



Nicotine Induces Progressive Properties of Lung Adenocarcinoma A549 Cells by Inhibiting Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Expression and Plasma Membrane Localization

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Abstract

Lung cancer remains one of the most common cancer-related deaths worldwide. The cigarette smoking is a risk factor for lung cancer development. Interestingly, the cystic fibrosis transmembrane conductance regulator encoded by *CFTR* gene, an ATP-binding cassette transporter-class ion channel that conducts chloride and bicarbonate anions across membrane of epithelial cells, has recently been suggested to play a role in the development and progression of many types of cancer. It has been well-documented that mutations of *CFTR* gene are the cause of cystic fibrosis, the most common fatal hereditary lung disease in *Caucasian* population; the function of cystic fibrosis transmembrane conductance regulator in the development of lung cancer however has not yet been established. In the present study, we aimed to interrogate the impact of cystic fibrosis transmembrane conductance regulator on the nicotine-promoted progressive potency in lung adenocarcinoma cells by assessing capacities of cystic fibrosis transmembrane conductance regulator to cell migration, invasion, and clonogenicity and the expression of markers of cell proliferation and lung stem cell-related transcription factors in lung adenocarcinoma A549 cells. The exposure of nicotine exhibited an ability to enhance progressive properties of adenocarcinoma cells including A549 cells, HCC827 cells, and PC-9 cells, alone with an inhibition of cystic fibrosis transmembrane conductance regulator protein expression. Remarkably, an overexpression of cystic fibrosis transmembrane conductance regulator significantly inhibited the progressive potency of A549 cells, including capacity of cell migration and invasion and clonogenicity, along with a decreased expression of cell proliferative markers Ki67, p63, and proliferating cell nuclear antigen, and cancer stem cell marker CD133, stem cell pluripotency-related transcription factors octamer-binding transcription factor 3/4, and sex-determining region Y-box 2, regardless of the presence of nicotine. In contrast, opposite effects were observed in A549 cells that the cystic fibrosis transmembrane conductance regulator was knockdown by short hairpin RNA to cystic fibrosis transmembrane conductance regulator. This study thus suggests that cystic fibrosis transmembrane conductance regulator may play a tumor suppressor role in lung cancer cells, which may be a novel therapeutic target warranted for further investigation.

Keywords

lung cancer, nicotine, cystic fibrosis transmembrane conductance regulator, cancer cell progression, cell proliferation, cancer stem cells

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Abbreviations

ABC, adenosine triphosphate-binding cassette; ALDH, aldehyde dehydrogenase; CCK-8, Cell Counting Kit-8; CD133, cluster of differentiation-133; cDNA, complementary DNA; CFTR, cystic fibrosis transmembrane conductance regulator; CSCs, cancer stem cells; DEAB, N,N-diethylaminobenzaldehyde; EMT, epithelial-to-mesenchymal transition; HA, hemagglutinin; IgG, immunoglobulin G; nAChRs, nicotinic acetylcholine receptors; NPC, nasopharyngeal carcinoma; NSCLC, non-small cell lung cancer; OCT^{3/4}, octamer-binding transcription factor^{3/4}; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; Ph⁺ ALL, Philadelphia chromosome-positive acute leukemia; SD, standard deviation; shRNA, short hairpin RNA; SOX2, SRY (sex-determining region Y)-box 2.

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Introduction

Lung cancer is a respiratory system malignancy and one of the leading causes of cancer-related death worldwide.^{1,2} In this respect, the non-small cell lung cancer (NSCLC) accounts for 80% to 85% of lung cancer cases.^{3,4} Nowadays, introduction of novel agents and combinatory therapeutic strategies, such as therapies targeting oncogenic pathways (tyrosine kinase inhibitors, TKIs targeting epithelial growth factor receptor pathway) or immune checkpoints (immune checkpoint blockers, anti-PD1/PD-L1 therapies), together with the employment of routine lung cancer diagnosis with a combination of histomorphological, immunohistochemical, and genetic analysis to stratify patients into clinically relevant subgroups for tailored treatment algorithms, has significantly improved the outcome of NSCLC treatments.^{5,6} However, the metastatic lung cancer and development of drug resistance to therapeutic agents result in the poor prognosis in patients with NSCLC having a 5-year survival rate less than 20%.^{1,7} Therefore, it is an unmet need to identify novel targets and develop effective therapeutic agents and/or strategies for NSCLC treatments.

Adenosine triphosphate-binding cassette (ABC) proteins are a large family of active transporters across extracellular and intracellular membranes. An accumulated numbers of study demonstrated the implications of ABC activity in the cancerogenesis and development of therapeutic resistance in various cancers.⁸ Indeed, a recent study by analyzing gene expression profile using tissues from 151 patients with cancer having various types of carcinomas revealed a significant relation between the profile of ABC gene expression and clinicopathological features of cancers, including the tumor size, histological grade, or clinical stage. Among these ABC transporters, the cystic fibrosis transmembrane conductance regulator (CFTR), A3, A8, A12, and C8 were the most regulated ABC genes. In particular, the prevailed CFTR gene in the most regulated expression profile of ABC genes implied a crucial role of CFTR protein in cancer development.⁸

As a member of ABC transporter protein family, CFTR is an anion channel responsible for the transportation of Cl⁻ and HCO₃⁻ anions across epithelial cell membrane.⁹ It has been defined that mutations of CFTR gene are the cause of cystic fibrosis disease, a heterogeneous recessive genetic disorder.¹⁰ However, emerging evidences have suggested that the CFTR

may be implicated in the pathogenesis of other diseases beyond the CF, such as chronic obstructive pulmonary disease¹¹ and cancers.¹² In this regard, CFTR has been demonstrated to exert either a tumor suppressor role or an oncogenic role in distinct cancer types. For example, an increased expression of CFTR suppressed the epithelial-to-mesenchymal transition (EMT) in breast cancer cells,¹³ the proliferation and migration of endometrial carcinoma cells,¹⁴ and the progression of prostate cancer,¹⁵ intestinal cancers,¹⁶ and nasopharyngeal carcinoma (NPC).¹⁷ These findings suggest a tumor suppressor role of CFTR in these types of cancer. Conversely, the increased CFTR abundance was found in prostate cancer tissues from patients with chemoresistance and in the cisplatin-resistant cell line LNCaP/CP. A knockdown of CFTR enhanced the sensitivity of prostate cancer cells to cisplatin.¹⁸ Such an oncogenic role of CFTR was also observed in ovarian cancer,¹⁹ in which the CFTR expression was associated with the aggression of tumor *in vivo* and knockdown of CFTR inhibited the progressive potency of cancer cells *in vitro*.^{17,19}

In the case of lung cancer, an early case-control study on the correlation between the genetic variants of the CFTR gene and the risk of lung cancer demonstrated that the deltaF508 mutation and genotypes with minor alleles of rs10487372 and rs213950 single-nucleotide polymorphism of CFTR gene were inversely associated with lung cancer risk.²⁰ In this context, participants with “deletion-T” (DeltaF508/rs10487372) haplotype exhibited a 68% reduced risk for lung cancer in comparison with those who carry a common haplotype “no-deletion-C,” indicating that genetic variations in CFTR gene might have an impact on the risk of lung.²⁰ Epigenetically, methylations of the promoter of CFTR gene were quantitatively higher, and the expression of CFTR gene was significantly lower in NSCLC tissues relative to normal lung tissues. The 5-aza-2'-deoxycytidine-induced demethylation could increase CFTR gene expression. Moreover, a more methylation of CFTR gene was determined in squamous cell carcinomas than in adenocarcinomas. Interestingly, the hypermethylation of CFTR gene was associated with a significantly poorer survival in young patients with NSCLC, but not in elderly patients.²¹ These studies imply that CFTR gene may be a tumor suppressor in NSCLC; however, its function and mechanism in the development and metastasis of NSCLC need further exploration.

Tobacco smoking is the main risk factor for lung cancer. There are more than 7000 chemicals in tobacco smoke, and more than 70 of them are able to cause cancer in humans or animals. Among these chemicals, nicotine is the primary addictive component, but a recent study indicated that the tar rather than the nicotine is the cause of lung cancer, even though a high dose of nicotine may be fatal.²² However, there is a compelling body of studies in lung cancer cells and animal models demonstrated that nicotine could enhance progressive capacity of cancer cells, such as cell proliferation, migration, invasion, and EMT, and promote tumor growth-associated autocrine loops.²³⁻²⁵ In addition, nicotine exhibits an ability to inhibit cell apoptosis and decrease the biologic effectiveness of target therapy, chemotherapy, and radiotherapy.²⁶⁻²⁸ These evidences clearly suggest that nicotine may be a carcinogen or cocarcinogen. Mechanistically, nicotine can bind to nicotinic acetylcholine receptors (nAChRs) and/or β -adrenergic receptors, activating its downstream of parallel signal transduction pathways to promote tumor progression and resistance to treatments.²⁹⁻³¹

In view of the above carcinogenic role of nicotine, and tumor suppressor role of *CFTR* gene in lung cancer cells and animal models, the objective of the present study was to interrogate the impact of CFTR on the nicotine-enhanced progressive potency of lung adenocarcinoma A549 cells *in vitro*.

Materials and Methods

Cell Lines and Reagents

The adenocarcinoma lung cancer cell lines A549 (CCL#185) and HCC827 (CRL-2868) were purchased from American Type Culture Collection (Manassas, Virginia). The adenocarcinoma lung cancer cell line PC-9 was purchased from Sigma (Cat# 90071810; St Louis, Missouri). The cells were cultured and maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 1% pen/strep. L-nicotine (Cat# HY-B0683) was purchased from MedChem Express (Beijing, China), which was dissolved in dimethyl sulfoxide at a concentration of 1.0 M/L in stock, and was used at a final concentration of 200 nM/L.

Adenoviral Vectors and Infection

Adenoviral vectors expressing human CFTR (Ad.CFTR), short hairpin RNA (shRNA) to CFTR (Ad.CFTRi), and adenoviral backbone vector control (Ad.BglIII) were kindly provided by Dr John F. Engelhardt at University of Iowa (Iowa City, Iowa). The vectors were generated using pacAd5.CMV.K-N.pA or pacAd5.K-N.pA shuttle plasmid backbone (Vector Core of Center of Gene and Cell Therapy, University of Iowa). For Ad.CFTR, human CFTR complementary DNA (cDNA; M28668.1;GI:180331) was inserted into the backward position of cytomegalovirus immediate-early promoter element of shuttle plasmid (pacAd5.CMV.K-N.pA); and for generation of Ad.CFTRi, a shRNA targeting the sequence of human CFTR

messenger RNA 5'GGAAGAATTCTATTCTCAATCCAAT3' was first generated and cloned into the downstream of murine U6 promoter, the U6-shRNA-CFTR was then subcloned into the promoter-free pacAd5.K-N.pA shuttle plasmid; the Ad.BglIII was generated using the pacAd5.CMV.K-N.pA shuttle plasmid. The adenoviral expression vector was produced by cotransfecting the shuttle plasmid with E1/E3-deleted adenoviral DNA into 293 cells using calcium phosphate precipitation as described elsewhere.³² The Ad.BglIII was used as the viral sham control. A549 cells were infected with adenoviral vectors at multiplicity of infection of 100 for 24 hours before they were cultured for additional 24 hours in the presence or absence of nicotine at a final concentration of 200 nM/L. In addition, plasmid pacAd5.huCFTR-ECL4HA was a shuttle vector expressing human CFTR cDNA that a 3× hemagglutinin (HA) encoding sequence was in frame fused in the extracellular domain 4,³³ which was generated in Engelhardt Lab at University of Iowa.

Immunofluorescent Staining

The A549 cells cultured in collagen-coated cover slides were transfected with pacAd5.huCFTR-ECL4HA plasmid for 12 hours and cultured in the presence or absence of nicotine for additional 24 hours. The cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 minutes, washed in PBS for 3× 5 minutes, and permeabilized with 0.3% Triton X-100 for 10 minutes at room temperature. Nonspecific antibody binding was blocked using 5% normal donkey serum in PBS for 1 hour at room temperature, after which primary antibodies of mouse anti-CFTR and rabbit anti-HA were applied at a 1:100 dilution in PBS and applied to probe proteins of interest by incubating slides at 4°C overnight. The primary antibody binding was detected using the Alexa Fluor 488-labeled donkey-anti-mouse immunoglobulin G (IgG) secondary antibody (1:500) and Alexa Fluor 588-conjugated donkey antirabbit IgG (1:500; Jackson ImmunoResearch Lab, West Grove, Pennsylvania). After extensively washing, the slides were mounted for fluorescence in Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Lab, Burlingame, California). Images were acquired using a Leica TCS SP2 A0BS Confocal System and processed on Leica Confocal Software version 2.6.1 (Leica, Wetzlar, Germany). Detailed information of antibodies used in this study is listed in Table 1.

Cell Viability (Cell Counting Kit-8) Assay

Cell proliferation was determined by assessing cell viability using Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instruction (Beyotime Company of Biotechnology, Shanghai, China). In brief, cells (5×10^3 cells/well) were seeded in 96-well microplates and cultured overnight at 37°C. The cells were then infected with the adenoviral vector for 24 hours prior to being cultured for additional 24 hours in the presence or absence of nicotine. Subsequently, the CCK-8

Table 1. Information of Antibodies Used in the Present Study.

Antigen	Host	Vendor	Cat #	MW (kDa)
ALDH1A	Rabbit	Boster, China	BA3672	55
CD133	Rabbit	Abcam	ab19898	110
CFTR	Mouse	Millipore	M3A705583	140-170
Collagen type I	Rabbit	Proteintech	14695-1-AP	130-250
Fibronectin	Rabbit	Proteintech	15613-1-AP	250-270
HA	Rabbit	Abcam	Ab9110	NA
Ki67	Rabbit	Thermo Fisher	RB9403	395
MMP2	Rabbit	Proteintech	10373-2-AP	72
MMP7	Rabbit	Proteintech	10374-2-AP	28-30
OCT 3/4	Rat	B&D Systems	MAB1759	46
p63	Mouse	BioCare Medical	CM163a	77
PCNA	Mouse	Abcam	ab29	30
SNAL1	Rabbit	Proteintech	10399-1-AP	29
SOX2	Goat	R&D Systems	AF2018	34
TTF2	Rabbit	Proteintech	13681-1-AP	18-20
Beta-actin	Rabbit	Proteintech	20536	42

Abbreviations: ALDH1A, aldehyde dehydrogenase 1A; CD133, cluster of differentiation 133 (prominin-1); CFTR, cystic fibrosis transmembrane conductance regulator; HA, hemagglutinin; MMP2, matrix metalloproteinase-2; MMP7, matrix metalloproteinase-7; OCT^{3/4}, octamer-binding transcription factor 3/4; PCNA, proliferating cell nuclear antigen; SNAL1, snail homolog 1; SOX2, SRY (sex-determining region Y)-box 2; TTF2, transcription termination factor 2.

solution (10 μ L) was added to each well and incubated for additional 4 hours. The absorbance was measured at wavelength of 450 nm using an enzyme-linked immunosorbent assay reader (Bio-Rad Laboratories, Richmond, California).

Cell Scratch Assay

The adenovirally infected A549 cells were seeded at 80% confluence for 24 hours (cells were grown to confluence) in 6-well culture plates. The cells were then scratched with a 200 μ L pipette tip. The resultant unattached cells were removed by rinsing with prewarmed PBS for 3 times, and the wounded monolayers were cultured for additional 24 hours in the presence or absence of nicotine prior to staining with 0.1% crystal violet solution. The closure of the wounded areas was observed under a microscope at a magnification of $\times 10$ (Leica) and photographed. The percentage of area of closure was quantified and calculated with the image processing Pro Plus 6 program. These experiments were performed in triplicate, and each experiment was repeated at least 3 times.

Transwell Assay

The invasive capacity of A549 infected with different adenoviral vectors was ascertained by a transwell assay using transwell migration chambers (Cat#3413, 8.0 μ m pore; Corning Incorporated, Corning, New York). The diameter of 6.5-mm filters was coated with 100 μ L Matrigel (BD Biosciences, San Jose, California), which was diluted to 1:8 concentration using serum-free 1640 medium, incubated at 37°C in a 5% CO₂ atmosphere for 30 minutes for gelling; 10⁴ adenovirus-

infected cells in 100 μ L were seeded in the top chamber, and 700 μ L of culture medium was added in the bottom chamber. The culture was then incubated in a 5% CO₂ atmosphere at 37°C for 12 hours in the presence or absence of nicotine. The medium was then removed, and the cells were washed twice with cool PBS. Cells were then fixed with 4% paraformaldehyde for 20 minutes, prior to being stained with 1% crystal violet for 20 minutes.

Clonogenic Assay

For clonogenicity assay, 1 $\times 10^3$ /well adenovirus-infected A549 cells were seeded on 6-well plates. Cells were continuously cultured with refreshment of medium with or without nicotine with a 3-day interval for 10 days. For colony counting, the medium was removed and the cells were rinsed with PBS prior to being fixed with 4% paraformaldehyde at room temperature for 5 minutes. After removing the fixation solution, the cells were then stained with 0.5% crystal violet solution and incubated at room temperature for 30 minutes. The staining solution was carefully removed, and the cells were rinsed with H₂O to remove residual staining solution before they were air-dried at room temperature for up to 1 day. The number of colonies was counted and calculated under a light microscope. Each condition was tested in duplicate and each experiment was repeated for 3 times.

Immunoblotting Analysis

Whole-cell extract was prepared by homogenizing cells in a lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40) for 60 minutes on ice. The cell lysates (70 μ g) were resolved in a 10% to 12% sodium dodecyl sulfate polyacrylamide gel and before there were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, Massachusetts). The membrane was blocked in 4% fat-free dry milk in PBS containing 0.2% Tween-20 and probed using antibodies against proteins of interest. The protein expression levels were quantified by optical densitometry using NIH ImageJ Fiji Software (<https://imagej.net/Fiji>) if applicable. Fold change was calculated as the ratio between the net intensity of each sample divided by the respective internal controls (β -actin) as previously described.³⁴ Antibodies used in this study are listed in Table 1.

Cytometric Assay for Aldehyde Dehydrogenase-Positive Cells (ALDEFLUOR Assay)

In order to assess the fraction of aldehyde dehydrogenase (ALDH)-positive cells, the ALDEFLUOR kit from Stemcell Technologies Inc (Vancouver, British Columbia, Canada) was used per the manufacturer's protocol. In brief, A549 cells were infected with different viruses for 24 hours and cultured in the presence or absence of nicotine for additional 24 hours. Cells were then collected and 10⁶ cells were placed in cytometry tubes containing the appropriate ALDEFLUOR buffer and the

ALDH substrate, BODIPY-aminoacetaldehyde, and incubated for 45 minutes in darkness at 37°C. The enzyme inhibitor N,N-diethylaminobenzaldehyde (DEAB) was used to abolish the fluorescent signal of ALDH-positive cells and compensate the flow cytometry signal (BD FACSCalibur, San Jose, California). For the analysis of ALDH-positive cells, DEAB-treated sample was used as a negative control and ALDH activity in presence of DEAB was considered as a baseline. Flow cytometry profiling was performed on a FACScan flow cytometer and data were analyzed using FlowJo software (Ashland, Covington, Kentucky).

Statistical Analysis

All data collected in this study were obtained from at least 3 independent experiments for each condition. SPSS19.0 analysis software (SPSS Inc, Chicago, Illinois) and PRISM 6 (GraphPad software, La Jolla, California) were used for statistical analysis. Statistical evaluation of the data was performed by 1-way analysis of variance when more than 2 groups were compared with a single control and *t* test for comparison of differences between the 2 groups. Significant differences were assigned to *P* values <.05, <.01, and <.001 denoted by *, **, and ***, respectively. Data were presented as the mean (standard deviation, SD).

Results

Nicotine Impairs the Ectopic Expression of CFTR Protein in A549 Cells

In order to examine the effect of nicotine in CFTR expression of lung cancer cells, including lung adenocarcinoma A549 cells, HCC827 cells, and PC-9 cells, the abundance of CFTR protein was first evaluated in adenovirus-transduced cells by immunoblotting assay and immunofluorescent staining. Immunoblotting results demonstrated a significantly less abundant CFTR protein in Ad/CFTR-infected cells exposed to nicotine as compared to those Ad/CFTR-infected cells in the absence of nicotine in all examined 3 cell types ($P < .01$; Figure 1). Interestingly, nicotine mainly affected the expression and translocation of endogenous CFTR (from plasma membrane to cytoplasm) but less the translocation of exogenous CFTR-HA in A549 transfected with plasmid expressing HA-tagged human CFTR (stained with HA) as determined by the immunofluorescent staining assay HA (Figure 2), albeit the overall CFTR (both exogenous CFTR-HA and endogenous CFTR) protein level was decreased in cells exposed to 200 nM of nicotine (Figure 2). These results suggest that nicotine may be able to reduce the expression and/or impair the translocation of CFTR protein into membrane of A549 cells. Since the A549 cells have been widely used for studies of lung adenocarcinomas *in vitro*, this cell line was employed as the model for further investigation of CFTR function in cancer cell progressive potency.

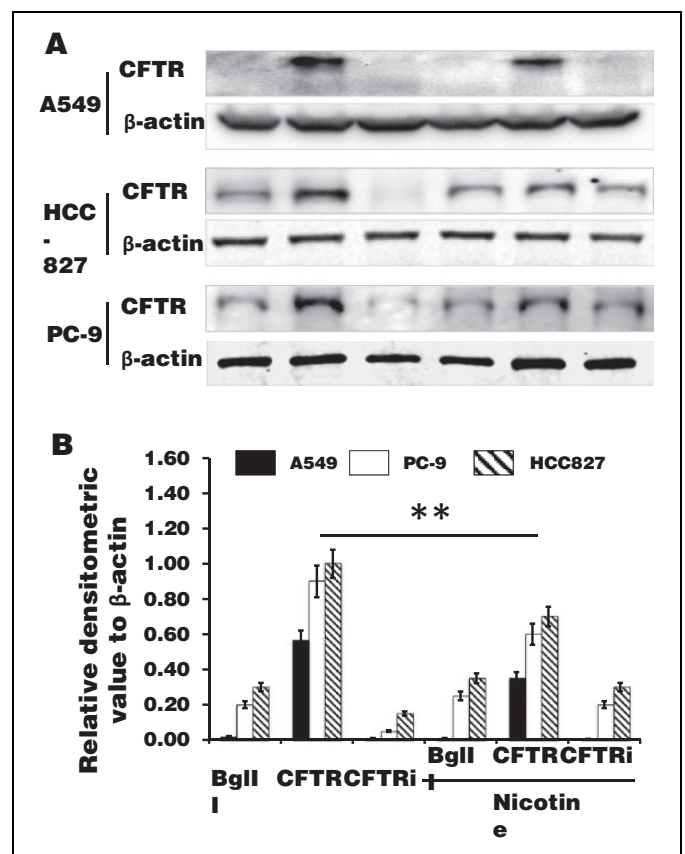


Figure 1. Nicotine suppresses cystic fibrosis transmembrane conductance regulator (CFTR) protein expression in lung adenocarcinoma cells. A, Lung adenocarcinoma cell lines A549 cells, HCC827 cells, and PC-9 cells were infected with Ad/BgII (BgII), Ad/CFTR (CFTR), and Ad/CFTRi (CFTRi) for 24 hours before they were exposed to nicotine or control for additional 24 hours. Immunoblots showed a remarkable reduction of CFTR protein in Ad/CFTR-infected cells exposed to nicotine as compared to controls. B, Densitometric assay showed a statistical difference of the abundance of CFTR proteins between the Ad/CFTR-infected cells exposed to nicotine and naive control medium ($P < .01$). Compared between indicated groups, $**P < .01$. Data represented the mean (SD) of 3 independent experiments. Comparison of differences between 2 groups was performed by a *t* test.

Nicotine Restores the CFTR-Decreased Migration and Invasion of A549 Cells

A compelling body of studies has revealed that nicotine is capable of inducing progressive potency and chemoresistance of lung cancer cells.^{25,27,31,35} In order to assess the potential impact of CFTR on the nicotine-induced progressive properties of A549 cells, effects of ectopic expression of CFTR on the cell migration and invasion in A549 cells in the presence of nicotine were examined in terms of scratch healing assay for cell migration (Figure 3) and transwell assay for cell invasion (Figure 4). Interestingly, neither ectopic expression of CFTR nor shRNA-mediated knockdown of CFTR exhibited a significant effect on cell proliferation as determined by cell viability assay, although a trend of inhibition and a promotion of cell proliferation were

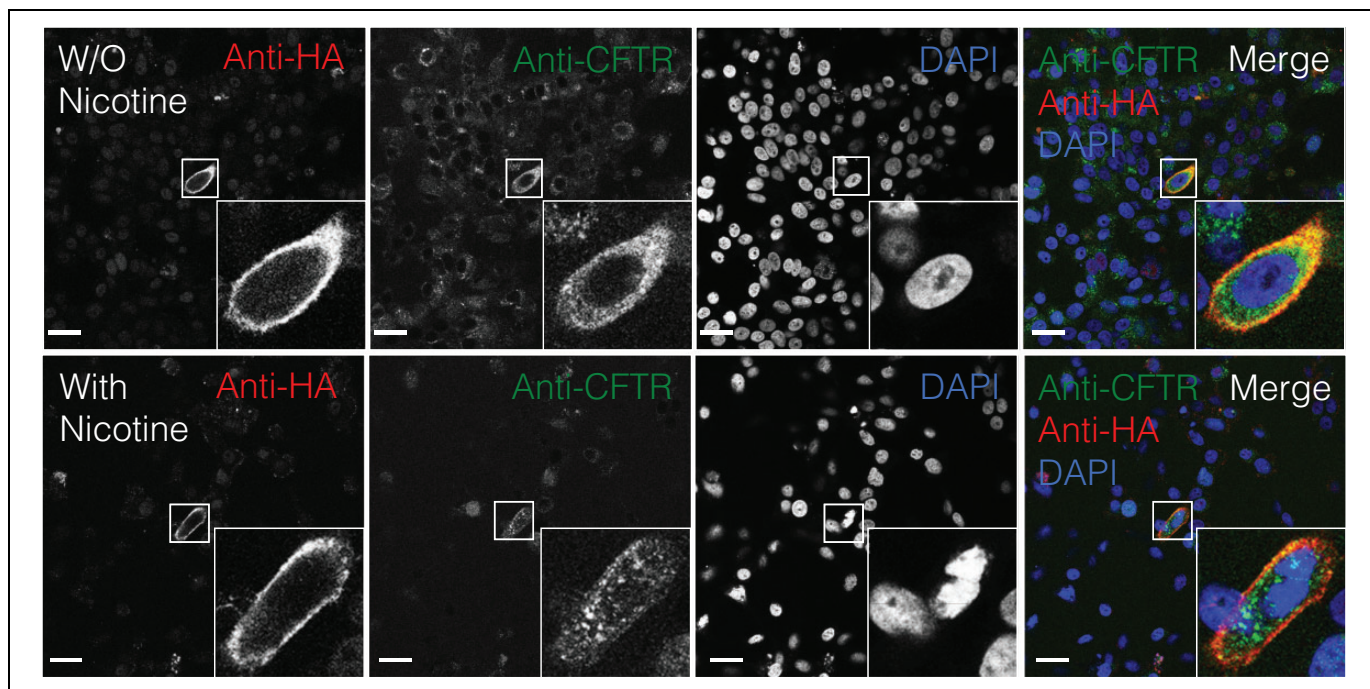


Figure 2. Nicotine inhibits cystic fibrosis transmembrane conductance regulator (CFTR) protein in A549 cells characterized by an immunofluorescent assay. A549 cells were transfected with pacAd5.huCFTR-ECL4HA plasmid for 12 hours and cultured in medium with or without 200 nm/L of nicotine for additional 24 hours. The cells were then used for assessing CFTR protein and hemagglutinin (HA) with mouse anti-CFTR antibody (1:100) and rabbit polyclonal antibody to HA (1:100), respectively. Alexa Fluor 488-labeled donkey-anti-mouse immunoglobulin G (IgG) secondary antibody (green) and Alexa Fluor 588-conjugated donkey-anti-rabbit IgG antibody (red) were used as secondary antibodies for visualizing respective proteins of interest. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. A-F, Representative images showed that nicotine was able to suppress the abundance of both membrane and cytoplasmic CFTR in transduced A549 cells (cells with both red and green staining). Of note, the overall intensity of green fluorescence was also reduced in cells exposed to nicotine compared to no nicotine-treated cells, implying a suppression of endogenous CFTR in nicotine-treated cells. Insets were the enlarged boxed regions of respective images. Bars: 50 μm .

observed in cells infected with Ad/CFTR and Ad/CFTRi in all 3 tested cell types (Supplemental Figure S1). Nevertheless, as expected, an enhanced cell migration (Figure 3) and invasion (Figure 4) were determined in A549 cells exposed to 200 nm/L of nicotine. Of importance, the ectopic expression of CFTR significantly inhibited the capacity of migration and invasion of A549 cells, regardless of the presence of nicotine ($P < .01$; Figures 3 and 4). In contrast, knockdown of CFTR by shRNA showed a dramatically enhanced potency of migration and invasion in both nicotine-treated and untreated A549 cells ($P < .01$; Figures 3 and 4). These results suggest that CFTR may play an inhibitory role in progressive characteristics in lung adenocarcinoma A549 cells, and ectopic expression of CFTR is able to suppress the migration and invasion of A549 cells.

Nicotine Restores the CFTR-Inhibited Clonogenicity of A549 Cells

Colony-forming ability is a hallmark of progressive property of cancer cells. Next we therefore sought to explore whether an overexpression of CFTR has an effect on the nicotine-enhanced clonogenic capacity of A549 cell in terms of a clonogenic

assay. Of note, an exposure of A549 cells to nicotine resulted in a strikingly enhanced capacity of colony formation in comparison with cells cultured in the absence of nicotine ($P < .01$; Figure 5). Intriguingly, the adenovirus-transduced overexpression of CFTR led to a remarkable reduction of colony formation in A549 cells, regardless of the presence of nicotine. Equally noteworthy, the nicotine-enhanced clonogenicity could be significantly inhibited by the increased CFTR expression ($P < .01$; Figure 5). In contrast, the shRNA-mediated knockdown of CFTR resulted in a significantly increased clonogenicity in A549 cells ($P < .01$; Figure 5). Since the clonogenic capacity is an important feature of stem cells, above results imply that CFTR-mediated inhibition of progressive properties of A549 cell may through a mechanism by suppressing the potency of lung cancer stem cells (CSCs).

Cystic Fibrosis Transmembrane Conductance Regulator Reduces the Expression of Cell Proliferative and Stem Cell Markers in A549 Cells

In order to better understand the molecular mechanism underlying CFTR-mediated inhibition of progressive potency in lung cancer cells, the expression of cell proliferation marker, stem

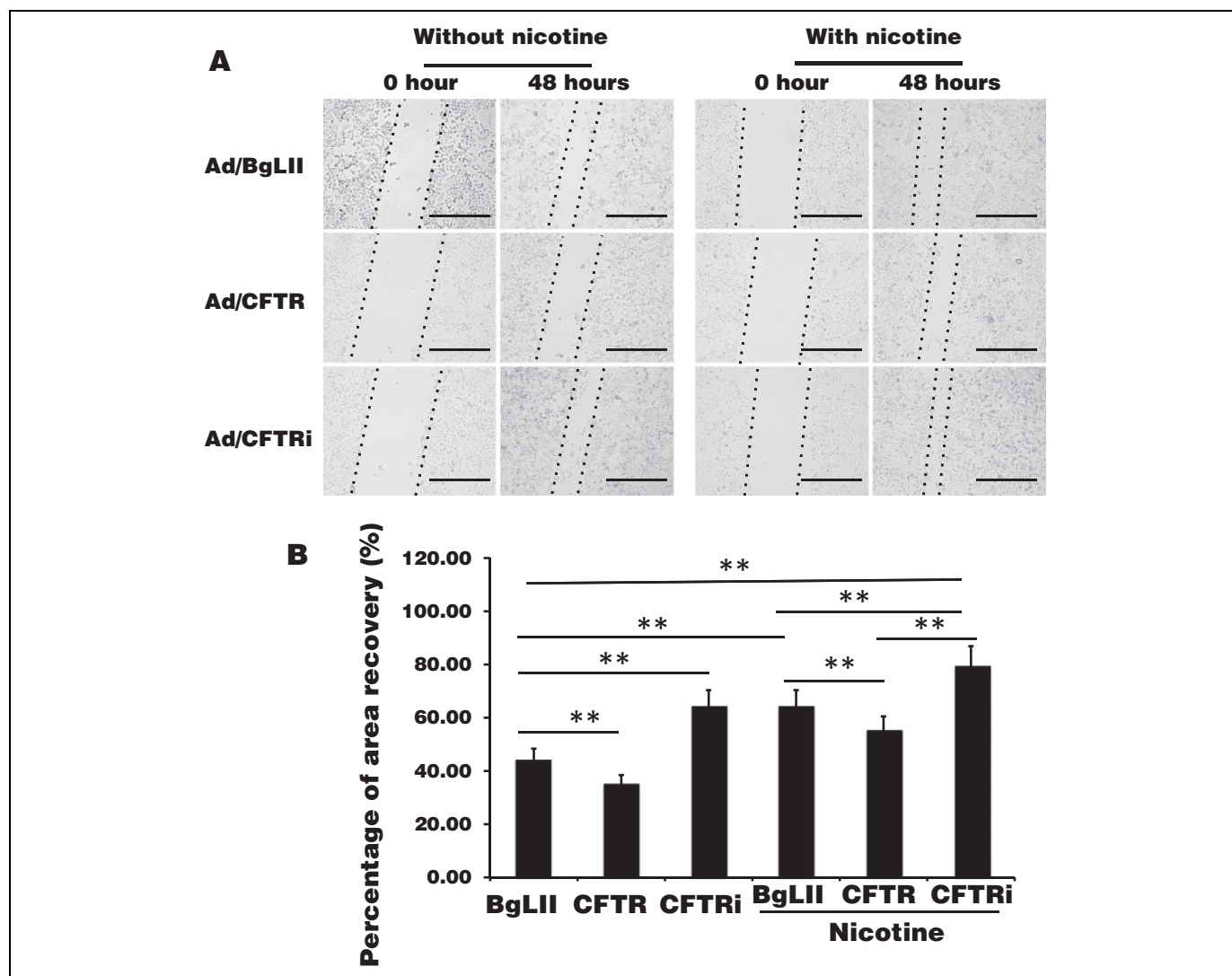


Figure 3. Impact of cystic fibrosis transmembrane conductance regulator (CFTR) on the migration of A549 cells, *in vitro*. Lung adenocarcinoma A549 cells were infected with Ad/BgLII (BgLII), Ad/CFTR (CFTR), and Ad/CFTRi (CFTRi) for 24 hours, and the capability of cell migration was assessed in terms of a scratch assay in the absence or presence of nicotine for additional 48 hours. A, Representative images of scratch assays for A549 cells cultured in indicated conditions. B, The relevant quantification of the results of cell migration index in (A). Areas between dashed lines of each image in (A) were measured using image processing Pro Plus 6 software for quantitative analysis. An overexpression of CFTR showed an ability to significantly inhibit cell migration in A549 cells. In contrast, an exposure of nicotine or knockdown of CFTR by short hairpin RNA (shRNA) strikingly promoted cell migration in A549 cells. Compared between indicated groups (B), $**P < .01$. Data represented the mean (SD) of 3 independent experiments (N = 9). Comparison of differences between 2 groups was performed by a *t* test. Bars in (A): 50 μ m.

cell-related transcriptional factors, CSC, and EMT markers was evaluated by the immunoblotting assay. Results showed an apparently less abundant of stem cell marker CD133, stem cell-related transcription factor octamer-binding transcription factor (OCT)^{3/4} and SRY (sex-determining region Y)-box 2 (SOX2), cell proliferation marker Ki67, p63, and proliferating cell nuclear antigen (PCNA; Figure 6), but there was not an overt alteration of EMT-related marker MMP7 and SNAI1 proteins and other examined proteins, including the ALDH1A that was observed (data not shown). In addition, ALDEFLUOR assay also showed no significant alteration in ALDH-expressing cell fraction between A549 cells expressing

different levels of CFTR in the presence or absence of nicotine (data not shown). These results underscored a necessity to further interrogate the mechanism underpinning CFTR-mediated suppression of progressive potency in lung cancer cells.

Discussion

Lung cancer is a leading cause of cancer-related mortality in the world. Despite advances in anticancer therapies such as targeted therapies and immunotherapies, the 5-year survival rate in patients with lung cancer remains poor, mainly owing to the relapse, metastasis, and development of therapeutic

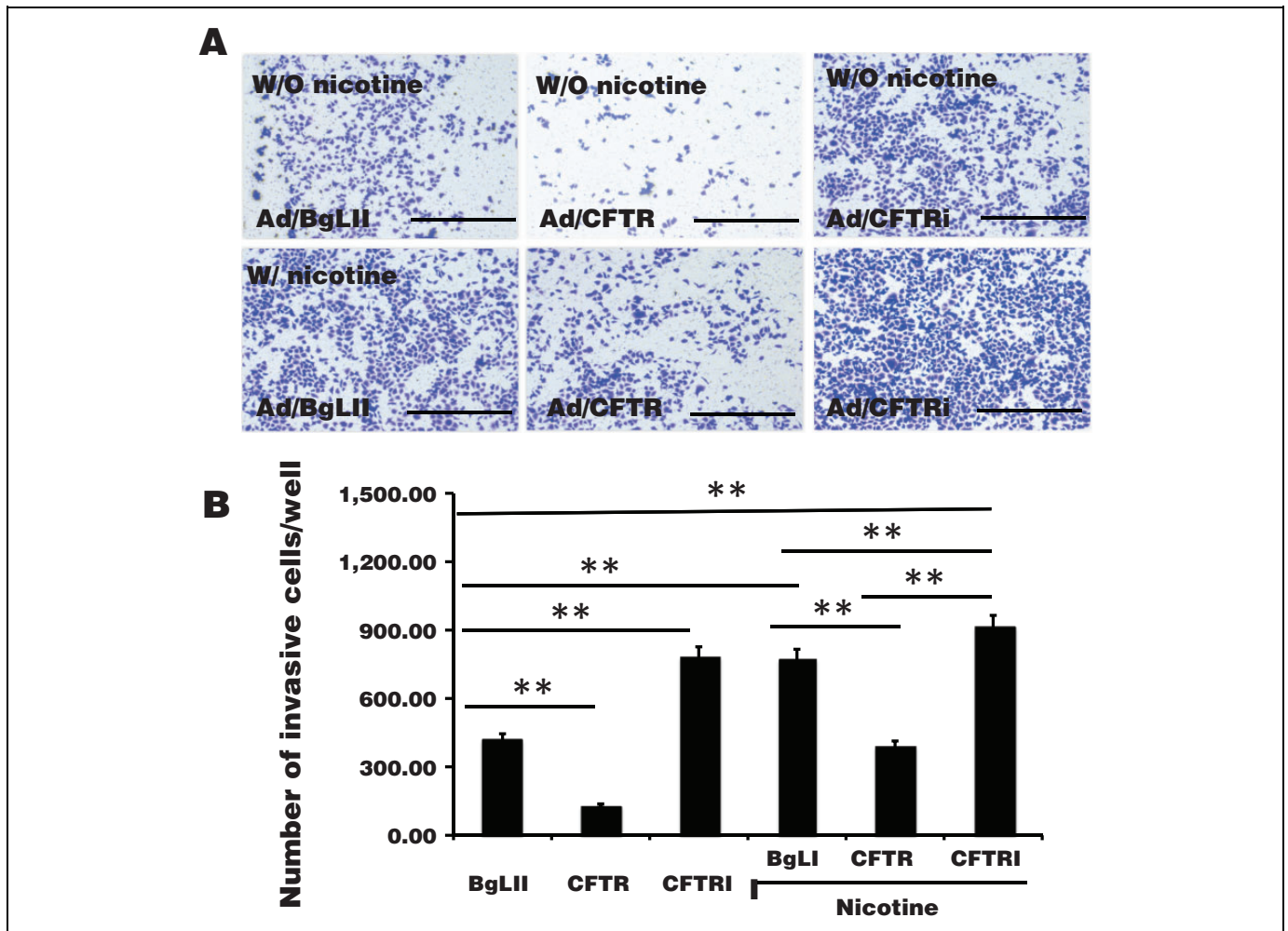


Figure 4. Impact of cystic fibrosis transmembrane conductance regulator (CFTR) on the invasion of A549 cells *in vitro*. A549 cells were infected with Ad/BgLI (BgLI), Ad/CFTR (CFTR), and Ad/CFTRi (CFTRi) for 24 hours; the capability of cell invasion was assessed by a transwell analysis in the absence or presence of nicotine. A, Representative images of transwell assay for A549 cells cultured in indicated conditions. B, Relevant quantification of the numbers of invasive cells in (A). An overexpression of CFTR showed an ability to significantly suppress cell invasion in A549 cells. In contrast, an exposure of nicotine or knockdown of CFTR by short hairpin RNA (shRNA) dramatically enhanced cell invasion in A549 cells. Compared between indicated groups (B), $**P < .01$. Data represented the mean (SD) of 3 independent experiments (N = 9). Comparison of differences between 2 groups was performed by a *t* test. Bars in (A): 50 μ m.

resistance. In the present study, the effect of *CFTR* gene in the nicotine-induced metastatic characteristics in lung cancer cells was examined in lung adenocarcinoma A549 cells. The results demonstrated that nicotine could enhance progressive properties of A549 cells in part by inhibiting *CFTR* gene expression. Interestingly, an overexpression of CFTR could remarkably inhibit the progressive potency of A549 cells, including capacities of cell migration and invasion and clonogenicity. In contrast, a knockdown of CFTR expression by shRNA led an opposite effect, that is, reduction or loss of CFTR promoted progressive abilities of A549 cells, regardless of the exposure of nicotine. Molecularly, the reduction in progressive capacity of cancer cells was along with a decreased expression of CSC marker CD133, and transcription factor SOX2 and OCT $\frac{3}{4}$, and cell proliferation markers Ki67, p63, and PCNA, but had no impact on the expression of putative lung CSC marker ALDH1

in A549 cells. The present study therefore provides an experimental evidence of that CFTR may exert a tumor suppressor role in lung cancer cells.

Tobacco use has been demonstrated to associate with the failure of cancer treatment and decrease survival rate in patients with cancer. Nicotine is one of compounds in tobacco smoke which has been recognized as an important factor to promote the development, progression, and therapeutic resistance in cancer treatments. In this regard, nicotine and its metabolites are able to promote metastatic capacity of cancer cells, including the cell migration, invasion, and EMT.^{23,25,36} Mechanistically, the nicotine binds to its nAChRs and/or β -adrenergic receptors and activates its downstream parallel signaling to promote cancer metastasis and resistance to treatment.^{23,27,35,37,38} Indeed, a blockade of nicotine binding to its receptor exhibited an ability to inhibit the

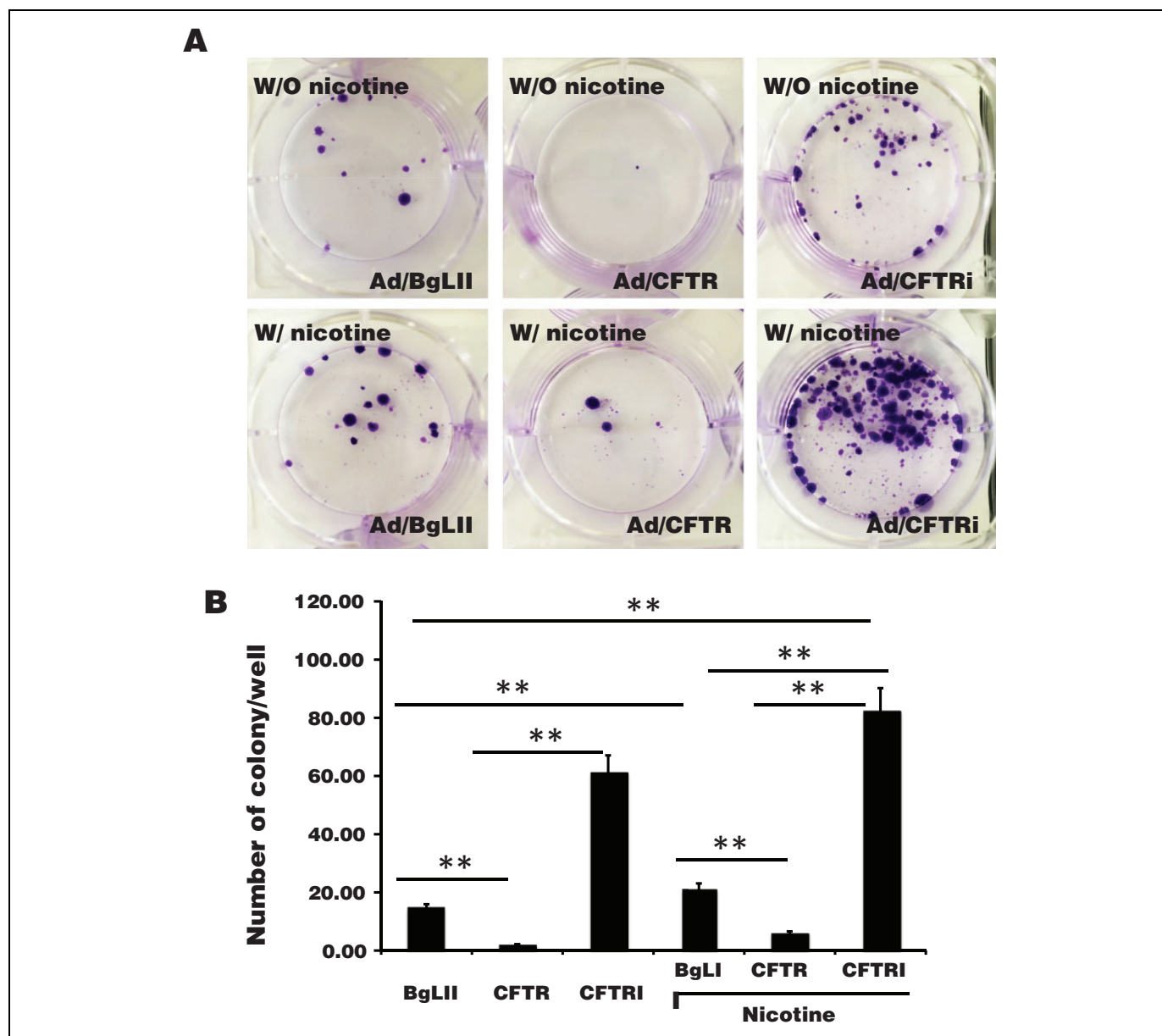


Figure 5. Cystic fibrosis transmembrane conductance regulator (CFTR) reduces the clonogenicity of A549 cells *in vitro*. A549 cells were infected with Ad/BgLI (BgLI), Ad/CFTR (CFTR), and Ad/CFTRi (CFTRI) for 24 hours; the capability of clone formation was analyzed using a clonogenic assay in 6-well plates cell in the absence or presence of nicotine for addition 10 days. A, Representative images of clonogenic assay for A549 cells cultured in indicated conditions. B, Relevant quantification of the number of colonies in (A). An overexpression of CFTR showed an ability to repress the clone formation in both A549 cells, and an exposure of nicotine and short hairpin RNA (shRNA)-mediated knockdown of CFTR significantly increased the clone formation. Compared between indicated groups (B), $**P < .01$. Data represented the mean (SD) of 3 independent experiments (N = 9). Comparison of differences between 2 groups was performed by a *t* test.

nicotine-induced tumor growth and vimentin expression in NSCLC cells.³⁹ In line with these findings, results presented in this study also showed that nicotine promoted the metastatic ability of migration, invasion, and colony formation in lung cancer A549 cells. Of note, a reduced cell proliferation was also observed in A549 cells exposed to a 200 nm/L of nicotine, suggesting a marginal cytotoxicity of nicotine at this concentration. Such a dose-dependent toxicity of nicotine in A549 cells was consistent with a previous observation by others.⁴⁰

Interestingly, signaling pathways activated by the binding of nicotine and its receptors (nAChRs) have been found to mediate many of the tobacco-related deleterious effects in the lung. In this respect, nAChRs are also regulators of CFTR in airway epithelial cells,^{37,41,42} and the loss or impaired CFTR function has been implicated in chronic lung diseases and cancers.^{11,16-19,21,43} For example, mouse-deficient $\alpha 7$ -nAChR exhibited phenotypes of bioelectric properties and mucociliary transport relevant to a deficient CFTR activity in airway epithelial cells.

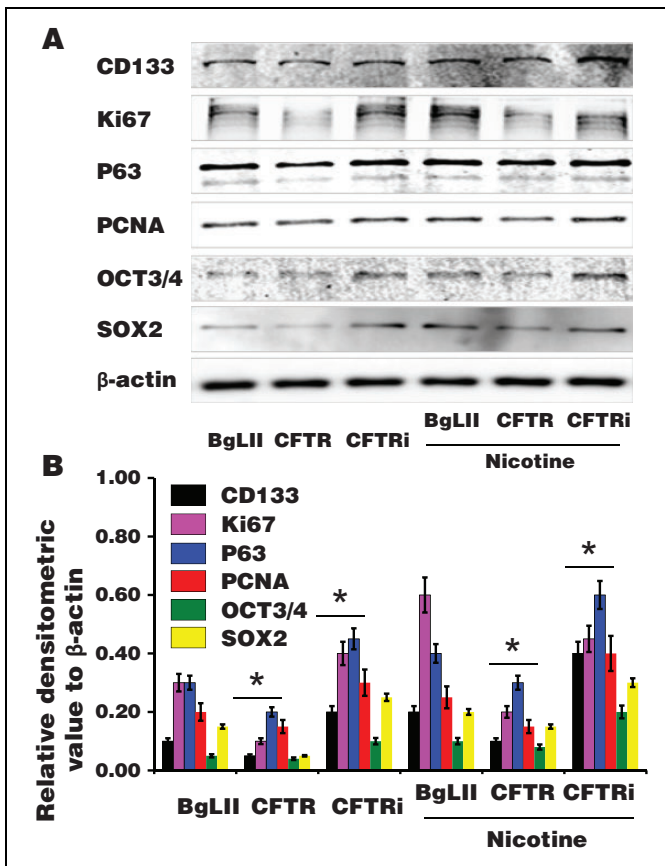


Figure 6. Cystic fibrosis transmembrane conductance regulator (CFTR) alters the expression of cell proliferative markers in A549 cells. A, A549 cells were infected with Ad/BgLII (BgLII), Ad/CFTR (CFTR), and Ad/CFTRi (CFTRi) for 24 hours before they were exposed to nicotine or control for additional 24 hours. The cells were then harvested for analyzing the expression of proliferative markers and cancer stem cell-related markers. The image showed representative image of immunoblots for cell proliferative markers Ki67, p63, and proliferating cell nuclear antigen (PCNA), putative lung cancer stem cell marker CD133, octamer-binding transcription factor (OCT) $\frac{3}{4}$, and SRY (sex-determining region Y)-box 2 (SOX2), and internal housekeeping gene control of β -actin in A549 cells treated with different conditions. An apparently less abundant examined protein was observed in Ad/CFTR-infected A549 cells relative to Ad/CFTRi-infected cells, regardless of the presence of nicotine. B, Densitometric analysis of the blots in (A) showed a statistical difference of the abundance of CFTR proteins between the Ad/CFTR-infected cells exposed to nicotine and naive control medium ($P < .01$). Compared between indicated groups, $*P < .05$. Data represented the mean (SD) of 3 independent experiments. Comparison of differences between 2 groups was performed by a *t* test.

Vice versa, a chronic exposure of nicotine could mimic the phenotype of the deficiency of $\alpha 7$ -nAChR in mice or its inactivation in human airway epithelial cells *in vitro*.³⁷ Consistently, data from this study also showed a reduction in both transduced CFTR expression and endogenous CFTR in A549 cell exposed to nicotine. Of note, nicotine alone was able to enhance the progressive capacity in A549 cells, regardless of the ectopic expression of CFTR, despite the overexpression of

CFTR could reduce the nicotine induced the progressive property of this NSCLC cell type.

Many lines of evidence recently demonstrated that CFTR played roles in the development, progression, and therapeutic resistance in a variety of cancer types, in which it may act as either a tumor suppressor or oncogene. For example, CFTR was found to exert a tumor suppressor role in murine and human intestinal cancer,¹⁶ endometrial carcinoma,¹⁴ breast cancer,¹³ NPC,¹⁷ and possibly the NSCLC.²¹ Conversely, a high expression of CFTR was reported to correlate with progressive capacity in ovarian cancer cells¹⁵ and Philadelphia chromosome-positive acute leukemia (Ph⁺ ALL).⁴⁴ A knock-down of CFTR in ovarian cancer cells and Ph⁺ ALL cells suppressed their metastatic capability, suggesting that CFTR played an oncogenic role in these 2 types of malignancies. Of interest, controversial functions of CFTR were reported in prostate cancer. In this regard, an ectopic expression of CFTR was first reported to suppress tumor progression in prostate cancer cells through miR-193b targeting urokinase plasminogen activator.¹⁵ However, a recent study demonstrated that the knockdown CFTR led an enhancement of sensitivity of prostate cancer cells to cisplatin via an inhibition of autophagy.¹⁸ These studies imply that CFTR may be multifaceted protein with both of tumor suppressor role and oncogenic role in distinct malignancies or cancer states. In the present study, an ectopic expression of CFTR was found to significantly inhibit the migration, invasion, and clonogenicity in lung cancer A549 cells, and knockdown of CFTR displayed an opposite effect, that is, enhancing the progressive properties in this lung cancer cell type, regardless of the presence of nicotine. Together with these findings and others,²¹ these results indicate that CFTR likely plays a tumor suppressor role in NSCLC cells.

There is growing body of evidence demonstrated that lung CSCs are responsible for the initiation, progression, metastasis, relapse, and therapeutic resistance in patients with lung cancer. Despite CSCs have been reported in a wide spectrum of human tumors, studies in biology of CSCs in NSCLC remain a challenge, partially owing to lacking of CSC-specific markers in lung cancers. Currently, the side population, ALDH, cluster of differentiation-133 (CD133), CD87, CD90, CD44, CD166 have been explored and considered as markers for NSCLC.⁴⁵ Among them, CD133 and ALDH1 are 2 of the most investigated ones.⁴⁶⁻⁴⁸ Transplantation of CD133⁺ cells isolated from NSCLC and SCLC tumor samples into severe combined immune-deficient mice could regenerate tumors phenotypically identical to the original tumor and develop chemoresistant phenotype following treatment with chemotherapeutic agents.⁴⁶ Aldehyde dehydrogenases are putative stem cell markers and are correlated with therapeutic resistance in many cancer types, including NSCLC.^{47,48} In addition, pluripotency transcription factors, such as SOX2 and OCT4 are responsible for stemness properties in stem cells, have also been suggested as CSC markers in lung cancer.⁴⁹ In the present study, although the overexpression of CFTR failed to alter the expression of EMT markers and ALDH; however, the abundance of stem cell pluripotency-related transcription factors, cell proliferation

markers, and lung CSC marker CD133 was reduced in Ad/CFTR-infected A549 cells. Of note, our results present in this study could not rule out the impact of CFTR on CSC phenotype in NSCLC cells in general, and A549 cells in particular. Further studies are required to investigate underlying mechanisms of CFTR in the development, progression, and therapeutic resistance in NSCLC.

Collectively, in the present study, we showed that nicotine could inhibit both the adenovirus-transduced ectopic expression of CFTR and endogenous CFTR expression in lung adenocarcinoma A549 cells, HCC827, and PC-9 cells. An overexpression of CFTR led to suppress the progressive properties of migration, invasion, and clonogenicity in NSCLC A549 cells, regardless of nicotine. Conversely, a knockdown of CFTR expression by shRNA resulted in an enhanced progressive potency of A549 cells. This study thus suggests that *CFTR* gene may be a tumor suppressor and a novel target for treatment in NSCLC and deeply investigates the underlying molecular mechanism of function of CFTR in NSCLC that may offer an insight into our understanding of the metastasis of lung cancers.

Authors' Note

H.L. and N.M. contributed equally to this work. J.S. and X.L. conceived and designed the experiments. H.L. and N.M. analyzed the data and drafted the manuscript. H.L., N.M., Y.W., C.Y., J.W., M.L., and Y.J. performed experiments and acquired data. J.W. and J.C. collected human samples and performed RT-PCR analysis. J.C., J.S., and X.L. interpreted data and critically revised the manuscript. All authors read and approved the final version of the manuscript. The authors are solely responsible for the contents and writing of this paper. The study and protocol were approved by the ethics committee for conduction of human research at General Hospital of Ningxia Medical University (NXMU-2017-004). The ages of all the patients analyzed ranged 40 to 72 years old. All participants gave informed consent. Biopsies of human lung were obtained from the patients with lung adenocarcinoma undergoing cancer resection in General Hospital of Ningxia Medical University. The biopsies of normal tissue, tumor adjacent tissue, and tumor tissue of lung of patient with lung cancer undergoing cancer resection were snap frozen in liquid nitrogen, before they were homogenized in TRIzol reagent for RNA extraction per manufacturer's instruction (Invitrogen, Carlsbad, California). Total RNAs isolated from lung biopsies of 20 pathologically confirmed lung adenocarcinoma tissues were analyzed in this study.

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Supplemental Material

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