

Baicalin suppresses the migration and invasion of breast cancer cells via the TGF- β /lncRNA-MALAT1/miR-200c signaling pathway

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Abstract

Metastasis is the major cause of death and failure of cancer chemotherapy in patients with breast cancer (BC). Activation of TGF- β /lncRNA-MALAT1/miR-200c has been reported to play an essential role during the metastasis of BC cells. The present study aimed to validate the suppression of BC-cell migration and invasion by baicalin and explore its regulatory effects on the TGF- β /lncRNA-MALAT1/miR-200c signaling pathway. We found that baicalin treatment inhibited cell viability and migration and invasion. Mechanistically, baicalin treatment significantly downregulated the expression of TGF- β , ZEB1, and N-cadherin and upregulated E-cadherin on both mRNA and protein levels. Additionally, baicalin treatment significantly downregulated the expression of lncRNA-MALAT1 and upregulated that of miR-200c. Collectively, baicalin significantly suppresses cell viability, migration, and invasion of BC cells possibly by regulating the TGF- β /lncRNA-MALAT1/miR-200c pathway.

Abbreviations: BC = breast cancer, EMT = epithelial–mesenchymal transition, lncRNAs = long noncoding RNAs.

Keywords: baicalin, breast cancer, invasion, migration, TGF- β /lncRNA-MALAT1/miR-200c

1. Introduction

Breast cancer (BC) is one of the most common cancers with high morbidity and mortality worldwide.^[1] Although multiple strategies, including surgical resection, radiotherapy, endocrine therapy, chemotherapy, and immunotherapy, have been used in the clinical treatment of BC, patients' mortality remains high.^[2–3] BC has a tendency to target the bones, brain, liver, and lungs (organ tropism).^[4] For patients with BC with metastases, 30% to 60% have lesions in the bones, 4% to 10% in the brain, 15% to 32% in the liver, and 21% to 32% in the lungs.^[5] Moreover, >90% of the deaths caused by BC are attributed to metastasis-related complications.^[6]

The migration and invasion of cancer cells into surrounding tissues is an important initial step in cancer metastasis. Metastasis is the leading cause of cancer-related death, and thus it is crucial to inhibit metastasis.^[7–9] As a multifunctional cytokine belonging to the transforming growth factor superfamily, transforming growth factor-beta (TGF- β) is involved in regulating several processes, including cellular proliferation, differentiation, apoptosis, migration, and invasion. In tumor development, the TGF- β signaling pathway can promote

epithelial–mesenchymal transition (EMT), which facilitates tumor cell invasion and metastasis.^[10,11]

TGF- β induces EMT by promoting the expression of a group of transcription factors, including ZEB1, which further represses epithelial gene expression (including E-cadherin)^[12,13] while increasing mesenchymal gene expression (including N-cadherin).^[14] Recently, studies revealed that TGF- β stimulation significantly downregulated the expression of miR-200 members, including miR-200c, which is involved in EMT by targeting ZEB1.^[15–17] Moreover, long noncoding RNAs (lncRNAs) have been demonstrated to be implicated in many important cellular processes, such as cell proliferation, migration, invasion, and apoptosis.^[18]

lncRNAs have been reported to act as important biomarkers for the diagnosis, prognosis, and therapy of BC. Increasing evidence suggests that lncRNA-MALAT1 plays an important role in the occurrence and development of tumors, including BC.^[19] More importantly, MALAT1 regulates TGF- β -induced EMT by directly regulating ZEB1.^[20] Moreover, MALAT1 is involved in the regulation of cell migration and invasion by negatively regulating miR-200c expression.^[21] These studies demonstrate that MALAT1 may be a potential target for

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All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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the treatment of BC and that the suppression of the TGF- β /lncRNA-MALAT1/miR-200c pathway might present a novel therapeutic strategy for BC.

Baicalin is a benzylisoquinoline alkaloid from the Chinese herbal medicine *Scutellaria lateriflora* Georgi,^[22] which has been demonstrated to play antiproliferative, anti-inflammatory, and antimetastatic roles in human cancers.^[23] Its anti-cancer properties are mainly attributed to its antiproliferative potential^[24] and its ability to inhibit the mobility of cancer cells.^[25] The prominent antitumor activity of baicalin has been proven in various cancer types, such as colon,^[26] liver,^[27] and bladder cancers^[28] and glioblastoma.^[29] Several studies have also evaluated the cytotoxic effects of baicalin against BC cells either in vitro or in vivo.^[30–32] Related studies have found that baicalin inhibits TGF- β 1-mediated EMT in breast epithelial cells and suppresses the tumorigenesis of BC cells.^[33] Additionally, baicalin inhibited the TGF- β 1-induced increase in cell migration, invasion, and anoikis resistance in TGF- β 1-induced U2OS cells.^[30] However, the regulatory effects of baicalin on BC metastasis from the perspective of lncRNA and miRNA should be explored further. Therefore, the present study aimed to explore the underlying mechanisms by which baicalin suppresses cell migration and invasion via the TGF- β /lncRNA-MALAT1/miR-200c pathway.

2. Material and Methods

2.1. Materials and reagents

Fetal bovine serum, trypsin-EDTA (0.25%), and Pierce TM BCA Protein Assay kits were purchased from Thermo Fisher Scientific (Waltham, MA). Cell Counting Kit-8 was purchased from Abbkine (Wuhan, Hubei, China). Antibodies against E-cadherin, N-cadherin, and TGF- β were purchased from CST (Danvers, MA). Anti-ZEB1 antibodies were purchased from SAB (College Park, MA). Transwell assay kits with and without matrigel were purchased from Becton Dickinson (BD, Franklin Lakes, NJ) and Corning (Lowell, MA). Hsa-miR-200c and U6 qPCR primers were purchased from Takara Biotechnology Co., Ltd (Dalian, Liaoning, China).

2.2. Preparations of baicalin

Baicalin was obtained and authenticated by Sigma-Aldrich (Louis, MO). Stock solutions of baicalin were prepared by dissolving baicalin powder in DMSO to a concentration of 20 mM. Baicalin was diluted using RPMI-1640 culture medium.

2.3. Cell culture

Human BC MDA-MB-231 cells were obtained from the Cell Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Hyclone, Logan, UT, USA). All cells were maintained in a humidified incubator at 37°C and 5% CO₂.

2.4. Cell viability

Cell viability was measured using Cell Counting Kit-8. MDA-MB-231 cells were seeded into 96-well plates at a density of 8000 cells per well overnight and then exposed to different concentrations of baicalin (0, 12.5, 25, or 50 μ M) for 24, 48, or 72 hours. At the end of the treatment, 10 μ l of Cell Counting Kit-8 solution was added into each well, followed by incubation at 37°C for 2 hours. The absorbance was measured at 450 nm using a microplate reader.

2.5. Migration and invasion assays

For the migration and invasion assays, 24-well Transwell chambers with (BD) or without matrigel (Corning) were used. Cells treated or untreated with baicalin were reseeded into the up-chamber of Transwell at a density of 1×10^4 cells/well for the migration assay or 5×10^4 cells/well for the invasion assay for 16 hours. At the end of the experiment, migrated or invaded cells were stained with 0.01% crystal violet at room temperature for 15 minutes. The stained cells in three random fields were counted under an inverted microscope (Leica, Heidelberg, Germany).

2.6. Real-time polymerase chain reaction analysis

To determine the expression of related genes or lncRNA-MALAT1 at the RNA level, total RNA was extracted from cultured cells using TRIzol (Takara, Dalian, Liaoning, China) and converted to cDNA by reverse transcriptase polymerase chain reaction (PCR) (Takara). Quantitative PCR (qPCR) analysis of mRNA expression was conducted using 2 μ l of cDNA. Fast SYBR Green Master Mix (Takara) was used to detect the expression of related genes at the mRNA level under the following conditions: initial denaturation at 95°C for 20 seconds, followed by 40 cycles of 15 s at 95°C and 30 sec at 60°C. Relative quantification was performed according to the comparative Ct method with normalization to GAPDH or U6. The primer sequences are listed in Table 1.

The RNA levels of miR-200c were determined by qPCR. The PCR reaction included the following constituents: SYBR premix Ex Taq II (10 μ l), PCR forward primer (10 μ M; 0.8 μ l), Uni-miR qPCR primer (10 μ M; 0.8 μ l), ROX reference dye II (50X; 0.4 μ l), cDNA (2 μ l), and dH₂O (6 μ l). Initial denaturation was performed at 95°C for 30 seconds, followed by 40 cycles at 95°C for 3 seconds, and then annealing at 60°C for 30 seconds.

2.7. Western blot analysis

Total proteins were extracted from cells using a lysis buffer (Beyotime Biotechnology, Nanjing, Jiangsu, China) supplemented with protease inhibitor PMSF (Amresco, Solon, Ohio, USA). The cells were treated with the lysis buffer for 30 minutes on ice, followed by centrifugation at 14,000 g for 20 min at 4°C. The supernatants were collected, and then the protein concentration was measured with a BCA protein assays kit (Thermo Fisher Scientific). An equal amount of protein (50 μ g) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) and then transferred onto polyvinylidene fluoride membranes. The blots were blocked with blocking buffer (Thermo Fisher Scientific) for 2 hours at room temperature and then were incubated with primary antibodies against E-cadherin, N-cadherin, TGF- β , or ZEB1 (dilution: 1:1000 for all) overnight

Table 1

Sequences of primers for each gene and lncRNA-MALAT1.

Name	Primers (5'-3')
TGF-beta	F: GACTTCAGCCTGGACAACGAGR: GGTGAGGAAATGGCTATCTTGC
E-cadherin	F:5'-CGAGAGCTACACGTTACCGG-3'R:5'- GGGTGTCGAGGGAAAAATAGG-3'
N-cadherin	F:5'-AAGAAGCCAGGCCAACAC-3'R:5'- CTGGCTCAAGTCATAGTCTGGTCT-3'
ZEB1	F:5'-TTACACCTTTGCATACAGAACCC-3'R:5'- TTTACGATTACACCCAGACTGC-3'
GAPDH	F:5'-ACAACCTTTGGTATCGTGAAGG-3'R:5'- GCCATCAGCCACAGTTC-3'
lncRNA-MALAT1	F: 5'-AAAGCAAGGTTCTCCCCACAAG-3'R: 5'-GGTCTGTGCTAGATCAAAGGCA-3'

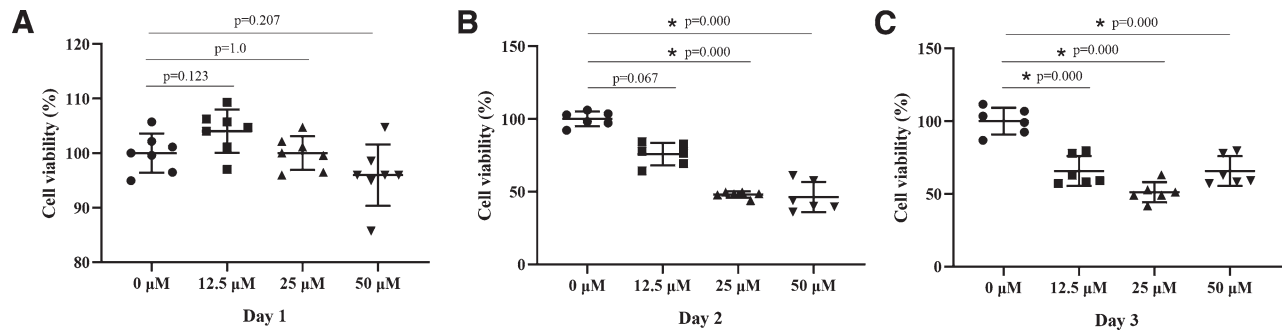


Figure 1. Effect of baicalin treatment on the viability of MDA-MB-231 cells. MDA-MB-231 cells were treated with 0, 12.5, 25, or 50 μM of baicalin for (A) 24 h, (B) 48 h, or (C) 72 h. The cell viability was determined using the Cell Counting Kit-8 assay. The cell viability of untreated cells was set as 100%. Results are presented as mean ± standard deviation, **P* < .05 vs untreated cells.

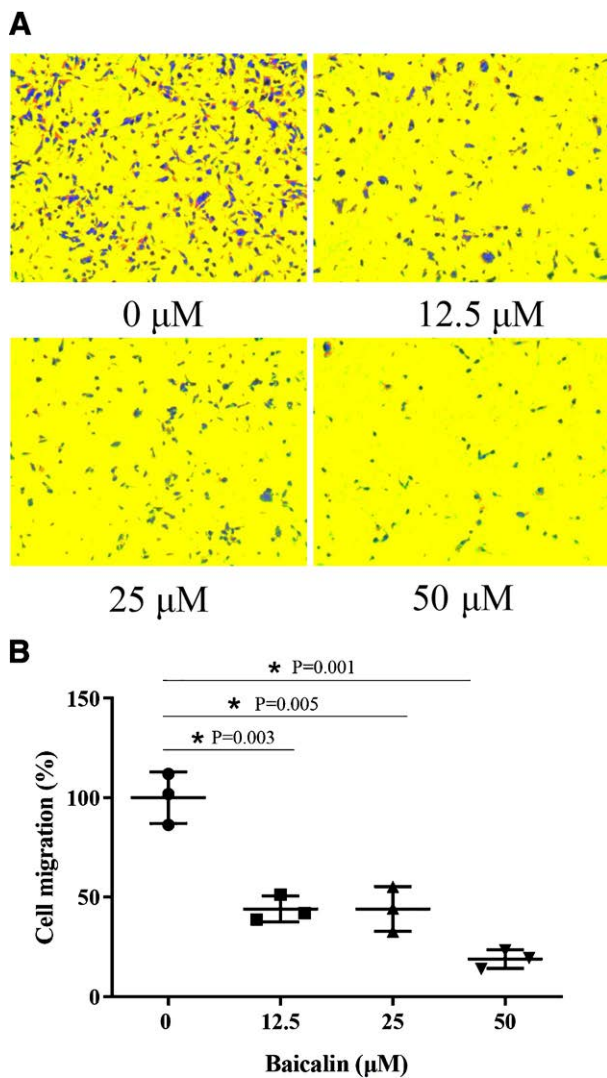


Figure 2. Effect of baicalin treatment on the migration of MDA-MB-231 cells. MDA-MB-231 cells were treated with 0, 12.5, 25, or 50 μM of baicalin for 24 h. Cells were suspended and reseeded into a Transwell chamber for 16 h. The migrated cells were stained with 0.01% crystal violet. (A) The cells were observed with an inverted microscope (× 100 magnifications) and images of the same were captured. (B) The numbers of migrated cells were counted. The cell viability of untreated cells was set as 100%. Results are presented as mean ± standard deviation, **P* < .05 vs untreated cells.

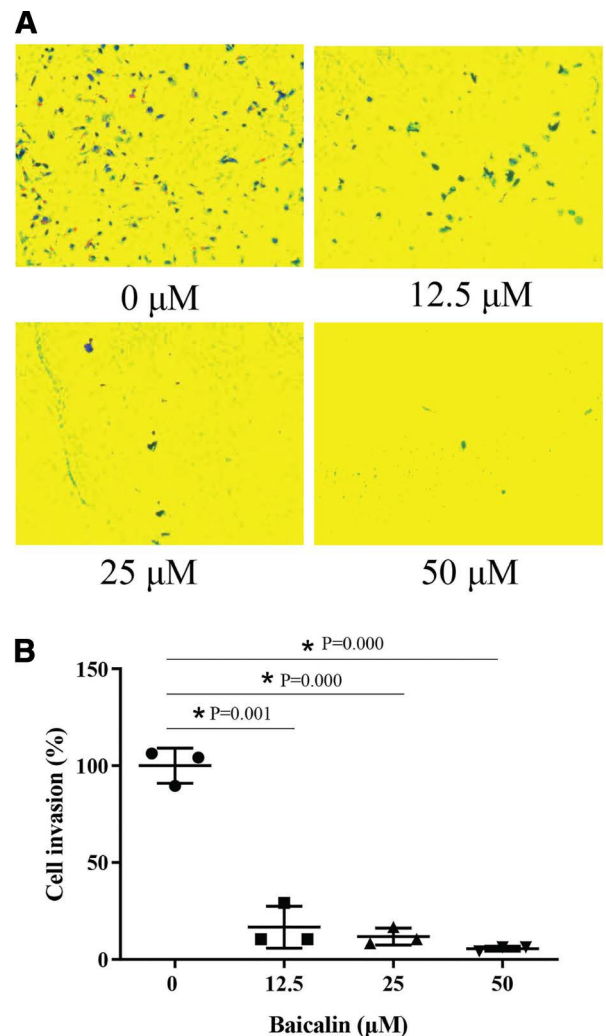


Figure 3. Effect of baicalin treatment on the invasion of MDA-MB231 cells. MDA-MB-231 cells were treated with 0, 12.5, 25, or 50 μM of baicalin for 24 h. Cells were suspended and reseeded into a Transwell chamber precoated with matrigel for 16 h. The invaded cells were stained with 0.01% crystal violet. (A) The cells were observed with an inverted microscope (× 100 magnifications) and images of the same were captured. (B) The numbers of invaded cells were counted. The cell viability of untreated cells was set as 100%. Results are presented as mean ± standard deviation, **P* < .05 vs untreated cells.

at 4°C. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (dilution: 1:5000). Protein bands were detected with a chemiluminescence kit (Thermo Fisher Scientific) and analyzed using the ImageLab software. GAPDH was used as a loading control.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 26.0 software. Data are presented as mean and standard deviation. Differences among three or more groups were assessed using one-way ANOVA. $P < .05$ was considered significant.

2.9. Ethics approval statement

Because the experiment does not involve animals and clinical research, this study does not need to be approved by moral and ethical clerks.

3. Results

3.1. Baicalin reduces the viability of MDA-MB-231 cells

To investigate the effects of baicalin on cell viability, we determined the cell viability of MDA-MB-231 cells after baicalin treatment at the indicated time points. As shown in Figure 1A, treatment with 12.5, 25, or 50 μM baicalin for 24 hours did not affect the cell viability of MDA-MB-231 cells ($P > .05$; compared with untreated cells). However, the cell viability was significantly decreased after 48 or 72 hours in cells treated with baicalin (Fig. 1B and 1C; $P < .05$; compared with untreated cells). Therefore, we chose 24 hours of baicalin treatment to

further investigated the effect of baicalin on the migration or invasion of MDA-MB-231 cells.

3.2. Baicalin inhibits cell migration in MDA-MB-231 cells

To assess the migration of BC cells after baicalin treatment, we performed the Transwell assays. The number of migrated cells for different concentration of baicalin treatment shown significantly reduction (Fig. 2; $P < .05$; compared to untreated cells), which revealed that baicalin plays a crucial role in suppressing BC cell migration.

3.3. Baicalin inhibits cell invasion in MDA-MB-231 cells

We further determined the effects of baicalin on cell invasion in MDA-MB-231 cells. The results showed that baicalin treatment significantly suppressed the invasion of MDA-MB-231 cells (Fig. 3; $P < .05$). Moreover, treatment with 25 and 50 μM baicalin blocked approximately 80% to 90% of invasion, demonstrating the potential of baicalin as a strong suppressor of BC cell invasion.

3.4. Baicalin inhibits the activation of the TGF- β /ZEB1 pathway in MDA-MB-231 cells

To further explore the underlying mechanism of baicalin on reducing cell migration and invasion, we further determination the activation of TGF- β /ZEB1 pathway. By performing qPCR analysis, we detected the expression of TGF- β and ZEB1 as well as their downstream regulators at the mRNA level. As shown in Figure 4, baicalin treatment downregulated TGF- β 1, ZEB1 and N-cadherin expression, but upregulated E-cadherin

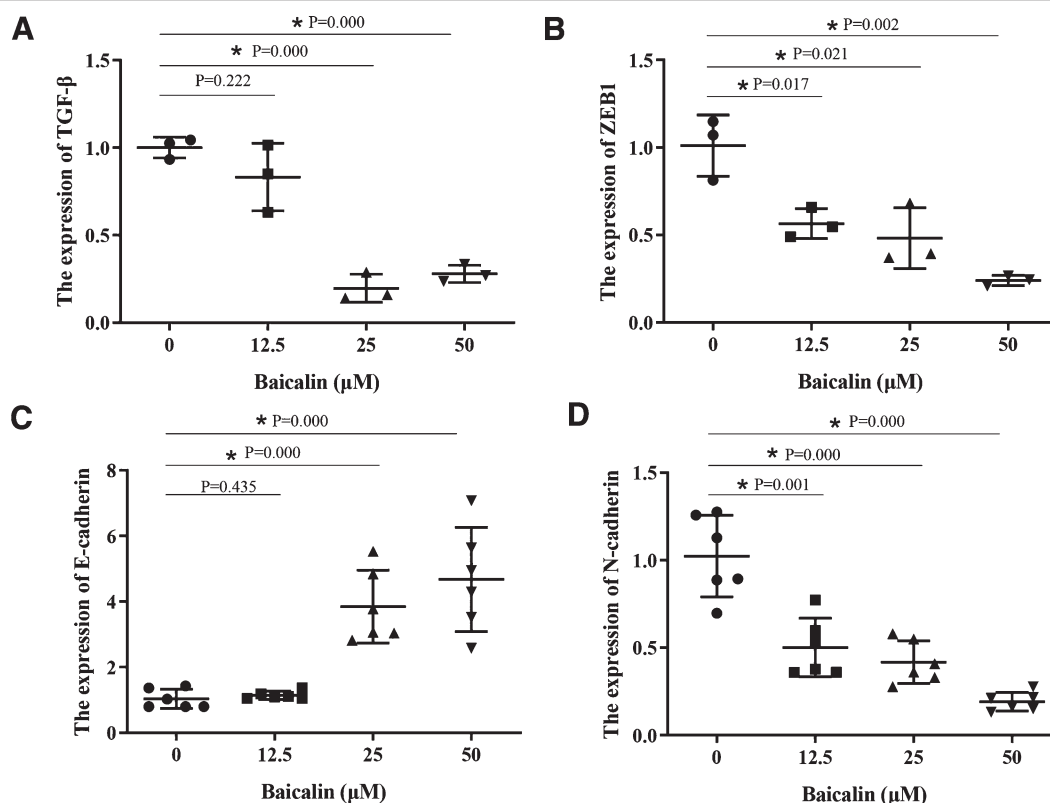


Figure 4. Effect of baicalin treatment on the relative expression of genes involved in the TGF- β pathway in MDA-MB-231 cells evaluated by qPCR. MDA-MB-231 cells were treated with the indicated concentrations of baicalin for 24 h. The mRNA-level expression of (A) TGF- β , (B) ZEB1, (C) E-cadherin, and (D) N-cadherin was determined by qPCR analysis. GAPDH was used as an internal control. The expression of those genes in untreated cells was set as 1. Results are presented as mean \pm standard deviation, * $P < .05$ vs untreated cells.

expression at the mRNA level ($P < .05$; compared with untreated cells). Consistently, western blot analysis revealed that baicalin treatment downregulated the expression of TGF- β 1, ZEB1, and N-cadherin, but upregulated the expression of E-cadherin at the protein level (Fig. 5; $P < .05$; compared with untreated cells).

3.5. Baicalin downregulates lncRNA-MALAT1 and upregulates miR-200c expression in MDA-MB-231 cells

Due to the essential role of lncRNA-MALAT1 in TGF- β 1-mediated tumor metastasis, we further determined the expression of lncRNA-MALAT1 by qPCR analysis. As shown in Fig. 6A, the expression of lncRNA-MALAT1 was significantly downregulated after baicalin treatment ($P < .05$; compared with untreated cells). As an intermediary of lncRNA-MALAT1 and

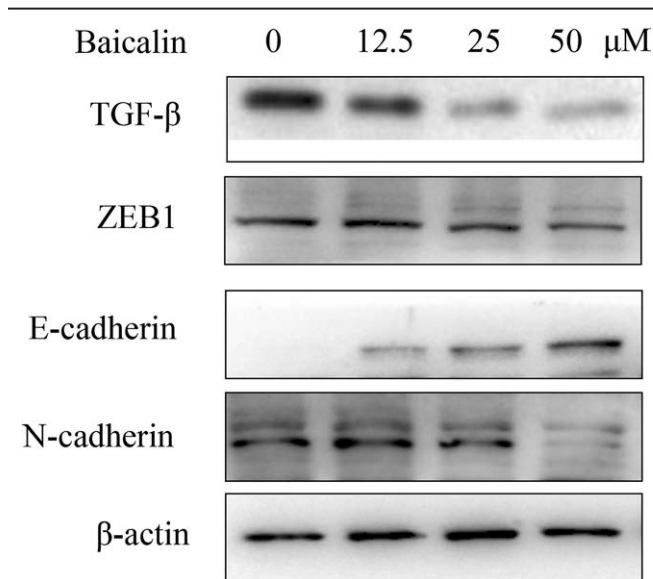


Figure 5. Effects of baicalin treatment on the relative expression of genes involved in the TGF- β pathway in MDA-MB-231 cells evaluated by western blot analysis. MDA-MB-231 cells were treated with the indicated concentrations of baicalin for 24h. The protein-level expression of TGF- β , ZEB1, E-cadherin, and N-cadherin was determined by western blot analysis. β -actin was used as the internal control. Images are representative of three independent experiments.

ZEB1, miR-200c expression was determined by qPCR analysis. As shown in Fig. 6B, miR-200c expression was significantly upregulated after baicalin treatment ($P < .05$; compared with untreated cells).

4. Discussion

Proliferation and metastasis are two major challenges in cancer treatment. The majority of deaths from BC have been known to be due to metastasis to distant organs.^[34] Although emerging findings indicate that the microenvironment in the host organs plays an important role in survival, seeding, and tumor regeneration, the metastatic cancer cells are still the driving force of metastasis.^[35,36] Early studies demonstrated that baicalin could induce apoptosis in human BC MCF-7 cells.^[37] Its anticancer properties are mainly attributed to its ability to inhibit the mobility of cancer cells.^[25] Our present study also showed that baicalin treatment significantly suppressed the proliferation, migration, and invasion of MDA-MB-231 cells. More importantly, our study also demonstrated that baicalin treatment significantly suppresses the activation of the TGF- β /lncRNA-MALAT1/miR200c signaling pathway.

EMT is a biological process in which epithelial cells lose their polarity and cell-cell adhesion and acquire migratory and invasive properties of mesenchymal cells.^[38-40] Furthermore, the epithelial marker E-cadherin and the mesenchymal marker N-cadherin are regarded as important markers of EMT^[41-43] and are widely used in cancer invasion and metastasis research. Using western blot analysis, we found that baicalin treatment reduced the protein-level expression of the mesenchymal marker N-cadherin but increased that of the epithelial marker E-cadherin, indicating that the antimetastasis activity of baicalin was associated with its inhibitory effect on EMT. miRNAs are a class of noncoding RNA molecules with 18–25 nucleotides and have been reported to act as important biomarkers for the diagnosis, prognosis, and therapy of BC at early stages.^[44] Studies have reported that miRNAs can regulate the TGF- β signaling pathway, which in turn inhibits cancer metastasis.^[45] Increasing evidence suggests that miR-200c functions as a tumor suppressor by inhibiting EMT via the downregulation of ZEB1 and ZEB2 expression in cancer cells.^[16,40,46,47] Western blot and qPCR analyses revealed that baicalin treatment suppressed the expression of TGF- β , ZEB1, and ZEB2, but upregulated the expression of miR-200c.

lncRNAs, which are noncoding RNA molecules with a length of >200 nucleotides, regulate physiologic functions of organisms from the epigenetic, transcriptional, and

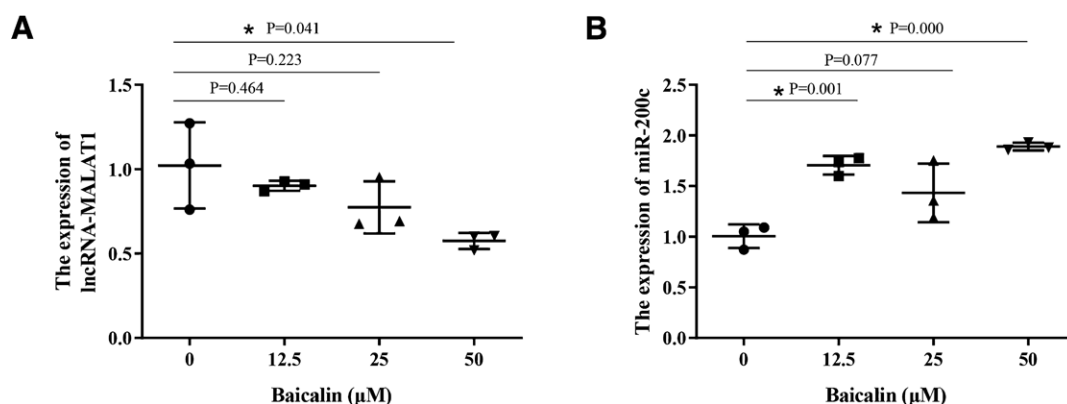


Figure 6. Effect of baicalin treatment on the expression of lncRNA-MALAT1 and miR-200c in MDA-MB-231 cells. MDA-MB-231 cells were treated with the indicated concentrations of baicalin for 24 h. (A) The expression of lncRNA-MALAT1 was determined by qPCR analysis. GAPDH was used as an internal control. The expression of lncRNA-MALAT1 in untreated cells was set as 1. Results are presented as mean \pm standard deviation, * $P < .05$ vs untreated cells. (B) The expression of miR-200c was determined by qPCR analysis. U6 was used as an internal control. The expression of miR-200c in untreated cells was set as 1. Results are presented as mean \pm standard deviation, * $P < .05$ vs untreated cells.

post-transcriptional perspectives.^[448] LncRNA-MALAT1 is a highly conserved lncRNA that is highly expressed in several types of cancers, including BC.^[449] It is particularly noteworthy that a recent study using genetic interventions with MALAT1 antisense nucleotides reported promising effects for suppressing cancer development in mouse models with luminal B BC.^[50] Data from qPCR analyses indicated that the suppression of lncRNA-MALAT1 expression might be a mechanism by which baicalin exerts its inhibitory effect on the migration and invasion of BC cells. However, it remains unknown whether baicalin can inhibit BC in vivo. This issue could be addressed in future studies to fully elucidate the mechanism by which baicalin is involved in cancer treatment.

5. Conclusion

In conclusion, we report that baicalin can inhibit the migration and invasion of human BC cells and suppress the TGF- β /lncRNA-MALAT1/miR200c signaling pathway, which might be one of the mechanisms of its anticancer function.

Author contributions

Conceptualization: Guolong Chen, Huajiao Chen, Jiafeng Li.
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 Writing – original draft: Guolong Chen, Hongjuan Liao, Huajiao Chen, Huajiao Chen, Jiafeng Li.
 Writing – review & editing: Guolong Chen, Huajiao Chen, Jiafeng Li, Liya Liu.

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