Kaempferol Suppresses Transforming Growth Factor-β1–Induced Epithelial-to-Mesenchymal Transition and Migration of A549 Lung Cancer Cells by Inhibiting Akt1-Mediated Phosphorylation of Smad3 at Threonine-179

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Abstract
Kaempferol, a natural dietary flavonoid, is well known to possess chemopreventive and therapeutic anticancer efficacy; however, its antimetastatic effects have not been mechanistically studied so far in any cancer model. This study was aimed to investigate the inhibitory effect and accompanying mechanisms of kaempferol on epithelial-to-mesenchymal transition (EMT) and cell migration induced by transforming growth factor-β1 (TGF-β1). In human A549 non–small lung cancer cells, kaempferol strongly blocked the enhancement of cell migration by TGF-β1–induced EMT through recovering the loss of E-cadherin and suppressing the induction of mesenchymal markers as well as the upregulation of TGF-β1–mediated matrix metalloproteinase-2 activity. Interestingly, kaempferol reversed TGF-β1–mediated Snail induction and E-cadherin repression by weakening Smad3 binding to the Snail promoter without affecting its C-terminus phosphorylation, complex formation with Smad4, and nuclear translocation under TGF-β1 stimulation. Mechanism study revealed that the phosphorylation of Smad3 linker region induced by TGF-β1 was required for the induction of EMT and cell migration, and selective downregulation of the phosphorylation of Smad3 at Thr179 residue (not Ser204, Ser208, and Ser213) in the linker region was responsible for the inhibition by kaempferol of TGF-β1–induced EMT and cell migration. Furthermore, Akt1 was required for TGF-β1–mediated induction of EMT and cell migration and directly phosphorylated Smad3 at Thr179, and kaempferol completely abolished TGF-β1–induced Akt1 phosphorylation. In summary, kaempferol blocks TGF-β1–induced EMT and migration of lung cancer cells by inhibiting Akt1-mediated phosphorylation of Smad3 at Thr179 residue, providing the first evidence of a molecular mechanism for the anticancer effect of kaempferol.

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Introduction
Non–small-cell lung cancer (NSCLC) is the most common type of lung cancer. NSCLC shows a poor prognosis and accounts for the leading cause of cancer-related death every year worldwide [1]. Because of the lack of robust and dependable molecular markers for the early diagnosis, most patients with NSCLC present locally...
advanced and metastatic cancer disease at the time of diagnosis. Moreover, the metastasis of lung cancer cells is a major contributor in aggressiveness of NSCLC and is responsible for the main cause of deaths in lung cancer patients. Therefore, identifying of the key factors that contribute to the lung metastatic process and a better understanding of the molecular mechanisms underlying lung cancer metastasis are crucial in providing a promising approach for lung cancer therapy that target metastasis.

Tumor metastasis is a dynamic multistep cascade process. In the metastatic process, epithelial-to-mesenchymal transition (EMT) is an important morphogenetic event for triggering metastasis from primary tumors and is characterized by the loss of E-cadherin-mediated cell-cell junction and the upregulation of mesenchymal markers including N-cadherin, vimentin, and fibronectin [2,3]. Therefore, changes during EMT lead to the transition of a polarized epithelial phenotype to a migratory mesenchymal phenotype, and then cells degrade extracellular matrix by activating matrix metalloproteinases (MMPs) and have invasive characteristics.

Transforming growth factor-β1 (TGF-β1), a prototypical member of the TGF-β superfamily, is a multifunctional cytokine that regulates cell proliferation, differentiation, apoptosis, and migration [4]. In normal physiologic condition, TGF-β1 has tumor-suppressive functions through inhibiting cell proliferation and promoting apoptosis. However, TGF-β1 acts also as a metastatic inducer by promoting EMT in late-stage tumor progression [5]. Molecular signaling mechanism studies on TGF-β1-induced EMT indicate crucial roles of Smad3 signaling pathway. Depletion of Smad3 completely abolishes TGF-β1 induction of EMT [6–8]. Smad3 acts as a transcriptional activator of E-cadherin repressors such as Snail, Slug, and Twist [9–11]. Smad3 also negatively regulates E-cadherin by upregulating ZEB1 and ZEB2 via microRNA-200 pathway [12]. Smad3 is a key mediator of TGF-β signaling pathway. Upon TGF-β1 stimulation, TβRI is activated by TβRII and mediates the phosphorylation of the conserved COOH-tail serine residues of Smad3. The phosphorylated Smad3 interacts with Smad4 and translocates from the cytosol into the nucleus, where it regulates transcription of target genes [13,14]. However, the regulatory mechanisms by which Smad3 determines the functional outcome of TGF-β1 responses under physiologic and pathologic conditions have yet to be fully elucidated. Smad3 linker region is a less conserved intermediate region that connects between conserved Mad-homology (MH) 1 and MH2 domains and contains several threonine and serine residues (Thr179, Ser204, Ser208, and Ser213) that phosphorylated by fundamental signaling kinases in a strongly cell context-dependent manner [15–19]. Several lines of recent evidence indicate the phosphorylation of the linker region of Smad3 as a crucial determinant of distinct cellular responses to TGF-β1 in normal and cancer cells. For example, in early carcinogenic process, hepatitis B virus X protein shifts hepatocytic TGF-β1 signaling from the tumor-suppressive Smad3 pathway to the oncogenic Smad3 pathway through inducing c-Jun NH2-terminal kinase–mediated phosphorylation of Smad3 linker region [20]. Constitutively activated Ras confers a selective advantage on benign tumors by inducing the phosphorylation of Smad3 linker region, leading to carcinoma in situ [21]. Hydrogen peroxide–mediated phosphorylation of Smad3 linker region opposes the antiproliferative responses of epithelial cells induced by TGF-β1 [22]. Therefore, the pharmacologic inhibition of Smad3 phosphorylation in the linker region may prevent progression to advanced carcinoma by suppressing oncogenic TGF-β1 signaling.

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a common natural flavonoid (chemical structure as shown in Figure 1A) that is abundant in tea, grapes, berries, and cruciferous vegetables [23]. In several types of human cancer cells, kaempferol acts as a potent antitumor growth agent that inhibits phosphatidylinositol-3-kinase (PI3K) and ribosomal S6 kinase activities [24–26] and increases the expression of tumor suppressor, phosphatase and tensin homolog [27]. Kaempferol also inhibits tumor angiogenesis and expression of MMP-2 [28,29]. However, the effect of kaempferol on the cancer metastasis of NSCLC, as well as the underlying mechanisms of the effect, has not yet been reported. In the present study, we investigated the molecular mechanisms by which kaempferol elicits its anticancer activity against metastatic potentials induced by TGF-β1 in human NSCLC cell lines. Our data clearly demonstrate that kaempferol inhibits TGF-β1–induced EMT, migration, and invasiveness of A549 lung cancer cells by suppressing Akt1-mediated phosphorylation of Smad3 at Thr179 residue in the linker region.

**Experimental Procedures**

**Materials**

Recombinant TGF-β1 was purchased from R&D systems (Minneapolis, MA). Kaempferol was obtained from Sigma-Aldrich (St. Louis, MO). AktIV, LY294002, U0126, and SB431542 were purchased from Calbiochem (San Diego, CA). Small interfering RNAs for control, Smad2, and Smad3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture**

A549, human non–small lung cancer cell line, was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in monolayers at 37°C in a 5% CO2 incubator in RPMI1640 medium supplemented with 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin.

**Lentiviral Vector Production and Infection**

The lentivectors carrying human Smad3 wild type, Smad3 (EPSM), and Smad3 (T179V) were cloned from pCMV-Myc-Smad3 wild type, pCMV-Myc-Smad3 (EPSM), and pCMV-Myc-Smad3 (T179V) (a gift from Dr. Fang Liu; The State University of New Jersey). The lentivector pCAG was digested with MluI and EcoRI; reverse, 5'-GATCACGCGTGGATCCCATCGATTTAAAGCT-3'; reverse, 5'-GATCCCTAGCCCCCTCTAGATGCTG-3'. For the production of lentivirus, 293T cells were co-transfected with pCAG vector together with pPAX2 and pMD2. G (Addgene, Cambridge, MA) by FuGENE6. The viral supernatant was collected 72 hours after transfection, centrifuged through a 0.45-μm filter (Millipore, Billerica, MA). Target cells (1 x 10^5/well) were seeded in six-well plate, and after incubation at 37°C for 24 hours, the medium of each well was replaced with 1-ml viral suspension supplemented with 8 mg/ml Polybrene (Sigma-Aldrich). Then, the plates were centrifuged at 1200 rpm for 30 minutes at room temperature, followed by 12-hour incubation in standard cell culture condition, and media were replaced with fresh Dulbecco's modified Eagle's medium. After 48 hours of additional incubation, the protein levels of each gene were analyzed by immunoblotting.
Luciferase Reporter Assay

The pSG5-p110α (K227E), which encodes a constitutively active p110α (CA-p110α), the catalytic subunit of PI3K, and pSG5-p85ΔSH2-N (deleted for amino acids 478-513 and widely referred to as pSG5-Δp85), which encodes a dominant-negative mutant of p85 (DN-p85), a regulator of PI3K, were gifts from Dr. J. Downward (Cancer Research, UK). The pCMV2-Akt1 (E40K), which encodes a constitutively active Akt1 (CA-Akt1), was provided by Dr. Naoya Fujita (University of Tokyo, Japan). The pcDNA3-HA-Akt1 (K179M), which encodes a dominant-negative mutant of Akt1 (DN-Akt1), was a gift from Dr. Kwon Young-Guen (Yeonse University, Korea). A549 cells in six-well plates (5 × 10⁵ cells per plate) were co-transfected with 0.5 μg of a luciferase reporter plasmid containing the human E-cadherin promoter (gift from Dr. Kyung Lib Jang, Pusan National University, Korea), the human Snail promoter (gift from Dr. Guhung Jung, Seoul National University), or an artificial SBE4-Luc reporter plasmid containing four tandem repeats of Smad-binding elements together with 0.2 μg of the β-galactosidase expression plasmid pCMV-LacZ with the use of FuGENE 6 reagent (Roche, Mannheim) according to the manufacturer’s instructions. Luciferase reporter activity was assessed on a luminometer with a dual-luciferase Reporter Assay System (Promega, Madison, WI). The activity of β-galactosidase was also measured and was used to normalize luciferase activity. The results represent the average and SD of three independent experiments.

Gelatin Zymography

The activity of MMP2 in culture medium was assessed by gelatin zymography. After serum starvation for 24 hours, A549 cells were subjected to treatment and then incubated further for 24 hours. The supernatants were collected, centrifuged at 3000×g for 10 minutes, concentrated using Amicon Ultra Centrifugal Filter Units (Millipore), and quantified using Bio-Rad protein assay reagent. Proteins (30 μg) were subjected to SDS-PAGE on a 7.5% gel containing gelatin (2 mg/ml). For detection of gelatinolytic activity, the gel was incubated for overnight at 37°C in a solution containing 50 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 150 mM NaCl, and 0.02% sodium azide; stained with solution containing 0.1% Comassie Brilliant Blue, 25% methanol, and 0.7% acetic acid for 30 minutes; and destained with a solution of 30% methanol and 10% acetic acid.

Scratch Wound Migration Assay

Confluent monolayers of A549 cells were scratched using a sterile 200-μl pipette tip to generate a cell-free gap between two adjoining areas. After removal of the loose cells by twice washes with PBS, cells were subjected to treatment. Scratched wound healing was monitored.

Figure 1. Kaempferol (KF) inhibits TGF-β1–induced EMT in A549 lung cancer cells. (A) Chemical structure of KF. (B) Phase contrast images of cells treated with DMSO, 5 ng/ml of TGF-β1, or 5 ng/ml of TGF-β1 plus 25 μM of KF for 24 hours. (C) A549 cells were pretreated with 25 μM of KF at the indicated concentrations for 30 minutes and then stimulated with 5 ng/ml of TGF-β1 for 48 hours. Then, the cells were subjected to Western blot analysis for E-cadherin, N-cadherin, and smooth muscle α-actin (SMα actin). β-Actin levels were monitored as a loading control for whole-cell extracts. (D) A549 cells were pretreated with DMSO or 25 μM of KF for 30 minutes before 5 ng/ml of TGF-β1 treatment for 48 hours and then subjected to immunofluorescence staining for E-cadherin (green), N-cadherin (red), and vimentin (red). DAPI (blue) was used to stain nuclei.
under phase-contrast microscopy immediately after incision and after 24 hours of treatment. The area of migrating cells was randomly photographed at three separate sites along the length of the scratch.

**Transwell Migration Assay**

A549 cells uninfected or infected with lentivirus were serum starved (0.1% FBS) overnight and then trypsinized and resuspended in RPMI1640 (serum free) medium at 2 × 10⁵ cells/ml. A total of 250 μl of suspension was added to the upper chamber of Transwell insert (8-μm pore size, Corning). Cells were immediately exposed to treatment and allowed to migrate for 24 hours toward chemotacttracting complete medium present in the bottom chamber. Unmigrated cells on the upper surface were removed, and migrated cells at the bottom of the insert were fixed in methanol and stained with eosin and hematoxylin for cell counting. The number of migrated cells was counted in five randomly selected fields per filter. The results represent the average and SD of three independent experiments.

**Western Blotting and Immunoprecipitation**

Cellular contents were extracted in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA, 2 mM DTT, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 1 μM microcystin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin] at 4°C. Cell lysates were clarified by centrifugation at 12,000 rpm/min at 4°C for 10 minutes. The leupeptin, 10 μM anti-(Cell Signaling Technology, Danvers, MA), and anti-E-cadherin and anti-phospho-Smad3 (Ser423/425) and anti-phospho-Akt (Ser426) (Santa Cruz Biotechnology), anti-Smad2 and anti-Smad3 (Zymed), incubated with appropriate antibodies [anti-myc and anti-vimentin 0.05% (v/v) Tween 20 and 5% (w/v) nonfat dry milk and then subsequently blocked for 1 hour with Tris-buffered saline containing 50 mM Tris-HCl, pH 8.1, and 1 mM EDTA]. The following rabbit or mouse polyclonal antibodies were used: e-Myc (9E10) and control IgG from Santa Cruz Biotechnology and Smad3 from Invitrogen (Carlsbad, CA). The antibody-bound protein/DNA complexes were precipitated and washed sequentially for 10 minutes each in TSE I [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], TSE II [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl], and buffer III [0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)]. The captured genomic DNA was eluted, cross-linking was reversed at 94°C for 15 minutes, and proteins were removed by treatment with Proteinase K at 37°C for 1 hour. Ten percent of total genomic DNA from the nuclear extract was used as input. The primer used for detection of Snail promoter sequence as follows: forward primer, 5'-CGCTCGTAAACACTGGATAA-3'; reverse primer, 5'-GAAGCGAGGAAGGGACAC-3'. The samples were amplified by conventional polymerase chain reaction (PCR) using the above Smad3 Snail promoter-specific forward and reverse primers and Platinum Taq DNA polymerase (Invitrogen).

**Semi-quantitative Reverse Transcriptase (RT)-PCR**

Total cellular RNA was extracted from cells using the phenol-guanidinium isothiocyanate method [30]. Two micrograms of RNA was reverse transcribed for 1 hour at 42°C and amplified by PCR using specific primers for human Snail (sense, 5'-GGGCAGGTATGGAGAGGAAGA-3'; antisense, 5'-TTCTTCTGGCGTACTGCTGC-3') and β-actin (sense, 5'-ACGTGTCTATCCAGGCTGTG-3'; antisense, 5'-GGAGCGTAGCACAGCTTCTC-3'; internal control). The detailed PCR condition for Snail involved 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 60 seconds. Amplified PCR products were subjected to electrophoresis on 1% agarose gel and were visualized by ethidium bromide staining and photographed with Polaroid 667 film (Cambridge, MA).

**Indirect Immunofluorescence Microscopy**

Cells were grown on coverslips, rinsed in PBS, fixed with 3.7% formaldehyde in PBS for 10 minutes at 4°C, and permeabilized with PBS supplemented with 0.5% Triton X-100 for 10 minutes at room temperature. Permeabilized cells were rinsed three times in PBS supplemented with 0.5% Tween 20 and blocked with blocking solution (0.1% BSA in PBS, pH 7.4) for 90 minutes. Cells were rinsed three times in PBS and incubated with primary antibodies against E-cadherin, N-cadherin, vimentin, or Smad3 in humidified chamber overnight at 4°C. After rinsing three times in PBS supplemented with 0.5% Tween 20, cover slips were incubated with the secondary antibodies Alexa-488–conjugated goat anti-rabbit or anti-mouse IgGs (Invitrogen Molecular Probes, Eugene, OR) for 1 hour at 37°C. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). The intracellular distribution of the protein was analyzed using confocal fluorescence microscopy (Fluoview, FV-300; Olympus, Melville, NY) with excitation at 488 nm (for Alexa-488) and 340 nm (for DAPI).

**Statistical Analysis**

The data are representative of three independent experiments. Results are presented as the means ± SD. Analyses were performed...
with Student’s *t* test for paired data using SigmaPlot 8.0 software. *P* values less than .05 were considered significant.

## Results

**Inhibition of TGF-β1–induced EMT, Cell Migration, and MMP2 Activation by Kaempferol**

Because TGF-β1 has been reported to induce metastatic responses, including EMT, motility, and invasiveness, in A549 lung cancer cells [31], we used it as an experimental system to evaluate the antimetastatic potential of kaempferol in cancer cells. As previously reported, treatment with TGF-β1 dramatically induced a loss of cell contact and acquired spindle-like shape in A549 cells, but this morphological change was clearly blocked by pretreatment with kaempferol (Figure 1B). In line with this result, kaempferol completely abolished TGF-β1–mediated repression of E-cadherin and upregulation of N-cadherin and smooth muscle α-actin in a concentration-dependent manner (Figure 1C). Immunofluorescence staining of E-cadherin, N-cadherin, and vimentin in A549 cells also revealed that the changes of those EMT marker expression induced by TGF-β1 were significantly reduced by treatment with kaempferol (Figure 1D).

EMT is a key event in metastatic tumor progression by which epithelial cancer develops toward more aggressive phenotype with enhanced motile and invasive capabilities [2,3]. We thus examined whether kaempferol affects the migration of A549 cells induced by TGF-β1. TGF-β1–induced cell migration (Figure 2A) and wound closure (Figure 2B) were substantially attenuated in cells pretreated with kaempferol compared with kaempferol-untreated cells. Upregulation of MMP2 is also known to promote the invasion of cancer cells through the degradation of basement membrane and extracellular matrix [32]. Gelatin zymography and reporter gene assay revealed that TGF-β1–induced increases of secreted MMP2 (Figure 2C) and MMP2 gene promoter activities (Figure 2D) were considerably abolished by treatment with kaempferol. All these results confirm the potential of kaempferol as an inhibitor of TGF-β1–induced EMT, cell migration, and invasiveness in A549 lung cancer cells.

![Figure 2](image-url). Kaempferol inhibits TGF-β1–induced migration and MMP2 activation in A549 lung cancer cells. (A) A549 cells treated as in Figure 1D were subjected to wound closure assay, and the wells were imaged every 12 hours with Nikon90i microscope, 100×. (B) A549 cells (1 × 10⁴) seeded on 8-mm porous Transwell chambers were treated as in Figure 1D. Transmigrating cells were stained with hematoxylin and eosin and counted for each of the indicated cells. Quantitative data are shown as the mean ± SD of three independent experiments. *P < .05, compared with vehicle-treated controls; **P < .01, compared with TGF-β1–treated cells. (C) A549 cells were treated as in Figure 1C, the culture medium from indicated cells was collected, and MMP2 activities were measured by gelatin zymography. (D) A549 cells were transiently transfected with MMP2 promoter-reporter construct. The cells were pretreated with 25 μM of KF at the indicated concentrations for 30 minutes and then stimulated with 5 ng/ml of TGF-β1 for 24 hours. All quantitative data are the mean ± SD of three independent experiments. *P < .05; **P < .01, compared with TGF-β1–treated cells.
Kaempferol reverses TGF-β1–mediated E-cadherin repression by weakening Smad3 binding to Snail promoter without affecting canonical Smad3 activation

Receptor-activated Smads, including Smad2 and Smad3, act independently as the major intracellular mediators of TGF-β signal transduction pathway [14]. Therefore, we analyzed the role of Smad2 and Smad3 in TGF-β1–mediated down-regulation of E-cadherin in A549 cells. Western blot analysis revealed that depletion of Smad3 with siRNA markedly reduced the changes in E-cadherin and Snail expression induced by TGF-β1, whereas depletion of Smad2 with siRNA had no such effect (Figure 3A). Similar to this result, overexpression of Smad3 (3SA), but not Smad2 (3SA), mutant lacking phosphorylation sites in the COOH-terminal region significantly reduced TGF-β1–induced Snail promoter activity (Figure 3B). TGF-β1–induced Snail expression at the mRNA level was also completely abolished by treatment with Smad3 siRNA (Figure 3C). We next examined whether kaempferol affects the binding of Smad3 to Snail promoter in response to TGF-β1. ChIP was applied to measure the enrichment of Smad3 on Snail promoter with two primers spanning its binding site. As shown in Figure 3D, kaempferol treatment completely inhibited TGF-β1–induced recruitment of Smad3 on the −616/−623 region of Snail promoter, and the inhibitory effect was comparable to that of SB431542, a specific TβRI inhibitor. Consistent with this result, RT-PCR showed that TGF-β1–induced Snail expression was significantly attenuated by treatment with kaempferol (Figure 3E). Furthermore, overexpression of Smad3 (3SA) markedly attenuated TGF-β1–induced migration of A549 cells compared with TGF-β1–treated control cells (data not shown). These results strongly suggest that Smad3 has a functional importance in TGF-β1–induced EMT and migration and can be a target of kaempferol.

In the canonical TGF-β signaling pathway, activation of Smad3 by TGF-β1 is a multistep process involving its phosphorylation in the COOH-terminal SSXS motif, complex formation with Smad4, and translocation into the nucleus [13]. We therefore evaluated the effect of kaempferol on TGF-β1–induced Smad3 activation in A549 cells. Interestingly, no difference on TGF-β1–induced Smad3 C-terminus phosphorylation was observed in cells pretreated with kaempferol compared with kaempferol-untreated cells (Figure 4, A and B). Immunoprecipitation followed by Western blotting also showed that TGF-β1–induced endogenous Smad3/Smad4 complex formation was not inhibited by treatment with kaempferol.

**Figure 3.** Kaempferol inhibits TGF-β1–induced Smad3-mediated Snail induction and E-cadherin repression in A549 lung cancer cells. (A) A549 cells were transfected with control (siCon), Smad2 (siS2), or Smad3 (siS3) siRNAs for 24 hours before 5 ng/ml of TGF-β1 treatment for 48 hours, after which the expressions of E-cadherin, Snail, Smad2, and Smad3 were determined by Western blot analysis. β-Actin levels were monitored as a loading control for whole extracts. (B) A549 cells were transiently co-transfected with Snail promoter-reporter construct (Snail-Luc) and vector for Smad2-3SA (S23SA), Smad3-3SA (S33SA), or the corresponding empty vector for 16 hours and then stimulated with 5 ng/ml of TGF-β1 for 24 hours. All cells were then assayed for relative luciferase activity. All quantitative data are the mean ± SD of three independent experiments. **P < .01. (C) A549 cells were transfected with control (siCont) or Smad3 (siS3) siRNAs for 24 hours before treatment with 5 ng/ml of TGF-β1 for 3 hours, after which the expressions of Snail and Smad3 mRNAs were determined by RT-PCR analysis. β-Actin levels were monitored as a loading control for total RNA. (D) A549 cells were pretreated with DMSO, 1 μM of SB431542, or 25 μM of KF for 30 minutes before treatment with 5 ng/ml of TGF-β1 for 24 hours and then subjected to ChIP assay using anti-Smad3 antibody. The purified DNA was analyzed by RT-PCR using primers spanning the Smad3-binding sites at Snail promoter. (E) A549 cells were pretreated with DMSO or 25 μM of KF for 30 minutes before treatment with 5 ng/ml of TGF-β1 for 3 hours. Expression of Snail mRNA was analyzed as in C. β-Actin levels were monitored as a loading control for total RNA.
Kaempferol Inhibits TGF-β1–Induced EMT and Cell Migration Through Downregulation of the Phosphorylation of Smad3 at Thr179 in the Linker Region

There is a growing body of evidence pointing to an important role of Smad3 linker region phosphorylation (pSmad3L) in the regulation of Smad3 function under physiologic and pathologic conditions [15,20]. To verify the requirement of pSmad3L in TGF-β1–mediated induction of EMT and migration of A549 cells, we used recombinant lentivirus constitutively expressing Smad3 EPSM mutant, in which all four phosphorylation sites of linker region, including Thr179, Ser203, Ser208, and Ser213, are replaced with Val or Ala (Figure 5A). Western blotting and immunofluorescence staining revealed that lentivirus-mediated overexpression of Smad3 EPSM greatly reversed the changes in epithelial and mesenchymal marker expression induced by TGF-β1 (Figure 5, B and C). TGF-β1–induced cell migration was also significantly attenuated in cells infected with lentivirus carrying Smad3 EPSM gene compared with cells infected with lentivirus carrying empty vector or Smad3 WT gene (Figure 5D). In addition, TGF-β1–mediated increases of secreted MMP2 (Figure 5E) and MMP2 gene promoter activity (Figure 5F) were fully impaired by overexpression of Smad3 EPSM but not by overexpression of Smad3 WT. These results indicate that pSmad3L is critically required for metastatic responses induced by TGF-β1 in A549 cells.

We next evaluated the effects of TGF-β1 and kaempferol on pSmad3L in A549 cells. As expected, treatment with TGF-β1 strongly induced the phosphorylation of Smad3 at Thr179, Ser203, Ser208, and Ser213 in the linker region (Figure 6, A and B). Interestingly, we found that kaempferol specifically inhibited TGF-β1–induced pSmad3L at Thr179 without affecting the phosphorylation of the remaining linker region sites (Figure 6, A and B). To assess the role of Smad3 phosphorylation at Thr179 in TGF-β1–induced EMT and migration of A549 cells, we used recombinant lentivirus constitutively expressing Smad3 Thr179V mutant, in which Thr179 phosphorylation site is replaced with Val. Western blotting (Figure 6C) and immunofluorescence staining (Figure 6D) of E-cadherin showed that TGF-β1–induced repression of E-cadherin was significantly abolished in cells infected with lentivirus carrying Smad3 Thr179V gene compared with cells infected with empty vector lentivirus. Consistent with this result, TGF-β1–mediated suppression of E-cadherin promoter activity was markedly rescues by co-transfection with Smad3 Thr179V, but not with Smad3 WT, plasmid DNA (Figure 6E). In line with these results, ChIP assay revealed that TGF-β1–induced enrichment of Smad3 on the -616/-623 region of Snail promoter was completely abolished in cells infected with lentivirus carrying Smad3 Thr179V.
mutant gene compared with cells infected with lentivirus carrying empty vector or Smad3 wild-type gene (Figure 6F). In parallel, TGF-β1–induced Snail promoter activity was markedly decreased by overexpression of Smad3T179V, but not of Smad3WT, plasmid DNA (Figure 6G). Furthermore, lentivirus-mediated ectopic expression of Smad3 linker mutant in A549 cells resulted in significant attenuation of cell migration (Figure 6H) induced by TGF-β1. TGF-β1–induced increases of MMP2 gene promoter activity were also fully impaired by overexpression of Smad3T179V (Figure 6I). These results clearly suggest that the ablation of Smad3 phosphor-ylation at Thr179 is responsible for the suppression by kaempferol of TGF-β1–induced EMT, cell migration, and MMP2 activation in A549 lung cancer cells.

**Kaempferol Suppresses TGF-β1–Induced pSmad3L at Thr179 and Metastatic Responses Through Akt Inhibition**

Previous study had identified PI3K as a direct target molecule for the inhibitory effects of kaempferol on neoplastic cell transformation [25]. It has been also reported that Akt, a downstream effector of PI3K, plays an important role in the regulation of TGF-β signaling through cross talk with canonical Smad pathway [33]. These prompted us to ask whether the ablation of PI3K-Akt pathway by kaempferol is responsible for the inhibition of TGF-β1–induced pSmad3L and metastatic cellular responses. Treatment of A549 cells with TGF-β1 resulted in rapid phosphorylation of Akt, but the effect was completely abrogated by kaempferol (Figure 7A). Pretreatment with AktIV, a specific inhibitor of Akt, completely reversed changes in TGF-β1–induced EMT marker expression in a concentration-dependent manner (Figure 7B). Consistent with this result, TβRI(T204D)-induced repression of E-cadherin promoter activity was significantly rescued by co-transfection with dominant negative forms of p85α or Akt (Figure 7C). ChIP and RT-PCR also revealed that TGF-β1–induced Smad3 binding to the Snail promoter (Figure 7D) and Snail expression at mRNA level (Figure 7E) were effectively weakened by treatment with AktIV or LY294002, a specific inhibitor of PI3K. In addition, treatment with AktIV or LY294002 resulted in remarkable attenuation in TGF-β1–induced cell migration (Figure 7F), and TGF-β1–mediated increase of MMP2 gene promoter activity was also significantly abolished by co-transfection with dominant negative forms of p85αΔSH2-N or Akt1K179M (Figure 7G), indicating a crucial involvement of PI3K-Akt axis in metastatic TGF-β signaling pathway.
We further addressed whether Akt activation is necessary for TGF-β1–induced phosphorylation of Smad3 at Thr179. Similar to the kaempferol effect, treatment with AktIV resulted in selective inhibition of TGF-β1–induced phosphorylation of Smad3 at Thr179 compared with expression of control counterpart (Figure 8A). In contrast, overexpression of constitutively active form of Akt alone resulted in an increase of phosphorylation of Smad3 at Thr179 compared with expression of control counterpart (Figure 8B). Treatment with TGF-β1 resulted in increase of Akt binding with Smad3, and the binding reached a peak at 30 minutes and then declined to baseline at 60 minutes (Figure 8C). The TGF-β1–induced binding of Akt with Smad3 was completely abolished by pretreatment with kaempferol (Figure 8C). Given these findings that Akt controls phosphorylation of Smad3 at Thr179, we next assessed whether Akt-mediated phosphorylation of Smad3 at Thr179 would be sufficient to drive TGF-β1–induced repression of E-cadherin promoter activity. Reporter gene assay revealed that TGF-β1–induced repression of E-cadherin promoter activity was further augmented in cells transfected with constitutively active forms of p110αE227E or Akt1E40K compared with that in cells transfected with empty vector. In contrast, in cells co-transfected with Smad3T179V and p110αE227E or Akt1E40K, TGFRII204D–induced repression of E-cadherin promoter activity was significantly rescued compared with that in cells.
co-transfected with control vector (Figure 8D), demonstrating Akt1 as a key mediator in TGF-β signaling pathway leading to E-cadherin repression through inducing the phosphorylation of Smad3 at Thr179.

**Discussion**

Although kaempferol has been extensively studied as a natural flavonoid that possesses chemopreventive effect on cancer, its antimetastatic effect has been less addressed. The enhancement of EMT in cancer cells is known to increase the risk of metastasis and closely associated with poor prognosis of many types of cancer. Fibroblast growth factor (FGF) signaling is important in promotion of EMT and invasion of cancer cells [34]. Kaempferol inhibits tyrosine phosphorylation of FGF receptor by FGF stimulation [35]. The study by Huang et al suggested that estrogen-related receptor (ERR) α promotes the migration and invasion of lung cancer A549 cells by inducing EMT [36]. Kaempferol was reported to inhibit cancer cell growth by antagonizing ERRα and ERRγ activities [37]. These findings strongly suggest that kaempferol may exert its antimetastatic activity through targeting key components of several signaling pathways involved in tumor metastasis. TGF-β1 potently induces EMT in epithelial tumor cells, conferring them migratory and invasive properties. Our data showed that kaempferol strongly inhibited TGF-β1–induced EMT, migration, and MMP-2 activation in human A549 lung cancer cells, which strongly support its potential as an antitumor agent that suppresses tumor metastasis.

Smad2 and Smad3 are key mediators for TGF-β signaling in both normal and pathological conditions. It has been established that Smad2 and Smad3 have distinct functions in TGF-β1–induced cellular responses. For example, Smad3 is critical to inducing TGF-β1–mediated growth inhibition [38,39], whereas Smad2 is uniquely essential for TGF-β signaling in CD4 T cell differentiation.
Our data demonstrated that Smad3, but not Smad2, was required for TGF-β1–induced E-cadherin repression and up-regulation of snail gene expression in A549 lung cancer cells. It was also notable that kaempferol significantly inhibited Smad3 binding to the promoter of snail gene and the induction of snail expression upon TGF-β1 stimulation, whereas it did not affect the canonical activation of Smad3 (C-terminal phosphorylation, complex formation with Smad4, and translocation into the nucleus) induced by TGF-β1. These results imply the possibility that Smad3 activity can be inhibited noncanonically by kaempferol before or after it enters the nucleus in response to TGF-β1.

Smad3 can be regulated noncanonically through phosphorylation of its linker region. The recent discovery of C-terminal domain phosphatase 1 as a specific phosphatase for Smad3 dephosphorylation in the linker region [41,42] highlights the need to consider Smad-linker phosphorylation as a reversible and tightly controlled TGF-β signaling event. Actually, the study by Matsuura et al. demonstrated that phosphorylation of Smad3 linker region by cyclin-dependent kinase inhibits its antiproliferative function, thus facilitating cell cycle progression [43]. Furthermore, increased phosphorylation of Smad3 linker region has been identified in many types of cancer cells and was proven to contribute to the TGF-β1–mediated signaling events to facilitate protumorigenic outcomes [44,45]. In line with these observations, our data demonstrated that TGF-β1 induced Smad3 linker phosphorylation, and Smad3 mutant lacking linker phosphorylation sites could reverse metastatic responses to TGF-β1 in A549 lung cancer cells. A remarkable finding is that the inhibitory effect of kaempferol on increased Smad3 linker phosphorylation by TGF-β1 treatment was specifically limited to Thr179 residue, but not to other linker sites, and it was sufficient to reverse TGF-β1–induced EMT and migration in A549 cells by expression of Smad3T179V alone. TGF-β1–induced Smad3 binding to the Snail promoter and subsequent repression of E-cadherin were also dependent on Smad3 phosphorylation at Thr-179. However, TGF-β1–induced p21WAF1 expression and cell cycle arrest in A549 cells were not affected by kaempferol treatment and overexpression of Smad3T179V (Supplementary Figure S1, A and B). These results newly characterized an important role of the Thr179 phosphorylated form of Smad3 in the metastatic TGF-β signaling in A549 lung cancer cells. However, we do not exclude the possibility that other Smad3 linker sites for phosphorylation could contribute to TGF-β1–induced metastatic responses.

A growing body of evidence has shown that the linker domain of Smad3 undergoes regulatory phosphorylation by various intracellular signaling kinases, including mitogen-activated protein kinase, cyclin-dependent kinase, glycogen synthase kinase 3-β, and protein kinase A [40].
kinase C, in normal and pathological conditions [46]. Because a of TGF-

EMT and cell migration in human lung cancer cell by targeting phosphorylation of Smad3 linker region. The presenting data also highlight a loss of PI3K/Akt-mediated Smad3 phosphorylation at Thr179 as a key mechanism of inhibition by kaempferol of oncogenic TGF-β1 signaling (Figure 9) and suggest that the pharmacologic inhibition of Smad3 phosphorylation at Thr179 can be an effective strategy to impede lung cancer progression.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2015.06.004.

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