RESEARCH ARTICLE

Tea polyphenol (−)-epigallocatechin-3-gallate inhibits nicotine- and estrogen-induced α9-nicotinic acetylcholine receptor upregulation in human breast cancer cells

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Received: June 3, 2010
Revised: August 8, 2010
Accepted: September 3, 2010

Scope: The aim of this research was to explore whether the tea-polyphenol (–)-epigallocatechin-3-gallate (EGCG) could be used as a potential agent for blocking smoking (nicotine, Nic)- or hormone (estradiol, E2)-induced breast cancer cell proliferation through inhibition of a common signaling pathway.

Methods and results: To explore whether Nic (>0.1 μM, 24 h) and E2 (>1 nM, 24 h) significantly increased α9-nicotinic acetylcholine (α9-nicotinic acetylcholine receptor (nAChR)) mRNA and protein expression levels, real-time PCR and immunoblotting analysis experiments were performed in human breast cancer (MCF-7) cells. Luciferase promoter activity experiment was performed to test the α9-nAChR promoter activity affected by Nic, E2 or EGCG. The results indicate that treatment with EGCG (1 μM) profoundly decreases Nic- and E2-induced MCF-7 proliferation by down regulating α9-nAChR expression. The α9-nAChR promoter activity is significantly induced by 24-h treatment with Nic (10 μM) or E2 (10 nM) (>1.8 and ~2.3-fold, respectively) in MCF-7 cells. Pretreatment with EGCG eliminated the Nic- and E2-induced α9-nAChR promoter-dependent luciferase activity. We further demonstrate that combined treatment with EGCG profoundly inhibits [3H]-Nic/ α9-nAChR binding activity in breast cancer cells.

Conclusions: We found that the EGCG could be used as an agent for blocking smoking (Nic)- or hormone (E2)-induced breast cancer cell proliferation by inhibiting of α9-nAChR signaling pathway. This study reveals the novel antitumor mechanisms of EGCG, and these results may have significant applications for chemopreventive purposes in human breast cancer.

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Abbreviations: ChIP, chromatin immunoprecipitation; **E2,** estradiol; **EGCG,** (−)-epigallocatechin-3-gallate; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; **nAChR,** nicotinic acetylcholine receptor; **Nic,** nicotine; **NNK,** 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; **PI3K,** PI3 kinase; **Sc,** scrambled vector control; **siRNA,** small interfering RNA

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Keywords:
Breast cancer / (−)-Epigallocatechin-3-gallate / Estradiol / Nicotine / Nicotinic acetylcholine receptor

1 Introduction

Breast cancer is the second leading cause of cancer-related deaths among women in the United States. Large cohort studies performed in the United States and Japan have indicated that the risk of developing breast cancer is associated with active and passive smoking [1, 2]. Cigarette smoke is a complex mixture of over 4000 chemical constituents and contains nicotine (Nic) as a major carcinogenic component. Studies performed using ^{14}C-Nic showed that 80–90% of inhaled Nic is absorbed systemically during smoking [3]. Nic concentrations in plasma average about 15 ng/mL immediately after smoking and are extremely high in the saliva and gastric juices (>1300 and >800 ng/mL, respectively) [4, 5]. Repeated exposure of noncancerous human breast epithelial (MCF-10A) cells to the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) results in xenografted tumor formation in nude mouse models and anchorage-independent colony formation in soft agar [6, 7]. *In vivo* studies have demonstrated that Nic promotes the growth of solid tumors, suggesting that Nic might contribute to the progression of cell proliferation, invasion, and angiogenesis in tumors [8]. These results imply that Nic alteration of normal breast epithelial cells may also contribute to breast tumorigenesis.

Human neuronal tissues have been reported to have a high level of nicotinic acetylcholine receptor (nAChR) subunit expression upon chronic Nic exposure [9]. The natural ligand of nAChRs is acetylcholine; however, tobacco components, such as Nic and NNK, are known to be high-affinity agonists of nAChRs [10]. Our earlier studies confirmed the previous reports that cigarette smoking is the chief risk factor in lung [11], colon [12], bladder [13], and breast cancers [14], suggesting that agents such as Nic and NNK may exert their carcinogenic functions in a receptor-dependent manner. Animal experiments have been used to demonstrate that overexpression of cyclin D3 is detected concomitantly in α9-nAChR-overexpressing MCF-10A-Nic (DOX)-xenografted tumor tissues in BALB/c-nu/nu mice [14]. We further demonstrated that agents that were isolated from natural edible plants could inhibit human breast cancer cell proliferation by specifically targeting human nAChRs [14, 15]. These results implied that nAChRs detected in human breast cancer cells could be used as a therapeutic molecular target for clinical applications.

The green tea extract (−)-epigallocatechin-3-gallate (EGCG) has been studied previously for its antioxidant and free radical scavenging activity [16, 17]. EGCG is also effective in inducing breast cancer apoptosis and cell-cycle arrest [18, 19]. Another study also suggested that many of the cancer chemopreventive properties of green tea are mediated by EGCG, which induces apoptosis and promotes cell growth arrest by altering the expression of cell-cycle regulatory proteins, activating killer caspases, and suppressing nuclear factor κB activation [20]. In this study, we demonstrate that the nicotinic receptor α9-nAChR is significantly induced by Nic and estradiol (E2) in human breast cancer cells. We also found that E2- and Nic-induced α9-nAChR protein expression is blocked by EGCG. These findings suggest a possible chemopreventive ability of EGCG through the inhibition of E2- or Nic-induced α9-nAChR protein expression, which is known to confer smoking-mediated breast tumorigenesis.

2 Materials and methods

2.1 Chemicals

Curcumin was purchased from E. Merck (Darmstadt, Germany). Magnolol, dibenzoylmethane, EGCG, 1-(2-hydroxyphenyl)-3-phenyl-1,3-propanedione and 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA). Aqueous stock solutions of 10 mM Nic and NNK (Chemsyn, Lenexa, KS, USA) were prepared in double-distilled H_2O. The other chemicals used in this study were dissolved in DMSO as described in our previous studies [21, 22].

2.2 Cell culture

Human mammary gland epithelial adenocarcinomas (MCF-7, MDA-MB-231, AU-565, MDA-MB-453, and BT-483) and normal human mammary gland epithelial fibrocystic cell lines (MCF-10A and HBL-100) were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). MCF-7, MDA-MB-231, and MDA-MB-453 cells were maintained in DMEM, whereas BT-483 cells were maintained in RPMI-1640. Cell growth, proliferation, and viability were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The formazan product was measured by absorbance at 540 nm.

2.3 RNA isolation and RT-PCR analysis

Total RNA was isolated from human cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The α9-nAChR subunit-specific primers were synthesized by MB Mission BioTech (Taipei,

Taiwan) as described in our previous reports [14, 15]. The PCR amplicons were analyzed on 1.2% agarose gels (Amresco, Solon, OH, USA) stained with ethidium bromide. The α9-nAChR mRNA intensity was measured and normalized to β-glucuronidase expression. Pictures of the bands were taken using an INFINITY-α digital imaging system (Vilber Lourmat, France), and band intensities were determined using PhotoCapt Version 11.01 software.

2.4 Protein extraction, Western blotting, and antibodies

To prepare protein samples, cells were washed once with ice-cold PBS and lysed on ice in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM $NaCl_2$, 0.5% Nonidet P-40, 100 mM sodium fluoride, and 200 μM sodium orthovanadate) containing protease inhibitors as described previously [15]. Protein (50 μg) from each sample was resolved by 12% SDS-polyacrylamide gel electrophoresis, transferred, and analyzed by Western blotting. Antibodies were purchased from the following vendors: anti-total AKT and protein A/G agarose beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-GAPDH and anti-α9-nAChRantibodies were from Abcam (Cambridge, UK); anti-phospho AKT (Ser^{473}), anti-c-Jun, and anti-c-Fos were from Cell Signaling Technology (Beverly, MA, USA). The secondary antibodies, alkaline phosphatase-coupled anti-mouse, and anti-rabbit IgG were purchased from Santa Cruz Biotechnology. The specific protein complexes were identified with the chromogenic substrates nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl-phosphate from KPL (Gaithersburg, MD, USA). In each experiment, proteins were also probed with anti-GAPDH antibodies as a protein-loading control.

2.5 Soft agar cloning assay

The base layer consisted of 0.9% low gelling point SeaPlaque agarose (Sigma, St. Louis, MO, USA) in culture medium [15]. Soft agar, consisting of 0.4% SeaPlaque agarose in culture medium, was mixed with 1×10^4 MCF-7 cells and plated on top of the base layer in 60-mm diameter culture dishes. Soft agar cultures were maintained at 37°C and counted for the appearance of colonies with a Leica DMI 4000B Microscope Imaging System (Leica Microsystems, Wetzlar, Germany).

2.6 RNA interference

Expression of α9-nAChR was ablated in breast cancer cells with at least two independent small interfering RNAs (siRNAs) [14]. The target sequences of α9-nAChR mRNA were selected to suppress α9-nAChR gene expression. Scrambled sequences of each siRNA were used as controls [14]. After BLAST analysis to verify that there were no significant sequence homologies with other human genes, the selected sequences were inserted into *Bgl*II/*Hin*dIII-digested pSUPER vectors to generate the pSUPER-Si α9-nAChR and pSUPER-scramble vectors. All constructs were confirmed by DNA sequence analysis. The transfection protocol utilized has been described previously [14, 15]. Briefly, 1.5×10^5 cells were washed twice with PBS and mixed with 0.5 μg of plasmid. Then, one pulse was applied for 20 ms under a fixed voltage of 1.6 kV on a pipette-type MP-100 microporator (Digital Bio, Seoul, Korea).

2.7 Generation of stable nAChR-knockdown cell lines

At least three clones of the MCF-7 cell lines stably expressing the siRNA were generated. All experiments were performed using multiple subclones of each cell line with the consistent results. The pSUPER-Si α9-nAChR and pSUPER-scramble vectors were transfected and stable integrants were selected 72 h later with Geneticin (4 mg/mL). After 30 days in selective medium, two geneticin (G418)-resistant clones, referred to as pSUPER-Si α9-nAChR, were isolated. These clones demonstrated >80% reduction in mRNA and protein levels when compared with the control clones (pSUPER-scramble).

2.8 Plasmid constructions

All α9-nAChR promoter–luciferase gene fusions were made in the pGL3-Basic vector (Promega, Madison, WI, USA) by introducing the appropriate α9-nAChR promoter fragments into the polylinker upstream of the luciferase gene. These fragments were generated with restriction enzymes and cloned directly into pGL3-Basic or subcloned first in pBluescript and then transferred to pGL3-Basic. Deletion analysis of the most promoter-proximal region was performed by generating either appropriate restriction enzyme fragments or PCR fragments with suitable sense oligonucleotides and an antisense primer (5′-TATA-GAGGCTCAGGAAAAAG-3′) that anneals to the pGL3-Basic vector downstream of the site of transcription initiation.

2.9 Luciferase activity assay

MCF-7 cells were plated in 6-well plates. Cells were transiently cotransfected the next day with 1.5 μg of PGL3-Basic plasmid containing the full-length α9-nAChR promoter and 0.1 μg of the RLTK plasmid (Promega) using an MP-100 microporator (Digital Bio), according to the manufacturer's instructions. After incubation for 24 h, the medium was

changed to culture medium containing 10% FBS or 0.1% FBS with or without Nic. Cells were lysed 24 h later with 1 × Reporter Lysis Buffer (Promega) and stored frozen at −20°C overnight. Luciferase activity was determined by testing 50 μL of the cell lysate and 50 μL of the Luciferase Assay Reagent (Promega) using a HIDEX Chameleon Microplate Reader. Relative luciferase units were normalized to renilla luciferase from the same cell lysates. Each luciferase assay experiment was performed three times.

2.10 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays of cultured cells were performed as described previously [14, 15]. Briefly, after various regimens of Nic treatment, the cells were fixed with a final concentration of 1% formaldehyde directly added to the cell culture medium at 25°C for 15 min. The cross-linking reaction was terminated with 0.125 M glycine for 5 min, and the cells were collected into a new Eppendorf tube. The cell lysate was sonicated three times with 10 s bursts to yield input DNA enriched for fragments around 1000 bp in size. A c-Jun (AP-1) antibody (Santa Cruz Biotechnology) was used for the immunoprecipitation reactions. Primers, which detect the α9-nAChR promoter regions, amplified a region from −960 to −1 for 32 cycles (forward: 5′-ATGCAATG-CAAGCCTGAGCT-3′ and reverse: 5′-TATA-GAGGCTCAGGAAAAAG-3′). The PCR products were then separated and analyzed by agarose gel electrophoresis.

2.11 [^{3}H]-Nic equilibrium binding

L-(−)-[*N*-methyl-^{3}H]-Nic ([^{3}H]-Nic) (71–75 Ci/mmol) was purchased from Dupont/NEN Research Products (Boston, MA, USA), and free-base Nic (99% pure) was purchased from The Eastman Kodak (Rochester, NY, USA). To study the uptake of [^{3}H]-Nic in MCF-7 cell monolayers (2×10^6 cells/well), cells were rinsed three times with a buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM Hepes/Tris (pH 7.4). Saturation binding studies were conducted in 6-well plates using at least eight different concentrations of [^{3}H]-Nic ranging from 1 to 15 nM for 2 h at 37°C. Nonspecific binding was determined by the addition of 10 nM Nic. Total binding was evaluated for each concentration in quintuplicate, and blanks were determined in triplicate. The uptake of [^{3}H]-Nic was halted by aspiration of the uptake medium and washing of the wells three times with ice-cold buffer. The cells were lysed in 1 mL of 0.5% Triton X-100, and aliquots of the cell lysates were transferred to scintillation vials to determine the incorporated radioactivity by scintillation counting. Inhibition studies were carried out by adding various concentrations of unlabeled compounds to the uptake medium.

2.12 Statistics

All data are expressed as the mean value ± SE. Comparisons were subjected to a one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was set at $p < 0.05$.

3 Results

3.1 Nic- and E2-induced nAChR expression in a human breast cancer cell line

Smoking and hormones are two important etiological factors involved in breast cancer formation [23]. Our recent study revealed that the expression of the α9-nAChR subunit in MCF-7 cells is largely responsible for the observed Nic-induced proliferation [14]. To test whether nicotinic-induced cell proliferation signaling was also influenced by crosstalk from hormones (such as E2) through the induction of α9-nAChR, MCF-7 cells were treated with different doses of Nic, its metabolically active carcinogenic compound NNK, and E2 (Fig. 1A). The results demonstrate that theα9-nAChR protein level was significantly induced in MCF-7 cells by either smoking components (NNK, Nic) or the hormone (E2) in a dose-dependent manner (Fig. 1A). To test whether the α9-nAChR protein induction in E2-induced MCF-7 cells was ER-status specific, human breast cancer cells with (MCF-7 and BT483) or without (MDA-MB-231 and MDA-MB-453) ER expression were treated with E2 (10 nM) for 24 h (Fig. 1B). The E2-induced α9-nAChR protein induction was clearly detected in the ER+(MCF-7 and BT483) cells compared with the ER-(MDA-MB-231 and MDA-MB-453) cells (Fig. 1B). Time course experiments demonstrated that significant increases in the α9-nAChR mRNA and protein levels in MCF-7 cells were detected 6 h after Nic treatment (Fig. 1C).

3.2 Nic- and E2-induced α9-nAChR expression was blocked by the tea polyphenol (EGCG) in MCF-7 cells

We then hypothesized that agents with inhibitory effects on α9-nAChR protein levels could be used to block Nic- or E2-mediated carcinogenic signals. Several natural polyphenolic compounds derived from edible plants were evaluated. All these compounds have been reported in the previous articles to have antiproliferative or apoptosis-inducing activities in human cancer cells [21, 24]. We found that α9-nAChR mRNA and protein were downregulated by EGCG (1 μM) in Nic-treated cells (Figs. 2A and B). We then tested whether E2-induced overexpression of α9-nAChR could also be blocked by EGCG. MCF-7 cells were treated with E2 in the presence or absence of EGCG (Fig. 2B). Our results demonstrate that α9-nAChR protein expression, which was induced by either Nic or E2, was significantly inhibited by EGCG in MCF-7 cells (Fig. 2B). In

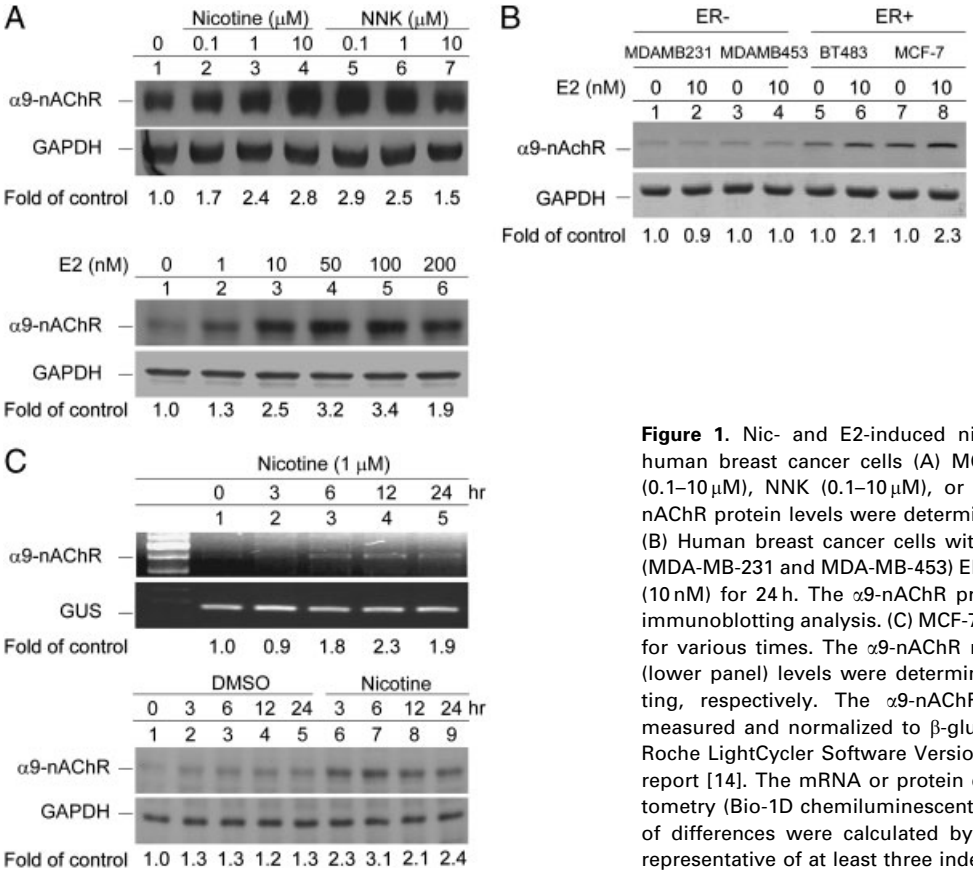

Figure 1. Nic- and E2-induced nicotinic receptor upregulation in human breast cancer cells (A) MCF-7 cells were treated with Nic (0.1–10 μM), NNK (0.1–10 μM), or E2 (1–200 nM) for 24 h. The α9-nAChR protein levels were determined by immunoblotting analysis. (B) Human breast cancer cells with (MCF-7 and BT483) or without (MDA-MB-231 and MDA-MB-453) ER expression were treated with E2 (10 nM) for 24 h. The α9-nAChR protein levels were determined by immunoblotting analysis. (C) MCF-7 cells were treated with Nic (1 μM) for various times. The α9-nAChR mRNA (upper panel) and protein (lower panel) levels were determined by RT-PCR and immunoblotting, respectively. The α9-nAChR mRNA expression level was measured and normalized to β-glucuronidase expression using the Roche LightCycler Software Version 4, as described in our previous report [14]. The mRNA or protein changes were analyzed by densitometry (Bio-1D chemiluminescent imaging system) and the results of differences were calculated by statistical methods. Results are representative of at least three independent experiments.

order to evaluate the quantitative effects of EGCG-induced α9-nAChR gene downregulation, we performed additional real-time PCR experiments to detect the α9-nAChR mRNA expression level in the EGCG- and Nic-treated human breast cancer cells. The results demonstrated that the α9-nAChR mRNA expression was significantly upregulated in response to Nic (10 μM) or E2 (10 nM) for 24-h treatment (Fig. 2C, left and right figures, bars 1 *versus* 2; $*p<0.05$). However, such effects were completely attenuated in the EGCG (10 μM)-treated cells (Fig. 2C, left and right figures, bars 2 *versus* 3; $*p<0.05$). Such results demonstrated that the Nic-induced α9-nAChR cell growth proliferation signal was attenuated by EGCG through downregulation of α9-nAChR mRNA expression.

3.3 EGCG pretreatment attenuated Nic-and E2-induced α9-nAChR protein expression through inhibition of AKT signals in MCF-7 cells

A previous study demonstrated that AKT is activated through phosphorylation of serine (Ser^{473}) or threonine (Thr^{308}) by different upstream stimulatory factors involved in breast cancer cell proliferation [25]. To test whether PI3 kinase (PI3K)-mediated signals are involved in Nic- and E2-induced α9-nAChR activation, MCF-7 cells were pretreated with a PI3K/AKT inhibitor (LY294002, 25 μM) or EGCG (10 μM) for 24 h and were subsequently treated with Nic (10 μM) or E2 (10 nM) for an additional 24 h. Immunoblotting analysis was performed, and the results show that Nic and E2 treatment significantly increased the levels of p-AKT (Ser^{473}) (Figs. 4A and B, lane 2). However, our study shows that Nic- and E2-induced upregulation of α9-nAChR is not significantly inhibited by LY294002 through inactivation of pAKT (Fig. 3A, lane 4 and Fig. 3B, lane 3). On the contrary, both α9-nAChR and pAKT protein expressions, which were induced by either E2 or Nic, were significantly inhibited by EGCG (Fig. 3A, lane 6 and Fig. 3B, lane 4). This result indicates that other signaling proteins are involved in the transcriptional activation of α9-nAChR protein expression.

3.4 Identification of transcription factor responses to EGCG-attenuated Nic-and E2-induced α9-nAChR protein expression in MCF-7 cells

To explore this hypothesis, a 1-kb genomic fragment encompassing the full-length human α9-nAChR promoter was inserted upstream of a luciferase reporter gene (Fig. 4A). Luciferase activity obtained with the full-length α9-nAChR promoter was compared with that obtained with the vector alone and defined as 100% (baseline level). Experiments utilizing the full-length construct demonstrated that α9-

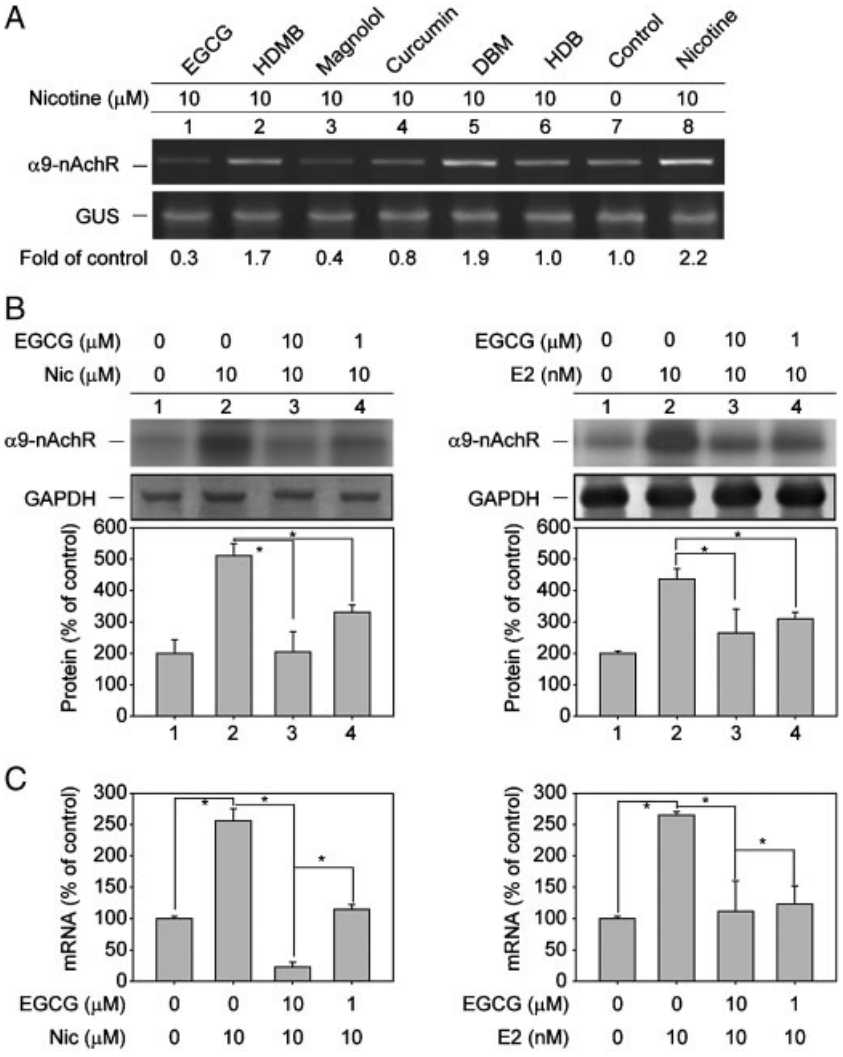

Figure 2. Nic- and E2-induced α9-nAChR expression is inhibited by EGCG in human breast tumor cell lines. (A) MCF-7 cells were treated with Nic (10 μM) in the presence or absence of natural polyphenolic compounds (1 μM, each compound) for 24 h. Expression of α9-nAChR mRNA was then detected by RT-PCR analysis. (B) MCF-7 cells were treated with Nic (10 μM) or E2 (10 nM) in the presence or absence of EGCG (1–10 μM) for 24 h. The α9-nAChR protein was then detected by immunoblotting. Results are representative of at least three independent experiments. (C) MCF-7 cells were treated with Nic (10 μM) in the presence or absence of EGCG (1–10 μM) for 24 h. The α9-nAChR mRNA expression levels were evaluated by real-time PCR analysis. Data are the mean ± SE of three independent experiments. Significance was set at $^{*}p < 0.05$.

nAChR promoter activity in MCF-7 cells was significantly induced by 24-h treatment with Nic (10 μM) or E2 (10 nM) (>1.8 and 2.3-fold, respectively) (Fig. 4B, $^{*}p < 0.05$). MCF-7 cells were also pretreated with EGCG (10 μM) for 24 h and then treated with Nic (or E2) for an additional 24 h. The results reveal that pretreatment with EGCG significantly reduces the Nic- and E2-induced α9-nAChR promoter luciferase activity (Fig. 4B, $^{*}p < 0.05$).

To define more precisely the regulatory elements that the α9-nAChR promoter required for Nic-induced transcriptional activation, transient transfection experiments were conducted with a series of 5′ promoter deletion constructs subcloned into the pGL3 vector. The resulting plasmids were transfected into MCF-7 cells. Specific response elements in the α9-nAChR promoter were eliminated in the shorter constructs. The Nic-induced responsiveness was preserved when the promoter fragments were constructed as pGL3(−44–1), pGL3(−125–1), and pGL3(−1000–700) (Fig. 4C). These results suggest that the Nic-induced transcription factor responsive elements were located at the AP-1 and p53 sites (Fig. 4A).

To determine whether the AP-1 or p53 transcription factors bind directly to the relevant α9-nAChR promoter-binding sites in response to Nic treatment, ChIP assays were performed in MCF-7 cells. The results demonstrate that increased binding of AP-1 to the α9-nAChR promoter was detected 24 h after Nic (10 μM) treatment in MCF-7 cells (Fig. 4D, lane 4). Pretreatment with EGCG (10 μM, 24 h) attenuated the Nic-induced AP-1 binding to the α9-nAChR promoter (Fig. 4D, lanes 5 *versus* 4). Interestingly, we found that p53 is a transcription factor that binds to the α9-nAChR promoter (−125–1) and is not affected by EGCG (Fig. 4D, lanes 5 *versus* 4).

3.5 EGCG affects Nic-induced breast cancer cell proliferation through the inhibition of α9-nAChR-mediated signals

To test whether α9-nAChR in human breast cancer cells could be used as a therapeutic molecular target, human breast cancer (MCF-7) and normal (MCF-10A) cells were

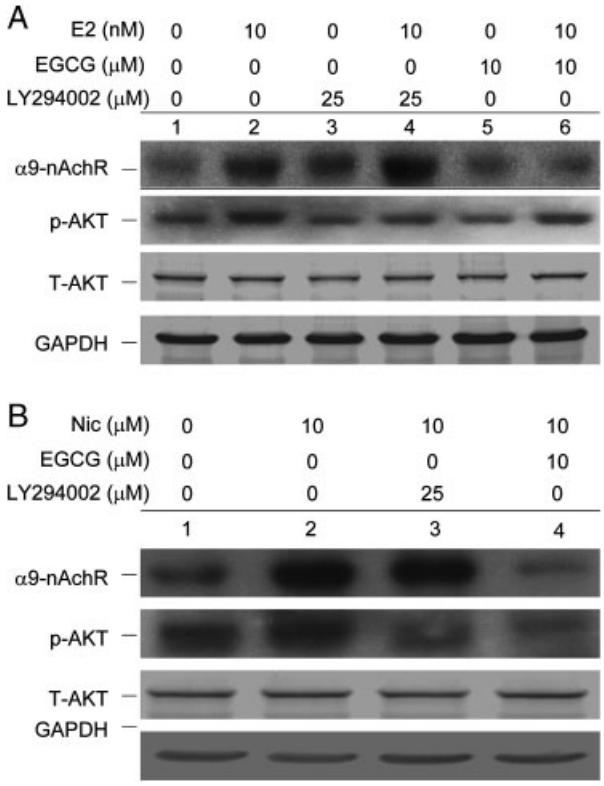

Figure 3. The effect of EGCG on Nic- and E2-induced PI3K activity in MCF-7 cells (A, B) MCF-7 cells were pretreated with a PI3K/AKT inhibitor (LY294002, 25 μM) or EGCG (10 μM) for 24 h and subsequently treated with (A) E2 (10 nM) or (B) Nic (10 μM) for an additional 24 h. The α9-nAChR, phosphorylated AKT (p-AKT), and total AKT proteins were then detected by immunoblotting.

treated with Nic (10 μM) in the presence or absence of EGCG (10–25 μM, 24 h) pretreatment, and the cell number was counted. We found that Nic significantly stimulated breast cancer cell growth, and this proliferation was blocked by EGCG in a dose-dependent manner (Fig. 5A, bars 3 *versus* 5 and bars 5 *versus* 9; $^{*}p<0.05$). On the contrary, no changes in cell number by either Nic or EGCG treatment in normal breast epithelial (MCF-10A) cells were observed (Fig. 5A). To explore the possible role of Nic-induced expression of the α9-nAChR subunit in human breast cell proliferation, we established a stable MCF-7 cell line [26] in which the expression of α9-nAChR was knocked down by RNA interference (Fig. 5B, bars 4 and 5). The cellular proliferation rates in wild-type MCF-7 cells (C) and cells containing the scrambled vector control (Sc) were significantly increased by treatment with Nic (10 μM) (Fig. 5B, bars 1 *versus* 2 and bars 1 *versus* 6; $^{*}p<0.05$). The EGCG (10 μM, 24 h) pretreatment reversed the Nic-induced cellular proliferation effects (Fig. 5B, bars 3 *versus* 2 and bars 7 *versus* 6; $^{*}p<0.05$). However, the growth effects observed in Nic-induced cells were almost completely attenuated in α9-nAChR siRNA knockdown (Si) cells (Fig. 5B, bars 4 *versus* 2). Interestingly, combined treatment with EGCG profoundly inhibited cell proliferation in the Nic-treated α9-nAChR (Si) cells when compared with the Sc cells (Fig. 5B, bars 5 *versus* 7; $^{*}p<0.05$).

These results suggest that breast cancer cell proliferation induced by Nic could be mediated through endogenous α9-nAChR. Therefore, agents that block the binding of Nic to α9-nAChR should be able to block the Nic-mediated cell proliferation effects. To test this hypothesis, MCF-7 cells were treated with [^{3}H]-Nic to determine its ligand-receptor binding activity. Our previous results demonstrated that the dissociation constant (Kd) of [^{3}H]-Nic binding is 3 nM, and that its maximum binding activity is attained at 60 min in MCF-7 cells [26]. The relative [^{3}H]-Nic binding activities were detected in cells with different treatment regimens: 24 h [^{3}H]-Nic treatment (control group); [^{3}H]-Nic+EGCG cotreatment for 24 h (cotreat group); EGCG pretreatment for 24 h followed by [^{3}H]-Nic cotreatment for an additional 24 h (pretreat group); or EGCG pretreatment for 24 h, followed by washing and then [^{3}H]-Nic treatment for an additional 24 h (pretreat, then wash group). Our results demonstrate that the relative [^{3}H]-Nic binding activity is significantly blocked in the EGCG pretreated group (Fig. 5C, bars 1 *versus* 4; $^{*}p<0.05$). On the contrary, the [^{3}H]-Nic binding activity was not significantly inhibited in the EGCG cotreated or the pretreated, then washed groups (Fig. 5C, bars 1 *versus* 2 and bars 1 *versus* 3). In addition, the mean [^{3}H]-Nic binding activity was significantly inhibited in MCF-7 (Si) cells compared with wild-type (C) or to (Sc) cells (Fig. 5D, bars 3 *versus* 1 or 3 *versus* 5; $^{*}p<0.05$). Interestingly, pretreatment with EGCG profoundly inhibited the [^{3}H]-Nic binding activity in the Nic-treated α9-nAChR (Si) cells when compared with the Sc or wild-type cells (Fig. 5D, bars 4 *versus* 2 or 4 *versus* 6; $^{*}p<0.05$).

3.6 Effects of EGCG on the inhibition of Nic-induced breast cancer cell colony formation in soft agar

Previous studies using a soft agar transformation assay and a xenografted nude mouse animal model demonstrated the transformation of noncancerous human breast epithelial (MCF-10A) cells by either a cigarette smoke condensate or the tobacco-specific carcinogen, NNK [6, 7]. To test whether Nic-induced formation of colonies through the activity of its cognate receptor (α9-nAChR), soft agar assays were performed with human breast cancer cells (MCF-7). Cells, α9-nAChR (Si) and (Sc), were treated with Nic (10 μM) in the presence or absence of EGCG (10 μM) (Figs. 6A and B). The results show that EGCG (10 μM) significantly inhibits the formation of transformed colonies when compared with Nic-treated cells (Fig. 6B, bars 2 *versus* 3, $^{*}p<0.05$). Interestingly, treatment with EGCG profoundly inhibited colony formation in α9-nAChR (Si) cells when compared with (Sc) cells (Fig. 6B, bars 5 *versus* 3 and 5 *versus* 7, $^{\#}p<0.05$).

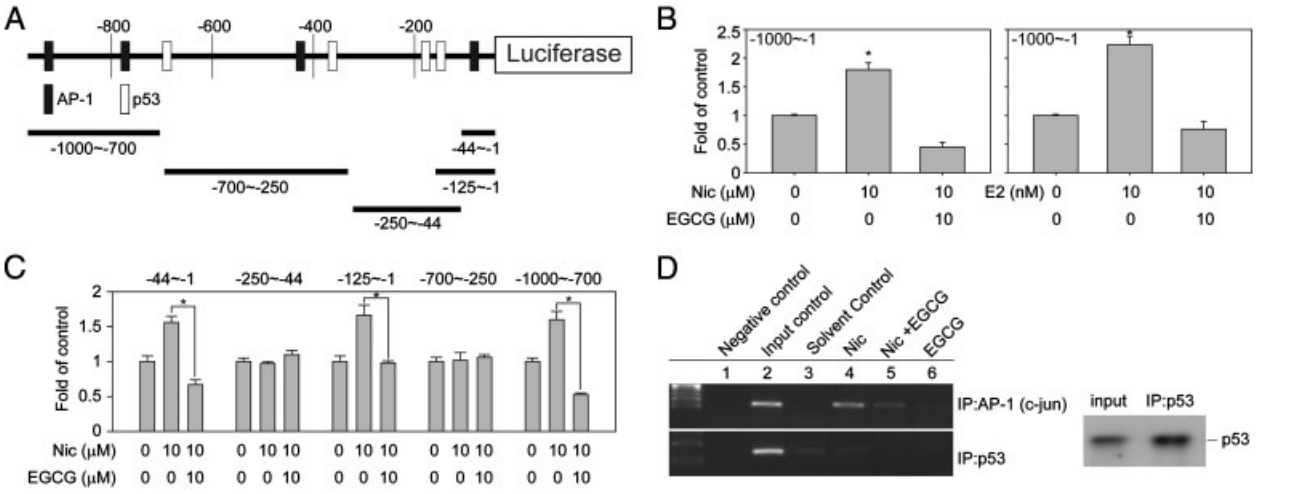

Figure 4. AP-1 mediates α9-nAChR transcriptional regulation by Nic and E2 in MCF-7 cells. (A) Schematic representation of the α9-nAChR promoter region (−1000∼−1) illustrating the putative AP1, and p53 transcription factor binding sites, (B) MCF-7 cells were transiently transfected with full-length (−1000∼−1) pGL3(α9-nAChR) and pRL-TK plasmid for 24 h. These cells were pretreated with EGCG (10 μM) for 24 h and then treated with Nic (10 μM) or E2 (10 nM) for an additional 24 h. Cell lysates were harvested, and relative firefly luciferase activities were measured and normalized to renilla luciferase from the same cell lysates. Luciferase activity obtained from the control cells treated only with vehicle (0.1% DMSO for E2 and ddH_2O for Nic) treatment was defined as a one-fold change. Data were analyzed using paired *t*-tests; all *p*-values are two sided. The comparisons were carried out as follows: the Nic- (or E2-) treated groups were compared between vehicle, EGCG+E2, or EGCG+Nic treatment groups ($^*p<0.05$). (C) MCF-7 cells were transiently transfected with different length pGL3 (α9-nAChR) promoters and pRL-TK plasmids for 24 h. The transfected cells were pretreated with EGCG (10 μM) for 24 h and then treated with Nic (10 μM) for an additional 24 h. Data were analyzed using paired *t*-tests; all *p*-values are two sided. The comparisons were carried out as follows: the Nic-treated groups were compared with the vehicle and EGCG+Nic combined treatment groups ($^*p<0.05$). (D) MCF-7 cells were treated with Nic (10 μM) for 24 h or pretreated with EGCG (10 μM), as described above. After treatment, the cell lysates were harvested and DNA bound by AP-1 or p53 was precipitated using specific antibodies for ChIP. To avoid false-negative results, immune-precipitation experiments were performed using antibody specific for p53. The p53 protein was detected by a p53-specific antibody (right panel). Data shown are representative of three independent experiments with similar results. Genomic DNA isolated from MCF-7 was used as a positive input control to ascertain the PCR conditions.

4 Discussion

Smoking and hormones are two important etiological factors involved in breast cancer formation. The aim of this research was to explore the molecular mechanisms of the antiproliferative effects of EGCG (10 μM), which was shown to completely inhibit Nic- (10 μM) and E2- (10 nM) induced transcriptional upregulation of α9-nAChR-mediated carcinogenic signals. We also demonstrate that EGCG prevents the binding of high concentrations of Nic (10 μM) to its receptor. These results are of clinical importance because a previous report demonstrated that the steady-state serum concentration of Nic in smokers was 200 nM and dramatically increased to 10–100 μM in serum or even to 1 mM in the saliva immediately after smoking [27]. Although many *in vitro* and animal studies have suggested a protective effect of green tea against breast cancer, findings from epidemiological studies have been inconsistent [28, 29]. For example, the possible protective role of EGCG in human breast cancer formation was investigated recently [28]. A long-term study (10.6 years) with a large cohort (24 226 women aged 40–69 years) was performed to explore the relationship between plasma levels of tea polyphenols, including (−)-epigallocatechin (EGC), (−)-epicatechin (EC), EGCG, and (−)-epicatechin-3-gallate (ECG), and breast cancer risk. The results did not support the hypothesis that tea polyphenols have protective effects. However, one study demonstrated that EGCG increased the sensitivity of human breast cancer cells to conventional chemotherapeutic agents [30], whereas another study demonstrated that EGCG suppressed the formation of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, in the lung DNA of mice given NNK [31]. These results indicated that the tea polyphenol EGCG could affect different pathways for the protection of smoking- or hormone-mediated disease (Fig. 7).

In this study, pretreatment with EGCG profoundly inhibited [^{3}H]-Nic binding activity in wild-type MCF-7 cells (Fig. 5D, bars 2 *versus* 1). We further demonstrate that siRNA knockdown of α9-nAChR significantly (>40%) inhibits [^{3}H]-Nic binding activity (Fig. 5D, bars 3 *versus* 1). Pretreatment with EGCG profoundly (>90%) inhibited [^{3}H]-Nic binding activity in (Si) cells when compared with Sc or wild-type cells (Fig. 5D, bars 4 *versus* 2 and 4 *versus* 6). We postulate that the EGCG-induced antitransforming activities were not mediated solely by α9-nAChR. Other nicotinic receptors (such as α5-, α7-, and α10-nAChR) detected in

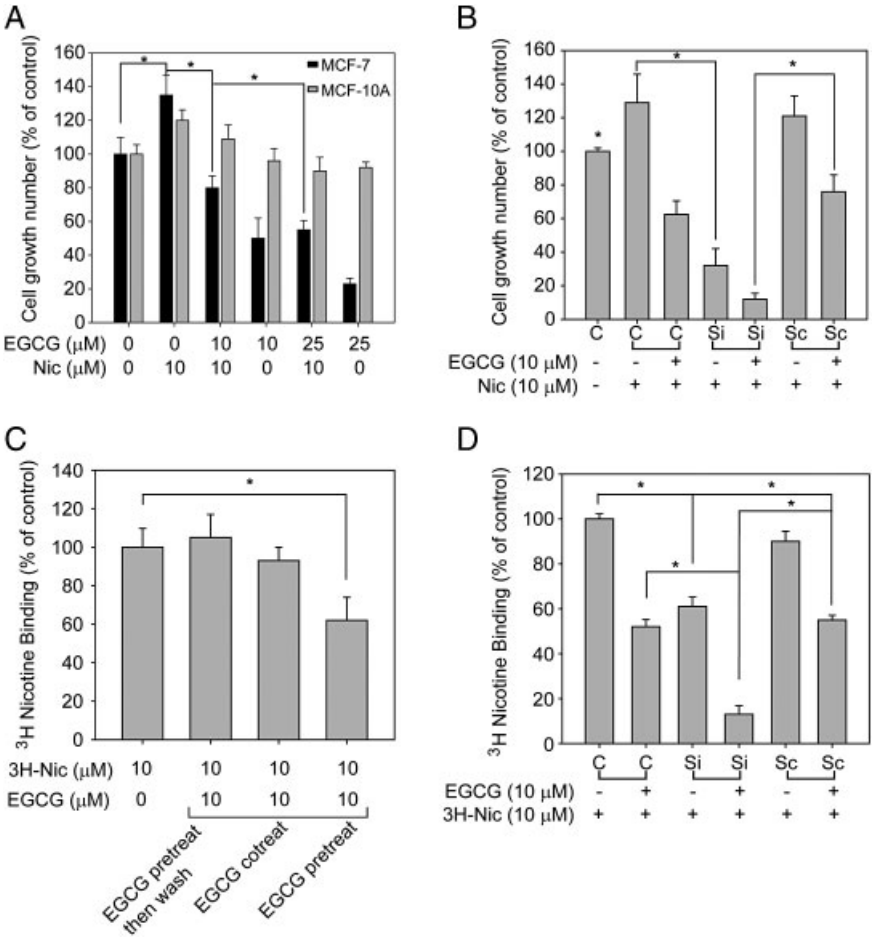

Figure 5. Effects of EGCG on the inhibition of Nic-induced breast cancer cell proliferation through blocking Nic binding to α9-nAChR (A) MCF-7 and MCF-10A cells were treated with Nic (10 μM, 24 h) or vehicle alone, with or without EGCG pretreatment (10 μM, 24 h). Cell growth was measured by the MTT assay at 24 h after drug treatment. (B) MCF-7, α9-nAChR (Si), and (Sc) cells were exposed to EGCG (10 μM) for 24 h then treated with Nic (10 μM) for an additional 24 h. Cell proliferation was assayed with MTT at the indicated times. (C) MCF-7 cells were pretreated with EGCG (10 μM) for 24 h. After treatment, [^{3}H]-Nic was added to the EGCG-exposed cells for an additional 24 h (EGCG pretreated group). In addition, the EGCG-treated cells were washed with PBS three times, and [^{3}H]-Nic was then added for an additional 24 h (EGCG pretreated then wash group). The cells that were treated with both EGCG and [^{3}H]-Nic for 24 h are designated as the EGCG cotreated group. The specific binding activity is presented as: total binding–nonspecific binding. (C) The α9-nAChR (Si), (Sc), and wild-type MCF-7 (C) cells were pretreated with EGCG (10 μM) for 24 h. After EGCG pretreatment, the cells were then treated with [^{3}H]-Nic (10 μM) for an additional 24 h and the [^{3}H]-Nic binding activity was then evaluated. Data are the mean ± SE of three independent experiments. Significance was set at $^*p < 0.05$.

MCF-7 cells could also play a role in the antitumorigenic effect of EGCG (unpublished data). In addition, E2- or Nic-induced overexpression of the α9-nAChR protein was almost completely inhibited in EGCG-pretreated cells (Fig. 3A, lane 6 and Fig. 3B, lane 4). However, the E2- and Nic-induced α9-nAChR protein levels were not completely blocked by a PI3K inhibitor (LY294002). These results demonstrate that α9-nAChR expression induced by E2 or Nic does not occur solely through PI3K activation (Figs. 3A, lane 4 *versus* 2 and Figs. 3B, lane 3 *versus* 2).

Our results are in contrast with the results of a previous study in which the pretreatment of cells with a PI3K/AKT inhibitor, LY294002, markedly inhibited Nic-stimulated nonsmall cell lung carcinoma cell proliferation through α7-nAChR-mediated signals [32]. Our results reveal that inhibiting PI3K signaling is not the best strategy for blocking proliferation in Nic-treated MCF-7 cells, which express α5- α7- α9-, and 10-nAChRs (unpublished data). To test whether inhibition of α9-nAChR by EGCG treatment is important for Nic-induced cellular proliferation and soft agar colony formation (Figs 5A, and 6B), α9-nAChR siRNA knockdown cells were exposed to EGCG and assayed for Nic-mediated effects. The antiproliferative effects were profoundly enhanced by EGCG treatment in α9-nAChR knockdown MCF-7 cells compared with wild-type or Sc control cells (Figs 5A, and 6B). Our recent study further demonstrated that inhibition of α9-nAChR protein expression by natural polyphenol compounds including garcinol, luteolin, and quercetin, which are isolated from edible fruits and vegetables, significantly reduced the Nic-mediated carcinogenic effects *in vivo* and *in vitro* [14, 15]. Our results may corroborate the EGCG inhibitory effects previously described in MCF-7 cells [18, 33–35]. To test whether tobacco smoking history, clinical staging criteria, and α9-nAChR mRNA expression fold ratios are correlated, 174 cases of breast tumor patients were examined, and the results from these studies suggested that Nic binding to α9-nAChR plays a direct role in the promotion and progression of human breast cancers [26]. These results suggested that diet-based protection against breast cancer may partly derive from synergy amongst dietary phytochemicals directed against specific molecular targets in responsive breast cancer cells. Our study also provides support for the feasibility of the development of a diet-based combinatorial approach in the prevention and treatment of breast cancer (Fig. 7).

Smoking and hormones are two important etiological factors of breast cancer formation. Previous studies have demonstrated that upregulation of PI3K/Akt signaling by E2

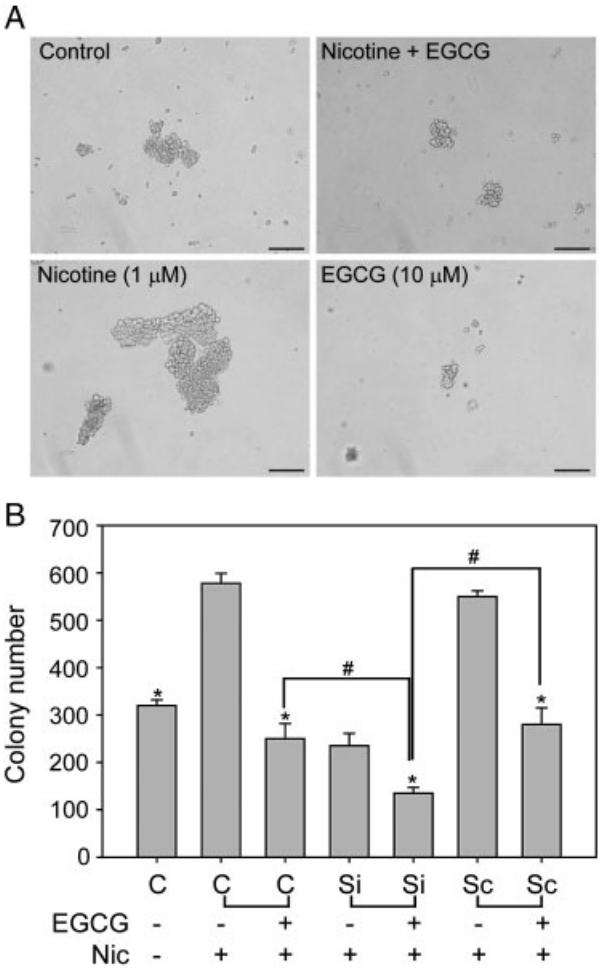

Figure 6. Effects of EGCG on the inhibition of Nic-induced breast cancer (MCF-7) cell colony formation in soft agar (A) MCF-7 cells were treated with Nic (10 μM) or vehicle alone with or without EGCG (10 μM). The gross morphology of MCF-7 cell colonies on culture plates is shown. These colonies exhibit subtle changes in their morphology, including a slight disaggregation compared with the untreated cells. Bar = 200 μm. (B) The number of colonies scored from the soft agar plates: the wild-type MCF-7 (C), α9-nAChR (Si), and (Sc) cells were seeded in soft agar and treated with Nic in the presence or absence of EGCG. The colonies in a 1 × 3 cm area were counted on each plate. Data are the mean ± SE of three independent experiments. Significance was set at $p < 0.05$. *The combined EGCG+Nic-treated groups were significantly different from the Nic-treated groups. ♯The combined EGCG+Nic-treated groups in the siRNA cells were significantly different from the EGCG+Nic-treated groups in the wild type (C) and (Sc) MCF-7 cells.

occurs through activation of ERα but not ERβ [36]. In response to E2 binding, $Ser^{104/106}$ and Ser^{118} are the major ERα sites [37] that are phosphorylated by the ERK1/2 MAP kinase [38, 39]. However, ERα (Ser^{167}) is the major site of phosphorylation by the PI3K/Akt pathway [40]. A recent study suggested that ERα phosphorylation at these different phosphorylation sites could affect the survival of ER-positive

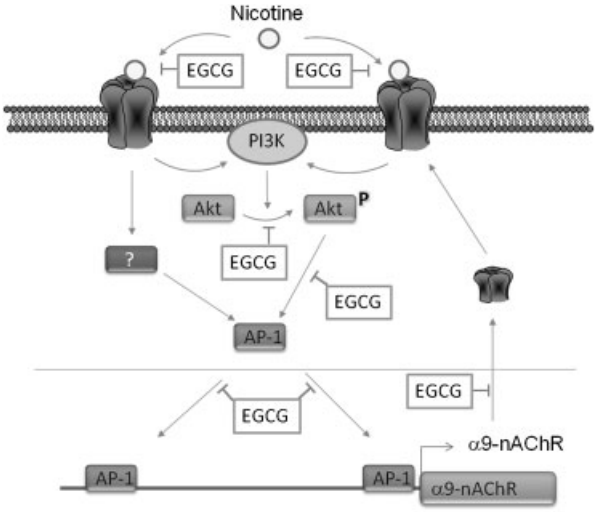

Figure 7. Schematic diagram of PI3K-mediated AP-1 transcriptional activation of the α9-nAChR gene promoter in MCF-7 cells Nic administration to human MCF-7 cells induces PI3K activation. The subsequently activated kinases (such as pAKT) induce the activation of AP-1, which then translocates to the nucleus in a concerted fashion, resulting in binding to the AP-1 sites of the α9-nAChR promoter. Expression of the α9-nAChR protein is thus induced, leading to amplification of the Nic-induced carcinogenic signals, which then lead to increased MCF-7 cell proliferation.

breast cancer patients undergoing endocrine therapy [41]. Accordingly, induction of phosphorylation of ERα at different sites by carcinogenic factors (such as hormones and smoking) may play an important role in breast cancer formation. Our study demonstrates that α9-nAchR mRNA expression is upregulated by either E2 or Nic (Fig. 3). The PI3K/Akt signals are shown to be important for α9-nAchR protein upregulation. Importantly, our study provides evidence that E2 (10 nM) significantly increases the α9-nAchR protein expression levels in MCF-7 cells.

This work was supported by the National Science Council, NSC 96-2628-B-038-003-MY3(1-3), NSC 98-2320-B-038-006-MY3(1-3), and DOH99-TD-C-111-008 to Dr. Ho and (NSC 97-2314-B-038-034-MY3(1-3)) to Dr. Wu, and by Cathay Medical Center (96CGH-TMU-02 and 97CGH-TMU-02).

The authors have declared no conflict of interest.

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