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EGCG Inhibits Transforming Growth Factor-β-Mediated Epithelial-to-Mesenchymal Transition via the Inhibition of Smad2 and Erk1/2 Signaling Pathways in Nonsmall Cell Lung Cancer Cells

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Supporting Information

ABSTRACT: Transforming growth factor-β (TGF-β)-mediated epithelial mesenchymal transition (EMT) of human lung cancer cells may contribute to lung cancer metastasis. It has been reported that EGCG can inhibit tumorigenesis and cancer cell growth in lung cancer; however, the effect of EGCG on EMT in nonsmall cell lung cancer (NSCLC) cells has not been investigated. In this study, we found that NSCLC cells A549 and H1299 were converted to the fibroblastic phenotype in response to TGF-β. Epithelial marker E-cadherin was down-regulated, and mesenchymal marker vimentin was up-regulated simultaneously. Our results illustrated that TGF-β was able to induce EMT in NSCLC cells, and EGCG would reverse TGF-β-induced morphological changes, up-regulate the expression of E-cadherin, and down-regulate the expression of vimentin. Immunofluorescent staining also demonstrated that E-cadherin was up-regulated and that vimentin was down-regulated by EGCG pretreatment. Moreover, wound-healing and the in vitro invasion assay showed that EGCG could inhibit TGF-β-induced migration and invasion of NSCLC cells. By using the dual-luciferase reporter assay, we demonstrated that EGCG inhibited TGF-β-induced EMT at the transcriptional level. EGCG decreased the phosphorylation of Smad2 and Erk1/2, inhibited the nuclear translocation of Smad2, and repressed the expression of transcription factors ZEB1, Snail, Slug, and Twist, and up-regulated the expression of E-cadherin. In summary, our results suggest that EGCG can inhibit TGF-β-induced EMT via down-regulation of phosphorylated Smad2 and Erk1/2 in NSCLC cells.

KEYWORDS: *EGCG, EMT, nonsmall cell lung cancer, Smad2, TGF-β*

INTRODUCTION

Lung cancer is the most common type of cancer present worldwide and a leading cause of mortality of more than one million people annually. The majority of patients with nonsmall cell lung cancer (NSCLC) present with advanced stage disease at diagnosis. NSCLC is the most common type of cancer present in the United States, and has a miserable 5 years and 15% survival rate.[1] Treatments such as surgery and chemotherapy provide only limited improvement in the survival of patients with NSCLC.[2] A continuing problem in the management of NSCLC is tumor metastasis, pointing out the importance of gaining a better understanding of the biological changes that occur in tumor cells to promote the aggressive neoplastic phenotype. The transition of the immobile cancer cells to a motile mesenchymal-like phenotype is a landmark of carcinoma progression during the metastatic phases.

Tumor local invasion represents the first and essential step in the metastatic cascade of carcinomas, leading to the generation of usually fatal distant metastasis. Tumor invasion requires profound changes in cell adhesion and enables tumor cells to dissociate and migrate from the primary tumor.[4] The changes in cell adhesion and migration during tumor invasion are reminiscent of an important developmental process termed epithelial−mesenchymal transition (EMT). EMT significantly contributes to various fibrotic conditions and the conversion of early stage tumors into invasive malignancies.[3,4] Hallmarks of EMT include loss of cell−cell adhesion, reorganization of the actin cytoskeleton, and acquisition of increased migratory characteristics. EMT is characterized by the up-regulation of mesenchymal markers, including fibronectin and N-cadherin, and acquisition of fibroblast-like migratory and invasive phenotypes. It is important to understand the molecular

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mechanisms that drive EMT if we are to identify new targets for the prevention of metastasis.

EMT can be initiated by external signals, such as transforming growth factor (TGF)-β, hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF).[5,6] TGF-β is a multifunctional cytokine that induces EMT during embryonic development, wound healing, fibrotic diseases, and cancer pathogenesis.[7] TGF-β suppresses the growth of epithelial cells, whereas tumor cells frequently lose the responsiveness to growth inhibitory activity of TGF-β. However, TGF-β is the major mediator of EMT and is critically involved in epithelial-mesenchymal interactions during lung morphogenesis.[8]

The molecular mechanisms underlying EMT in the lung have not been explored in detail. TGF-β is known to signal through two main pathways: the canonical Smad-dependent pathway and the alternative mitogen-activated protein kinase (MAPK) pathway.[9] During TGF-β-mediated EMT, TGF-β binds to the TGF-β type II receptor (TβRII), and this binding facilitates activation of TGF-β type I receptor (TβRI) kinase, which contains a kinase domain that phosphorylates receptor-associated Smad2 and Smad3.[10] Phosphorylated Smads partner with cytosolic Smad4 and form a heteromeric Smad complex, which in turn translocates to the nucleus and mediates gene transcription by binding to Smad binding elements (SBE) in the promoters of its target genes.[11] Recent studies revealed that several transcription factors, including Snail, Slug, δEF-1 (ZEB1), and SIP1, are involved in the induction of EMT.[12,13] These transcription factors repress expression of E-cadherin and induce EMT when overexpressed in epithelial cells.

There is increasing interest in the role of green tea polyphenols in maintaining health and treating disease. (−)-Epigallocatechin-3-gallate (EGCG) generally accounts for >40% of the total polyphenols in green tea and is the most widely studied polyphenol for disease prevention.[14] Previous studies showed that EGCG inhibited lung metastasis in mouse B16 melanoma and Lewis lung carcinoma 3LL cells.[15] The repression of EGCG on tumor invasion of lung carcinoma 95-D cells was shown to be attributed to the decrease of the expression of MMP-9 and NF-kappa B, which might result from the decrease of intracellular oxidants.[16] However, the underlying mechanisms of the inhibitory effects of EGCG on lung metastasis have not been clearly elucidated.

In the present study, we use a well-validated model of EMT in the A549 human NSCLC cell line to explore the ability of EGCG in inhibiting the TGF-β1-mediated changes associated with EMT in human lung cancer. Our results demonstrate that EGCG inhibits several TGF-β-induced changes in markers of EMT.

MATERIALS AND METHODS

Cell Lines and Cell Cultures. A549 and NCI-H1299 cells were purchased from American Type Culture Collection. A549 cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). NCI-H1299 cells were grown in DMEM supplemented with 10% FBS. Cells were grown in a humidified incubator at 37 °C under 5% CO_2 in air. Cell culture materials were obtained from Invitrogen (Burlington, Ontario, Canada).

Reagents and Antibodies. EGCG, PD98059, and SB431542 were purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant TGF-β was purchased from R&D Systems (Minneapolis, MN). Primary antibodies against phospho-Smad2, Smad2, phospho-Erk1/2, Erk1/2, phospho-JNK, JNK, phospho-p38, p38, Snail, E-cadherin, and Slug were purchased from Cell Signaling Technology (Beverly, MA). Primary antibody against vimentin was purchased from Abcam Inc. (Cambridge, MA). Primary antibodies against ZEB1 and Twist were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody against β-actin was purchased from Sigma Chemical Co. (St. Louis, MO). Secondary antibodies, HRP-conjugated Goat anti-Mouse IgG and Goat anti-Rabbit IgG, were obtained from Millipore (Billerica, MA).

Scattering Assay. Cells (2×10^4) were seeded in each well of a 24-well plate and incubated in a 37 °C incubator with 5% CO_2 overnight. Cells were pretreated with the indicated concentrations of EGCG for the appropriate time. TGF-β was added to each well with the final concentration of 2 ng/mL. Cells were then incubated at 37 °C for 24 h. Representative photographs were taken at 200× magnification using a NIKON TE2000-U Inverted Microscope.

Wound Healing Assay. Cells were plated on a 6-well plate to form a confluent monolayer in serum-containing media. The monolayer was pretreated with the indicated concentrations of EGCG for 2 h before being scratched. The monolayers were scratched by a plastic tip and washed by PBS to remove cell debris. Serum-containing DMEM/F12 (0.5%), indicated concentrations of EGCG, and 2 ng/mL TGF-β were then added to each well, and the scratched monolayer was incubated in a 37 °C incubator with 5% CO_2 for 24 h. Wound closure was measured in 10 random fields at 200× magnification using Image-Pro Express software and a NIKON TE2000-U Inverted Microscope. Data of three independent experiments were analyzed by *t*-test using GraphPad Prism 5 software. The distance between groups was considered statistically significant when $p < 0.05$.

In Vitro Invasion Assay. The matrigel invasion assay was performed in 24-well transwell culture plates. Briefly, 25 μL BD Matrigel Basement Membrane Matrix (BD Biosciences; Los Angeles, CA) was resolved at 4 °C for overnight and coated on the transwell insert membrane. The inserts were then incubated at 37 °C for 30 min to gel. After matrigel coating, 2×10^4 cells in 100 μL of DMEM/F12 medium with 0.1% serum and 20 μM EGCG were added to the top chamber. Then, 500 μL of serum-containing DMEM/F12 and 2 ng/mL TGF-β were plated in the bottom chamber. The cells were incubated in a 37 °C incubator with 5% CO_2 for 24 h. After incubation, the medium was aspirated, and noninvading cells were scrubbed by a wet cotton swab. The cells were washed by PBS and fixed by 4% paraformaldehyde at room temperature for 15 min. Fixed cells were washed three times by PBS and stained by 0.5% Toluidine Blue O (Sigma-Aldrich; St. Louis, MO) in 2% Na_2CO_3 for 10 min. Excess stain was removed by distilled water wash 3 times. The invading cells were counted in five random fields at 400× magnification. Three independent experiments were conducted, and data were analyzed by *t*-test using GraphPad Prism 5 software. The distance between groups was considered statistically significant when $p < 0.05$.

Western Blot Analysis. Cells (2×10^6) were seeded onto a 100-mm tissue culture dish containing 10% FBS DMEM/F12 and cultured for 24 h. Then cells were incubated in 10% FBS DMEM/F12 and treated with various agents as indicated in figure legends. After treatment, cells were placed on ice, washed with cold PBS, and lysed in the lysis buffer. Western Blot was done as described previously.[17]

Confocal Microscopy. After treatment, cells were fixed with methanol, blocked with 3% bovine serum albumin, first stained with anti-E-cadherin, antivimentin, or Smad2 monoclonal antibody and then FITC-conjugated antimouse IgG antibody. Nuclear staining was done with DAPI. Cells were imaged with a Leica TCS SP2 Spectral Confocal System.

RNA Extraction and Quantitative Real-Time PCR. RNA extraction was performed as previously described.[18] Quantitative real-time PCR was performed by the StepOnePlus real-time PCR system (Applied Biosystems) with the preset PCR program, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was applied as an internal control. The sequences of primers used for real-time PCR experiments are 5′-TGCTCTTCCAGGAACCTCTGTG-3′ (forward) and 5′-GGTGACCACACTGATGACTCCTG-3′ (reverse).

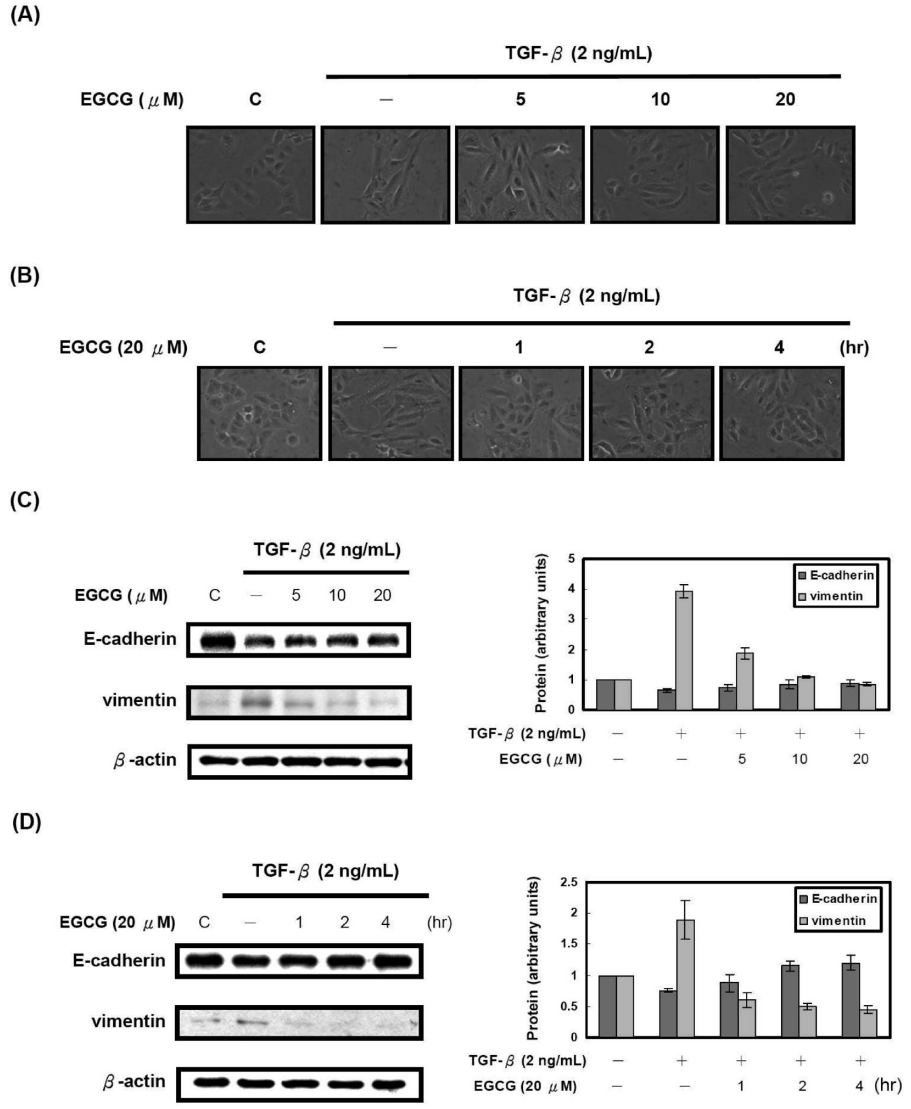

Figure 1. EGCG blocks TGF-β-induced cell scattering and EMT in A549 cells. (A) A549 cells were pretreated with DMSO (control) or increasing EGCG concentrations (5−20 μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. (B) A549 cells were incubated with DMSO (control) or 20 μM EGCG for 1 h, 2 h, or 4 h prior to TGF-β stimulation (2 ng/mL). EMT was examined by phase contrast photomicrographs. (C) A549 cells were pretreated with DMSO (control) or increasing EGCG concentrations (5−20 μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. (D) A549 cells were incubated with DMSO (control) or 20 μM EGCG for 1 h, 2 h, or 4 h prior to TGF-β stimulation (2 ng/mL). The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. Western blot data presented are representative of those obtained in at least 3 separate experiments. Immunoblots were quantified, and the right panel presents the average ± SD of three independent experiments. The value of the control cells was set to 1.

Generation of the Reporter Construct, Transient Transfection, and Dual-Luciferase Reporter Assay. The regulatory region of the *E-cadherin* gene was cloned by PCR amplification of genomic DNA and inserted into the *Hind*III/*Bgl*II sites of the pXP2 vector to generate the pXP2-*E-cadherin* (wt) construct. The pXP2-*E-cadherin* (mut-E1E2E3) constructs were generated by mutation of three E-boxes of the *E-cadherin* promoter.[44] For reporter the assay, one day before transfection, 1×10^5 A549 cells were plated onto 24-well plates and grown to 90% confluent and then cotransfected with pRL-*CMV* (containing the Renilla luciferase reporter gene) and pXP2-*E-cadherin* by using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA). After 6 h of incubation, the medium was replaced with 10% FBS. After transfection, the A549 cells were treated with EGCG at various concentrations with or without TGF-β-stimulation. For luciferase assays, cells were lysed in the lysis buffer (Promega, Madison, W), and the firefly luciferase and Renilla luciferase activities were detected with dual luciferase assay kits (Promega, Madison, WI).

Statistical Analysis. All values were expressed as the mean ± SD. Each value is the mean of at least three separate experiments in each group. The results were subjected to the one way ANOVA test using GraphPad Prism software. Asterisks indicate that the values were significantly different from those of the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS

TGF-β Induced A549 and NCI-H1299 Cells to Undergo EMT. We first determined the optimum concentrations required for TGF-β to initiate EMT in cultures of the NSCLC cells, A549 and NCI-H1299. Changes in cell morphology were assessed following treatment of A549 and NCI-H1299 cells with various concentrations (1−10 ng/mL) of TGF-β for 24 h. A549 and NCI-H1299 cells underwent morphological changes on exposure to the TGF-β (Figure S1A

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and B, Supporting Information). A549 and NCI-H1299 cells cultured in the absence of TGF-β maintained a classic cobblestone epithelial morphology and growth pattern, but after stimulation with various concentrations of TGF-β for 24 h, the cells adopted a more fibroblast-like morphology and reduced their cell−cell contact (Figure S1A and B, Supporting Information). Downregulation of E-cadherin and upregulation of vimentin are often associated with EMT.[19] The expression of the epithelial phenotype marker, E-cadherin, and of the mesenchymal phenotype marker, vimentin, was also determined following treatment of A549 and NCI-H1299 cells with various concentrations (1−10 ng/mL) of TGF-β for 24 h. TGF-β decreased E-cadherin expression in a dose-dependent manner (Figure S1C and D, Supporting Information). In parallel with the marked decrease in the E-cadherin epithelial marker, TGF-β induced expression of the mesenchymal marker vimentin in a dose-dependent manner (Figure S1C and D, Supporting Information). These results indicated that TGF-β initiated EMT in NSCLC cells.

EGCG Blocked TGF-β-Induced Scattering of NSCLC Cells. The activation of TGF-β signaling significantly caused the polarization of cell shape in NSCLC cells (Figure S1, Supporting Information). To determine whether EGCG could affect the TGF-β-induced scattering, A549 and NCI-H1299 cells were pretreated with EGCG prior to stimulation with TGF-β. Recently, Li et al. reported that EGCG inhibited A549 and NCI-H1299 cells proliferation.[20] The 50% cell growth inhibitory concentration (IC_{50}) of EGCG was ~40 μM on A549 cells and ~20 μM on NCI-H1299. To ensure that EGCG-blocked TGF-β-induced scattering was not due to cell death or inhibition of proliferation, we chose low toxicity concentrations of EGCG for further experiments. A549 cells were pretreated with EGCG for 2 h with a range of EGCG concentrations (5−20 μM) prior to stimulation with TGF-β (2 ng/mL) for 24 h. NCI-H1299 cells were pretreated with EGCG for 2 h with a range of EGCG concentrations (2.5−7.5 μM) prior to stimulation with TGF-β (2 ng/mL) for 24 h. EGCG blocked TGF-β-induced scattering in a dose-dependent fashion (Figures 1A and 2A). To address this pretreatment time-dependent effect, we pretreated A549 cells with 20 μM EGCG for varying periods of time from 1 to 4 h prior to TGF-β addition. An increase in inhibition of TGF-β-induced scattering was observed beginning at 1 h of EGCG pretreatment with a greater percentage of cells remaining adherent in colonies with 4 h of pretreatment time (Figure 1B). When NCI-H1299 cells were pretreated with 7.5 μM EGCG for varying periods of time prior to TGF-β addition, an increase in inhibition of TGF-β-induced scattering was also observed (Figure 2B). Moreover, trypan blue staining revealed that EGCG had no effect on cell viability (results not shown).

EGCG Suppressed TGF-β-Induced EMT by Downregulation of Vimentin and Upregulation of E-Cadherin. To further clarify whether abolishment of TGF-β-induced scattering in EGCG treated NSCLC cells resulted from dysregulation of EMT related proteins, we examined the expression of EMT marker proteins vimentin and E-cadherin in A549 and NCI-H1299 cells. Pretreatment with EGCG significantly suppressed the TGF-β-induced upregulation of vimentin and downregulation of E-cadherin in a dose and time-dependent manner (Figures 1C and D, and 2C and D). To further confirm that EGCG suppressed the TGF-β-induced upregulation of vimentin and downregulation of E-cadherin, A549 cells were imaged using confocal microscopy. As shown

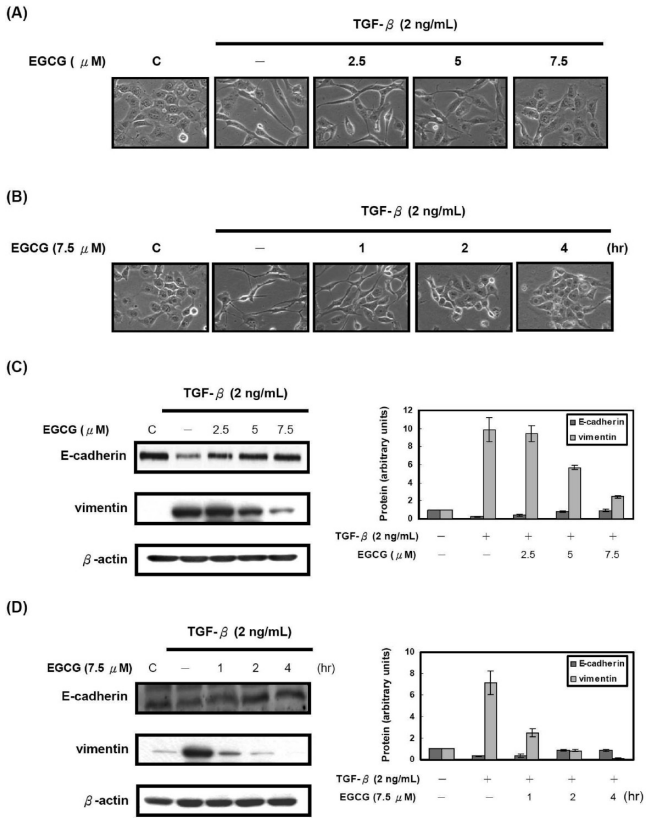

Figure 2. EGCG blocks TGF-β-induced cell scattering and EMT in NCI-H1299 cells. (A) NCI-H1299 cells were pretreated with DMSO (control) or increasing EGCG concentrations (2.5−7.5 μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. (B) NCI-H1299 cells were incubated with DMSO (control) or 7.5 μM EGCG for 1 h, 2 h, or 4 h prior to TGF-β stimulation (2 ng/mL). EMT was examined by phase contrast photomicrographs. (C) NCI-H1299 cells were pretreated with DMSO (control) or increasing EGCG concentrations 2.5−7.5) μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. (D) NCI-H1299 cells were incubated with DMSO (control) or 7.5 μM EGCG for 1 h, 2 h, or 4 h prior to TGF-β stimulation (2 ng/mL). The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. Western blot data presented are representative of those obtained in at least 3 separate experiments. Immunoblots were quantified, and the right panel presents the average ± SD of three independent experiments. The value of the control cells was set to 1.

in Figure 3, confocal microscopy using E-cadherin and vimentin antibodies indicated that TGF-β treatment resulted in upregulation of vimentin and downregulation of E-cadherin. Pretreatment with various concentrations of EGCG for 2 h significantly suppressed the TGF-β-induced upregulation of vimentin and downregulation of E-cadherin in a dose-dependent manner (Figure 3A and B).

EGCG Blocked TGF-β-Induced Cell Migration and Invasion. We further examined whether EGCG also affected TGF-β-induced cell migration. We pretreated confluent A549 cells with various concentrations of EGCG for 2 h; cells were scratched by pipet tips and washed to remove the debris, followed by the fresh medium containing 0.5% serum with various concentrations of EGCG. Cells were then incubated with 2 ng/mL of TGF-β for 24 h. TGF-β-induced cell migration was determined by measuring wound closure. The dotted lines in Figure 4A indicate the edges at the T_0 point. Comparing to cells treated with DMSO (control), TGF-β

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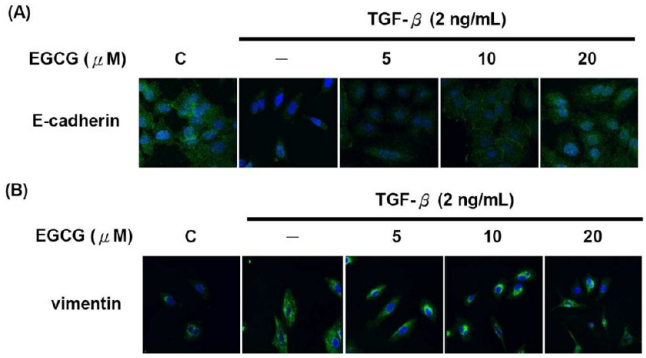

Figure 3. EGCG blocks TGF-β-induced EMT by downregulation of vimentin and upregulation of E-cadherin in A549 cells. A549 cells were pretreated with DMSO (control) or increasing EGCG concentrations (5–20 μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. Cells were fixed, permeabilized, and stained with (A) anti-E-cadherin monoclonal antibody (green) and DAPI (blue), and (B) antivimentin monoclonal antibody (green) and DAPI (blue). Cells were analyzed by confocal microscopy.

significantly induced cell migration (Figure 4A). Notably, the TGF-β-induced cell migration was abrogated in the presence of EGCG (Figure 4A). The data of cell motility, obtained from Figure 4A, were plotted by mean ± SD (Figure 4B). A modified invasion assay was performed to further determine whether EGCG was also capable of blocking TGF-β-induced invasion. Cells were cultured in 0.1% serum-containing DMEM/F12 ± 20 μM EGCG in the 24-well transwell chambers. Then, 500 mL of serum-containing DMEM/F12 ± 2 ng/mL TGF-β was plated in the lower chamber. The cells were incubated for 24 h. The number of invasive cells induced by TGF-β was 2.8-fold than that of the DMSO control or EGCG alone, and treatment of cells with 20 μM EGCG significantly decreased the number of TGF-β induced invasive cells to 1.4-fold compared to the control cells. Consistent to results obtained from Figure 4B, pretreatment of EGCG reduced A549 invasion induced by TGF-β (Figure 4C).

Effect of EGCG on TGF-β Inducible E-Cadherin Downregulation. Since pretreatment with EGCG significantly suppressed the TGF-β-induced downregulation of E-cadherin, we next determined whether the pretreatment with EGCG would suppress the TGF-β-induced downregulation of E-cadherin mRNA. Real-time PCR analysis was used to assess the effect of EGCG on E-cadherin mRNA expression. Consistent with Western blotting, TGF-β-induced downregulation of E-cadherin mRNA expression was restored by pretreatment with EGCG (Figure 5A). We further explored whether EGCG influenced E-cadherin transcription. The luciferase assay showed that TGF-β repressed the human *E-cadherin* promoter activity (Figure 5B). Pretreatment with EGCG significantly restored the TGF-β-repressed *E-cadherin* promoter activity in a dose-dependent manner.

EGCG Inhibited TGF-β-Inducible Smad2 Phosphorylation and Smad2 Nuclear Import in NSCLC Cells. TGF-β was reported to be a major secretory ligand stimulating Smads

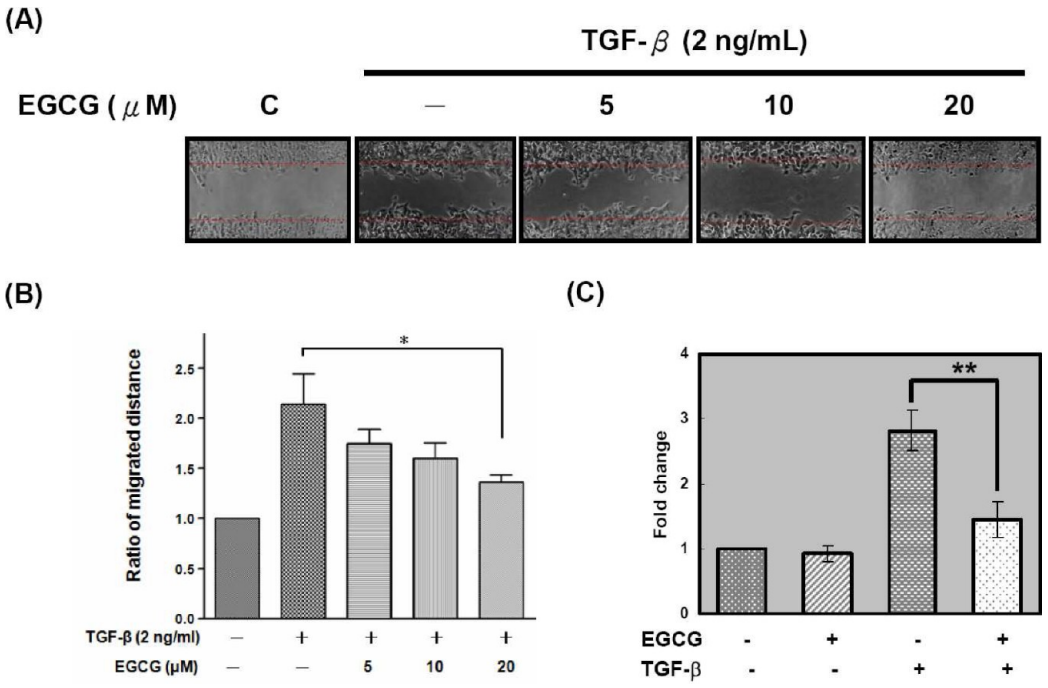

Figure 4. Effects of EGCG on TGF-β-induced cell migration and invasion. (A) For the wound healing assay, the confluent A549 monolayer was pretreated with DMSO (control) or with various concentrations of EGCG for 2 h, cells were scratched by pipet tips, and washed to remove the debris followed by fresh medium containing 0.5% serum with various concentrations of EGCG. Cells were then incubated with 2 ng/mL TGF-β for 24 h. Dotted lines indicate the cell edges at the T_0 point. Representative pictures are shown. (B) TGF-β-induced cell motility was determined by measuring the closure of the wound. Data were plotted by mean ± SD (n = 3). The closure distance of the control cells was set to 1. EGCG significantly inhibited TGF-β-induced cell motility (*, $p < 0.05$). (C) After matrigel coating, 2×10^4 A549 cells were cultured in 0.1% serum-containing DMEM/F12 ± 20 μM EGCG in the 24-well transwell chambers. Then 500 mL of serum-containing DMEM/F12 ± 2 ng/mL TGF-β was plated in the lower chamber. The cells were incubated for 24 h. Invading cells were than fixed, stained with toluidine blue, and counted in five random fields. Data were plotted by mean ± SD (n = 3). The number of control cells was set to 1. Treatment of EGCG reduced A549 cells invasion induced by TGF-β (**, $p < 0.01$).

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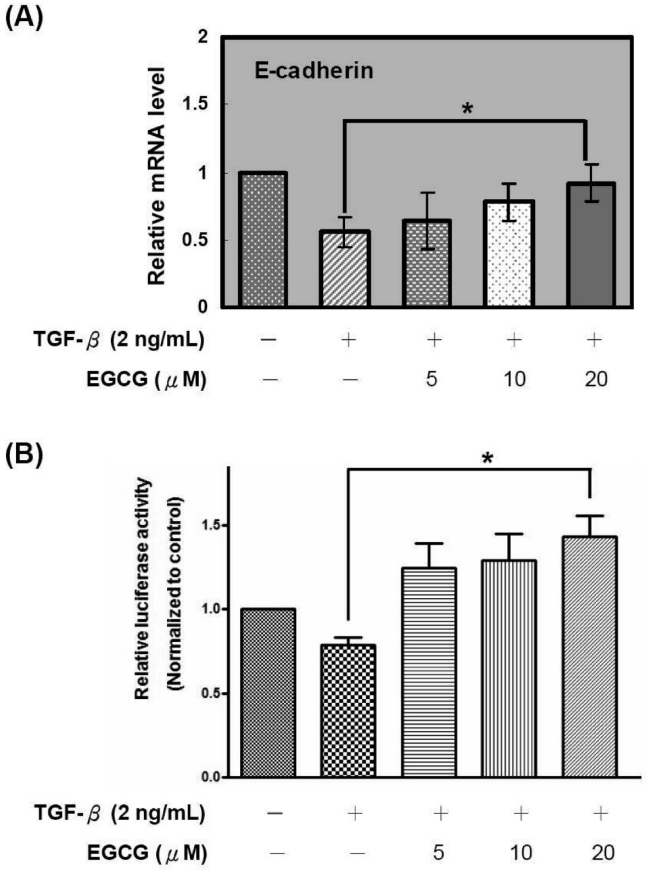

Figure 5. EGCG blocks TGF-β-induced EMT at the transcriptional level. (A) A549 cells were pretreated with DMSO (control) or increasing EGCG concentrations (5−20 μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. Quantitative real-time PCR was performed by the StepOnePlus real-time PCR system, and GAPDH was applied as an internal control. Data were plotted by mean ± SD (n = 3). The value of the control was set to 1. Asterisk, values significantly different from the TGF-β stimulation. *, $p < 0.05$. (B) After cotransfecting with pRL-*CMV* and pXP2-*E-cadherin* in A549 cells, cells were pretreated with DMSO (control) or increasing EGCG concentrations (5−20 μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. *E-cadherin* promoter activity was analyzed by luciferase reporter assays. Data were plotted by the mean ± SD (n = 3). The value of the control was set to 1. Asterisk, values significantly different from the TGF-β stimulation. *, $p < 0.05$.

activation by acting on TβRI. Since TGF-β is a growth factor for NSCLC and induces Smad2 Ser465/467-phosphorylation, we investigated whether EGCG could inhibit this activity. Interestingly, 2 ng/mL of TGF-β-induced Ser465/467-phosphorylation of Smad2 within 15 min of stimulation, and the level of Smad2 phosphorylation reached a maximum between 30−60 min after treatment and remained elevated for the duration of the experiment without affecting total Smad2 expression (Figure 6A). Pretreatment with 20 μM EGCG for 2 h significantly suppressed the TGF-β-induced Ser465/467-phosphorylation of Smad2 (Figure 6A). Under resting state, Smad2 is unphosphorylated and retained in the cytoplasm. Upon activation, Smad2 is phosphorylated and undergoes dimerization with Smad3, thus permitting its translocation into nucleus. Thus, we also confirmed the subcellular location of Smad2 in NSCLC cells under EGCG treatment. A549 cells were grown on a glass slide and pretreated with EGCG for 2 h with a range of EGCG concentrations prior to stimulation with TGF-β (2 ng/mL) for 24 h and followed by immunostaining with the Smad2 antibody. Figure 6B clearly demonstrated that after treatment with TGF-β, Smad2 signals were mainly present in the nuclei; however, these were shifted to the cytoplasm of A549 cells by EGCG treatment. These results indicated that EGCG has the ability to suppress not only the phosphorylation of Smad2 but also its presence in the nucleus. Consequently, Smad2 cannot bind to and activate the promoter of its downstream genes. In order to further confirm whether Smad2 is involved in EGCG-inhibited, TGF-β-mediated EMT, SB431542, a potent and specific inhibitor of TβRI kinases (ALK-4,-5,-7), was used to inhibit TGF-β1-induced Ser465/467-phosphorylation of Smad2 in A549 cells. Western blot analysis revealed that TGF-β1-induced Ser465/467 phosphorylation of Smad2 (Figure 6C), upregulation of vimentin, and downregulation of E-cadherin (Figure 6D) were significantly inhibited by pretreatment with 10 μM SB431542. We also tested for the effect of copretreatment with SB431542 (10 μM) and EGCG (20 μM) on phosphorylated Smad2, vimentin, and E-cadherin protein levels after TGF-β treatment. In the copresence of SB431542 (10 μM) and EGCG (20 μM), TGF-β-induced Ser465/467-phosphorylation of Smad2 (Figure 6C), upregulation of vimentin, and downregulation of E-cadherin (Figure 6D) were more potent than pretreatment with SB431542 or EGCG alone. To confirm whether Smad2 is involved in EGCG inhibited TGF-β-mediated cell migration and invasion, cell migration was determined by measuring wound closure, and a modified invasion assay was performed. Cell migration and invasion analysis revealed that TGF-β1-induced cell migration and invasion were significantly inhibited by pretreatment with 10 μM SB431542. In the copresence of SB431542 (10 μM) and EGCG (20 μM), TGF-β-induced cell migration and invasion were more potent than pretreatment with SB431542 or EGCG alone (Figure 6E and F).

EGCG Inhibits TGF-β-Inducible Erk1/2 Phosphorylation in NSCLC Cells. The phosphorylation of Erk1/2 was examined at various time points after adding TGF-β (2 ng/mL) to A549 cells. The phosphorylation of Erk1/2 was increased slightly at 15 min after stimulation, and this effect lasted for at least 12 h without alteration of total Erk1/2 protein (Figure 7A). Pretreatment with 20 μM EGCG for 2 h significantly suppressed the TGF-β-induced phosphorylation of Erk1/2 (Figure 7B). To investigate whether Erk1/2 is involved in EGCG inhibited TGF-β1-mediated EMT, the MEK inhibitor PD98059 was used to inhibit TGF-β-induced phosphorylation of Erk1/2 in A549 cells. Western blot analysis revealed that TGF-β-induced phosphorylation of Erk1/2 (Figure 7B), upregulation of vimentin, and downregulation of E-cadherin (Figure 7C) were significantly inhibited by pretreatment with 10 μM PD98059. We also tested for the effect of copretreatment with PD98059 (10 μM) and EGCG (20 μM) on phosphorylated Erk1/2, vimentin, and E-cadherin protein levels after TGF-β treatment. In the copresence of PD98059 (10 μM) and EGCG (20 μM), TGF-β-induced phosphorylation of Erk1/2, upregulation of vimentin, and downregulation of E-cadherin (Figure 7C) were more potent than pretreatment with PD98059 or EGCG alone (Figure 7B). To further confirm whether Erk1/2 is involved in EGCG inhibited TGF-β-mediated cell migration and invasion, cell migration was determined by measuring wound closure, and a modified invasion assay was performed. Cell migration and invasion analysis revealed that TGF-β1-induced cell migration and invasion were significantly inhibited by pretreatment with 10

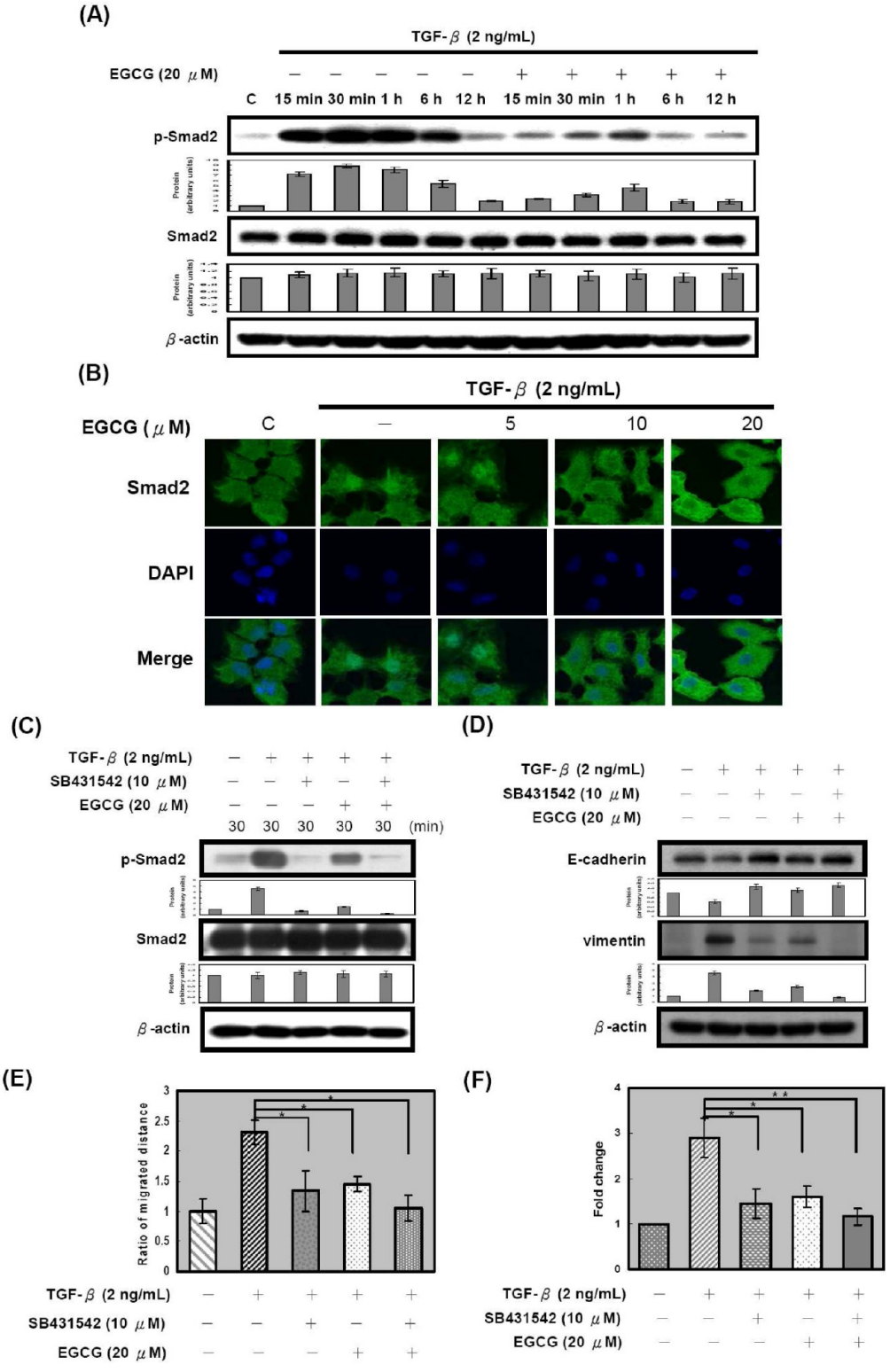

Figure 6. EGCG blocks the TGF-β-induced Smad2 pathway in a time-dependent manner. (A) A549 cells were preincubated in the presence or the absence of 20 μM of EGCG for 2 h prior to TGF-β1 stimulation (2 ng/mL) for various times. The cells were then harvested and lysed for the detection of p-Smad2, Smad2, and β-actin. (B) A549 cells were pretreated with DMSO (control) or increasing EGCG concentrations (5−20 μM) for 2 h prior to TGF-β stimulation (2 ng/mL) for 24 h. Cells were fixed, permeabilized, and stained with anti-Smad2 monoclonal antibody (green) and DAPI (blue). Cells were analyzed by confocal microscopy. (C) A549 cells were pretreated with 20 μM EGCG or 10 μM SB431542, or a combination of the two for 2 h and then treated with TGF-β for 30 min. The cells were then harvested and lysed for the detection of p-Smad2, Smad2, and β-actin. (D) A549 cells were pretreated with 20 μM EGCG or 10 μM SB431542, or a combination of the two for 2 h and then treated with TGF-β for 24 h. The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. Western blot data presented are representative of those obtained in at least 3 separate experiments. Immunoblots were quantified, and the lower panel presents the average ± SD of three independent experiments. The value of the control cells was set to 1. (E) Cell motility was determined by measuring the closure of the wound.

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Figure 6. continued

Data were plotted by the mean ± SD (n = 3). EGCG or SB431542, or a combination of the two significantly inhibited TGF-β-induced cell motility (*, $p < 0.05$). (F) After matrigel coating, 2×10^4 A549 cells were cultured in 0.1% serum-containing 20 μM EGCG or 10 μM SB431542, or a combination of the two in the 24-well transwell chambers. Then, 500 mL of serum-containing 2 ng/mL TGF-β was plated in the lower chamber. The cells were incubated for 24 h. Invading cells were than fixed, stained with toluidine blue, and counted in five random fields. Data were plotted by mean ± SD (n = 3). The number of the control cells was set to 1. EGCG or SB431542, or a combination of the two significantly inhibited TGF-β-induced cell migration (*, $p < 0.05$; **, $p < 0.01$).

μM PD98059. In the copresence of PD98059 (10 μM) and EGCG (20 μM), TGF-β-induced cell migration and invasion were more potent than pretreatment with PD98059 or EGCG alone (Figure 7D and E). Our data suggest that the inhibition of Smad2 and Erk1/2 signaling pathways is involved in EGCG-inhibited, TGF-β-mediated EMT, cell migration, and invasion in A549 cells.

EGCG Inhibits TGF-β-Mediated Transcription Factors That Are Involved in the Induction of EMT via the Inhibition of Smad2 and Erk1/2 Signaling Pathways. Recent studies revealed that several transcription factors, including Snail, Slug, ZEB1, and Twist1, are involved in the induction of EMT.[11,12] These transcription factors repress the expression of E-cadherin and induce EMT in response to TGF-β. In the present study, Western blot analysis revealed that TGF-β-induced expression of Snail, Slug, ZEB1, and Twist1 were significantly inhibited by pretreatment with EGCG (20 μM) or SB431542 (10 μM) (Figure 8A and B). To investigate whether Smad2 or Erk1/2 was involved in EGCG-inhibited TGF-β-mediated transcription factors, SB431542 and PD98059 were used to inhibit TGF-β-mediated transcription factors in A549 cells. The copresence of SB431542 (10 μM) and EGCG (20 μM) more potently inhibited TGF-β-mediated transcription factors than pretreatment with SB431542 or EGCG alone (Figure 8A). Similarly, the copresence of PD98059 (10 μM) and EGCG (20 μM) more potently inhibited TGF-β-mediated transcription factors than pretreatment with PD98059 or EGCG alone (Figure 8A and B). Interestingly, all of these factors bind to the same elements in the *E-cadherin* gene: three E-boxes with a core 5′-CACCTG-3′ sequence placed in the proximal promoter. We further investigated whether EGCG inhibited the binding of these factors to these E-boxes and repressed the *E-cadherin* promoter. Promoter activity was analyzed by transfection of the *E-cadherin* promoter-containing three E-boxes constructed into A549 cells. A promoter-activity assay showed that TGF-β repressed the human *E-cadherin* promoter activity. Pretreatment with EGCG (20 μM) or SB431542 (10 μM) restored the TGF-β-repressed *E-cadherin* promoter activity. The copresence of SB431542 (10 μM) and EGCG (20 μM) significantly restored the TGF-β-repressed *E-cadherin* promoter activity more potently than pretreatment with SB431542 or EGCG alone (Figure 8C). Similarly, pretreatment with EGCG (20 μM) or PD98059 (10 μM) restored the TGF-β-repressed *E-cadherin* promoter activity. The copresence of PD98059 (10 μM) and EGCG (20 μM) significantly restored the TGF-β-repressed *E-cadherin* promoter activity more potently than pretreatment with PD98059 or EGCG alone (Figure 8D). Mutation of all three E-boxes abrogated repression by TGF-β. Pretreatment with EGCG (20 μM), SB431542 (10 μM), or the copresence of SB431542 (10 μM) and EGCG (20 μM) cannot restore the E-box-mutated *E-cadherin* promoter activity (Figure 8E). Moreover, pretreatment with EGCG (20 μM), PD98059 (10 μM), or the copresence of PD98059 (10 μM) and EGCG (20 μM) also cannot restore the E-box-mutated *E-cadherin* promoter activity (Figure 8F). Nevertheless, our data suggest that the inhibition of Snail, Slug, ZEB1, and Twist1 expression is involved in EGCG-inhibited TGF-β-mediated EMT in A549 cells.

DISCUSSION

Herein, in agreement with previous reports,[21,22] we showed that TGF-β induced EMT in A549 cells, suggesting that A549 cells are highly sensitive to inducers of EMT. TGF-β treatment caused A549 cells to lose their polygonal appearance and cell−cell contacts leading to the acquisition of elongated, spindle-shaped morphology consistent with fibroblasts (Figure S1, Supporting Information). TGF-β also altered the expression of cell adhesion molecules consistent with EMT as reported previously.[23] Significant reductions in E-cadherin, a protein expressed only by epithelial cells and diminished during EMT, were evident even in the absence of obvious morphological changes, suggesting that a loss of E-cadherin is required before the alteration of cell morphology (Figure S1, Supporting Information). We also showed a similar phenomenon using NCI-H1299 cells. NCI-H1299 cells underwent EMT in response to TGF-β, indicating that extracellular stimuli play key roles in lung cancer progression (Figure S1, Supporting Information). In addition, our data also showed that TGF-β was a strong inducer of tumor cell metastasis. Overexpression of TGF-β has been found in advanced stages of mouse and human carcinomas.[30] Taken together, these data indicate that TGF-β can start EMT in NSCLC cells and promote tumor cells metastasis.

EGCG, the major catechins in green tea, inhibits cell proliferation and induces apoptosis in various types of cancer cells, by blocking the activation of growth factor signaling pathways.[24] EGCG inhibits the activation of the epidermal growth factor receptor (EGFR) family, insulin-like growth factor-1 receptor (IGF-1R), and vascular endothelial growth factor receptor 2 (VEGFR2), the other members of the RTK family, and this effect is also associated with its anticancer and chemopreventive properties.[25−29] The present study shows that TGF-β induces EMT in NSCLC cells and that this process can be effectively blocked by EGCG. Pretreatment with EGCG blocks TGF-β-induced scattering and causes changes in cell morphology (reduction in stress fibers and cell flattening). How EGCG affects these changes remains to be determined. Actin stress fibers are important for cell motility, and disruption of actin stress fibers caused by EGCG treatment may account for the compound's ability to block TGF-β induced scattering and motility, although this remains to be tested.

TGF-β signaling is conducted through heteromeric receptor complexes composed of TβRI and TβRII. TβRI and TβRII form tight complexes and lead to phosphorylation of Smad, thereby activating many downstream signaling pathways.[31] Smad2 phosphorylation has been noted in EMT processes for several cell types including breast and renal epithelial cells.[32,33]

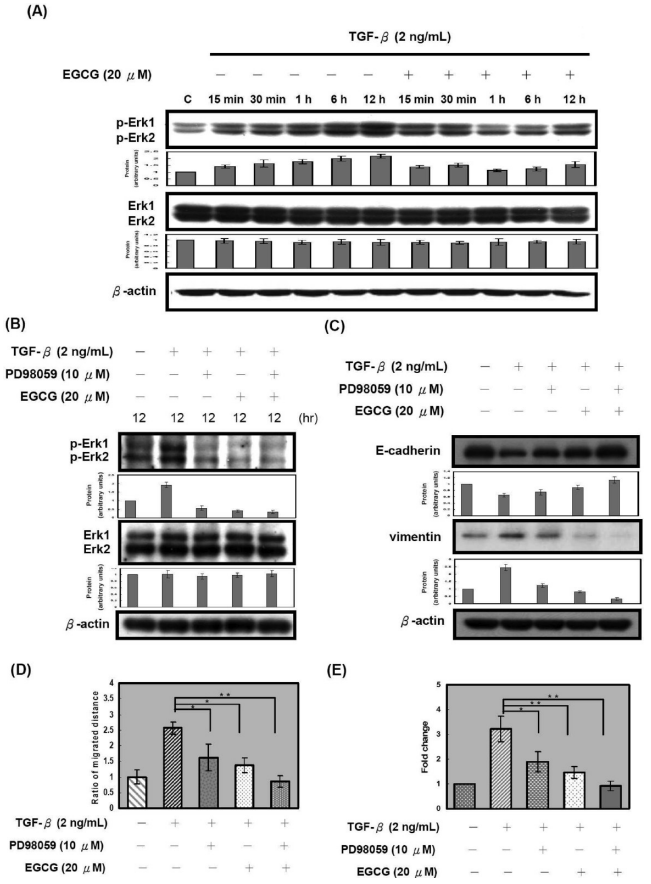

Figure 7. EGCG blocks TGF-β-induced Erk1/2 pathway in a time-dependent manner. (A) A549 cells were preincubated in the presence or the absence of 20 μM of EGCG for 2 h prior to TGF-β1 stimulation for various times. The cells were then harvested and lysed for the detection of p-Erk1/2, Erk1/2, and β-actin. (B) A549 cells were pretreated with 20 μM EGCG or 10 μM PD98059, or a combination of the two for 2 h and then treated with TGF-β for 12 h. The cells were then harvested and lysed for the detection of p-Erk1/2, Erk1/2, and β-actin. (C) A549 cells were pretreated with 20 μM EGCG or 10 μM PD98059, or a combination of the two for 2 h and then treated with TGF-β for 24 h. The cells were then harvested and lysed for the detection of E-cadherin, vimentin, and β-actin. Western blot data presented are representative of those obtained in at least 3 separate experiments. Immunoblots were quantified, and the lower panel presents the average ± SD of three independent experiments. The value of the control cells was set to 1. (D) Cell motility was determined by measuring the closure of the wound. Data were plotted by mean ± SD (n = 3). EGCG or PD98059, or a combination of the two significantly inhibited TGF-β-induced cell motility (*, $p < 0.05$; **, $p < 0.01$). (E) After matrigel coating, 2×10^4 A549 cells were cultured in 0.1% serum-containing 20 μM EGCG or 10 μM PD98059, or a combination of the two in the 24-well transwell chambers. Then, 500 mL serum-containing 2 ng/mL TGF-β was plated in the lower chamber. The cells were incubated for 24 h. Invading cells were then fixed, stained with toluidine blue, and counted in five random fields. Data were plotted by mean ± SD (n = 3). The number of the control cells was set to 1. EGCG or PD98059, or a combination of the two significantly inhibited TGF-β-induced cell migration (*, $p < 0.05$; **, $p < 0.01$).

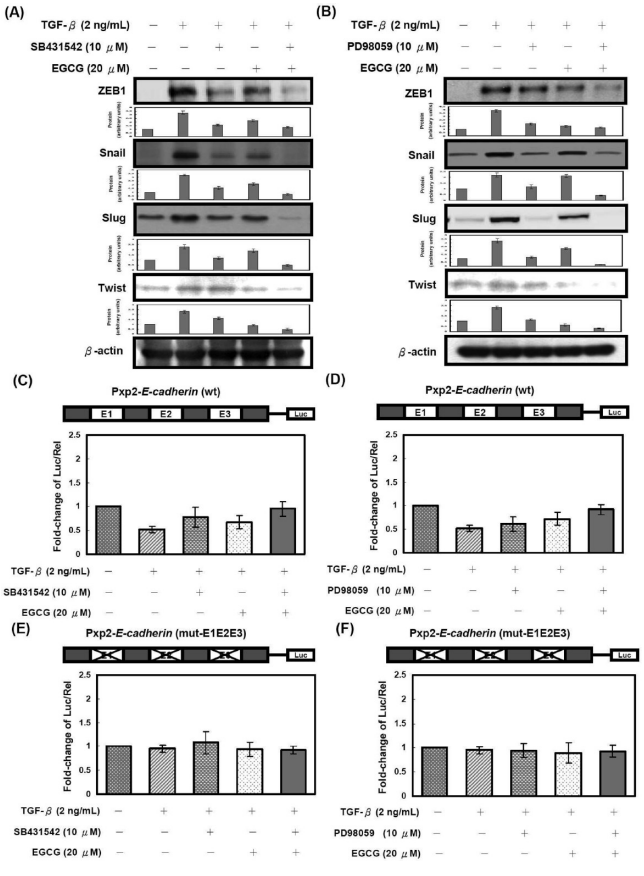

Figure 8. EGCG inhibits TGF-β-mediated transcription factors that are involved in the induction of EMT. (A) A549 cells were pretreated with 20 μM EGCG or 10 μM SB431542, or a combination of the two for 2 h and then treated with TGF-β for 24 h. (B) A549 cells were pretreated with 20 μM EGCG or 10 μM PD98059, or a combination of the two for 2 h and then treated with TGF-β for 24 h. The cells were then harvested and lysed for the detection of ZEB1, Snail, Slug, Twist, and β-actin. Western blot data presented are representative of those obtained in at least 3 separate experiments. Immunoblots were quantified, and the lower panel presents the average ± SD of three independent experiments. The value of the control cells was set to 1. (C) After cotransfecting with pRL-*CMV* and pXP2-*E-cadherin* in A549 cells, cells were pretreated with 20 μM EGCG or 10 μM SB431542, or a combination of the two for 2 h and then treated with TGF-β for 24 h. (D) After cotransfecting with pRL-*CMV* and pXP2-*E-cadherin* in A549 cells, cells were pretreated with 20 μM EGCG or 10 μM PD98059, or a combination of the two for 2 h and then treated with TGF-β for 24 h. Pxp2-*E-cadherin* promoter activity was analyzed by luciferase reporter assays. Data were plotted by mean ± SD (n = 3). The value of the control was set to 1. Asterisk, values significantly different from the TGF-β1 stimulation. *, $p < 0.05$. (E) After cotransfecting with pRL-*CMV* and Pxp2-*E-cadherin* (mutE1E2E3) in A549 cells, cells were pretreated with 20 μM EGCG or 10 μM SB431542, or a combination of for 2 h and then treated with TGF-β for 24 h. (F) After cotransfecting with pRL-*CMV* and Pxp2-*E-cadherin* (mutE1E2E3) in A549 cells, cells were pretreated with 20 μM EGCG or 10 μM PD98059, or a combination of the two for 2 h and then treated with TGF-β for 24 h. Pxp2-*E-cadherin* (mutE1E2E3) promoter activity was analyzed by luciferase reporter assays. Data were plotted by mean ± SD (n = 3). The value of the control was set to 1.

Here, our results show that TGF-β-induced Ser465/467-phosphorylation of Smad2, upregulation of vimentin, and downregulation of E-cadherin were significantly inhibited by pretreatment with EGCG. These data suggest that EGCG inhibited TGF-β-inducible Smad2 phosphorylation and Smad2 nuclear import in NSCLC cells.

TGF-β signaling is not limited to the Smad signaling pathway, but they can mediate through non-Smad signaling

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pathways, a number of which have an impact on EMT.[34,35] Recently, various research results support the activation of Erk1/2 signaling in response to TGF-β. The activation of Erk1/2 signaling enhances TGF-β-induced EMT, accompanied by the morphological changes and loss of E-cadherin expression.[36,37] Blocking the function of MEK1/2 using a special inhibitor resulted in inactivation of the Erk1/2 and inhibited TGF-β-induced EMT.[38] Our data also showed that the Erk1/2 was activated with TGF-β1 treatment, while EGCG and the MEK inhibitor, PD98059, blocked this response, which also prevented the upregulation of vimentin and the downregulation of E-cadherin. These results indicate that the MAPK/Erk1/2 signaling pathway is also a critical mediator for TGF-β induction of EMT.

The main hallmark of EMT is the down-regulation of the adherens junction protein E-cadherin due to transcriptional repression. An EMT can be induced by several transcription factors; specifically, Snail, Slug, Twist1, Zeb1, SIP1, and E12/E47 can promote this transition.[39] The expression of some of these transcription factors has been found to be induced during tumor progression. Overexpression of Snail in epithelial cells causes a complete EMT and down-regulates E-cadherin through its binding to the E-cadherin promoter.[40] Moreover, up-regulation of Snail RNA is observed in many cellular systems when EMT is induced.[13] Besides Snail, other cellular factors such as the Slug,[41] the basic helix−loop−helix protein E12/E47,[42] or two members of the Zeb family, Zeb1/EF-1 and Zeb2/Sip1,[43] are capable of repressing E-cadherin (*CDH1*) promoter activity and RNA levels. All of these factors bind to the same elements in the *CDH1* gene: three E-boxes with a core 5′-CACCTG-3′ sequence placed in the proximal promoter. In this study, we further verified that corresponding variations in gene expression were obtained after treatment with TGF-β in A549 cells. TGF-β acts via the Smad proteins or through ERK signaling pathways, to induce the expression of multiple E-box repressors including Snail, Slug, ZEB1, and Twist1. Pretreatment with EGCG significantly inhibited TGF-β-induced expression of Snail, Slug, ZEB1, and Twist1. Our observations draw attention to EMT-related proteins as a therapeutic target. Significantly, we identified that EGCG can inhibit TGF-β-induced EMT via the down-regulation of phosphorylated Smad2 and Erk1/2.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A549 and NCI-H1299 cells undergoing EMT in response to TGF-β. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EGCG, (−)-epigallocatechin-3-gallate; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF-1R, insulin-like growth factor-1 receptor; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSCLC, nonsmall cell lung cancer; TGF-β, transforming growth factor-β; TβRI, TGF-β type I receptor; VEGFR2, vascular endothelial growth factor receptor 2

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