

# Nicotine Promotes Mammary Tumor Migration via a Signaling Cascade Involving Protein Kinase C and *cdc42*

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## Abstract

**Nicotine, one of the major components in tobacco, is at high concentrations in the bloodstream of cigarette smokers. However, the mechanisms of how nicotine affects tumor development and whether nicotine is a potential carcinogen for malignancies induced by secondhand smoking are not fully understood yet. Here, we investigate the signaling pathways by which nicotine potentiates tumorigenesis in human mammary epithelial-like MCF10A or cancerous MCF7 cells. We show that human MCF10A and MCF7 cells both express four subunits of nicotine acetylcholine receptor (nAChR). The treatment of these cells with nicotine enhances the activity of protein kinase C (PKC)  $\alpha$  without altering the expression level of this kinase. Nicotine also stimulates [<sup>3</sup>H]thymidine incorporation into the genome of these cells as well as forces serum-starved cells to enter S phase of the cell cycle, resulting in growth promotion. Importantly, on nicotine treatment, the mobility of MCF10A and MCF7 cells is enhanced, which can be blocked by the addition of nAChR or PKC inhibitor. Experiments using small interfering RNA knockdown or ectopic expression of *cdc42* showed that *cdc42* functions as a downstream effector of PKC and is crucial in the regulation of nicotine-mediated migratory activity in the cells. Together, our findings suggest that nicotine, through interacting with its receptor, initiates a signaling cascade that involves PKC and *cdc42* and consequently promotes migration in mammary epithelial or tumor cells.** [Cancer Res 2008;68(20):8473–81]

## Introduction

Tobacco contains various components, of which many are carcinogenic and/or mutagenic (1, 2). Although studies have linked cigarette smoking with various onsets of human malignancies, little is known about how secondhand smoking promotes tumor development or causes the onset of cancer. Nicotine has been identified as one of the important constituents of tobacco (3). Through interacting with nicotine acetylcholine receptor (nAChR), nicotine functions either on the motor end plate of muscle or at the central nervous system responsible for tobacco addiction (4–6). Recently, it has been recognized that nicotine is able to activate various intracellular signaling pathways in nonneuronal cells, indicating that nicotine may possess a function for tumor promotion (7–11). After engaging its receptor, nicotine rapidly

activates mitogenic-related, intracellular signaling pathways in endothelial cells or keratinocytes (11, 12). Emerging evidence showed that nicotine potently induces secretion of different types of calpain from lung cancer cells, which then promotes cleavage of various substrates in the extracellular matrix to facilitate metastasis and tumor progression (10).

nAChR is composed of nine  $\alpha$  subunits ( $\alpha 2$ – $\alpha 10$ ) and two  $\beta$  subunits ( $\beta 2$  and  $\beta 4$ ; refs. 13, 14). The receptor is expressed on the surface of various nonneuronal cells, such as lung epithelial cells, keratinocytes, and vascular smooth muscle cells (7–14). On the surface of these cells, the  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 7$ ,  $\alpha 9$ ,  $\beta 2$ , and  $\beta 4$  subunits are expressed. Among the subunits,  $\alpha 3$ ,  $\alpha 5$ ,  $\beta 2$ , and  $\beta 4$  form heteromeric channels in different combinations (11, 15–18). The homomeric channel often is composed by several  $\alpha 7$  or  $\alpha 9$  subunits. These heteromeric or homomeric channels are highly  $\text{Ca}^{2+}$  permeable, which allow releases of  $\text{Ca}^{2+}$  from intracellular stores to the cytosol of cells (19, 20). In human epidermal cells, the interaction of nicotine and its receptor has been shown to activate calmodulin-dependent protein kinase II, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K)/Akt, and Rac/*cdc42* that are often involved in the regulation of cell adhesion, migration, and invasion (11, 21, 22). The activation of nicotine receptor is able to activate Ras/Raf/mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase/ERK signaling (11). Recently, it has also been reported recently that on the ligation of nAChR by nicotine, the tyrosine kinase Janus-activated kinase-2 and transcription factor signal transducer and activator of transcription (STAT) 3 in macrophages are activated (22).

PKC consists of more than 12 different isoforms of protein-serine/threonine kinases that are important components of phospholipase-coupled growth factor receptor signaling pathways (23–26). The enzymatic activities of PKC isoforms are regulated by diacylglycerol (DAG) or  $\text{Ca}^{2+}$ , which divides these kinases into three subclasses: DAG-dependent (PKC $\delta$ , PKC $\epsilon$ , PKC $\theta$ , and PKC $\mu$ ), DAG/ $\text{Ca}^{2+}$ -dependent (PKC $\alpha$ , PKC $\beta$ I, and PKC $\beta$ II), and atypical (PKC $\zeta$ , PKC $\lambda$ , and PKC $\tau$ ) isoforms. These isoforms have different tissue distributions and are involved in different biological processes, including cell growth, migration, apoptosis, and differentiation (23–26). In the bloodstream of smokers, nicotine is found to be at pharmacologic concentrations of 90 to 1,000 nmol/L, which is able to activate PKC in cultured human or murine lung cancer cells (7, 27). The activation of PKC is responsible for Bcl-2 phosphorylation, which, in turn, antagonizes drug-induced apoptosis in lung cancer cells (27). It has been reported that nicotine can elicit the activity of Ras or Raf-1 (9, 11, 28). In addition, studies have shown that Akt was phosphorylated in the lungs of nicotine-treated mice and in human lung cancer tissues derived from smokers (29). All these observations suggest that nicotine is able to promote prosurvival activity in cells and is important for tumorigenesis.

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Cell migration or tumor metastasis is a crucial process in tumor development. Numerous factors play a role in the regulation of this process, which includes growth factors, kinases, phosphatases, as well as extracellular matrix components. Growth-related receptors, when activated by corresponding ligands, contribute to cell proliferation and migratory or invasive capacity of cells. After recruiting downstream effectors, growth-related receptors often exert their functions by organizing their downstream effectors, such as PKC, PI3K, or cdc42, to initiate signaling cascades, which affect various biological processes, including cell migration and cancer cell invasion (30–35). Nicotine has been shown to induce the phosphorylation of different subtypes of calpains, resulting in enhanced cell migration or, more specifically, lung cancer metastasis (10, 33).

Although numerous studies indicated the role of nicotine exposure in tumor promotion, little is known about the effect of nicotine on breast tumor development, especially on the metastatic process of breast cancer. Here, we showed that four different subunits of nAChRs were expressed in MCF10A and MCF7 cells, and the expression of these subunits was not affected by nicotine exposure. However, the treatment with nicotine augmented the activity of PKC in these cells. Although nicotine stimulated DNA synthesis as well as the S-phase entry of these cells under serum-starved conditions, the suppression of PKC activity did not significantly block the growth-promoting effects of nicotine. Furthermore, nicotine exposure induced both “normal” mammary epithelial and mammary cancer cells to migrate under serum-starved conditions, which could be suppressed by either nAChR or PKC inhibitor. Acting as a downstream effector of PKC, cdc42 was identified as a major effector in this nicotine-mediated action to promote migratory activity. Together, our investigation suggests that nicotine mobilizes PKC and cdc42 signaling in both normal mammary epithelial and mammary cancer cells to promote cell migratory activity.

## Materials and Methods

**Materials.** Nicotine and the nAChR inhibitor mecamylamine hydrochloride (MCA) were purchased from Sigma-Aldrich, Inc. The PKC inhibitor 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3- $\alpha$ )pyrrolo(3,4-*c*)-carbazole (GO6976) was obtained from EMD Chemicals, Inc. The anti-PKC antibody was purchased from BD PharMingen. Wortmannin and UO16 were obtained from Cell Signaling Technology (Calbiochem).

**Cell culture and transfection.** Human breast cancer MCF10A and MCF7 cells were purchased from the American Type Culture Collection. MCF10A cells were cultured in DMEM/F12 medium supplemented with 5% donor horse serum, 20 ng/mL epidermal growth factor (EGF), 10  $\mu$ g/mL insulin, 0.5  $\mu$ g/mL hydrocortisone, 100 ng/mL cholera toxin, and antibiotics. MCF7 cells were maintained in DMEM with 10% FCS, 4 mmol/L L-glutamine, and antibiotics.

*cdc42* was inserted into MSCV retroviral vector and subsequently transiently infected into the cells. Small interfering RNAs (siRNA) were chemically synthesized and inserted into a lentiviral vector. The vector carrying targeted siRNA sequence was introduced into the cells. The antisense strand siRNA was targeted against GTPase using 21-nucleotide sequences 5'-AAGAAGTCAAGCATTCTGTC-3' for RhoA, 5'-AAGATAACT-CACCACTGTCCA-3' for *cdc42*, and 5'-AAGTCTTAATTTGCTTTTCC-3' for Rac1 (34).

**PCR and real-time PCR.** Total RNAs were extracted from cultured cells with or without treatments using RNeasy Mini kit (Qiagen) following the protocol provided by the manufacturer. Primers for the genes encoding the subunits of nAChR were designed with the assistance of the Primer Express software (Applied Biosystems). For real-time PCR, the gene expression was normalized using *glyceraldehyde-3-phosphate dehydrogenase* as the control.

**PKC enzymatic assay.** After various treatments, cells were lysed in the lysis buffer [25 mmol/L Tris-HCl (pH 7.5), 1% NP40, 20 mmol/L MgCl<sub>2</sub>, 150 mmol/L NaCl]. The lysates were normalized for protein concentration. The equal amount of total proteins was immunoprecipitated with anti-PKC $\alpha$  antibody and the immunoprecipitates were analyzed for PKC $\alpha$  activity using a PKC enzymatic kit (Millipore). Briefly, the immunoprecipitates were mixed with the substrate cocktail, the inhibitor cocktail, and the lipid activator in microcentrifuge tubes. After adding Mg<sup>2+</sup>/ATP cocktail containing [ $\gamma$ -<sup>32</sup>P]ATP, the samples were incubated at 30°C for 10 min and then spotted on P81 phosphocellulose papers. The radioactivity of the <sup>32</sup>P-incorporating substrate was measured by a scintillation counter.

**[<sup>3</sup>H]thymidine incorporation.** MCF10A or MCF7 cells were grown in Petri dishes until 60% to 70% confluence. Controls and treated cells were done in triplicate. The cells were cultured in the medium containing 0.5% serum for 24 h. Subsequently, the cells were grown in the medium containing 0.5% or 10% serum plus 4  $\mu$ Ci/mL of [<sup>3</sup>H]thymidine (Perkin-Elmer Life Sciences) with or without nicotine treatment. The cells were labeled for 4 h at 37°C. After precipitation with cold 10% trichloroacetic acid, the cells were dissolved in 0.5 mL of 0.1 mol/L NaOH overnight at 4°C. The amount of radioactivity in each sample was counted using a scintillation machine.

**Cell proliferation assay.** The cells ( $2 \times 10^5$ ) were plated in 12-well plates and cultured in the medium containing 0.5% serum, which is designated as day 1. Subsequently, cells treated with or without nicotine or refed with 10% serum were grown for another 4 d. The numbers of viable cells were determined by trypan blue staining and counted daily using a hemocytometer.

**Cell cycle analysis.** Cell cycle distribution of DNA content was measured using a flow cytometer as described (35). The cells, after the treatments, were fixed in 65% DMEM and 35% ethanol for 2 to 4 h at 4°C and stained with propidium iodide. Subsequently, the DNA profiles were analyzed using CellQuest software.

**Cell migration and invasion assay.** Following various treatments, cell migration was analyzed using Boyden cell migration assay plates (Neuro Probe, Inc.) according to the manufacturer's instructions. Untreated or treated cells were labeled with DiI-labeled acetylated low-density lipoprotein (10  $\mu$ g/mL) for 4 h. Cells were trypsinized and resuspended in phenol red-free medium at 10,000/25  $\mu$ L. Each experiment was performed in triplicate and three separate experiments were performed in each experimental group.

**Monolayer wound-healing assay.** Cells were seeded and allowed to grow to 90% confluent. A cell scraper was used to wipe away the cell monolayer on one side of the start line that had been drawn on the bottom of the plate. Wounded monolayer was washed four times with the medium to remove cell debris. Cells were then treated. Twenty-four hours later, the wounded areas were photographed at various time points with the assistance of the landmarks drawn on the undersurface of the plate.

**Experimental lung metastasis assay.** MCF7 cells ( $1 \times 10^6$ ) were injected into the tail veins of nude mice. A group of 15 mice was peritoneally injected with nicotine (30  $\mu$ g/mouse) every 2 d (36). Another group of the same numbers of mice was left untreated. Five mice from each group were sacrificed at 3 and 4 wk after tumor cell injection. The lung tissues were isolated and slides were prepared. After staining with H&E, the approximate number of foci of blue-stained metastatic tumor cells present in the lung was estimated with an Olympus dissecting microscope. The scoring system was used to score the degree of metastasis based on the estimated counts: –, no blue cells; +, about 1 to 50 blue cells; ++, about 50 to 100 blue cells; +++, about 100 to 200 blue cells (36).

**Immunoblotting.** Following treatments, cell lysates were prepared and proteins were separated by SDS-PAGE gels. Membranes were incubated with the designated primary antibody (1:1,000 for all antibodies) overnight in a cold room at 4°C. Bound primary antibodies were reacted with corresponding second antibodies for 2 h and detected by chemiluminescence.

**Statistical analysis.** Three to five independent repeats were conducted in all experiments. Error bars represent these repeats. A Student's *t* test was used and a *P* value of <0.05 was considered significant.

## Results

**Activation of PKC by nicotine treatment in breast epithelial and cancer cell lines.** It is known that nAChRs are expressed on the surface not only of neuronal cells but also of lung epithelia and vascular endothelial cells, through which nicotine affects various biological or pathobiological processes, including angiogenesis, ischemia, or growth promotion (7–12). To explore whether nicotine could influence breast cancer development, the gene expression of nAChRs in normal breast epithelial-like MCF10A cells and breast cancer MCF7 cells was examined using PCR technique. Total mRNAs from these cells were isolated, and subsequently, the gene expression of each nAChR subunit was analyzed (Fig. 1A). The results from PCR revealed that nAChR  $\alpha$ 5,  $\alpha$ 7,  $\alpha$ 9, and  $\beta$ 4 were consistently expressed in both cells. The expressions of the genes of other nAChR subunits were undetectable in these cells (data not shown). To determine whether nicotine could stimulate nAChR expression, the cells were treated with nicotine and the levels of nAChR  $\alpha$ 5,  $\alpha$ 7,  $\alpha$ 9, and  $\beta$ 4 expression were measured by real-time PCR (Fig. 1B). The amount of the gene expression of each nAChR subunit in untreated cells was similar as that in treated cells.

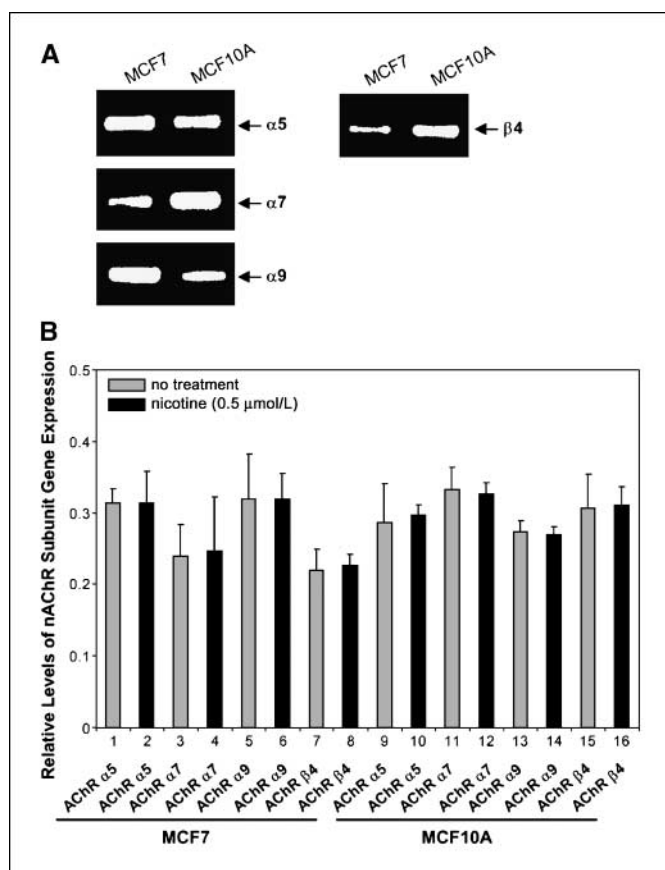
Nicotine exposure has been shown to rapidly increase ERK activation as well as serine phosphorylation of STAT1 and STAT3. Studies also showed that nicotine is able to stimulate PI3K/Akt and

Ras/Raf pathways (21, 22). We previously showed that nicotine treatment up-regulates PKC activity in mouse lung epithelial cells (9, 28). It has been reported that nicotine receptor engagement correlates well with the migration promotion of human lung cancer cell, in which PKC plays a crucial role (10). Because the phorbol ester and calcium-dependent isoforms of PKC are predominantly expressed in many types of cells and their activity is involved in growth promotion (23–26), the expression of PKC $\alpha$  in MCF7 or MCF10A cells with regard to nicotine treatment was first examined by immunoblotting (Fig. 2A). A similar level of PKC $\alpha$  expression was recognized by the antibody in both breast cancer cell lines with or without the treatment, indicating that nicotine plays no role in the expression of this kinase. To determine whether nicotine treatment affects PKC $\alpha$  activity, the kinase activity was measured using a PKC-specific kinase activity assay (Fig. 2B). After growing the cells in the medium containing 0.5% serum for 24 h, a basal activity of this kinase was detected in MCF7 and MCF10A cells (Fig. 2B, lanes 1 and 6). In contrast, the kinase activity was dramatically increased after nicotine exposure under serum-starved conditions (about 4- to 5-fold; Fig. 2B, lanes 2 and 7) or refed with 10% serum (about 5- to 6-fold; Fig. 2B, lanes 4 and 9). The addition of MCA (a nAChR inhibitor) abrogated the induction of PKC $\alpha$  activity mediated by nicotine in serum-starved cells (Fig. 2B, lanes 3 and 8) but had no negative effects on the cells refed with serum (Fig. 2B, lanes 5 and 10). The expression and activity of other PKC isoforms in nicotine-treated cells did not change in comparison with that in untreated cells (data not shown). Therefore, the data suggest that nicotine, through its receptor, activates PKC $\alpha$  in these breast cells.

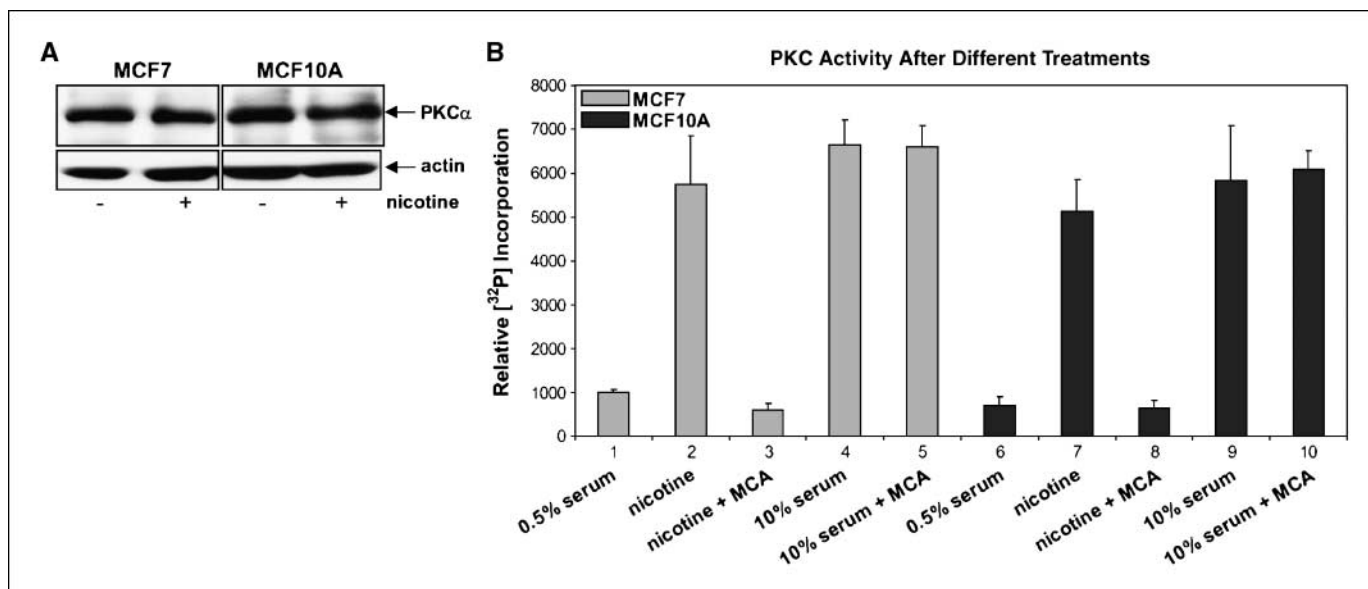
### Effect of nicotine engagement on breast cancer growth.

Emerging evidence shows the potential growth-promoting activity rendered by nicotine (21, 22). Increase in cell proliferation is a crucial step in tumorigenesis. Therefore, we used [ $^3$ H]thymidine incorporation assay to test if nicotine treatment has an effect on DNA uptake in MCF7 (Fig. 3A, left) and MCF10A (Fig. 3B, right) cells. After growing in the medium containing 0.5% serum for 48 h, MCF7 and MCF10A cells were treated with nicotine under serum-starved conditions or after refeeding with 10% serum in the presence of [ $^3$ H]thymidine. Subsequently, rates of DNA synthesis were measured. Under serum-depleted conditions, little [ $^3$ H]thymidine incorporation was observed in either MCF7 or MCF10A cells (Fig. 3A, left and right, lane 1). A moderate amount of [ $^3$ H]thymidine (about 2- to 3-fold) was incorporated in nicotine-treated cells under serum-starved conditions (Fig. 3A, left and right, lane 2). The higher rate of [ $^3$ H]thymidine intake (4- to 5-fold) was seen in the cells refed with 10% serum (Fig. 3A, left and right, lane 3). However, the addition of nicotine and 10% serum into starved cells caused an even greater increase of [ $^3$ H]thymidine incorporation into their genomes (about 5- to 6-fold; Fig. 3A, left and right, lane 4). We further determined the role of nAChR or PKC in nicotine-mediated DNA synthesis promotion. The addition of MCA or GO6976 blocked [ $^3$ H]thymidine incorporation in nicotine-treated MCF7 and MCF10A cells (Fig. 3A, left and right, lanes 5 and 6). The effect of nicotine on cell proliferation was confirmed by the assay for cell growth. After 24 h of serum starvation, MCF7 or MCF10A cells were grown in the medium containing 0.5% or 10% serum or with nicotine or for 4 consecutive days (Fig. 3B). The cells exposed to nicotine, such as those cultured in the growth medium, were consistently dividing in the medium lacking serum over the testing period.

Next, we tested if nicotine was able to promote cell cycle progression in the cells. After serum depletion for 24 h, MCF10A



**Figure 1.** Expression of the subunits of nAChR in MCF10A and MCF7 cells. *A*, the total RNA from each cell line was isolated. Equal amounts of RNA were reverse transcribed, and the expressions of nAChR  $\alpha$  and  $\beta$  subunits were identified by PCR. *B*, with or without nicotine treatment (0.5  $\mu$ mol/L) for 24 h, the total RNAs were obtained. After reverse transcription, real-time PCR was performed. The abundance of nAChRs was normalized to actin. Columns, mean of three independent experiments; bars, SD.



**Figure 2.** PKC activation in nicotine-treated MCF cells. *A*, the cells were treated with nicotine (0.5  $\mu$ mol/L) for 30 min, and cell lysates were extracted for immunoblotting analysis.  $\beta$ -Actin was used to determine equal loading of total protein per lane. *B*, after nicotine treatment in the presence or absence of MCA (50 nmol/L), the cells were harvested and lysates were prepared for PKC $\alpha$  activity assay. *Columns*, mean of three independent experiments; *bars*, SD.

and MCF7 cells were stimulated with nicotine for 4 h and cell cycle analysis was performed to measure the percentage of the cells in the S phase (Fig. 3C and D). Under serum depletion, there were in general very low percentages of MCF10A and MCF7 cells in the cell cycle beyond S phase (<4%; Fig. 3D, lanes 1 and 4). In contrast, nicotine exposure caused a more than 2-fold increase in the S-phase population of both MCF10A and MCF7 cells in comparison with untreated controls (Fig. 3D, lanes 2 and 5). After refeeding with 10% serum, the percentage of both cells in the S phase was increased to  $\sim$ 3-fold, as expected (Fig. 3D, lanes 3 and 6). Together, the data indicate that nicotine partially mimics mitogenic stimulation and promotes cell cycle progression or proliferation in MCF10A or MCF7 cells.

**Nicotine treatment promotes the migratory activity of MCF10A and MCF7 cells.** Growth factors, such as EGF or insulin-like growth factor, stimulate not only cell proliferation but also migration. Nicotine is able to activate various intracellular signaling pathways that promote cell growth or survival (21, 22). We further examined whether nicotine exposure affects migration of MCF breast cells using the Boyden chamber and cell wound-healing assays. The Boyden chamber assay revealed that the migration phenomenon in MCF10A or MCF7 cells was undetectable when growing in the medium containing 0.5% serum (Fig. 4A, lanes 1 and 7). The addition of nicotine, under serum-depleted conditions, increased the number of the migratory cells (>2-fold; Fig. 4A, lanes 2 and 8), the magnitudes of which are similar as those refed with 10% serum (Fig. 4A, lanes 3 and 9). The addition of GO6976 dramatically suppressed the nicotine-induced migratory activity in serum-starved MCF10A and MCF7 cells (Fig. 4A, lanes 4 and 10), but the inhibition of PI3K/Akt by wortmannin (Fig. 4A, lanes 5 and 11) or MAP/ERK signaling by UO16 (Fig. 4A, lanes 6 and 12) had no effect on the promotion of migration mediated by nicotine. Collectively, the data indicate that nicotine, through mobilizing PKC signaling pathway, promotes migration in the breast cancer cells.

We then tested the effect of nicotine on MCF7 cell migration using a wound-healing assay (Fig. 4B). MCF7 cells were unable to migrate

under serum-starved conditions. Following nicotine treatment, the starved cells displayed a high capacity to migrate to the wounded area. However, in the presence of MCA or GO6976, the migration mediated by nicotine was completely suppressed. The addition of UO16 had no effect on nicotine-induced MCF7 cell migration. Consistently, the treatment with wortmannin to block PI3K/Akt signaling did not affect nicotine-induced wound healing (data not shown). Together, the data strongly suggest that nicotine, like growth factors, functions through its receptor and governs multiple downstream pathways. Among these signaling pathways, PKC seems to be involved in the regulation of the activity of cell migration.

To further test whether nicotine is able to promote metastasis, experimental lung metastasis assay was performed, which has been used frequently to detect micrometastases (36–38). MCF7 cells that are known to be poorly metastatic or invasive in nude mice (36–38) were used. After injecting MCF7 cells into the tail veins of nude mice, a group of mice was peritoneally injected with nicotine every 2 days for 28 days and another group with the same amount of mice was left untreated. Subsequently, a metastatic phenotype was examined in the lungs harvested from mice treated with nicotine for 21 and 28 days or from untreated mice (Table 1). Four weeks after tumor cell injection, there were abundant blue-stained tumor cells deposited around vessels in the lung from nicotine-treated mouse (Fig. 4C). In comparison, there was no micrometastasis in the lungs of mice without the administration of nicotine. Thus, the *in vivo* data further support the notion that nicotine facilitates the process of tumor metastasis.

**cdc42 functions as a downstream effector of PKC to promote the migratory activity mediated by nicotine.** GTPases have been reported to act downstream of PKC to regulate cell adhesion or migration (34). Therefore, it led us to explore the involvement of GTPases in nicotine-induced migratory promotion. To evaluate the individual role of each GTPase, the expression of RhoA, Rac1, and cdc42 and the inhibition of their expression by siRNAs were first examined by immunoblotting (Fig. 5A and B). MCF7 cells express RhoA, Rac1, and cdc42. After introducing the

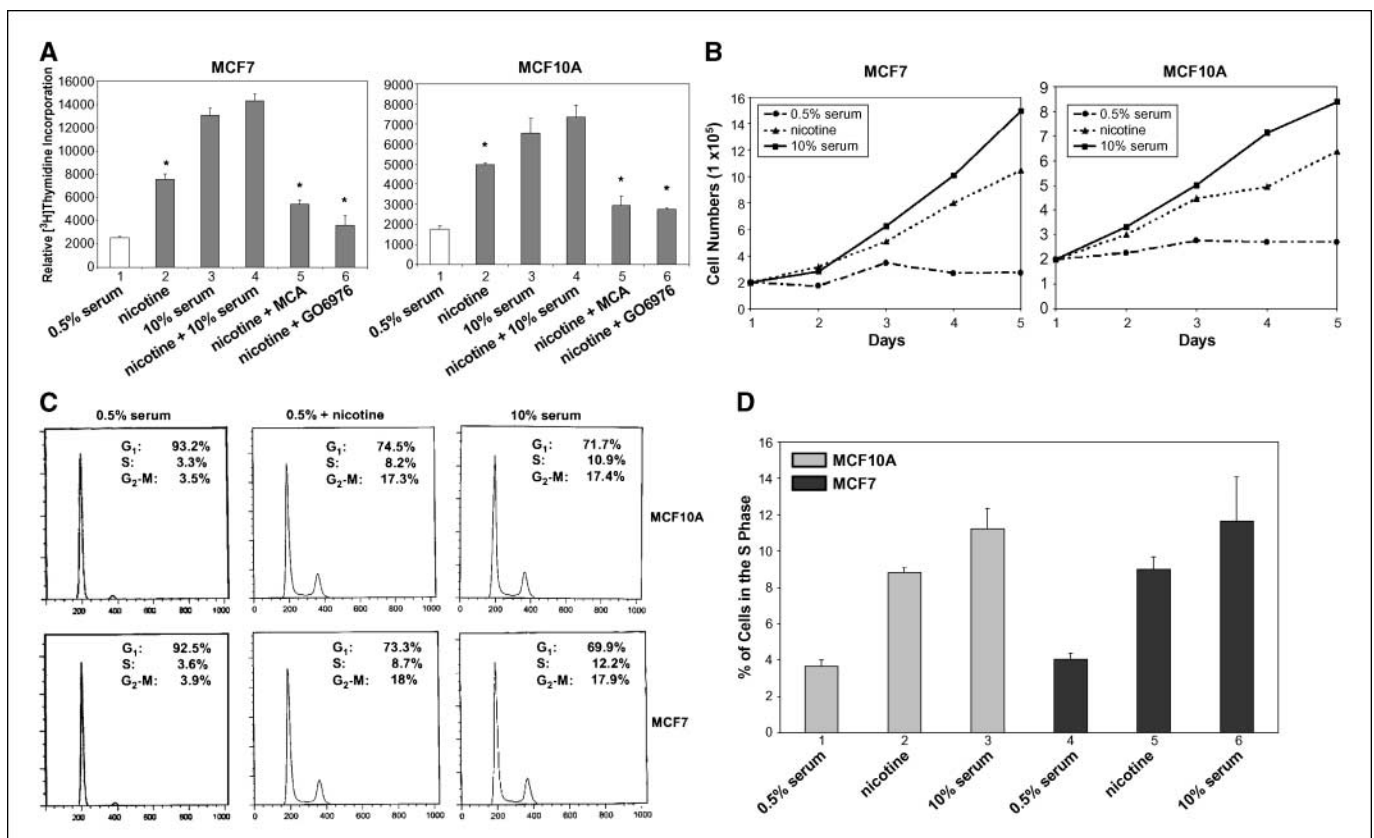
corresponding siRNAs into MCF7 cells, the levels of protein expressions of these GTPases were dramatically suppressed (6- to 8-fold). The expression of *cdc42* in MCF10A cells, after the introduction of *siRNA-cdc42*, was also examined and the amount of the protein was reduced (~7-fold) in comparison with the control.

Before testing the effect of these GTPases on nicotine-induced migratory activity, *cdc42* was ectopically expressed in MCF7 or MCF10A cells (Fig. 5C). A significant increased amount of exogenous *cdc42* (~4-fold) was detected by the antibody in both cells. Subsequently, Boyden chamber assays were then conducted (Fig. 5D). The suppression of *cdc42* by siRNA dramatically blocked the nicotine-induced migration in serum-starved MCF7 cells (Fig. 5D, left, lane 4). The introduction of siRNA into the cells to suppress either RhoA or Rac1 did not affect nicotine-mediated migratory activity (Fig. 5B, left, lanes 5 and 6). Transient overexpression of *cdc42* in MCF7 cells further augmented the magnitude of nicotine-mediated migratory activity (Fig. 5B, left, lane 7). Because the inhibitory effect of *cdc42* siRNA on migration was similar as that occurred after treatment with the PKC inhibitor GO6976 (comparing Fig. 3A, lanes 4 and 8), it indicates that both molecules are involved in this nicotine-mediated action. To determine the possible linear relationship between PKC and *cdc42*, serum-starved MCF7 cells with transient overexpression of *cdc42* were treated with nicotine in the presence of GO6976 and

then subjected to the Boyden chamber assay. The suppression of PKC dramatically suppressed the nicotine-mediated migratory effect of the cells regardless of the augmented expression of *cdc42* (Fig. 5B, left, lane 8). The similar results were obtained from MCF10A cells (Fig. 5B, right). Overall, the data suggest that *cdc42* participates in nicotine-mediated migration and its activation depends on the existence or activity of PKC.

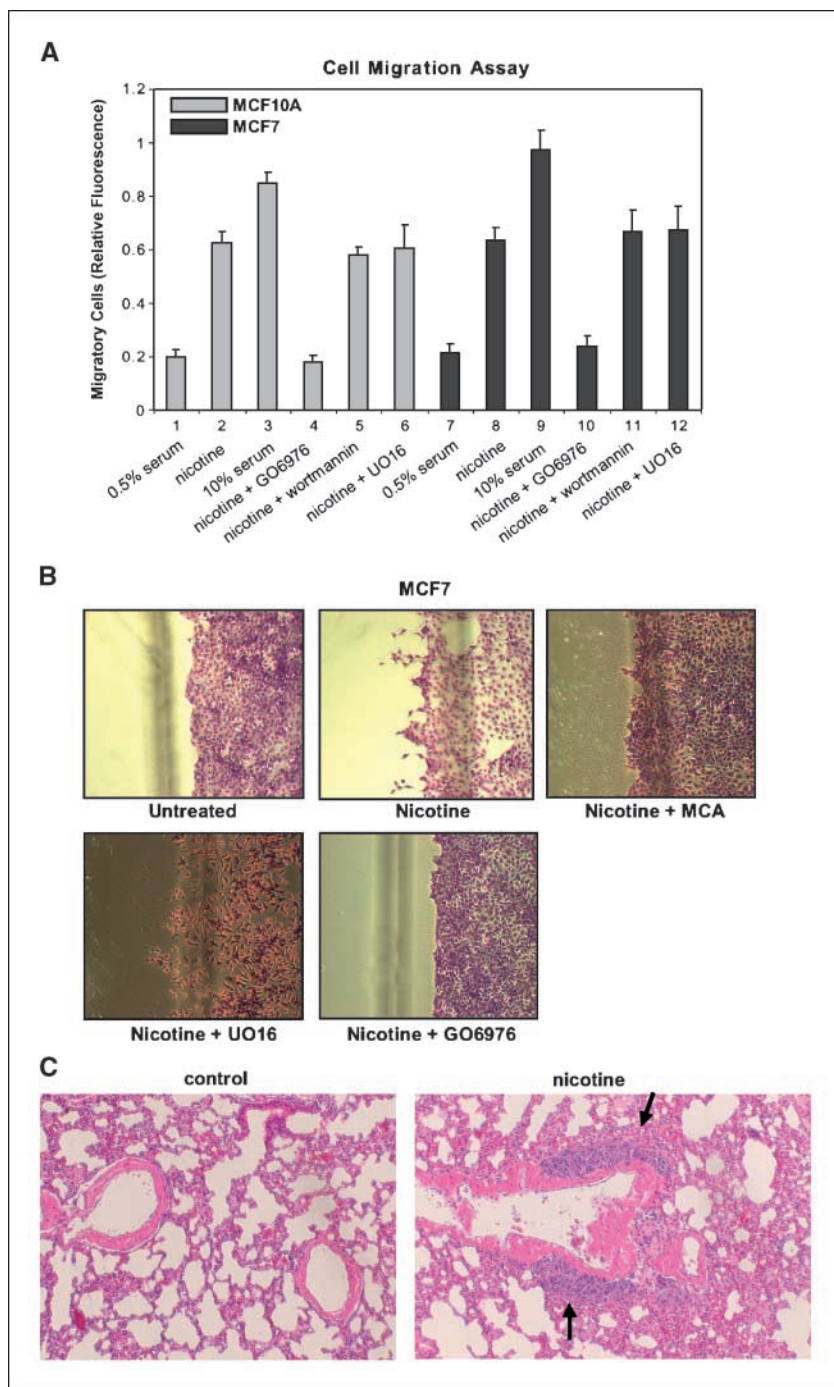
## Discussion

Tobacco smoke has been implicated in the promotion of the onset of not only lung cancer but also malignancies in various other organs (1-3). Increasing incidences of human cancers caused by secondhand smoking have drawn a lot of attention for the needs to understand the etiology of the malignancies induced by such environmental smoking pollution. Nicotine is originally thought to be mainly responsible for tobacco addiction. However, many studies now reveal that this tobacco component is able to modulate various key biological activities in nonneuronal tissues (7-12). In particular, nicotine has been shown to promote survival of many cell types, including keratinocytes and head or neck tumor cells (11, 39). Through associating with nicotine receptor, nicotine has been shown to modulate the expressions of a diverse set of genes, the products of which are involved in the regulation of gene transcription, RNA processing, and protein modification (21). In



**Figure 3.** Effect of nicotine exposure on the proliferation and cell cycle progression in MCF10A and MCF7 cells. **A**, cells were grown in the medium containing 0.5% serum for 24 h and then subjected to various treatments. Subsequently, the rate of <sup>3</sup>H incorporation was measured by a scintillation counter. Columns, mean of five independent experiments ( $n = 5$ ); bars, SD. \*,  $P < 0.05$ . **B**, after culturing in the medium containing 0.5% serum, cell proliferation of MCF7 and MCF10A with or without nicotine treatment was measured. Points, mean of five independent experiments; bars, SD. **C**, after culturing in the medium containing 0.5% serum, cells were treated with nicotine or refed with 10% serum for 4 h, and DNA profiles of the cells were analyzed by a flow cytometer. **D**, the plot represents the percentages of the cells in the S phase of the cell cycle. Columns, mean of five independent experiments; bars, SD.





**Figure 4.** Acceleration of cell migration and wound healing by nicotine exposure. **A**, after culturing in the medium containing 0.5% serum for 24 h, cells were untreated (*lanes 1 and 7*) or treated with nicotine (*lanes 2 and 8*), 10% serum (*lanes 3 and 9*), nicotine plus GO6976 (*lanes 4 and 10*), nicotine plus wortmannin (*lanes 5 and 11*), and nicotine plus UO16 (*lanes 6 and 12*). Cells then were labeled with 10  $\mu$ g/mL DiI-labeled acetylated low-density lipoprotein for 4 h at 37°C. Subsequently, cells were loaded in Boyden chamber and incubated overnight. The migratory cells at the bottom wells were measured by fluorescence. Columns, mean of three independent experiments; bars, SD. **B**, cells were partially wiped away and then subjected to various treatments. Photos are cells migrating to the wiping-off area at 48 h. The line represents the edge of the cell monolayer immediately after wiping away the cells. **C**, 4 wk after the injection of MCF7 cells through the tail veins of mice, the lung tissues from mice without nicotine treatment (*left*) or with nicotine exposure (*right*) were isolated, coated on slides, and stained with H&E. Arrows point to the area with metastatic tumor cells.

lung cancer, studies indicate that nicotine exposure facilitates the spread of the cancer in the body (10, 33). In the process of tumor promotion, nicotine has been shown to stimulate phosphorylation of  $\mu$ -calpains and m-calpains to enhance proteolytic activity and further to accelerate cell invasion (10, 33). Therefore, nicotine exposure seems to be able to promote not only cancer cell survival but also tumor metastasis.

Here, our study shows that human breast cancer MCF10A and MCF7 cells, like lung epithelial cells or keratinocytes, express at least four subunits of nAChR. Based on the similarity of the expression patterns of nAChR subunits in human breast cancer cells and cells from other types of tumors, we have examined the effect of

nicotine in regulation of cell migration as well as on growth. We showed in this study that nicotine exposure is moderately mitogenic in serum-starved MCF10A and MCF7 cells. Furthermore, nicotine treatment, via stimulating PKC and cdc42 signaling cascade, promotes cell migration and invasion as measured by the Boyden chamber and wound-healing assays. This nicotine-mediated migratory activity requires the engagement of nicotine receptor. Therefore, from the experiments using human breast cancer cell lines, we conclude that one way for nicotine to promote breast tumor development may be through potentiating metastasis.

Cell surface growth factors and adhesion molecules are often involved in regulation of mitogenesis and metastasis that are

**Table 1.** Nicotine exposure promotes i.v. injected MCF7 cells to metastasize to the lungs of nude mice

Group	Cell line	Time after injection (d)	Lung metastases	
			Incidence*	Extent <sup>†</sup>
Control	MCF7	21	0/5	—
Control	MCF7	28	0/5	—
Exposed	MCF7	21	0/5	—
Exposed	MCF7	28	1/5	+++

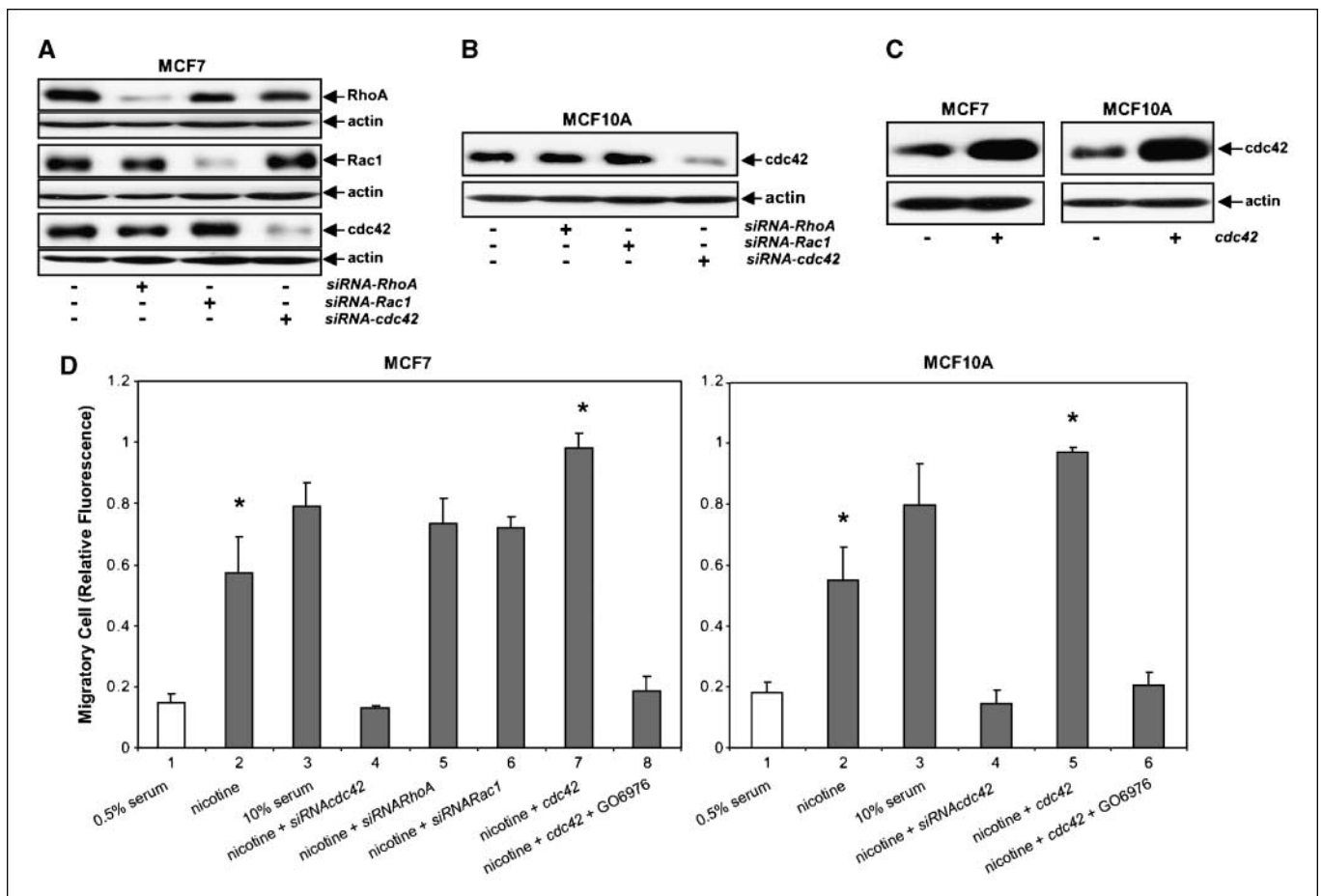
NOTE: One million MCF7 cells were injected into the tail veins of nude mice. One group of mice was injected with nicotine and another group was left untreated. Mice were sacrificed at the indicated times, lungs were harvested and stained, and metastases were quantified. Scoring system: —, no blue cells; +, about 1 to 50 blue cells; ++, about 50 to 100 blue cells; +++, about 100 to 200 blue cells.

\*Number of mice with metastases/number of mice injected.

<sup>†</sup>Extent of metastases according to scoring system.

critical steps during tumor development. It has been shown that the addition of nicotine mobilizes Ras and its downstream signaling pathways in mouse lung epithelial cells, resulting in the up-regulation of cyclin D1 and subsequent entry of cell cycle following G<sub>1</sub> stimulation (9). Furthermore, on nicotine exposure,

the lung cells were unable to arrest in G<sub>1</sub> phase of the cell cycle, which renders the cells a genetic background for the establishment of genetic instability (28). Our current study using breast epithelial-like or cancer cell lines again shows that nicotine is able to mobilize multiple intracellular signaling pathways to modulate



**Figure 5.** Involvement of *cdc42* in nicotine-mediated cell migration. *A*, after transfecting the siRNAs into MCF7 cells, the expression of RhoA, Rac1, and *cdc42* was examined by immunoblotting analysis. *B*, expression of *cdc42* in MCF10A cells after introducing *siRNA-cdc42*. *C*, expression of *cdc42* in MCF7 or MCF10A cells after transient introduction of *cdc42*. *D*, after ectopically expressing *cdc42* or introducing the siRNAs, MCF7 (left) or MCF10A (right) cells were cultured in the medium containing 0.5% serum for 24 h before nicotine treatment. Subsequently, the Boyden chamber assay was performed. Columns, mean of three independent experiments ( $n = 3$ ); bars, SD. \*,  $P < 0.01$ .

growth- or survival-related activities. Through binding to the receptor, nicotine disrupts the G<sub>1</sub> restriction point induced by serum starvation and, at the same time, stimulates cell migration or invasion. It is possible that nicotine, after binding to its receptor, recruits angiogenic or migration-related factors to the surface of cells and creates a high concentration of ligands near the receptor for the potentiation of cell migration. In such a cross-talk between nicotine receptor and angiogenic receptors, nicotine promotes interactions of intracellular angiogenic factors and their receptors. Our study also showed that nicotine has a profound effect on cell proliferation after the cells were refed with the growth medium containing 10% serum (Fig. 2A). It is also conceivable that nicotine synergizes with growth factor receptor-induced pathways, which often occur in the cooperation between vascular endothelial growth factor and other growth factors (40–42).

PKC has been reported to regulate the expression or structural change of cytoskeleton-associated proteins, which further affects cytoskeleton reorganization and cell invasion (34, 43, 44). On mitogenic stimulation, PKC, acting as a second messenger, associates with the plasma membrane and then transmits signals to downstream effectors, including GTPases (34, 43, 44). In human endothelial cells, *cdc42* has been shown to function downstream of PKC for reorganization of stress fibers after exposing to a stress environment (34). In this study, we showed that the suppression of PKC or *cdc42* blocks nicotine-mediated migratory activity in breast cancer cells. The overexpression of *cdc42* is able to further enhance the migration. In contrast, although *cdc42* was overexpressed, there were a few migratory cells after PKC activation is inhibited. Given the fact that PKC activation occurs at the early stage and the plasma membrane level on various stimulations and GTPases often function as intracellular effectors, our data suggest that PKC and *cdc42* work in a linear signaling relationship to transduce the signals initiated by nicotine to accelerate cell migration.

Many growth factors and their downstream effectors are aberrantly activated or overexpressed, which contribute to growth deregulation in cancer. It has been reported that the addition of nicotine increases EGF receptor (EGFR) expression in colon cells (14). Therefore, it is possible that nicotine could elevate the sensitivity of breast epithelial or cancer cells to growth factors and subsequently promote cell proliferation and metastasis. Because it

is known that src is responsible for the phosphorylation of both EGFR and nAChR, it would be interesting to determine if src acts as a mediator to transmit the signals elicited by nicotine to EGFR pathway.

MCF10A is a spontaneously immortalized but nontransformed human mammary epithelial cell line derived from the breast tissue of a patient with fibrocystic changes (45). MCF10A cells have been considered as normal breast epithelial cells because these cells cannot grow in soft agar and are dependent on growth factors and hormones in the cultured medium for growth and survival (45). Our investigations show that the mammary epithelial MCF10A cells, like MCF7 breast carcinoma cells, express the same subunits of nAChR, and nicotine treatment does not alter the expression pattern of each subunit. The magnitudes of growth and migratory promotion by nicotine in these two cell lines are very similar. Moreover, both PKC and *cdc42* are involved in nicotine-induced cell migration. Thus, the data suggest that the same machinery in these two different cell lines is used by nicotine to enhance the migratory or proliferation activity.

In summary, we have shown that nicotine treatment can stimulate human breast epithelial-like MCF10A as well as cancer MCF7 cells to proliferate, migrate, and wound heal. Metastasis plays a critical role in the process of tumorigenesis. The results also provided an insight into how nicotine potentiates cell migration in normal breast epithelial or cancer cells. Overall, our study provides evidence to suggest that nicotine is a possible component for the initiation of breast cancer induced by secondhand smoking. The present investigation also warrants a caution for the clinical use of nicotine to relieve chronic pain or aid in the cessation of cigarette smoking.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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