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*Original Article*

# Ginkgetin attenuates metastasis of breast cancer via inhibition of the Hedgehog signaling pathway

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**Abstract:** Objectives: To investigate the role and mechanism of Ginkgetin (GGT) in breast cancer metastasis. Methods: The anti-proliferative effects of GGT on breast cancer cell lines 4T1 and MDA-MB-231 were examined using the MTT assay and colony formation assay. The wound-healing assay and invasion assay were carried out to evaluate GGT's impact on the migration and invasion abilities of these cancer cells. The effect of GGT on the stemness of breast cancer cells was further analyzed by the tumorsphere assay. The expression levels of tumor stem cell (CSC) markers SOX2, OCT4, CD44, and ALDH1 were determined by qRT-PCR. The effect of GGT on breast cancer cell metastasis in vivo was studied in a mouse model. RNA sequencing analysis was performed to explore the mechanism of GGT action in breast cancer cells. Results: The MTT and colony formation assays showed that GGT exerted moderate anti-proliferative effects on the breast cancer cell lines 4T1 and MDA-MB-231. Moreover, the wound-healing and transwell invasion assays demonstrated that GGT significantly inhibited the migration and invasion capabilities of these cancer cells. Tumorsphere experiments further indicated that GGT substantially reduced the stemness of breast cancer cells. The qRT-PCR analysis results showed that GGT markedly suppressed the expression of cancer stem cell (CSC) markers, such as SOX2, OCT4, CD44, and ALDH1. In vivo experiments revealed that GGT significantly inhibited breast cancer cell metastasis and enhanced mouse survival. Preliminary mechanistic studies based on RNA sequencing analysis showed that GGT downregulated the Hedgehog (Hh) signaling pathway in breast cancer cells. Conclusion: The data suggest that GGT inhibits breast cancer stem cells by suppressing the Hh signaling pathway. This discovery provides a novel approach for breast cancer treatment.

**Keywords:** Ginkgetin, metastasis, triple-negative breast cancer, Hedgehog signaling pathway, cancer stem cells

## Introduction

Breast cancer is one of the most common malignancies among women, and its global incidence is on the rise [1]. Moreover, breast cancer is being diagnosed at increasingly younger ages, especially triple-negative breast cancer, which is more common in women under 40 years old [2]. Currently, the main treatment options for breast cancer patients include surgical resection, radiotherapy, and chemotherapy [3-5]. However, for patients with advanced breast cancer, surgical options are often not feasible, and they have to rely on chemotherapy, radiotherapy, endocrine therapy, and other methods to extend survival and improve quality of life. Nevertheless, the efficacy of these treatment modalities is limited and is often accompanied by severe side effects [6-9]. Therefore, the development of new treatment strategies to improve the outcomes and survival rates of advanced breast cancer patients is an urgent requirement in breast cancer treatment.

Ginkgetin (GGT) is a biflavonoid compound isolated from Ginkgo biloba leaves and exhibits a wide range of biological activities [10]. Previous reports have shown that GGT has significant anti-inflammatory properties by modulating inflammatory signaling pathways, especially through the NF-κB pathway [11]. It has also been demonstrated that GGT can significantly inhibit the expression of various factors both in vitro and in vivo, such as NF-κB, TLR4, COX-2, iNOS, and PGE2 [12-17]. Additionally, some studies have revealed that GGT is able to scavenge free radicals in the body, thereby protecting cells from oxidative-stress-related damage. It can inhibit inflammatory responses by reducing the release of inflammatory mediators, thus

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alleviating tissue damage and pain [17, 18]. Moreover, GGT has been reported to improve microcirculation in the cardiovascular and cerebrovascular systems, increasing blood flow and lowering blood pressure and lipid levels, thus showing preventive and therapeutic effects on cardiovascular diseases [19, 20]. Furthermore, GGT also shows moderate antifungal activity by inhibiting the growth and reproduction of fungi [21]. In addition, it has been shown that GGT can suppress the proliferation of different types of human cancer cells [22, 23]. This suppressive effect is achieved through the modulation of genes related to the cell cycle and programmed cell death. Specifically, GGT has been reported to suppress the activation of the NF-κB and Akt signaling pathways, which are crucial for maintaining the balance between cell survival and programmed cell death [24, 25]. GGT has also proven effective in inhibiting angiogenesis and metastasis, thereby preventing cancer cells from invading and spreading [26, 27].

In this study, it was shown that GGT could not only reduce the migration, invasion, and stemness of breast cancer cells in vitro but also decrease the metastasis of tumor cells in vivo, thus improving the survival rate of mice. Mechanistically, it was found that GGT inhibited the Hh signaling pathway. This suggests that GGT could be a promising treatment option for breast cancer.

## Materials and methods

### *Cell culture*

Cell lines, 4T1 (CL-0007) and MDA-MB-231 (CL-0150) were kindly provided by Wuhan Pricella Biotechnology Co., Ltd. 4T1 and MDA-MB-231 cells were respectively cultured in medium RPMI-1640 (PM150110) and Leibovitz's L-15 (PM151010) supplemented with 10% fetal bovine serum (FBS, 164210-50) and 1% Pen-Strep Solution (PB180120). These cells were maintained at 37°C with 5% $CO_2$.

### *MTT assay*

A total of $2.0 \times 10^3$ cells of the 4T1 cell line and $4.0 \times 10^3$ cells of MDA-MB-231 cell line were added to each well of a 96-well plate in a volume of 100 μL. Various concentrations of GGT were administered 24 hours after planting. After the treatment had been ongoing for 72 hours, 10 μL of MTT solution (5 mg/mL, MCE, HY-15924) was applied to every well. Dimethyl sulfoxide (DMSO, 100 μL) was used to dissolve the formazan crystals after 4 hours of incubation, after which the liquid above the crystals was discarded. Thermo Fisher Scientific's Multiskan FC microplate reader was used to measure the absorbance at 490 nm (Inhibition rate (%) = (drug experimental group OD - drug control hole average OD)/(cell control hole average OD - drug control hole average OD) × 100%).

### *Colony formation assay*

A total of 1,000 cells of 4T1 or MDA-MB-231 were added to each well of a 6-well plate. Media with different concentrations of drugs were changed every 3 days. The cells were washed twice with PBS after 14 days and then exposed to 4% paraformaldehyde for 15 minutes. After staining the colonies with 0.1% crystal violet for 15 minutes, photographs were taken.

### *Wound-healing assay*

The single-layer fusion of 4T1 cells and MDA-MB-231 cells reached 100% in the 6-well cell culture plate. After a 10 μl pipette tip was used to scratch directly in the monolayer of cells. Cells were washed three times with PBS and cultured in medium without FBS but containing different concentrations of drugs. The width of the wound was measured at 0 and 72 hours.

### *Transwell assay*

Cell invasion was assessed using a Millipore 24-well Millicell cavity with a pore size of 8 mm. The medium cell suspension ($5 \times 10^4$) without FBS were added to the upper cavity pre-coated with Matrigel (Sigma), and the lower cavity was filled with the medium containing 20% FBS. After incubation for 24 h, the cells were fixed, stained with 0.1% crystal violet (Solarbio), and the infiltrated cells were imaged and counted under a 10-fold inverted optical microscope.

### *Sphere-formation assay*

The development of tumor spheres was examined by seeding 4T1 and MDA-MB-231 cells into 96-well ultralow attachment plates (Corning) at a density of 500 cells per well. For this reason, a specific tumorsphere medium was

**Table 1.** Human qPCR primer pair

| Gene | Forward | Reverse |
|---|---|---|
| GAPDH | GTTTCTATAAATTGAGCCCGCAG | CGACCAAATCCGTTGACTCC |
| OCT4 | ACTGAGAGGCAACCTGGAGA | CAAAAACCCTGGCACAACT |
| SOX2 | GCTACAGCATGATGCAGGACCA | TCTGCGAGCTGGTCATGGAGTT |
| CD44 | CCAGAAGGAACAGTGGTTTGGC | ACTGTCCTCTGGGCTTGGTGTT |
| ALDH1 | CGGGAAAAGCAATCTGAAGAGGG | GATGCGGCTATACAACACTGGC |
| SHh | CCGAGCGATTTAAGGAACTCACC | AGCGTTCAACTTGTCCTTACACC |
| Ptch | GCTGCACTACTTCAGAGACTGG | CACCAGGAGTTTGTAGGCAAGG |
| SMO | TGCTCATCGTGGGAGGCTACTT | ATCTTGCTGGCAGCCTTCTCAC |
| Gli | AGCCTTCAGCAATGCCAGTGAC | GTCAGGACCATGCACTGTCTTG |

**Table 2.** Mouse qPCR primer pair

| Gene | Forward | Reverse |
|---|---|---|
| GAPDH | CATCACTGCCACCCAGAAGACTG | ATGCCAGTGAGCTTCCCGTTCAG |
| OCT4 | CAGCAGATCACTCACATCGCCA | GCCTCATACTCTTCTCGTTGGG |
| SOX2 | AACGGCAGCTACAGCATGATGC | CGAGCTGGTCATGGAGTTGTAC |
| CD44 | CGGAACCACAGCCTCCTTTCAA | TGCCATCCGTTCTGAAACCACG |
| ALDH1 | GGAATACCGTGGTTGTCAAGCC | CCAGGGACAATGTTTACCACGC |
| SHh | GGATGAGGAAAACACGGGAGCA | TCATCCCAGCCCTCGGTCACT |
| Ptch | CCTCGCTTACAAACTCCTGGTG | TGATGCCATCTGCGTCTACCAG |
| SMO | GAGGCTACTTCCTCATCAGAGG | GCTGAAGGTGATGAGCACAAAGC |
| Gli | CTCAAACTGCCCAGCTTAACCC | TGCGGCTGACTGTGTAAGCAGA |

utilized, which contained 20 ng/mL of epidermal growth factor, 10 ng/mL of basic fibroblast growth factor, 5 μg/mL of insulin, 0.4% bovine serum albumin, and DMEM/F12. After 3 days, the tumorspheres that had formed were subjected to different concentrations of GGT for another 72 hours. Changes in the morphology and number of spheres were meticulously documented.

*Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay*

After extracting total RNA via the Trizol Reagent (Yeasen), reverse transcription was carried out using the Hifair® III 1st Strand cDNA Synthesis Kit (Yeasen) to cDNA. The LightCycler®480 real-time PCR system conducted Real-time PCR with 2 × SYBR Green Premix PCR Master Mix (Bimake). The threshold cycle (ct) value of the purpose gene was normalized to GAPDH cycle threshold value. The sequence of primers is shown in **Tables 1** and **2**.

*Western blotting*

After protein concentration was determined using the BCA detection kits (Beyotime, China), equal amounts of protein lysates were resolved on 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to PVDF membranes. After blocking the membranes with 5% skim milk powder, the membranes were incubated with primary antibodies including anti-SHh (Proteintech, 20697-1-AP), anti-Ptch (Proteintech, 17520-1-AP), anti-SMO (Santa, sc-166685), anti-Gli (Proteintech, 66905-1-Ig) and anti-β-actin (Proteintech, 66009-1-Ig, to be used for control) at 4°C overnight. Then, they were incubated with a secondary antibody (1:8000, KPL, 5220-0336) at room temperature for 2 hours. Detection of protein bands was performed using a chemiluminescent WB luminol reagent provided by Santa Cruz Biotechnology, USA.

*Mouse xenograft models*

The mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were grown in an SPF clean animal room. The animal experiments were conducted by following the rules of the Animal Care and Use Committee of Cangzhou Disease Prevention

and Control Center, Hebei Province, China. Before the cells were injected, they were given 1 week to acclimate to the growth environment. The mice (BALB/c; 6-8 weeks; Female) were injected with 4T1 cells ($5 \times 10^5$ cells) into the tail vein. BALB/c mice injected with 4T1 cells were divided into two groups: a control group and a GGT (100 mg/kg) treatment group of 5 mice each, which began to be administered 7 days after the cells were injected and continued for 14 days. During this period, the weight of mice was recorded, and the survival period of mice was recorded after drug withdrawal, and the survival curve was drawn. At the experimental endpoint, euthanasia of mice was performed by inhalation of $CO_2$, which involves placing the mice in a sealed container and gradually increasing the concentration of $CO_2$ until the mice exhibit cessation of respiration and heartbeat.

*Hematoxylin and Eosin (H&E) staining*

The lung metastatic nodules were fixed with paraformaldehyde, embedded in paraffin, and sectioned into 5 μm slices. The slices were dewaxed in xylene and hydrated in 100%, 95%, 85%, and 75% ethanol for 5 minutes each. After being washed twice with PBS, the slices underwent hematoxylin and eosin staining (Beyotime). Subsequently, the slices were cleared through 75%, 85%, 95%, and 100% alcohol and xylene, air-dried, and mounted with neutral resin.

*Statistical analysis*

The data were presented as the means ± standard deviation (SD) from at least three independent assays. The analysis was performed using two-tailed paired Student's t-test between control and treated samples. Differences were considered significant when the *p* value was less than 0.05 (*P<0.05, **P<0.01, ***P< 0.001). SPSS 18.0 and GraphPad Prism 10.0 were used to perform statistical analysis.

## Results

*GGT inhibits the proliferation of breast cancer cells in vitro*

Ginkgetin (GGT) is a biflavonoid compound isolated from Ginkgo biloba leaves, and its chemical structure is shown in **Figure 1A**. To investigate its role in the treatment of advanced breast cancer, we first performed the MTT assay. As depicted in **Figure 1B**, GGT exerted moderate anti-proliferative effects on two triple-negative breast cancer cell lines. The IC50 values were 21.09 μM for 4T1 and 29.02 μM for MDA-MB-231, respectively. Furthermore, colony-formation assays verified that GGT significantly and dose-dependently inhibited cellular proliferation (**Figure 1C**). Collectively, these findings confirm that GGT effectively suppresses the growth of breast cancer cells *in vitro*.

*GGT inhibits metastasis and invasion in vitro*

To further explore the effect of GGT on advanced breast cancer cells, wound-healing and transwell invasion assays were carried out. The results showed that, compared with the control group, GGT significantly reduced the migration of breast cancer cells (**Figure 2A** and **2B**). Additionally, the transwell assay demonstrated that GGT remarkably decreased the invasion ability of 4T1 and MDA-MB-231 cells (**Figure 2C** and **2D**).

*GGT attenuates the stemness of breast cancer*

Numerous studies have indicated that cancer stem cells play a crucial role in tumor survival, growth, metastasis, and recurrence [28-30]. To explore whether GGT could reduce the stemness of breast cancer cells, tumorsphere experiments were conducted. The results demonstrated that GGT treatment not only disrupted the tumorspheres of 4T1 (**Figure 3A**) and MDA-MB-231 (**Figure 3B**) but also decreased the number of tumor cell micro-spheres. To further confirm the anti-breast cancer stem cell effects of GGT, qRT-PCR experiments were carried out. The findings revealed that GGT decreased the mRNA expression levels of cancer stem cell (CSC) transcription factors SOX2 and OCT4, as well as CSC markers CD44 and ALDH1 (**Figure 3C** and **3D**). Collectively, these results suggest that GGT may be a potential lead compound against cancer stem cells.

*GGT downregulates the Hedgehog signaling pathway*

To determine the potential mechanism through which GGT inhibits the progression of breast cancer stem cells, RNA-seq analysis was car-

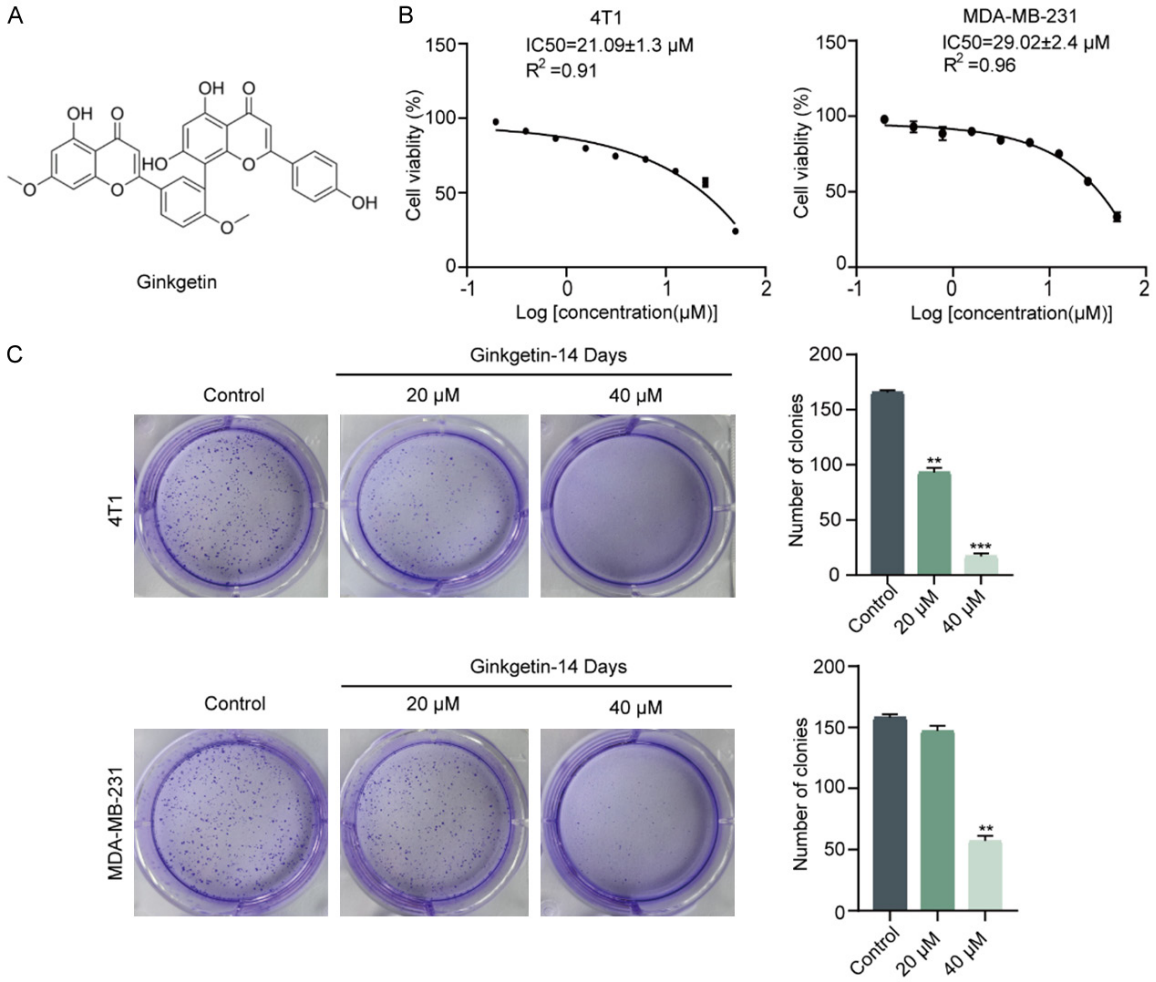


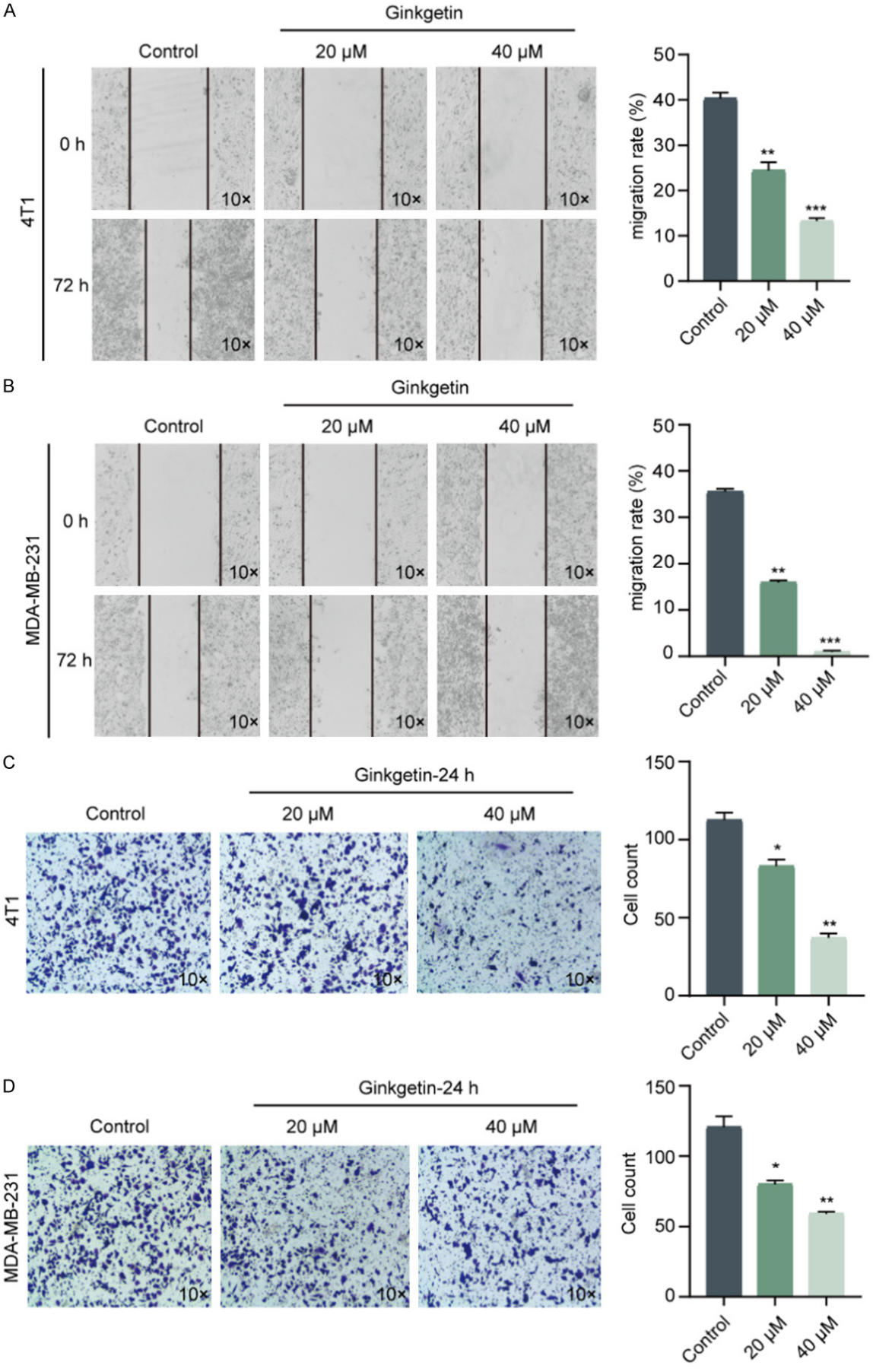

**Figure 1.** GGT suppresses the growth of breast cancer cells in *vitro*. A. Chemical structure of GGT. B. The suppressive impact of GGT on the proliferation of 4T1 and MDA-MB-231 cells. C. Analysis of colony formation in cells treated with varying concentrations of GGT.

ried out on breast cancer cells. The resulting data were subjected to gene set enrichment analysis (GSEA). The results show that GGT treatment is closely related to the Hedgehog (Hh) signaling pathway (**Figure 4A**). The Hh signaling pathway is vital for cell growth and differentiation and is crucial for the development of vertebrate embryos [31, 32]. In breast cancer, the Hh signaling pathway is frequently dysregulated [33, 34]. This abnormal activation can be caused by factors such as overproduction of Hh ligands, dysfunction of Ptch receptors, or activating mutations in Smo proteins [34-37]. These abnormalities lead to the continuous activation of the Hh signaling pathway in breast cancer cells, thus promoting cell growth and metastasis.

Consequently, qRT-PCR and western blot experiments were conducted on breast cancer cells to verify the expression of key proteins in the Hh signaling pathway. The findings revealed that, compared with the control group, the mRNA expressions of SHh, Ptch, SMO, and Gli in the GGT-treated group were significantly down-regulated after GGT treatment (**Figure 4B** and **4C**). Similarly, the protein expressions were also significantly reduced (**Figure 4D** and **4E**). This suggests that GGT may suppress the activity of cancer stem cells (CSCs) by inhibiting the Hh signaling pathway in breast cancer cells.

*GGT suppresses the metastasis of breast cancer cells in vivo*

A tail-vein xenograft model was utilized to perform in-vivo experiments to investigate the effect of GGT on breast cancer metastasis. Mice were injected with 4T1 cells via the tail


A
Ginkgetin
Control
20 μM
40 μM
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0 h
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**Figure 2.** GGT inhibits the metastasis and invasion in *vitro*. Wound healing experiments were conducted on 4T1 (A) and MDA-MB-231 (B) cells in the control group and the GGT treated group. Transwell assay of GGT-treated 4T1 (C) and MDA-MB-231 (D) cells.

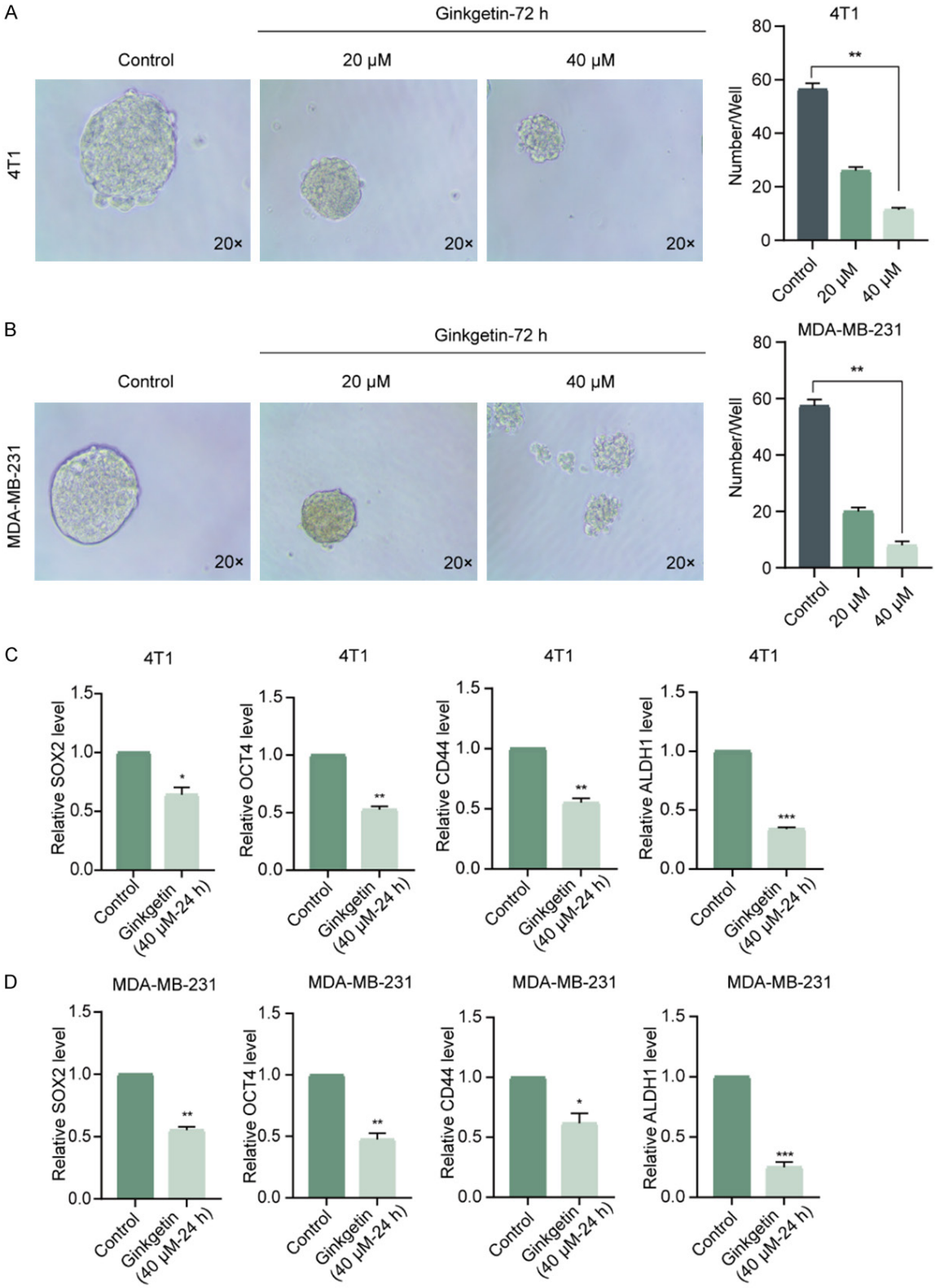

**Figure 3.** GGT attenuates the stemness of breast cancer. A and B. Representative images and statistics of tumorsphere formation in 4T1 and MDA-MB-231 cells exposed to two distinct concentrations of GGT. C and D. qRT-PCR assessment of mRNA levels of transcription factors and biomarkers of CSC after GGT treatment.

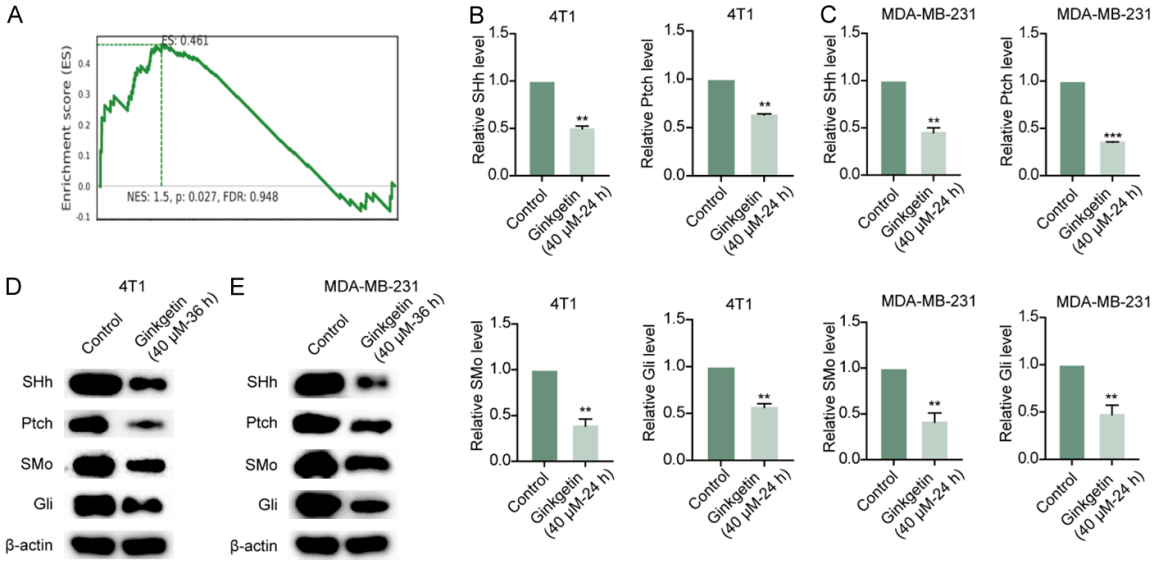


**Figure 4.** GGT downregulates Hedgehog signaling pathway. (A) Enrichment score plots for RNA-seq. The mRNA expression of Hh signaling pathway gene detected by qRT-PCR in 4T1 (B) and MDA-MB-231 (C) cells. The protein expression of the Hh signaling pathway gene in 4T1 (D) and MDA-MB-231 (E) cells was detected by western blot.

vein. Seven days later, they were randomly divided into two groups: the control group and the GGT-treatment group (**Figure 5A**). GGT was administered once daily for two weeks. During this period, the body weight of the mice was not significantly affected by the treatment, indicating that they tolerated GGT well in vivo (**Figure 5B**). The study further demonstrated a remarkable prolongation in the survival time of the mice in the GGT-treatment group compared to the control group (**Figure 5C**). Notably, lung-metastasis images (**Figure 5D**) and HE staining revealed that GGT could clearly reduce the lung metastasis of 4T1 cells in vivo (**Figure 5E** and **5F**).

## Discussion

Breast cancer stem cells (BCSCs) represent a distinct subset of cells within breast cancer, characterized by their self-renewal capacity, multilineage differentiation potential, and high tumorigenicity. These cells play a pivotal role in the initiation, progression, metastasis, and recurrence of breast cancer. As a result, therapeutic strategies targeting BCSCs are considered crucial approaches for enhancing the efficacy of breast cancer treatment [38-40].

GGT, also referred to as ginkgobilobin or ginkgetin flavone, is a biflavonoid compound isolated from Ginkgo biloba leaves. GGT has a significant association with tumors, mainly manifested through its antitumor effects. Multiple publications have reported that GGT inhibits the growth and proliferation of various tumor cell types and induces their apoptosis [23, 41, 42]. Studies have shown that GGT can effectively suppress the growth and proliferation of breast cancer cells and trigger apoptosis in these cells. Additionally, GGT inhibits the migration and invasion abilities of breast cancer cells, potentially impeding breast cancer metastasis [43, 44].

We carried out experiments using 4T1 and MDA-MB-231 cells. MTT and colony-formation assays demonstrated that GGT can inhibit the proliferation of breast cancer cells. Wound-healing and Transwell assays indicated that GGT can suppress the migration and invasion capabilities of breast cancer cells in vitro. Moreover, in-vivo experiments also revealed that GGT can inhibit cellular metastasis, which is consistent with previously reported findings. However, current research on the direct relationship between GGT and BCSCs, along with

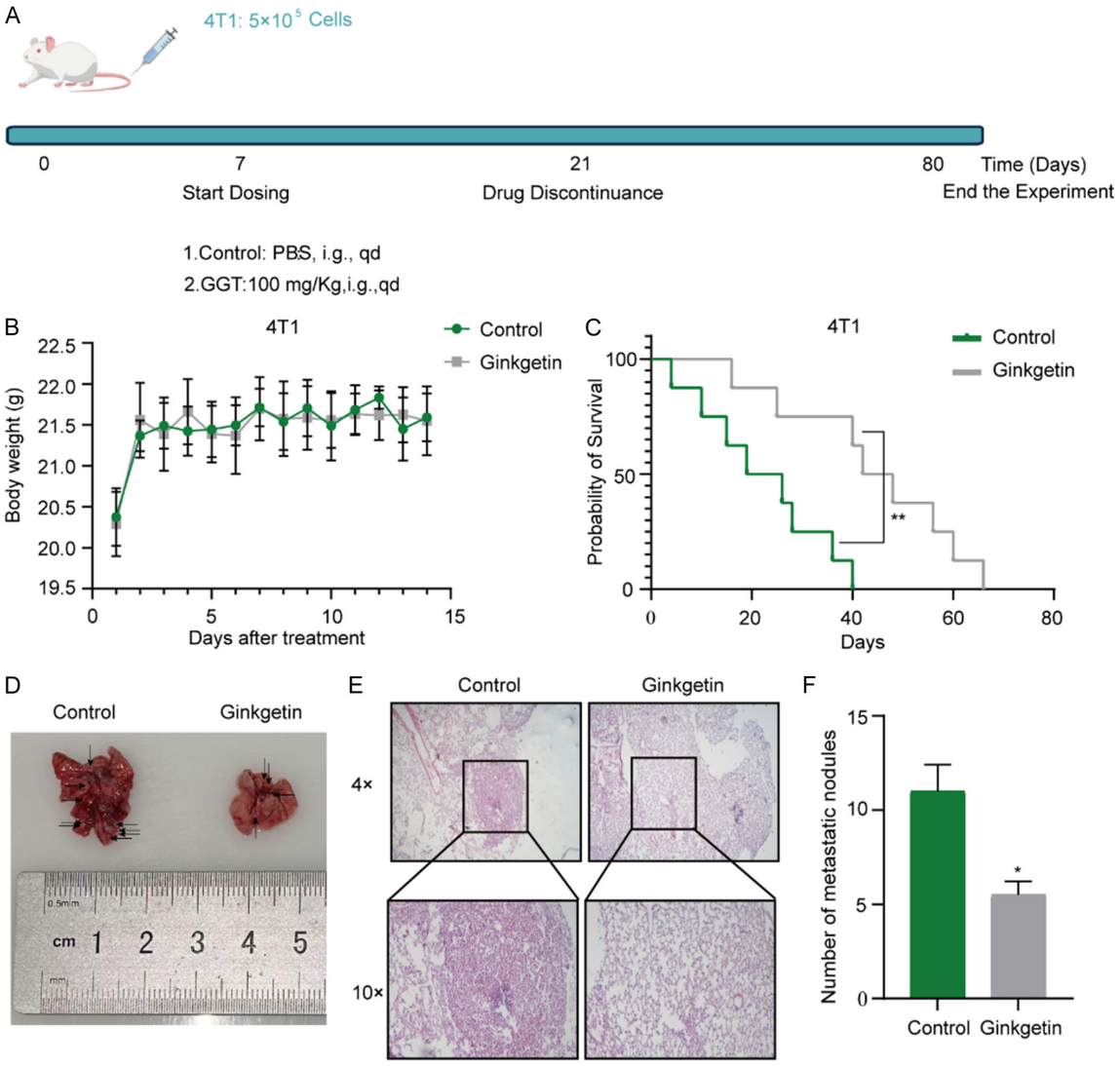


**Figure 5.** GGT suppresses the metastasis of breast cancer cells in *vivo*. (A) Schematic diagram of the mouse tail vein metastasis model. (B) Body weight of mice in control group and GGT administration group (100 mg/kg, n = 5). (C) Survival curve of mice. (D) Representative image of lung metastases, arrows indicate lung metastatic tumors (E) hematoxylin and eosin (H&E) staining of lungs; images were photographed under 4× and 10× objectives. (F) Statistical analysis of the number of lung metastases.

the underlying molecular mechanisms, remains limited.

## Conclusion

In this study, in addition to inhibiting the proliferation and migration of breast cancer cells, GGT has also shown its ability to suppress the stemness of cancer cells and downregulate the transcription factors and markers of cancer stem cells (CSCs). Furthermore, it was discovered that GGT inhibits breast cancer progression via the Hedgehog (Hh) signaling pathway. The results suggest that GGT inhibits breast cancer metastasis by inhibiting the Hedgehog signaling pathway. This research provides initial insights into the potential mechanisms of action of GGT as an anti-breast cancer agent, offering both theoretical and experimental bases for its future application in breast cancer treatment.

## Acknowledgements

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## Disclosure of conflict of interest

None.

## Abbreviations

BCSCs, Breast cancer stem cells; CSC, Cancer stem cell; FBS, Fetal bovine serum; H&E, Hematoxylin and Eosin; PBS, Phosphate-buffered saline; Hh, Hedgehog.

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