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Ginsenoside Rg3 Reduces Epithelial-Mesenchymal Transition Induced by Transforming Growth Factor-β1 by Inactivation of AKT in HMrSV5 Peritoneal Mesothelial Cells

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Background: Ginsenosides, including ginsenoside Rg3, are components of *Panax ginseng* C.A. Meyer (*Araliaceae*) used in traditional Chinese medicine. Long-term peritoneal dialysis induces peritoneal fibrosis that impairs ultrafiltration and is associated with epithelial-mesenchymal transition (EMT) of peritoneal cells. This study aimed to investigate the effects of ginsenoside Rg3 on EMT induced by transforming growth factor-β1 (TGF-β1) in HMrSV5 human peritoneal mesothelial cells.

Material/Methods: The cell counting kit-8 (CCK-8) assay measured HMrSV5 cell viability. The expression of EMT markers, E-cadherin, vimentin, and α-smooth muscle actin (α-SMA) were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The wound-healing assay determined cell migration. The S-phase of the cell cycle was assessed by 5-ethynyl-2'-deoxyuridine (EdU) labeling, and expression of phosphorylated AKT was measured by Western blot. The effect of ginsenoside Rg3 and the AKT activator SC79 on the TGF-β1-induced EMT of HMrSV5 cells were evaluated.

Results: Low concentration of ginsenoside Rg3 did not effect cell viability of HMrSV5 cells. TGF-β1 treatment decreased the expression of E-cadherin, and increased the expression of vimentin and α-SMA and promoted cell migration of HMrSV5 cells. However, co-treatment of ginsenoside Rg3 and TGF-β1 significantly reduced TGF-β1-induced EMT in HMrSV5 cells. TGF-β1 increased the phosphorylation of AKT and increased the expression of Smurf2. Ginsenoside Rg3 reduced TGF-β1-induced activation of AKT and Smurf2. SC79 reversed the effects of ginsenoside Rg3 on TGF-β1-induced EMT in HMrSV5 cells.

Conclusions: Ginsenoside Rg3 inhibited EMT induced by TGF-β1 in HMrSV5 human peritoneal mesothelial cells by inhibiting the activation of AKT.

MeSH Keywords: Epithelial-Mesenchymal Transition • Peritoneal Fibrosis • Proto-Oncogene Proteins c-akt

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Background

Chronic kidney disease is a global public health problem, both medically and economically, affecting more than 400,000 patients annually in China [1]. For the treatment of end-stage renal disease (ESRD), peritoneal dialysis (PD) is the first-line treatment to preserve residual renal function and is used for patients who await renal transplantation [2]. The normal semipermeable peritoneum allows the exchange of dialysis fluid [3]. However, continuous exposure to dialysis fluid, which has high osmolality and low pH and contains glucose degradation products leads to peritonitis, ultrafiltration failure, and progressive peritoneal fibrosis [4]. Although the precise mechanism of this process remains unclear, epithelial-mesenchymal transition (EMT) of human peritoneal cells is regarded as an important and early process in the occurrence and development of peritoneal fibrosis [5–7]. Therefore, the development of therapies that target EMT is of interest to reduce peritoneal fibrosis.

Some compounds with anti-inflammatory and anti-fibrotic functions have shown the capacity to reduce peritoneal fibrosis by regulating EMT of peritoneal cells *in vivo* and *in vitro* [8,9]. Ginsenosides are components of *Panax ginseng* C.A. Meyer (*Araliaceae*) and are used in traditional Chinese medicine. Among the various active compounds extracted from ginseng, ginsenoside Rg3 has shown several pharmacological functions, including reducing oxidation [10], inflammation [11], has also demonstrated effects in cancer [12]. Previous studies have shown that ginsenoside Rg3 can suppress EMT by several signaling pathways [12,13], suggesting the potential role of the ginsenoside Rg3 to reduce peritoneal fibrosis.

EMT includes complex changes in cell progression, including reduced expression of E-cadherin and increased expression of vimentin and α-smooth muscle actin (α-SMA) [14], leading to dysfunction of the peritoneum. Multiple signaling pathways, including Wnt, Notch, transforming growth factor-β (TGF-β), and the tyrosine kinase signaling pathways, are involved in the progression of EMT [14]. The TGF-β family signaling pathway triggers the downstream phosphatidylinositol 3-kinase kinase (PI3K)/AKT pathway to alter protein phosphorylation and expression, leading to the initiation of EMT [15]. Therefore, this study aimed to investigate the effects of ginsenoside Rg3 on EMT induced by TGF-β1 in HMrSV5 human peritoneal mesothelial cells.

Material and Methods

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical College, Qiqihar, Heilongjiang, China.

Chemicals, medium, and cell culture

Ginsenoside Rg3 (purity, ≥98%) and SC79 (purity, >97%) were dissolved in dimethyl sulfoxide (DMSO) (Sangon Biotech Co., Ltd., Shanghai, China) were provided by Sigma-Aldrich (St. Louis MO, USA). Human peritoneal mesothelial cells, HMrSV5 (Jennio Biotech Co., Ltd., Guangzhou, China) was routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermofisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermofisher Scientific, Waltham, MA, USA), 100 U/mL penicillin (Sangon Biotech Co., Ltd., Shanghai, China), and 100 U/mL streptomycin (Sangon Biotech Co., Ltd., Sangon, China) at 37°C in 5% CO_2. Transforming growth factor-β1 (TGF-β1) (10 ng/mL, Solarbio Science & Technology Co., Ltd., Beijing, China) was used to induce epithelial-mesenchymal transition (EMT). SC79 was used at a concentration of 4 μg/mL.

Cell viability assays

Cell counting kit-8 (CCK-8) assay (Solarbio Science & Technology Co., Ltd., Beijing, China) was used to explore the effect of ginsenoside Rg3 on cell viability. In a 96-well plate, human peritoneal mesothelial cells (5×10^3 cells/well) were incubated for 24 h. Then, fresh medium containing different concentrations of ginsenoside Rg3 was prepared to replace the medium, and the cells were further incubated for 72 h. Cell viability was measured according to the manufacturer's instructions. The final DMSO concentration was <0.1%. Treated cells were also labeled with 5-ethynyl-2´-deoxyuridine (EdU) using a Cell Light Edu Apollo 567 *In Vitro* Kit (Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol. Images were captured using an Axio Imager M2 fluorescence microscope (Zeiss GmbH, Jena, Germany).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

After 24 hours of treatment with different agents, total RNA of treated HMrSV5 cells was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol, and acted as the template to synthesize cDNA by using a PrimeScript™ RT reagent kit (Takara, Dalian, China) according to the manufacturer's protocol. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a SYBR Green kit (Takara, Dalian, China) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers used were listed in Table 1. Gene expression profiles were normalized to GADPH and calculated by the $2^{-\Delta\Delta Cq}$ method [16].

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Table 1. Primers used in this study.

Primers	Sequences (5'-3')
Vimentin (F)	TTGAACGCAAAGTGGAAT
Vimentin (R)	AGGTCAGGCTTGGAAACA
Smurf2 (F)	TAGCCCTGGCAGACCTCTT
Smurf2 (R)	CTTGTTGCGTTGTCCTCTGT
E-cadherin (F)	CAACGACCCAACCCAAGAA
E-cadherin (R)	CCGAAGAAACAGCAAGAGCA
α-SMA (F)	CATCATGCGTCTGGATCTGG
α-SMA (R)	GGACAATCTCACGCTCAGCA
GADPH (F)	TGCACCACCAACTGCTTAGC
GADPH (R)	GGCATGGACTGTGGTCATGAG

Wound-healing assay

A wound-healing assay was used to determine cell migration ability. Briefly, HMrSV5 cells (2×10^5 cells/well) were seeded in 6-well culture plates and were incubated until cell confluence reached 80%. Cells were scratched by a 100 µL pipette tip and washed using PBS to remove debris. Then, cells were cultured with fresh medium containing drug treatment. The cells were photographed after 48 h. Relative cell motility was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Each result was calculated from three random views to obtain the mean value.

Western blot

After 24 hours of treatment, total protein was extracted by Pierce cell lysis buffer and quantified by a BCA Protein Assay Kit (Takara, Dalian, China). Total protein (50 µg/lane) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Burlington, MA, USA), which was blocked with 5% dried skimmed milk powder for 1 h at room temperature and subsequently incubated with specific antibodies (Abcam, Cambridge, UK) overnight at 4°C, including antibodies to pan-Akt (cat. no. ab38449; 1: 500), total Akt, (cat. no. ab179463; 1: 10000), and β-actin (cat. no. ab8227; 1: 2,000). The membrane was probed with horseradish peroxidase-conjugated secondary antibody (cat. no. ab205718; 1: 2,000). Finally, the bands were visualized using the ECL Western blot substrate (Solarbio Science & Technology Co., Ltd., Beijing, China).

Immunofluorescence assay

Cells (5×10^4 cells/well) were incubated in an 8-well Nunc™ Lab-Tek™ II Chamber Slide™ (Thermofisher Scientific, Waltham, MA, USA) with the treatment of different drugs for 24 h. Cells were then washed twice with PBS, fixed with 4% paraformaldehyde, incubated with 0.1% Triton X-100, and blocked with 5% BSA. Cells were incubated with a primary antibody to Smurf2 (cat. no. ab94483; 5 µg/mL) and washed with PBS, followed by incubation with DyLight® 488 secondary antibodies (cat. no. ab96899; 1: 200). Images were acquired using an Axio Imager M2 fluorescence microscope (Zeiss GmbH, Jena, Germany).

Statistical analysis

Data were analyzed by SPSS version 19.0 software (SPSS, Inc., Chicago, USA) and presented as the mean ± standard deviation (SD). The differences between groups were analyzed by Student's t-test or one-way analysis of variance (ANOVA) with Newman–Keuls post hoc test. Each experiment was conducted in triplicate. A P-value <0.05 was considered to be statistically significant.

Results

The effects of ginsenoside Rg3 on the viability of HMrSV5 human peritoneal cells

We first determined the effect of ginsenoside Rg3 on cell proliferation of human peritoneal cells using the cell counting kit-8 (CCK-8) assay. As shown in Figure 1A, ginsenoside Rg3 showed no significant cytotoxicity at 2 µg/mL and significantly inhibited cell viability at higher concentrations in a dose-dependent manner. The S-phase of the cell cycle was assessed by 5-ethynyl-2'-deoxyuridine (EdU) labeling to determine whether 2 µg/mL of ginsenoside Rg3 inhibited cell proliferation. Counting of EdU positive cells showed that cell proliferation was not altered at ginsenoside Rg3 concentration of 2 µg/mL (Figure 1B). These results indicated that the low concentration of ginsenoside Rg3 did not affect cell viability. Therefore, 2 µg/mL was chosen as the dose of ginsenoside Rg3 for further studies.

The effects of ginsenoside Rg3 on the expression of epithelial-mesenchymal transition (EMT) markers in human peritoneal cells

The effects of ginsenoside Rg3 on the expression of EMT markers was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Treatment with transforming growth factor-β1 (TGF-β1) reduced the expression of E-cadherin and increased the expression of vimentin and α-smooth muscle actin (α-SMA) (Figure 2A). Ginsenoside Rg3 had no significant effect on the mRNA levels of these EMT markers. However, treatment with ginsenoside Rg3 reversed the TGF-β1-mediated aberrant expressions of E-cadherin, vimentin, and α-SMA. These

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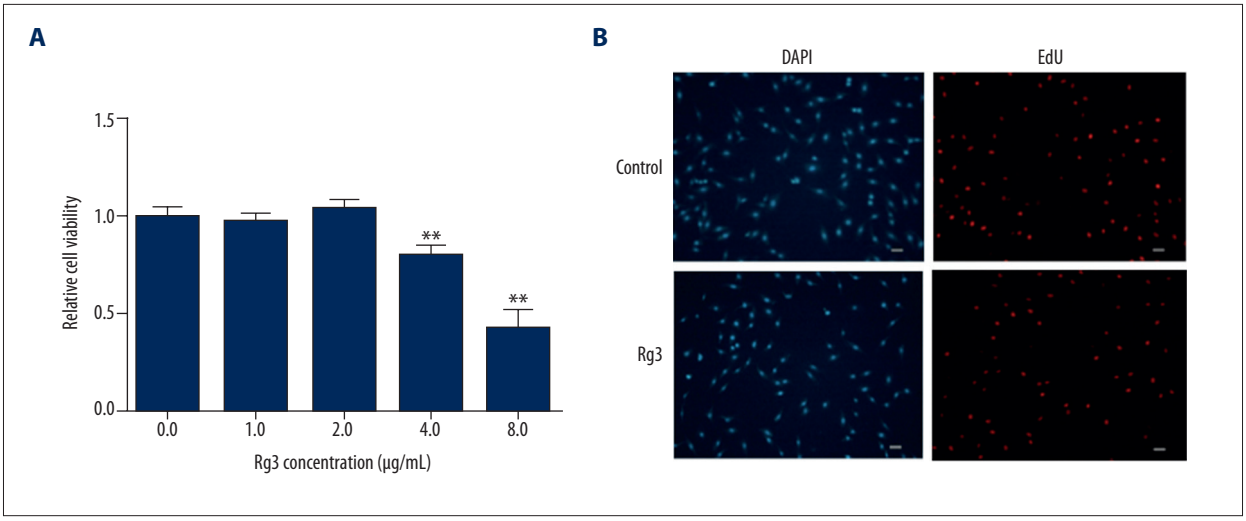

Figure 1. The effects of ginsenoside Rg3 on the viability of HMrSV5 human peritoneal mesothelial cells. (**A**) The effects of ginsenoside Rg3 on the cell viability of HMrSV5 cells were determined by the cell counting kit-8 (CCK-8) assay. (**B**) The effects of 2 μg/mL of ginsenoside Rg3 on cell viability were determined by 5-ethynyl-2'-deoxyuridine (EdU) labeling. Scale bar: 50 μm. The data are represented as the mean ±SD. ** P<0.01.

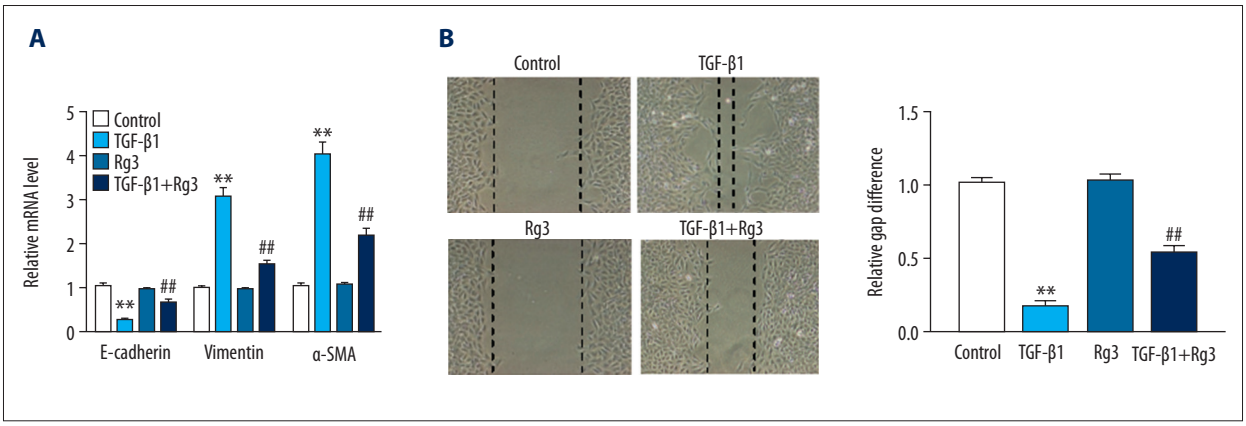

Figure 2. The effects of ginsenoside Rg3 on the expression of markers of epithelial-mesenchymal transition (EMT) induced by transforming growth factor-β1 (TGF-β1) and cell migration of HMrSV5 human peritoneal mesothelial cells. (**A**) The effects of ginsenoside Rg3 and TGF-β1 on the expression of markers of epithelial-mesenchymal transition (EMT) were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (**B**) The wound-healing assay determined the effects of ginsenoside Rg3 and TGF-β1 on cell migration. The data are presented as the mean ±SD. ** *vs.* Con, P<0.01; ## *vs.* TGF-β1, P<0.01.

results indicate that ginsenoside Rg3 reduced TGF-β1-induced expression of EMT markers in HMrSV5 human peritoneal cells.

The effects of ginsenoside Rg3 on cell migration in human peritoneal cells

To further determine the function of ginsenoside Rg3 on the TGF-β1-treated human peritoneal cells, a wound-healing assay was performed to analyze the changes in cell migration after treatment. As shown in Figure 2B, TGF-β1 induced a more rapid rate of cell migration compared with the control group. Ginsenoside Rg3 showed no influence on the migration capacity of human peritoneal cells. In contrast, combined treatment with ginsenoside Rg3 and TGF-β1 reduced cell migration compared with the TGF-β1-treated group. These results suggest that ginsenoside Rg3 treatment had a positive effect on the recovery of TGF-β1-induced cell migration in human peritoneal cells.

The effects of ginsenoside Rg3 on the expression of AKT and pAKT in human peritoneal cells

AKT is an important regulator of TGF-β1-mediated EMT. To determine the role of AKT on the pharmacological activity of ginsenoside Rg3, the expression and phosphorylation of AKT were analyzed by Western blot. The expression level of total AKT was similar in all the tested groups (Figure 3A). Ginsenoside Rg3 did

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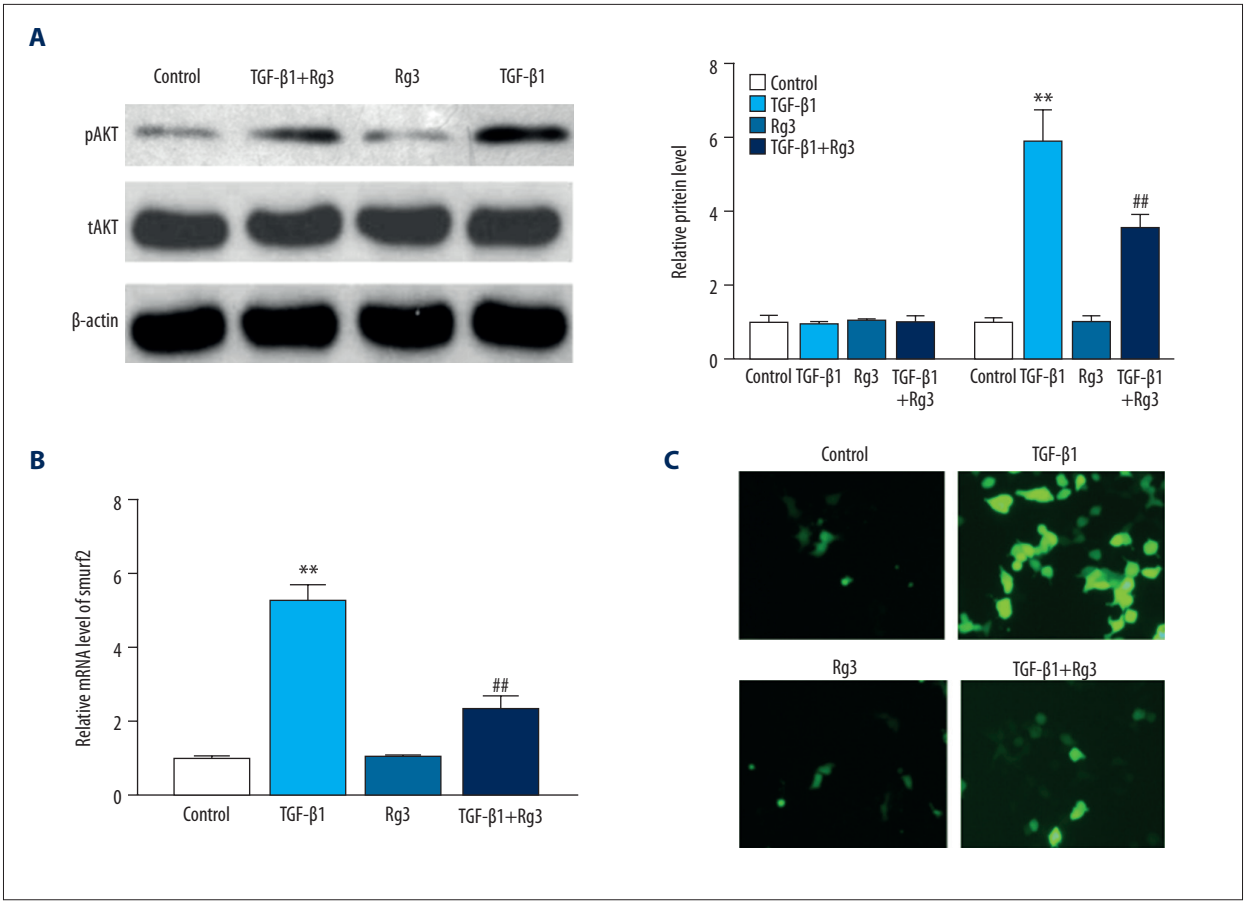

Figure 3. The effects of ginsenoside Rg3 and transforming growth factor-β1 (TGF-β1) on the expression of AKT, pAKT, and Smurf2 in HMrSV5 human peritoneal mesothelial cells. (**A**) The effects of ginsenoside Rg3 and TGF-β1 on the expressions of AKT and pAKT were determined by Western blot. (**B**) The effects of ginsenoside Rg3 and TGF-β1 on the expressions of Smurf2 were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (**C**) The immunofluorescence assay determined the effects of ginsenoside Rg3 and TGF-β1 on the expression of Smurf2. The data are represented as the mean ± SD. ** *vs.* Con, P<0.01; ## *vs.* TGF-β1, P<0.01.

not affect the expression level of pAKT. Treatment with TGF-β1 promoted the phosphorylation of AKT, and the combined treatment with ginsenoside Rg3 reversed this change. Smurf2 is a downstream regulator involved in EMT of the AKT pathway [15]. We further analyzed the expression of Smurf2 by qRT-PCR and immunofluorescence. As shown in Figure 3B and 3C, the expression level of Smurf2 in the TGF-β1 group was higher than the control group. Ginsenoside Rg3 did not affect the expression of Smurf2. However, the TGF-β1-mediated overexpression of Smurf2 was reduced by the addition of ginsenoside Rg3. These results indicated that ginsenoside Rg3 reduced the TGF-β1-induced activation of AKT and the overexpression of Smurf2 in human peritoneal cells.

Ginsenoside Rg3 attenuates the TGF-β1 induced EMT through AKT in human peritoneal cells

SC79, an AKT activator, was used to determine the potential role of AKT in the pharmacological activity of ginsenoside Rg3. Western blot showed the expression of AKT was not altered, but the phosphorylation of AKT was significantly improved by the addition of SC79 when compared with the TGF-β1+ginsenoside Rg3 group (Figure 4A). The mRNA level and fluorescence intensity of Smurf2 in the TGF-β1+ginsenoside Rg3 group were also increased with the addition of SC79 (Figure 4B, 4C). These results indicated that SC79 activated AKT and the downstream EMT-regulator Smurf2. Also, qRT-PCR analysis showed that TGF-β1+ginsenoside Rg3+SC79 group showed a reduced E-cadherin level and increased vimentin and α-SMA levels (Figure 4D). The combined treatment of SC79, TGF-β1, and ginsenoside Rg3 also led to an increased migration capacity of human peritoneal cells compared with the TGF-β1+ginsenoside Rg3 group (Figure 4E). These results suggest that ginsenoside Rg3 reduced TGF-β1-induced EMT in HMrSV5 human peritoneal cells *in vitro* by downregulating the phosphorylation of AKT.

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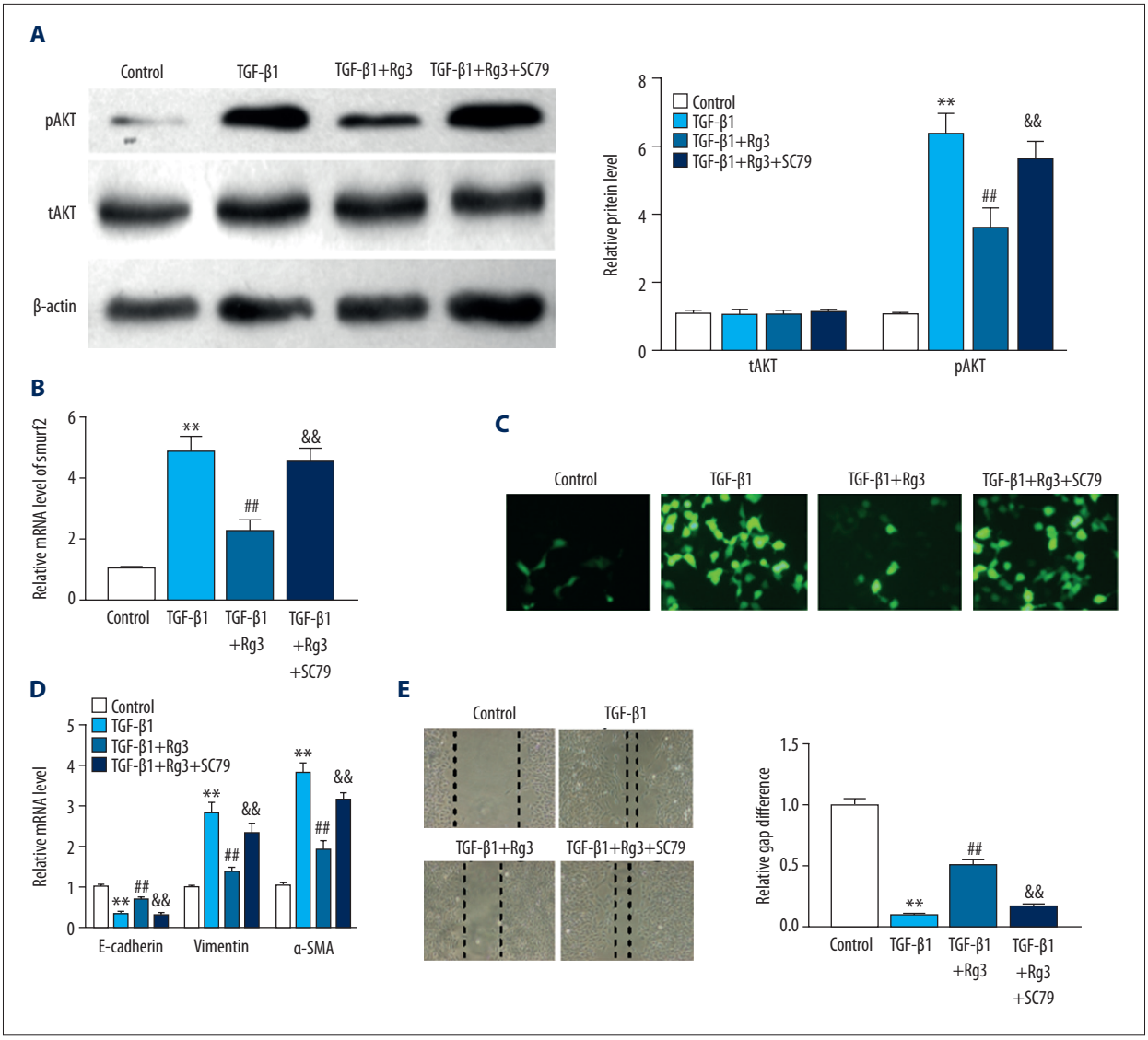

Figure 4. The AKT activator, SC79, reversed the effect of ginsenoside Rg3 on epithelial-mesenchymal transition (EMT) induced by transforming growth factor-β1 (TGF-β1) of HMrSV5 human peritoneal mesothelial cells. (**A**) The AKT activator, SC79, on the effects of ginsenoside Rg3 on the TGF-β1-induced expression of AKT and pAKT were determined by Western blot. (**B**) The effects of SC79 on ginsenoside Rg3 and TGF-β1-induced expression of Smurf2 were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (**C**) The effects of SC79 on ginsenoside Rg3 and TGF-β1-induced expression of Smurf2 were determined by immunofluorescence assay. (**D**) The effects of SC79 on ginsenoside Rg3 and TGF-β1-induced expression of EMT markers were determined by qRT-PCR. (**E**) The wound-healing assay determined the effects of SC79 on ginsenoside Rg3 and TGF-β1-induced cell migration. The data are represented as the mean ±SD. ** *vs.* Con, P<0.01; ## *vs.* TGF-β1, P<0.01; && *vs.* TGF-β1+Rg3, P<0.01.

Discussion

Long-term peritoneal dialysis results in peritoneal fibrosis, and there is a lack of an appropriate therapeutic strategy for the prevention of peritoneal fibrosis to improve the prognosis of end-stage renal disease (ESRD). Previous reports have shown that some compounds [15,17] and herbal medicines [18] are effective in the treatment of human peritoneal cells *in vitro* and *in vivo*. The capacity of ginsenoside Rg3 to prevent mesothelial cell epithelial-mesenchymal transition (EMT) has been observed in previous studies. In lung cancer cells, ginsenoside Rg3 inhibited EMT by down-regulating fucosyltransferase IV [12]. Ginsenoside Rg3 has been shown to block the activation of the NF-κB induced EMT to increase drug sensitivity of hypoxic lung cancer cells to cisplatin [13]. Similar results have also been reported for hypoxia-induced EMT by downregulating HIF-1α in ovarian cancer cells [19].

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The present study was the first to report that ginsenoside Rg3 also could reverse TGF-β1-induced EMT in human peritoneal cells. Although the herbal medicine astragaloside IV also reversed the TGF-β1-induced EMT in human peritoneal cells, its effective dose (400 μg/mL) [18] was significantly higher than that of ginsenoside Rg3 (2 μg/mL), suggesting improved pharmacological activity of ginsenoside Rg3. The antiproliferation activity of ginsenoside Rg3 has been reported in several types of cancer cells [13]. In the present study, a high level of ginsenoside Rg3 inhibited the viability of human peritoneal cells, and to avoid this effect, a relatively low dose of ginsenoside Rg3 was used.

EMT is important in cancer, wound healing, and fibrosis [14]. Several factors are involved in EMT and fibrosis, and TGF-β signaling plays a critical role and functions as a key mediator in peritoneal dialysis-induced fibrosis [20]. Our results also confirmed the positive effect of TGF-β on the EMT of human peritoneal cells. In the canonical TGF-β/Smad pathway, TGF-β can recruit and activate TβRI by binding to membrane TβRII and then phosphorylates and translocates Smad2/3 into the cell nucleus [21]. The activated Smad complexes then repress or activate the expression of EMT-related proteins, E-cadherin, vimentin, and α-smooth muscle actin (α-SMA).

Recently published studies have shown that some non-Smad signaling pathways also contribute to TGF-β-induced EMT [15,18]. A previous study showed that the TβRI can directly bind to PI3K with the induction of TGF-β1, leading to the activation of the PI3K/AKT pathway [22]. Our results also demonstrated phosphorylation and activation of AKT by TGF-β1 in human HMrSV5 peritoneal cells. Notably, this activation was reversed by ginsenoside Rg3, reducing the TGF-β1-induced expression of EMT marker proteins. A similar inhibitory effect of ginsenoside Rg3 on the PI3K/AKT pathway has been observed in lung cancer cells [23]. Also, the TGF-β1-induced expression of Smurf2 was also rescued by ginsenoside Rg3. As a ubiquitination regulatory factor, Smurf2 is positively regulated by pAKT and functions as a promoter of TGF-β-induced EMT [15,24]. Our results further indicated that Smurf2 might also participate in the pharmacological activity of ginsenoside Rg3 in the TGF-β1 treated HMrSV5 human peritoneal cells.

SC79 is a small molecule AKT activator that can promote the phosphorylation of AKT in some types of cells [25–27]. Here, the repressed phosphorylation level of AKT by ginsenoside Rg3 was enhanced by treatment of SC79, suggesting that SC79 also functions as an AKT activator in human peritoneal cells. Our results also showed that activated AKT promoted the expression of Smurf2, further confirming the activation of the downstream regulator of AKT. Also, the addition of SC79 reversed the pharmacological activity of ginsenoside Rg3, leading to the improved cell migration, reduced expression of E-cadherin, and promoted the expression of vimentin and α-SMA. These results showed that the inactivation of AKT participated in the pharmacological activity of ginsenoside Rg3 and TGF-β1-induced EMT in HMrSV5 human peritoneal cells.

Conclusions

Ginsenoside Rg3 reduced transforming growth factor-β1 (TGF-β1)-induced epithelial-mesenchymal transition (EMT) in HMrSV5 human peritoneal cells *in vitro*. Although AKT was identified as a target of ginsenoside Rg3, some other signal pathways might also be involved in the pharmacological activity of ginsenoside Rg3. Further *in vivo* studies are needed to analyze the potential function of ginsenoside Rg3 in preventing peritoneal EMT and fibrosis associated with peritoneal dialysis.

Conflict of interest

None.

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