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RESEARCH ARTICLE

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# Toosendanin, a natural product, inhibited TGF-β1-induced epithelial-mesenchymal transition through ERK/Snail pathway

Weiwei Luo | Xin Liu* | Wen Sun | Jin-Jian Lu | Yitao Wang | Xiuping Chen

State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau, China

**Correspondence**
Prof. Yitao Wang and Dr. Xiuping Chen, Institute of Chinese Medical Sciences, University of Macau, Avenida da Universidade, Taipa, Macau, China.
Email: ytwang@umac.mo; xpchen@umac.mo

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## Abstract

Epithelial-mesenchymal transition (EMT) plays important roles in the metastasis of solid tumors. In this study, the effect of toosendanin (TSN), a natural insecticide extracted from *Melia toosendan* Sieb et Zucc, on transforming growth factor-β1 (TGF-β1)-induced EMT was investigated. EMT was induced by TGF-β1 in A549 and H1975 lung cancer cells. The morphological alterations were observed with a microscopy. The protein expression and localization of EMT biomarkers were determined by Western blotting and immunofluorescence. The migration, invasion, and adhesion were determined by wound-healing, transwell, and adhesion assays. TGF-β1 treatment induced spindle-shaped alterations of cells, upregulation of N-cadherin, Vimentin, p-ERK1/2, and downregulation of E-cadherin. The abilities of migration, invasion, and adhesion were also enhanced. These effects were significantly reversed by TSN at very low concentration (<10 nM). Furthermore, silence Snail significantly reversed TGF-β1-induced EMT biomarkers. In addition, TGF-β1-induced phosphorylation of ERK1/2 without affecting p38 mitogen-activated protein kinases and Jun N-terminal kinase. PD98059 and U0126, inhibitors of ERK1/2, showed similar inhibitory effect to that of TSN. In summary, TSN significantly inhibited TGF-β1-induced EMT and migration, invasion, and adhesion through ERK/Snail pathway in lung cancer cells. This study provides novel anticancer effects and molecular mechanisms for TSN.

**KEYWORDS**

EMT, ERK1/2, lung cancer, Snail, toosendanin

## 1 | INTRODUCTION

Metastasis, one of the essential hallmarks of cancer, is the leading cause of cancer-related mortality world widely. Lung cancer, the most common cancer, could easily migrate to other organs such as brain, bone (D'Antonio et al., 2014; Hanibuchi, Kim, Fidler, & Nishioka, 2014). Metastasis is a rather complex process which requires several fundamental mechanisms such as angiogenesis, matrix barriers degradation, extravasation, and intravasation (Perlikos, Harrington, & Syrigos, 2013). Epithelial-mesenchymal transition (EMT) is a biologic process, during which polarized, immotile epithelial cells epithelial polarized cells convert to motile mesenchymal-appearing cells. Morphologically, EMT is characterized by the loss of cell-to-cell adhesion and cell polarity, the increased cell motility and invasiveness. Biochemically, decreased epithelial biomarkers (such as E-cadherin, and Claudin) and acquired mesenchymal biomarkers (such as Vimentin and N-cadherin) and cytoskeleton alterations as well as activation of a panel of transcription factor families (such as Snail, Twist, and Zeb) have been identified during the EMT process (Moustakas & Heldin, 2016; Puisieux, Brabletz, & Caramel, 2014; Yang & Weinberg, 2008). A plethora of experimental results have suggested that EMT is potential crucial driver of cancer progression by favoring metastatic, which provide novel strategies and targets for the antimetastatic therapy (Kaufhold & Bonavida, 2014; Khan, Chen, Zhang, & Fu,

*Co-first author

  

2013; Rafael et al., 2015). Furthermore, sufficient amounts of natural products demonstrate promising anti-metastasis activities by, at least in part, suppressing EMT (Chanvorachote, Chamni, Ninsontia, & Phiboonchaiyanan, 2016).

Toosendanin (TSN) is a natural triterpenoid isolated from *Melia toosendan* Sieb et Zucc, the fruits of which were used as anthelmintic in Traditional Chinese Medicine. TSN was used as an insect antifeedant to prevent and control of agricultural pests in China (Ma, Gulia-Nuss, Zhang, & Brown, 2013). In 1950s, TSN was the main component of an anti-roundworm drug prescribed in clinical in China. Especially, it showed potent inhibitory effects on the toxicity of botulinum toxin (Shi & Wang, 2004). Recently, TSN was reported to possess anticancer activities by inducing apoptosis in several types of cancer line cells, such as breast cancer MDA-MB-231 cells, hepatoma carcinoma Bel7402 cells, and promylocytic leukemia HL-60 cells (He et al., 2010; Ju et al., 2012; Zhang, Wang, Tang, & Shi, 2005). However, its effect on metastasis remains to be cleared. In this study, we reported that TSN inhibited transforming growth factor-β1 (TGF-β1)-induced EMT and adhesion, migration, and invasion in A549 and H1975 lung cancer cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents and antibodies

TSN (>98%) was purchased from Chengdu Preferred Biotech Co. Ltd. (Chengdu, China). Human recombinant TGF-β1 purchased from Cell Signaling Technology (Danvers, MA, USA) was activated and stored in accordance with the manufacturer's instructions. RPMI 1640, fetal

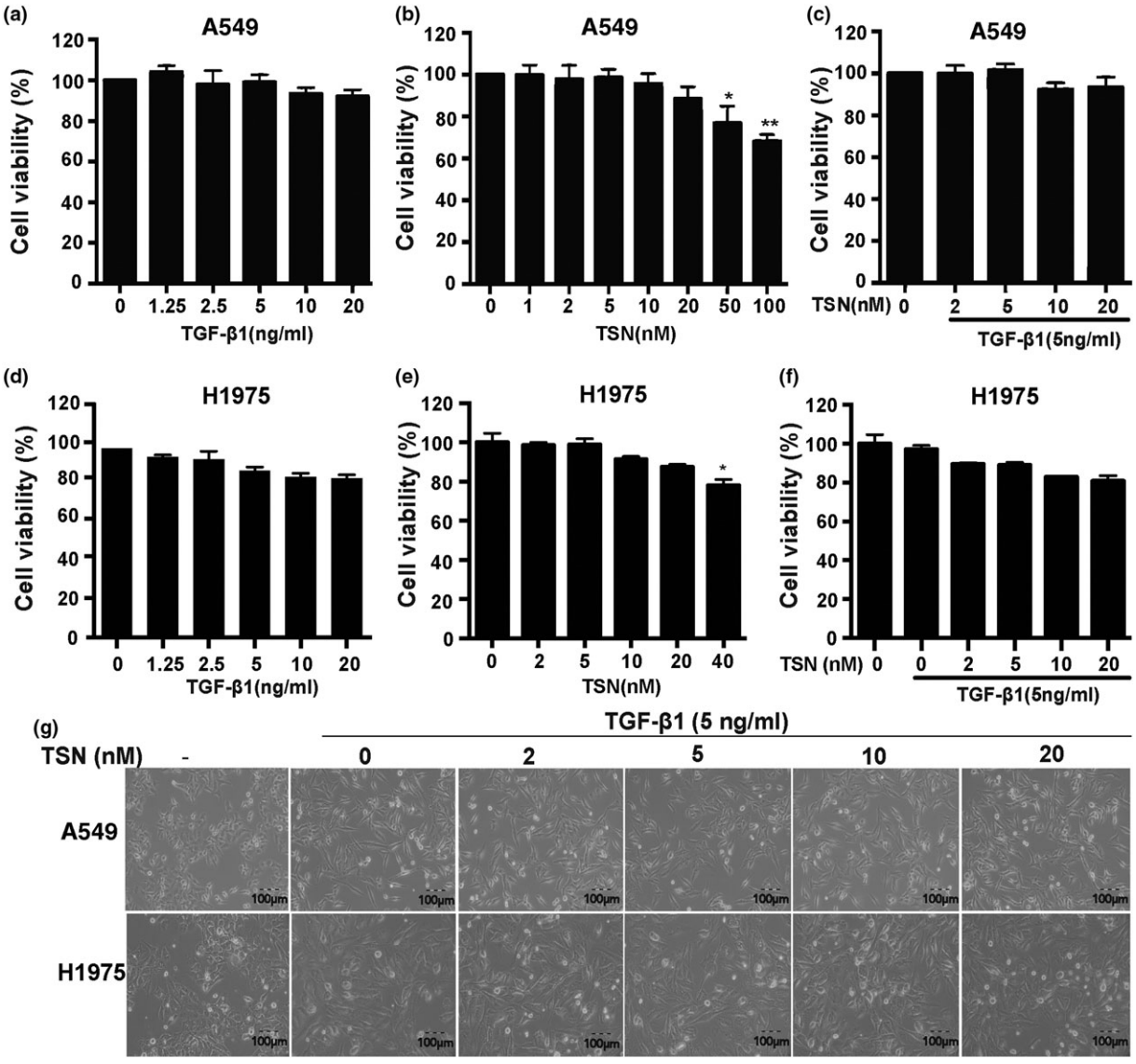


**FIGURE 1** The cytotoxic effect of TSN, TGF-β1, and the inhibitory effect of TSN on TGF-β1-induced morphological changes in lung cancer cells. A549 and H1975 cells were treated with TGF-β1 (a and d) or TSN (b and e) or TGF-β1 plus TSN (c and f) for 48 hr. The cell viability was determined by MTT assay. Cells were treated with TGF-β1 (5 ng/ml) with or without TSN co-treatment for 48 hr, the morphological changes of cells were observed (g). $^{*}p < 0.05$ versus control, $^{**}p < 0.01$ versus control. TSN: toosendanin; TGF-β1: transforming growth factor-β1 [Colour figure can be viewed at wileyonlinelibrary.com]

bovine serum (FBS), penicillin–streptomycin solution, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide, and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific antibodies against E-Cadherin, N-Cadherin, Vimentin, phosphorylated ERK1/2 (Thr202/Tyr204; p-ERK), phosphorylated c-Jun N-terminal kinase (JNK; Thr183/Tyr185; p-JNK), phosphorylated p38 mitogen-activated protein kinases (p38MAPK; Thr180/Tyr182; p-p38), and glyceraldehyde 3-phosphate dehydrogenase were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for Snail and Slug were purchased from Abcam (Cambridge, UK). Matrigel, fibronectin, and Type I collagen were purchased from BD Biosciences (Sparks Glencoe, MD, USA).

## 2.2 | Cell culture

Human lung cells lines A549 and H1975 obtained from American Type Culture Collection (ATCC, USA) were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin solution at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

## 2.3 | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells ($5 \times 10^3$ cells/well) cultured in 96-well plates were treated with TGF-β1 (0–20 ng/ml), TSN (0–100 nM), or TSN (0–20 nM) plus TGF-β1 (5 ng/ml) for 48 hr. Then, 100 μl of MTT mixture was added and co-cultured for another 4 hr. The formazan was solubilized with 100 μL dimethylsulfoxide, and the absorbance at 570 nm was determined using a Multilabel counter (Perkin Elmer, Singapore).

## 2.4 | Morphology observation

Cells ($1 \times 10^5$ cells/well) seeded in 12-well plates were treated with TGF-β1 (5 ng/ml) alone or in combination with TSN (0–20 nM) for 48 hr. The cells' morphology was captured by Olympus IX73 microscope (Japan).

## 2.5 | Western blotting assay

Cells were treated with TGF-β1 (5 ng/ml) alone or in combination with TSN (0–20 nM), PD98059 (25 μM), or U0126 (10 μM) for 48 hr. Then, the cells were harvested, and the total protein was extracted. BCA™ Protein Assay Kit was used to determine the protein content in each sample. Thirty micrograms of proteins from each sample were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc). After blocking with 5% nonfat milk in Tris-buffered saline with tween at room temperature for 1 hr, membranes were incubated with specific primary and secondary antibodies. The protein–antibody complexes were detected using an ECL Advanced Western Blot Detection Kit.

## 2.6 | Wound healing assay

Cells ($1 \times 10^6$ cells/well) were seeded in six-well plates and cultured overnight. The monolayers were gently and slowly scratched with a new pipette tip across the center of the well. After washed with PBS for twice, cells were treated with or without TGF-β1 (5 ng/ml) alone or in combination with TSN (0–20 nM), PD98059 (25 μM), or U0126 (10 μM) for another 48 hr. Images were taken by Olympus IX73 microscope at 0 and 48 hr.

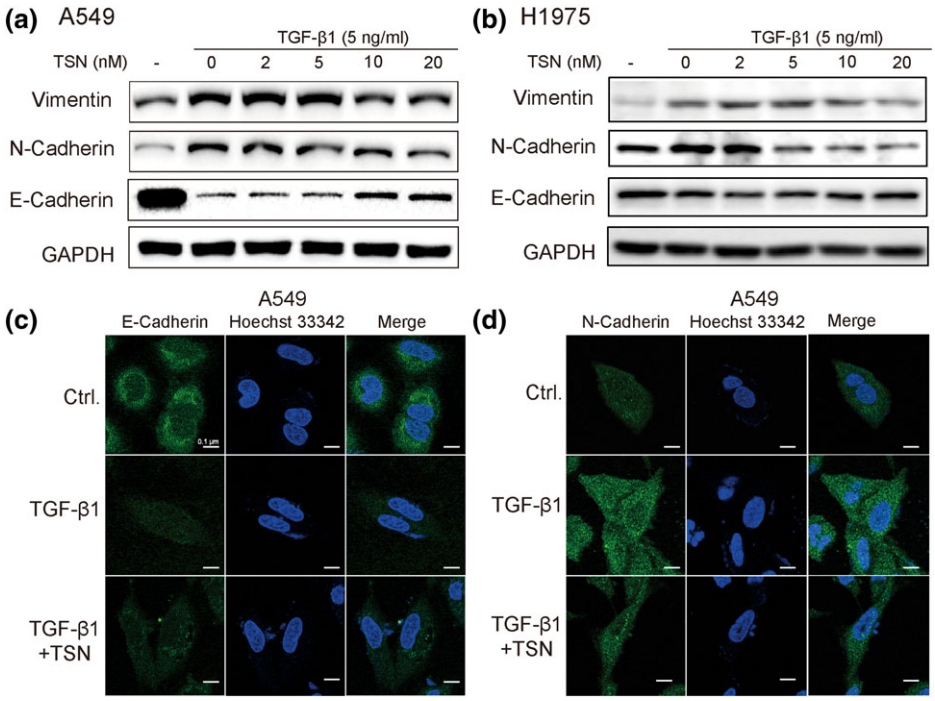


**FIGURE 2** Effect of TSN on expression of epithelial-mesenchymal transition biomarkers. A549 and H1975 cells were treated with TGF-β1 with or without TSN co-treatment for 48 hr. The protein expression was determined by Western blotting (a and b) and the localization of E-cadherin (c) and N-cadherin (d) in A549 cells was determined by immunofluorescence staining. TSN: toosendanin; TGF-β1: transforming growth factor-β1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase [Colour figure can be viewed at wileyonlinelibrary.com]

## 2.7 | Transwell assay

The migration and invasion were detected by transwell assay. Cells were digested by trypsin and diluted by 300 μl medium supplemented with 10% FBS and TGF-β1 (5 ng/ml) and then seeded in the transwell inserts ($1 \times 10^5$ cells/well). The 24-well plates were added with 500 μl medium supplemented with 10% FBS and TSN (0–20 nM) per well. Then, the transwell inserts and the 24-well plates were incubated with 5% $CO_2$ at 37°C together for 48 hr.

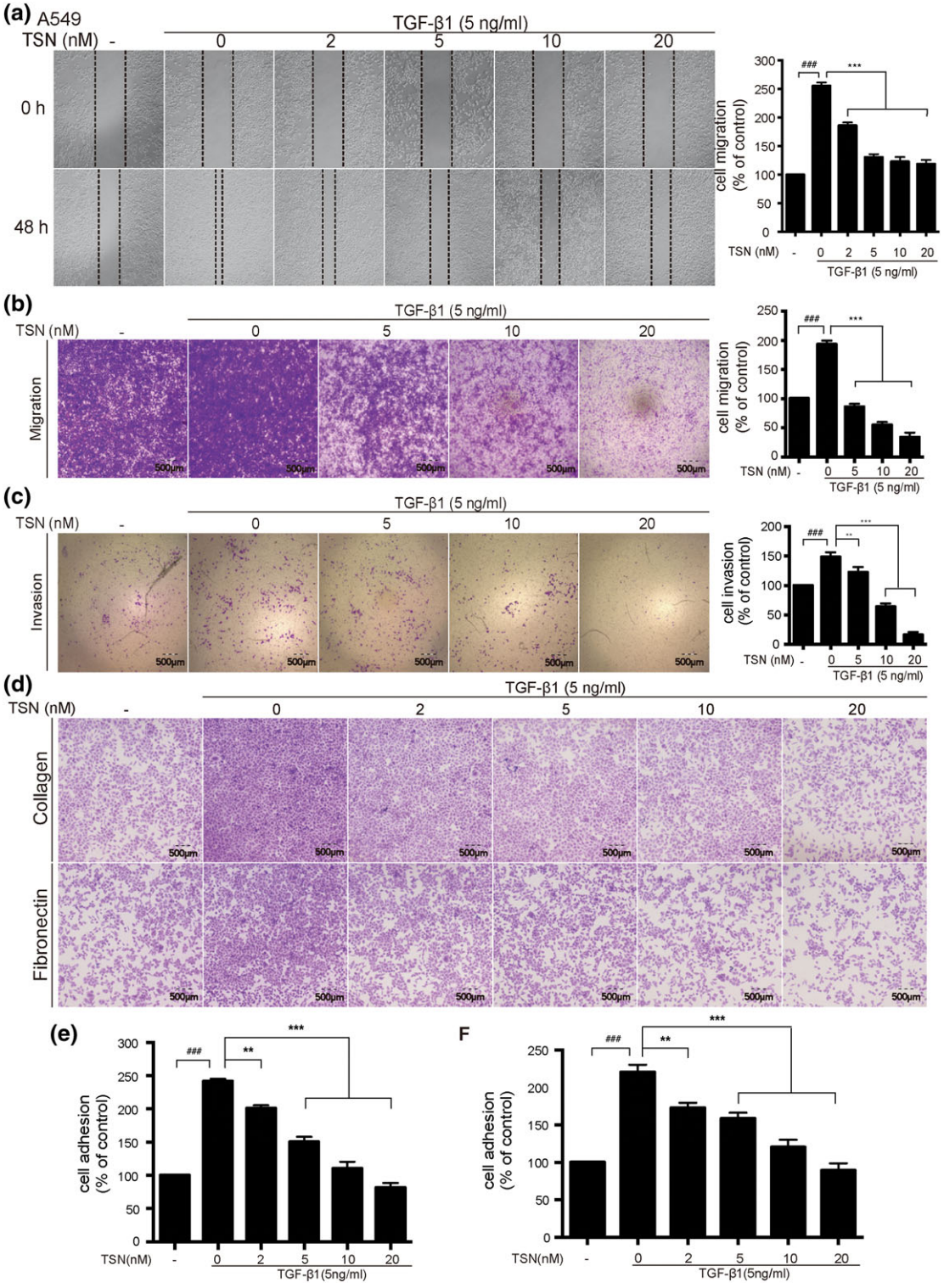


**FIGURE 3** TSN inhibited TGF-β1-induced migration, invasion, and adhesion in A549 cells. A549 cells were treated with TGF-β1 with or without TSN co-treatment for 48 hr. The migration and invasion capacities were measured by the wound healing (a) and transwell (b and c) assays, respectively. The adhesion capacities to Type I collagen (d and e) and fibronectin (d and f) were measured by the adhesion assays. $^{\#\#}p < 0.01$, $^{**}p < 0.01$, and $^{\#\#\#}p < 0.005$, $^{***}p < 0.005$. TSN: toosendanin; TGF-β1: transforming growth factor-β1 [Colour figure can be viewed at wileyonlinelibrary.com]

For invasion assay, matrigel (1:12) was plated at the bottom of the inserts. Cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. Images were taken by Olympus IX73 microscope after 48 hr, and the migrated cells were counted.

## 2.8 | Adhesion assay

Cells ($1 \times 10^6$ cells/well) cultured in six-well plates were co-treated with TGF-β1 (0–20 nM), PD98059 (25 μM), or U0126 (10 μM) for 48 hr. Then, cells were collected and cultured in fibronectin or Type

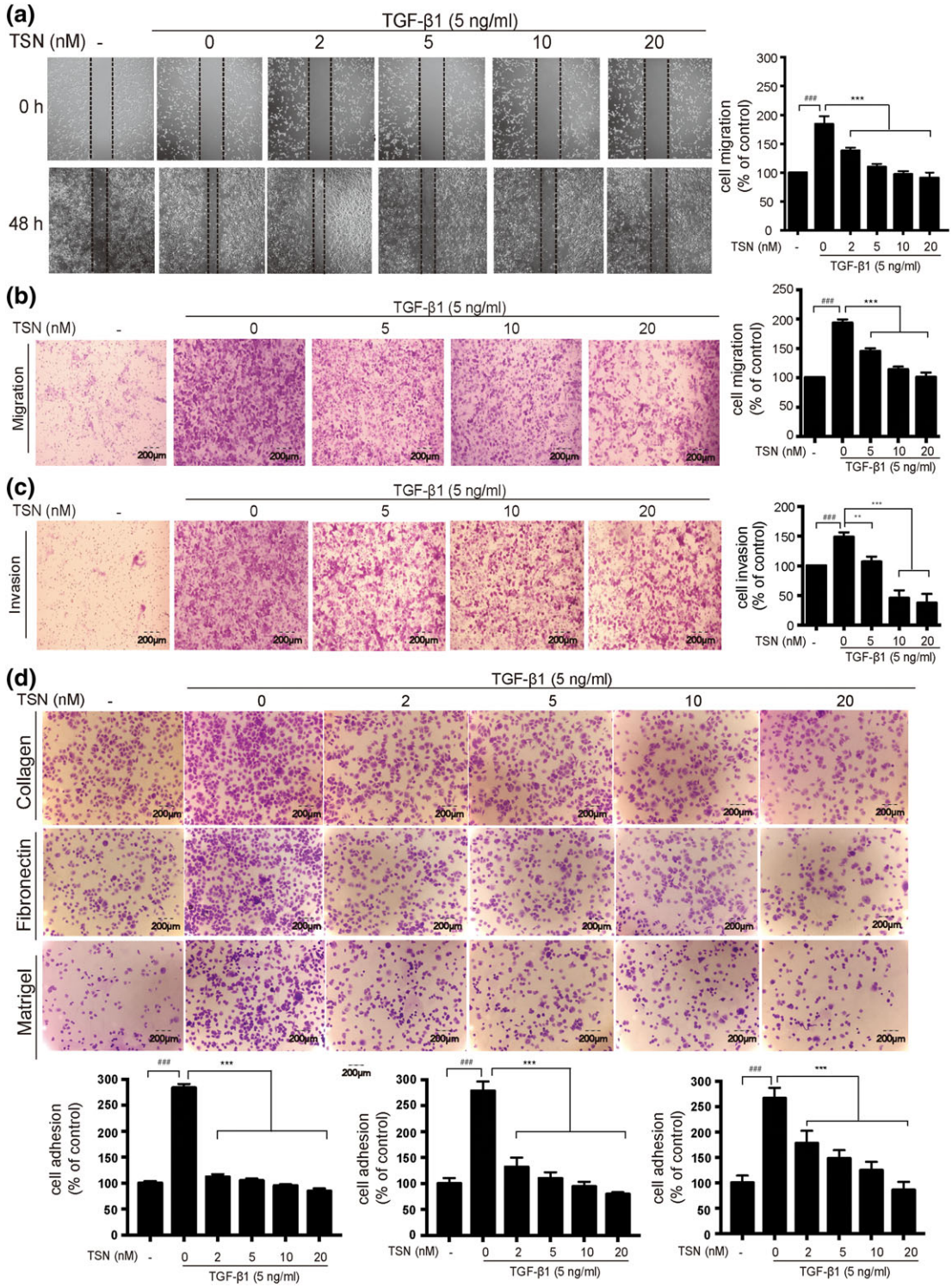


**FIGURE 4** TSN inhibited TGF-β1-induced migration, invasion, and adhesion in H1975 cells. H1975 cells were treated with TGF-β1 with or without TSN co-treatment for 48 hr. The migration and invasion capacities were measured by the wound healing (a) and transwell (b and c) assays, respectively. The adhesion capacities to Type I collagen, fibronectin, and matrigel (d) were measured by the adhesion assays. $^{\#\#}p < 0.01$, $^{**}p < 0.01$, and $^{\#\#\#}p < 0.005$, $^{***}p < 0.005$. TSN: toosendanin; TGF-β1: transforming growth factor-β1 [Colour figure can be viewed at wileyonlinelibrary.com]

I collagen precoated 96-well plates for 2 hr. After gently rinsed with PBS, the adhered cells were dyed with crystal violet, and images were taken by Olympus IX73 microscope.

### 2.9 | Immunofluorescence assay

Cells ($1 \times 10^5$ cells/well) were seeded in confocal dishes. After co-treatment with TGF-β1 and TSN as described above, slides were fixed with 4% paraformaldehyde at room temperature for 30 min. Slides were then permeabilized with PBS-T (containing 0.1% Triton x-100 in PBS solution) and blocked with PBS-B (containing 4% BSA in PBS solution). After consecutive incubation with the primary antibody (E-cadherin and N-cadherin, 1:500) overnight at 4°C and the secondary antibody (1:1,000) for 1 hr at room temperature, nucleus were stained with Hoechst 33342 in the dark for 30 min. Fluorescence images were taken using confocal system.

### 2.10 | Small interfering RNA (siRNA) silence

Cells were seeded at the density of $1 \times 10^6$ per well in six-well plates for overnight. Lipofectamine 3000 reagent was used to transfer siRNA for silencing Snail following manufacturer's instructions. The siRNA sequences for Snail were 5′-CAGAUGUCAAGAAGUACCATT-3′ and 5′-UGGUACUUCUUGACAUCUGTT-3′. The negative control sequences were 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3.

### 2.11 | Statistical analysis

Data were expressed as the means ± SD. The differences between groups were analyzed using Prism 5.0 (Graph Pad Software Inc, San Diego, CA), and the statistical analysis was performed by analysis of variance (one-way ANOVA) followed by Student Newman–Keuls test. $P < 0.05$ is considered statistically significant.

## 3 | RESULTS

### 3.1 | Toosendanin reversed transforming growth factor-β1-induced morphological changes

In order to exclude the impact of cell death, the cytotoxicity of TSN and TGF-β1 alone or in combination was examined. As shown in Figure 1a,d, TGF-β1 (0–20 ng/ml) showed no significant cytotoxicity to either A549 or H1975 cells as determined by MTT assay after 48 hr treatment. TSN showed no significant cytotoxicity at 20 nM in both A549 and H1975 cells but slightly decreased cell viability at 50 and 40 nM, respectively, after 48 hr treatment (Figure 1b,e). Thus, noncytotoxicity concentrations were chosen for the following experiments. Furthermore, combined TSN with TGF-β1 showed no obvious effect on cell viability (Figure 1c,f). TGF-β1 remarkably induced morphological changes of both A549 cells and H1975 cells. The spindle-like appearances were disappeared, whereas the fibroblast-like appearances with longed shape were observed. These morphological changes were partially reversed by TSN co-treatment in a concentration dependent manner (Figure 1g).

### 3.2 | Toosendanin reversed transforming growth factor-β1-induced expression of epithelial-mesenchymal transition biomarkers

In A549 and H1975 cells, TGF-β1 significantly inhibited the protein expression of epithelial marker E-cadherin and increased the mesenchymal marker N-cadherin and Vimentin, which was significantly reversed by TSN co-treatment in a concentration-dependent manner (Figures 2a,b). Immunofluorescence results from A549 cells showed that decreased green fluorescence was observed in TGF-β1-treated group suggesting the decreased expression of E-cadherin. TSN co-treatment partially increased the green fluorescence indicating the reversal effect of TSN on E-cadherin (Figure 2c). Similar reversal effect

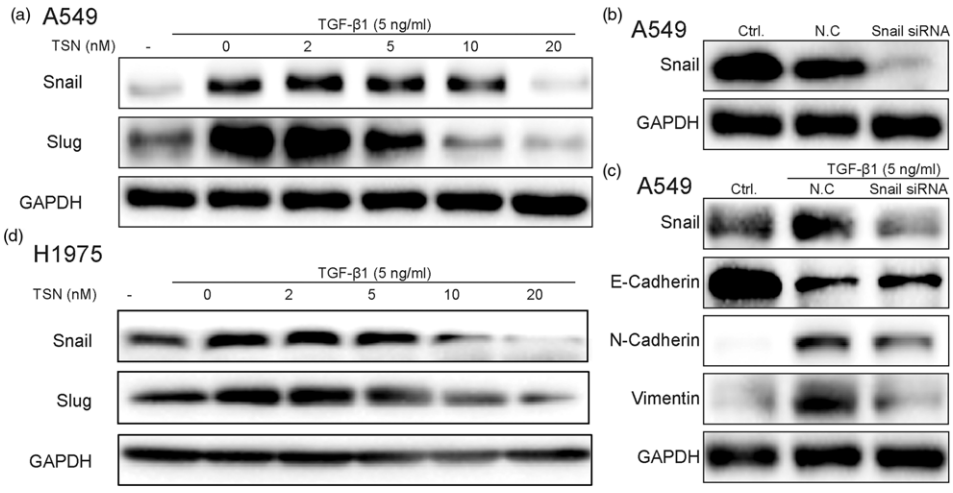


**FIGURE 5** TSN inhibited TGF-β1-induced epithelial-mesenchymal transition mediated by Snail. A549 and H1975 cells were treated with TGF-β1 with or without TSN co-treatment for 48 hr. The protein expression of Snail and Slug was determined by Western blotting (a and d). Snail was silenced with siRNA (b) and cells were treated with TGF-β1 (5 ng/ml) for 48 hr, and the protein expression was determined by Western blotting in A549 cells (c). TSN: toosendanin; TGF-β1: transforming growth factor-β1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; siRNA: small interfering RNA [Colour figure can be viewed at wileyonlinelibrary.com]

of TSN was observed on TGF-β1-induced upregulation of N-cadherin (Figure 2d).

### 3.3 | Toosendanin suppressed transforming growth factor-β1-induced migration and invasion

As shown in Figures 3a and 4a, TGF-β1 significantly induced the migration of A549 and H975 cells in the wound healing migration assay as evidenced by the narrowed healing borders, while TSN co-treatment dramatically reversed this phenomenon. To confirm the reversible effect of TSN on TGF-β1-induced migration, transwell migration assay was performed. Results showed that the migrations of A549 and H1975 cells were also remarkably increased by TGF-β1, which were significantly reversed by TSN co-treatment (Figures 3b and 4b). The invasion transwell assay results showed that TGF-β1 obviously increased the number of invaded cells, which could be

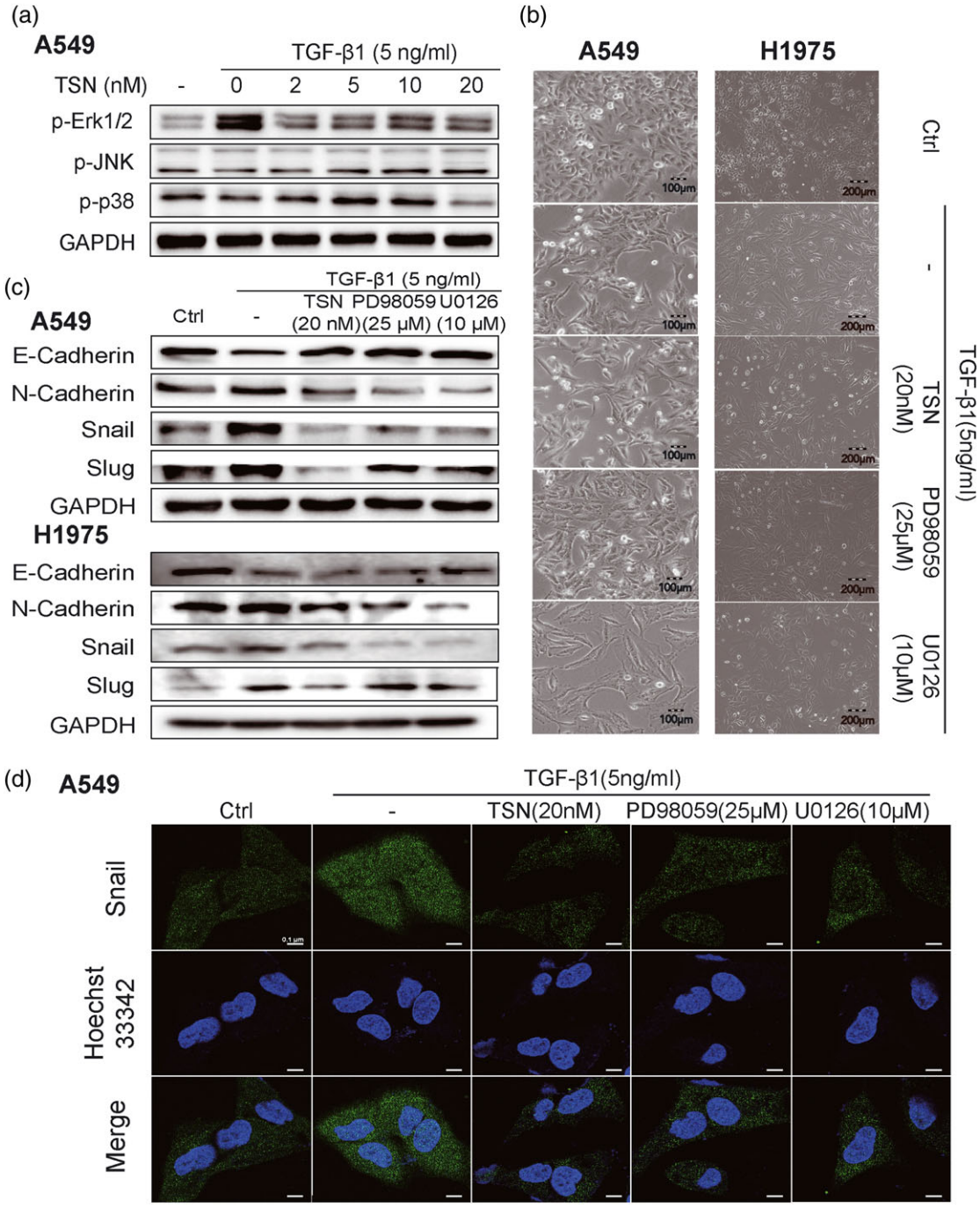


**FIGURE 6** The inhibitory effect of TSN on TGF-β1-induced epithelial-mesenchymal transition was mediated by Erk1/2 and Snail. A549 cells were treated with TGF-β1 with or without TSN co-treatment for 48 hr, then the protein expression of p-ERK1/2, p-JNK, and p-p38 was determined by Western blotting (a). A549 and H1975 cells were treated with TGF-β1 with or without TSN, PD98059 or U0126 co-treatment for 48 hr. The cell morphology was observed (b), and the protein expression was determined by Western blotting (c). Immunofluorescence staining was performed for detecting the Snail localization in A549 cells (d). TSN: toosendanin; TGF-β1: transforming growth factor-β1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase [Colour figure can be viewed at wileyonlinelibrary.com]

inhibited by TSN co-treatment in a concentration dependent manner (Figures 3c and 4c).

### 3.4 | Toosendanin inhibited transforming growth factor-β1-induced adhesion to Type I collagen and fibronectin

As shown in Figure 3d, compared with control group, TGF-β1 treatment dramatically enhanced the adhesion of A549 cells to Type I collagen and fibronectin. Meanwhile, it also enhanced the adhesion of H1975 cells to Type I collagen, fibronectin, and matrigel (Figure 4d). TSN co-treatment could significantly reverse TGF-β1-induced adhesion in a concentration-dependent manner in both lung cancer cell lines.

### 3.5 | Snail was involved in the suppression of transforming growth factor-β1-induced epithelial-mesenchymal transition by toosendanin

In both A549 and H1975 cells, compared with the control group, TGF-β1 treatment induced protein expression of Snail and Slug, which was concentration dependently inhibited by TSN (Figure 5a, d). Furthermore, the immunofluorescence results in A549 cells showed similar results (Figure 6d). When Snail was silenced by siRNA

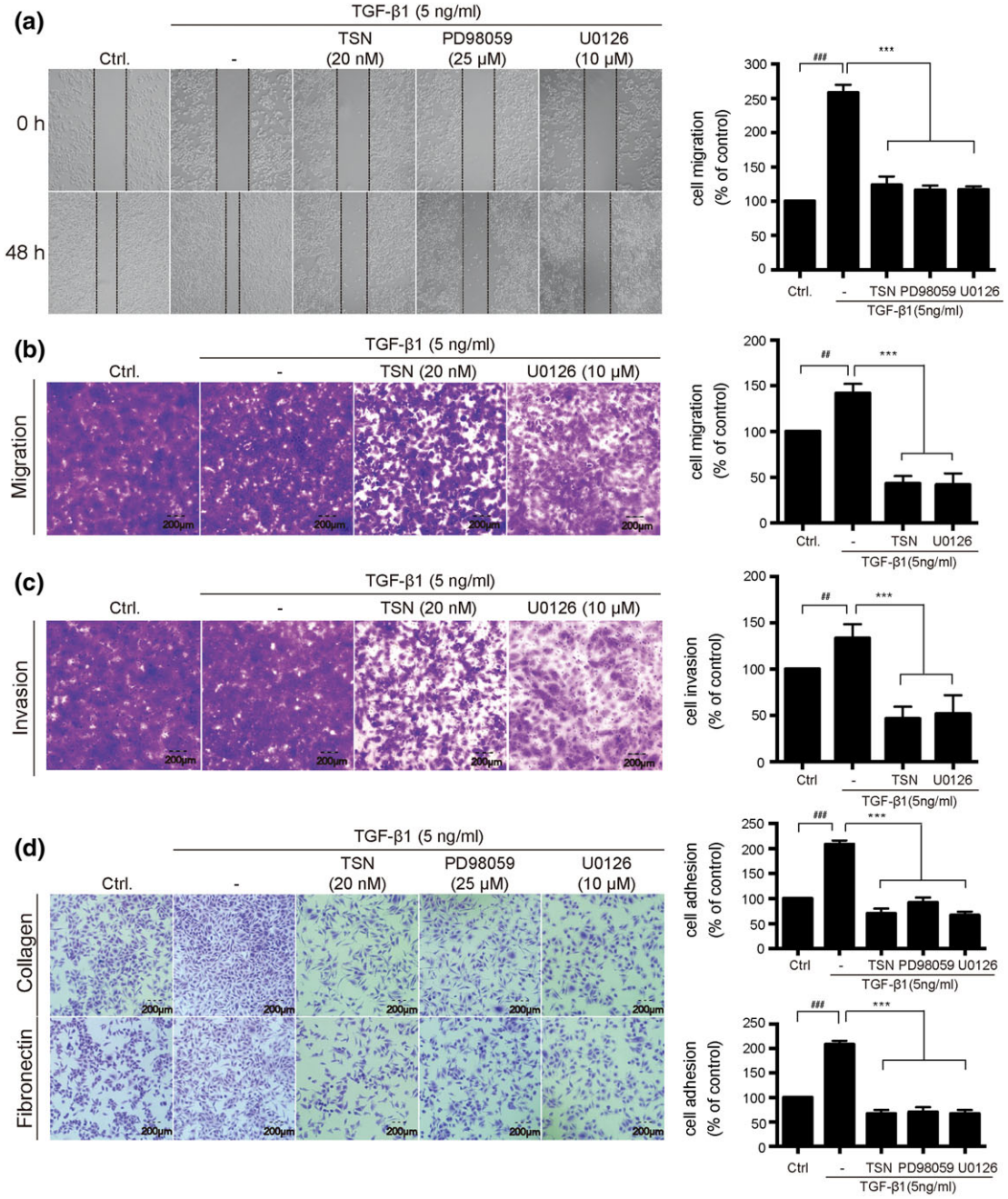


**FIGURE 7** ERK was involved in TGF-β1-induced migration, invasion, and adhesion in A549 cells. A549 cells were treated with TGF-β1 with or without TSN, PD98059 or U0126 co-treatment for 48 hr. The migration, invasion, and adhesion capacities were measured by the wound healing (a), transwell (b and c), and adhesion (d) assays. $^{\#\#}p < 0.01$, $^{**}p < 0.01$, and $^{\#\#\#}p < 0.005$,$^{***}p < 0.005$. TSN: toosendanin; TGF-β1: transforming growth factor-β1 [Colour figure can be viewed at wileyonlinelibrary.com]

in A549 cells (Figure 5b), TGF-β1-induced protein expression of both N-cadherin and Vimentin was partially reversed whereas the decreased E-cadherin was partially restored (Figure 5c).

### 3.6 | Extracellular signal-regulated kinase was involved in the suppression of transforming growth factor-β1-induced epithelial-mesenchymal transition by toosendanin

Compared with the control group, TGF-β1 treatment induced dramatically phosphorylation of ERK1/2 while showed no effect on the phosphorylation of JNK and p38MAPK in A549 cells. TSN concentration dependently inhibited TGF-β1-induced ERK1/2 phosphorylation without affecting phosphorylation of JNK and p38MAPK (Figure 6a). Interestingly, TGF-β1-induced morphological changes were significantly reversed by PD98059 but not by U0126 in A549 cells, whereas in H1975 cells, TGF-β1-induced morphological changes were significantly reversed by both PD98059 and U0126 (Figure 6b). Furthermore, TGF-β1-induced protein expression of N-cadherin, Snail, Slug was significantly reversed by both PD98059 and U0126. They also significantly restored TGF-β1-induced decreased expression of E-cadherin in both A549 and H1975 cells (Figure 6c). In addition, immunofluorescence results also showed that TGF-β1-induced Snail expression was reversed by both PD98059 and U0126 in A549 cells (Figure 6d).

### 3.7 | Toosendanin suppressed transforming growth factor-β1-induced adhesion, migration, and invasion by inhibiting extracellular signal-regulated kinase

In A549 and H1975 cells, similar to that of TSN, co-treatment PD98059 or U0126 with TGF-β1 significantly inhibited TGF-β1-

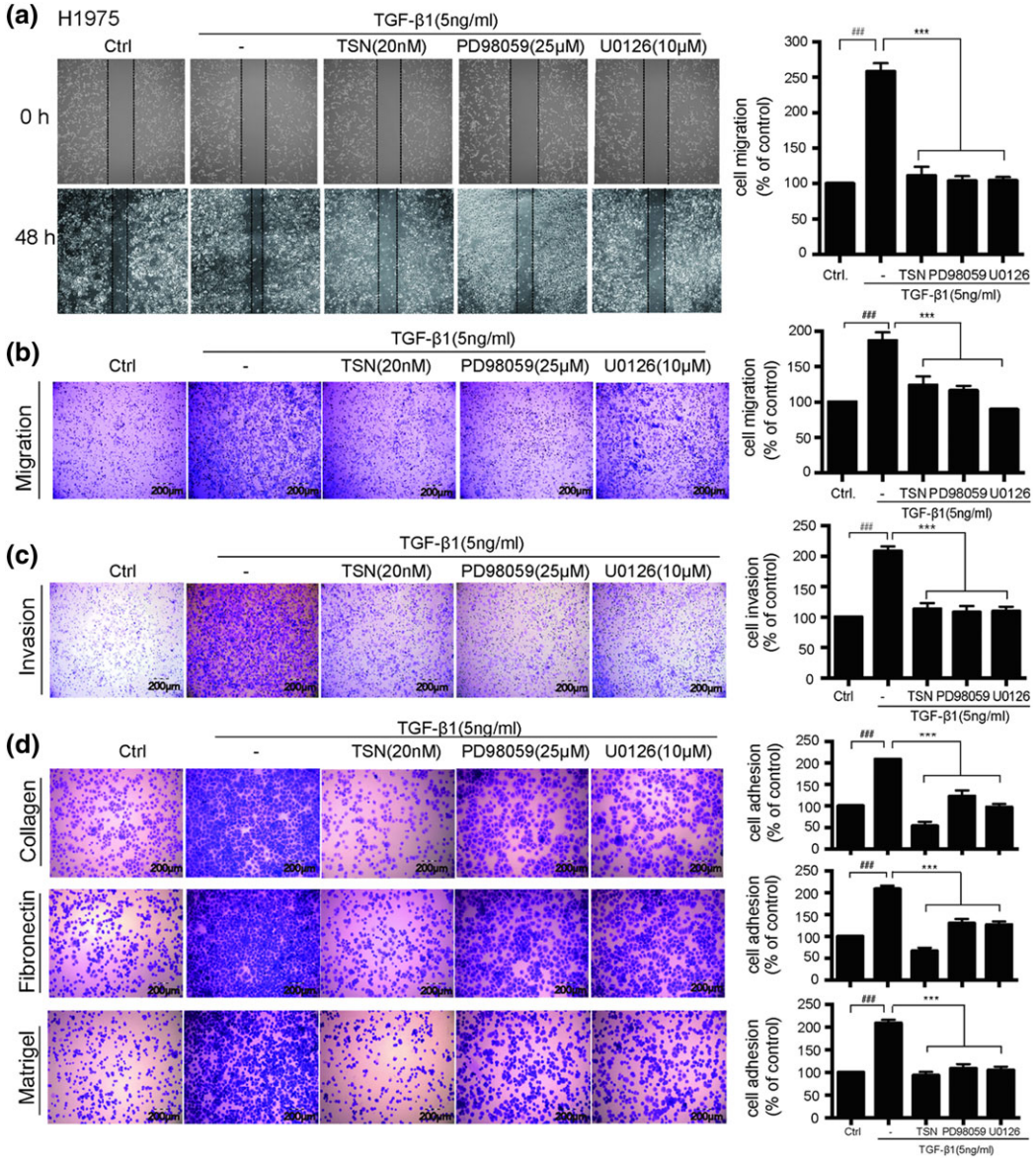


**FIGURE 8** ERK was involved in TGF-β1-induced migration, invasion, and adhesion in H1975 cells. H1975 cells were treated with TGF-β1 with or without TSN, PD98059 or U0126 co-treatment for 48 hr. The migration, invasion, and adhesion capacities were measured by the wound healing (a), transwell (b and c), and adhesion (d) assays. $^{\#\#}p < 0.01$, $^{**}p < 0.01$, and $^{\#\#\#}p < 0.005$, $^{***}p < 0.005$. TSN: toosendanin; TGF-β1: transforming growth factor-β1 [Colour figure can be viewed at wileyonlinelibrary.com]

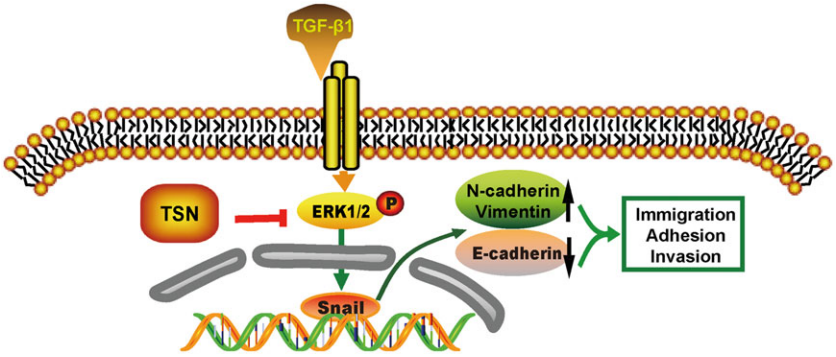


**FIGURE 9** Mechanism of the inhibitory effect of TSN on TGF-β1-induced epithelial-mesenchymal transition in lung cancer cells. TSN inhibit TGF-β1-induced epithelial-mesenchymal transition, adhesion, invasion, and migration in lung cancer cells through ERK1/2 and Snail pathways. TSN: toosendanin; TGF-β1: transforming growth factor-β1 [Colour figure can be viewed at wileyonlinelibrary.com]

induced migration and invasion in wound healing assay (Figures 7a and 8a), and transwell assay (Figures 7b,c and 8b,c). Furthermore, TGF-β1-induced adhesions of A549 cells to Type I collagen and fibronectin were suppressed by both PD98059 and U0126 (Figure 7d). In H1975 cells, similar to that of A549 cells, TGF-β1-induced adhesions to Type I collagen, fibronectin, and matrigel were suppressed by both PD98059 and U0126 (Figure 8d).

## 4 | DISCUSSION

Previous studies showed that TSN demonstrated anticancer effect by directly killing cancer cell through induction of apoptosis (He et al., 2010; Ju et al., 2012). Here, we reported its anticancer effect by inhibiting EMT. Our main findings are (a) TSN inhibited TGF-β1-induced EMT in A549 and H1975 cells with high efficacy (less than 10 nM); (b) TSN inhibited TGF-β1-induced adhesion, migration, and invasion of A549 and H1975 cells; and (c) ERK and Snail mediated the inhibitory effect of TSN on EMT.

As high concentrations of TSN showed cytotoxic effect of on both A549 and H1975 cells, low concentrations of TSN was selected to avoid the interference of cell death on metastasis. TGF-β1 is the most widely used stimulant to research EMT in various cells (Neuzillet et al., 2015). The morphological alterations of epithelial to mesenchymal appearing and the biochemical changes of decreased epithelial biomarkers and increased mesenchymal biomarkers provide simple methods to confirm the generation of EMT. Here, TGF-β1 treatment induced alterations in morphology, increased protein of Vimentin, N-cadherin, and decreased expression of E-cadherin as determined by Western blotting and immunofluorescence assays in A549 cells and H1975 cells suggesting that the mesenchymal transition occurred. TSN co-treatment dramatically inhibited and reversed these alterations morphologically and biochemically, suggesting that TSN inhibited TGF-β1-induced EMT. Especially, the inhibitory effect was clearly observed at as low as 5 nM in A549 cells and 2 nM in H1975 cells, which was more potent than that of on pancreatic cancer cells EMT (Pei, Fu, & Wang, 2017), suggesting the high efficacy of TSN in inhibiting EMT in lung cancer cells.

Transcription factor families, such as Snail, Twist, and Zeb, were identified as key regulators in mediating EMT and in regulating the expression of epithelial and mesenchymal proteins (Huber, Kraut, & Beug, 2005; Zavadil & Bottinger, 2005). Both Snail and Slug could be upregulated by TGF-β1, which contributed to the dysregulation of E-cadherin, N-cadherin, and Vimentin (Choi, Park, & Joo, 2007; Medici, Hay, & Olsen, 2008). Furthermore, transcription factors Snail, Smad2, and Smad3 were found to play important roles in TGF-β1-induced EMT in A549 cells (Kasai, Allen, Mason, Kamimura, & Zhang, 2005; Kim et al., 2007; Ko et al., 2013). Here, we found that TGF-β1 induced expression of Snail, which could be inhibited by TSN in a concentration-dependent manner in A549 and H1975 cells. In addition, silencing Snail dramatically reversed the expression of cadherins and Vimentin in A549 cells. Thus, Snail may play an important role in the inhibitory effect of TSN on TGF-β1-induced EMT.

Accumulating evidence suggests that EMT plays a critical role in various aspects of cancer progression, such as stem cell traits, chemoresistance, and especially in metastasis (Gomes, Terra, Sogayar, & Labriola, 2011; Meng & Wu, 2012). This was clearly confirmed by the increased invasion, migration, and adhesion of A549 and H1975 cells in response to TGF-β1. As invasion, migration, and adhesion assays were the classical methods to test the antimetastatic effect in vitro, the inhibitory effect of TSN on these assays suggested that TSN might have antimetastatic potentials in vivo, which needs further experiments to confirm.

Previous reports showed that the MAP kinases such as p38MAPK, JNK, and ERK1/2 actively participated in TGF-β1-induced EMT in A549 cells (Chen et al., 2012; Chen, Zhou, Shi, & Yang, 2013). Recent study showed that TSN inhibited pancreatic cancer cells EMT via deactivating protein kinase B/mammalian target of rapamycin signaling (Pei et al., 2017). Consistent with previous report, (Chen et al., 2012) TGF-β1 treatment dramatically induced phosphorylation of ERK1/2 after 48 hr treatment but showed no effect on p38MAPK and JNK. Thus, the contribution of ERK1/2 was further investigated. Similar to that of TSN, PD98059 and U0126, two commonly used ERK1/2 inhibitors, significantly reversed TGF-β1-induced on EMT biomarkers. Furthermore, they also inhibited TGF-β1-induced protein expression of Snail. Thus, ERK1/2 mediated, at least in part, TGF-β1-induced EMT in A549 and H1975 cells. Surprisingly, TGF-β1-induced morphological changes could be significantly restored by PD98059 while U0126 showed no obvious effect in A549 cells. This was possibly due to the differences of their specificity on MEK isoforms. PD98059 was a potential MEK1 inhibitor whereas U0126 potently inhibited both MEK1 and MEK2 (Bassil et al., 2007; Dudley, Pang, Decker, Bridges, & Saltiel, 1995). Recent evidence showed that MEK1 and MEK2 played different roles in cancer cell proliferation, differentiation, cell survival, and EMT (Lemieux et al., 2009; Liu et al., 2012; Scholl et al., 2009). In addition, PD98059 and U0126 also

significantly inhibited TGF-β1-induced A549 and H1975 cells migration, invasion, and adhesion. Of note, TSN showed similar inhibitory effects in these assays and at very low concentrations suggesting its high efficacy. Collectively, these results suggested that TSN inhibited TGF-β1-induced EMT in A549 and H1975 cells through ERK1/2 and Snail pathways.

In summary, this study demonstrated that TSN, a natural product, inhibited TGF-β1-induced EMT, adhesion, invasion, and migration in A549 and H1975 lung cancer cells possibly through ERK1/2 and Snail pathways (Figure 9). These findings showed new insights into the potential antimetastatic effect of TSN.

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## DISCLOSURES

We declare that none of the authors has any kind of conflict of interest related to the present work.

## ORCID

*Jin-Jian Lu* http://orcid.org/0000-0001-6703-3120
*Xiuping Chen* http://orcid.org/0000-0003-2675-7645

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