) and in the epithelial-to-mesenchymal transition (14, 24). Compared with these reports, our data showed that knockdown of TGF-β1 expression did not attenuate the induction of cytoplasmic accumulation; additionally, nuclear translocation of β-catenin in nicotine-treated HBECs demonstrated that β-catenin expression might be reg-
ulated mainly by Wnt3a upregulation. The increase in TGF-β1 production thus may be a secondary event that plays a role on nicotine-induced EMT in HBECs.

In summary, our results provide additional insight into EMT, a potentially important mechanism in airway remodeling. We demonstrate that nicotine can drive EMT in HBECs in vitro. We show that this effect is primarily mediated via a Wnt3a/β-catenin-dependent mechanism. Moreover, Wnt3a positively regulates TGF-β1 expression, and TGF-β1 expression may be further enhanced in EMT in HBECs. These results establish the basis for future investigations into the mechanism of bronchial epithelial cell-to-mesenchymal cell differentiation, which in turn will contribute both to a better understanding of COPD and to the development of new therapeutic approaches. Further in vivo manipulation of Wnt signaling is needed to characterize its role during EMT.

GRANTS

This work was supported by National Natural Science Foundation of China (81170043), National Natural Science Foundation of Guangdong (S2011020002789), and Doctoral Fund of Ministry of Education of China (20104423110001).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

W.Z., B.L., and P.R. conceived and designed the research; W.Z. performed experiments; W.Z., Y.Z., Z.Z., and P.R. analyzed data; W.Z. and Y.Z. drafted manuscript; Z.Z., B.L., and P.R. interpreted final version of manuscript; Y.Z. and P.R. prepared figures; W.Z., Y.Z., Z.Z., B.L., and P.R. edited and revised manuscript; W.Z., Y.Z., Z.Z., and P.R. interpreted results of experiments.

REFERENCES


