Continual Exposure to Cigarette Smoke Extracts Induces Tumor-Like Transformation of Human Nontumor Bronchial Epithelial Cells in a Microfluidic Chip

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Introduction: Heavy cigarette smoking-related chronic obstructive pulmonary disease is an independent risk factor for lung squamous carcinoma. However, the mechanisms underlying the malignant transformation of bronchial epithelial cells are unclear.

Methods: In our study, human tumor-adjacent bronchial epithelial cells were obtained from 10 cases with smoking-related chronic obstructive pulmonary disease and lung squamous carcinoma and cultured in an established microfluidic chip for continual exposure to cigarette smoke extracts (CSE) to investigate the potential tumor-like transformation and mechanisms. The integrated microfluidic chip included upstream concentration gradient generator and downstream cell culture chambers supplied by flowing medium containing different concentrations of CSE.

Results: Our results showed that continual exposure to low doses of CSE promoted cell proliferation whereas to high doses of CSE triggered cell apoptosis. Continual exposure to CSE promoted reactive oxygen species production in human epithelial cells in a dose-dependent manner. More importantly, continual exposure to low dose of CSE promoted the epithelial-to-mesenchymal transition process and anchorage-independent growth, and increased chromosome instability in bronchial epithelial cells, accompanied by activating the GRP78, NF-κB, and PI3K pathways.

Conclusions: The established microfluidic chip is suitable for primary culture of human tumor-adjacent bronchial epithelial cells to investigate the malignant transformation. Continual exposure to low doses of CSE promoted tumor-like transformation of human nontumor bronchial epithelial cells by inducing reactive oxygen species production and activating the relevant signaling.

Key Words: Smoking, Transformation, Epithelial cells, Reactive oxygen species, Microfluidic chip

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Cigarette smoking is the most important risk factor of lung cancer, and smokers have a 15- to 30-fold increased risk to develop lung cancer.[1] Long-term of heavy smoking can cause chronic obstructive pulmonary disease (COPD), and cigarette smokers account for more than 90% of COPD patients in developed countries.[2] Recently, a growing body of epidemiological studies has consistently demonstrated a link between cigarette smoking-related COPD and lung cancer. It has been reported that 50% to 70% of lung cancer patients also suffer from COPD[3] and that smokers with COPD had up to a four- to sixfold increased risk of lung cancer.[4,5] Therefore, COPD is strongly associated with lung cancer and may be a surrogate marker for patients' background with squamous cell lung carcinoma.[6]

However, the molecular mechanisms by which long-term smoking induces the malignant transformation of bronchial epithelial cells and the development of lung cancer have not been clarified. Previous studies have shown that heavy smoking-related COPD is characterized by excessive inflammation and extracellular matrix destruction[7] and shares many risk factors with lung cancer in humans. Cigarette smoke extract (CSE) can enhance the neoplastic transformation of human bronchial epithelial cells,[8] and tobacco smoking can promote lung tumorigenesis by activating the inflammatory signal pathways.[9] Other studies reveal that long-term smoking induces chronic lung inflammation and oxidative stress, and promote the process of epithelial-to-mesenchymal transition (EMT), DNA damage, and cellular proliferation.[3,10,11]

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Oxidative stress can induce abnormally higher levels of reactive oxygen species (ROS) production, which is a critical factor for tumorigenesis.[12,13] Cigarette smokers are exposed to many types of oxidants, which can cause mitochondrial dysfunction, resulting in ROS production and the NF-κB, STAT3, and PI3K activation that promote cell proliferation during the malignant transformation.[14,15] However, most of these studies are performed in cell lines or animal models, and the results may not reflect the real process of human bronchial epithelial cells. Currently, conventional in vitro models are based on culturing cells under static conditions, with a fixed concentration of media supplement, nutrition composition, or substances from cigarettes, which are far away from the real environments in vivo. Furthermore, there is no current ideal animal model to study on cigarette smoking-induced COPD and lung cancer,[16] and there are only few studies on the tumor-like transformation of bronchial epithelial cells from patients with COPD, because of limited resource. Therefore, there is an urgent need to establish a novel model with which we can investigate the molecular mechanisms underlying the process of continual cigarette smoking-induced tumor-like transformation of human nontumor bronchial epithelial cells from COPD patients.

Microfluidic chip technology has been used for sample preparation, biological and chemical reaction, separation, and tests with many advantages.[17,18] Microfluidic chip-based cell cultures can continually supply the cells with fresh medium of oxygen and nutrition at a controlled flow rate.[19] In addition, microfluidic chip can be sued for high-throughput tests and has been widely used in the field of drug screening, single cell analysis, and cell signal pathway research.[20] Thus, the microfluidic chip may provide a unique and ideal platform to study the continual exposure of primarily cultured human bronchial epithelial cells to CSEs.

In this study, we built a microfluidic chip for continual exposure of primarily cultured human bronchial epithelial cells to CSE to investigate their tumor-like transformation and potential molecular mechanisms. We employed human nontumor bronchial tissues from 10 patients with lung cancer and smoking-related COPD to obtain bronchial epithelial cells because these nontumor bronchial tissues surrounding the tumor mass are already exposed to cigarette smoking and chronic inflammation.[21] We found that continual exposure to CSE promoted the tumor-like transformation of primarily cultured human nontumor bronchial epithelial cells in the microfluidics by inducing endoplasmic reticulum stress and ROS production, and activating the NF-κB and PI3K/AKT/mTOR signaling.

MATERIALS AND METHODS

The Fabrication of Microfluidic Chip

A microfluidic chip contained four parallel operating modules for simultaneous culture of four cell samples (Fig. 1*A*) and one module including a concentration gradient generator (CGG), six main channels (width 300 μm, height 100 μm), and 18 cell chambers (diameter 2.5 mm, height 100 μm, Fig. 1*B*). The CGG included five cascaded-mixing stages that generated linear concentrations by adjusting the flow rate of two merging solutions in each stage through controlling channel length, which was proportional to fluidic resistance. When the cell culture medium and CSE were filled into channels by a syringe pump, the CGG theoretically produced at least six different concentrations of CSE media (with CSE proportions of 0:1:3:5:7:9). A microfluidic chip is shown in Figure 1*C*. The polydimethylsiloxane (PDMS) microfluidic chip was fabricated using a Sylgard 184 kit (Dow Corning, Midland, TX) by a standard soft lithography method. Briefly, PDMS was fabricated by replica molding against masters, and bonded to a glass slide that had been treated with oxygen plasma (150 mTorr, 50 W, 25s). The finished PDMS microfluidic chip was first treated with dehydrated alcohol then distilled water, and autoclaved at 120°C and 103.4kPa for 30 minutes.

Specimen Collection and Tumor-Adjacent Bronchial Epithelial Cell Culture

This study was approved by the Institutional Review Board and Human Ethics Committee of the First Affiliated Hospital of Dalian Medical University, and written informed consent was obtained from individual patients. A total of 10 patients with lung squamous carcinoma and surgical treatment were recruited in the First Affiliated Hospital of Dalian Medical University, China, from January 2013 to May 2013 (see Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A607, which demonstrates the demographic and clinical data). The criteria for the enrollment of patients were as follows: (1) pathological identification of a lung squamous carcinoma; (2) a history of COPD with current lung function (FVE1/FVC less than 70%, 2011 GOLD); (3) a history of cigarette smoking for more than 40 pack-years; and (4) currently without any other respiratory disease, such as tuberculosis.

After surgical removal of tumor, the tumor-adjacent bronchial tissues were dissected and the connective tissue, mucus, and blood clot in the samples were removed. The remaining bronchial tissues were cut into small pieces of approximately 1 mm^3 and cultured on six-well plates precoated with placenta collagen IV (Sigma Aldrich, St. Louis, MO) in serum-free airway epithelial cell medium (Promocell, Heidelberg, Germany) at 37°C in a humidified atmosphere of 5% of CO_2. The cells were exposed to fresh medium every other day until the cells reached 80% confluence. Subsequently, the structure of cells was examined by hematoxylin-eosin (H&E) staining, and the expression of cytokeratin 19 (CK19) was determined by immunofluorescent assay using specific antibody (Abcam, ab15463).

CSE Preparation

CSE was prepared, as described previously.[22] Briefly, the smoke from two lit cigarettes (Research Grade Cigarette, University of Kentucky) was collected into 50 ml serum-free medium using constant vacuum flow. The CSE solution was filtrated through a 0.22-μm pore filter and utilized in experiments within 30 min.

Continual Exposure of Human Tumor-Adjacent Bronchial Epithelial Cells to CSE in a Microfluidics

The tumor-adjacent human bronchial epithelial cells were harvested, adjusted to a density of 1×10^6 cells/ml in medium, and were injected into the cell inlets #1–#6 of the microfluidic chip using a syringe pump at a constant flow rate of 8 μL/min for 10 seconds. After the cells were attached, the

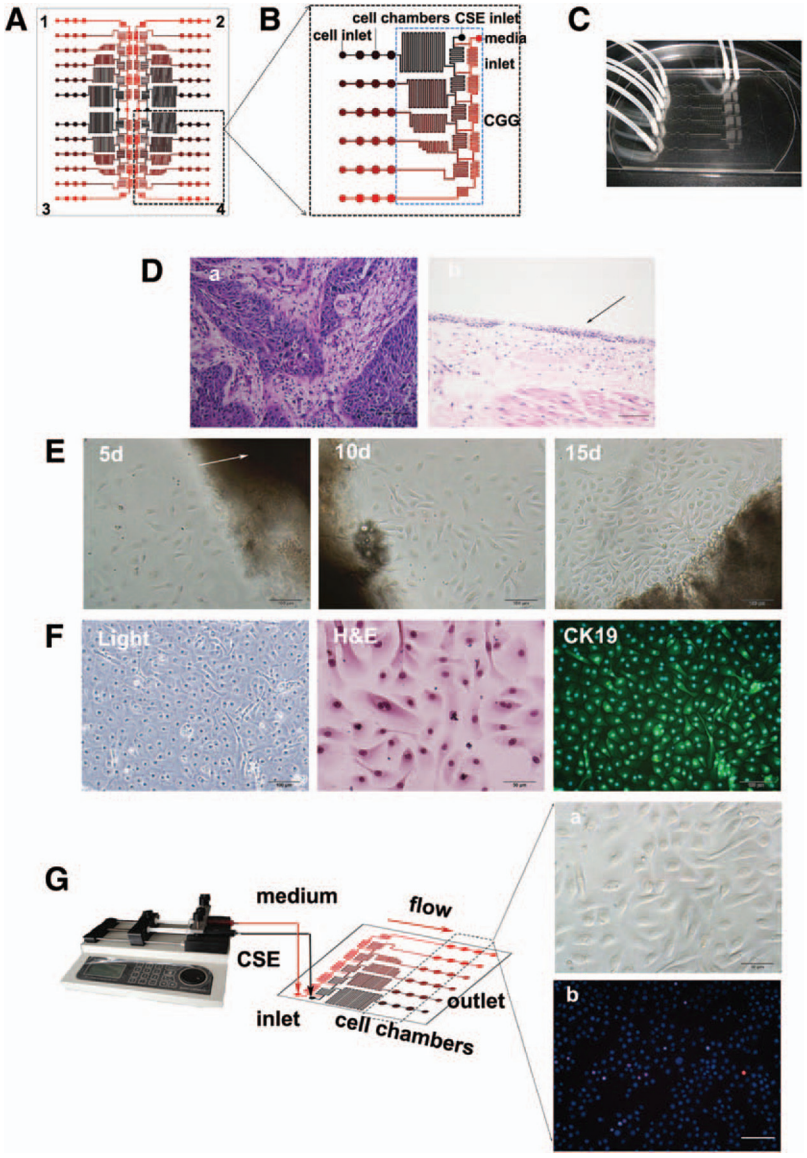

FIGURE 1. Fabrication of a microfluidic chip for cell culture. *A*, The designed microfluidic chip containing four parallel operating modules. (*B*) Magnified section of an operating module including one media inlet, one cigarette smoke extract (CSE) inlet, one concentration gradient generator (CGG), 18 parallel cell chambers, and six cell inlets. The fabricated CGG inside the microfluidic chip (in the blue box) can produce six different concentrations of CSE (theoretical proportion 0:1:3:5:7:9). The black represents CSE and the red represents medium. A concentration gradient of CSE was obviously shown from inlet one to six. (*C*) The image of an operating module in the microfluidic chip viewed from above.(D) Representative images of the histological examination of the surgical specimens of lung cancer tissues, including lung cancer area (a) and tumor-adjacent bronchus (b, black arrow, scale bar 100 μm). (E) The structure of the cells under a light microscope after cultured from day 5 to 15 (white arrow: the tumor-adjacent bronchus). (*F*) Cytological identification of the primarily cultured epithelial cells by H&E and anti-CK19 immunofluorescent staining. (*G*) The schematics of the primarily cultured bronchial epithelial cells that exposed to CSE on the microfluidic chip. The microfluidic chip with a syringe pump supplied medium and CSE simultaneously to the cells through the inlet. The CGG integrated in the microfluidic chip produced a concentration gradient of CSE. The primarily cultured cells (a) obtained from the case 1 grew in the microfluidic chip without CSE for 2 weeks and the cell apoptosis was assayed by Hoechst33342/propidium iodide staining and the percentages of red nuclei in blue nuclei was less than 10% (b, scale bar 100 μm).

cells were supplied with complete medium at a constant rate of 5 μL/min for 48 hours, by a syringe pump in a perfusion mode. When the cells reached 80% confluence, the cells were exposed to different concentrations of CSE in medium by simultaneously infusing medium and different concentrations of CSE stock solutions into the chip through the CSE inlet and medium inlet at a constant rate of 7 and 5 μL/min, respectively.

Proliferation and Apoptosis Assay of Human Bronchial Epithelial Cells

The impact of CSE on cell proliferation was determined using a Cell-Light EdU Apollo 488 in vitro Kit (Ribobio, Guangzhou Ribobio Company, China), according to the manufacturers' instruction. Briefly, cells (1000/chamber) were treated with, or without, CSE for 48 hours and labeled with EdU for 2 hours. The cells were fixed, stained for Apollo and DNA, and examined under a fluorescent microscope (Olympus IX71, Japan, magnification factor ×200). The effect of CSE on cell apoptosis was determined by staining with Hoechst33342 and propidium iodide staining (Sigma Aldrich).

The Detection of ROS and Its Related Signaling Pathways in the Cultured Human Bronchial Epithelial Cells

The levels of cytoplasmic ROS were characterized using a ROS Assay kit, according to the manufacturers' instruction (Beyotime Biotechnology, Jiangsu, China). Briefly, cells were treated with, or without, CSE for 48 hours and stained with DCFH-DA for 20 minutes by injecting fluorescent probe in medium (1:1000) into the chambers, followed by examining under a fluorescent microscope.

The impact of ROS on the GRP78, NF-κB, and PI3K expression was determined by immunofluorescent assays. Briefly, cells were pretreated with, or without, a ROS inhibitor N-acetylcysteine (NAC, 20 mM, Sigma) for 12 hours and exposed to CSE for 3 hours. The cells were fixed with

4% paraformaldehyde, permeabilized, and blocked by 5% goat serum. Subsequently, the cells were stained with 1:200 diluted anti-GRP78 (Santa Cruz), anti-phospho-IRE1α (Abcam, ab48187), anti-phospho-IKKα (Beijing Biosynthesis Biotechnology, China), anti-NF-κB p65 (Abcam, ab7970), anti-cyclin D1 (Abcam, ab16663), anti-PI3Kp85 (Abcam, ab86714), anti-phospho-AKT (Cell Signaling Technology), and anti-phospho-mTOR (Santa Cruz) overnight at 4°C, respectively. After being washed, the bound antibodies were detected using Dylight488 or Dylight594-labeled secondary antibodies (1:400, Jackson ImmunoResearch Laboratories) at 37°C for 1 hour. The cells were examined and imaged under a fluorescence microscope.

The expression of GRP78, NF-κB, and PI3K in the lung cancer tissues was determined by immunohistochemistry[23] using antibodies against GRP78, NF-κB, and PI3K and the SP-9000 Histostain TM-Plus Kits (ZYMED).

Detection of E-cadherin and Vimentin Expression

The impact of CSE on the E-cadherin and Vimentin expression was determined by immunofluorescent assays. Briefly, the cultured cells in the chambers were pretreated with, or without, 20 mMNAC, GRP78 inhibitor 4-phenylbutyrate (4-PBA, 5 mM, Sigma), NF-κB inhibitor BAY11-7082 (2 μM, InvivoGen) or PI3K inhibitor LY294002 (50 μM, Sigma) for 12 hours and then exposed to CSE for 48 hours twice a week for 8 weeks. The cells were fixed, permeabilized, and stained with 1:200 diluted anti-E-cadherin (Epitomics) or anti-Vimentin (Abcam), and were visualized using Dylight488-labeled secondary antibodies, followed by examining under a laser scanning confocal microscope.

In addition, the relative levels of E-cadherin and Vimentin expression were determined by Western blot. Briefly, cells at 10^5/well were cultured in six-well plates, pretreated, and treated for 8 weeks, as described earlier. The cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (Pall). After being blocked with 5% fat-free milk, the membranes were incubated with antibodies against E-cadherin (Epitomics, 1:1000), Vimentin (abcam, 1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma,1:5000), and the bound antibodies were detected using a Super Signal West Pico Kit (Thermo), followed by quantitatively densitometric analyses using the Eagle Eye II software.

Anchorage-Independent Growth Assay

The impact of CSE on anchorage-independent growth of human bronchial epithelial cells was examined. Briefly, after being exposed to CSE for 8 weeks, the cells at 2000/well were cultured in 0.3% low-melt agarose (Sigma) in complete medium that were overlaid on 0.6% agarose in six-well plates for 2 weeks. Individual cell colonies with more than 50 cells were counted under a microscope.

Chromosome Analysis

Cells were pretreated with, or without, inhibitors for 12 hours, as described above, and exposed to CSE for 48 hours twice a week for 8 weeks. The cells were treated with 0.04 μg/ml of colchicines for 4 hours and subjected to chromosome analysis.[24]

Statistic Analyses

All experiments were performed at least for three times. Data are expressed as the means ± SD. The difference among groups was analyzed by analysis of variance using SPSS13.0 for Windows software. p value less than 0.05 was considered statistically significant.

RESULTS

Culture of Human Bronchial Epithelial Cells on a Microfluidic Chip

To test the feasibility of a microfluidic chip for culturing cells, 10 surgical lung squamous carcinomas (Fig. 1*D*a) were obtained and their tumor-adjacent human bronchi (Fig. 1*D*b) were cut into small pieces. After culture of small pieces of nontumor tissues for varying periods, human bronchial epithelial cells migrated from the tissues, proliferated, and exhibited in single-layer growth (Fig. 1*E*). The cells displayed a typical structure of lung epithelial cells and expressed CK19, an indicative of epithelial character (Fig. 1*F*). After incubation of the cells in the microfluidic chambers supplied with continually flowing medium for 2 weeks, the cells remained in epithelial structure (Fig. 1*G*a) and approximately less than 10% of the cells only underwent apoptosis (Fig. 1*G*b). In addition, we observed that the cells from most of the samples survived about 10 weeks in the microfluidic chip. These observations clearly demonstrated that the established microfluidic chip was successful to culture human primary nontumor bronchial epithelial cells.

The Effect of CSE on Cell Proliferation and Apoptosis

To investigate the effect of continual CSE exposure on the survival, human bronchial epithelial cells were continually exposed to medium containing different percentages (2.37–91.88%) of CSE for 48 hours and the percentages of cell proliferation and apoptosis were quantitatively analyzed in Figure 2*A*. Treatment with lower doses (2.37–12.28%) of CSE seemed to stimulate human epithelial cell proliferation in a dose-dependent manner, but did not trigger cell apoptosis. In contrast, treatment with higher doses (19.86–91.88%) of CSE induced human bronchial epithelial cell apoptosis in a dose-dependent manner. A similar pattern of cell proliferation and apoptosis was observed by fluorescent staining (Fig. 2*B*,*C*). Interestingly, treatment with lower doses of CSE for 3 or 8 weeks continually stimulated human bronchial epithelial cell proliferation in the microfluidic chip (Fig. 2*D*). Therefore, continual exposure to different doses of CSE had opposite effects on the proliferation and survival of primarily cultured human bronchial epithelial cells.

Continual Exposure to CSE Induces ROS Production and Activates the Relevant Signal Pathways in Primarily Cultured Human Bronchial Epithelial Cells

Cigarette smoking induces oxidative stress. Next, we tested the effect of continual exposure to CSE on the ROS production and relevant signaling. The isolated bronchial epithelial cells were treated with different concentrations

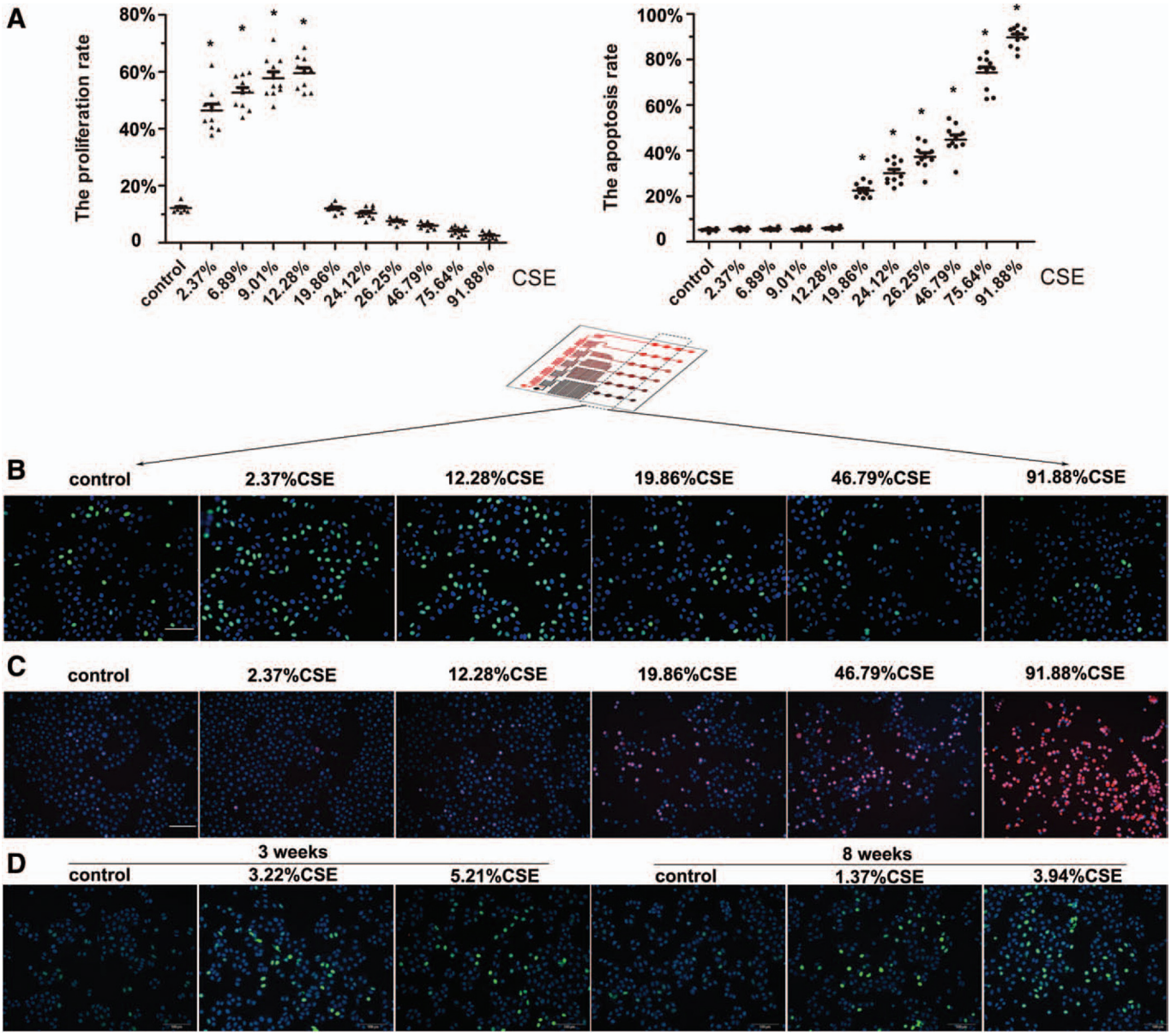

FIGURE 2. Continual exposure to different doses of cigarette smoke extract (CSE) affects the proliferation and apoptosis of human bronchial epithelial cells. Human bronchial epithelial cells were exposed to CSE at the indicated concentrations for 24 hours and the proliferation and apoptosis of cells were determined by EdU and Hoechst33342/propidium iodide staining, respectively. Additional cell proliferation assays were performed at 3 and 8 weeks post CSE exposure. Data are representative images and expressed as individual means of percentages of cell proliferation and apoptosis from 10 samples from 10 separate experiments. *A,* The percentages of cell proliferation and apoptosis. **p* less than 0.05 versus the controls. The horizontal lines reflect the median values for each group of cells. (*B*) Fluorescent assay of cell proliferation (the green nucleus cells). (*C*) Fluorescent assay of cell apoptosis (the red nucleus cells). (*D*) Fluorescent images of cell proliferation at the indicated periods (scale bar 100 μm).

of CSE for 3 hours and stained with DCFH-DA. The mean intensity of fluorescent staining in the cells from individual patients was quantitatively analyzed in Figure 3*A*. Apparently, treatment with CSE induced ROS production in a dose-dependent manner and a representative pattern of fluorescent images from the case 5 is shown in Figure 3*B*. Analysis of the ROS-related signaling revealed that increased levels of GRP78 expression and IRE1α phosphorylation were detected in the cells following treatment with CSE for 30 minutes. The IKKα phosphorylation, NF-κB nuclear translocation, and cyclin D1 expression were detected in the cells at 1 hour post CSE exposure. Similarly, increased levels of PI3K expression, AKT and mTOR phosphorylation were also detected in the cells at 1 hour post CSE exposure (Fig. 4*A*). However, in comparison with that in the cells without exposure to CSE or NAC, there was no obvious change

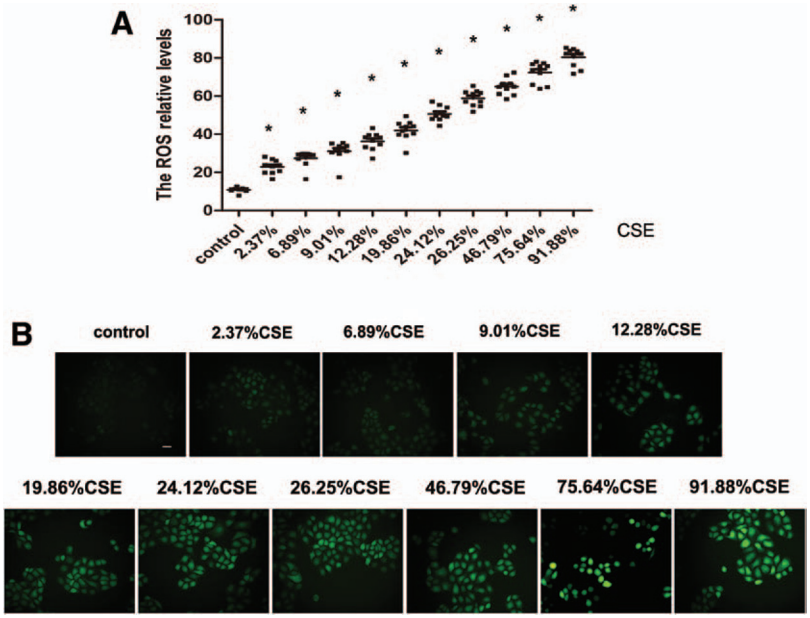

FIGURE 3. Continual exposure to different doses of cigarette smoke extract (CSE) induces reactive oxygen species (ROS) production in human bronchial epithelial cells in a dose-dependent manner. Human bronchial epithelial cells were exposed to CSE at the indicated doses for 3 hours and the contents of intracellular ROS were determined by fluorescent probe DCFH-DA. Data are representative images and expressed as individual means of the intensity of fluorescent staining per cell from 10 samples. (*A*) The levels of intracellular ROS after normalized to cell numbers. **p* less than 0.05 versus the controls. The horizontal lines reflect the median values for each group of cells. (*B*) Fluorescent images of intracellular ROS levels (scale bar 15 μm).

in the levels of GRP78 and PI3K expression and NF-κB phosphorylation in the cells that had been treated with NAC before exposure to CSE (Fig. 4*B*). Hence, these data suggest that continual exposure to CSE induced ROS production, which sequentially activated the GRP78/IRE1α, NF-κB, and PI3K/AKT/mTOR pathways in primarily cultured human bronchial epithelial cells in vitro.

To understand the importance of the activation of these pathways, we characterized the GRP78, PI3K, and NF-κB expression in 10 lung cancer samples by immunohistochemistry (see Figure, SDC 2, http://links.lww.com/JTO/A608, which demonstrates the expressions of GRP78, PI3K, and NF-κB in lung cancer tissues). There were obviously higher levels of GRP78, PI3K, and NF-κB expression in the carcinoma areas, when compared with that in the paracarcinoma areas. There were seven of 10 samples with positive staining for anti-GRP78, eight with positive staining for anti-NF-κB, and five with positive staining for anti-PI3K. These data suggest that aberrant activation of the GRP78/IRE1α, NF-κB, and PI3K/AKT/mTOR pathways induced by continual exposure to CSE may reflect the tumor-like transformation of primarily cultured human bronchial epithelial cells in our experimental conditions.

Low Dose of CSE Promotes the Process of EMT in Human Bronchial Epithelial Cells

Tumor cells can lose their epithelial characters and are subjected to the EMT process. Accordingly, we analyzed the relative levels of E-cadherin and Vimentin expression after continual exposure to CSE in human bronchial epithelial cells. We found that continual exposure to CSE for 3 weeks obviously reduced the levels of E-cadherin, but increased the levels of Vimentin expression in human bronchial epithelial cells, suggesting that continual exposure to CSE promoted the EMT process of human bronchial epithelial cells in the microfluidic chip (Fig. 5*A*). Furthermore, continual exposure to CSE for 8 weeks enhanced the EMT process in human bronchial epithelial cells (data not shown). However, the reduced E-cadherin and increased Vimentin expression induced by CSE were not detected in the cells that had been pretreated with NCA, BAY11-7082, 4-PBA, or LY294002. A similar pattern of E-cadherin and Vimentin expression was detected in the different groups of cells by Western blot assays (Fig. 5*B*). The modulation in the levels of E-cadherin and Vimentin induced by CSE exposure was abrogated by pretreatment with an inhibitor for ROS or relevant signaling. These two lines of evidence demonstrated that continual CSE exposure promoted the EMT process in human bronchial epithelial cells, which was mediated by ROS-related signaling.

Continual Exposure to CSE Induces Tumor-Like Transformation of Human Primary Bronchial Epithelial Cells

Tumor-like transformation of nontumor epithelial cells usually changes their structure. To determine the potential tumor-like transformation induced by continual exposure to CSE, we characterized the morphological changes in human bronchial epithelial cells longitudinally. After treatment with CSE (12.28%) for 3 weeks, there were some cells with condensed nuclei and abnormal nuclear-cytoplasmic ratios, accompanied by atypical mitoses in the CSE-exposed cells, but not in the cells that had been pretreated with the indicated inhibitor (Fig. 6*A*). The percentages of abnormal cells increased with prolonged CSE exposure (data not shown). Furthermore, continual exposure to CSE for 4–6 weeks promoted multiple layers of cell growth, an indicative of the loss of contact inhibition (Fig. 6*B*), which was not in the cells pretreated with an inhibitor indicated. In addition, continual exposure to CSE stimulated the colony formation and pretreatment with an inhibitor indicated either completely prevented or significantly reduced the numbers of cell colonies (Fig. 6*C* and *D*). These data indicated that continual exposure to CSE promoted the anchor-independent growth of human

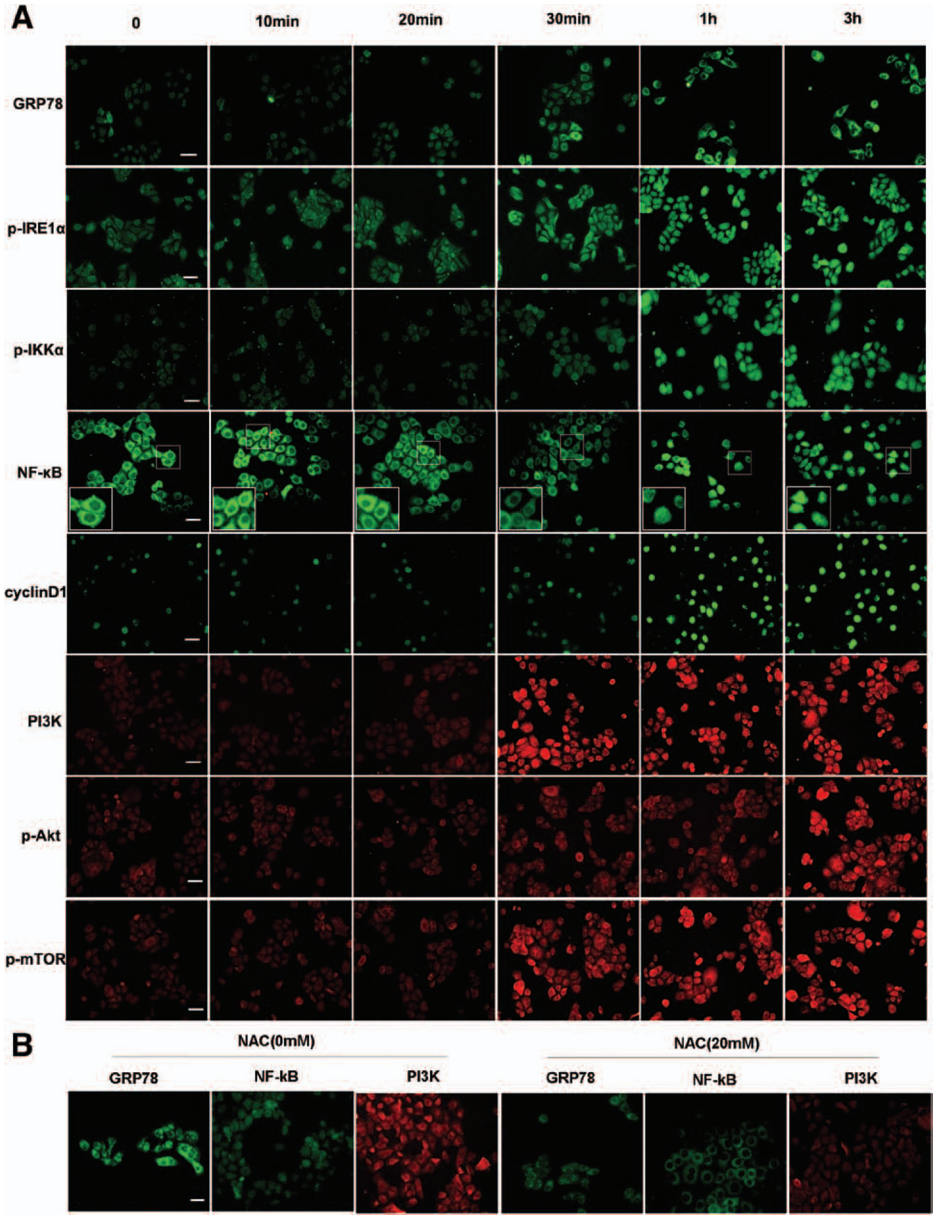

FIGURE 4. Continual cigarette smoke extract (CSE) exposure activates the GRP78, NF-κB, and PI3K pathways in human bronchial epithelial cells. Human bronchial epithelial cells were exposed to CSE (12.5%) and the GRP78, NF-κB, and PI3K signaling was detected by immunofuorescent staining at the indicated time points. Additional cells were pretreated with 20 mM NAC and exposed to CSE for 3 hours and the GRP78, NF-κB, and PI3K signaling was detected by immunofluorescent staining. Data shown are representative fluorescent images from 10 separate experiments. The GRP78 and NF-κB pathways were labeled by Dylight488 (green) and the PI3K pathway was labeled by Dylight594 (red, scale bar 10 μm). *A*, Longitudinal assays of the activation of GRP78, NF-κB, and PI3K pathways. (*B*) The activation of GRP78, NF-κB, and PI3K was abrogated by pretreatment with NAC.

bronchial epithelial cells, an indicative of tumor-like transformation of human nontumor epithelial cells, which were associated with low amount of ROS production and related signaling.

Low Dose of CSE Causes the Chromosome Instability of Human Bronchial Epithelial Cells

To further demonstrate the tumor-like transformation of human bronchial epithelial cells induced by CSE exposure, human bronchial epithelial cells were treated with, or without, CSE for 8 weeks and their chromosomes were analyzed. The cells without exposure to CSE displayed diploid, whereas the CSE-exposed cells were hypodiploid or hyperdiploid (Fig. 6*E*). Some CSE-treated cells exhibited abnormal chromosomes, such as dicentric chromosomes, and these abnormal chromosomes were not observed in the cells that had been pretreated with an inhibitor indicated. Quantitative analysis indicated that continual exposure to CSE induced a higher frequency of human bronchial epithelial cells with chromosome instability, which was abrogated by pretreatment with an inhibitor indicated (Fig. 6*F*). The morphological changes, anchor-independent growth, and higher frequency of cells with chromosome instability, together with high levels of the EMT process, suggest that continual exposure to CSE may induces tumor-like transformation of human nontumor bronchial epithelial cells in vitro.

DISCUSSION

In this study, we built a microfluidic chip and established a cellular model of continual CSE exposure of human nontumor bronchial epithelial cells from 10 surgical samples from patients with lung squamous carcinoma and smoking-related COPD to investigate the potential mechanisms underlying tumor-like transformation. Our studies lead to the after novel discoveries:

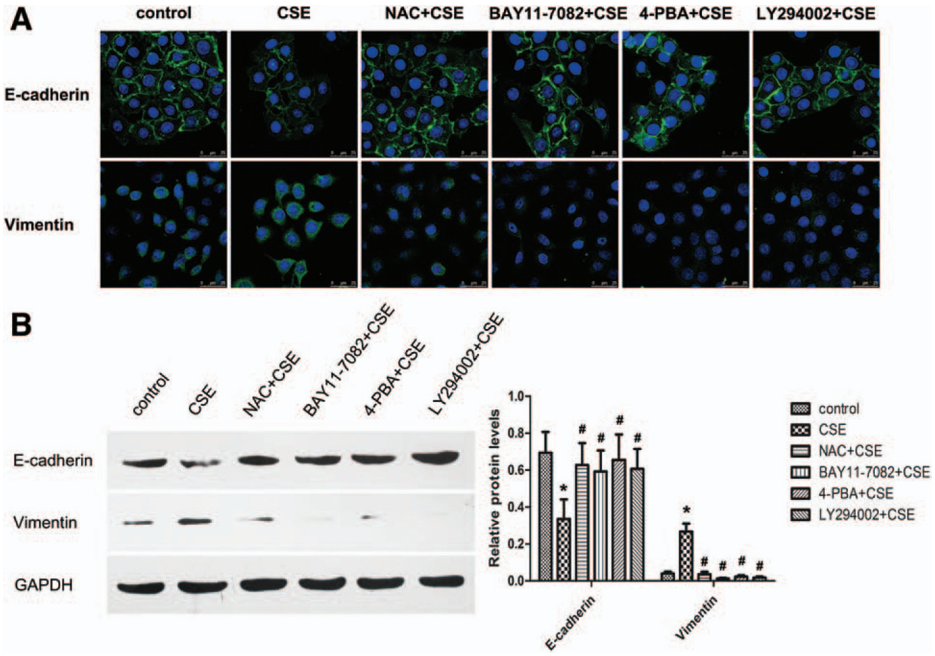

FIGURE 5. Continual cigarette smoke extract (CSE) exposure modulates the levels of E-cadherin and Vimentin expression in human bronchial epithelial cells. Human bronchial epithelial cells were pretreated with, or without, NAC, BAY11-7082, 4-PBA, or LY294002 for 12 hours and then exposed to CSE for 48 hours twice per week for 8 weeks. The relative levels of E-cadherin and Vimentin were determined by immunofluorescent staining and Western blot assays. Data are representative images and expressed as the means ± SD of each group cells from five separate experiments. (A) Immunofluorescent images of E-cadherin and Vimentin expression. (B) Western blot analysis of the relative levels of E-cadherin and Vimentin expression. *p less than 0.05 versus the controls. #p less than 0.05 versus the CSE-exposed cells.

First, continual exposure to different doses of CSE had dual effects on the proliferation and survival of primarily cultured human bronchial epithelial cells. Second, continual exposure to CSE induced ROS production in a dose-dependent manner and moderate levels of ROS were associated with tumor-like transformation of human nontumor bronchial epithelial cells by activating the GRP78, NF-κB, and PI3K pathways. Third, continual CSE exposure promoted the EMT process, anchor-independent growth, and higher frequency of cells with chromosome instability in human bronchial epithelial cells, which was abrogated by pretreatment with a ROS scavenger. Fourth, the microfluidic chip was an excellent platform for the investigation of malignant transformation of nontumor bronchial epithelial cells and had many advantages over conventional in vitro cellular models. Our results extended previous findings[25,26] and support the notion that heavy smoking-related oxidative stress, endoplasmic reticulum (ER) stress, and associated inflammation are crucial for inducing tumor-like transformation of human nontumor bronchial epithelial cells in vitro.

COPD is an inflammatory lung disease associated with heavy smoking. During the pathogenic process, heavy smoking induces oxidative stress and ROS production. Previous studies have shown that high levels of ROS can trigger epithelial cell apoptosis, whereas low levels of ROS can stimulate the proliferation, invasiveness, and metastasis of lung cancer cells by activating the NF-κB, AP-1, PI3K, MAPKs, and others.[27] In this study, we found that continual exposure to different doses of CSE induced ROS production in human nontumor bronchial epithelial cells in a dose-dependent manner. Although continual exposure to higher doses of CSE to induce high levels of ROS triggered human bronchial epithelial cell apoptosis, continual exposure to lower doses of CSE to induce moderate levels of ROS promoted the proliferation of human bronchial epithelial cells. More importantly, continual exposure to low dose of CSE activated the GRP78, NF-κB, and PI3K pathways in human bronchial epithelial cells, which was abrogated by pretreatment with NAC. In addition, we detected aberrant activation of these pathways in the corresponding lung cancer tissues of these patients. Previous studies have shown that aberrant activation of the NF-κB and PI3K is crucial for malignant transformation of epithelial cells,[28,29] and ER stress is associated with the development of COPD in heavy smokers and drug-resistance in lung cancer patients.[30,31] It is possible that continual CSE exposure to produce moderate levels of ROS may promote tumor-like transformation of human nontumor bronchial epithelial cells.[32]

It is well known that malignant cells can undergo the EMT process and anchorage-independent growth and show chromosome aberration and unequal structure. Although E-cadherin expression is usually attenuated in all three subtypes of EMT, Vimentin, a cytoskeletal marker, is often induced when EMT occurs. Recently, Vimentin is frequently used to indentify cells undergoing type 3 EMT in cancers.[33,34] We found that continual CSE exposure increased the levels of Vimentin expression, but reduced the levels of E-cadherin expression in human bronchial epithelial cells, an indicative of the EMT process. Furthermore, continual CSE exposure promoted anchorage-independent growth of human bronchial epithelial cells and increased the frequency of cells with chromosome instability, accompanied by some cells with condensed nuclei, abnormal nuclear-cytoplasmic ratios, and atypical mitoses. These several lines evidence clearly indicated that continual exposure to moderate dose of CSE promoted tumor-like transformation of human primarily cultured nontumor bronchial epithelial cells in vitro. However, these tumor-like changes were abrogated by pretreatment with NAC, GRP78 inhibitor 4-PBA, NF-κB inhibitor BAY11-7082, and PI3K inhibitor LY294002. These data suggested that moderate levels of ROS and associated GRP, NF-κB, and PI3K activation induced by continual CSE exposure are crucial for tumor-like transformation of human

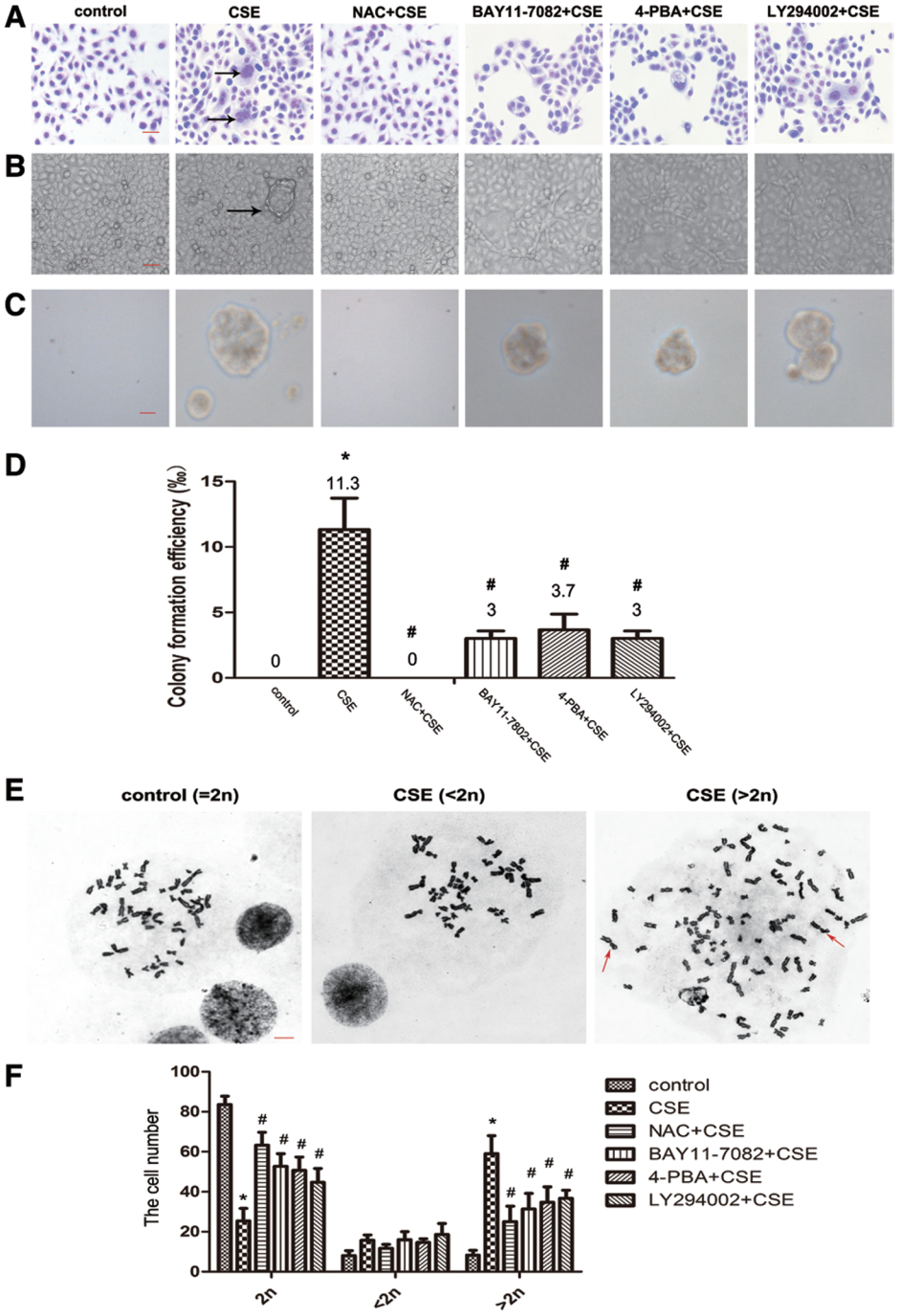

FIGURE 6. Continual cigarette smoke extract (CSE) exposure induces tumor-like transformation of human bronchial epithelial cells. Human bronchial epithelial cells were pretreated with, or without, NAC, BAY11-7082, 4-PBA, or LY294002 for 12 hours and then exposed to CSE for 48 hours twice per week for 8 weeks. The cell structure and anchor-independent growth of human bronchial epithelial cells were examined and the percentages of cells with chromosome instability were determined. Data are representative images and expressed as the means ± SD of each group of cells from five separate experiments. *A*, Morphological changes of the cells. Black arrow: the cells with condensed nucleus staining, high nuclear-cytoplasmic ratios and atypical mitoses (scale bar 10 μm). (*B*) Multilayer growth of cells (black arrow, scale bar 10 μm). (*C*) Colony formation of cells (scale bar 10 μm). (*D*) Quantitative analysis of the percentages of colonies. (*E*) Analysis of the cell karyotypes. Red arrow: the aberrant chromosome (scale bar 5 μm). (*F*) The distribution of the cell karyotype. **p* less than 0.05 versus the controls. #*p* less than 0.05 versus the CSE-exposed cells.

bronchial epithelial cells. Therefore, these findings may provide new insights into the molecular mechanisms underlying the link of heavy smoking-related COPD with lung squamous carcinoma in humans. We are interested in further investigating whether continual exposure to CSE can also induce tumor-like transformation of human nontumor bronchial epithelial cells from nonsmoking patients with lung squamous carcinoma, from nontumor patients, and from nonsmoking nontumor patients. To the best of our knowledge, this was the first report on that continual CSE exposure to induce tumor-like transformation of human nontumor bronchial epithelial cells from patients with heavy smoking-related COPD and lung squamous carcinoma. Conceivably, therapeutic target oxidative stress, ER stress, and aberrant activation of these pathways may be valuable for prevention from the development of squamous carcinoma in heavy smoking-related COPD patients.

The microfluidic chip used in this study was a unique and ideal platform for studying the tumor-like transformation of bronchial epithelial cells induced by continual CSE exposure because the process of CSE exposure is similar to heavy smoking

in humans. Its minute structure, high-oxygen permeable PDMS materials, and a continuously flowing medium can mimic a real microenvironment in vivo and meet the requirements of primarily cultured human bronchial epithelial cells.[18] Therefore, compared with that from conventional platform, the research results from the microfluidic chip may be more reliable and meaningful. Furthermore, this microfluidic chip we built can be sued for high-throughput screening for the potential signal pathways and therapeutic targets with time-lapse imaging. With one device, we tested four samples for several conditions simultaneously, which reduced the experimental errors. More importantly, the microfluidic chip allowed both the morphological tracing and the fluorescence marker detection to fulfill the dynamic observation of cultured cells and their signaling during the long-term exposure of a carcinogen. Indeed, we monitored three signal pathways, including eight factors in the cultured cells in real time only on a chip. The dynamics of the intracellular signaling in the cell from 10 specimens were recorded in a short period. Thus, this designed microfluidic chip is a unique platform for studying the proliferation, apoptosis, and tumor-like transformation of primarily cultured bronchial epithelial cells and other types of cells.

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REFERENCES

1. Sasco AJ, Secretan MB, Straif K. Tobacco smoking and cancer: a brief review of recent epidemiological evidence. *Lung Cancer* 2004;45 Suppl 2:S3–S9.
2. Pauwels RA, Buist AS, Ma P, Jenkins CR, Hurd SS; GOLD Scientific Committee. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: National Heart, Lung, and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary. *Respir Care* 2001;46:798–825.
3. Yao H, Rahman I. Current concepts on the role of inflammation in COPD and lung cancer. *Curr Opin Pharmacol* 2009;9:375–383.
4. Mannino DM, Aguayo SM, Petty TL, Redd SC. Low lung function and incident lung cancer in the United States: data From the First National Health and Nutrition Examination Survey follow-up. *Arch Intern Med* 2003;163:1475–1480.
5. Punturieri A, Szabo E, Croxton TL, Shapiro SD, Dubinett SM. Lung cancer and chronic obstructive pulmonary disease: needs and opportunities for integrated research. *J Natl Cancer Inst* 2009;101:554–559.
6. Powell HA, Iyen-Omofoman B, Baldwin DR, Hubbard RB, Tata LJ. Chronic obstructive pulmonary disease and risk of lung cancer: the importance of smoking and timing of diagnosis. *J Thorac Oncol* 2013;8:6–11.
7. Houghton AM, Mouded M, Shapiro SD. Common origins of lung cancer and COPD. *Nat Med* 2008;14:1023–1024.
8. Du H, Sun J, Chen Z, Nie J, Tong J, Li J. Cigarette smoke-induced failure of apoptosis resulting in enhanced neoplastic transformation in human bronchial epithelial cells. *J Toxicol Environ Health A* 2012;75:707–720.
9. Takahashi H, Ogata H, Nishigaki R, Broide DH, Karin M. Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. *Cancer Cell* 2010;17:89–97.
10. Adcock IM, Caramori G, Barnes PJ. Chronic obstructive pulmonary disease and lung cancer: new molecular insights. *Respiration* 2011;81:265–284.
11. Houghton AM. Mechanistic links between COPD and lung cancer. *Nat Rev Cancer* 2013;13:233–245.
12. Frohlich DA, McCabe MT, Arnold RS, Day ML. The role of Nrf2 in increased reactive oxygen species and DNA damage in prostate tumorigenesis. *Oncogene* 2008;27:4353–4362.
13. Xing M. Oxidative stress: a new risk factor for thyroid cancer. *Endocr Relat Cancer* 2012;19:C7–11.
14. West KA, Brognard J, Clark AS, et al. Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. *J Clin Invest* 2003;111:81–90.
15. Ho YS, Chen CH, Wang YJ, et al. Tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces cell proliferation in normal human bronchial epithelial cells through NFkappaB activation and cyclin D1 up-regulation. *Toxicol Appl Pharmacol* 2005;205:133–148.
16. Churg A, Wang RD, Tai H, Wang X, Xie C, Wright JL. Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med* 2004;170:492–498.
17. Kim SM, Lee SH, Suh KY. Cell research with physically modified microfluidic channels: a review. *Lab Chip* 2008;8:1015–1023.
18. Tanaka Y, Sato K, Shimizu T, Yamato M, Okano T, Kitamori T. Biological cells on microchips: new technologies and applications. *Biosens Bioelectron* 2007;23:449–458.
19. Wang S, Li E, Gao Y, et al. Study on invadopodia formation for lung carcinoma invasion with a microfluidic 3D culture device. *PLoS One* 2013;8:e56448.
20. Xu Z, Gao Y, Hao Y, et al. Application of a microfluidic chip-based 3D co-culture to test drug sensitivity for individualized treatment of lung cancer. *Biomaterials* 2013;34:4109–4117.
21. Bota S, Auliac JB, Paris C, et al. Follow-up of bronchial precancerous lesions and carcinoma in situ using fluorescence endoscopy. *Am J Respir Crit Care Med* 2001;164:1688–1693.
22. Carp H, Janoff A. Possible mechanisms of emphysema in smokers. In vitro suppression of serum elastase-inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. *Am Rev Respir Dis* 1978;118:617–621.
23. Wang Q, He Z, Zhang J, et al. Overexpression of endoplasmic reticulum molecular chaperone GRP94 and GRP78 in human lung cancer tissues and its significance. *Cancer Detect Prev* 2005;29:544–551.
24. Tatsuka M, Nikaido O, Tatsumi K, Takebe H. X-ray-induced G2 arrest in ataxia telangiectasia lymphoblastoid cells. *Mutat Res* 1989;214:321–328.
25. Luppi F, Aarbiou J, van Wetering S, et al. Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells in vitro: role of glutathione. *Respir Res* 2005;6:140.
26. Zhao Y, Xu Y, Li Y, et al. NF-kappaB-mediated inflammation leading to EMT via miR-200c is involved in cell transformation induced by cigarette smoke extract. *Toxicol Sci* 2013;135:265–276.
27. Allen RG, Tresini M. Oxidative stress and gene regulation. *Free Radic Biol Med* 2000;28:463–499.
28. Zhao JJ, Gjoerup OV, Subramanian RR, et al. Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. *Cancer Cell* 2003;3:483–495.
29. Ren Q, Kari C, Quadros MR, et al. Malignant transformation of immortalized HaCaT keratinocytes through deregulated nuclear factor kappaB signaling. *Cancer Res* 2006;66:5209–5215.
30. Ribeiro CM, O'Neal WK. Endoplasmic reticulum stress in chronic obstructive lung diseases. *Curr Mol Med* 2012;12:872–882.
31. Wang Y, Wang W, Wang S, Wang J, Shao S, Wang Q. Down-regulation of GRP78 is associated with the sensitivity of chemotherapy to VP-16 in small cell lung cancer NCI-H446 cells. *BMC Cancer* 2008;8:372.
32. Azad N, Rojanasakul Y, Vallyathan V. Inflammation and lung cancer: roles of reactive oxygen/nitrogen species. *J Toxicol Environ Health B Crit Rev* 2008;11:1–15.
33. Yoon Y, Liang Z, Zhang X, et al. CXC chemokine receptor-4 antagonist blocks both growth of primary tumor and metastasis of head and neck cancer in xenograft mouse models. *Cancer Res* 2007;67:7518–7524.
34. Paccione RJ, Miyazaki H, Patel V, et al. Keratin down-regulation in vimentin-positive cancer cells is reversible by vimentin RNA interference, which inhibits growth and motility. *Mol Cancer Ther* 2008;7:2894–2903.

