Nobiletin inhibits invasion via inhibiting AKT/GSK3β/β-catenin signaling pathway in Slug-expressing glioma cells

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Abstract. Epithelial-mesenchymal transition (EMT) is a pivotal event in tumor progression during which cancer cells undergo dramatic changes acquiring highly invasive properties. In this study, we found that nobiletin, a polymethoxylated flavone, suppressed migration and invasion in both U87 and U251 glioma cells. Expression of epithelial markers (E-cadherin and occludin) was upregulated; mesenchymal markers (N-cadherin, fibronectin) and the transcriptional factor Slug were downregulated after nobiletin treatment. Transforming growth factor β (TGF-β) was applied to stimulate EMT and the results showed that nobiletin not only influenced basal level cell migration but also prevented TGF-β-triggered migration and EMT, with the AKT/GSK3β/β-catenin signaling pathway greatly involved. Furthermore, nobiletin remarkably diminished TGF-β-induced β-catenin nuclear translocation and the binding to the Slug promoter. It is worth noting that nobiletin almost blocked invasion in Slug-expressing U87 and U251 cells, and only exhibiting faint effect on non-Slug-expressing U343 glioma cells. Reinforced Slug expression in U343 cells by transfecting Slug plasmid was significantly attenuated by nobiletin, demonstrating the essential role of Slug in the anti-metastasis effect of nobiletin. Nobiletin repressed tumor growth *in vivo* and abrogated EMT in nude mice bearing U87-Luc xenografts, as demonstrated by Xenogen IVIS imaging and immunohistochemistry assay. Our findings suggested that nobiletin might have a great potential for treating glioblastoma.

Introductions

As the most frequent tumors affecting humans, glioma originates in the cerebral glia of brain tissue. Glioblastoma (GBM) is the most common malignant form of glioma with a high mortality. The survival rate at present for GBM patients is only one year (1), and the dilemma has not been greatly overcome over the last decade. Specifically, the available treatments including targeted drugs, chemotherapy, radiotherapy and surgical resection, have displayed slight improvement on survival of GBM patients (2). No distinct borderline exists between brain tissue and glioma due to the nature of aggressive invasion (3). Uncovering the molecular mechanism of malignant glioma invasion will be beneficial for the clinical diagnosis and therapy of malignant gliomas. This will not only prolong the progression-free survival but also suggest new approaches for the innovation of potent anti-glioma drugs.

As a crucial step in the initiation of metastatic status, epithelial-mesenchymal transition (EMT) has drawn worldwide attention for therapeutic interventions targeting metastasis. During the process of EMT, cells gradually replace their epithelial phenotypes with a mesenchymal characteristic, which is accompanied with augmented migration and invasion. Decrement in epithelial junction protein (E-cadherin) as well as an increment in mesenchymal markers (N-cadherin and vimentin) is commonplace in tumors with metastatic potential (4). An emerging hypothesis is that EMT endows cancer cells to invade, migrate and subsequently disseminate to form distant metastases.

It is well known that the complex interactions between the microenvironment and tumor are critical to orchestrate the destiny of carcinoma development (5). A tumor microenvironment is composed of an intricate crosstalk of various cell types and cytokines and growth factors, which have been proposed to modify the EMT procession. Among them, the polypeptide cytokine transforming growth factor beta (TGF-β) is widely involved in cell biological events such as migration, invasion, differentiation and proliferation (6,7). TGF-β1 has been demonstrated to facilitate the expression of extracellular matrix proteins, thus inducing EMT. Particularly in glioma, high level and secretion of TGF-β1 has been observed, indicating the important role of TGF-β1 in glioma invasion. Moreover, TGF-β1 is closely associated with the regulation of epithelial or mesenchymal markers, such as fibronectin and E-cadherin (8).

Both phosphatidylinositol 3-kinase (PI3K)/AKT and Smad pathways play important roles in translational regulating effect

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of TGF-β during EMT. p-AKT also functions in differentiation, apoptosis, and proliferation of glioma cells. Glycogen synthase kinase-3β (GSK-3β), a ubiquitously expressed serine/threonine kinase, is a downstream target of p-AKT and can inactivate various substrates, including HIF-1, cyclin D1, glycogen synthase and β-catenin (9). GSK-3β is active in resting epithelial cells (10). β-catenin is a crucial structure adaptor. PI3K/AKT signaling inhibited the GSK3β-mediated degradation of β-catenin. This fosters the translocation of cytoplasmic β-catenin into the nucleus where it can interact with the lymphoid enhancer factor and T-cell factor (LEF/TCF) transcriptional complexes (11,12). This interaction will further modulate the expression of various downstream target genes including Slug and E-cadherin.

Bioactive components emerging from traditional medicine have exhibited potential anticancer efficacy in recent years. Also known as heterogeneous polyphenols, flavonoids are rich in fruits, vegetables, tea and wine. Nobiletin (3',4',5,6,7,8-hexamethoxyflavone) is a polymethoxy flavonoid found in citrus fruits, which have been widely used in traditional Chinese medicine with potent anti-inflammatory, antidepressant, antioxidant, anti-hypertension and antibacterial effects. Previous studies have demonstrated the growth inhibitory effects of nobiletin in various cancer cell lines and tumor models such as glioma cancer, lung cancer, and breast cancer. Anticancer activities of nobiletin involve repression of proliferation, migration, invasion and angiogenesis.

Recently, it was reported that nobiletin regulates the cell cycle via MAPK and AKT pathways, accompanied with the suppression of glioma cell proliferation (13). Nobiletin inhibits tumor growth and angiogenesis of ovarian cancers via the AKT pathway (14). Moreover, metastasis was also attenuated via both ERK and PI3K/AKT pathways in HGF-treated liver cancer $HepG_2$ cells (15). In summary, these data proved the essential role of AKT in the anticancer effect of nobiletin, as an encouraging bioactive compound with distinguishing anticancer effects. However, the downstream effector and the molecular mechanism need to be explored. Given that EMT plays a fundamental role in metastasis of glioma tumors, we therefore examined the potential of nobiletin to prevent EMT, migration, and invasion. We demonstrated that nobiletin inhibited EMT of U87 and U251 cells, in which expression of Slug can be observed, while exerting weak effects on U343 cells which expresses rather low level of Slug. Nobiletin inhibited TGF-β-induced EMT *in vivo* and *in vitro*, with AKT/GSK3β/β-catenin signaling pathway greatly involved. Collectively, our data indicated that nobiletin may have potential in glioma cancer treatment.

Materials and methods

Reagents. Nobiletin, LY294002, TGF-β1 was from Merck Millipore (Darmstadt, Germany). AZD1080, SKL2001 and diamidino-phenyl-indole (DAPI) were purchased from Funakoshi (Tokyo, Japan). Slug plasmid (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was transfected into U343 cells according to the manufacturer's protocol. Antibodies against p38, p-p38, ERK, p-ERK, p-AKT, AKT, β-catenin, GSK-3β, p-GSK-3β, p-Smad3 were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against E-cadherin, N-cadherin, occluding, fibronectin, Slug, Twist1, and Snail were obtained from Santa Cruz Biotechnology.

Cell culture and viability assay. U87, U251 and U43 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained at 37˚C, 5% CO_2, in DMEM supplemented with 10% fetal bovine serum, penicillin (500 U/ml) and streptomycin (100 μg/ml). Cell proliferation was determined using the CCK-8 (Beyotime Institute of Biotechnology, Shanghai, China) method. U87 and U251 cells ($3x10^3$ cells/well) were plated in 96-well plates. At 24 h incubation with nobiletin, CCK-8 reagents were added and incubated for another two hours. The absorbance at 450 nm represents the number of viable cells with a Multiskan Spectrum microplate reader.

Migration and invasion. In the migration assays, scratches were made in the cells and presented by the average of distance differences between 24 and 0 h. To assess invasion potential, Matrigel-coated Transwell was used. Nobiletin-treated cells were seeded in the upper chamber of the Transwell insert. The migration capacity over the Matrigel and the membrane towards the bottom chamber containing 10% FBS was examined. Cells were fixed in formaldehyde solution, and stained with crystal violet and counted.

Immunofluorescence staining. Cells were grown on glass coverslips and blocked with 5% normal fetal bovine serum containing 0.1% Triton X-100 for 2 h at room temperature, thereafter, coverslips were incubated with anti-E-cadherin antibodies overnight with DAPI (Molecular Probes). Fluorescence was visualized with a microscope and Image-Pro Plus 4.0 software.

Signaling experiments. Signaling experiments were performed as described previously with slight modifications (16). Nuclear and cytosolic fractions were carried out according to manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). In western blot assays, protein samples were separated on SDS polyacrylamide gel and then blotted onto nitrocellulose membranes using a Semi-dry electrotransfer mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) and transfer buffer. The membranes were incubated overnight at 4˚C with primary antibodies and then incubated with anti-IgG Antibody. Protein G-agarose beads (Invitrogen) were used for immunoprecipitations. Pierce Clean Blot IP Detection reagent (Thermo Fisher Scientific, Rockford, IL, USA) were applied for immunoprecipitation experiments. ECL substrates were used for protein band visualization (Bio-Rad). The bands densitometry was analyzed with ImageJ software.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed as previously described with slight modification (17). β-catenin antibody was used, and qPCR was performed with specific primers to the slug promoter. PCR amplifications were conducted using Blend Taq DNA polymerase and the PCR products were analyzed by electrophoresis on agarose gel and then qPCR. Cross-link released chromatin was saved and reversed by proteinase K digestion and phenol/chloroform extraction. Immunoprecipitated samples were compared to the housekeeping gene RPL30.

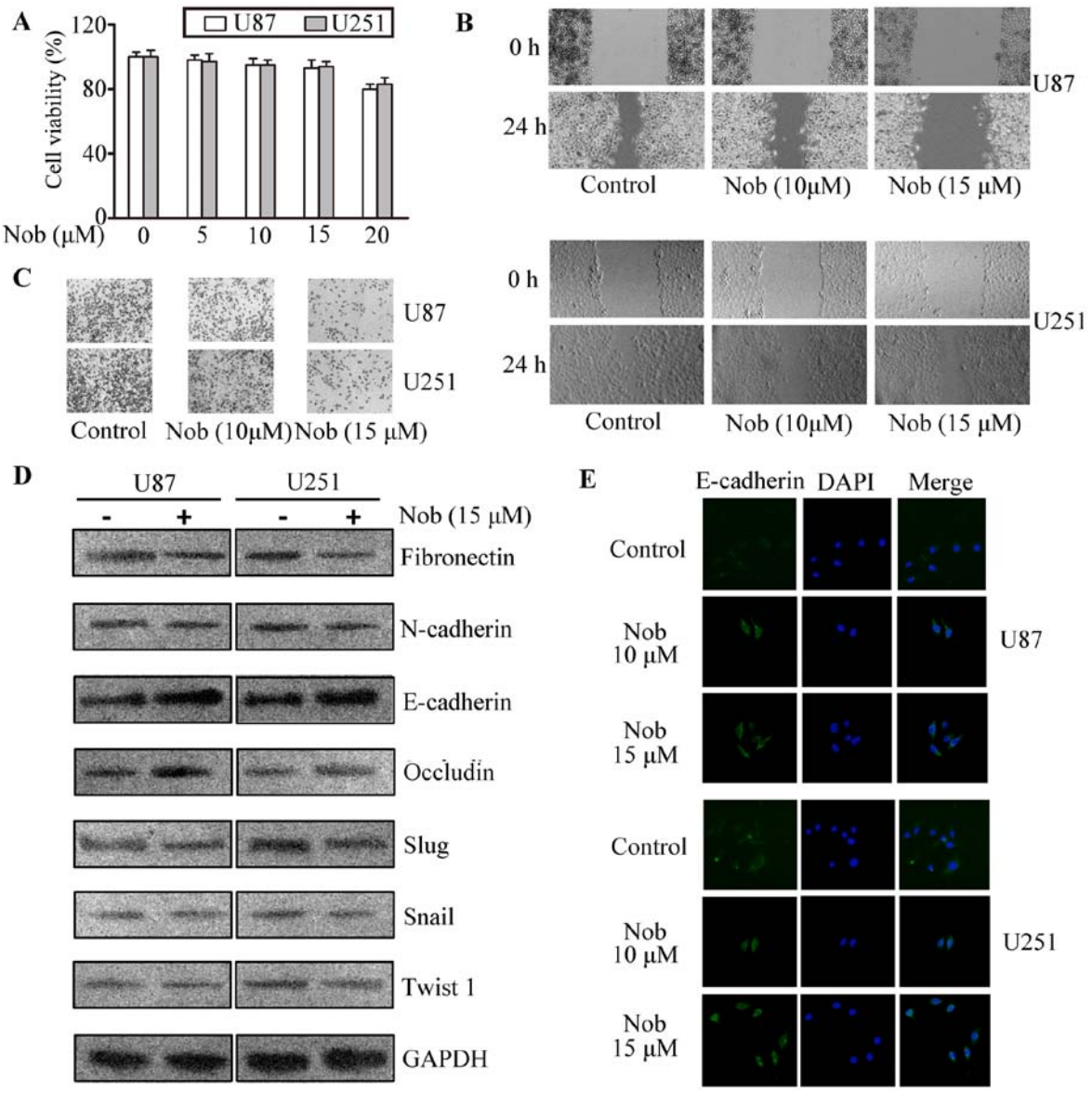

Figure 1. Nobiletin repressed invasion, migration and EMT in glioma cancer cells. (A) CCK-8 assays of cell viability after 48 h of incubation with nobiletin at indicative concentrations. $p<0.05$; $p<0.01$, versus control. (B) Migration of U87 and U251 cells was assessed with scratch-wound healing experiments. (C) The invasion of U87 and U251 cells were quantified by calculating the crystal violet-stained cells invading to the lower surface of the membrane. (D) U87 and U251 cells were treated with nobiletin (15 μM) for 24 h. Cell lysates were subjected to western blot analysis for E-cadherin, occludin, N-cadherin, fibronectin, Slug, Snail, Twist1 and GAPDH. (E) U87 and U251 cells treated with nobiletin (15 μM) for 24 h. Then the cells were incubated with anti-E-cadherin antibodies, respectively, and assessed by immunofluorescence. The nucleus was stained with DAPI (blue).

Antitumor efficiency. Athymic nude mice (4 weeks old) were maintained under pathogen-free conditions. U87-Luc cells were harvested and resuspended. Suspended cells ($1x10^7$) were subcutaneously injected into the flank of each nude mouse. U87-Luc-bearing nude mice were randomly divided into four groups as follows: Vehicle group (saline daily); Cyclophosphamide positive group (20 mg/kg, i.p. every 3 days); nobiletin group (15 and 30 mg/kg, oral administration daily). The tumor growth was examined with a caliper every 3 days and calculated by the following equation: V = length x width^2/2. Mice were observed by the IVIS *in vivo* imaging system after 24 day-treatment.

Immunohistochemical analysis (IHC). The primary tumors were immunostained for E-cadherin, N-cadherin and Slug as previously described (18).

Statistical analysis. Statistical analysis was conducted with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA was used to compare between multiple groups. All results are expressed as mean ± SD. $p<0.05$ and $p<0.01$ were considered to indicate statistically significant differences.

Results

Nobiletin inhibits viability, migration, invasion and EMT of glioma cells. In the CCK-8 assays, nobiletin at 5, 10 and 15 μM exhibited no significant cytotoxic effect to U87 and U251 cells (Fig. 1A). Based on our previous data, these concentrations intervene with neither metabolism nor function of mitochondria, so they were then used in the following experiments. Wound healing models were used to assess the effect of nobiletin on

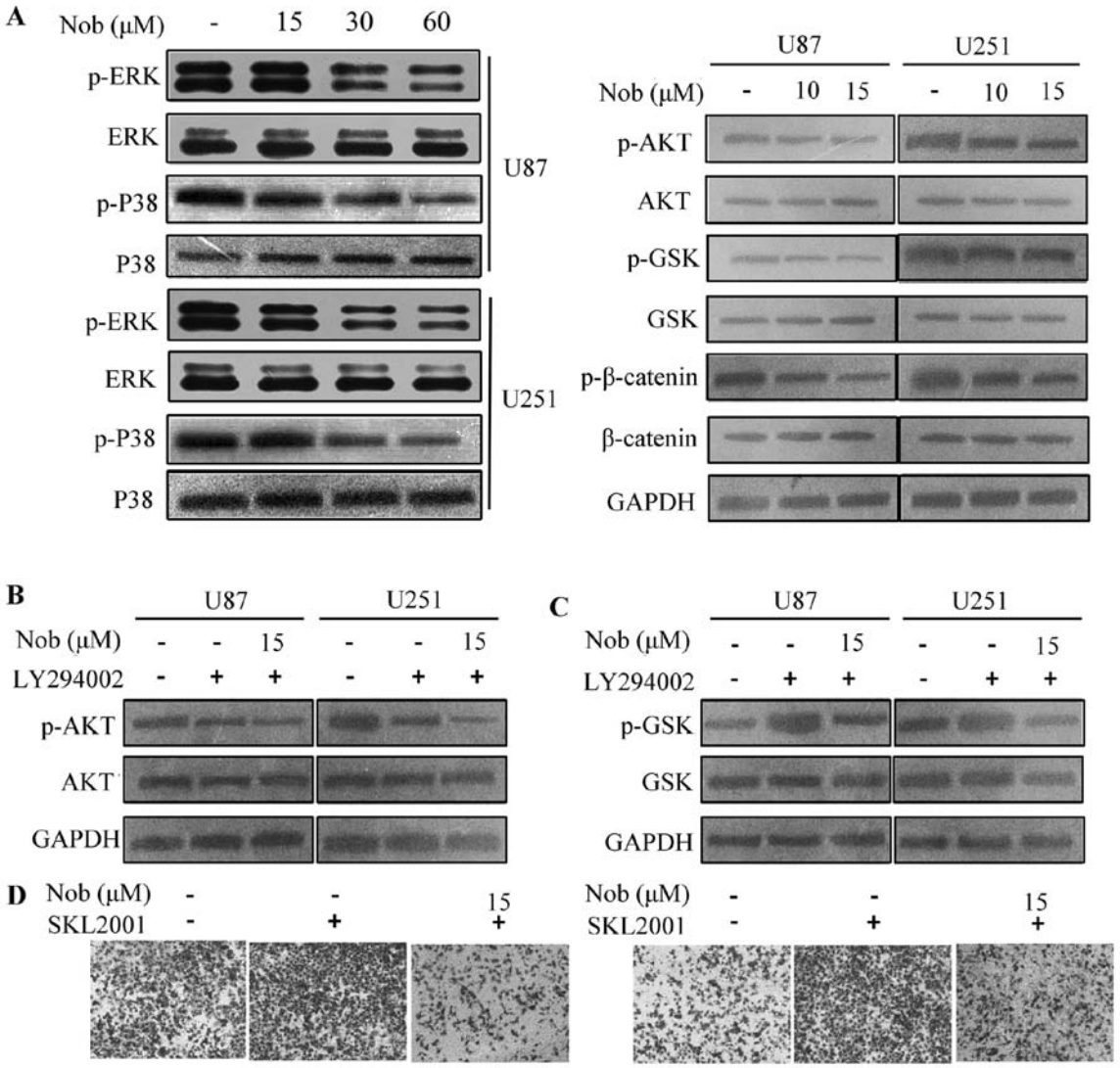

Figure 2. Nobiletin inhibits cell migration by inhibiting the AKT/GSK-3β/β-catenin signaling pathway. U87 and U251 cells were treated with nobiletin (10 and 15 μM) for 24 h. (A) ERK/p38, PI3K/AKT and Wnt/β-catenin signaling was evaluated via western blotting. (B) After exposure to the combination of nobiletin and LY294002 (a specific PI3K inhibitor) for 24 h, western blotting was applied to analyze p-AKT proteins with GAPDH as a loading control. (C) After exposure to nobiletin, cells were further incubated with AZD1080, an inhibitor of GSK3β, for 12 h. p-GSK-3β was detected by western blotting. (D) Cells were co-incubated with nobiletin and SKL2001 (40 μM), a small molecule inhibitor of β-catenin degradation and further subjected to cell invasion assays. Each value represents the mean ± SD from triplicates.

the migratory of glioma cells. It can be seen from Fig. 1B that the migratory potential was remarkably inhibited by nobiletin. Twenty-four hours after the scratch, cells in the vehicle group almost filled the scratched area, whereas an obvious gap was observed in the wound in nobiletin-treated cells. The quantification by scratch width showed that the inhibition rate of nobiletin (10 and 15 μM) was approximately 25 and 45% in U251 cells and 33 and 50% in U87 cells, respectively. Next, the regulatory effect of nobiletin on the invasion of U87 and U251 cells were validated via matrigel transmembrane assay. The results showed that cells in the control group invaded through the matrigel faster than nobiletin-treated cells, and the inhibitory rate of nobiletin (15 μM) reached approximately 65 and 60% in U87 and U251 cells, respectively (Fig. 1C).

E-cadherin, occluding, N-cadherin, and fibronectin play an essential role during the progress of cancer metastasis. In western blotting assays, the expression of EMT-related proteins in glioma cells were examined. In Fig. 1D-E, the epithelial markers occludin and E-cadherin were increased while mesenchymal markers such as fibronectin and N-cadherin were reduced in both cell lines. As is known, Snail, Slug and Twist1, are potent repressors of E-cadherin. Herein, transcriptional factors involved in EMT were further tested to prove the efficacy of nobiletin. We found nobiletin considerably inhibited Slug expression while displaying negligible effect on the levels of Snail and Twist1.

Nobiletin inhibits cell migration by blunting the AKT/GSK-3β/β-catenin signaling pathway. To uncover the molecular mechanisms of nobiletin, essential and crucial pathways manipulating cancer invasion are examined, such as ERK/p38, PI3K/AKT and Wnt/β-catenin axis. The ERK/p38 signaling was only subtly affected by nobiletin treatment at 15 μM. At the concentration of 30 and 60 μM, nobiletin displayed a potent inhibitory effect on ERK and p38 phosphorylation. Moreover, PI3K/AKT pathway was remarkably

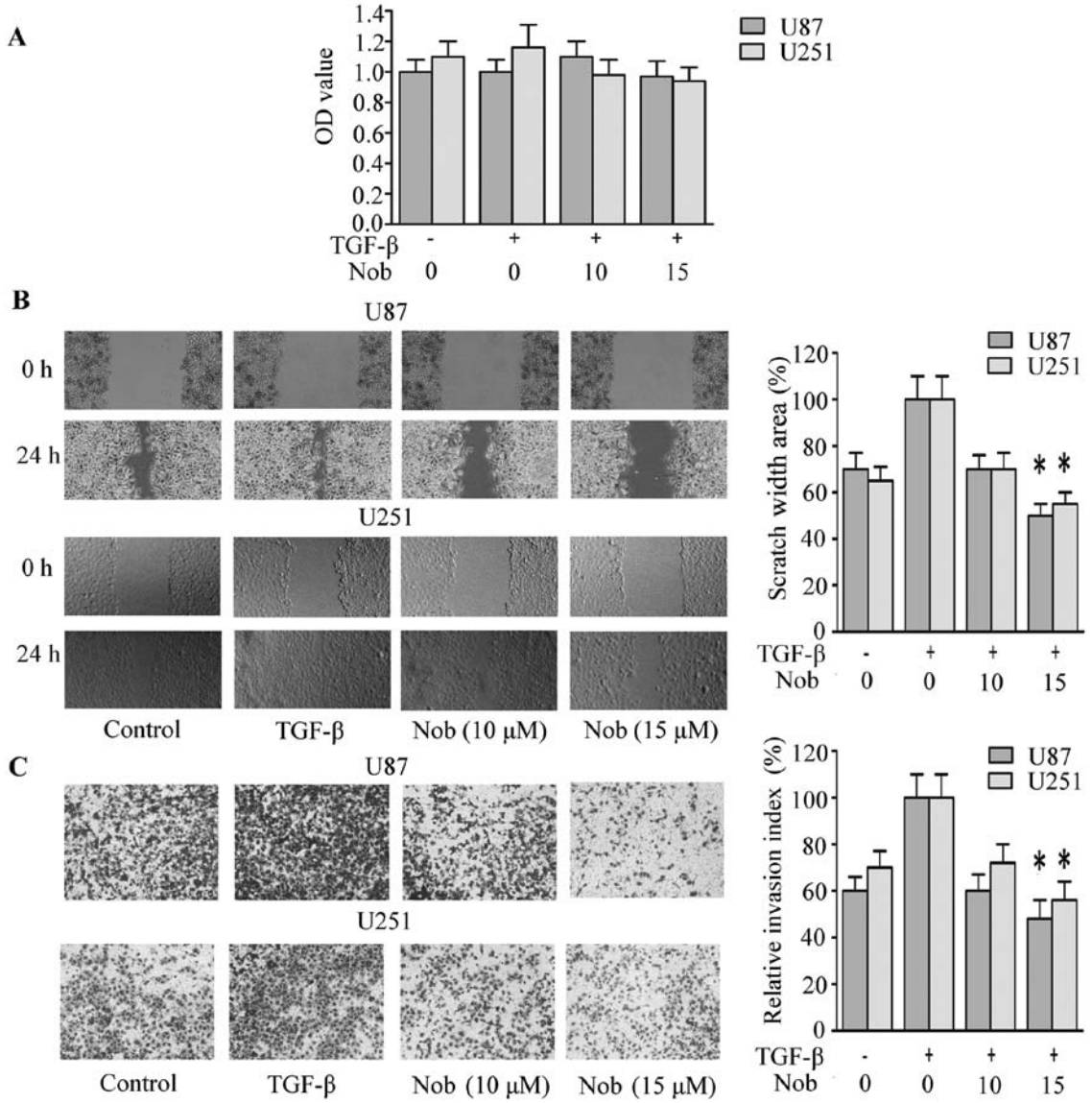

Figure 3. Nobiletin suppresses TGF-β1-stimulated migration and invasion in glioma cancer cells. (A) CCK-8 assays of cell viability after 24 h of co-incubation with TGF-β1 (10 ng/ml) and nobiletin at indicative concentrations. (B) Migration was examined with scratch-wound healing experiments. (C) Cell invasion was measured by calculating the crystal violet-stained cells invading to the lower surface of the membrane. Images were captured with microscope. *p<0.05; versus control. #p<0.05 versus TGF-β1-treated cells.

suppressed at 15 μM. Decrement in the phosphorylation of AKT, GSK3β and β-catenin proteins was observed in nobiletin-treated U87 and U251 cells (Fig. 2A). Combination of nobiletin and LY294002 (a specific PI3K inhibitor) synergistically repressed the activation of AKT pathway in glioma cells (Fig. 2B). As a potent inhibitor of GSK3β, AZD1080 was also used in the study. Nobiletin reversed AZD1080-triggered GSK-3β phosphorylation in both U87 and U251 cells. SKL2001, a small molecule inhibitor of β-catenin degradation, was chosen to promote β-catenin stabilization (19). SKL2001 (40 μM) enhanced cell invasion, and the increment was partially abrogated by nobiletin (Fig. 2D). In summary, these results imply that the AKT/GSK-3β/β-catenin axis plays a critical role in the function of nobiletin on glioma cell migration.

Nobiletin inhibits TGF-induced cell invasion and EMT in glioma cells. Amplified secretion of TGF-β by carcinoma cells, and augmented TGF receptor expression, leading to autocrine TGF-β loop, are regarded to drive or to be requisite for EMT progression in tumor cells. Matrigel invasion and wound-healing assay was performed to assess whether nobiletin inhibits invasion stimulated by TGF-β. In the CCK-8 analysis, medium containing TGF-β (10 ng/ml) and nobiletin (10, 15 μM) exerted no distinguished cytotoxicity on cell viability (Fig. 3A). Therefore, TGF-β (10 ng/ml) and nobiletin (10, 15 μM) were used for following assays. After exposure to TGF with or without nobiletin for 24 h, U87 cells were harvested and reseeded into a Transwell-coated with matrigel. After 24 h, cells that penetrated through the inserts was stained with crystal violent and assessed under microscopy. TGF-β enhanced both invasion and migration, but the tendency was reversed by nobiletin (Fig. 3B and C). Cells that had undergone EMT in response to TGF-β accompanied with fibronectin and N-cadherin increment and E-cadherin and occludin decrement. In line with inhibitory effect on basal

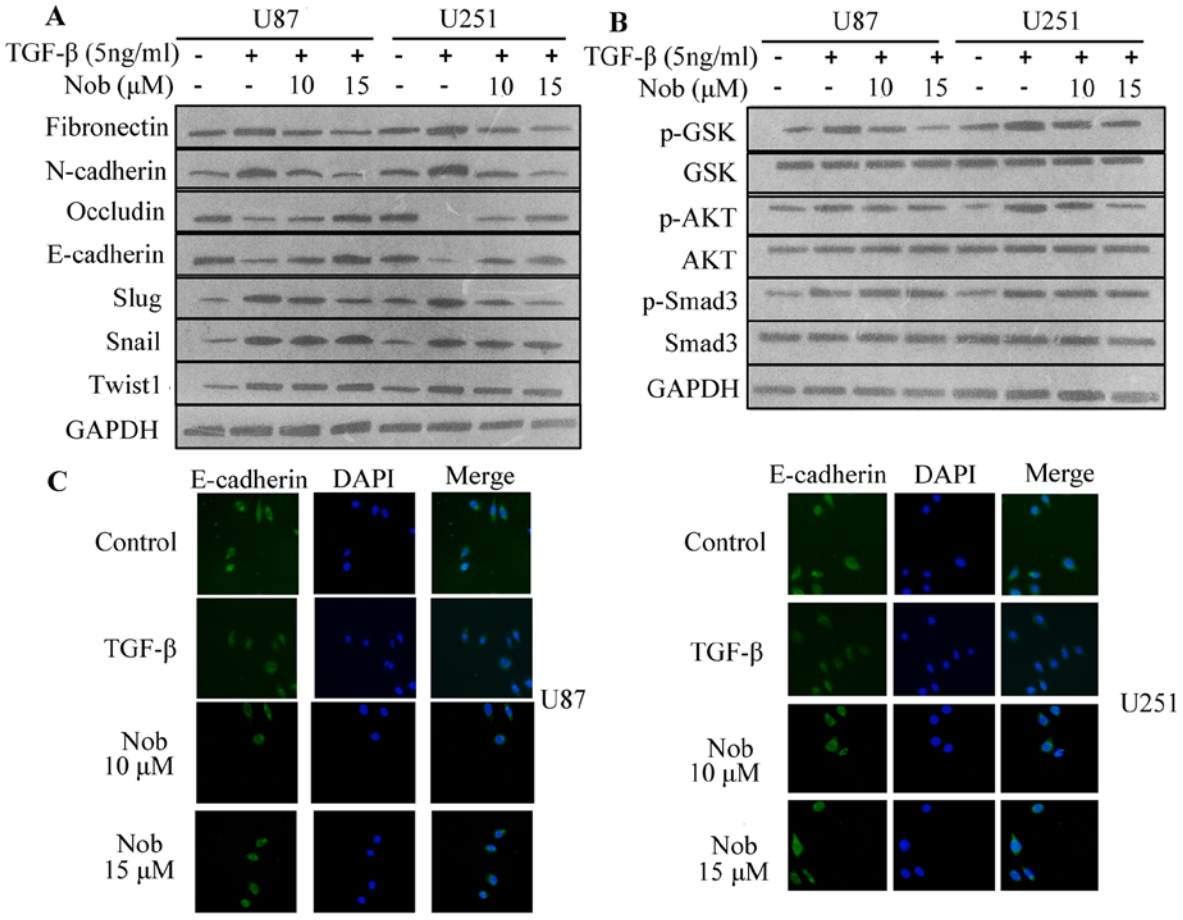

Figure 4. Nobiletin inhibits TGF-β1-induced EMT in glioma cancer cells. (A) Cells were treated with TGF-β1 (10 ng/ml) and nobiletin (15 μM) for 24 h. EMT-related proteins were assessed by western blotting. (B) TGF-β/smad and non-canonical pathways were measured via western blotting. (C) The expression of epithelial marker E-cadherin was evaluated by immunofluorescence.

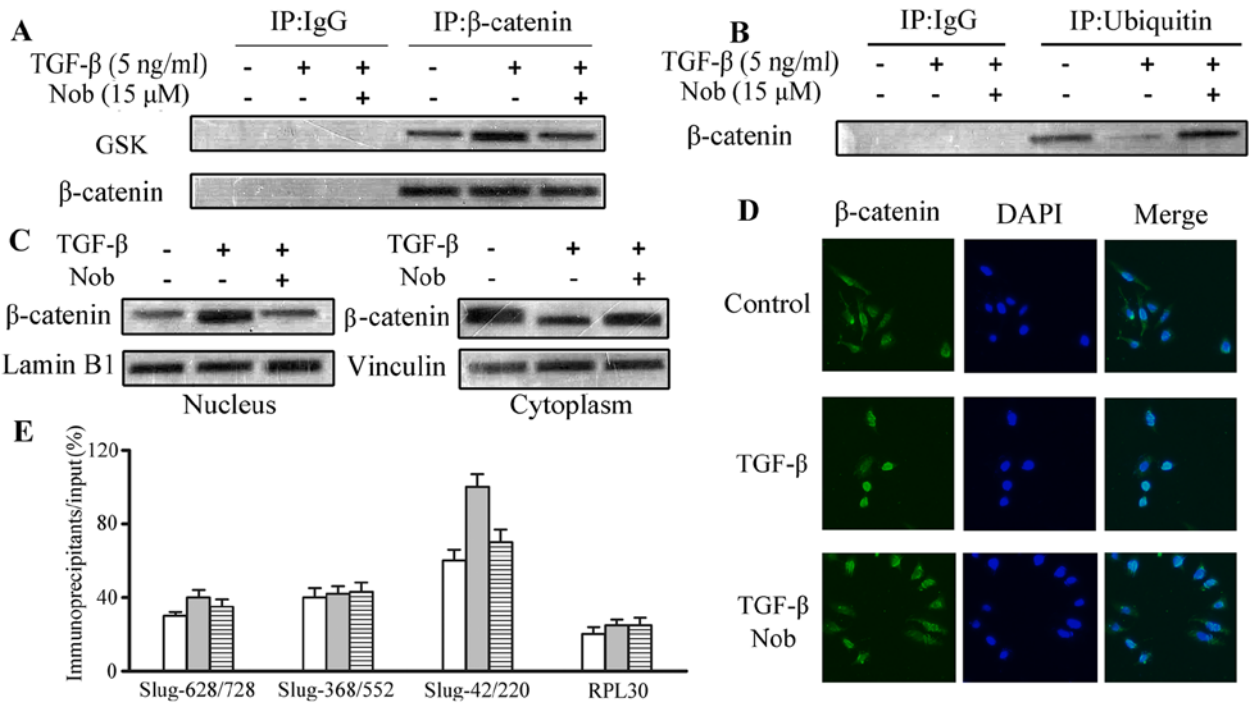

Figure 5. Nobiletin abrogates the activation of β-catenin induced by TGF-β1. (A) Immunoprecipation assay was used to examine the β-catenin/GSK-3β complex in TGF-β treated U87 cells. (B) Western blot assays for β-catenin ubiquitination. (C and D) Western blot and immunofluorescence staining were used to detect nucleus translocation of β-catenin. (E) ChIP tests were carried out to explore the binding of β-catenin to the promoter of Slug. $p<0.05$; $p<0.01$, versus control. $p<0.05$; $p<0.01$, versus TGF-β1-treated cells.

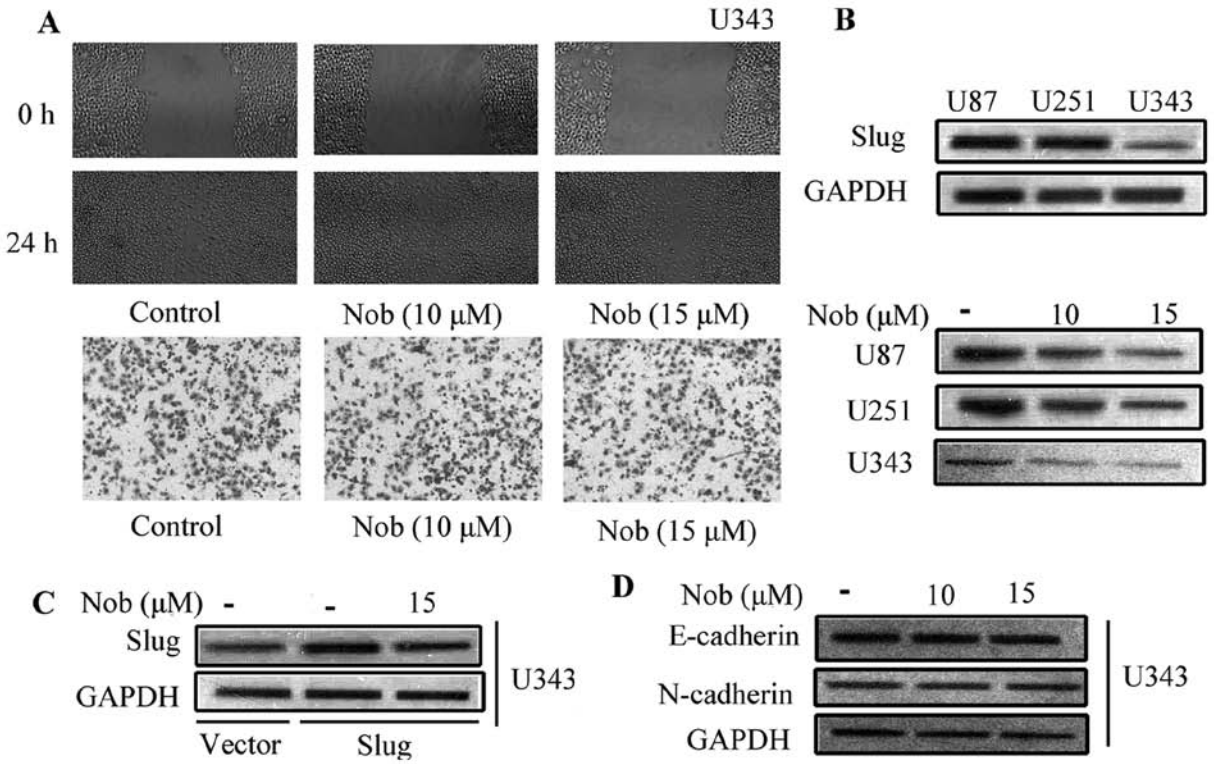

Figure 6. Nobiletin fails to inhibit the migration and invasion of glioma U343 cells. (A) Effect of nobiletin on the migration and invasion of U343 cells at 10 and 15 μM. (B) The protein expression of Slug in U343, U87 and U251 cells. (C) Effect of nobiletin on Slug expression of U343 cells or Slug plasmid-transfected U343 cells. (D) Effect of nobiletin on EMT-related protein levels in U343 cells.

level of mesenchymal markers, nobiletin reversed TGF-β-induced epithelial-mesenchymal transition (Fig. 4A).

TGF/Smads regulates the activities of EMT transcription factors such as Slug (Snai2), which suppresses E-cadherin transcription. TGF-β also activates non-Smad pathway, such as AKT and GSK-3β. The role of above-mentioned pathways in regulating TGF-β-stimulated EMT was assessed. Consistently, nobiletin blunted the acitivity of AKT/GSK-3β axis induced by TGF-β, however, showing little effect on TGF-β-activated Smad3 phosphorylation (Fig. 4B). Collectively, these data indicated that nobiletin did not retard EMT via directly targeting Smad3 signaling.

Nobiletin intervenes with TGF-β-induced β-catenin nuclear translocation, and abolishes interactions between β-catenin and Slug. By interacting with E-cadherin, β-catenin is important in EMT progression. On the other hand, active GSK-3β will form a complex with β-catenin to hinder the translocation of β-catenin into nucleus. Phosphorylated GSK-3β is an inactive form of GSK-3β, which would abolish the interaction with β-catenin, subsequently promoting nuclear translocation and inhibiting β-catenin degradation. As revealed in the immunoprecipation assay, an increase was observed in the β-catenin/GSK-3β complex in TGF-β treated U87 cells (Fig. 5A). Western blot assays for β-catenin ubiquitination displayed that TGF-β considerably reduced β-catenin proteasome degradation, and the decrement was greatly alleviated by nobiletin treatment (Fig. 5B). Furthermore, nucleus translocation of β-catenin was also upregulated due to TGF stimuli. Nevertheless, the function was abolished after nobiletin treatment (Fig. 5C), as demonstrated in the immunofluorescence staining. Fractionation followed by western blot also showed similar results (Fig. 5D). ChIP tests were carried out to explore the binding of β-catenin to the promoter of Slug. Noteworthy, nobiletin successfully prevented the binding of β-catenin to the Slug promoter, induced by TGF (Fig. 5E). It can be concluded that nobiletin might influence EMT via regulating GSK-3β and the downstream effector β-catenin.

Nobiletin fails to inhibit the migration and invasion of glioma U343 cells, which do not express Slug. To confirm the essential role of Slug in the anti-metastasis effect of nobiletin, a non-Slug-expressing glioma cell line was applied to compare the effect of nobiletin on migration and EMT. According to preliminary research, these three cell lines have divergent Slug protein levels. The protein expression of Slug was rather low, almost undetectable in U343 cells, and high level of Slug was observed in both U87 and U251 cells (Fig. 6A). Nobiletin had little effect on the invasion of U343 cells at 10 and 15 μM. However, at the same concentration, the inhibitory rate reached 45 and 60% in U87 and U251 cell, respectively (Fig. 6B). Moreover, nobiletin failed to change Slug protein expression in U343 cells. Notably, nobiletin treatment brought down Slug protein expression in Slug plasmid-transfected U343 cells (Fig. 6C). The phenomenon that nobiletin enlarged E-cadherin expression and inhibited the level of N-cadherin in both U87 and U251 cells was not observed in U343 cells (Fig. 6D). EMT-related proteins remained unaffected in U343 cells. These data implied that Slug, either at transcriptional or

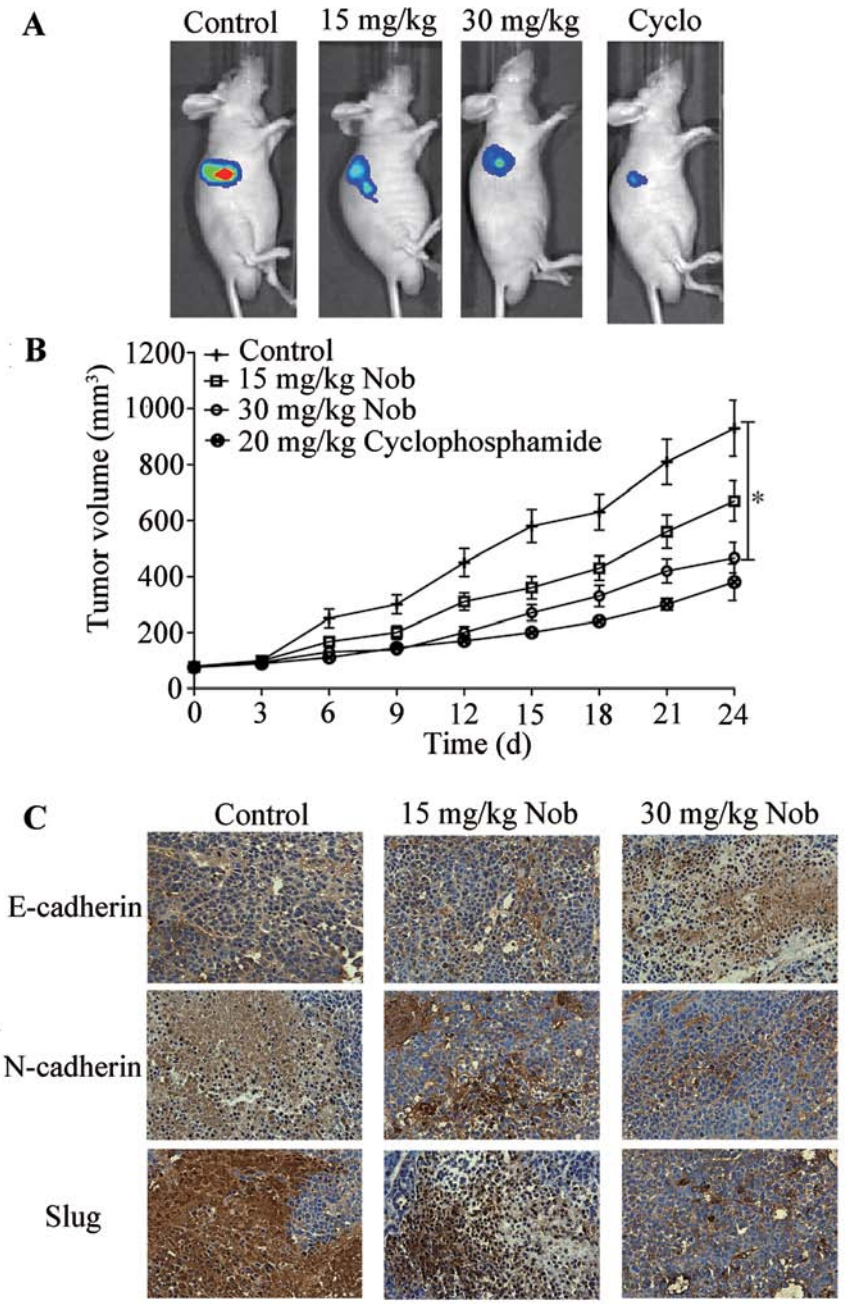

Figure 7. Nobiletin suppresses tumor growth in nude mice. (A) Representative xenografts of U87-Luc tumor images at the end of the treatment are shown. (B) Tumor volumes were measured every other day using electronic vernier calipers. (C) Representative images of E-cadherin, N-cadherin and Slug-immunostained tumor sections. *p<0.05, versus the vehicle group.

post-translational level, is greatly involved in the regulatory effect of nobiletin.

Nobiletin suppresses tumor growth in vivo. To further assess the antitumor efficiency of nobiletin *in vivo*, athymic nude mouse model bearing U87-Luc implanted xenografts were used. After 24-day treatment, the bioluminescence imaging results demonstrated impressive repression of tumor volume in nobiletin-treated nude mice ($p<0.01$, versus the vehicle group). Tumor sections were then subjected to IHC analysis for detection of E-cadherin, N-cadherin and Slug. E-cadherin expression was enhanced while N-cadherin and Slug was inhibited in nobiletin-treated group (Fig. 7). All these date indicated that nobiletin prevented EMT and growth of glioma cancer cells *in vivo*.

Discussion

Amplified TGF-β expression secreted by tumors is a critical hallmark in cancer progression, and it makes contributions to tumor growth and metastasis via autocrine and paracrine signaling (20). Cells motivated with TGF-β will be equipped with a greater invasive capacity than that of the control cells. An essential role of TGF-β in cancer progression is attributed to the ability of inducing EMT, along with cell phenotype changes and enhanced cell invasion and migration (21). Using

human glioma cells models such as U87 and U251 that undergo EMT triggered by TGF-β, we found that enhanced cell invasion in response to TGF-β stimuli go along with an increment in protein content of EMT-related markers. Cells treated with TGF-β and nobiletin showed weaken invasive ability than cells triggered with TGF-β only. A number of studies indicate a critical role for PI3K in TGF-β signaling (16-18). Besides, TGF-β activates AKT/GSK3β/β-catenin pathway and that this signaling is involved in the anti-metastasis effect of nobiletin. In addition to modulating EMT through above-mentioned signaling, nobiletin remarkably reduced TGF-β-induced β-catenin nuclear translocation and the binding to the Slug promoter. Finally, we demonstrated that nobiletin repressed the tumor growth and EMT progression in nude mice bearing glioma cell xenografts. Our findings suggested that nobiletin might have great potential for treating glioma.

Glioblastoma multiform (GBMs) represents the most lethal and most aggressive central nervous system tumor (22). GBM is characteristic of invasion all through the brain, distinguished heterogeneity in appearance and gene expression. Surgical resection followed by traditional radio-chemotherapy and newly-developed targeted approaches remains the standard therapy, but tumor recurrence is unavoidable and almost always causes mortality owning to their infiltrative nature. EMT is a multiple process which is regarded a crucial event in tumor progression and is responsible for introducing pathological variation in organisms. EMT reprogramming is characteristic of loss of cell-cell adhesions and apical polarity which ultimately leads to the formation of spindeloid morphology with cytoskeleton reorganization and increment in migratory capacity. A hallmark of EMT is the loss of E-cadherin, which is an important constituent of adhesion junctions that plays a vital role in maintaining the epithelial integrity (23).

A group of specific transcription factors, including Slug, Snail, Twist and ZEB, have been implicated in the manipulating of EMT, acting as transcriptional repressors of the E-cadherin (24). Snail family proteins regulate transcription of molecules for cell-cell adhesion during EMT. As a mediator of EMT and metastasis, Slug/Snail2 expression is strengthened in glioblastoma and can be stimulated by TGF-β and HGF (25). TGF-β-induced EMT, which is fundamentally studied as a model, incorporates classic Smad and non-Smad signaling, and transmits signaling via downstream PI3K/AKT and ERK/MAPK pathways (26). Invasion and EMT of TGF-induced cells as well as normal cells were all reversed by nobiletin in glioma cells. It is obvious that the protein levels of epithelial markers E-cadherin and occludin were upregulated after incubated with nobiletin for 24 h, along with reduced mesenchymal markers such as fibronectin and N-cadherin.

The oncogenic kinase AKT encourages EMT as well as the stabilization of β-catenin via phosphorylating GSK-3β. β-catenin was first discovered as a component of a mammalian cell adhesion complex (27). Phosphorylation of β-catenin by AKT promotes the dissociation of β-catenin from cell junctions and the gathering in nucleus (28). Additionally, β-catenin, which initially conglomerates with the cytoplasmic tail of E-cadherin at the C-terminus, exhibits nuclear translocation to promote expression of EMT-related genes and molecules associates with PI3K/AKT signaling (29).

To uncover the antitumor mechanisms of nobiletin, the effects of nobiletin during normal, repressed and motivated AKT phosphorylation protein levels was examined in glioma cells. The data showed that nobiletin can hinder AKT phosphorylation in all of these conditions. Combined treatment of nobiletin and LY294002 (a specific PI3K inhibitor) synergistically blocked AKT activation in glioma cells. GSK-3β phosphorylation induced by AKT obstructs GSK-3β activity. Accordingly, nobiletin downregulated the level of p-GSK-3β and p-β-catenin. We further used AZD1080, an inhibitor of GSK-3β, and found that nobiletin reversed AZD1080-triggered GSK-3β phosphorylation in glioma cells. Herein, it can be concluded that nobiletin might influence EMT via regulating GSK-3β and the downstream effector β-catenin. Our study indicated that nobiletin can abrogate the aberrant glioma cell invasion via blunting the activity of the AKT/GSK-3β/β-catenin signaling.

As is known, β-catenin mediated activation of Slug (30). AKT inhibition impeded Slug accumulation and blocked EMT and stemness of human thyroid cancer cells (31). TGF-β1 also regulates the degradation of the Slug protein, probably through GSK-3β inactivation (32). Overexpression of Slug will, in turn, repress E-cadherin. Mutation of GSK-3β phosphorylation sites (S92/96A or S100/104A) promoted the Slug-mediated EMT properties of E-cadherin suppression and vimentin stimulation, compared with wild-type Slug.

In clinical samples, both Slug and nuclear β-catenin scores were considerably higher in the sarcomatous components than carcinomatous elements (30). In the ChIP tests, nobiletin reversed TGF-induced binding of β-catenin to the Slug promoter. Nobiletin considerably inhibited Slug expression, however, without significant effect on Snail and Twist1 expressions. Interactions between β-catenin and Slug were also greatly abolished by nobiletin treatment. To further explore the role of Slug, U343 cell line was applied, which do not express Slug. However, high level of Slug was observed in both U87 and U251 cells. Of note, nobiletin failed to inhibit the migration and invasion of U343 cells. However, Slug protein expression was brought down by nobiletin treatment in Slug plasmid-transfected U343 cells. These data demonstrated the essential role of Slug in the progression of EMT and invasion of glioma cells, and suggested that nobiletin inhibits invasion probably via inhibiting AKT/GSK3β/β-catenin signaling pathway in Slug-expressing glioma cells.

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References

1. Wirsching HG and Weller M: The role of molecular diagnostics in the management of patients with gliomas. Curr Treat Options Oncol 17: 51, 2016.
2. Huse JT: Establishing a robust molecular taxonomy for diffuse gliomas of adulthood. Surg Pathol Clin 9: 379-390, 2016.
3. Zou M, Zhu W, Wang L, Shi L, Gao R, Ou Y, Chen X, Wang Z, Jiang A, Liu K, *et al*: AEG-1/MTDH-activated autophagy enhances human malignant glioma susceptibility to TGF-β1-triggered epithelial-mesenchymal transition. Oncotarget 7: 13122-13138, 2016.

4. Khan Z and Marshall JF: The role of integrins in TGFβ activation in the tumour stroma. Cell Tissue Res 365: 657-673, 2016.
5. Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C and Marini FC: Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res 18: 84, 2016.
6. Smith BN and Bhowmick NA: Role of EMT in metastasis and therapy resistance. J Clin Med 5: 5, 2016.
7. Guo L, Zhang Y, Zhang L, Huang F, Li J and Wang S: MicroRNAs, TGF-β signaling, and the inflammatory microenvironment in cancer. Tumour Biol 37: 115-125, 2016.
8. Cervantes-Arias A, Pang LY and Argyle DJ: Epithelial-mesenchymal transition as a fundamental mechanism underlying the cancer phenotype. Vet Comp Oncol 11: 169-184, 2013.
9. Xu W, Yang Z and Lu N: A new role for the PI3K/Akt signaling pathway in the epithelial-mesenchymal transition. Cell Adhes Migr 9: 317-324, 2015.
10. Papkoff J and Aikawa M: WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells. Biochem Biophys Res Commun 247: 851-858, 1998.
11. Clevers H and Nusse R: Wnt/β-catenin signaling and disease. Cell 149: 1192-1205, 2012.
12. Angers S and Moon RT: Proximal events in Wnt signal transduction. Nat Rev Mol Cell Biol 10: 468-477, 2009.
13. Lien LM, Wang MJ, Chen RJ, Chiu HC, Wu JL, Shen MY, Chou DS, Sheu JR, Lin KH and Lu WJ: Nobiletin, a polymethoxylated flavone, inhibits glioma cell growth and migration via arresting cell cycle and suppressing MAPK and Akt pathways. Phytother Res 30: 214-221, 2016.
14. Chen J, Chen AY, Huang H, Ye X, Rollyson WD, Perry HE, Brown KC, Rojanasakul Y, Rankin GO, Dasgupta P, *et al*: The flavonoid nobiletin inhibits tumor growth and angiogenesis of ovarian cancers via the Akt pathway. Int J Oncol 46: 2629-2638, 2015.
15. Shi MD, Liao YC, Shih YW and Tsai LY: Nobiletin attenuates metastasis via both ERK and PI3K/Akt pathways in HGF-treated liver cancer $HepG_2$ cells. Phytomedicine 20: 743-752, 2013.
16. Ma HW, Xie M, Sun M, Chen TY, Jin RR, Ma TS, Chen QN, Zhang EB, He XZ, De W, *et al*: The pseudogene derived long noncoding RNA DUXAP8 promotes gastric cancer cell proliferation and migration via epigenetically silencing PLEKHO1 expression. Oncotarget: Aug 5, 2016 (Epub ahead of print).
17. McCormick SM, Gowda N, Fang JX and Heller NM: Suppressor of cytokine signaling (SOCS)1 regulates interleukin-4 (IL-4)-activated insulin receptor substrate (IRS)-2 tyrosine phosphorylation in monocytes and macrophages via the proteasome. J Biol Chem 291: 20574-20587, 2016.
18. Navarro-Villarán E, Tinoco J, Jiménez G, Pereira S, Wang J, Aliseda S, Rodríguez-Hernández MA, González R, Marín-Gómez LM, Gómez-Bravo MA, *et al*: Differential antitumoral properties and renal-associated tissue damage induced by tacrolimus and mammalian target of rapamycin inhibitors in hepatocarcinoma: In vitro and in vivo studies. PLoS One 11: e0160979, 2016.
19. Gwak J, Hwang SG, Park HS, Choi SR, Park SH, Kim H, Ha NC, Bae SJ, Han JK, Kim DE, *et al*: Small molecule-based disruption of the Axin/β-catenin protein complex regulates mesenchymal stem cell differentiation. Cell Res 22: 237-247, 2012.
20. Kucuksayan H, Ozes ON and Akca H: Downregulation of SATB2 is critical for induction of epithelial-to-mesenchymal transition and invasion of NSCLC cells. Lung Cancer 98: 122-129, 2016.
21. Larsen JE, Nathan V, Osborne JK, Farrow RK, Deb D, Sullivan JP, Dospoy PD, Augustyn A, Hight SK, Sato M, *et al*: ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. J Clin Invest 126: 3219-3235, 2016.
22. Gao F, Cui Y, Jiang H, Sui D, Wang Y, Jiang Z, Zhao J and Lin S: Circulating tumor cell is a common property of brain glioma and promotes the monitoring system. Oncotarget 7: 71330-71340, 2016.
23. Lau MT, So WK and Leung PC: Fibroblast growth factor 2 induces E-cadherin down-regulation via PI3K/Akt/mTOR and MAPK/ERK signaling in ovarian cancer cells. PLoS One 8: e59083, 2013.
24. Shirai K, Hagiwara N, Horigome T, Hirose Y, Kadono N and Hirai Y: Extracellularly extruded syntaxin-4 binds to laminin and syndecan-1 to regulate mammary epithelial morphogenesis. J Cell Biochem: Jul 27, 2016 (Epub ahead of print).
25. Nagaishi M, Paulus W, Brokinkel B, Vital A, Tanaka Y, Nakazato Y, Giangaspero F and Ohgaki H: Transcriptional factors for epithelial-mesenchymal transition are associated with mesenchymal differentiation in gliosarcoma. Brain Pathol 22: 670-676, 2012.
26. Yang G, Liang Y, Zheng T, Song R, Wang J, Shi H, Sun B, Xie C, Li Y, Han J, *et al*: FCN2 inhibits epithelial-mesenchymal transition-induced metastasis of hepatocellular carcinoma via TGF-β/Smad signaling. Cancer Lett 378: 80-86, 2016.
27. McCrea PD, Turck CW and Gumbiner B: A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. Science 254: 1359-1361, 1991.
28. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T and Lu Z: Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. J Biol Chem 282: 11221-11229, 2007.
29. Spano D and Zollo M: Tumor microenvironment: A main actor in the metastasis process. Clin Exp Metastasis 29: 381-395, 2012.
30. Inoue H, Takahashi H, Hashimura M, Eshima K, Akiya M, Matsumoto T and Saegusa M: Cooperation of Sox4 with β-catenin/p300 complex in transcriptional regulation of the Slug gene during divergent sarcomatous differentiation in uterine carcinosarcoma. BMC Cancer 16: 53, 2016.
31. Visciano C, Liotti F, Prevete N, Cali' G, Franco R, Collina F, de Paulis A, Marone G, Santoro M and Melillo RM: Mast cells induce epithelial-to-mesenchymal transition and stem cell features in human thyroid cancer cells through an IL-8-Akt-Slug pathway. Oncogene 34: 5175-5186, 2015.
32. Kim JY, Kim YM, Yang CH, Cho SK, Lee JW and Cho M: Functional regulation of Slug/Snail2 is dependent on GSK-3β-mediated phosphorylation. FEBS J 279: 2929-2939, 2012.