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Taxifolin inhibits breast cancer cells proliferation, migration and invasion by promoting mesenchymal to epithelial transition via β-catenin signaling

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ABSTRACT

Aim: To investigate the effects and underlying mechanisms of taxifolin on proliferation, migration and invasion of highly aggressive breast cancer in vitro and in vivo.

Main methods: The antineoplastic activity of taxifolin was evaluated in MDA-MB-231 and 4 T1 cells by crystal violet assay and colony formation assay. The effects of taxifolin on migration and invasion were determined by wound healing assay and Transwell assay, respectively. mRNA and protein expression of genes were assayed respectively with qRT-PCR and western blot, and the protein expression and location was also detected by immunofluorescence and immunohistochemistry. β-catenin overexpression was performed with adenovirus infection. The effects of taxifolin on growth and metastasis of breast cancer in vivo were investigated in BALB/c mice bearing 4T1 xenografts.

Key findings: We found that taxifolin had the potential to inhibit proliferation, migration and invasion of highly aggressive breast cancer cells in a dose-dependent manner. In addition, taxifolin promoted the MET process, the reversed process of EMT, as evaluated by EMT markers and EMT-transcriptional factors in breast cancer cell lines. Meanwhile, the protein and mRNA expressions of β-catenin were dose-dependently downregulated by taxifolin, and overexpression of β-catenin by adenoviruses abrogated these beneficial effects of taxifolin above-mentioned. Furthermore, within a 4T1 xenograft mouse model, taxifolin markedly inhibited the growth of primary tumors and reduced lung metastasis of breast cancer.

Significance: Our findings provide a theoretical foundation for the possibility of taxifolin used as a promising agent in the clinical treatment of highly aggressive breast cancer patients.

1. Introduction

Breast cancer has become the most commonly diagnosed malignancy and a prime leading cause of cancer death in global women [1]. Although the cancer mortality rate has dropped 39% from 1989 to 2015 and the 5-year relative cancer survival rate has been raised up to approximately 90% for female breast cancer partly because of the advances in early detection and holistic treatment [2], there are still about 40% of patients suffer from metastasis and recurrence, especially for those patients with highly aggressive biological subtypes, such as HER-2 positive and triple negative breast cancer [3,4]. Hence, novel sufficient agent to suppress breast cancer excessive proliferation and metastasis is urgently needed.

Epithelial-to-mesenchymal transition (EMT) and its reversed process, mesenchymal-to-epithelial transition (MET), are fundamental processes participating in normal biological development and disease progression. The EMT process can be classified into three different types according to its distinct functions, including the EMT during implantation, embryogenesis and organogenesis, the EMT during wound healing, tissue regeneration and organ development, and the EMT during tumor metastasis [5]. When EMT process occurs, the epithelial cancer cells change its flat and polygonal shape, lose the apical-basal polarity, cell-cell adhesion and basement-membrane, instead, acquire mesenchymal-like properties, that is spindle-like shape, front-to-back

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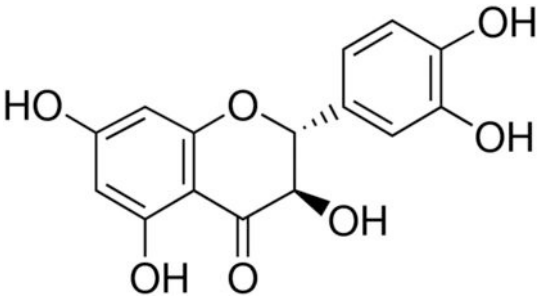

Fig. 1. Chemical structure of taxifolin.

polarity and increased motility potential [6]. Meanwhile, the epithelial markers such as E-cadherin and Claudin are downregulated, while mesenchymal markers such as N-cadherin and Vimentin are upregulated [7]. All these transformations endow cancer cells with the motivation to escape from the primary lesion into surrounding healthy tissues, lymphatic and hematological system, subsequently resulting in tumor locoregional recurrence and distant metastasis. It is apparent that EMT is tightly regulated by several so-called EMT-activating transcription factors (EMT-TFs), mainly of the Snail, Twist and ZEB families and numerous signaling pathways such as Hedgehog (Hh), Wnt/β-catenin, Notch and transforming growth factor-β (TGF-β), which plays pleiotropic roles from cancer initiation to metastasis and recurrence [8,9].

In recent years, along with the continuous exploration of traditional Chinese medicine, naturally extractive dietary substances, as additional treatment strategies, have exhibited unique attractiveness in the treatment of cancers mainly because they are easy to be obtained, low in toxicity and well-tolerated in human bodies [10,11]. Taxifolin, also called dihydroquercetin ((2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one) (Fig. 1), is a type of flavonoid commonly plentiful in onions, citrus fruits, grapes, olive oil, milk thistle, French maritime bark and Douglas firbar [12,13]. It has been demonstrated that taxifolin possesses an extensively pharmacological activities, including anti-oxidant, anti-parasite, anti-amyloid-beta oligomer formation to prevent Alzheimer's disease, anti-hyperuricemic, cardioprotection, hepatoprotection, anti-angiogenesis, antinociceptive, anti-oedematogenic and so on [14–21]. Hitherto, taxifolin has been identified as a potential antineoplastic agent in a various kinds of cancers, such as breast carcinogenesis, colon carcinogenesis, skin carcinogenesis, osteosarcoma, Ewing's sarcoma and prostate cancer [22–27]. However, the effects and underlying mechanisms of taxifolin on highly aggressive breast cancer still remain undetermined.

Here, we selected two highly aggressive breast cancer cell lines MDA-MB-231 and 4 T1 to explore the potential effects and mechanisms of taxifolin on breast cancer in vitro and in vivo [28].

2. Materials and methods

2.1. Reagents and antibodies

Taxifolin (purity > 99%) was obtained from Push Bio-Technology (Chengdu, China). Crystal violet was purchased from Sigma (St. Louis, USA). Vectastain ABC kit was bought from Vector (Burlingame, CA, USA), and liquid DAB + Substrate Chromogen System were purchased from Dako (California, USA). BCA protein assay kit was from Thermo Fisher Scientific (Waltham, MA USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's (DMED) medium, RPMI-1640 medium, trypsin-EDTA, phosphate buffer saline (PBS) and penicillin/streptomycin used for cell culture were all the products of Hyclone (Los Angeles, USA). RNA extraction, PrimeScript RT and PCR reagent kits were purchased from TaKaRa (Dalin, China). The primary antibodies involved in this research, including anti-E-cadherin, anti-Claudin anti-Vimentin, anti-N-cadherin, anti-Slug, anti-Snail, anti-β-catentin were from Cell Signaling Technology (New England Bio labs, Ipswich, MA), anti-ki67 was from Abcam (Cambridge, UK), and anti-GAPDH was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies, HRP-labeled goat-anti-rabbit monoclonal IgG were from Abcam (Cambridge, UK), Alexa Fluor 594 goat anti-rabbit IgG and Dylight 488 goat anti-mouse IgG were from Molecular Probes (Oregon, USA). ActinGreen™ 488 Ready Probes® Reagent was from Molecular Probes (Oregon, USA).

2.2. Cell culture and treatment

Human breast cancer cell line MDA-MB-231 and Balb/c mouse mammary cancer cell line 4 T1 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). They were separately maintained in DMEM and RPMI-1640 medium added with 10% FBS and 1% penicillin/streptomycin. Cells were grown at 37 °C with 5% CO_2 in humidified air. All experiments were done using cells in exponential growth, and 24 h after seeding, cells were treated with culture medium with or without various concentrations of taxifolin for indicated time.

2.3. Cell proliferation assay

The effect of taxifolin on cell proliferation was evaluated by crystal violet assay. Briefly, exponentially growing MDA-MB-231 and 4 T1 cells were plated in 24-well plates at an initial density of 2×10^4 per well and were treated with various concentrations of taxifolin (1, 3, 10, 30, 100 μM for the former and 3, 10, 30, 100, 150 μM for the latter) for 72 h. Each concentration set 3 replicates. At the end of the drug exposure duration, cells were fixed and stained with 4% paraformaldehyde containing 0.25% crystal violet. Then cleaned up the redundant fixing and staining solution with PBS and dissolved the crystal. Solubilized formazan products were quantified by using a microplate reader (ELx800, BioTek, USA) at 570 nM.

2.4. Colony formation assay

This assay was used to assess the influence of taxifolin on cell proliferation. MDA-MB-231 and 4 T1 cells were digested, counted and seeded in six-well plates at a density of 1000 cells per well. After adhering to the surface, the cells were treated with culture medium or taxifolin in different concentrations. After 10 days of drug exposure, the visible colonies formed. Colonies were washed with PBS for 3 times, then fixed with 4% paraformaldehyde and stained with 0.25% crystal violet. The colonies were counted using a light microscope.

2.5. Wound healing assay

MDA-MB-231 and 4T1 cells were cultured in 6-well plates within culture medium containing 10% FBS and grown to nearly confluent cell monolayer. Then made a linear wound by scratching the center of well with a 10 μM plastic pipette tip. The monolayer cells were washed with PBS for twice to remove the detached cells and the remaining cells were maintained in the serum-free medium with or without taxifolin (10, 30, 100 μM) for more 24 h. The gap distance of the wound was measured and photographed by using a light microscope with cameras (Nikon, Japan).

2.6. Transwell invasion assay

The invasion of MDA-MB-231 and 4 T1 cells was evaluated through the use of Matrigel-coated Transwell chamber (Corning, MA, USA). In short, cells were trypsinized, washed, and suspended in a serum-free medium with or without taxifolin (10, 30, 100 μM) and seeded at a density of 5×10^4 cells/well onto the upper chambers, the lower chamber were filled with culture medium added 10% FBS and the same

concentration of taxifolin with the upper chamber. After 8 h incubation, the cells on top surface of the chamber was rubbed off, while the cells on the lower surface were fixed with 4% paraformaldehyde for 10 min and stained with 0.25% crystal violet for 15 min. Then four random fields of every chamber were scanned to record the cells at the lower membrane side at 200× magnifications using a microscope (Nikon, Japan), and calculated the invasion rate of the two cancer cell lines.

2.7. Immunofluorescence (IF) analysis

For the immunofluorescence experiments, MDA-MB-231 and 4 T1 cells pretreated by various concentrations of taxifolin were fixed, permeabilized and incubated with primary antibody against Vimentin and ki-67 in an appropriate dilution overnight at 4 °C. Then incubated with Alexa 594 secondary antibodies against rabbit IgG in darkness. Following incubation with anti-F-actin 488 at the recommended dilution in darkness. At last DAPI was added to counterstain the nucleus. The primary tumor tissues were blocked in PBS containing 1% BSA before incubating with the primary antibody. The primary antibodies against Vimentin (mouse mAb) and slug (rabbit mAb) were mixed and covered the tissues for 2 h at room temperature, followed by incubation of the mixed Dylight 488 against mouse monoclonal IgG and Alexa Fluor 594 against rabbit monoclonal IgG secondary antibodies. Other procedures were performed according to the same protocol in cells. Images of cells and tissues were captured using fluorescence microscope (Nikon, Japan).

2.8. Western blot analysis

Total proteins of cells were extracted according to the standard protocol and its concentrations were detected by BCA protein assay kit. Briefly, the proteins were electrophoresed on 10% SDS gel and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked in Tris-Buffered Saline (TBS) containing 5% BSA and 0.1% Tween-20, the membranes were incubated with the primary monoclonal antibodies against E-cadherin, Claudin, Vimentin, N-cadherin, Slug, Snail and β-catenin at 4 °C overnight, then incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). At last use the enhanced chemiluminescent system and X-ray to make the binding antibodies visualized.

2.9. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Briefly, total RNA was extracted from cells following the manufacturer's instruction of the RNA extraction kit. Total RNA (1 μl) was reverse transcribed to complementary DNA (cDNA) by using the PrimeScript RT reagent Kit. At last qPCR was performed by using the PCR kit following the instruction. The specific primer sequences we used were as following: E-cadherin: 5′-TCCTGGGCAGAGT GAATTTTG AAGA-3′ (forward), 5′-AAACGGAGGCCTGATGGGG-3′ (reverse); Claudin: 5′-CCTCCTGGGAGTGATAGCAAT-3′ (forward), 5′-GGCAACT AAAATA GCCAGACCT-3′ (reverse); Vimentin: 5′-TACAGGAAGCTGCT GGAAGG-3′ (forward), 5′-ACCAGA GGGAGTGAATCCAG-3′ (reverse); N-Cadherin: 5′-AGCCAACCTTAACTGAGG AGT-3′ (forward), 5′-GGCA AGTTGATTG GAGGGATG-3′ (reverse); Snail: 5′-TCG GAAGCCTAACT ACAGCGA-3′ (forward), 5′-AGATGAGCATTGGCAGCGAG-3′ (reverse); Slug: 5′-GGGGAG AAGCCTTTTTCTTG-3′ (forward), 5′-TCCTCATGTT TGTGCAGGAG-3′ (reverse); β-catenin: 5′-AGCGAGCAGCCCCCAAA GTT-3′ (forward), 5′-GGGCACGAA GGCTCATCATT-3′ (reverse); GAPDH: 5′-TGTTGC CATCAATGACCCCTT-3′ (forward), 5′-CTCCACGA CGTACTCAGCG-3′ (reverse). GAPDH primers were used as internal control and equal loading.

2.10. Adenovirus infection

The adenovirus expressing β-catenin was used following the procedures described previously [29]. Besides the expression of transgenes, Ad-β-catenin also expressed GFP as a marker for monitoring transfection efficiency. An analogous adenovirus expressing only GFP (Ad-GFP) was used as a control, and expression efficiency was evaluated by western blotting.

2.11. Immunohistochemistry (IHC) analysis

Tumor tissues were fixed with 4% paraformaldehyde, embedded in paraffin and cut into four microns-thick sections. Then the IHC experiments were performed using the standard avidin-biotin-peroxidase complex (ABC) method with the Vectastain ABC kit according to the manufacturer's instructions. Briefly, tissues were deparaffinized, rehydrated, antigen repaired, endogenous peroxidases quenched and blocked. Followed by incubation with primary antibodies against ki-67 and β-catenin at 4 °C overnight and signal amplification with biotin-conjugated second antibody, avidin and HRP-conjugated biotin. Finally, the binding antibodies were visualized with DAB staining and imaged.

2.12. Animal experiments

The present study was performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023), and the protocol was approved by the Ethics Committee of Chongqing Medical University. Six to eight-week-old female Balb/c mice were bought from the Experimental Animal Center of Chongqing Medical University and raised in specific pathogen free (SPF) laboratory environment. All mice were injected subcutaneously with 1×10^6 4T1 cells at bilateral gluteal regions. Until the tumor reached 1 cm^3 in volume, mice were divided randomly into sham-treated group and taxifolin-treated group with 10 mice in each. The mice were perorally gavaged with either 100 μl CMC-Na control or taxifolin (100 mg/kg) every four days. After 28 days, all mice were sacrificed under anesthesia, and the tumors and lungs were excised, weighted, counted for the tumor nodules and fixed for further analysis.

2.13. Statistical analysis

All experiments were repeated at least three times. Data was presented as mean values ± standard deviation (SD). Student's *t*-test and one-way ANOVA analysis were used to analyze the variances between groups. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Taxifolin suppresses highly aggressive breast cancer cells proliferation

To explore the antitumor effect of taxifolin in highly aggressive breast cancer cells, we treated the human breast cancer cells MDA-MB-231 and Balb/c mouse mammary cancer cells 4T1 with increasing concentrations of taxifolin. To begin with, we assessed their proliferative activities by using crystal violet assay for 72 h and colony formation assay for 10 days. The results revealed that taxifolin suppressed the proliferation of both two cancer cell lines in a dose-dependent way (Fig. 2A & 2B). Furthermore, in order to support this finding at a molecular level, IF assay was performed to detect the protein expression level of ki-67, which is a representative marker of cell proliferation. As shown in Fig. 2C, the ki-67 expression level was significantly higher in control group comparing with 30 μM taxifolin-treated group.

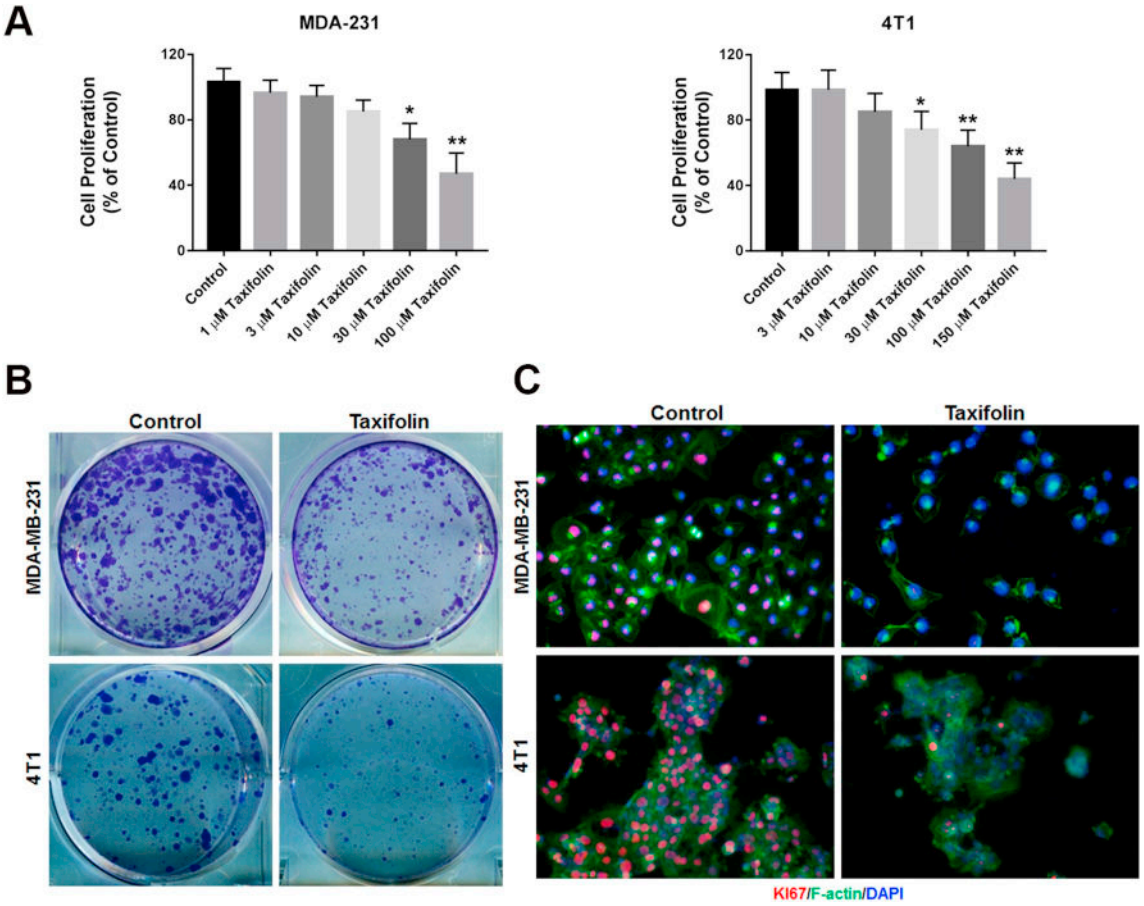

Fig. 2. Taxifolin suppresses breast cancer cells proliferation in vitro. (A) The breast cancer cells viability was determined by crystal violet assay. The highly invasive breast cancer cells MDA-MB-231 were pretreated with 1, 3, 10, 30, 100 μM of taxifolin and 4T1 with 3, 10, 30, 100, 150 μM of taxifolin for 72 h. (B) The breast cancer cells proliferation was determined by colony formation assay. MDA-MB-231 and 4T1 cells were treaded with taxifolin (30 μM) for 10 days. (C) IF analysis shown the down-regulation of ki-67 in breast cancer cells treated with taxifolin (30 μM) for 24 h (original magnification, 400 ×). Nucleus is stained with DAPI (blue), ki-67 with Alexa Fluor 549 (red) and F-actin with ActinGreen™ 488 Ready Probes® Reagent (green). All experiments were performed thrice in triplicate. Mean ± SD, $*P < 0.05$ and $**P < 0.01$, vs. control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Taxifolin inhibits the migration and invasion of highly aggressive breast cancer cells

To determine whether taxifolin had the potential to restrict the migration and invasion of highly aggressive breast cancer cells, wound healing and Transwell invasion assays were performed on MDA-MB-231 and 4T1 cells. In the wound healing experiment, the width of wound scratches in taxifolin-treated groups (10, 30, 100 μM) were much wider than it presented in control group at 24 h, indicating that taxifolin had the potential to inhibit the migration of breast cancer cells, meanwhile, this inhibitory action exhibited an obvious does-dependent increasing tendency (Fig. 3A). In the Transwell invasion assay, as same as the results of wound healing assay, the numbers of invaded cells were does-dependently decreased in taxifolin-treated groups (10, 30, 100 μM) comparing with control group (Fig. 3B & C).

3.3. Taxifolin promotes highly aggressive breast cancer cells MET process

It has been demonstrated that EMT is a considerable process contributed to the metastasis of cancer cells, but MET process acts in the opposite way. Based on the above results that taxifolin could inhibit MDA-MB-231 and 4T1 cells migration and invasion, we devised experiments to figure out the relationship between taxifolin and EMT/MET. The indirect IF analysis illustrated that compared with untreated breast cancer cells, the taxifolin-treated cells exhibited a visibly weaker expression of mesenchymal marker Vimentin protein at the concentration of 30 μM, and the cytoskeletal protein F-actin turned to be depolymerized diffusely in the cytoplasm (Fig. 4A). Furthermore, western blot and qRT-PCR assays were determined to quantitate the protein and mRNA expression of EMT-representative markers, respectively. As shown in Fig. 4B & C, at the concentrations of 10, 30, 100 μM, taxifolin resulted in an up-regulation of the epithelial markers E-cadherin and Claudin expression along with the augmentation of drug concentration, whereas the mesenchymal markers N-cadherin and Vimentin changed in the opposite way. Meanwhile, Snail and Slug which were two main TFs involved in the activation of EMT program were simultaneously down-regulated (Fig. 4B & C).

3.4. β-Catenin conduces to the beneficial effects of taxifolin with regard to the proliferation, metastasis and EMT of highly aggressive breast cancer cells

A previous research has proved that taxifolin inhibits the breast carcinogenesis by regulating AhR/CYP1A1 signaling pathway [23]. And in the present study we wondered what molecular mechanism did taxifolin adopts to restrain metastasis and EMT process of the highly aggressive breast cancer cells. Since β-catenin accumulation in the cytoplasm or translocation to the nucleus has been reported in approximately 50% of breast cancers and has been correlated with the activation of EMT program [9,30], we firstly resolved whether taxifolin influence the β-catenin signaling pathway. Western blot and qRT-PCR

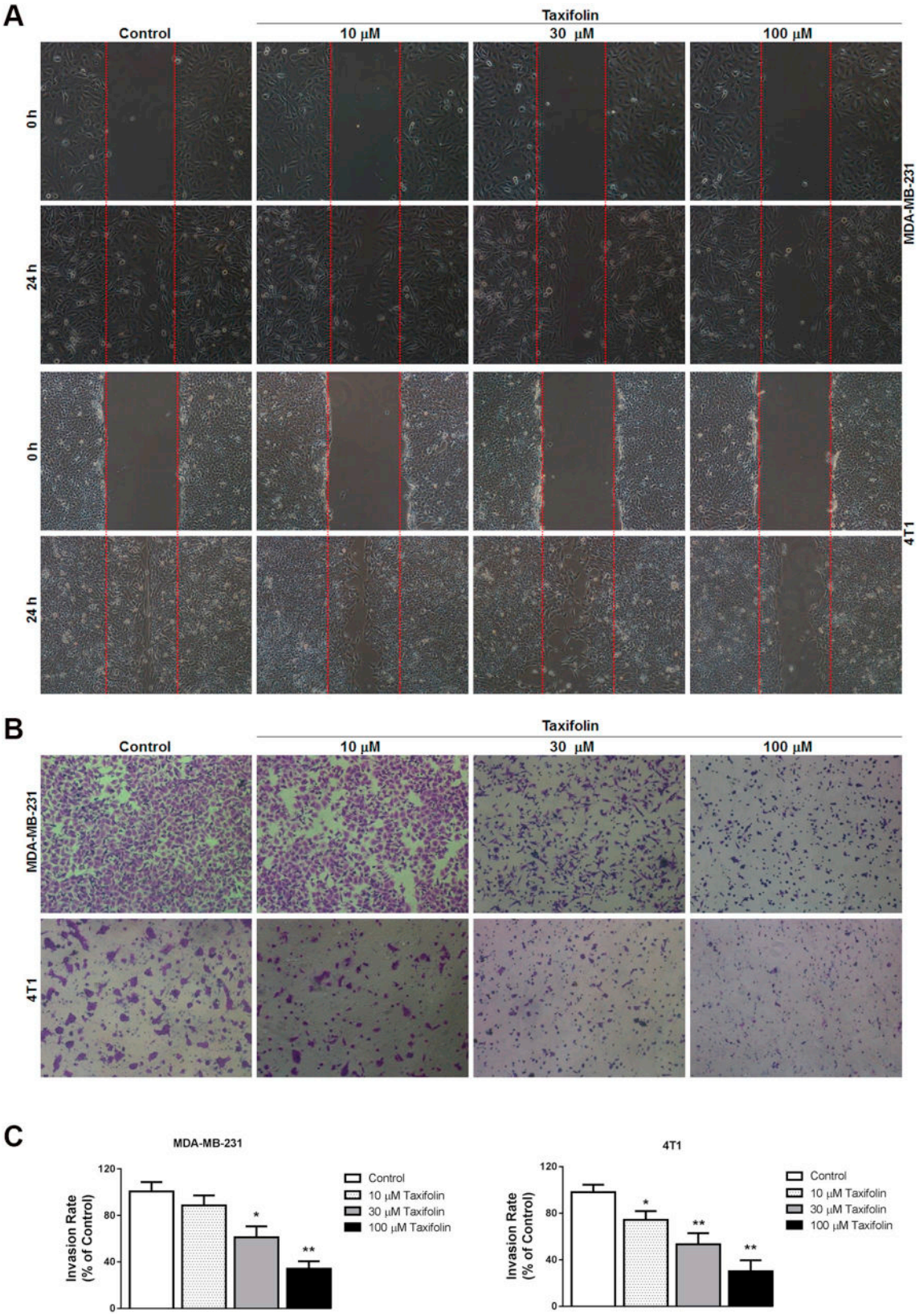

Fig. 3. Taxifolin inhibits the migration and invasion of breast cancer cells in vitro. The highly aggressive breast cancer cells MDA-MB-231 and 4T1 were pretreated with different concentrations of taxifolin (10, 30, and 100 μM) for indicated time. (A) (A) Cell migration was determined by wound healing assay. (B) Cell invasion was determined by Transwell invasion assay. (C) Quantification of the invasive cells. All experiments were performed thrice in triplicate. Mean ± SD, $^{*}P < 0.05$ and $^{**}P < 0.01$, vs. control group.

results shown that comparing with the marked expression of β-catenin in control group, taxifolin does-dependently downregulated the protein and mRNA expression of β-catenin (Fig. 4B & C). Furthermore, in order to verify whether suppressing β-catenin signaling pathway contributes to the anticancer activity of taxifolin, adenovirus overexpressing β-catenin was transfected into both MDA-MB-231 and 4T1 cells. Intriguingly, we found that overexpression of β-catenin markedly passivated

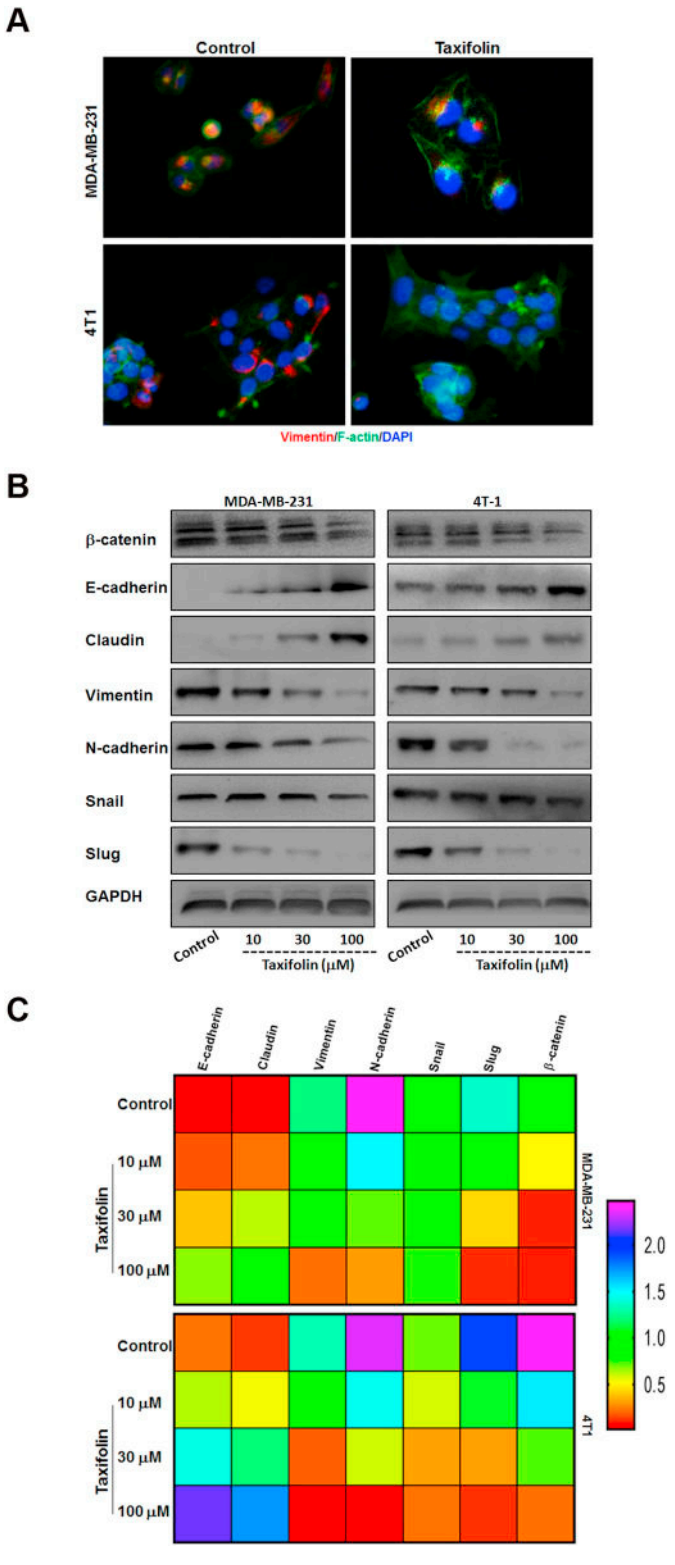

Fig. 4. Taxifolin reverses breast cancer cells EMT process and inhibits the activation of β-catenin signaling pathway. MDA-MB-231 and 4T1 cells were pretreated with various concentrations of taxifolin (10, 30, and 100 μM) for 24 h. (A) The mesenchymal marker vimentin and cytoskeletal protein F-actin was detected by IF staining (original magnification, 400×). Nucleus was stained with DAPI (blue), vimentin with Alexa Fluor 549 (red) and F-actin with ActinGreen™ 488 Ready Probes® Reagent (green). (B) The effect of taxifolin on the protein expressions of EMT markers, related transcriptional factors and β-catenin were detected by Western blotting. GAPDH was used as an internal loading control. (C) The effect of taxifolin on the mRNA expressions of EMT markers, related transcriptional factors and β-catenin were detected by qRT-PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the inhibitory effects of taxifolin on breast cancer cells proliferation, migration and invasion (Fig. 5A-C). Simultaneously, in the taxifolin pretreated breast cancer cells, the EMT-related markers and its modulated TFs were upregulated in the Ad-β-catenin group comparing with the Ad-GFP group, indicating that the promoted effect of taxifolin on MET was also abrogated by β-catenin overexpression (Fig. 5D).

3.5. Taxifolin inhibits the growth of breast tumor and promotes MET in vivo

4 T1 xenograft tumor model was established to examined the antitumor effects of taxifolin in vivo. We observed that the primary tumors of taxifolin-treated mice exhibited an obvious diminution in volume and weight (Fig. 6A) as compared with the control mice. As shown in Fig. 6B, IHC staining of ki-67 in the primary tumor specimens also clarified that taxifolin could dramatically restrict the proliferation of breast tumor in vivo. Furthermore, lower expression of Vimentin and Slug were observed in the orthotopic tumor tissues of taxifolin-treated mice by IF staining (Fig. 6C), and the same change of β-catenin was obtained in taxifolin-treated group by IHC staining (Fig. 6B). All these results are fully consistent with our findings in vitro, demonstrating that taxifolin performs a remarkable antitumor activity against breast cancer.

3.6. Taxifolin restricts the metastasis to lung of highly aggressive breast cancer cells in vivo

4 T1 xenograft metastasis tumor model was also used to determine the effects of taxifolin on breast cancer metastasis in vivo. We found the number of prominent metastatic nodules on the surface of lung in taxifolin-treated mice were much less than it in the control mice (Fig. 7A & B). H&E staining of the lungs also exhibited that taxifolin dramatically decreased the number of metastases and diminished the lesion area in lung tissues caused by metastatic breast cancer cells (Fig. 7C).

4. Discussion

As is known to all, sustaining ceaseless proliferation and activating invasion and metastasis are two main hallmarks of cancer cells [31]. The ability to sustain chronic proliferation is the most fundamental trait of cancer cells, which is the evil consequence of deregulating the normal growth-promoting signals that maintain dynamic equilibrium of cell numbers [31]. This uncontrollable proliferation of cancer cells usually results in the enlargement of the tumor volume and the destruction of the architecture and function of the surrounding healthy tissues. Invasion and metastasis of cancer cells pose a more severe threat to patients, which inevitably lead to cancer recurrence and even induce to death. In the past few years, although the treatments of breast cancer have made an unprecedented development, there are still numbers of patients suffer from the dilemma that the proliferation and metastasis of breast cancer can't be controlled, partly because of the ineffective therapy or therapeutic resistance [32]. So, we urgently need

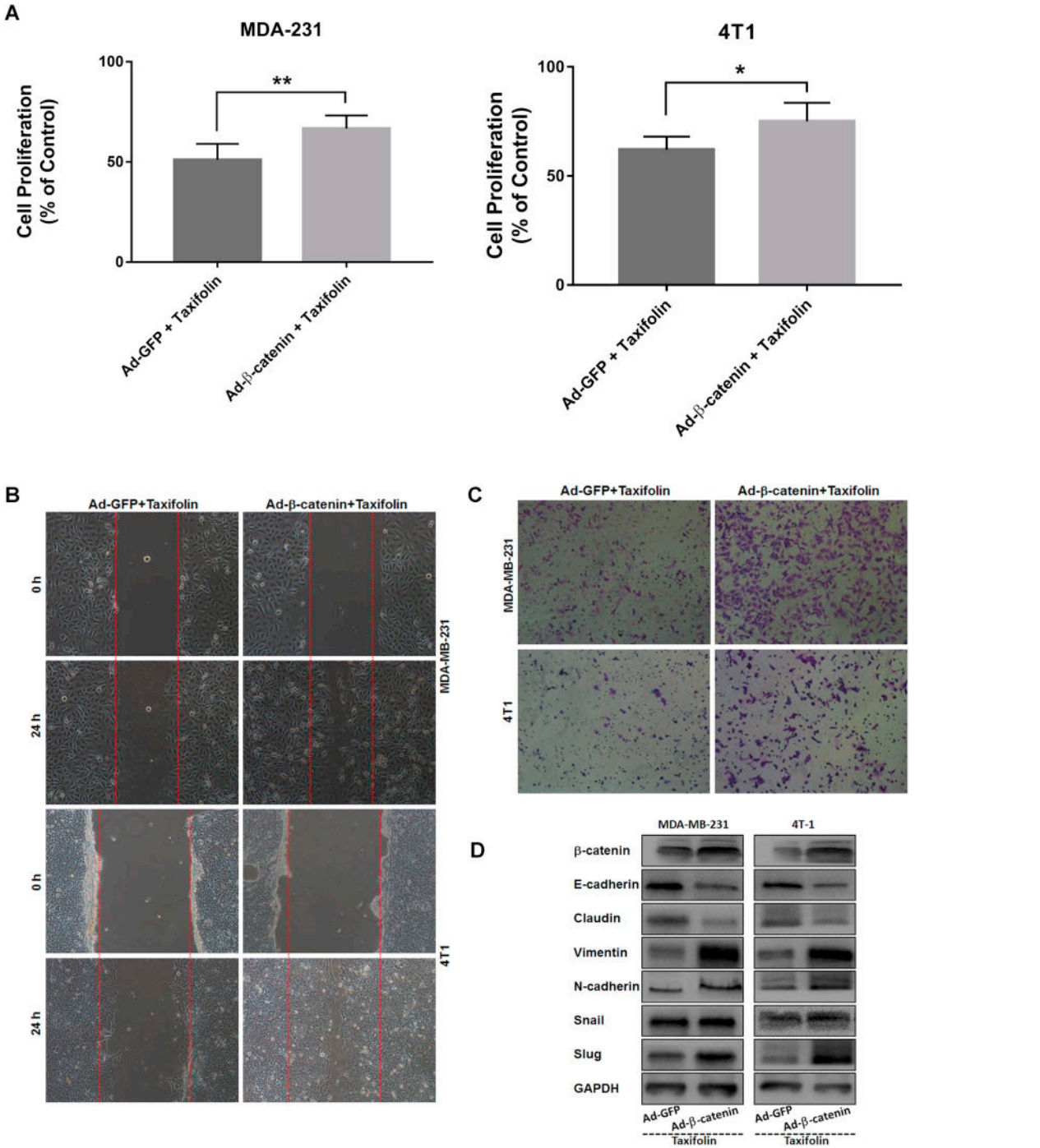

Fig. 5. Overexpression of β-catenin abrogates the beneficial effects of taxifolin in regard to the proliferation, metastasis and EMT of breast cancer cells. MDA-MB-231 and 4 T1 cells were transfected with Ad-β-catenin or Ad-GFP and then all pretreated with taxifolin (100 μM) for indicated time. (A) The cell proliferation was determined by crystal violet assay. (B) The cell migration was determined by wound-healing assay. (C) The cell invasion was determined by Transwell invasion assay. (D) The protein expressions of EMT markers, related transcriptional factors and β-catenin were detected by Western blotting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to find an effective agent to break this dilemma. Taxifolin has been reported to possess certain anticancer properties. However, its effects on highly aggressive breast cancer have not been clarified.

In the present study, we selected two highly aggressive breast cancer cell lines, MDA-MB-231, which is a human breast cancer cell in non-expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER)-2, and 4T1, which is a mouse mammary cancer cell enable to metastasize from the primary tumor to multiple distant sites spontaneously to investigate the potential effects of taxifolin in breast cancer and revealed the underlying molecular mechanisms. Treatment of highly aggressive breast cancer cells with taxifolin resulted in decreased cell viability and proliferation in a dose dependent approach which was demonstrated by crystal violet assay and colony formation assay. Furthermore, at the molecular level, the expression of ki-67 was significantly decreased with the taxifolin treatment which was detected by IF assay. Results from the wound healing and Transwell invasion assays revealed that taxifolin treatment inhibited the migration and invasion abilities of

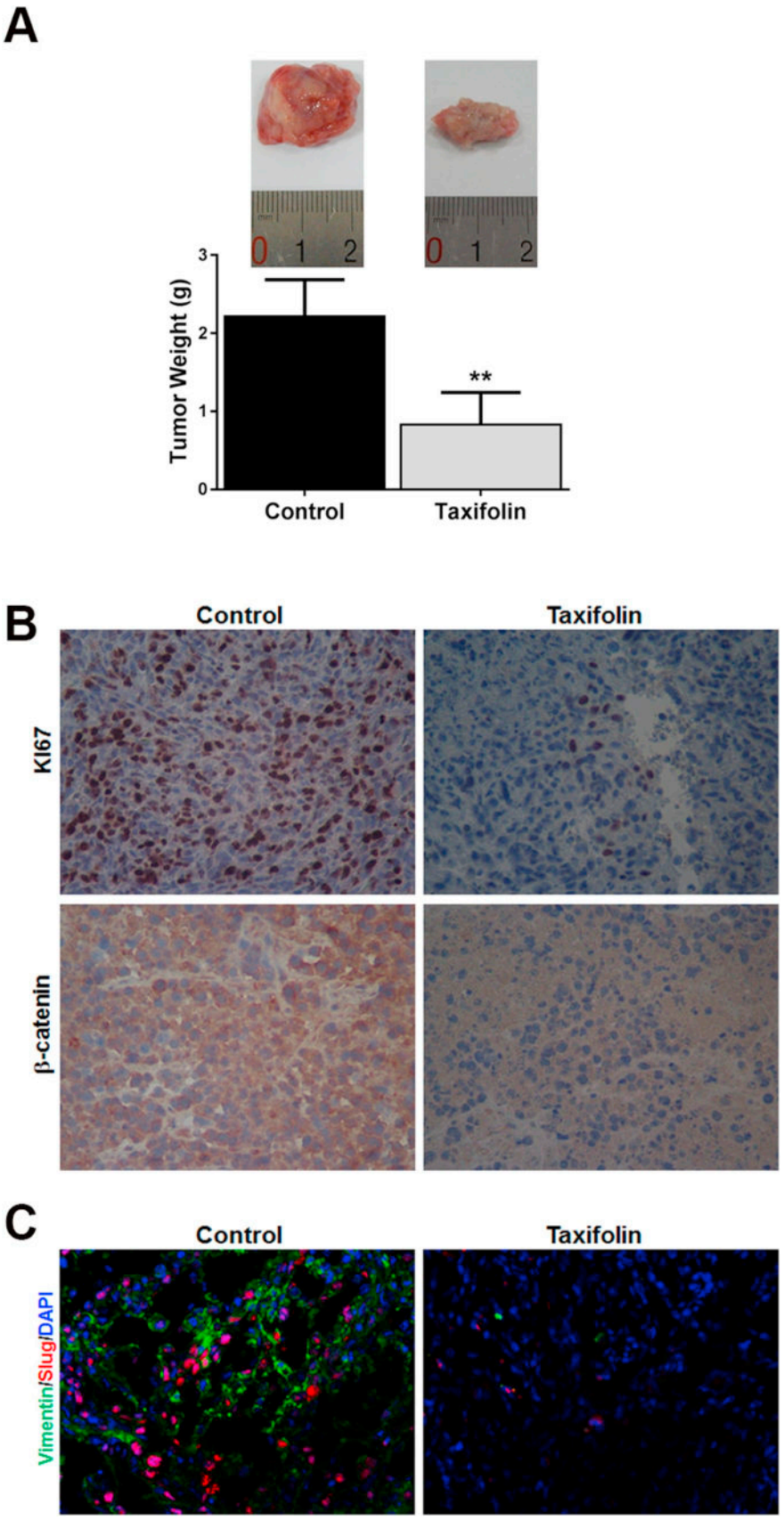

Fig. 6. Taxifolin inhibits the growth of breast tumor and reverses EMT in vivo. (A) Tumor volume and weight were measured in different groups. (B) Ki-67 staining for cell proliferation and β-catenin staining in primary tumor tissues were assessed by IHC analysis (original magnification, ×400). (C) The expression of vimentin and slug in primary tumor tissues were evaluated by IF analysis. All data were presented as the Mean ± SD, $n = 10$, $^{*}P < 0.05$ and $^{**}P < 0.01$, vs. control group.

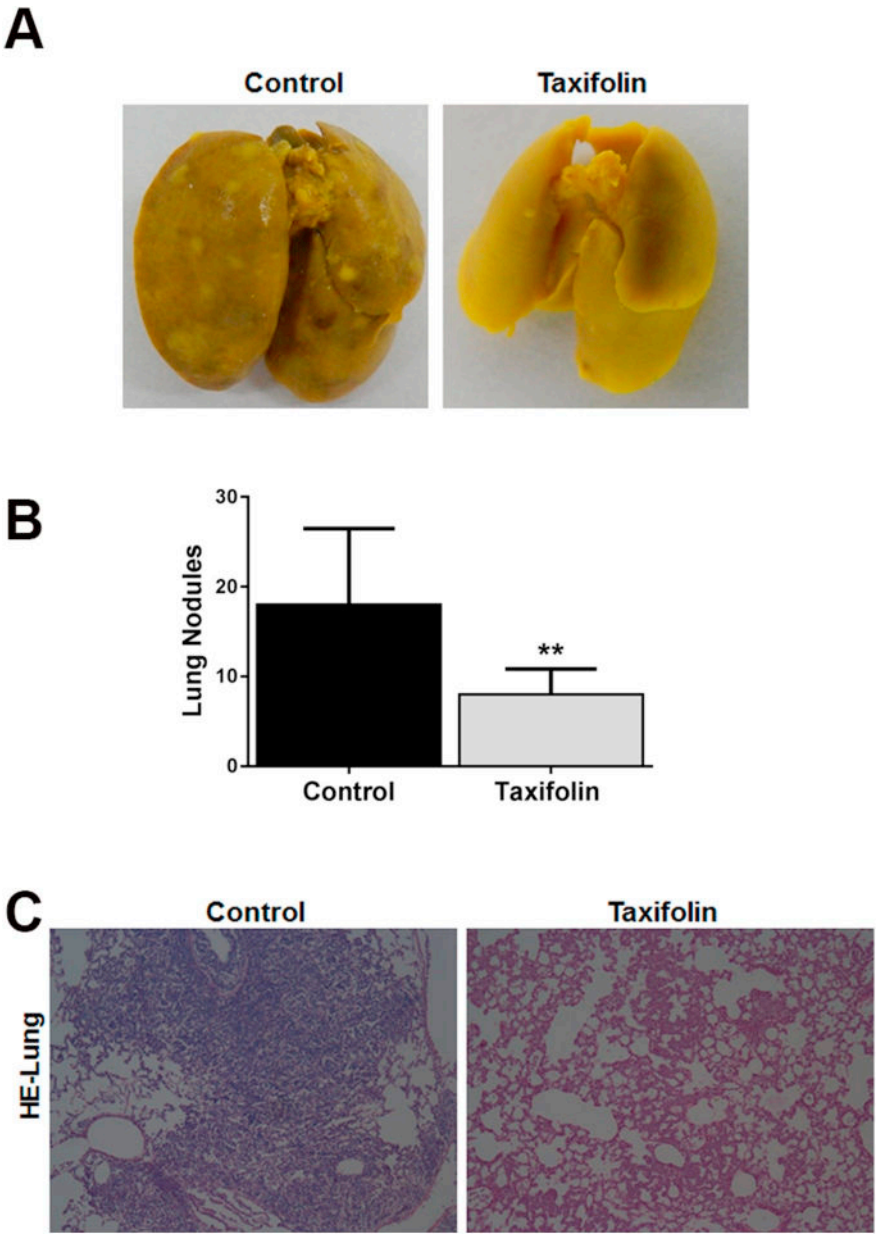

Fig. 7. Taxifolin restricts the metastasis to lung of highly aggressive breast cancer cells in vivo. (A) and (B) Metastatic tumor nodules in the lung were counted in different groups. (C) The metastatic tumor nodules were shown in the images of the H&E stained sections of the lung. All data were presented as the Mean ± SD, $n = 10$, $^{*}P < 0.05$ and $^{**}P < 0.01$, vs. control group.

both MDA-MB-231 and 4T1 breast cancer cells dose-dependently. In addition, the in vivo studies demonstrated that taxifolin treatment significantly inhibited tumor growth and lung metastasis in mice bearing 4T1 cell-derived xenograft tumors. These results suggest that taxifolin may serve as a potential therapeutic agent in the treatment of breast cancer.

In order to explore the mechanisms underlying the anti-metastasis effects of taxifolin further, western blot and qRT-PCR analysis were performed, which showed that the protein and mRNA levels of E-cadherin and Claudin were increased, whereas those levels of Vimentin and N-cadherin were decreased markedly in both two breast cancer cell lines following treatment with taxifolin, and the IF staining showed the suppression of Vimentin expression in the primary tumor tissues in vivo, indicating that taxifolin significantly promoted the mesenchymal to epithelial transition (MET) process in highly aggressive breast cancer, which is the reversed process of EMT. A great quantity of evidence has confirmed that the EMT and its reversed process, MET, are fundamental processes in embryonic development and tissue repair but confer malignant properties to carcinoma cells, especially invasive behavior. The spatiality and temporality of the occurrences of EMT/MET are strict and critical for cancer metastasis. At the primary tumor and for the pre-metastasis stages, EMT acts as the leading role, whereby tumor cells achieve the shift from adhesive, aplanatic, epithelial-like phenotype to invasive, motorial, mesenchymal-like phenotype, which endows them with the power of degrading and expanding out of the surrounding microenvironment [33]. Subsequently, at the site of metastases and for the later stages, MET is more critical, whereby the disseminated mesenchymal tumor cells undergo the reversed transition to form the metastatic lesions [34]. The inhibitory effects of taxifolin is mainly for the primary cancer cells, inhibiting the EMT but promoting the MET process, to avoid the escape of cancer cells from primary tumors by regaining cell-cell adhesion and restraining cells motility. A number of transcription factors (TFs) have been reported to drive the EMT process, and many of them act by repressing epithelial-related

genes. Snail and Slug, as two major EMT-TFs, modulate the expression of epithelial, mesenchymal and proteases genes to promote an invasive phenotype by coordinating histone hypermethylation and deacetylation through recruiting factors such as Polycomb Repressive Complex 2 (PRC2) [5,35]. In the present study, we found that the protein and mRNA expression of Snail and Slug, especially Slug, were decreased after intervention of taxifolin, meanwhile, the translocation to nucleus of Slug was suppressed by taxifolin in vivo. All these data confirmed that the inhibitory effects of taxifolin on the migration and invasion of breast cancer cells might result from the promotion of MET process.

EMT is triggered by a number of signaling molecules, which not just act alone, but interact with each other to form a cross talk net [9]. One of the most important motivators of EMT is the cytokine transforming growth factor-β (TGF-β), acting through the Smad proteins with an intrinsic receptor tyrosine-kinase activity (RTK). In addition, Notch, Hedgehog and ErbB/EGF are also able to activate EMT [36–38]. In this study, we found that the expression of β-catenin was dose-dependently downregulated by taxifolin at both protein and mRNA levels. Consistently, IHC staining showed that the expression of β-catenin in primary tumor tissues dissected from 4T1 xenograft tumor model was also suppressed by taxifolin. Actually, the Wnt/β-catenin signaling pathway is another important inducer of EMT. In normal states, cytoplasmic β-catenin is depleted by the destruction complex consisting of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), Axin, and casein kinase-1α, which induces the phosphorylation of GSK3β, resulting in an ubiquitin-mediated degradation of β-catenin [39]. Once the Wnt ligands bind to Fz receptors and LRP5/6 coreceptors, cytoplasmic disheveled (Dvl), a downstream protein of the receptor complex, is phosphorylated and the activity of the destruction complex is inhibited, allowing accumulation and translocation of β-catenin to the nucleus, where it interacts with the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) complex, leading to the transcription of a number of targeted genes associated with cell proliferation, apoptosis, migration, angiogenesis, and more importantly, the mesenchymal phenotype induction [40–42]. Therefore, taking the such important effect of β-catenin into consideration, it was overexpressed by an adenovirus vector system. Along with the increase of β-catenin expression, the suppressive effects of taxifolin on migration, invasion and EMT were markedly abrogated in MDA-MB-231 and 4 T1 cells, suggesting that these beneficial effects of taxifolin may be involved in the downregulation of β-catenin signaling.

5. Conclusion

In summary, our experimental results showed the dramatically inhibitory effects of taxifolin are contributed to the suppression of proliferation, migration and invasion in highly aggressive breast cancer both in vitro and in vivo, which might be achieved by promoting the MET process through decreasing the expression of β-catenin. All these findings provide a theoretical foundation for the possibility of taxifolin used as a promising agent in the clinical treatment of breast cancer, to sufficiently improve the prognosis of breast cancer patients.

Declaration of Competing Interest

The authors declare no competing financial interest.

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