Rhapontigenin inhibits TGF-β-mediated epithelial-mesenchymal transition via the PI3K/AKT/mTOR pathway and is not associated with HIF-1α degradation

YEN-HSIU YEH[1], SHIH-WEI WANG[4], YEN-CHENG YEH[5], HO-FU HSIAO[3] and TSAI-KUN LI[1,2]

[1]Department and Graduate Institute of Microbiology, College of Medicine, Taipei; [2]Center for Biotechnology, National Taiwan University, Taipei; [3]Department of Emergency Medicine, Sijhih Cathay General Hospital, New Taipei City; [4]Department of Medicine, Mackay Medical College, New Taipei City; [5]Department of Internal Medicine, Kaohsiung Armed Forces General Hospital, Kaohsiung, Taiwan, R.O.C.

Received October 20, 2015; Accepted November 26, 2015

DOI: 10.3892/or.2016.4664

Abstract. The epithelial-mesenchymal transition (EMT) is a pivotal event in cancer cell invasion and metastasis. Emerging evidence suggests that rhapontigenin (Rha) may impede the progression of cancer by disrupting angiogenesis and the EMT. However, the underlying mechanism of Rha has not yet been clarified. In this study, we used transforming growth factor β (TGF-β) to trigger EMT in diverse types of cancer cells and revealed that Rha inhibited TGF-β-induced EMT and derived-cell invasiveness. The effects of TGF-β were blocked by Rha via interference with the PI3K/AKT/mTOR/GSK3β/β-catenin signaling pathway. Furthermore, Rha also inhibited TGF-β-induced expression of transcription regulators Snail and hypoxia-inducible factor 1α (HIF-1α) by causing their degradation by the 26S proteasome. Surprisingly, although HIF-1α was degraded with Snail as a result of Rha exposure, HIF-1α was not a key factor involved in TGF-β-mediated EMT induced by Rha. Knocking-down Snail expression, but not HIF-1α expression, by RNA interference dramatically reversed TGF-β-mediated EMT. Moreover, Rha abolished TGF-β-triggered cell invasiveness. Our results demonstrate that Rha inhibits TGF-β-induced EMT in cancer cells by suppressing the activity of the PI3K/AKT/mTOR pathway. Therefore, Rha may represent a new route for therapeutic intervention in cancer patients and merits future studies to assess its potential.

Correspondence to: Dr Tsai-Kun Li, Department and Graduate Institute of Microbiology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Ren'ai Road, Zhongzheng, Taipei 10051, Taiwan, R.O.C.
E-mail: tsaikunli@ntu.edu.tw

Dr Ho-Fu Hsiao, Department of Emergency Medicine, Sijhih Cathay General Hospital, No. 2, Ln. 59, Jiancheng Road, Xizhi, New Taipei City 22174, Taiwan, R.O.C.
E-mail: hsiaohofu@gmail.com

Key words: rhapontigenin, epithelial-mesenchymal transition, transforming growth factor β

Introduction

Recent research into the inflammatory microenvironment of malignant cancer tissues demonstrates that a tight association exists between inflammation and tumor metastasis progression (1,2). Despite attempts in the last few decades to elucidate the inflammatory cytokines underlying invasion and metastasis in the tumor micro-environment (TME) (3), knowledge of the role of pro-inflammatory cytokines in cancer is limited. Mounting evidence demonstrates that inflammation induces activity by several factors in the TME, including growth factors, survival factors, and extracellular matrix-modifying enzymes, which control proliferative signaling, leading to activation of the epithelial-to-mesenchymal transition (EMT), a crucial process in cancer cell migration and metastasis. Transforming growth factor-β (TGF-β) was the first factor described as an inducer of EMT (4). TGF-β is a pleiotropic cytokine that has a wide variety of biological functions and is activated by Smad2/3/4, which induces translocation of TGF-β from the receptor to the nucleus. Dysregulation of the TGF-β pathway contributes to a variety of diseases, including fibrotic kidney diseases, colorectal cancer, Alzheimer's disease, and diabetes (5,6). Based on the importance of the TGF-β pathway in the progression of cancer, a better understanding of its mechanisms and targets could facilitate the development of improved chemopreventive agents.

EMT is a fundamental process in embryogenesis and a pivotal event in the initial steps of the metastatic cascade that allows cells to acquire migratory, invasive, and stem cell-like properties. During EMT, epithelial cells lose polarity and cell-to-cell contacts, followed by dramatic remodeling of the cytoskeleton. Epithelial markers such as E-cadherin promote cell-to-cell contact, whereas mesenchymal markers such as vimentin, fibronectin, and N-cadherin induce spindle-shaped, fibroblast-like morphology and cell invasion (7,8). In addition, several key transcriptional repressors, including Snail,

Slug, and Twist, are activated via multiple cellular signaling pathways, including NF-κB, Wnt, and Hedgehog, during EMT (9-11). Therefore, reversing and/or preventing the EMT process is thought to be an effective strategy for limiting the dissemination of cancer.

Recently, dietary chemopreventive agents have gained much attention in the area of cancer research. Grape seed extract and red wine contain strong antioxidants and polyphenols that reduce free radical damage. Adequate intake of grape seed extract and red wine has a preventive effect against some cancers. Among dietary chemopreventive agents, rhapontigenin, a stilbene derivative isolated from the rhizome of traditional herbal medicine *Rheum undulatum* (Polygonaceae), has been recognized as an analog of resveratrol with multiple biological functions, including anti-allergic, anti-hyperglycemic, and anti-angiogenic activities. Although several studies have described the diverse effects and mechanisms of dietary chemoprevention, the mechanism underlying prevention of cancer invasion and metastasis by Rha has not been reported. We hypothesize that Rha has diverse biological functions that contribute to its anticancer effects.

Based on a previous study (4) showing that TGF-β induces EMT in cancer cells, TGF-β was used in the present study to induce a pro-inflammatory microenvironment, which allowed dissection of the anticancer mechanism of Rha via assessment of changes produced in the TME. The aim of this study was to assess inhibition of TGF-β-triggered EMT in cancer cells as a mechanism for the anti-metastatic effect of Rha. We discovered that Rha inhibits TGF-β-induced EMT and derived invasion in renal cancer cells while suppressing activation of the PI3K/AKT/mTOR/Snail signaling pathway.

Materials and methods

Cell culture. Human 769-P renal carcinoma cells, A549 human lung epithelial cells, HeLa cervix adenocarcinoma cells, and PC3 prostate adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, USA) and cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 37˚C CO_2 incubator.

Antibodies and chemical reagents. LY294002 (LY), SB203580 (SB), PD98059 (PD), and MG132 were obtained from Sigma (USA). SP600125 was obtained from Cayman. β-catenin inhibitor BH535 was obtained from Calbiochem. TGF-β was obtained from Peprotech (USA). Antibodies against HIF-1α, E-cadherin, N-cadherin, and β-catenin were obtained from BD Biosciences. Antibodies against fibronectin, vimentin, tubulin, and actin were obtained from Sigma. Antibodies against HA were obtained from Covance. Antibodies against CA9, S6K, S6, cyclin D1, and GAPDH were obtained from GeneTex. Antibodies against mTOR, mTOR-Ser2448, Akt-Ser473, Akt, S6K-Thr389, S6-Ser235/236, GSK-3β, GSK3β-Ser9, Smad2-Ser465/467, active β-catenin, and Snail were obtained from Cell Signaling Technology.

RNA interference. The sequences targeted by the anti-Snail siRNA were 5'-GGUGUGACUAAUGCAATT-3' and 5'-UUG CAUAGUUAGUCACACCTT-3'. siRNAs were synthesized by and purchased from Biotools (USA). Transfection of siRNA was conducted using Lipofectamine RNAiMAX (Invitrogen, USA) at a final concentration of 0.1-0.3 μM. ON-TARGETplus SMARTpool siRNAs (Dharmacon, Chicago, IL, USA) targeting human Akt or mTOR were transfected as siRNA duplexes for 48 h. Knockdown of HIF-1α achieved by transfection with the pSUPER-based plasmid expressing sh-HIF-1α, which was a generous gift from Dr K.J. Wu (Taiwan). Transfection of sh-HIF-1α siRNA was performed according to the manufacturer's protocol. Briefly, cells in the exponential phase of growth were plated in 6-well tissue culture plates at a density of $1.5x10^5$ cells/well, grown for 24 h, and transfected with siRNA using Lipofectamine 2000 and OPTI-MEM serum free medium. After 48 h, cells were harvested and lysed for western blotting.

Immunoblotting and immunoprecipitation. Cells were lysed in TEGN buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 420 mM NaCl, 10% glycerol, and 0.5% Nonidet P-40) containing 1 mM Na_3VO_4 and 1 mM protease inhibitor cocktail (Roche). For immunoprecipitation, lysates were diluted 1:1 with TEG buffer (10 mM Tris, pH 7.5, 1 mM EDTA, and 20% glycerol) and rocked with antibodies and 20 μl of 50% protein G beads (Upstate) for 3 h at 4˚C. The immunoprecipitates were washed four times in TEG:TEGN buffer (1:1), boiled in protein sample dye (2 M β-mercaptoethanol, 12% SDS, 0.5 M Tris, pH 6.8, 0.5 mg/ml bromophenol blue, and 30% glycerol), analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and subjected to western blotting.

Transient transfections. Transient transfections of cancer cell lines were performed using Lipofectamine 2000 (Invitrogen). Whole cell extracts were prepared at specific time-points after transfection and subjected to SDS-PAGE/western blotting or immunoprecipitation assays as described previously (12).

Immunofluorescence. Cells were plated on cover slips 1 day before drug exposure. After treating the cells with TGF-β with or without Rha or LY for the specified times, the cells were fixed with 3% paraformaldehyde and 2% sucrose in PBS for 10 min. Coverslips were permeabilized with Triton X-100 buffer (0.05% Triton X-100, 20 mM HEPES-KOH, pH 7.9, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) for 10 min and blocked in PBS containing 1% BSA for 1 h at room temperature, after which the cells were washed three times with PBS and incubated with Alexa Fluor 488- or rhodamine-conjugated secondary antibodies (Molecular Probes) in Hoechst 33342 stain containing PBS for 1 h. Finally, the treated cells were washed with PBS three times and mounted in Fluoromount™ Aqueous Mounting Medium (Sigma).

Invasion assay. Cells ($5x10^5$) in 6-well plates were pre-treated with or without LY294002 for 30 min, followed by exposure to TGF-β for 24 h. Later, the cells were trypsinized and counted, after which $1.25x10^5$ cells were seeded in Matrigel-coated Transwell inserts (24-well, 8-μm pore size; BD Biosciences, Canada) with serum-free medium with inhibitors or inhibitors/TGF-β. DMEM with 10% FBS was added to the lower chamber to act as a chemo-attractant. After incubation for 48 h, the inner cells that did not invade the lower chamber via the pores were removed with a cotton swab. The cells that adhered

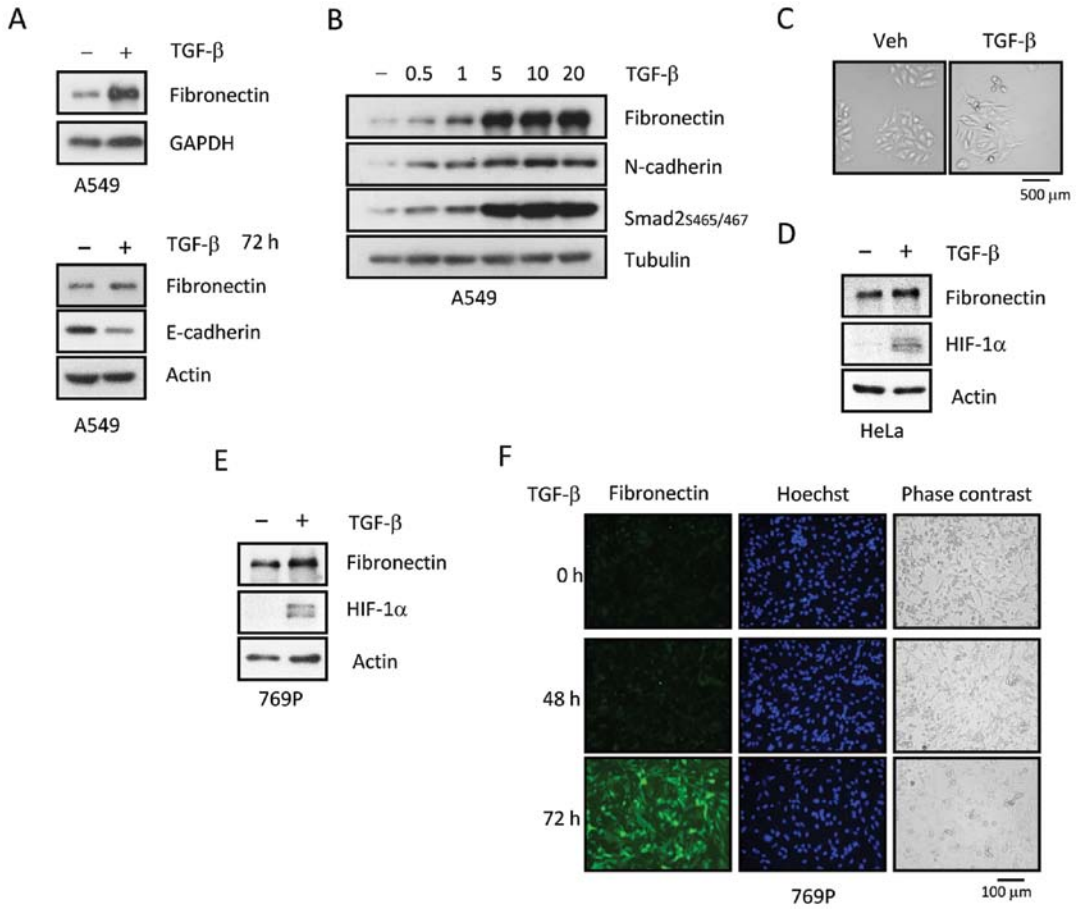

Figure 1. A549 cells treated with TGF-β undergo EMT. (A) TGF-β treatment for 24 h led to fibronectin upregulation (A, upper panel) and E-cadherin downregulation (A, lower panel). (B) TGF-β treatment for 24 h led to upregulation of FN and N-cadherin expression, as well as phosphorylation of Smad2, in a dose-dependent manner. (C and D) TGF-β administration for 5 days led to upregulation of FN expression and fibroblastic morphology (C, phase contrast microscope images; D, western blotting data of the upregulation of HIF-1α and FN expression by TGF-β. (E and F) TGF-β administration for 2 or 3 days led to upregulation of FN and HIF-1α in 769-P cells (E). Cells grown on coverslips were treated with 5 nM TGF-β for the indicated time. After fixing the cells in 3% paraformaldehyde and 2% sucrose in PBS, the cells were incubated with rabbit anti-fibronectin antibodies and Alexa 594-labeled anti-rabbit secondary antibodies, mounted on glass slides with mounting medium containing Hoechst 33342, and observed using a fluorescence microscope (F).

to the underside of the membrane were fixed in methanol and stained with 0.5% crystal violet. For each sample, six random fields were counted and examined under a microscope with a x100 objective to determine the number of cells that had invaded across the membrane. Three independent experiments were conducted.

Quantitative measurements and statistical analyses. Quantitative measurements of the obtained results were performed using ImageQuant software following the manufacturer's protocol (Molecular Dynamics) and presented as mean + standard error of the mean (SEM) for three independent experiments (n=3). Statistical analyses were performed using Student's t-test. Results were considered significant at p-value <0.05 (P<0.05).

Results

Cancer cells treated with TGF-β undergo EMT. We investigated the mechanism underlying TGF-β-induced EMT in A549 (lung), HeLa (cervical), and 769-P (renal) cancer cells, all of which have been established as suitable models for studying EMT *in vitro* (12-15). As expected, TGF-β treatment resulted in the acquisition of spindle-fibroblastic morphology, downregulation of epithelial marker E-cadherin, and upregulation of mesenchymal markers fibronectin (FN), vimentin and N-cadherin (Fig. 1A and C). FN induction was observed at day 1 (Fig. 1A, upper panel). TGF-β dose-dependently induced expression of FN and N-cadherin, as well as phosphorylation of Smad2 (Fig. 1B). E-cadherin reduction by TGF-β occurred on day 3 in in A549 cells (Fig. 1A, bottom panel). Additionally, TGF-β treatment for 2 days led to upregulation of HIF-1α expression in 769-P cells (Fig. 1E). Changes in protein expression were confirmed by immunofluorescence (IFA) experiments. When 769-P cells were stimulated with TGF-β for 2 days, FN expression was slightly increased. IFA experiments also confirmed upregulation of FN in 769-P cells (Fig. 1F). Similar protein alterations were observed in HeLa cells (Fig. 1D).

TGF-β mediated EMT alteration and HIF1a expression via the PI3K/AKT pathway. Several studies suggest a role for PI3K in TGF-β signaling (16-18). The involvement of various signaling pathways in regulating TGF-β-induced EMT was assessed. A series of pharmacological inhibitors, including LY (PI3K inhibitor), PD (MAPK inhibitor), SB (p38 inhibitor), and

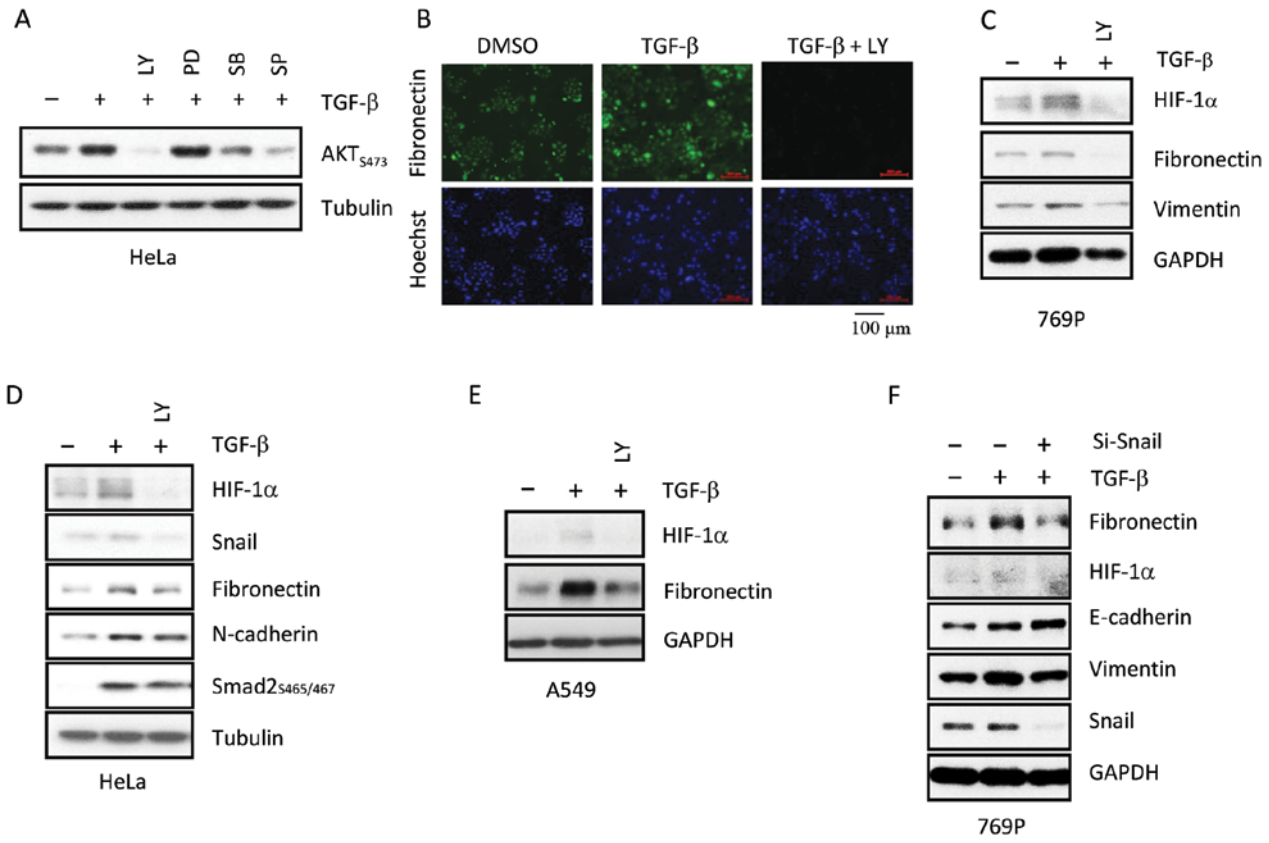

Figure 2. Rhapontigenin-induced degradation of Snail is involved in the regulation of PI3K/AKT pathway. (A) PI3K inhibitor LY294002 abrogated TGF-β-induced phosphorylation of AKT at serine 473. (B-E) The PI3K pathway is involved in regulation of TGF-β mediated expression of HIF-1α and EMT markers. Rhapontigenin treatment led to downregulation of TGF-β-mediated Snail expression. The decrease in Snail expression was due to degradation by the 26S proteasome. Western blotting and immunofluorescence assays were performed to assess LY-induced reductions in protein levels of HIF-1α and EMT-related markers, including Snail, fibronectin, N-cadherin, and vimentin, in diverse types of cancer cells. (F) Knockdown of Snail via siRNA led to decreased protein levels of fibronectin, HIF-1α, and vimentin, whereas the E-cadherin protein level was increased.

SP (JNK inhibitor), were used to determine the involvement of various signaling pathways in regulating TGF-β-mediated responses. Serine/threonine kinase Akt, a well-known downstream effector of PI3K, is involved in cell survival signaling. Akt phosphorylation at serine 473 was strongly activated by TGF-β. When cells were treated with LY, PD, SB, or SP, AKT phosphorylation at serine 473 was downregulated in HeLa cells (Fig. 2A). Relative to SB and SP, LY more effectively abolished phosphorylation of Akt, indicating that TGF-β activation may occur via the PI3K/Akt signaling.

Next, in order to verify the importance of the PI3K pathway in TGF-β regulation, 769-P cells were pre-treated with or without PI3K inhibitor LY for 30 min, followed by co-treatment with TGF-β for 20 h. As shown in Fig. 2B and C, LY dramatically blocked TGF-β-mediated expression of EMT markers in 769-P cells. Additionally, TGF-β-induced expression of HIF-1α and EMT-related genes Snail, FN, and N-cadherin were abrogated by LY in HeLa and A549 cells, implicating the PI3K signaling pathway in the regulation of TGF-β-mediated responses (Fig. 2D and E). To assess the role of Snail in TGF-β-induced EMT (16,18), Snail protein was knocked-down via small interfering RNA (siRNA) in 769-P cells. In 769-P cells lacking Snail protein, TGF-β-induced EMT was abolished, demonstrating the crucial role of Snail in the EMT (Fig. 2F). These results suggest that Snail may play a crucial role in TGF-β-induced EMT.

The 26S proteasome is responsible for Rhapontigenin-induced degradation of Snail. To verify our hypothesis regarding Rha, we assessed the impact of Rha on TGF-β-mediated Snail expression, which is a transcriptional regulator active during EMT. Therefore, various cancer cell types were exposed to Rha in the presence or absence of TGF-β. Together with previous studies, the results described above show that TGF-β can alter expression of EMT-related genes, including Snail (10,11,19,20). As shown in Fig. 3A-C, TGF-β triggered increased Snail expression in diverse cancer cells. However, when cancer cells were pre-treated with Rha, TGF-β-induced Snail expression was blocked by Rha in all tested types of cancer cells. To examine whether the proteasome degradation pathway is involved in Rha-induced HIF-1α protein degradation, 769-P cells were stimulated with TGF-β or co-treated with Rha for 20 h, followed by exposure to 26S proteasome inhibitor MG132 for 6 h. As shown in Fig. 3D, MG132 completely blocked the reduction in TGF-β-induced Snail expression induced by Rha, suggesting that Snail was recruited to the 26S proteasome for degradation (Fig. 3D, upper). Similar results were observed in PC3 cells (Fig. 3D, bottom), indicating that the ability of MG132 to reverse Snail expression by Rha was ubiquitous.

Rha induces ubiquitination and degradation of HIF-1a. In previous studies, Rha was recognized as an inhibitor of HIF-1α accumulation in PC3 cells. Therefore, TGF-β mediated down-

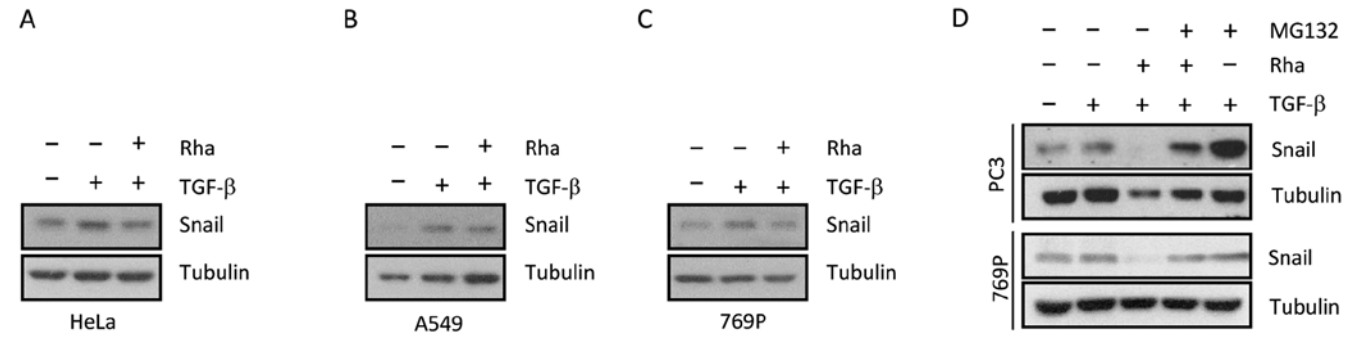

Figure 3. The 26S proteasome is responsible for Rhapontigenin-induced degradation of Snail. (A-C) Rhapontigenin (Rha) inhibited TGF-β-mediated Snail expression in diverse cell lines, including (A) HeLa, (B) A549, and (C) 769-P cells. (D) 26S proteasome inhibitor MG-132 prevented Rha-induced degradation of Snail in 769-P (upper panel) and PC3 (bottom panel) cells.

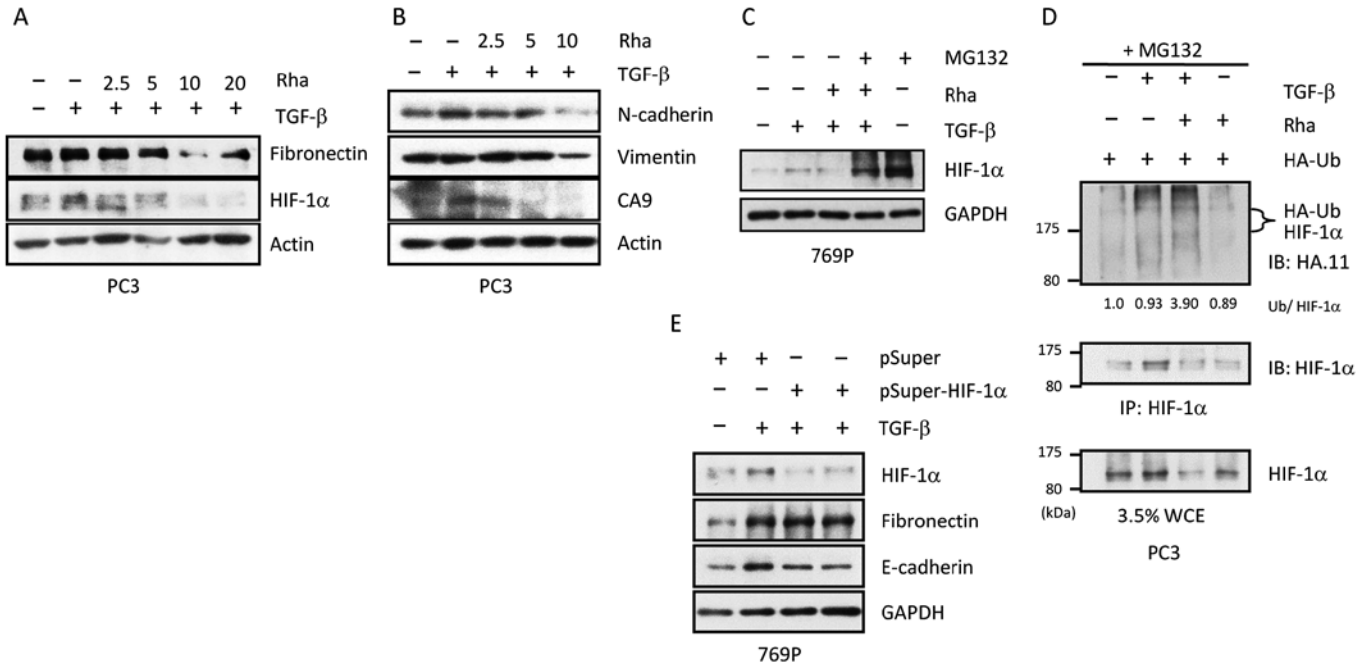

Figure 4. Rhapontigenin induces ubiquitination and degradation of HIF-1α. (A) Rhapontigenin (Rha) inhibited HIF-1α expression in a dose-dependent manner. (B) Rha inhibited TGF-β-induced expression of N-cadherin, vimentin, and CA9. (C) Pre-treatment with 26S proteasome inhibitor MG132 prevented induction of TGF-β-mediated degradation of HIF-1α by Rha. (D) Rha enhanced ubiquitination of HIF-1α via TGF-β signaling. PC3 cells were transfected with HA-tagged ubiquitin (HA-Ub) as indicated. At 24 h post-transfection, cells were pre-treated with Rha for 30 min, followed by TGF-β stimulation overnight. After 24 h, the cells were treated with 10 μM MG132 for 4 h and cell lysates were extracted for immunoprecipitation (IP). HA-Ub-conjugated HIF-1α was detected using anti-HA antibodies. (E) Knocking down HIF-1α expression via siRNA had little effect on TGF-β-mediated EMT.

regulation of HIF-1α expression by Rha was investigated. Rha reduced protein levels of HIF-1α in a concentration-dependent manner (Fig. 4A). In addition, protein levels of EMT-related markers N-cadherin, vimentin, and the noted HIF-1α target gene carbonic anhydrous IX (CA9) were reduced by Rha in a concentration-dependent manner (Fig. 4B). The mechanism underlying the effect of Rha on HIF-1α was assessed. As shown in Fig. 4C, MG132 completely abolished Rha-induced degradation of HIF-1α, indicating that the reduction in HIF-1α expression induced by Rha was partially due to degradation by the 26S proteasome. Next, to determine whether HIF-1α was recruited to the 26S proteasome as a result of ubiquitination, an immunoprecipitation (IP) experiment was performed, in which PC3 cells were transfected with a plasmid containing HA-tagged ubiquitin for 24 h, followed by exposure to TGF-β in the presence or absence of Rha for 24 h. Finally, the cells were treated with MG132 for 6 h. As shown in Fig. 4D, PC3 cells co-treated with TGF-β and Rha had approximately 3-fold the level of HA-tagged ubiquitin as those treated only with TGF-β. Because of the changes in EMT markers that accompanied HIF-1α expression, we assessed the involvement of HIF-1α in TGF-β-mediated changes in EMT markers. Therefore, 769-P cells were transfected with pSuper-HIF-1α or the pSuper control plasmid for 24 h, followed by exposure to TGF-β with or without Rha for 24 h. As shown in Fig. 4E, HIF-1α silencing had little impact on alterations in EMT markers, indicating that HIF-1α was not a critical factor in TGF-β-mediated EMT. Collectively, these results indicate that Rha treatment promotes TGF-β-induced HIF-1α ubiquitination, subsequently leading to HIF-1α degradation by the

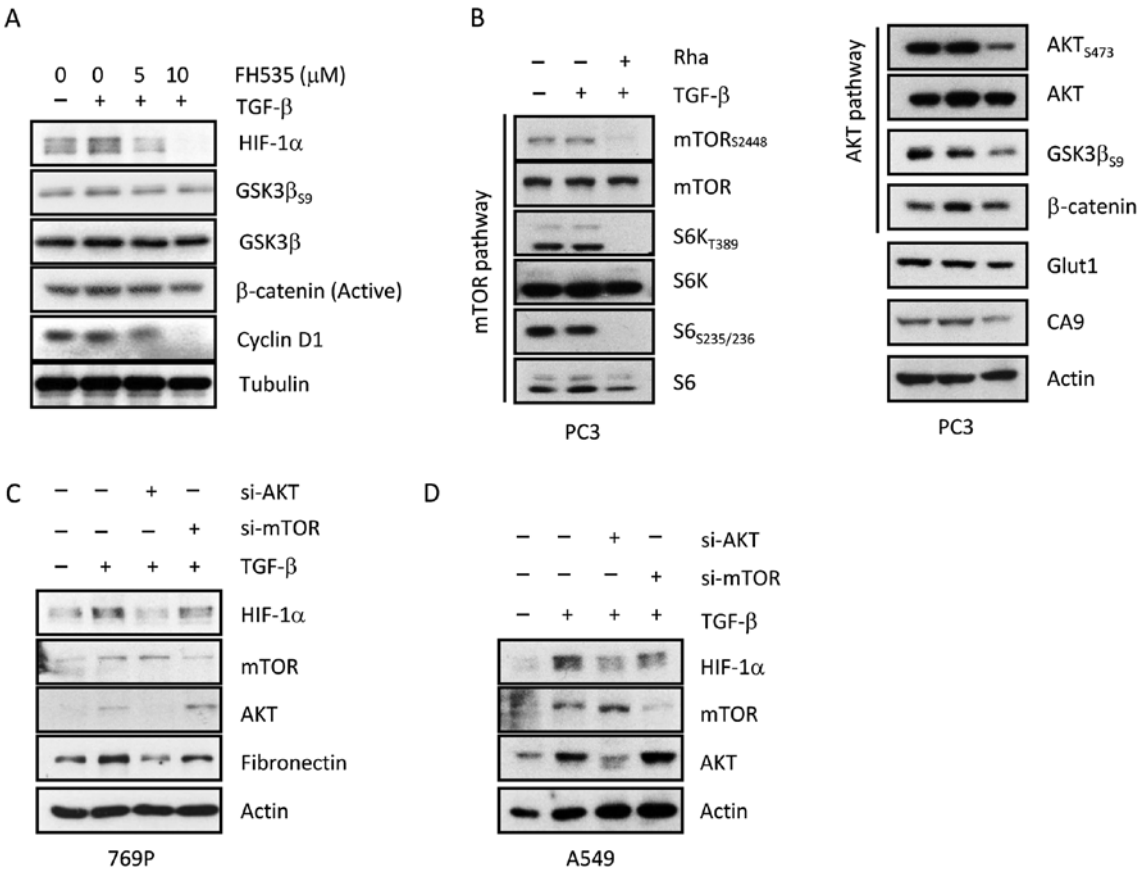

Figure 5. TGF-β-induced HIF-1α expression requires AKT and mTOR. (A) β-catenin inhibitor BH535 inhibited TGF-β-mediated HIF-1α expression. GSK3β phosphorylation at serine 9, GSK3β expression, and expression of β-catenin and cyclin D1 (downstream of GSK3β) were also downregulated by BH535 treatment. (B) Rhapontigenin inhibited TGF-β-mediated phosphorylation of AKT at serine 473, mTOR at serine 2448, S6K at threonine 389 and serine 235/236, and GSK3β at serine 9. (C) Knockdown of AKT and mTOR via siRNA led to downregulation of HIF-1α and fibronectin in 769-P cells. (D) Knockdown of AKT and mTOR via siRNA led to downregulation of HIF-1α in A549 cells.

26S proteasome. However, HIF-1α itself is not required for TGF-β-induced EMT.

TGF-β-induced HIF-1α expression requires AKT and mTOR. Previous studies demonstrated the importance of glycogen synthase kinase-3β (GSK-3β) and mTOR as downstream effectors of Akt following activation by TGF-β in diverse cell types (21,22). However, the functional relevance of Akt and its downstream effector pathways in TGF-β signaling remains largely unknown. We tested the involvement of pathways downstream of Akt in the regulation of TGF-β-mediated HIF-1α expression. The role of GSK-3β in the regulation of TGF-β-mediated HIF-1α expression was investigated. When cells are exposed to Wnt, GSK-3β is phosphorylated and becomes inactive. When Wnt signaling is blocked, active GSK-3β leads to phosphorylation, ubiquitination, and degradation of β-catenin. In brief, β-catenin retains the GSK-3β consensus motif for ubiquitination (23,24). As shown in Fig. 5A, Wnt/β-catenin inhibitor FH535 inhibited TGF-β-induced expression of HIF-1α and phosphorylation of GSK-3β at serine 9 in a dose-dependent manner. As the concentration of FH535 was increased, the decreased protein level of HIF-1α was concomitant with the decrease in phosphorylation of GSK-3β at serine 9 and active (non-phosphorylated) β-catenin in PC3 cells. In addition, FH535 treatment also significantly decreased the protein level of cyclin D1 in a dose-dependent manner in PC3 cells, demonstrating the association between β-catenin and cyclin D1 (25,26). We next examined the inhibitory effect of Rha on the mTOR and Akt signaling pathways. As shown in Fig. 5B, Rha abolished mTOR signaling by reducing phosphorylation of mTOR at Ser2448, S6K at Thr389, and S6 at Ser235/236. In addition, phosphorylation of molecules involved in Akt signaling, such as Akt (Ser473), GSK-3β (Ser9), and β-catenin, was also attenuated by Rha treatment. Furthermore, expression of HIF-1α downstream gene CA9 was also reduced by Rha treatment. These results suggest that Akt and mTOR may be important for TGF-β-induced expression of HIF-1α; therefore, to confirm this hypothesis, 769-P cells were transfected with siRNA against Akt and mTOR for 24 h, followed by exposure to TGF-β in the presence or absence of Rha for 24 h, after which the cells were collected for western blotting. As shown in Fig. 5C, knockdown of Akt and mTOR by siRNA markedly attenuated TGF-β mediated HIF-1α expression in 769-P cells, indicating that Akt and mTOR are required for TGF-β mediated expression of HIF-1α. However, the effect of Akt inhibition was more significant than that of mTOR inhibition. Similar effects were observed in A549 cells (Fig. 5D).

Rha inhibits TGF-β-mediated EMT via the PI3K/AKT pathway. Taken together with previous studies, the results described above prompted us to investigate whether the alterations in

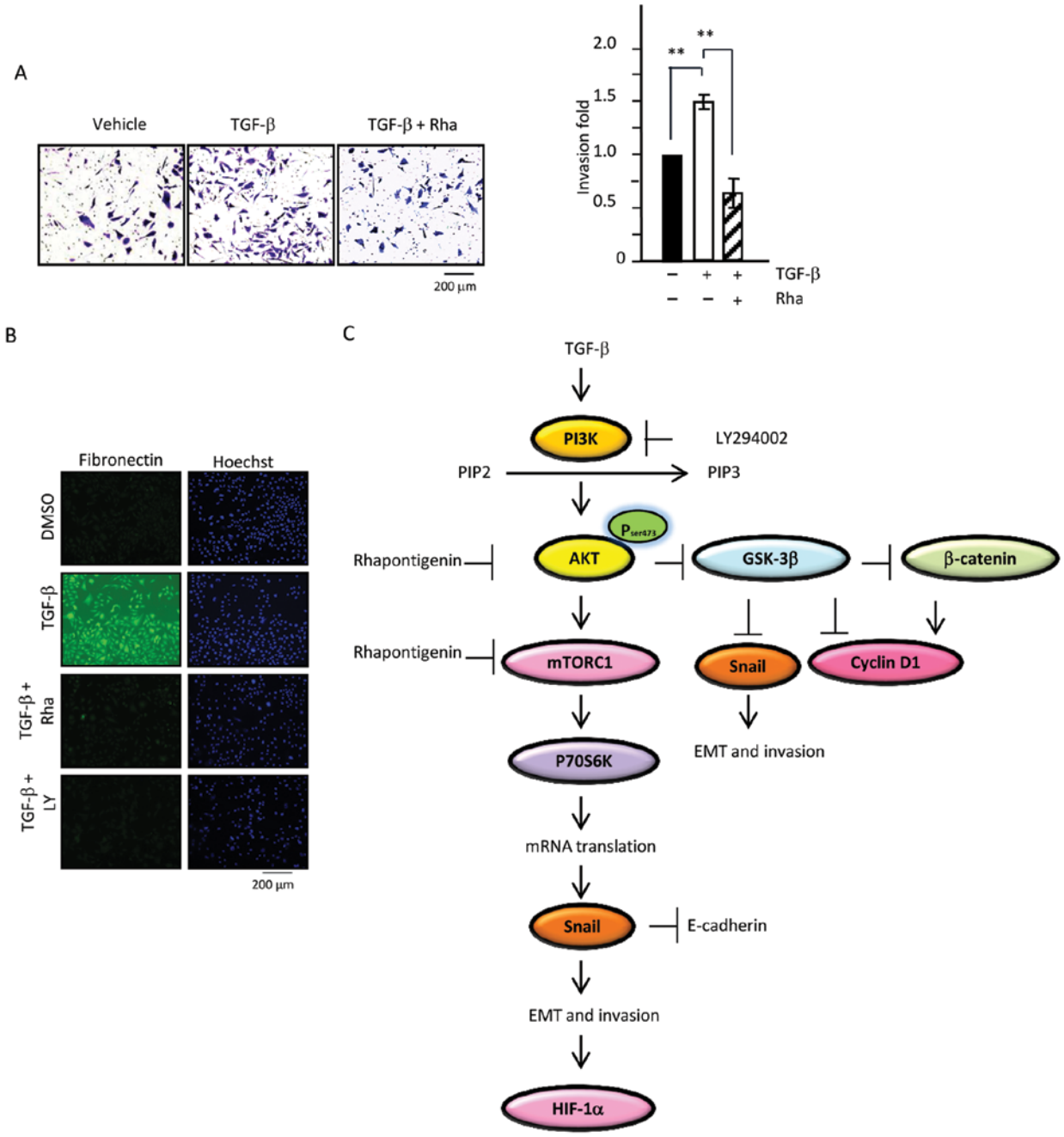

Figure 6. Rhapontigenin inhibits TGF-β-mediated EMT via the PI3K/AKT pathway. (A) Rhapontigenin (Rha) treatment inhibits TGF-β induced cell invasion. 769-P cells were treated with TGF-β for 1 day, followed by trypsinization and re-seeding into the Transwell chamber. Crystal violet was used to stain cells that penetrated through the Transwell insert [left panel, representative image from the invasion assay; right panel, quantitative results (n=4, $^{**}P<0.01$ vs. control)]. (B) Inhibition of fibronectin (FN) expression in PC3 prostate cancer cells by Rha. Cells were pre-treated with Rha (10 μM) for 30 min, followed by co-incubation with TGF-β for 48 h and staining with antibodies against FN. Reduced expression of FN as a result of Rha exposure was observed under a microscope. (C) Schematic representation of the molecular mechanism by which Rha inhibits EMT by suppressing AKT and mTOR, reducing the activity ofthe PI3K/AKT/mTOR/GSK3β/β-catenin pathway.

EMT induced by TGF-β enhanced invasiveness and metastasis (5,15,16,27-30). Therefore, IFA was performed. As shown in Fig. 6B, cells exposed to TGF-β for 48 h had higher expression of mesenchymal marker FN. In contrast, cells treated with LY or Rha in the presence of TGF-β lacked FN protein. Because of the connection between increased FN expression and cell invasiveness, cell invasion assays were employed to confirm the inhibitory effect of Rha. 769-P cells were exposed to TGF-β in the presence or absence of Rha for 20 h, followed by re-trypsinization and re-seeding of the treated cells into an invasion chamber harboring Matrigel ($1.25x10^5$ cells). After 48 h of incubation, the invasive ability of the cells that had penetrated through the inserts was assessed by crystal violent staining microscopic examination. Cells stimulated with TGF-β had an enhanced invasive ability in comparison with that of the control cells. Moreover, cells exposed to TGF-β and Rha had less invasive ability than cells treated with TGF-β only (Fig. 6A). Taken together, our results demonstrate that TGF-β is a contributing factor in cancer progression and metastasis. Moreover, our results suggest that Rha may suppress TGF-β-mediated inva-

sion via inhibition of the PI3K/Akt/mTOR/GSK-3β/β-catenin signaling pathway (Fig. 6C).

Discussion

In this study, we investigated the potential link between inflammation and the TME. In 1863, Virchow reported that cancers tended to occur at sites of chronic inflammation (2). Later, a wide array of evidence confirmed the connection between inflammation and cancer, as best demonstrated by inflammatory bowel disease (IBD) and renal fibrosis (27,31,32). As a result of such studies, it has been accepted that the state of the TME plays an important role in cancer development.

The results described above demonstrate that TGF-β plays a pivotal role in the EMT and subsequent cancer cell invasion. In order to survive and adapt to stressful environments, stromal cells secrete cytokines that allow them to avoid detection by the immune system, thus promoting the development of inflammation-related cancer. Furthermore, invasion and metastasis are crucial biological processes of malignant tumors. During the progression to metastasis, the EMT allows cancer cells to acquire mesenchymal-like characteristics that enhance invasiveness.

For decades, the transcription factor HIF-1α has been implicated in cell survival, the EMT, and anticancer drug resistance (33,34). Therefore, HIF-1α is an attractive target for chemopreventive agents. Under normoxic conditions, HIF-1α is activated by hormones, cytokines, and other signaling molecules (33,35). The mechanism underlying cytokine-induced HIF-1α expression is different from that by which it is upregulated during hypoxia. Growth factor-mediated HIF-1α activation is associated with increased protein synthesis and reduced protein degradation via regulation of PI3K and MAPK signaling (36-39), in accordance with our observation that TGF-β mediated HIF-1α expression in the cancer cells tested in our study. In contrast, hypoxia-mediated HIF-1α expression is usually achieved by decreasing HIF-1α degradation and blocking recognition of HIF-1α by E3 ligase VHL (33).

Numerous studies have demonstrated that the stability of Snail is regulated primarily by Akt/GSK3β and NF-κB (19,40,41). We found that Rha decreased the stability of Snail by suppressing PI3K/Akt/mTOR signaling. Snail-mediated FN upregulation and E-cadherin downregulation are hallmarks of the EMT. In addition, activation of PI3K/Akt signaling has been reported as a characteristic of EMT (42,43). Our results indicate that the ubiquitin-proteasome pathway was involved in regulating turnover of HIF-1α and Snail in diverse cell lines. Furthermore, our findings demonstrate that Snail and HIF-1α were recruited to the 26S proteasome for degradation as a result of Rha exposure. Taken together, our results revealed that Snail is required for TGF-β induced EMT, whereas HIF-1α is not.

The present study demonstrates that Snail plays a pivotal role in TGF-β mediated EMT and is associated with TGF-β-driven cell invasion. Based on these results, we have identified mechanisms that underlie the effects of Rha on TGF-β and HIF-1α signaling in cancer cells. First, Rha inhibited TGF-β-triggered invasion. Second, Rha, like LY, strongly inhibited TGF-β-induced EMT via PI3K/Akt/mTOR signaling (Fig. 6C). Therefore, targeting PI3K/Akt/mTOR and HIF-1α could be useful therapeutic strategies for preventing tumor metastasis (Fig. 6D). Our experiments demonstrated the link between PI3K/Akt/mTOR signaling and TGF-β, which can be regulated by Rha. We showed that Rha increased proteolytic degradation of HIF-1α and suppressed TGF-β-mediated EMT. Our results demonstrate for the first time that Rha inhibits cancer progression and metastasis by suppressing the EMT via regulation of the PI3K/Akt/mTOR pathway. Additionally, Rha was found to reduce HIF-1α protein expression in a dose-dependent manner. Given the multiple functions of Rha, future studies should be aimed at confirming its potential as a chemopreventive agent.

Acknowledgements

This study was supported by the grants from the Ministry of Science and Technology (MOST 104-2325-B-002-040) as well as (Most-103-2320-B-002-063-MY3).

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