Baicalin inhibits the metastasis of highly aggressive breast cancer cells by reversing epithelial-to-mesenchymal transition by targeting β-catenin signaling

TAO ZHOU[1,2*], AIJIE ZHANG[1,2*], GE KUANG[2], XIA GONG[3], RONG JIANG[4], DAN LIN[2], JIE LI[1,2], HONGZHONG LI[5], XIANG ZHANG[1], JINGYUAN WAN[2] and HONGYUAN LI[1]

[1]Department of Endocrine and Breast Surgery, The First Affiliated Hospital of Chongqing Medical University; [2]Chongqing Key Laboratory of Biochemistry and Molecular Pharmacology, Chongqing Medical University; [3]Department of Anatomy, Chongqing Medical University; [4]Laboratory of Stem Cell and Tissue Engineering, Chongqing Medical University; [5]Molecular Oncology and Epigenetics Laboratory, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. Metastasis is the main cause of death in breast cancer patients, which is due partly to the lack of effective treatment. Baicalin, a flavonoid compound isolated from the roots of *Scutellaria lateriflora* Georgi (Huang Qin), has recently been confirmed as an effective agent for the treatment of a variety of cancers. Yet, the effects and underlying molecular mechanisms of baicalin in regards to the metastasis of breast cancer remain unclear. In the present study, we found that baicalin had the potential to suppress the migration and invasion of highly aggressive breast cancer cells in a dose-dependent manner but had no impact on the viability of these cancer cells. Additionally, baicalin reversed the epithelial-to-mesenchymal transition (EMT) process, as evaluated by EMT markers in breast cancer cell lines with a change from a mesenchymal feature to an epithelial type. At the same time, the expression of β-catenin mRNA and protein was dose-dependently downregulated by baicalin in highly invasive breast cancer cell lines, and overexpression of β-catenin by adenoviruses abolished these beneficial effects of baicalin in regards to the migration and invasion, and EMT of breast cancer cells. Furthermore, using a xenograft mouse model, baicalin markedly reduced liver and lung metastasis of breast cancer, inhibited expression of β-catenin, and degraded the EMT molecules vimentin and Slug in the orthotopic tumor tissues. Taken together, all these results indicate that baicalin effectively suppresses the metastasis of breast cancer by reversing EMT, which may be mediated by downregulation of β-catentin expression.

Correspondence to: Dr Hongyuan Li, Department of Endocrine and Breast Surgery, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing 400016, P.R. China
E-mail: hongy_li@hotmail.com

Dr Jingyuan Wan, Chongqing Key Laboratory of Biochemistry and Molecular Pharmacology, Chongqing Medical University, Chongqing 400016, P.R. China
E-mail: jywan@cqmu.edu.cn

*Contributed equally

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Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death among women, and is a major health concern for women around the world. There were an estimated 1.7 million new cases (25% of all cancer cases in women) and 0.5 million breast cancer deaths (15% of all cancer-related deaths in women) globally in females in 2012 (1). In China, among the 5 most commonly diagnosed cancers, breast cancer alone accounted for 15% of the cases (2). Although there are numerous adjuvant treatments with new drugs and systematic treatment schedules to be applied in the clinic, metastasis, the mainly cause of death in breast cancer patients partly due to the lack of effective treatment, is still a huge barrier to overcome for therapy (3). Thus, to identify agents which can effectively inhibit breast cancer metastasis and explore the related mechanisms may provide a solution for this issue.

Epithelial-to-mesenchymal transition (EMT), which is involved in embryonic development, reconstruction of wounded tissues and fibrosis, plays an important role in tumor formation and metastasis (4). EMT is a process that guides the transformation of adhesive, non-mobile, polar, epithelial-like tumor cells into cells with a mobile, invasive, non-polar mesenchymal-like phenotype, which provides the potential for tumor cells to migrate to distant sites and form metastatic tumors. During this process, molecular biomarkers of epithelial cells such as E-cadherin and claudin are downregulated while markers of mesenchymal cells including vimentin and N-cadherin are altered in an opposite way (5). A series of transcriptional factors (TFs) have been identified as EMT

regulators, directly or indirectly repressing the encoding of E-cadherin. These TFs such as ZEB, Snail, Slug, Twist, are regulated by various complex signaling pathway networks such as Hedgehog (Hh), Wnt/β-catenin, Notch, or transforming growth factor-β (TGF-β), by acting as isolated units or cross-talking to provide tumor cells with an additional mechanism with which to escape the effects of chemotherapy (6).

In recent years, due to the resistance of tumor cells to traditional therapy, dietary chemopreventive agents, as additional treatment strategies against cancers, have received increased attention for their excellent effects to suppress, reverse, or retard the process of tumorigenesis (7). Baicalin (Fig. 1A), a flavonoid compound isolated from the roots of *Scutellaria baicalensis* Georgi, has been demonstrated to possess many different pharmacological actions including antioxidant, anti-inflammation, anti-HIV-1 and antitumor activity (8-12). In regards to the antitumor activities, accumulating evidence reveals that baicalin exhibits its function in a wide range of cancers such as hematological malignancies, hepatic cancer, gallbladder carcinoma, lung cancer, colorectal cancer, breast cancer and bladder cancer (13-19), by inducing cell apoptosis, cycle arrest and autophagy, suppressing cell proliferation and tumor growth, and inhibiting migration, invasion and metastasis of cancer cells (15,18,20,21). Although research has reported the protective activities of baicalin in malignancies, its underlying detailed mechanisms and its relationship with EMT remain unclear. Furthermore, the upstream intracellular-signaling cascades of metastatic breast cancer cells with high malignancy remain indeterminate.

MDA-MB-231 is an estrogen-independent fibroblastic human triple-negative breast cancer cell line lacking the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER)-2 (22). 4T1 is a mouse mammary cancer cell line, possessing the characteristic of resistance to 6-thioquanine and metastasizes from the primary tumor to multiple distant sites spontaneously including lymph nodes, blood, liver and lung (23). In the present study, we selected these two highly aggressive breast cancer cell lines to investigate the potential effects and mechanisms of baicalin on the metastasis of breast cancer *in vitro* and *in vivo*.

Materials and methods

Reagents and antibodies. Baicalin (purity >99%) was obtained from Zelang Medical Technology Co. (Nanjing, China). Crystal violet was purchased from Sigma (St. Louis, MO, USA). Vectastain ABC kit and liquid DAB+ Substrate Chromogen system for immunohistochemistry were purchased from Vector Laboratories Inc. (Burlingame, CA, USA) and Dako (Carpinteria, CA, USA), respectively. RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, phosphate-buffered saline (PBS) and penicillin/streptomycin were the products of Hyclone Laboratories Inc. (Los Angeles, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Antibodies used in this study were anti-E-cadherin, anti-claudin, anti-vimentin, anti-N-cadherin, anti-β-catentin, anti-Snail and anti-Slug from Cell Signaling Technology (New England Biolabs, Ipswich, MA, USA); anti-Ki67 (rabbit monoclonal), secondary antibody (rabbit monoclonal IgG) from Abcam (Cambridge, UK); anti-GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-F-actin-Green 488, and Alexa Fluor 594 (goat-anti-rabbit IgG) from Molecular Probes (Oregon, Eugene, OR, USA).

Cell lines and culture. Human breast cancer cell line MDA-MB-231 and mouse mammary cancer cell line 4T1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM and RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin, respectively. Both of the cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37˚C.

Cell viability assay. Cell viability was determined by crystal violet assays. MDA-MB-231 and 4T1 breast cancer cells were harvested with 0.05% trypsin-EDTA and seeded in 24-well plates (Corning, MA, USA) at a density of $6x10^5$/well and $1x10^6$/well, respectively. Four hours later, the cells were treated with different concentrations of baicalin (10, 30, or 100 μM) dissolved in fresh medium or vehicle, and incubated for another 72 h. Then cells were fixed with 10% formaldehyde solution and stained with 0.25% crystal violet. After being washed with PBS, crystal violet was dissolved and the absorbance was read at 570 nM using a microplate reader (ELx800; BioTek, Winooski, VT, USA).

Wound healing assay. MDA-MB-231 and 4T1 breast cancer cells were seeded in 6-well plates (Corning, MA, USA) at a density of $2x10^5$/well. Scrapes were made over the cells by a sterile toothpick until cells formed a confluent monolayer. After scraping, the cells were washed with PBS twice, and then replaced with fresh medium with or without various concentrations of baicalin and continued to culture. The width of the wound was monitored and photographed using a microscope (Nikon, Tokyo, Japan) at 100-fold magnification until the wound of the control group healed or almost healed.

Transwell migration assay. The migration assay was performed in 24-well Transwell chambers (Corning, MA, USA). Briefly, complete DMEM with 10% FBS with or without various concentrations of baicalin was added into the lower chambers, and MDA-MB-231 (10^5) and 4T1 breast cancer ($6x10^4$) cells suspended in serum-free DMEM were added into the upper chambers. After incubation for 18 h, the cells were fixed with 10% formaldehyde solution and stained with 0.25% crystal violet. Then the non-migration cells on the upper chamber were wiped off and the migrated cells in the lower chamber were photographed under a microscope (Nikon) at 100-fold magnification.

Immunofluorescence (IF) analysis. MDA-MB-231 and 4T1 breast cancer cells were seeded in 24-wells and treated with 100 μM baicalin for 24 h. After being fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 at room temperature, the cells were incubated with primary antibodies anti-vimentin (1:100) or anti-Slug (1:50) overnight at 4˚C. After a further wash with PBS, the cells were incubated with Alexa Fluor 594-conjugated secondary antibody (dilution 1:500) for 1 h. Then, anti-F-actin-Green 488 (dilution 1:500) was added to stain the cytoskeleton and the nuclei were stained

by DAPI. Images were captured at x200 under a fluorescence microscope (Nikon).

Immunohistochemical (IHC) analysis. Tissues were fixed, dehydrated, embedded and cut into 5-μm sections in accordance with standard procedures. After deparaffinization, rehydration, antigen retrieval and blockage, the sections were incubated with primary antibodies overnight at 4˚C. Then the sections were blocked the endogenous peroxidase with hydrogen peroxide and incubated with the biotinylated goat anti-rabbit secondary antibodies (1:100). After signal amplification with avidin and horseradish peroxidase (HRP)-conjugated biotin, the sections were counterstained with DAB to visualize the nuclei. Finally, the sections were mounted and imaged.

Protein isolation and western blot analysis. Cells were lysed in cell lysis buffer containing various enzyme-protecting agents to collect total proteins. The concentration of protein was determined by the BCA kit. Total proteins (40 μg) were separated on 10% SDS gel and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocked with 5% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20, the membranes were incubated with the primary monoclonal antibody at 4˚C overnight. The membranes were washed with TBST three times, followed by incubation with the secondary antibodies labeled with horseradish peroxidase (HRP). Finally, the membranes were visualized with an enhanced chemiluminescent system.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA from cancer cells was extracted by using the RNA isolation kit according to the manufacturer's instructions, and cDNA was synthesized using PrimeScript RT reagent kit with 1 μg total RNA. qPCR was performed by using the PCR kit according to the instructions. The following primer sequences were used: E-cadherin sense, 5'-TCCTGGG CAGAGTGAATTTTGAAGA-3' and antisense, 5'-AAACGG AGGCCTGATGGGG-3'; claudin sense, 5'-CCTCCTGGGA GTGATAGCAAT-3' and antisense, 5'-GGCAACTAAAATA GCCAGACCT-3'; vimentin sense, 5'-TACAGGAAGCTGCT GGAAGG-3' and antisense, 5'-ACCAGAGGGAGTGAATC CAG-3'; N-cadherin sense, 5'-AGCCAACCTTAACTGAGG AGT-3' and antisense, 5'-GGCAAGTTGATTGGAGGG ATG-3'; Snail sense, 5'-TCGGAAGCCTAACTACAGCGA-3' and antisense, 5'-AGATGAGCATTGGCAGCGAG-3'; Slug sense, 5'-GGGGAGAAGCCTTTTTCTTG-3' and antisense, 5'-TCCTCATGTTTGTGCAGGA G-3'; and GAPDH sense, 5'-TGTTGCCATCAATGACCCCTT-3' and antisense, 5'-CTCCACGACGTACTCAGC G-3'. Relative quantification was achieved by normalization to GAPDH.

Xenograft model. Six- to eight-week-old female BALB/c mice were obtained from the Laboratory Animal Center of Chongqing Medical University and housed in a specific pathogen-free (SPF) laboratory environment. BALB/c mice were injected subcutaneously with $1x10^6$ 4T1 cells into the bilateral gluteal region. One week after inoculation, the mice were divided randomly into a sham-treated group and a baicalin-treated group. The former received PBS while the latter received 100 mg/kg baicalin in an intraperitoneal injection approach every 3 days. After 6 weeks, all mice were sacrificed under anesthesia, the tumors and lungs were excised, weighed, counted for tumor nodules and fixed for further analysis.

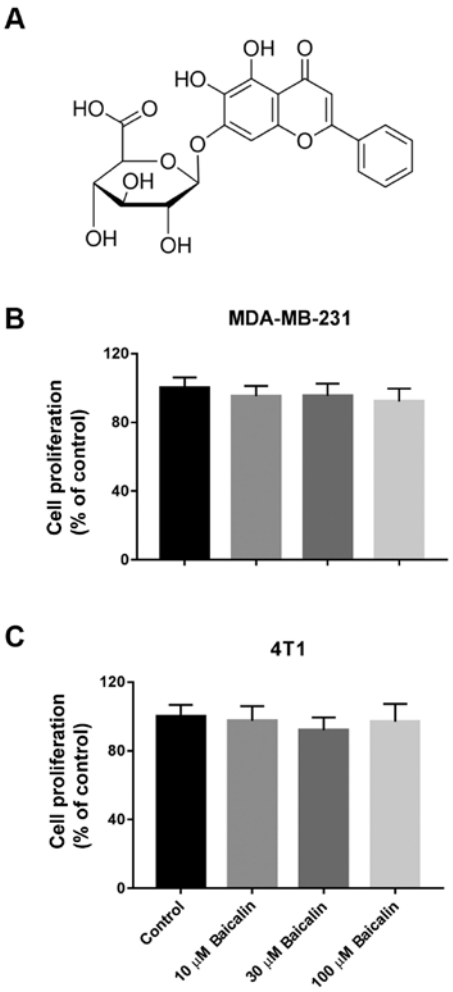

Figure 1. Baicalin does not affect the viability of breast cancer cells *in vitro*. The highly invasive breast cancer cells were pretreated with different concentrations of baicalin for 72 h. (A) Chemical structure of luteolin. The viability of (B) MDA-MB-231 and (C) 4T1 cells was assayed by crystal violet assay. The results are shown as the mean ± SD (n=3).

Statistical analysis. All data wre analyzed with SSPS 13.0. Data are expressed as mean ± SD. Variances between groups were analyzed using the Student's t-test and one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant result.

Results

Baicalin does not affect the viability of breast cancer cells. Previous studies have revealed that baicalin exerts beneficial effects on inhibiting cell proliferation and inducing apoptosis in various cancer types (24-26). To evaluate whether baicalin affects the viability of breast cancer cells, the crystal violet assay was performed in MDA-MB-231 and 4T1 cells. As shown in Fig. 1B and C, the cell viability had no significant difference when the breast cancer cells were treated with increasing concentrations of baicalin up to 100 μM.

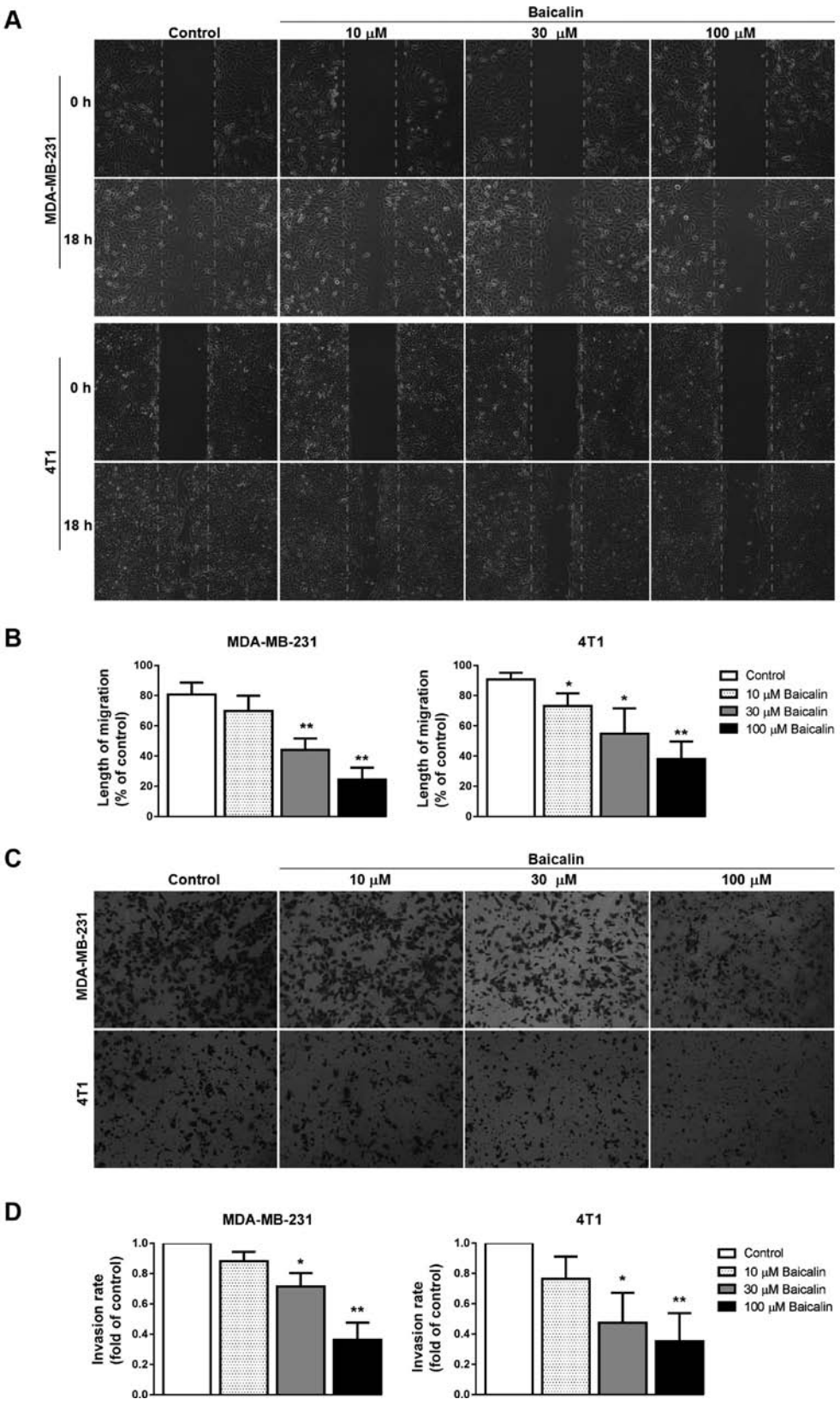

Figure 2. Baicalin suppresses breast cancer cell migration and invasion *in vitro*. The highly invasive breast cancer cells were pretreated with different concentrations of baicalin for the indicated time. (A) Cell migration was determined by wound healing assay. (B) Quantification of the migrated cells. (C) Cell invasion was determined by Transwell invasion assay. (D) Quantification of the invasive cells. The results are shown as the mean ± SD (n=3), *P<0.05, **P<0.01 compared with the control.

Baicalin suppresses breast cancer cell migration and invasion. To determine whether baicalin has the potential to inhibit breast cancer cell migration and invasion, wound healing and transwell migration assays were performed using two highly aggressive breast cancer cell lines MDA-MB-231 and 4T1. In the wound healing assay, following pretreatment

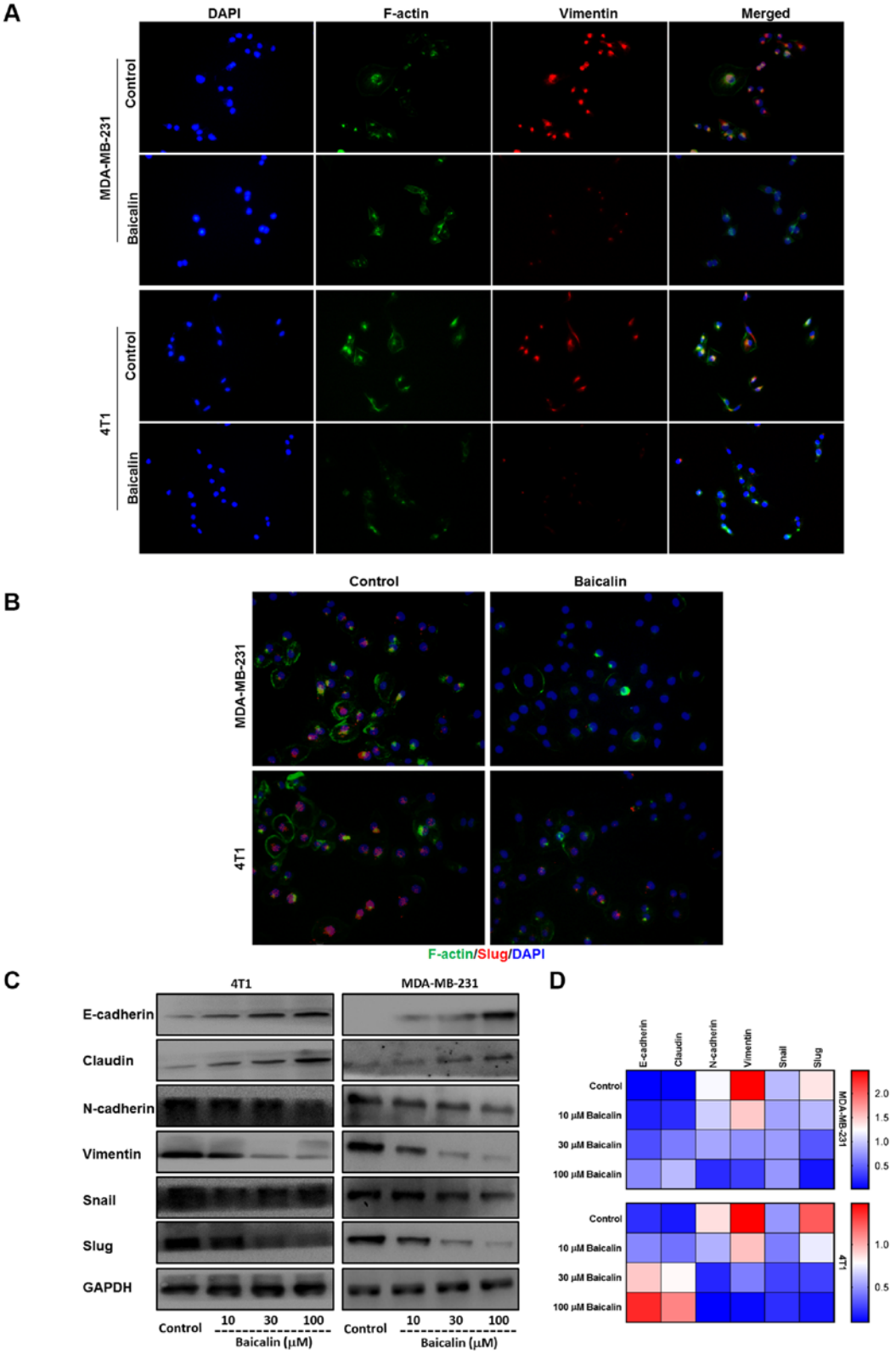

Figure 3. Baicalin reverses epithelial-to-mesenchymal transition (EMT) in breast cancer cells. The highly invasive breast cancer cells were pretreated with different concentrations of baicalin for 24 h. (A) The breast cancer cells were subjected to immunofluorescence assay for vimentin and F-actin. (B) Slug was determined by immunofluorescence assay. (C) The breast cancer cells were subjected to western blot analysis for the indicated proteins. (D) The breast cancer cells were subjected to qRT-PCR for the indicated mRNAs.

with baicalin (10, 30, or 100 μM), the wound scratches in the baicalin-treated groups were wider than those noted in the control group at 18 h, and had a dose-dependent increasing trend (Fig. 2A and B), indicating that baicalin inhibited cell migration of breast cancer cells. In the cell invasion experiment by Transwell assay, results similar to those of the wound healing assay were observed in the baicalin-treated breast cancer cells (Fig. 2C and D).

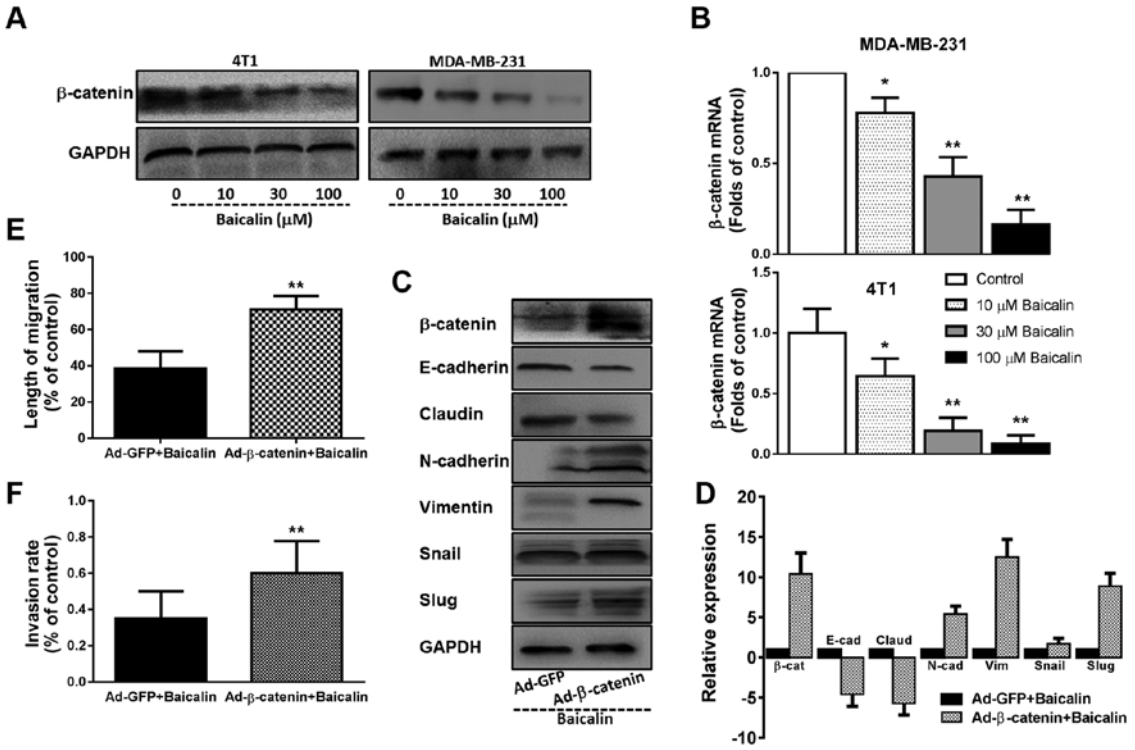

Figure 4. Baicalin downregulates the expression of β-catenin to mediate its anti-metastatic effect *in vitro*. The highly invasive breast cancer cells with or without Ad-β-catenin overexpression were pretreated with different concentrations of baicalin for 24 h. (A) β-catenin protein was determined by western blotting. (B) β-catenin mRNA was detected by qRT-PCR. (C) EMT markers and transcriptional factors were determined by western blotting. (D) EMT markers and transcriptional factors were detected by qRT-PCR. (E) Cell migration was determined by wound healing assay. (F) Cell invasion was determined by Transwell invasion assay. The results are shown as the mean ± SD (n=3), *P<0.05, **P<0.01. EMT, epithelial-to-mesenchymal transition.

Baicalin reverses breast cancer cell EMT process. It has been confirmed that EMT plays a major role in tumor metastasis. In order to explore the relationship between baicalin and EMT, we used varying concentrations of baicalin to treat MDA-MB-231 and 4T1 breast cancer cells, respectively. IF assay indicated that the mesenchymal marker vimentin was degraded and the cytoskeletal protein F-actin was remolded after the two breast cancer cell lines were treated with 100 μM baicalin (Fig. 3A), and the expression of Slug, one of the major EMT TFs that is usually maintained at a high level in highly invasive breast cancer cells (27), was downregulated in the baicalin-treated MDA-MB-231 and 4T1 cells (Fig. 3B). Furthermore, western blotting and quantitative RT-PCR also showed that epithelial markers E-cadherin and claudin were upregulated while mesenchymal markers N-cadherin and vimentin and relative TFs Snail and Slug were downregulated in a dose-dependent manner by baicalin in the two highly invasive breast cancer cell lines (Fig. 3C and D).

β-catenin contributes to the beneficial effects of baicalin in regards to the metastasis and EMT of breast cancer cells. Prior studies have demonstrated that baicalin can inhibit breast cancer cell migration through the p38 MAPK signaling pathway (18). Cancer metastasis and EMT are complex processes which are influenced by numerous signaling pathways through crosstalk (6). In view of the important status of β-catenin in cancer metastasis and EMT, we first determined whether baicalin affects the β-catenin signaling pathway. Western blotting and quantitative RT-PCR analysis found that β-catenin was markedly expressed in the MDA-MB-231 and 4T1 breast cancer cells, and pretreatment with baicalin dose-dependently downregulated the expression of β-catenin protein and mRNA (Fig. 4A and B). In addition, overexpression of β-catenin in the baicalin-treated 4T1 cells by adenovirus vector system, significantly blunted the role of baicalin in regards to the expression levels of EMT-related molecules and TFs, returning cells back to a mesenchymal type (Fig. 4C and D). Simultaneously, the inhibitory effects of baicalin on the migration and invasion of highly invasive breast cancer cells were also reversed with the overexpression of β-catenin (Fig. 4E and F).

Baicalin suppresses the metastasis of breast cancer cells in vivo. Finally, we constructed a xenograft metastasis tumor model of 4T1 breast cancer cells to investigate the effects of baicalin on breast cancer metastasis *in vivo*. As shown in Fig. 5A and B, we found that the numbers of metastatic nodules on the surface of the liver and lung in the baicalin-treated group were less than these numbers in the control group. H&E staining of the liver and lung also indicated that baicalin reduced the metastatic lesions of breast cancer cells in the liver and lung tissues (Fig. 5C). Similar to the *in vitro* experiment, the expression levels of mesenchymal marker vimentin and EMT-activating TF Slug in the orthotopic tumor tissues were downregulated by baicalin (Fig. 5D). Finally, immunohistochemistry showed that baicalin inhibited β-catenin expression in the orthotopic tumor tissues (Fig. 5E).

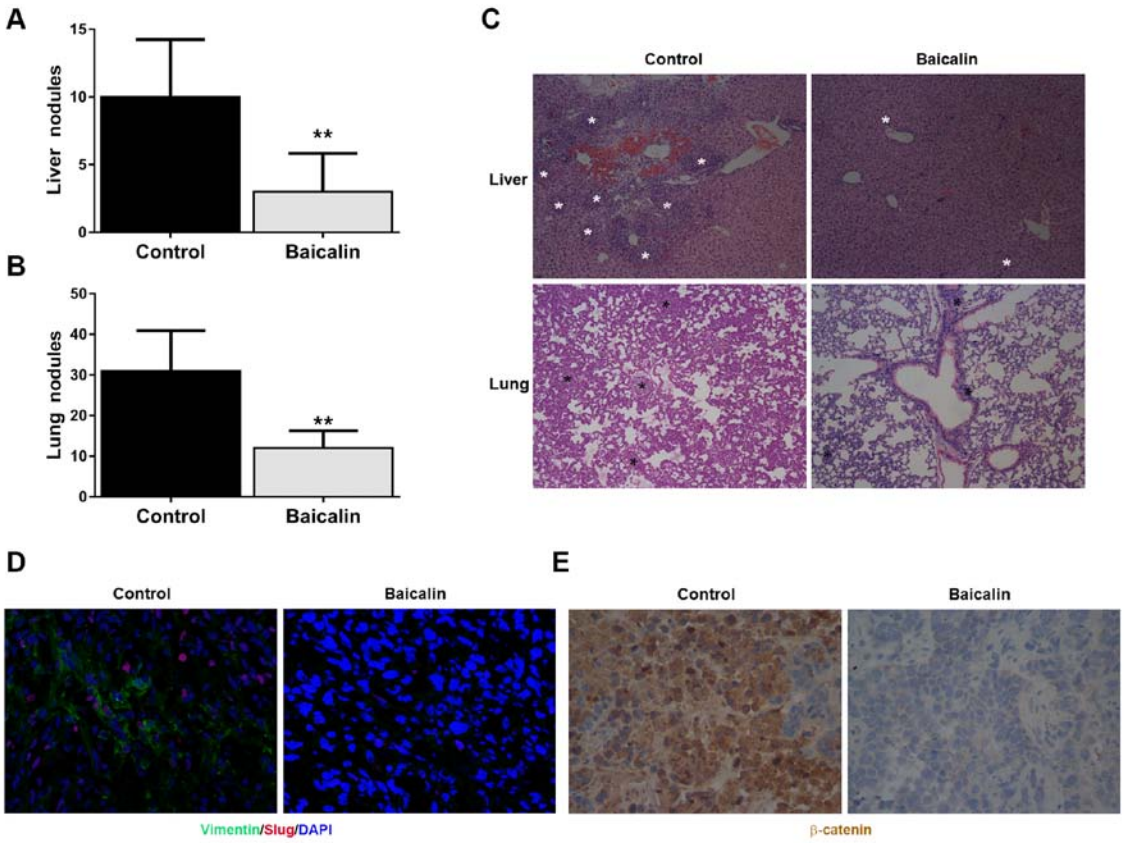

Figure 5. Baicalin inhibits the metastasis of breast cancer *in vivo*. Female BALB/c mice were injected subcutaneously with $1x10^6$ 4T1 cells into the bilateral gluteal region. One week after inoculation, the mice received PBS or 100 mg/kg baicalin in an intraperitoneal injection approach every 3 days. After 6 weeks, all mice were sacrificed under anesthesia. Tumors, liver and lungs were excised and tumor nodules were counted and tissues were fixed for analysis. (A) Tumor nodules in the liver were counted. (B) Tumor nodules in the lung were counted. (C) The metastatic nodules are shown as asterisks in the images of the H&E-stained sections of the liver and lung. (D) Vimentin and Slug in the primary tumors were examined by immunofluorescence. (E) β-catenin in the primary tumors was examined by immunohistochemistry. The results are shown as the mean ± SD (n=5), $^{**}P<0.01$.

Discussion

Although the mortality rate of breast cancer is decreasing along with more efficacious adjuvant treatments and systematic treatment schedules, the mortality rate is still high and breast cancer is the leading cause of cancer-related mortality among middle-age women (28). As is known, the main cause of cancer death is not the primary tumor but its metastasis to distal sites and metastasis-related diseases. Partly due to ineffective therapy or therapeutic resistance, the incidence of metastasis is becoming more and more frequent, which causes a high percentage of recurrence and poor prognosis for patients. In the present study, we investigated the potential effect of baicalin on the inhibition of metastasis in breast cancer cells and elucidated the underlying molecular mechanisms. Our findings revealed that baicalin effectively inhibited the migration and invasion of breast cancer cell lines MDA-MB-231 and 4T1, and suppressed the lung and liver metastasis of breast cancer xenograft tumors derived from 4T1 breast cancer cells, suggesting that baicalin may serve as a rational therapeutic strategy to suppress the metastasis of breast cancer.

A compelling body of evidence has confirmed that EMT is not only a crucial morphogenic process normally activated during embryogenesis and reconstruction of wounded tissues, but also plays an important role during the metastasis of cancers, allowing dynamic and reversible transition from adhesive, non-mobile, epithelial-like phenotype to mobile, invasive mesenchymal-like phenotype, which is utilized by cancer cells to promote their capabilities of local invasion and distant metastasis (29). Indeed, EMT exhibits an essential manifestation of molecular changes, namely loss of epithelial markers E-cadherin and claudin and gain of mesenchymal markers vimentin and N-cadherin (30). Previous studies have elucidated that EMT is associated with clinicopathological features, resistance to apoptosis, evasion of the immune response and poor prognosis of breast cancer (31). Based on its anti-migratory potential, we aimed to ascertain whether baicalin has an influence on EMT in highly aggressive breast cancer cell lines. Our study found that MDA-MB-231 and 4T1 cancer cells acquired epithelial features and lost mesenchymal phenotype at the same time following treatment with baicalin, indicating that baicalin could effectively reverse EMT in highly aggressive breast cancer cells. A large number of previous studies have shown that Slug is an important transcription factor of EMT, and promotes the process of EMT and cancer metastasis (32,33). Thus, we analyzed the expression and nuclear translocation of Slug, and found that the expression and nuclear translocation of Slug was decreased after intervention of baicalin. All these data confirmed that the effect of baicalin on the migration of breast cancer cells resulted from the reversion of EMT.

To date, numerous studies have demonstrated that multiple signaling pathways participate in the regulation of EMT, cell migration, invasion and tumor metastasis, including the Wnt/β-catenin, NF-κB and p38MAPK signaling pathways (18,34-36). Among them, the aberrant activation of the Wnt/β-catenin signaling pathway has been found in many human malignancies. In terms of the canonical Wnt/β-catenin signaling pathway, β-catenin is a key component as a TF. Upon Wnt ligand engagement, β-catenin is activated and disrupted, followed by its translocation from the cytoplasm into the nucleus, where it facilitates the transcription of genes involved in EMT, such as Twist, Snail, Slug, which repress the expression of E-cadherin, influencing cell junction and polarity (37). Previous studies have reported that overexpression and hyperactivation of β-catenin are observed in aggressive basal-like breast cancer, and are positively correlated with poor patient clinical outcome (38-40). Therefore, β-catenin may be a novel therapeutic target with which to overcome breast cancer metastasis and improve the prognosis of breast cancer patients. In the present study, we found that β-catenin was robustly expressed in MDA-MB-231 and 4T1 breast cancer cells which was dose-dependently inhibited by baicalin at the protein and mRNA levels. Using a xenograft mouse *in vivo* model, baicalin markedly downregulated the expression of β-catenin in primary tumor tissues. Furthermore, overexpression of β-catenin by an adenovirus vector system markedly blunted the suppressive effects of baicalin on metastasis and EMT in breast cancer cells, suggesting that these beneficial effects of baicalin may be involved in the downregulation of β-catenin signaling.

In conclusion, our experimental data showed that baicalin exhibited significant effects on the suppression of migration and metastasis in highly aggressive breast cancer both *in vitro* and *in vivo*, which may be through reversal of the EMT process and downregulation of β-catenin expression. Therefore, application of baicalin in conjunction with currently conventional adjuvant treatments may provide a novel therapeutic strategy for patients with metastatic breast cancer.

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