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TGF-β1-induced epithelial–mesenchymal transition and acetylation of Smad2 and Smad3 are negatively regulated by EGCG in Human A549 lung cancer cells

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ABSTRACT

Transforming growth factor-β1, the key ligand of Smad-dependent signaling pathway, is critical for epithelial–mesenchymal transition during embryo-morphogenesis, fibrotic diseases, and tumor metastasis. In this study, we found that activation of p300/CBP, a histone acetyltransferase, by TGF-β1 mediates Epithelial–mesenchymal transition (EMT) via acetylating Smad2 and Smad3 in TGF-β1 signaling pathway. We demonstrated that treatment with EGCG inhibited p300/CBP activity in human lung cancer cells. Also, we observed that EGCG potently inhibited TGF-β1-induced EMT and reversed the up-regulation of various genes during EMT. Our findings suggest that EGCG inhibits the induction of p300/CBP activity by TGF-β1. Therefore, EGCG inhibits TGF-β1-mediated EMT by suppressing the acetylation of Smad2 and Smad3 in human lung cancer cells.

1. Introduction

Epithelial–mesenchymal transition (EMT) is a morphological change of polarized epithelial cells from an epithelial phenotype to a mesenchymal fibroblastoid phenotype in a process characterized by dissolution of cell–cell junctions, cytoskeletal rearrangements, increased cell motility, and synthesis of extracellular matrix. These are accompanied by the acquisition of mesenchymal properties, such as cell migratory and invasive behaviors. According to these properties, many EMT biomarkers have been identified, some of which have been used to detect EMT in clinical samples [1,2]. EMT occurs during tissue fibrosis, embryonic development, organogenesis, wound repair, certain diseases, and often during cancer invasion and metastasis.

TGF-β1-activated cells become spindle-shaped and undergo morphological changes, such as a decrease in cell–cell adhesion and loss of structural polarity. EMT is defined by the loss of cell–cell adhesion molecules, such as E-cadherin, and epithelial differentiation markers, such as cytokeratins, and up-regulation of mesenchymal markers, such as N-cadherin, vimentin, fibronectin, and α-smooth muscle actin [3–5]. The repression of E-cadherin expression is an important and essential event in TGF-β1-mediated EMT. In this process, TGF-β1 acts directly by reducing E-cadherin expression [6]. However, it is still unclear how the cells acquire the capacity to migrate.

TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) regulate the expression of genes that play roles in a large variety of biological phenomena, ranging from tissue remodeling to tumor initiation and progression [7,8]. These effects include regulation of proliferation and apoptosis, and stimulation of EMT. In the lung, the precise role of TGF-β1-mediated EMT during the response to injury and pathogenesis of fibrosis in the lung remains to be identified. However, there is increasing evidence that it may play a substantial role in a variety of pathogenic processes, and in fact may be a major source of pathogenic mesenchymal cell types, such as myofibroblasts, during fibrogenesis. In the liver, TGF-β1 is released to act as a critical mediator of responses to injury. TGF-β1 controls the proliferation of hepatocytes, induces hepatocyte apoptosis, and activates EMT [9–11]. The Smad-group of proteins has been shown to be specifically activated in response to TGF-β1 superfamily members [12]. The activation of serine/threonine kinase TGF-β1 receptors by TGF-β1 binding induces phosphorylation of Smad2 and Smad3 (Receptor-Regulated Smads), and these form heteromeric complexes with Smad4 (Common-mediator Smad).

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Then, the Smad complexes translocate to the nucleus, where they regulate transcription by directly binding to promoters of target genes, associating with various DNA-binding proteins, and recruiting transcriptional coactivators or corepressors, such as p300/CBP (histone acetyltransferase; coactivators) and HDACs (histone deacetylases; corepressors). Recent studies have revealed that the transcriptional coactivator p300/CBP may also play a role in TGF-β1/Smad signaling [13,14]. However, the role of p300/CBP in TGF-β1/Smad pathway-mediated transcriptional activation of EMT target genes is still unknown in human lung cancer cells.

The activation of histones (Histone 3 and Histone 4) and non-histone proteins (Smads, p53, p65, etc.) is tightly regulated at the post-translational level by phosphorylation, acetylation, sumoylation, and ubiquitylation. The acetylation of non-histone proteins is mediated by the histone acetyltransferases (HATs; p300/CBP, PCAF, and Tip60). Conversely, the HDACs or NAD-dependent HDAC SIRTs negatively regulate the acetylation of non-histone proteins. Protein acetylation influences a broad set of cellular signaling pathways, including diverse aspects of transcriptional regulation, through the recruitment of HDACs and HATs [15]. Thus, dysregulation of protein acetylation and deacetylation can lead to pathological conditions and diseases, including inflammation and cancer [16,17]. According to previous reports, among the small molecules that are capable of modulating epigenetic status, HDAC inhibitors have been extensively studied and several are currently in clinical trials. On the other hand, there is little information available on HAT inhibitors [18]. As suggested in recent reports, the development of a HAT inhibitor from natural compounds is a key step in the development of a novel therapeutic agent. Several natural dietary factors, such as garcinol, curcumin, anacardic acid, and epigallocatechin-3-gallate (EGCG), have the ability to inhibit HAT activity. Garcinol has been found to inhibit p300 and PCAF *in vitro* and *in vivo*; anacardic acid inhibits TIP60 as well as p300 and PCAF; curcumin inhibits p300 and PCAF; and EGCG inhibits p300 and CBP. These natural compounds are associated with the prevention of cancer and other diseases [19–22].

Recently, green tea has been found to contain several polyphenolic components known as catechins, including EGCG, epigallocatechin, epicatechin gallate, and epicatechin. EGCG, the major polyphenol found in green tea, has been shown to impart anti-proliferative and chemopreventive effects against several forms of cancer, including colon cancer, lung cancer, and breast cancer, and to inhibit the production of nitric oxide synthase by blocking the NF-κB signaling pathway in chronic inflammation. In addition, several recent studies have shown that polyphenols from green tea significantly improved the quality of wound healing and scar formation in a rat model, remarkably suppressed both collagen production and collagenase activity in hepatic stellate cells, and dramatically attenuated experimental cholestasis-induced liver fibrosis [23,24].

In this study, our aims was to investigate whether the inhibition of TGF-β1-induced EMT and hyper acetylation of Smad2 and Smad3 by EGCG directly blocked the activity of p300/CBP protein and to represent a new molecular mechanism for novel anti-EMT of EGCG and chemopreventive drugs in human lung cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

Human lung cancer A549 cells were obtained from the American Type Culture Collection and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotics–antimycotics in a humidified 5% CO_2 atmosphere at 37 °C. A549 cells were stimulated with TGF-β1 (PEPPROTECH) and were treated with Epigallocatechin-3-gallate (EGCG) (Sigma–Aldrich, St. Louis, MD, USA). DMEM, antibiotics–antimycotics and FBS were purchased from Hyclone, USA. The Lipofectamine 2000 transfection reagent was purchased from Invitrogen, USA.

2.2. HAT activity assays

Histone acetyltransferase (HAT) activity assay was carried out according to the manufacturer's instruction by using an available commercial kit (Biovision Biotechnology, USA). For HAT activity assays, p300 and CBP proteins were immunoprecipitated by using anti-p300 and anti-CBP with A549 nuclear extracts. Immunoprecipitated complexes were collected and washed with HAT assay buffer (50 mmol/l Tris pH 8.0, 10% glycerol, 0.1 mmol/l EDTA).

2.3. Immunoprecipitation and western blot analysis

For immunoprecipitation and western blot, after treatment with TGF-β1 and/or EGCG for 48 h, A549 cells were washed with cold PBS, scraped off, and harvested. Cell extracts were prepared with lysis buffer (50 mmol/l Tris–Cl (pH 7.5), 150 mmol/l NaCl, 1% NP40, 10 mmol/l NaF, 10 mmol/l sodium pyrophosphate, and protease inhibitors), incubated for 30 min on ice. The lysates were centrifuged at 20,000*g* for 10 min at 4 °C. Total cell lysate protein incubated with the anti-Smad2 or anti-Smad3 (Cell signaling, USA), and with 20 μl of protein A/G agarose overnight at 4 °C. After washing three times with agarose beads washing buffer, Immunoprecipitated protein-antibody complexes were separated on 8% SDS–PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked by incubating for 2 h in 5% w/v non-fat Difco™ skim milk blocking buffer with 1X PBST. The blocked membranes were incubated overnight at 4 °C with acetyl-lysine antibody. After washing with 1X PBST, the membranes were incubated with secondary HRP-conjugated antibody for 1 h. The results were subjected to western blot analysis and visualized by developer.

2.4. RNA extraction and reverse transcription-PCR quantitative

Total RNA was isolated with the RNA Easyspin kit according to the instructions of the manufacturer (Intron, Korea). Total RNA from each sample was reverse transcribed with random primers using a StrataScript™ reverse transcriptase kit (Stratagene, USA) according to the protocols of the manufacturer. All samples were normalized to human GAPDH and expressed as fold induction. All reactions were done in triplicate. Relative expression levels and SDs were calculated using the comparative method. Primers for amplification of ITGA5 transcript were 5′-GACTGGCCATGATGAGTTTG-3′ and 5′-GGATATCCATTGCCATCCAG-3′. Primers for MMP2 amplification were 5′-ACATCAAGGGCATTCAGGAG-3′ and 5′-GCCTCGTATACCGCATCAAT-3′. Primers for SERPINE1 (PAI1) amplification were 5′-CTCTCTCTGCCCTCACCAAC-3′ and 5′-GTGGAGAGGCTCTTGGTCTG-3′. Primers for SNAI1 (Snail) amplification were 5′-GCGAGCTGCAGGACTCTAAT-3′ and 5′-CCCACTGTCCTCATCTGACA-3′. Primers for SNAI2 (Slug) amplification were 5′-GAGCATACAGCCCCATCACT-3′ and 5′-GGGTCTGAAAGCTTGGACTG-3′. Primers for SPARC amplification were 5′-ATGATGGTGCAGAGGAAACC-3′ and 5′-AAGTGGCAGGAAGAGTCGAA-3′. Primers for Vimentin amplification were 5′-CCCTCACCTGTGAAGTGGAT-3′ and 5′-TCCAGCAGCTTCCTGTAGGT-3′. Primers for GAPDH amplification were 5′-CGCGGGGCTCTCCAGAACATCATCC-3′ and 5′-CTCCGACGCCTGCTTCACCACCTTCTT-3′.

2.5. Report assays

To measure Smad transcriptional activity, A549 cells were transiently cotransfected with reporter construct pSBE-Luc and pSV40 plasmids. The Renilla luciferase reporter plasmid was included as an internal control. Cells were harvested and Total cell extracts were prepared and dual luciferase activity was measured, according to the manufacturer's instruction (Promega, USA). All reporter activities were normalized relative to *Renilla* luciferase activities and are presented as the means (±SD) of three independent experiments.

2.6. Matrigel invasion assays and wound healing assays

For Matrigel invasion assays, *in vitro* cell invasiveness was determined by the ability of cells to transmigrate through a layer of extracellular matrix in Biocoat Matrigel invasion chambers (SPL Lifescience, Korea). A549 cells were seeded at a density of 2.0×10^4 per insert and cultured for 12 h. The cells were placed in wells containing the same medium plus TGF-β1 (5 ng/ml) with or without EGCG. After 48 h, non-invading cells were removed with cotton swabs. Invading cells were fixed with 100% methanol and stained with 1% crystal violet (Sigma–Aldrich) before enumeration under an inverted microscope (40×, three random fields per well). Data are expressed as the mean ± SD of at least three independent experiments. For Wound Healing Assays, A549 cells were transfected with pEGFP-C1 expression plasmid using the Lipofectamin 2000 transfection reagent. A549 cells were treated with TGF-β1 and/or EGCG. When cell confluence had reached about 90% for 48 h, the wounds of monolayer were scratched in confluent cells using a 200 μl pipette tip and washed with media twice prior to incubation to remove any free-floating cells and debris. Culture media was then added, and the culture plates were incubated at 37 °C. Wound healing was observed within the scrape line at different time points, and representative scrape lines for each cell line were photographed. Duplicate wells for each condition were examined for each experiment, and each experiment was repeated three times.

2.7. Immunofluorescence analysis

A549 cells were plated at a density of 2×10^5 cells per 22-mm glass coverslip for 24 h before TGF-β1 and EGCG treatment. After incubation with TGF-β1 for 24 h, cells were treated with EGCG (0, 50, 100 μM) for 24 h, and fixed for 30 min in cold 4% (weight/volume) paraformaldehyde, permeabilized for 2 min at 25 °C in 0.25% (volume/volume) Triton X-100. Expressed p300 and CBP were detected by incubation at 4 °C with anti-p300, anti-CBP rabbit polyclonal antibody (Santa Cruz Biotechnology), and the decrease of E-cadherin and increase of vimentin were detected by anti-E-cadherin (Santa Cruz Biotechnology) and anti-Vimentin rabbit polyclonal antibody (Cell signaling). After three washes in PBS, coverslips were incubated for 1 h at 37 °C with the secondary antibody AlexaFluor 555 goat anti-rabbit (Invitrogen). Coverslips were then mounted in medium containing 4′, 6-diamino-2-phenylindole (Vector Laboratories). Cells were examined with a DeltaVision RT Imaging Microscope System (Applied Precision).

2.8. Cell viability assay

Cells viability was measured to determine the cytotoxicity of EGCG on A549 cells. Cell viability was determined with the conventional MTT reduction assay. Briefly, A549 cells were seeded at 5×10^3–1×10^4 cells in a 96-well plate. After 12 h of incubation, cells were pre-incubated for 24 h with or without TGF-β1, and then cells were incubated with EGCG (0, 50, 100 μM) for another 24 h. Cells were then treated with 15 ml MTT solution (2 mg/ml) for 90 min at 37 °C, the absorbance was recorded at 570 nm, and a reference was recorded at 630 nm with a micro plate reader (Model 550, BIO-RAD Laboratories, CA, USA). All MTT assay were presented as the means (±SD) of three independent experiments.

2.9. ChIP assays and real-time PCR analysis

ChIP assays were performed with the indicated antibodies as described previously [25], but without SDS in all buffers. Eluted DNA was amplified with specific primers using the SYBR green PCR master mix (Applied Biosystems, CA, USA). Real-time PCR analysis was carries out with an ABI Prism 7300 Sequence Detection System. Primers used in PCR were as follows: PAI-1 (forward (F): 5′-CCTCCAACCTCAGCCAGACAAG-3′; reverse (R): 5′-CCCAGCCCAACAGCCACAG-3′). All reactions were normalized relative to input activities and are presented as means (±SD) of three independent experiments.

2.10. Statistical analysis

Statistical analysis was done using Student's *t*-test with the SPSS program. $P < 0.05$ was considered to be significant.

3. Results

3.1. EGCG inhibits TGF-1-mediated EMT in A549 cells

First, we measured the effect of EGCG on cell viability with the MTT assay. We examined that cell viability did not change with EGCG treatment. We determined that concentrations of EGCG below 100 μM did not induce cell toxicity in A549 cells (Fig. 1A). To more fully investigate the effect of TGF-β1 with or without EGCG treatment in A549 cells, we stimulated the cells with 5 ng/ml TGF-β1. After 24 h, we treated with 100 μM EGCG for an additional 24 h. As shown in Fig. 1B, TGF-β1 stimulated A549 cells changed cell morphology, but EGCG treatment suppressed TGF-β1-induced cell morphologic changes. We sought to determine whether EGCG suppresses TGF-β1-induced migration of A549 cells using wound healing and migration assays. For the migration assay, A549 cells were cultured in a migration chamber. We treated the cells in transwell with TGF-β1, and other cells in transwell treated with TGF-β1 and EGCG for 48 h. The cells were then fixed and migration was assayed. TGF-β1-treated cells migrated across the transwell. In contrast, TGF-β1-stimulated A549 cells with EGCG showed impaired migration to the lower surface of the transwell chamber.

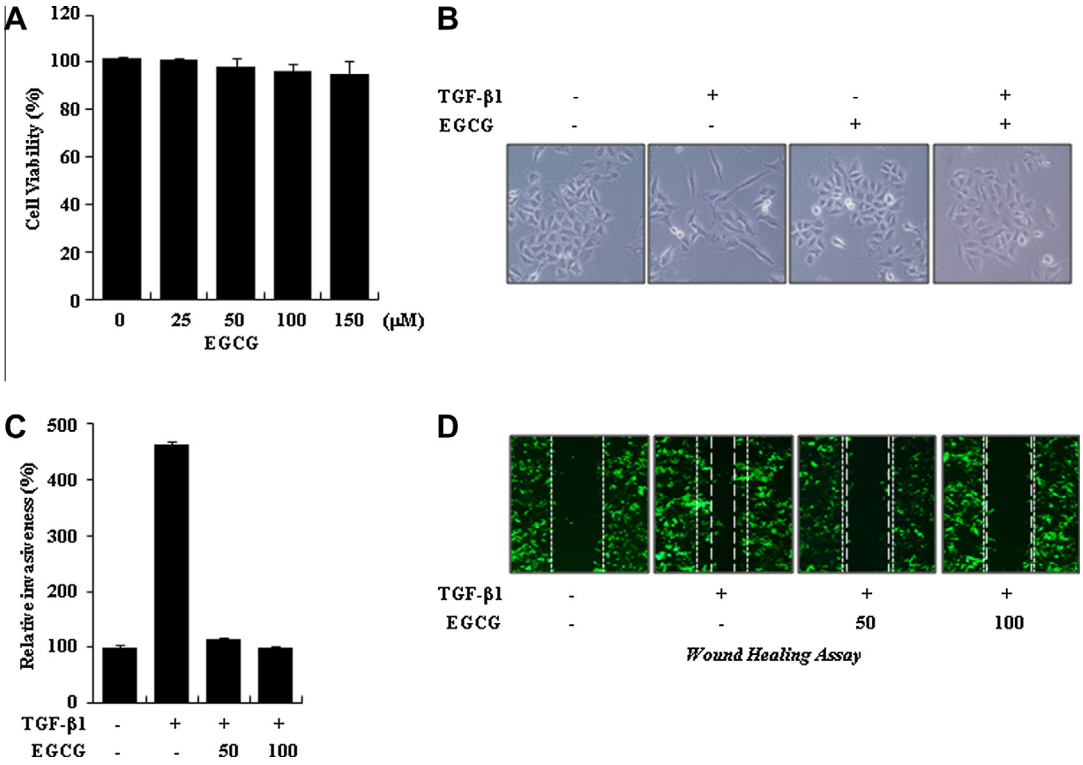

Fig. 1. TGF-β1-induced EMT is inhibited by EGCG in dose dependent manner. (A) Effect of increasing concentrations of EGCG on the viability of A549 cells, as measured with the MTT assay. (B) Morphological changed between TGF-β1-treated A549 cells and TGF-β1-treated A549 cells with EGCG. Cells were cultured in the complete media for 48 h. (C) *In vitro* Invasiveness of A549 cells were measured by counting cells that migrated through the extracellular matrix layer of Biocoat Matrigel invasion chambers. The data are expressed as mean ± SD for triplicates. (D) EGCG antagonized the TGF-β1-induced migration of A549 cells. When cell confluence had reached about 90%, the cells were scratched with a 200 μl pipette tip and washed with DMEM media to remove any free-floating cells and debris. Then Culture media was added, and the culture plates were incubated at 37 °C with TGF-β1 or with TGF-β1 and EGCG. The cells were photographed under a microscope 12 h after scratching. The data are expressed as mean ± SD for triplicates.

Thus, the effect of TGF-β1 on migration was antagonized by EGCG (Fig. 1C). In accordance with the results of the migration assay, the wound healing assay showed that TGF-β1 treatment promoted the mobility of A549 cells, whereas the mobility of TGF-β1-stimulated A549 cells was decreased in the presence of EGCG. These results indicate that EGCG antagonizes the migration of A549 cells permitted by TGF-β1-induced EMT (Fig. 1D).

3.2. EGCG inhibits the switching of expression from E- cadherin to N-cadherin

To demonstrate the inhibitory role that EGCG plays in TGF-β1-induced EMT in A549 cells, we measured the switching of expression from E-cadherin to N-cadherin by western blot (Supplementary Fig. S1) and E-cadherin promoter activity by E-cadherin-Luc reporter assay. A549 cells were incubated with 5 ng/ml TGF-β1. After 24 h, these cells were incubated with or without EGCG (0, 50, 100 μM) for 24 h. TGF-β1-stimulated A549 cells showed a significant decrease in E-cadherin expression and a concomitant increase in the mesenchymal marker N-cadherin, Vimentin expression compared with control cells (Fig. 2A). TGF-β1 stimulation decreased E-cadherin promoter activity, whereas EGCG treatment significantly rescued the decrease of E-cadherin promoter activity by TGF-β1, indicating that EGCG blocked TGF-β1-mediated EMT by enhancing E-cadherin expression (Fig. 2B). To investigate the effects of EGCG on the distribution of E-cadherin, N-cadherin and vimentin in A549 cells, A549 cells were stimulated with TGF-β1 for 24 h and then treated with 100 μM EGCG for another 24 h. These cells were fixed and stained for E-cadherin, N-cadherin and vimentin. Immunofluorescence showed that E-cadherin expression was completely lost at the membrane of TGF-β1-stimulated A549 cells. In contrast, the cells that were treated with TGF-β1 and EGCG had less inhibition of E-cadherin expression than TGF-β1-stimulated A549 cells (Fig. 2C). On the other hand, vimentin expression was strongly increased and uniformly distributed in TGF-β1-stimulated cells. Interestingly, up-regulated N-cadherin and Vimentin expression by TGF-β1 was clearly inhibited by EGCG (Fig. 2D and E). These results strongly suggest that EGCG promoted E-cadherin expression and suppressed N-cadherin and vimentin expression during TGF-β1-induced EMT.

3.3. EGCG antagonizes up-regulated genes during TGF-β1-induced EMT

To strengthen our finding that TGF-β1-induced EMT is antagonized by EGCG, we examined EMT marker gene expression during TGF-β1-induced EMT with and without EGCG treatment by quantitative real-time polymerase chain reaction (qPCR), reverse transcription (RT)-PCR, and western blot. The loss of E-cadherin induced by TGF-β1 is associated with an increase in several EMT markers. Therefore, we investigated whether EGCG inhibited the expression of these genes during TGF-β1-induced EMT. To determine the major genes associated with TGF-β1-induced EMT, we isolated total RNA from A549 cells treated with or without TGF-β1 for 48 h, or treated with TGF-β1 for 24 h and then treated with EGCG for 24 h. We examined the transcriptional changes of up-regulated target genes during EMT using an iQ5 Bio-Rad qPCR machine (Fig. 5A). Relative to TGF-β1-induced EMT, we found significant decreases in the expression levels of 7 genes (ITGA5, MMP2, SERPINE1 [PAI1], SNAI1 [Snail], SNAI2 [Slug], SPARC, and VIMENTIN) by EGCG. As shown in Fig. 5B and C, the expression levels of target genes were measured by RT-PCR and western blot. Furthermore, we examined whether the effect of EGCG could be generalized in other lung cancer cell lines (EKVX, H226, H23 and

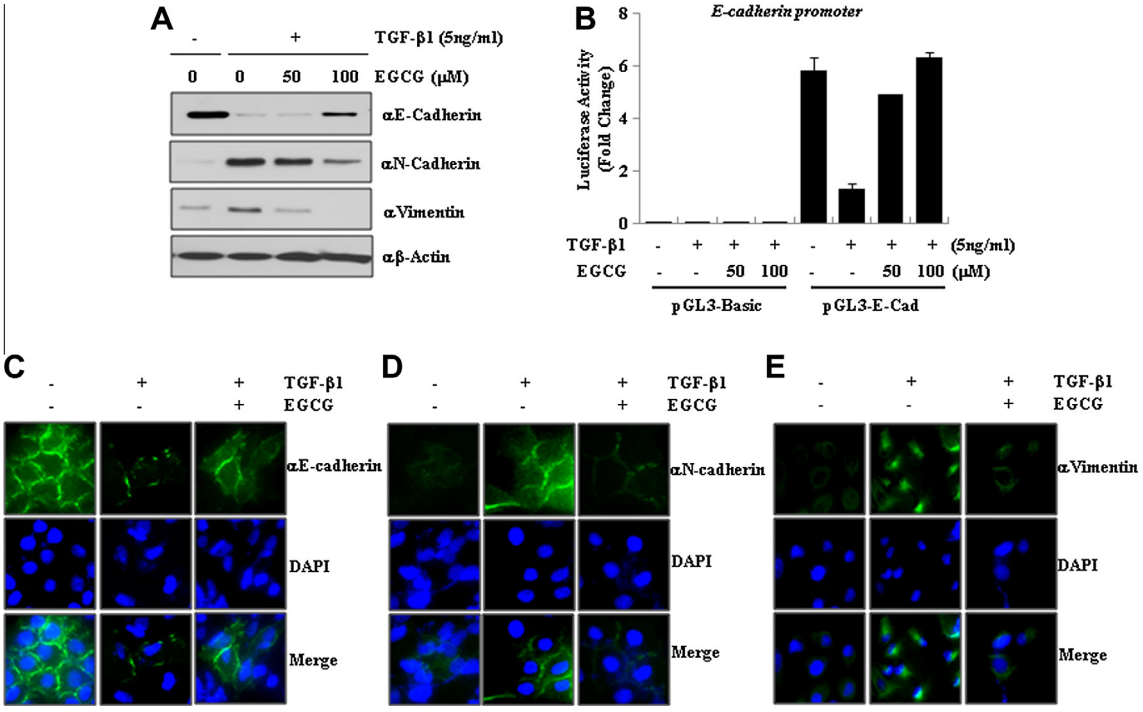

Fig. 2. EGCG inhibits TGF-β1-induced EMT markers, E-Cadherin, N-Cadherin and Vimentin, in A549 cells. (A) N-cadherin to E-cadherin switching by TGF-β1. A549 cells were stimulated by TGF-β1 (5 ng/ml), and other A549 cells were stimulated by TGF-β1 with EGCG in dose dependent manner of 48 h. Western blots of whole cell lysates were detected with anti-E-cadherin, anti-N-cadherin and anti-β-actin. (B) Reporter assay of E-cadherin transcription activity in A549 cells. Cells were transfected with pGL3-E-cad-Luc or pGL3-Basic. Reporter assay was performed under the same conditions. The data are expressed as mean ± SD for triplicates. (C) EGCG increased E-cadherin expression, and (D and E) EGCG decreased N-cadherin and Vimentin expression. E-cadherin, N-cadherin and Vimentin were detected by using Immunofluorescence analysis. A549 cells were cultured in the TGF-β1 with EGCG or without EGCG for 48 h. The expression of E-cadherin, N-cadherin and Vimentin were visualized by applying primary anti-E-cadherin, anti-N-cadherin and anti-Vimentin. Images shows nucleus stained by DAPI.

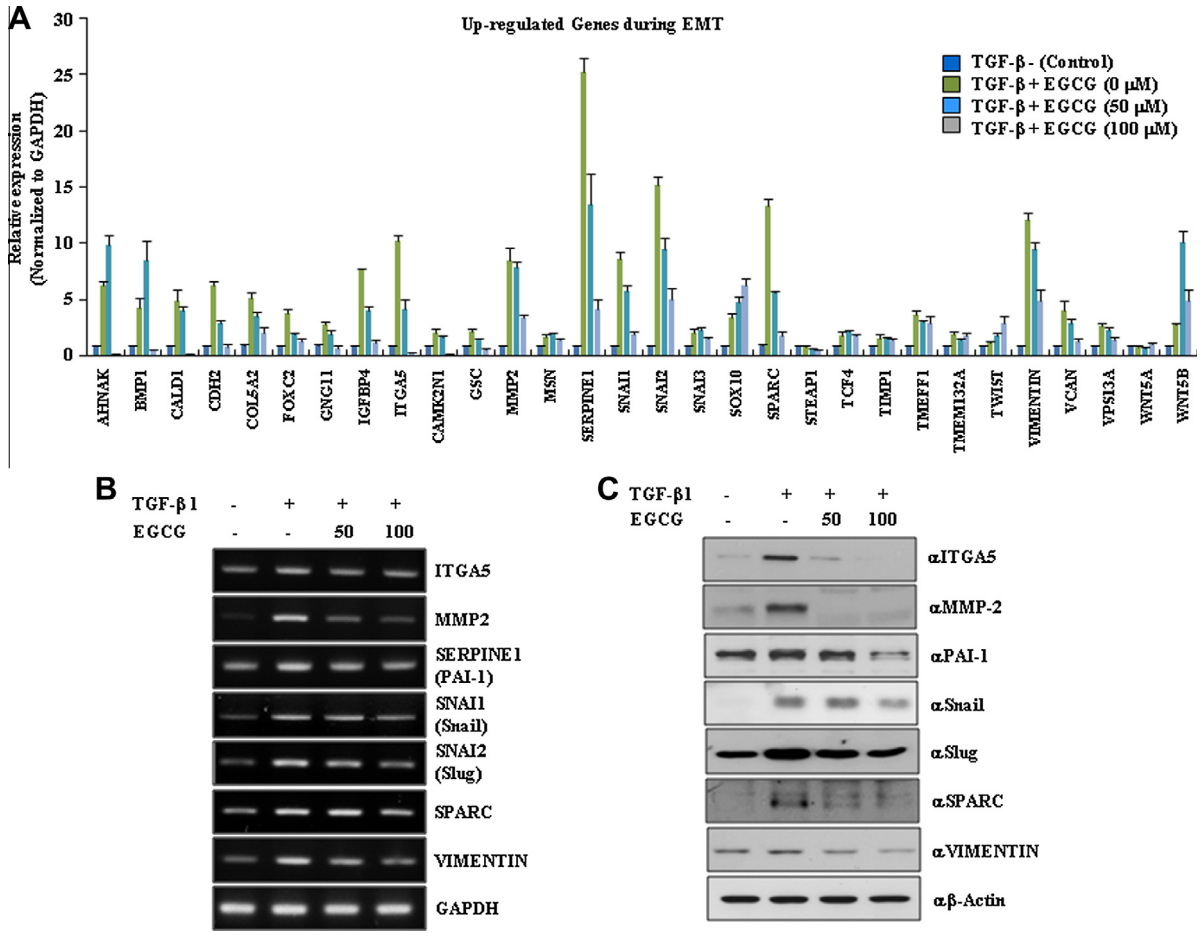

Fig. 3. EGCG suppressed the TGF-β-induced EMT dependent genes. (A) EGCG downregulated TGF-β1-induced EMT markers. A549 cells were treated with TGF-β1 (5 ng/ml) or with TGF-β1 and EGCG (0, 50, 100 μM) for 48 h. The expression level of each gene was analyzed by Quantitative real-time PCR using total mRNA of A549 cells and compared with TGF-β1 alone (Control) of same gene. The data are expressed as mean ± SD for triplicates. (B and C) A549 cells were treated with TGF-β1 (5 ng/ml, 48 h) or with TGF-β1 and EGCG (0, 50, 100 μM), and the expression levels of indicated genes were analyzed by RT-PCR (B) and western blot analysis (C).

H460) with or without *k*-Ras mutation by western blot and qPCR (Supplementary Fig. S2). As expected, EGCG treatment efficiently suppressed the expression of up-regulated EMT markers. From these data, we suggest that most of the EMT markers induced by TGF-β1 decreased when TGF-β1-stimulated A549 cells were also treated with EGCG for 24 h. Thus, EGCG antagonized EMT markers, and these results provide a possible therapeutic approach for using EGCG as an inhibitor of HAT activity.

3.4. EGCG inhibits the acetylation activity of increased p300/CBP during TGF-β1-induced EMT

We reported that EGCG potently inhibited HAT activity in the NF-κB inflammatory signaling pathway. On the basis of these reports, we decided to examine whether EGCG inhibited HAT activity in a dose-dependent manner during TGF-β1-induced EMT. To examine enzyme specificity, we sought to assess the effect of EGCG on HAT activity. Firstly, extracts of A549 cells treated with TGF-β1 were measured by using a HAT activity colorimetric assay. Histone acetylation induced by TGF-β1 was suppressed in the presence of EGCG (Fig. 4A). To understand the mechanism underlying EGCG inhibition of HAT activity, we examined the activities of immunoprecipitated p300 and CBP acetyltransferases by HAT assay (Fig. 4B). EGCG was a highly efficient inhibitor of p300/CBP activity. These results therefore established that EGCG has the key biological function for inhibition of TGF-β1-induced EMT. Also, as shown in Fig. 4C, TGF-β1 increased the expression of p300 and CBP from nuclear extracts of A549 cells treated with TGF-β1 and its inhibition by EGCG. We next determined whether EGCG suppressed TGF-β1-stimulated nuclear expression of p300 and CBP by immunofluorescence. EGCG dramatically suppressed p300 (Fig. 4D) and CBP (Fig. 4E) protein expression levels in TGF-β1-stimulated A549 cells compared with control cells. These results prompted us to investigate whether p300 and CBP expression is directly involved in the TGF-β1 signaling pathway. Taken together, these remarkable findings show that EGCG strongly inhibited the enhancement of p300 and CBP expression by TGF-β1 and suppressed HAT activity.

3.5. Acetylation of Smad2 and Smad3 is directly inhibited by EGCG

Smad2 and Smad3 are essential in the TGF-β1 signaling pathway and are phosphorylated and translocated to the nucleus on TGF-β-dependent receptor activation. According to recent reports, Smad2 and Smad3 are direct substrates of p300/CBP, and this acetylation is stimulated by TGF-β1. The translocated Smad2 and Smad3 are acetylated to activate the transcription of target genes by p300/CBP in the nucleus. To demonstrate that endogenous nuclear Smad2 and Smad3 are acetylated in response to TGF-β1, these proteins were immunoprecipitated with anti-Smad2 and anti-Smad3 antibodies from nuclear extracts of A549 cells treated with TGF-β1 and/or SB431542, an inhibitor of TGF-β1 type I receptor kinases, for 48 h and detected by western blotting with acetyllysine antibody. SB431542 blocked the acetylation of Smad2 and

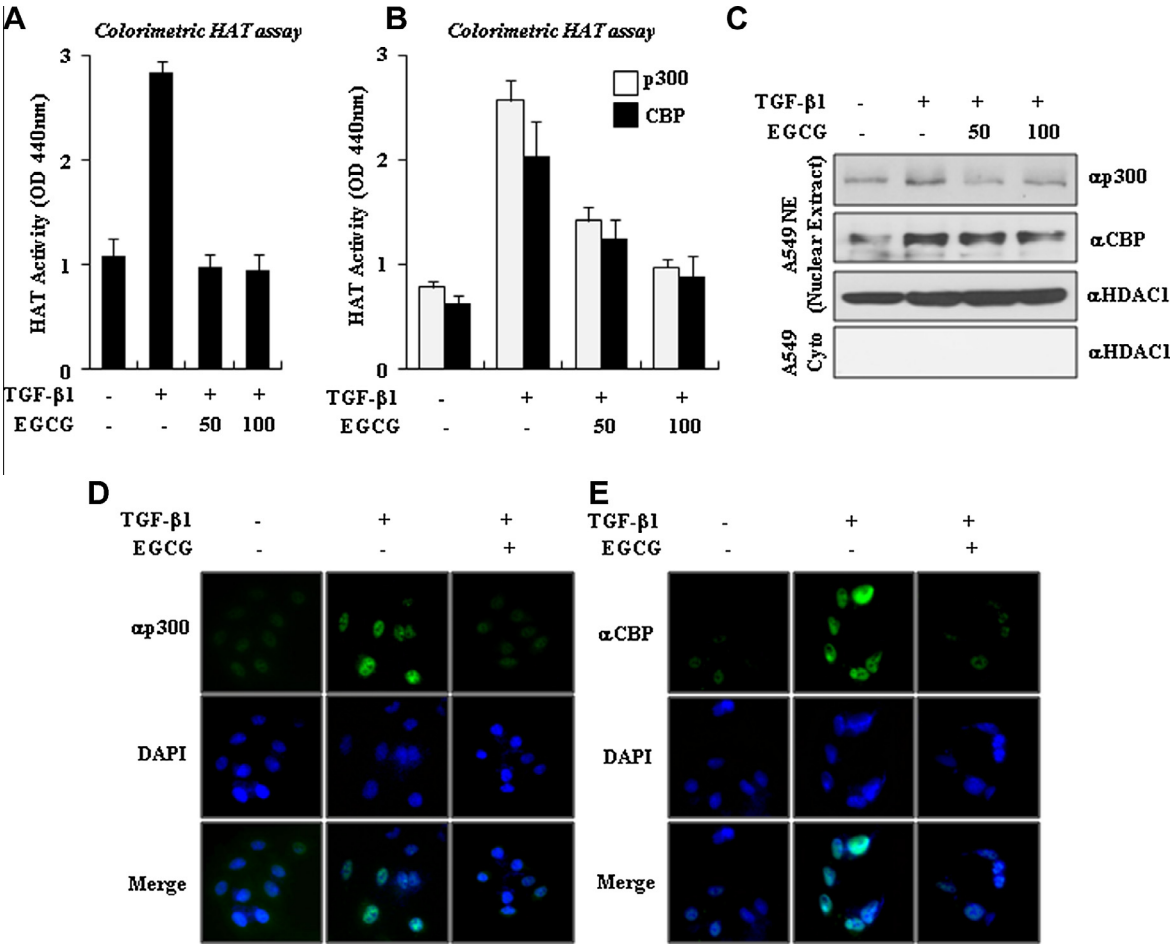

Fig. 4. EGCG antagonized the increasing of HAT activity by TGF-β1. (A) EGCG has anti-HAT activity. HAT activity was assayed with a colorimetric HAT activity assay kit. HAT activity was blocked EGCG in dose dependent. (B) EGCG inhibits p300 and CBP activity in TGF-β1 stimulated A549 cells. The p300 and CBP enzymes were immunoprecipitated by using anti-p300 and anti-CBP antibodies. *In vitro* HAT activity assays were performed with a colorimetric HAT activity assay kit. The data are expressed as mean ± SD for triplicates. (C) TGF-β1 accumulated p300 and CBP expression in the nuclear. EGCG treatment led to loss of p300 and CBP. A549 cells were treated with TGF-β1 or with TGF-β1 and EGCG. Nuclear and cytoplasm extracts were prepared and processed for western blot analysis using the indicated antibodies. (D) p300 and (E) CBP were accumulated by TGF-β1 in the nuclear, and EGCG treatment blocked a nuclear accumulation of p300 and CBP. p300 and CBP were detected by using Immunofluorescence analysis.

Smad3 in TGF-β1-stimulated A549 cells compared with levels in the presence of TGF-β1 only (Fig. 5A). To more assess Smad2 and Smad3 acetylation by p300/CBP, we examined the *in vivo* acetylation of Smad2 and Smad3 by using an antibody against acetyl-lysine (Ac-K) in A549 cells. The acetylation of Smad2 and Smad3 was significantly increased by TGF-β1, but was inhibited by EGCG treatment. These results show that the acetylation of Smad2 and Smad3 by p300/CBP downstream of TGF-β1 was efficiently inhibited by EGCG (Fig. 5B). Additionally, as shown in Fig. 5C, reporter assay analysis with SBE-Luc, which includes Smad binding elements (SBE), verified that the transcriptional activation of Smad2 and Smad3 by TGF-β1 appeared to be determined by phosphorylation and acetylation. We transiently transfected A549 cells with SBE-Luc reporter plasmids and treated with TGF-β1 or with EGCG for 48 h. Thus, these results demonstrated the transcriptional activation of Smad2 and Smad3 by a SBE-Luc reporter assay. We strongly suggest that activated p300/CBP by TGF-β1 is induced the hyperacetylation of Smad2 and Smad3 in the nucleus of A549 cells.

3.6. The Samd-p300/CBP complex to chromatin for TGF-β1 signaling activation enhanced the recruitment of acetylated Smad2 and Smad3

Recent studies demonstrated that acetylation targets Smad2 and Smad3 to the Smad binding site of EMT target genes, including PAI-1, Snail, and Slug, in a TGF-β1-dependent manner [26]. To investigate the role of acetylation in Smad2 and Smad3 recruitment to the PAI-1 promoter, we performed ChIP assays in TGF-β1-stimulated A549 cells (Fig. 6A). The recruitment of acetylated Smad2 and Smad3 to PAI-1 promoters was enhanced by TGF-β1 and was inhibited by EGCG in a dose-dependent manner. The increased association of acetylated Smad2 and Smad3 with the PAI-1 promoter coincided with increased activity and recruitment of p300/CBP at the promoter (Fig. 6B). Thus, our results demonstrate that Smad2 and Smad3 are acetylated in response to TGF-β1 signaling, and the acetylated Smad2 and Smad3 associate with the promoters of EMT target genes. Taken together, these results importantly suggest that EGCG blocked acetylation of Smad2 and Smad3 by p300/CBP in the nucleus during TGF-β1-induced EMT, and EGCG is a strong HAT inhibitor (Fig. 6C).

4. Discussion

EMT is an essential process in developmental biology. The process was initially identified in normal tissue development, such as during embryogenesis and organogenesis. However, recent results identified EMT functions and its regulatory factors as major contributors to wound healing by fibrosis and invasive tumor cell behavior [27–29]. In the EMT process, epithelial cells undergo phenotypic conversion to mesenchymal cells by activating the

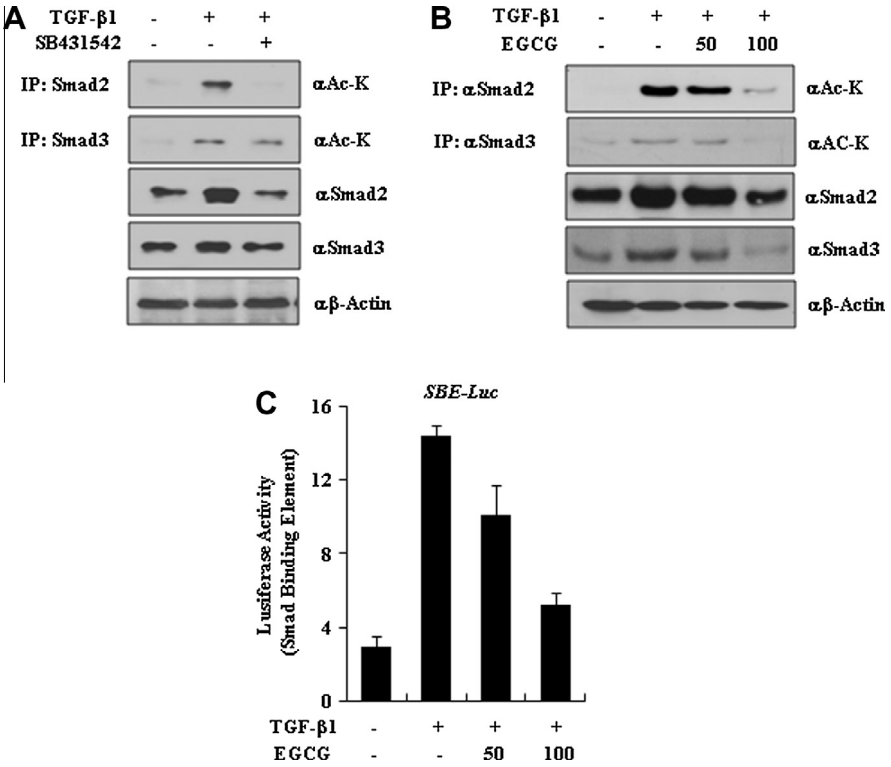

Fig. 5. Hyperacetylation of Smad2 and Smad3 by p300/CBP is reduced by EGCG. (A) TGF-β1 directly enhanced the acetylation of Smad2 and Smad3. A549 cells were treated with TGF-β1 and/or SB431542 (10 μM, Sigma–Aldrich), an inhibitor of TGF-β type I receptor (TβRI) kinases, for 24 h. The nuclear lysates of A549 cells were immunoprecipitated by anti-Smad2 and anti-Smad3 antibodies, and acetylated Smad2 (Ac-Smad2) and Smad3 (Ac-Smad3) were detected by western blot analysis using the anti-Ac-K antibody. The expression of each protein was measured by western-blotting of nuclear lysates with anti-Smad2, anti-Smad3 and anti-β-Actin antibodies. (B) The acetylation of TGF-β1-mediated Smad2 and Smad3 was prevented by EGCG in dose dependent manner. A549 cells were treated with TGF-β1 or with TGF-β1 and EGCG (0, 50, 100 μM). The hypoacetylation of Smad2 and Smad3 by EGCG was processed for western blot analysis using antibody against acetyl-K (lysine). Immunoprecipitation was performed by nuclear lysates of A549 cells. (C) The transactivation of TGF-β1-response promoter, pSBE-Luc, was decreased by EGCG. A549 cells were transiently tranfected with the pSBE-Luc reporter gene, and treated with TGF-β1 or with TGF-β1 and EGCG. The data are expressed as mean ± SD for triplicates.

expression of EMT marker genes. The major function of these genes is transcriptional repression of the main epithelial molecules, notably the cell–cell adhesion protein E-cadherin, leading to a morphological change from cytoskeletal rearrangement. Complementary to the epithelial gene repression is simultaneously induced expression of mesenchymal genes coding for N-cadherin, vimentin, and matrix proteins. Loss of E-cadherin disrupts adherens junctions, allowing individual cell motility, which is a hallmark feature of TGF-β1-induced EMT [30,31]. In this study, our results confirm that A549 cells with TGF-β1 treatment enhanced cell mobility by cell invasion and wound healing assays. In addition, these cells converted to a spindled mesenchymal morphology. On the other hand, when the TGF-β1-stimulated A549 cells were treated with EGCG, EGCG inhibited EMT functions and characteristics. Thus, we suggest that EGCG is directly involved in preventing EMT and invasion and metastasis of cancer cells.

TGF-β1 triggered both the increase of mesenchymal markers and the decrease of epithelial markers. TGF-β1 also constructs a preferential environment for this phenotypic transition and is responsible for inducing the invasiveness of cancer cells. Smad2 and Smad3 are directly phosphorylated by TGF-β type I receptor kinases in the TGF-β1 signaling pathway. TGF-β1 stimulation results in the phosphorylation of Smad2 and Smad3 on the SSXS motif in the C-terminal residue, leading to the formation of a complex with the common mediator Smad4 and nuclear translocation. Both Smad2 and Smad3 directly interact with the transcriptional coactivators p300/CBP in nucleus, and Then the acetylation of Smad2 and Smad3 is induced by TGF-β1. In this study, we identified the acetylation of Smad2 and Smad3 by p300/CBP in EMT. This activity of p300/CBP is considered a novel target for the prevention of EMT and fibrosis [32–34]. Our results show that TGF-β1 strongly upregulated acetyltransferase activity of p300/CBP in human lung cancer cells. The induction of p300/CBP contributed to the hyperacetylation of Smad2 and Smad3. Thus, the regulation of Smad signaling by EGCG is an important pharmacologic tool for chemoprevention of invasion and metastasis in cancer.

Green tea is one of the most widely consumed teas in the world and is reported to have significant benefits for human health. The health-promoting effects of green tea are mainly attributed to EGCG, the most abundant polyphenol compound in green tea. EGCG has global biological activity and potentially acts as an inflammation inhibitor. In addition, EGCG as an antioxidant prevents oxidative stress-related diseases, including cancer, cardiovascular disease, and fibrosis. Previous studies also show that EGCG has a role in improving wound healing, scarring, and other fibrotic diseases. The inhibitory effects of EGCG on MMP-2 expression and activation of fibroblasts and vascular smooth muscle cells have recently been investigated. Animal studies have reported that EGCG suppressed bleomycin-induced pulmonary fibrosis and injury-induced liver fibrosis. EGCG regulates multiple signal transduction pathways, such as the NF-κB and TGF-β1 signaling pathways. Recently, EGCG was reported to suppress TGF-β1-induced ROS production and ERK activation in fibroblasts [35,36] and we previously showed EGCG completely inhibits NF-κB activation in inflammatory diseases [21].

Although EGCG was reported to inhibit the expression of TGF-β1-mediated EMT markers, the role of EGCG as a HAT inhibitor in the inhibition of Smad signaling in TGF-β1-stimulated EMT has

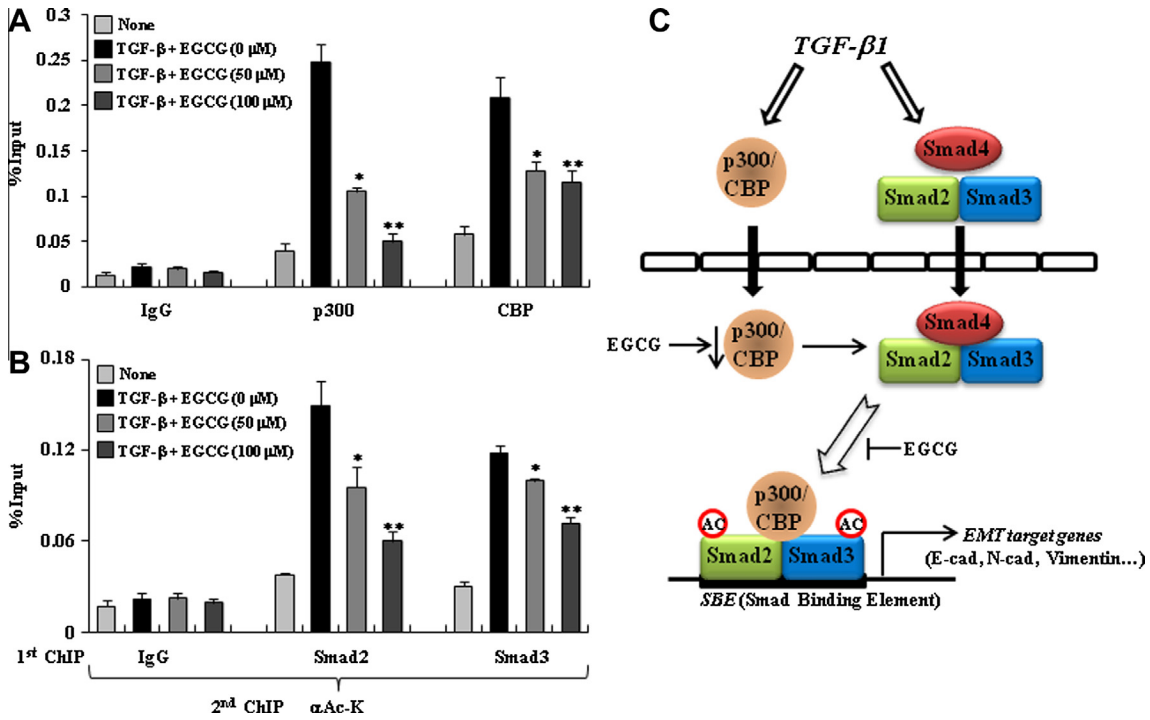

Fig. 6. Samd-p300/CBP Complex enhanced to the promoter regions of EMT marker gene by TGF-β1. (A) TGF-β1 treatment increased the recruitment of p300 and CBP to the promoter regions of EMT target gene, PAI-1, but the recruitment of p300 and CBP decreased by EGCG. (B) Acetylated Smad2 and Smad3 by p300 and CBP recruited to PAI-1 promoter regions. A549 cells were treated with TGF-β1 (5 ng/ml) or with TGF-β1 and EGCG (0, 50, 100 μM) for 48 h, and 1st ChIP and 2nd ChIP assays were performed with the indicated antibodies. The data are expressed as mean ± SD for triplicates. (C) Model of our findings. In cancer cells with TGF-β1 treatment, active p300/CBP acetylates Smad2 and Smad3 to activate EMT-marker transcription, enhancing invasiveness and migration of cancer cells. Importantly, EGCG antagonizes acetylation of Smad2 and Smad3 to repress EMT-marker expression via a TGF-β1 signaling pathway. Thus, EGCG is a HAT inhibitor for cancer prevention.

seldom garnered attention. To gain more information into the effects of EGCG on the suppression of EMT, we demonstrated the effects of EGCG on Smad acetylation by TGF-β1, as well as regulation of the histone acetylation. In addition, we reported for the first time that EGCG significantly suppressed TGF-β1-stimulated EMT marker expression in human lung cancer cells (Fig. 3). Our results also demonstrated that EGCG completely blocked TGF-β1-induced EMT through inhibition of p300/CBP activation (Fig. 4). These findings provided further understanding of the molecular mechanisms underlying the anti-HAT effects of EGCG and helped to further outline the targets of therapeutic intervention and prevention of EMT in cancer cells and other fibrotic diseases.

The transcriptional coactivators p300/CBP have acetyltransferase activity that functions in epigenetic regulation by loosening the condensed structure of chromatin and promoting the accessibility of the target DNA to the transcriptional coactivator complex [17]. Recently, several HAT inhibitors may be useful for drug development. These HAT inhibitors may be associated with the prevention of diabetes, cancer, and other diseases. In our previous studies, we found that EGCG possessed a universal specificity for HAT enzymes not but HDACs, SIRTs, or HMTases [21]. EGCG also induced hypoacetylation of p65. Therefore, we strongly advocated that EGCG is a novel inhibitor of HATs along with curcumin and anacadic acid. In this study, we investigated the role of p300/CBP in TGF-β1-induced EMT and enhancement of EMT- and fibrosis-related target genes. The coactivator p300/CBP also directly interacts with Smad2 and Smad3 and induces acetylation of Smad2 and Smad3 in the TGF-β1 signaling pathway. We also showed that EGCG inhibited the stimulated p300/CBP activity by TGF-β1. Thus, our findings highlight that p300/CBP and Smad2/Smad3 in the TGF-β1 signaling pathway are essential components for the regulation of TGF-β1-induced transcriptional activation of EMT markers in human lung cancer cells (Fig. 6).

In conclusion, we demonstrate that the activation of p300/CBP in TGF-β1-induced EMT is directly involved in Smad2 and Smad3 acetylation and that the decrease of p300/CBP activity by EGCG is induced by deacetylation of Smad2 and Smad3 in A549 cells (Fig. 6C). We also found that the transcriptional activity of Smad2 and Smad3 was significantly decreased by EGCG treatment. Thus, EGCG decreased EMT marker gene expression. As a result, our findings revealed a new inhibitory mechanism of TGF-β1-induced EMT through inhibition of p300/CBP activity in human lung cancer cells.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.02.018.

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