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# *Ganoderma lucidum* (Reishi) suppresses proliferation and migration of breast cancer cells via inhibiting Wnt/β-catenin signaling

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ABSTRACT

The medical mushroom *Ganoderma lucidum* (Reishi), a traditional Chinese medicine, has exhibited a promising anti-cancer effect. However, the molecular mechanism of its action on cancer cells remains unclear. Aberrant activation of Wnt/β-catenin signaling pathway is the cause of many types of cancer, including breast cancer. Here we investigated the effect of Reishi on Wnt/β-catenin signaling pathway and elucidated the molecular mechanism of its function in inhibiting breast cancer cells. We found that Reishi blocked Wnt/β-catenin signaling through inhibiting the phosphorylation of Wnt co-receptor LRP6. In human (MDA-MB-231) and mouse (4T1) breast cancer cell lines, Reishi significantly decreased the phosphorylation of LRP6 and suppressed Wnt3a-activated Wnt target gene Axin2 expression. Administration of Reishi inhibited Wnt-induced hyper-proliferation of breast cancer cells and MDA-MB-231 cell migration. Our results provide evidence that Reishi suppresses breast cancer cell growth and migration through inhibiting Wnt/β-catenin signaling, indicating that Reishi may be a potential natural inhibitor for breast cancer.



## 1. Introduction

Wnt signaling pathway plays an important role in embryonic development and tissue homeostasis [1,2]. As for the canonical Wnt/β-catenin pathway, in the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, adenomatosis polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK3β) and is phosphorylated by CK1 and GSK3β at the N-terminal region, resulting in degradation through ubiquitination-proteasome pathway. In the presence of Wnt ligand, Wnt binds to receptor Frizzled (Fzd) and its co-receptor low-density-lipoprotein receptor related protein 5/6 (LRP5/6) [3]. The formation of this complex, together with recruitment of Dishevelled (Dvl), leads to LRP5/6 phosphorylation and Axin recruitment. These events disrupt Axin-mediated degradation complex and allow β-catenin to accumulate and translocate into nucleus where it cooperates with T-cell factor/lymphoid-enhancing factor (Tcf/Lef) to activate Wnt target genes expression. Aberrant activation of Wnt signaling pathway by gene mutations and epigenetic changes is closely associated with various human diseases, especially with many types of cancer [4,5]. Therefore, targeting Wnt/β-catenin signaling pathway represents a great opportunity to develop novel drugs for cancer therapy.

*Ganoderma lucidum*, also commonly known as Reishi or Lingzhi, is one of the most intriguing traditional Chinese medicines and has been recognized as a potent medicinal mushroom for more than 2000 years in China [6]. Accumulated studies have reported that Reishi exerted great anti-cancer activities in various cancer cell lines [7–9] and in some tumor-bearing mice [10,11]. In addition, observed activities of Reishi have prompted its usage in cancer patients as an adjunct to chemotherapy or radiotherapy [12]. Another two epidemiological studies from Asia suggest that a high dietary intake of Reishi is negatively associated with breast cancer risks [13,14]. However, despite the fact that several mechanisms have been proposed, the biological pathways by which it exhibits anti-cancer effects remain poorly understood.

In the present study, we demonstrate for the first time that Reishi inhibit Wnt/β-catenin signaling pathway at the cell surface level, and this activity is closely related to its suppression of breast cancer cells proliferation and migration.

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## 2. Materials and methods

### 2.1. Reishi preparation

A crude powder of *Ganoderma lucidum* (400 mg) was dissolved in 8 ml double-distilled water ($ddH_2O$). After vortex, the mixture was stirred at room temperature for 2 h and centrifuged (10,000 rpm) for 5 min to remove the insoluble materials. Then 32 ml of 99% ethanol was added to the supernatant (final ethanol concentration is 80%) at 4 °C for 24 h and centrifuged (3000 rpm) for 5 min, followed by collecting the brown precipitation and dissolved in 8 ml $ddH_2O$. The resulting solution was concentrated to about 3–4 ml at 50 °C using rotary evaporator (EYELA N-1100) and lyophilized to generate 154 mg dark brown powder using freeze dryer (Four-Ring Science Co.). The sample was dissolved in 8 ml $ddH_2O$, dialyzed to remove excessive salts by dialysis bag (Spectrums), concentrated and then lyophilized to get 112 mg Reishi powder. Stock solution was prepared by dissolving treated Reishi in $ddH_2O$ at a final concentration of 50 mg/ml and stored at 4 °C.

### 2.2. Cell culture and transfection

Human embryonic kidney epithelial HEK293T and HEK293FT cells, human breast cancer cell MDA-MB-231 and mouse breast cancer cell 4T1 were maintained in Dulbecco's minimum essential medium (DMEM) (Coring) supplemented with 10% fetal bovine serum (FBS) (Hyclone) at 37 °C in a humidified 5% $CO_2$ incubator. Transfection was accomplished with PEI (polyethylenimine), following the instructions of manufacturers.

### 2.3. Antibodies and reagents

Anti-pLRP6, anti-LRP6 and anti-Dvl2 antibodies were purchased from Cell Signaling Technology. Anti-β-catenin antibody was purchased from Santa Cruz Biotechnology. Recombinant human TGF-β and mouse Wnt3a proteins were purchased from R&D Systems Inc.

### 2.4. Luciferase reporter assay

Cells were seeded in 24-well plates overnight before transfection. HEK293T or HEK293FT cells were transfected with *Renilla* and TopFlash or CAGA-Luc together with other indicated plasmids. After 12 h, the cells were treated with specific amount of Reishi, Wnt3a conditioned medium (Wnt3a CM) from L Wnt-3A cell line (ATCC CRL-2647) or LiCl (an inhibitor of GSK3β) for 24 h. The cells in each well were lysed in 100 μl passive lysate buffer (PLB), and 20 μl lysate were placed into 96-well plates respectively, followed by measurement of luciferase and *Renilla* activity by luciferase assay system (Promega). Reporter assays were all conducted with three independent repeats. After normalizing to *Renilla* activity, the final luciferase activity was presented as means ± SD.

### 2.5. Immunoblotting

Cells cultured in 6 cm dish were harvested and lysed with 200 μl TNE buffer (10 mM Tris-HCl, pH 7.5, 0.5% NP-40, 1 mM EDTA, 150 mM NaCl) containing protease inhibitor cocktail (Roche) for 30 min at 4 °C. After centrifugation, the supernatants added with loading buffer were separated on 10% SDA-PAGE gel and then transferred to nitrocellulose membranes (Pall). The membranes were blocked with milk for 1 h and washed three times with TBST (TBS + 0.05% Tween 20). And then membranes were sequentially incubated with primary antibodies overnight at 4 °C, and finally with secondary antibodies at room temperature for 1 h. The proteins were detected with chemiluminescent substrate.

### 2.6. RNA extraction, reverse transcription, quantitative real-time PCR (qRT-PCR)

Cells in 6-well plate were harvested and total RNA was extracted with 500 μl Trizol reagent (Invitrogen). Then cDNA was synthesized through 1 μg RNA, oligo-dT and reverse transcriptase. Finally qRT-PCR was carried out using EvaGreen dye (Biotium) on LC480 (Roche Applied Science) system. The primers used were as follows: for human GAPDH, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′; for human Axin2, 5′-TTATGCTTTGCACTACGTCCCTCCA-3′ and 5′-CGCAACATGGTCAACCCTCAGAC-3′; for mouse GAPDH, 5′-AAGAAGGTGGTGAAGCAG-3′ and 5′-TCATACCAGGAAATGAGC-3′; for mouse Axin2, 5′-GCTCCAGAAGATCACAAAGAGC-3′ and 5′-AGCTTTGAGCCTTCAGCATC-3′.

### 2.7. Cell proliferation assay

MDA-MB-231 and 4T1 cells were seeded into 24-well plates ($8 \times 10^3$ cells/well for MDA-MB-231 and $3 \times 10^3$ cells/well for 4T1). After overnight incubation, cells were treated with 200 μg/ml Reishi or 20 ng/ml Wnt3a. Then we counted cell numbers every 24 h or 12 h. Each sample was counted three times.

### 2.8. Wound healing assay

Cells were seeded in 12-well plates and cultured until confluent. Then the straight scratch wound was made using a yellow pipette tip. After washing with PBS and changing with fresh medium, 20 ng/ml Wnt3a or 200 μg/ml Reishi were added into medium followed by photographing the positions chosen. Photographic images were taken at 0 h, 24 h and 48 h.

### 2.9. Statistical analysis

Statistical analyses were conducted using Student's t-test. The values were presented as mean ± SD and differences at $p < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Inhibition of Wnt/β-catenin signaling by Reishi

To clarify the inhibitory effect of Reishi and at what levels it acts in Wnt/β-catenin signaling, we first carried out the luciferase reporter assay. The TopFlash luciferase reporter, which contains three copies of Tcf response elements (CCTTTGATC) driven by the *c-fos* promoter and upstream of a luciferase reporter gene [15], is widely used as a transcriptional reporter for β-catenin-dependent signaling events. HEK293T cells were transfected with TopFlash reporter plasmid together with Wnt1, Myc-LRP6ΔE1-E4 (constitutively active LRP6), Dvl2 and β-catenin expression plasmids (Fig. 1A–D). Treating these cells with Reishi dose-dependently blocked Wnt signaling activated by Wnt1 (Fig. 1A), as well as Myc-LRP6ΔE1-E4 (Fig. 1B). Wnt3a conditioned medium (Wnt3a CM) could also induce Wnt pathway activation, and Reishi treatment inhibited Wnt transcription in a dose-dependent manner (Fig. 1E).

However, Reishi did not suppress Dvl2 (Fig. 1C), LiCl (an inhibitor of GSK3β) (Fig. 1F) or β-catenin (Fig. 1D)-induced Wnt signaling pathway, indicating that Reishi may inhibit Wnt signaling upstream

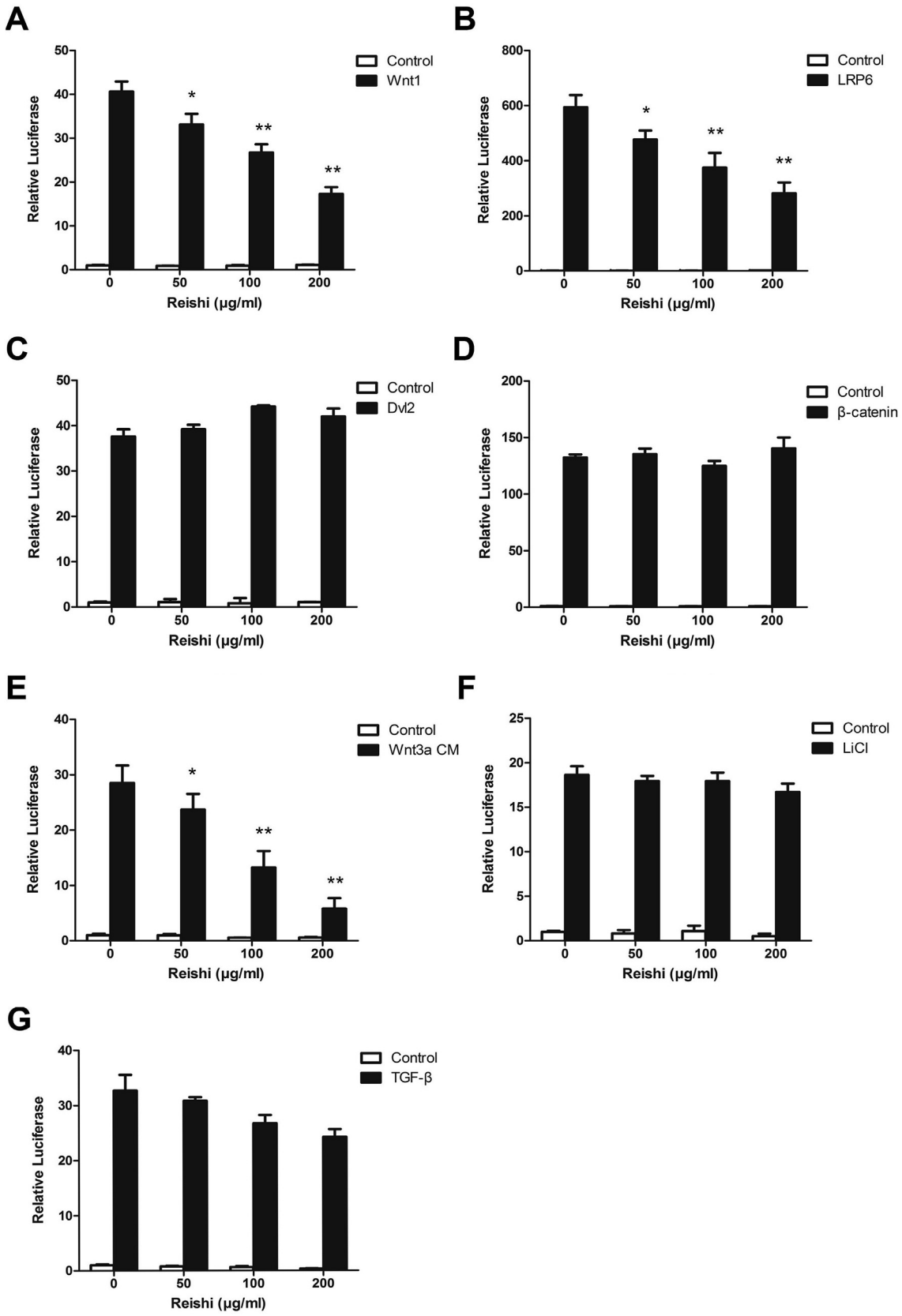


**Fig. 1.** Reishi specifically inhibits Wnt/β-catenin signaling. (A–D) The TopFlash reporter plasmid was transfected into HEK293T cells with Wnt1 (A), Myc-LRP6ΔE1-E4 (B), Dvl2 (C) and β-catenin (D). (E–F) The TopFlash reporter plasmid was transfected into HEK293T cells, then the cells were treated with control or Wnt3a CM (E), LiCl (F). (G) HEK293FT cells were transfected with CAGA-Luc reporter plasmid and then treated with 100 pM TGF-β. The transfected cells were incubated with vehicle or 50–200 μg/ml Reishi for 24 h. Data represent the mean of three independent experiments, and error bars indicate standard deviations from the means. Statistical analyses were conducted with Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

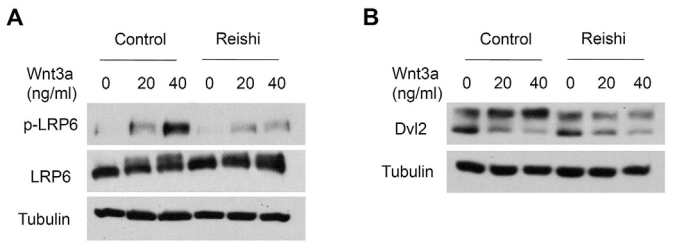


**Fig. 2.** Reishi inhibits Wnt/β-catenin signaling in HEK293T cells. (A) HEK293T cells were treated with indicated amounts of Wnt3a or 200 μg/ml Reishi for 12 h. Then phosphorylated LRP6 and total LRP6 were detected by immunoblotting. (B) Administration conditions were as in A. The shifted band of Dvl2 was the phosphorylated form on SDS-PAGE gel.

of Dvl2 and be at the cell surface level. In control experiment, Reishi at Wnt inhibitory concentrations did not obviously influence the CAGA-Luc reporter (a Smad-dependent luciferase reporter) [16] activated by TGF-β (Fig. 1G).

### 3.2. Inhibition of Wnt/β-catenin signaling in HEK293T cells by Reishi

To further confirm the target of Reishi inhibiting Wnt signaling, we did western blotting assay in HEK293T cells. As 200 μg/ml Reishi obviously inhibited Wnt signaling in reporter assay, this concentration was used for following experiments. Cells were treated with increasing concentrations of Wnt3a or 200 μg/ml Reishi for 12 h. As expected, Wnt3a increased the phosphorylation of LRP6 and Dvl2 (Fig. 2A–B) in a Wnt3a dose-dependent manner. The phosphorylated Dvl2 showed slower mobility detected by Dvl2 antibody on SDS-PAGE gel. Compared to control group, Reishi treatment significantly decreased the phosphorylation of LRP6 and Dvl2, indicating that Reishi may block Wnt/β-catenin signaling by targeting LRP6 and Dvl.

### 3.3. Suppression of Wnt/β-catenin signaling in breast cancer cells by Reishi

As mutations of Wnt signaling pathway in breast cancer cells often occurred at the cell surface level [4], we chose human MDA-MB-231 and mouse 4T1 cell lines to test if Reishi inhibits the Wnt/β-catenin signaling in breast cancer cells. In both cell lines, 200 μg/ml Reishi could decrease the phosphorylation levels of LRP6 and Dvl2 (Fig. 3A–B), which were activated by Wnt3a CM. Additionally, β-catenin expression level was reduced after Reishi treatment.

Activation of Wnt pathway increases target genes transcription, such as Axin2, CyclinD1 and c-Myc [2]. In MDA-MB-231 and 4T1 cells, Wnt3a CM significantly activated Axin2 transcription (Fig. 3C–D), which was suppressed by 200 μg/ml Reishi after incubating for 4 h.

### 3.4. Effect of Reishi on breast cancer cell proliferation and migration

To investigate the effectiveness of Reishi targeting Wnt pathway in breast cancer cells, we first tested its inhibitory effect on MDA-MB-231 and 4T1 cells by cell proliferation assay. As shown in Fig. 4A and Fig. 4B, 20 ng/ml Wnt3a promoted proliferation of MDA-MB-231 after 2 days and of 4T1 after 36 h compared to control group. Wnt3a and 200 μg/ml Reishi treatment inhibited the proliferation of both cell lines obviously. Here we noticed that Reishi alone could inhibit proliferation of breast cancer cells.

Since MDA-MB-231 cells have migration ability, we further tested the effect of Reishi on cell migration by wound healing assay. As shown in Fig. 4C, 200 μg/ml Reishi decreased the migration of MDA-MB-231 cells to the scratched area compared to untreated cells after two days of culture. Compared with control group,

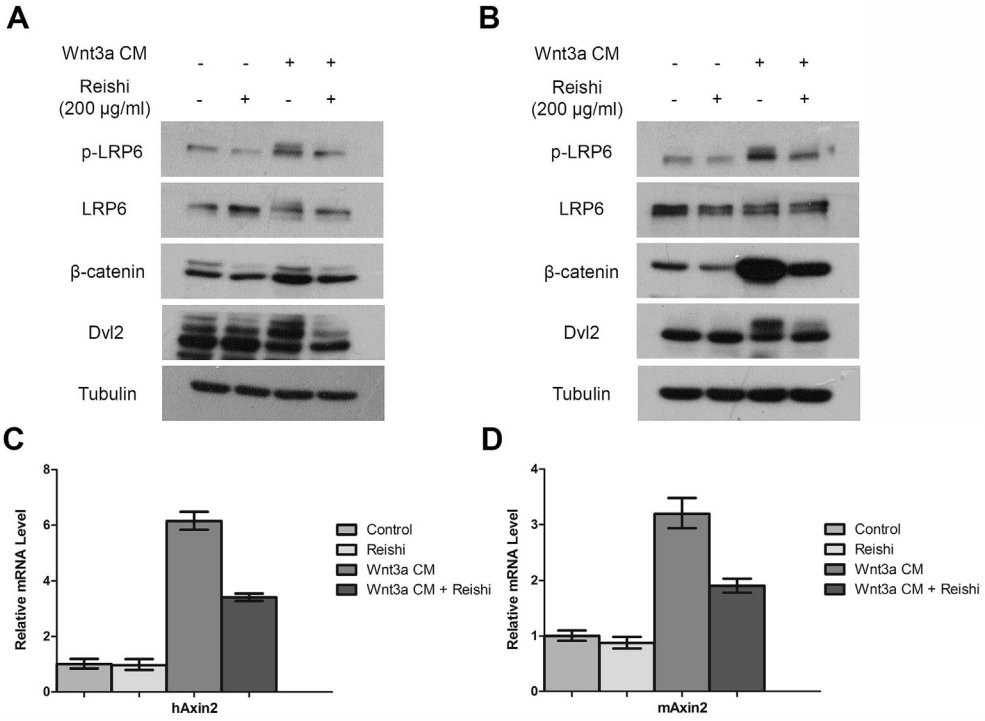


**Fig. 3.** Reishi inhibits Wnt/β-catenin signaling in breast cancer cells. (A–B) MDA-MBA-231 and 4T1 cells were treated with Wnt3a CM or 200 μg/ml Reishi for 12 h. Phosphorylated LRP6, total LRP6, phosphorylated Dvl2 and β-catenin were detected by immunoblotting. (C–D) The transcription levels of hAxin2 and mAxin2 were measured by qRT-PCR respectively in MDA-MBA-231 and 4T1 cells, which were treated with Wnt3a CM or 200 μg/ml Reishi for 4 h.

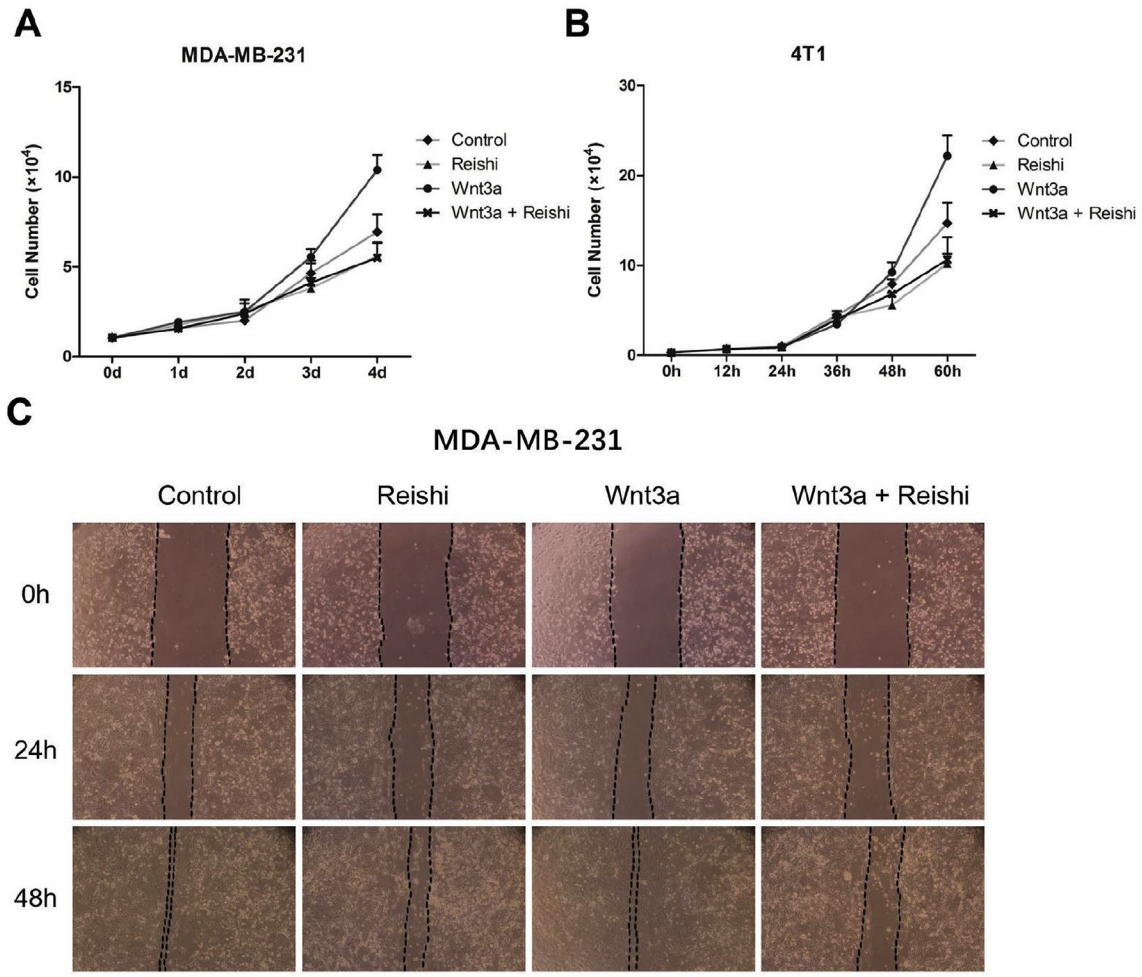


**Fig. 4.** Reishi inhibits proliferation and migration of breast cancer cells. (A) Cell proliferation assay was conducted in MDA-MB-231 cells treated with 20 ng/ml Wnt3a or 200 μg/ml Reishi. (B) Cell proliferation assay was conducted in 4T1 cells treated with Wnt3a or Reishi. (C) MDA-MB-231 cell migration was detected through wound healing assay after 20 ng/ml Wnt3a or 200 μg/ml Reishi treatment.

Wnt3a administration seemingly had no further effect on migration of MDA-MB-231 cells probably due to its high basic level of Wnt signaling. These results demonstrated that Reishi effectively inhibited breast cancer cell migration.

## 4. Discussion

Aberrant Wnt activation is found in 40%–60% breast carcinomas [17]. It has been reported that ectopic expression of Wnt1 induced mammary hyperplasia and tumorigenesis even in the absence of ER in female and male mice [18]. Further studies validated that LRP6 could be a potential therapeutic target in breast cancer treatment as it was up-regulated in human breast carcinomas [19]. Similarly, amplification and increased expression of Dvl were observed in breast cancer tissues [20]. Therefore, inhibition of Wnt signaling, especially the upstream of Wnt signaling, could display a promising means to treat breast cancer. Multiple Wnt inhibitors have been reported to exert anticancer activity in breast cancer cells. Prodigiosin, a natural red pigment, acted as a potent Wnt/β-catenin antagonist to block the phosphorylation of LRP6 and Dvl2 and activate GSK3β in breast cancer [21]. Moreover, Silibinin was a natural compound isolated from milk thistle seed extracts and it suppressed Wnt co-receptor LRP6 expression [22]. In addition, it was reported that Niclosamide (trade name Niclocide), a kind of teniacide in the antihelmintic family, suppressed breast and prostate cancer cell growth by inducing LRP degradation [23].

Our results here demonstrated that Reishi suppressed Wnt/β-catenin signaling pathway and inhibited proliferation and migration of breast cancer cells. Meanwhile, the effect of Reishi on Wnt pathway is at the same concentration with those required to inhibit breast cancer cell proliferation and migration. Remarkably, Reishi has traditionally been used as a medicinal mushroom with little side effects and even high doses are tolerated by mice [10,11]. There have been many studies about Reishi inhibiting breast cancer cells. In the matter of inflammatory breast cancer, Reishi inhibited cell viability, cell metastasis and invasion, and induced cell apoptosis by downregulating the PI3K/AKT/mTOR pathway [11,24]. Furthermore, Reishi increased Erlotinib (EGFR Tyrosine Kinase Inhibitor) sensitivity by inactivating AKT and ERK signaling pathways in inflammatory breast cancer [25]. Reishi also inhibited constitutively active transcription factors AP-1, NF-κB and decreased expression of uPA and uPA receptor (uPAR), as well as secretion of uPA [26]. Another study reported that the inhibition of ER and NF-κB pathways resulted in the down-regulation of c-Myc, and finally suppressed the breast cancer cell proliferation and breast-to-lung metastasis [27,28].

In this study, we found for the first time that the inhibitory effect of Reishi on breast cancer could attribute to its suppression of Wnt/

β-catenin signaling at the cell surface level. Thus, the combination of Reishi with other targeted drugs or antiestrogen could enhance the treatment of breast cancer with mutations in Wnt pathway. As a natural product with little side effects, Reishi may be an interesting compound to be optimized and tested in vivo for its preventive and even therapeutic functions in the future.

## Conflict of interest

We have no conflicts of interest to disclose.

## Acknowledgments

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