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Ezetimibe suppresses the migration and invasion of triple-negative breast cancer cells by targeting TGFβ2 and EMT

Qinyu He
National Institute of Healthcare Data Science at Nanjing University, Jiangsu Key Laboratory of Molecular Medicine, Medical School, Nanjing University
Ding Ma
National Institute of Healthcare Data Science at Nanjing University, Jiangsu Key Laboratory of Molecular Medicine, Medical School, Nanjing University
konglingkai Kong
National Institute of Healthcare Data Science at Nanjing University, Jiangsu Key Laboratory of Molecular Medicine, Medical School, Nanjing University
Weiwei Shi
National Institute of Healthcare Data Science at Nanjing University, Jiangsu Key Laboratory of Molecular Medicine, Medical School, Nanjing University
Chunping Jiang
National Institute of Healthcare Data Science at Nanjing University, Jiangsu Key Laboratory of Molecular Medicine, Medical School, Nanjing University
Junhua Wu (wujunhua@nju.edu.cn)
National Institute of Healthcare Data Science at Nanjing University, Jiangsu Key Laboratory of Molecular Medicine, Medical School, Nanjing University
Qilei Xin
Jinan Microecological Biomedicine Shandong Laboratory, Shounuo City Light West Block, Qingdao Road 3716#, Huaiyin District, Jinan City, Shandong Province, China.

Article

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Abstract

In recent years, the important role of cholesterol in tumor metastasis has been revealed. Ezetimibe is the only selective cholesterol uptake inhibitor currently on the market. Here, we aimed to explore the effect of ezetimibe on breast cancer metastasis. The effects of ezetimibe on breast cancer cell migration, invasion, and EMT were examined by wound healing assay, invasion assay, and western blotting assay. After ezetimibe treatment of breast cancer cells, differentially expressed genes were investigated by transcriptome sequencing and validated by western blotting assay and qRT-PCR. Breast cancer cells overexpressing TGFβ2 were constructed, and the effect of TGFβ2 on the migration and invasion of ezetimibe-treated breast cancer cells was examined by wound healing and invasion assays. The study showed that ezetimibe inhibited the migration, invasion, and EMT of breast cancer cells and significantly suppressed the expression of TGFβ2 in breast cancer cells. Enhanced TGFβ2 expression reversed the inhibitory effect of ezetimibe on the migration and invasion of breast cancer cells. This study demonstrates that ezetimibe is a potential drug for the treatment of breast cancer metastasis.

Introduction

Cancer ranks second in mortality among chronic noncommunicable diseases worldwide [1]. In 2020, breast cancer had the highest mortality rate among female malignancies [2]. Current common treatments for breast cancer include surgical resection, radiotherapy, and chemotherapy [3–6]. However, current chemotherapeutics for breast cancer often cause drug toxicity, such as gastrointestinal adverse effects and liver and kidney function damage, while killing tumor cells [7], which brings great difficulties for clinical treatment. Therefore, it is urgent to develop safe and effective drugs for breast cancer.

Tumor metastasis is one of the major causes of clinical treatment failure and recurrence in breast cancer [8,9]. Studies have shown that cholesterol can promote tumor metastasis [10,11]. Cholesterol, a key component of cell membranes, is closely related to tumor progression [12–14]. Various studies have shown that high cholesterol can promote the growth and metastasis of lung adenocarcinoma and breast cancer [15–19]. In addition, high cholesterol levels are associated with breast cancer recurrence [8,20]. These results suggest that cholesterol promotes the metastasis of breast cancer.

Currently, drugs that interfere with cholesterol metabolism through different mechanisms have been used in antitumor research [21]. Statins, the most common class of drugs that intervene in cholesterol synthesis, act by inhibiting HMG CoA reductase, the rate-limiting enzyme of the mevalonate pathway [22]. Statins affect cancer cell plasticity, increasing metastatic seeding but reducing tumor formation and metastatic growth [23]. Studies have shown that breast cancer patients using lipophilic statins have significantly reduced rates of recurrence [24] and mortality [25]. These results illustrate that cholesterol-lowering drugs can inhibit not only breast cancer growth but also breast cancer metastasis.

Ezetimibe, the only selective cholesterol absorption inhibitor currently on the market, can block the exogenous absorption pathway of cholesterol [26], and its cancer-suppressing effects have been reported. In vivo experiments demonstrated that ezetimibe inhibited tumor growth in prostate cancer-bearing mice fed a high-cholesterol diet by reducing angiogenesis [27]. Kristine Pelton et al. found that in the breast cancer model of SCID mice, ezetimibe can improve the tumor microenvironment of breast cancer by inhibiting angiogenesis, thereby inhibiting the development of tumors [28]. These studies demonstrated in different tumor models that ezetimibe inhibits tumor growth mainly by reducing angiogenesis. However, the effect of ezetimibe on tumor metastasis was not investigated.

As described earlier, cholesterol promotes breast cancer metastasis, and cholesterol-lowering drugs, such as statins, can inhibit breast cancer metastasis and recurrence. Therefore, we were curious whether ezetimibe, also a cholesterol-lowering drug, would have similar effects. In this study, we will observe whether ezetimibe can inhibit the migration and invasion of breast cancer cells and explore the possible mechanisms.

Materials and Methods

Cell Culture. MDA-MB-231 (human, female) cells were maintained in DMEM (WISENT, catalog # 319-005-CL) with 10% fetal bovine serum (FBS) (WISENT, catalog # 086150035) and penicillin (WISENT, catalog # 450-201-EL): streptomycin solution (Gibco, catalog # 15140163). 4T1 (mouse) cells were maintained in RPMI 1640 (WISENT, catalog # 350-000-CL) with 10% FBS and penicillin: streptomycin solution. All cells were cultured at 37°C in a humid incubator with 5% CO^2.

Preparation of ezetimibe. The preparation of ezetimibe used and working concentrations was as follows: ezetimibe (MedChemExpress, catalog #HY-17376) was added to the sterile tube and dissolved in DMSO before being stored at 4°C for long-term use.

Cytotoxicity assay. Cells were first treated with different concentrations of ezetimibe for 48 h. Then, 10 μL of CCK8 solution was added, and the cells were incubated for 1 h at 37°C. The absorbance of each well was measured at OD450 nm using a multiwell plate reader.

Wound Healing Assay. Wounding healing assays were performed in 24-well culture plates with the culture insert (Ibidi, catalog #80206). A total of 4×10^4 cells were seeded in every culture insert for 24 h. After pulling out the insert, the cells were washed with PBS twice and cultivated with serum-free medium containing different concentrations of ezetimibe at 37°C. The cells were photographed in four random fields by microscopy every 24 h.

Transwell Assays. Transwell assays were performed in 24-well culture plates with a Transwell chamber (Millipore, catalog #PIHP01250), and the upper compartment of the chamber was precoated with Matrigel (Corning, catalog #354230). A total of 8×10^4 cells were suspended in 300 μL serum-free medium and plated in the upper compartment when 1 mL medium with 10% FBS was added to the lower

compartment, and then the cells were treated with different concentrations of ezetimibe. After the cells were allowed to invade at 37°C for 24 h, the Transwell membranes were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet at room temperature for 1 h. Cells invading the membranes were calculated in four random fields under the microscope.

Lentivirus overexpression. Cells were inoculated overnight in 12-well plates at a density of 5×10^4 cells per well until cell confluency was 30%. According to the complex of infection (MOI) of the cells, a purified high-titer lentiviral suspension (GENEChem) was prepared with the appropriate virus dilutions, and the cells were subsequently infected for 24 hours (MOI = 10 for the two cell lines utilized) with virus dilutions. After changing the culture medium, the cell lines stably overexpressing TGFβ2 were screened by adding 2 μM puromycin. The protein expression level of TGFβ2 was detected by Western blotting.

Quantitative RT-PCR. Cells were inoculated overnight in 12-well plates at a density of 2 × 105 cells per well and cultured for 48 h. Total RNA was extracted using TRIzol reagent (Life Technologies, 15596-018), and reverse transcription was performed using the Transcriptase cDNA Synthesis Kit (Vazyme, R323) according to the manufacturer's instructions. Real-time PCR analysis was performed in an Applied Biosystems 7500 Real-Time PCR System using ChamQ SYBR qPCR Master Mix (Vazyme, Q341) according to the manufacturer's instructions. The primers (F, TGTCCCTGCTGCACTTTTGTA, and R, GGTGCCATCAATACCTGCAAATC) were synthesized to detect TGFB2 mRNA expression by GenScript.

Western blotting analysis. Cells were lysed in NP40 buffer on ice, and protein was extracted. The concentration of total protein was measured by the BCA method. Equal amounts of harvested total proteins were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes. Then, the membranes were blocked at room temperature for 2 h and incubated with primary antibodies at 4°C overnight, followed by HRP-conjugated secondary antibodies at room temperature for 2 h. After washing three times in TBST, protein bands were observed using an ECL chemiluminescence system (Bio-Rad). Primary antibodies against N-Cadherin(CST, 13116, 1:1000), E-Cadherin(CST,3195, 1:1000), Vimentin (CST, 5741, 1:1000), Snail(CST,3879, 1:1000), GAPDH(Biogot technology, MB001, 1:1000), TGFβ2(Abcam, ab167655, 1:1000).

Statistical analysis. The statistics were analyzed by GraphPad Prism 8.0. The variations between the two groups were compared using the independent t-test, and a statistically significant difference was defined as $P < 0.05$.

Results

Ezetimibe inhibits the migration of MDA-MB-231 and 4T1 cells.

To determine the safe concentration of ezetimibe on MDA-MB-231 and 4T1 cells and evaluate its effect on the migration and invasion of MDA-MB-231 and 4T1 cells, we tested the effect of ezetimibe on the viability of MDA-MB-231 and 4T1 cells through CCK8 experiments. The viability plots of MDA-MB-231 and 4T1 cells after treatment with different concentrations of ezetimibe for 48 hours are shown in

(Fig. 1a).The IC_{50} of ezetimibe on MDA-MB-231 and 4T1 cells for 48 hours was 45.71 μM and 34.67 μM, respectively. When the concentration of ezetimibe was 10 μM for 48 hours of treatment, the cell viability of MDA-MB-231 and 4T1 cells was 88.17% and 85.99%, respectively. When the concentration of ezetimibe was 20 μM for 48 hours of treatment, the viability of MDA-MB-231 and 4T1 cells was 81.83% and 76.83%, respectively. Therefore, 10 μM and 20 μM ezetimibe had little effect on the viability of MDA-MB-231 and 4T1 cells. These two concentrations were selected to study the effect of ezetimibe on the migration and invasion of MDA-MB-231 and 4T1 cells. Then, the wound healing assay was used to assess the effect of ezetimibe on the migration of MDA-MB-231 and 4T1 cells, and we found that treatment with 10 μM and 20 μM ezetimibe on MDA-MB-231 and 4T1 cells for 24 h resulted in a significant decrease in the healing rate compared with the untreated group, and the cell healing rate showed a dose-dependent manner (Fig. 1b). The results indicated that ezetimibe could inhibit the migration ability of triple-negative breast cancer cells.

Ezetimibe inhibits the invasion of MDA-MB-231 and 4T1 cells.

After observing that ezetimibe could inhibit the migration of MDA-MB-231 and 4T1 cells, we further explored the effect of ezetimibe on the invasion of MDA-MB-231 and 4T1 cells. We evaluated the effect of ezetimibe on the invasion of MDA-MB-231 and 4T1 cells using transwell invasion assays. As shown in Fig. 2, the numbers of MDA-MB-231 and 4T1 cells treated with ezetimibe for 24 h that passed through the Matrigel were significantly fewer than those of untreated cells in a dose-dependent manner. The results illustrate that the invasive ability of MDA-MB-231 and 4T1 cells treated with ezetimibe was significantly reduced compared to that of untreated cells, and ezetimibe inhibited the invasion of triple-negative breast cancer cells.

Ezetimibe inhibits EMT in MDA-MB-231 and 4T1 cells.

We found that ezetimibe can inhibit the migration and invasion abilities of MDA-MB-231 and 4T1 cells, and epithelial-mesenchymal transition (EMT) plays an important role in the occurrence of invasion and subsequent metastasis of tumor cells. Therefore, we examined whether ezetimibe affects EMT in MDA-MB-231 and 4T1 cells. We first observed the cell morphology of ezetimibe-treated MDA-MB-231 and 4T1 cells after 24 hours by microscopy, as shown in (Fig. 3a). The ezetimibe-treated cells exhibited a cobblestone-like shape as opposed to the spindle-shaped DMSO-treated control cells, with a low infiltrative and migratory competent phenotype, indicating that ezetimibe slows the morphological transition of triple-negative breast cancer cells toward EMT. We detected the expression of EMT-related markers by Western blot assay in MDA-MB-231 and 4T1 cells. As shown in (Fig. 3b and Fig S2), compared with untreated cells, the levels of the mesenchymal markers N-cadherin, vimentin, and snail protein were significantly downregulated, while the expression of the epithelial marker E-cadherin was significantly increased in the ezetimibe-treated group. These results indicate that MDA-MB-231 and 4T1 cells treated with ezetimibe display lower levels of EMT and that ezetimibe can significantly inhibit EMT in triple-negative breast cancer cells, thereby affecting the migration and invasion abilities of triple-negative breast cancer cells.

Ezetimibe inhibits TGFβ in triple-negative breast cancer cells.

We have shown that ezetimibe can inhibit the migration and invasion abilities as well as EMT of triple-negative breast cancer cells, and to define the mechanism of ezetimibe's effect, we used transcriptome sequencing to compare the differential gene expression profiles between ezetimibe-treated and untreated MDA-MB-231 cells, and the gene expression profiles by transcriptome sequencing are shown in Table S1 and Fig S1. We screened 146 differentially expressed genes based on the fold change values and p values less than 0.05. Of these, 44 genes had a difference multiple of more than 2 times, and 102 genes had a difference multiple of less than 0.5. The differential gene cluster analysis plot and volcano plot of transcriptome sequencing is shown in(Fig. 4a, 4b, and Table S2). Based on the fold change values of upregulated gene ploidy greater than 2 and downregulated gene ploidy less than 0.52, we identified 6 upregulated genes and 14 downregulated genes in ezetimibe-treated cells with FPKM more significant than 1 and p values less than 0.05. We then further subjected these differentially expressed genes to pathway enrichment analysis.

The Gene Ontology analysis results are shown in (Fig. 4c and 4d), and according to the enrichment degree q value of the pathway, the upregulated gene is mainly related to the process of toxin metabolism (Fig. 4c), which may be a normal metabolic process after the treatment of cells with the cholesterol-lowering drug ezetimibe. This study focused on tumor migration and invasion; therefore, the downregulated genes were explored. Based on the enrichment degree q value of the pathway, the downregulated genes were related to cell morphology pathways, which are related to breast cancer cell migration, invasion, and EMT that we previously studied. We then filtered out two downregulated genes associated with cell morphology: il7r (interleukin 7 receptor) and tgfb2 (transforming growth factor beta 2) (Fig. 4d). Since il7r is associated with inflammation and immunity [29], by reviewing the literature to analyze the correlation between each downregulated gene and tumor migration and invasion and considering the ranking of the fold change of genes, we found that TGFβ2 in downregulated genes is closely related to the metastasis of tumors. TGFβ2, as a member of the TGFβ family, plays a critical role in the EMT of cells [30–32]. Multiple studies have demonstrated that increased expression of TGFβ2 promotes breast cancer metastasis [33,34]. Notably, recent studies have noted that a high-cholesterol diet significantly induces TGFβ expression in the livers of mice [35,36]. These results guide us to further probe the relationship between ezetimibe and TGFβ in breast cancer cells. We validated the effect of ezetimibe on TGFβ2 mRNA by qRT-PCR, which showed a significant decrease in TGFβ2 mRNA in MDA-MB-231 and 4T1 cells treated with ezetimibe compared to untreated cells (Fig. 4e). We next tested the effect of ezetimibe on TGFβ2 protein expression by Western blot assay. The expression levels of TGFβ in MDA-MB-231 and 4T1 cells treated with ezetimibe were significantly decreased compared with those in untreated cells (Fig. 4f and Fig S3). These results demonstrate that the mRNA and protein expression levels of TGFβ were significantly decreased in MDA-MB-231 and 4T1 cells treated with ezetimibe.

Overexpression of TGFβ2 reverses the inhibitory effect of ezetimibe on the migration and invasion of MDA-MB-231 and 4T1 cells.

Sequencing results and validation experiments implicated the role of TGFβ2 in the ezetimibe-mediated inhibition of migration and invasion in triple-negative breast cancer cells. Given that previous studies have shown that increased TGFβ2 expression promotes breast cancer metastasis, we hypothesized that ezetimibe inhibits the migration and invasion of triple-negative breast cancer cells through TGFβ2.

First, we used lentiviral transfection to achieve TGFβ2 overexpression in MDA-MB-231 and 4T1 cells and verified it by real-time PCR and Western blot experiments. As shown in (Fig. 5a and Fig S4), TGFβ2-overexpressing MDA-MB-231 and 4T1 cells were constructed successfully. We next treated TGFβ-overexpressing MDA-MB-231 and 4T1 cells and control cells with ezetimibe. The migration ability of the cells was assessed by a scratch assay, and the healing rate of cells overexpressing TGFβ2 was significantly higher than that of control cells (Fig. 5b), illustrating that overexpression of TGFβ2 in triple-negative breast cancer cells resisted the inhibitory effect of ezetimibe on cell migration.

Similarly, we treated TGFβ2-overexpressing MDA-MB-231 and 4T1 cells and control group cells with ezetimibe, and then the invasive ability of the cells was assessed by Transwell invasion assay. We found that the number of TGFβ2-overexpressing MDA-MB-231 and 4T1 cells crossing the Matrigel was significantly higher than that of control cells (Fig. 5c), indicating that TGFβ overexpression significantly attenuated the inhibitory effect of ezetimibe on the invasion of triple-negative breast cancer cells. Overall, we demonstrated that overexpression of TGFβ2 can reverse the inhibitory effect of ezetimibe on the migration and invasion of triple-negative breast cancer cells. Thus, ezetimibe's ability to inhibit the migration and invasion of triple-negative breast cancer occurs through TGFβ2.

Discussion

Tumor metastasis is a significant cause of clinical therapy failure and cancer recurrence, especially in breast cancer [8,9]. Multiple studies have demonstrated a positive association between high cholesterol levels and the risk of developing breast cancer [11,37,38]. Targeting cholesterol metabolism may be an effective way to inhibit cancer progression [21]. For instance, ezetimibe, a drug that stops cholesterol absorption, has been found to inhibit the growth of prostate, breast, and liver cancers by inhibiting angiogenesis [27,28,39]. Our study showed that ezetimibe effectively inhibited triple-negative breast cancer cell migration, invasion, and EMT. Additionally, we discovered that ezetimibe significantly decreased TGFβ2 mRNA and protein levels. This finding implies that TGFβ2 may be a crucial factor in preventing breast cancer metastasis by ezetimibe. We further observed that overexpressing TGFβ2 in triple-negative breast cancer cells counteracted the inhibitory effects of ezetimibe on tumor cell invasion and metastasis. Our results suggest that TGFβ2 plays a critical role in metastasis and that ezetimibe prevents the metastasis of triple-negative breast cancer by suppressing the expression of TGFβ2.

Regarding the effect of ezetimibe on negative breast cancer cells, Kristine Pelton et al. [28] found that inhibiting angiogenesis in triple-negative breast cancer with ezetimibe could block the promotion of tumor metastasis caused by a high cholesterol diet. This implies that high cholesterol concentrations are closely related to tumor vascularization and that cholesterol-lowering drugs could help prevent tumor

metastasis by inhibiting tumor vascularization. However, our study discovered a novel mechanism for inhibiting tumor metastasis with ezetimibe: inhibiting the migration and invasion of triple-negative breast cancer cells by suppressing TGFβ2 expression. These findings suggest that ezetimibe might inhibit tumor metastasis independently of its cholesterol-lowering function.

In recent years, there has been some progress in the study of drugs targeting cholesterol metabolism in breast cancer metastasis [21]. Colin H. Beckwitt et al. [40] found that atorvastatin at a concentration of 20 μM inhibited migration by significantly inhibiting mesenchymal breast cancer cell proliferation. While the drug used in our study was ezetimibe, which directly inhibits cholesterol absorption, and ezetimibe did not affect breast cancer cell survival, the results indicated that ezetimibe inhibits the cholesterol absorption pathway of tumor cells and can directly inhibit EMT, migration, and invasion abilities of breast cancer cells. We speculate that breast cancer cell migration and invasion may be more dependent on exogenous cholesterol uptake and that directly inhibiting this pathway might play a more significant role in inhibiting these abilities.

In addition to statins, which inhibit cholesterol synthesis, and ezetimibe, which inhibits cholesterol absorption, inhibition of exogenous lipid uptake by inhibiting proprotein convertase subtilisin/kexin type-9 (PCSK9) is also a common strategy to lower cholesterol [41]. Studies have demonstrated that overexpression of PCSK9 inhibited EMT in colorectal cancer cells to promote tumor metastasis [42]. The ezetimibe used in this study can bind with high efficiency to the cholesterol carrier protein NPC1L1 and lower cholesterol in the blood by inhibiting the uptake of exogenous cholesterol. The results illustrate that inhibition of cholesterol synthesis or absorption pathways can effectively inhibit tumor metastasis.

Numerous studies have indicated that TGFβ2 facilitates breast cancer metastasis by promoting EMT and lipid storage in tumor cells [33,34,43]. To investigate how ezetimibe can impede TGFβ2 expression in breast cancer cells, we conducted RNA SEQ experiments on drug-treated cells. Our results showed that ezetimibe inhibits TGFβ2 expression in triple-negative breast cancer cells. Previous studies have established that agents inhibiting the TGFβ2 pathway can impede tumor metastasis [44–46]. For instance, ITD-1, a drug that suppresses TGFβ2, prevents Smad2/3 phosphorylation, thereby inhibiting glioma cell and gastric cancer cell invasion [44,45]. Similarly, our data revealed that ezetimibe restrains breast cancer cell migration and invasion, conceivably by targeting TGFβ2 and its downstream target Smad2/3, warranting further exploration.

Another mechanism by which TGFβ2 triggers tumor metastasis is by promoting angiogenesis [47]. Studies have found that inhibitors of TGFβ2, such as Trabedersen, can obstruct TGFβ2's interaction with angiogenesis-promoting factors to halt pancreatic tumor growth and angiogenesis [46]. Moreover, Kristine Pelton et al. demonstrated that ezetimibe stabilizes the vascular structure in a breast cancer mouse model by increasing pericyte coverage on vessels, thereby inhibiting angiogenesis and consolidating the tumor vasculature[28]. These observations led us to hypothesize that ezetimibe restrains breast cancer cell migration and invasion by nullifying the angiogenic consequences of TGFβ2 and increasing perivascular

cell coverage by trimming cholesterol absorption levels. Our findings about the novel potential effects of ezetimibe on tumor angiogenesis warrant further study.

In conclusion, this study demonstrates that ezetimibe inhibits breast cancer cell migration, invasion, and EMT and identifies TGFβ2's key role in this process. For the first time, this study reveals the effects of ezetimibe on tumor cell migration and invasion, and the underlying mechanism is the inhibition of TGFβ and EMT in cancer cells. This study provides new evidence for the regulation of cholesterol metabolism in tumor cells, which can inhibit their migration and invasion. Additionally, it presents new evidence for the use of drugs that regulate cholesterol metabolism to control the metastasis and recurrence of tumors. Furthermore, this study offers a basis for the clinical translation of ezetimibe for the treatment of breast cancer metastasis and recurrence.

Declarations

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

J.W.: Conceptualization (lead); Writing-review and editing (equal). C.J.: Conceptualization (supporting); Writing-review and editing (equal). Q.H.: Investigation-performing the experiments (lead); Writing-original draft (lead). D.M.: Investigation-performing the experiments (supporting). L.K.: Writing-original draft (supporting). W.S.: Visualization-data presentation (equal). Q.X.: Investigation-performing the experiments (supporting). All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Competing interests

The authors declare no competing interests.

Correspondence and requests for materials should be addressed to J.W.

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Supplementary Information

The online version contains supplementary material

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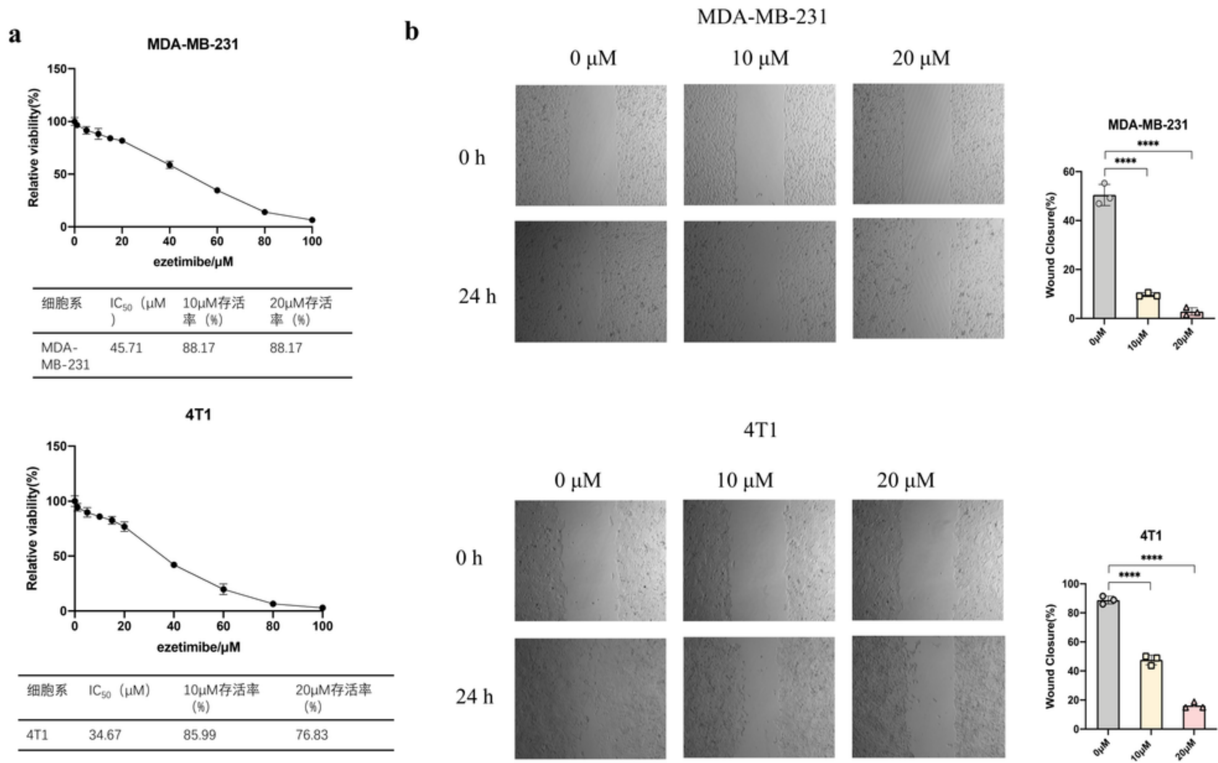
Figures



细胞系	IC_{50}（μM）	10μM存活率（%）	20μM存活率（%）
MDA-MB-231	45.71	88.17	88.17

细胞系	IC_{50}（μM）	10μM存活率（%）	20μM存活率（%）
4T1	34.67	85.99	76.83

Figure 1

Effect of ezetimibe on the viability and migration ability of MDA-MB-231 and 4T1 cells.(a) MDA-MB-231 and 4T1 cells were treated with different concentrations of ezetimibe (0, 1, 5, 10, 15, 20, 40, 60, 80, 100 μmol/L) for 48 hours, and cell viability was assessed using the CCK-8 assay. (b) MDA-MB-231 and 4T1 cells were treated with different concentrations of ezetimibe (0, 10, 20 μmol/L) for 24 hours, and then a scratch healing assay was used to determine the cell healing rate. Cell images at 0 h and 24 h were taken under a light microscope at a magnification of × 40 in three random fields for each experimental setting. The results shown are representative of three independent experiments and are shown as the mean ± standard deviation (mean ± SD): * P < 0.05, * * P < 0.01, * * * P < 0.001, * * * * P < 0.0001.

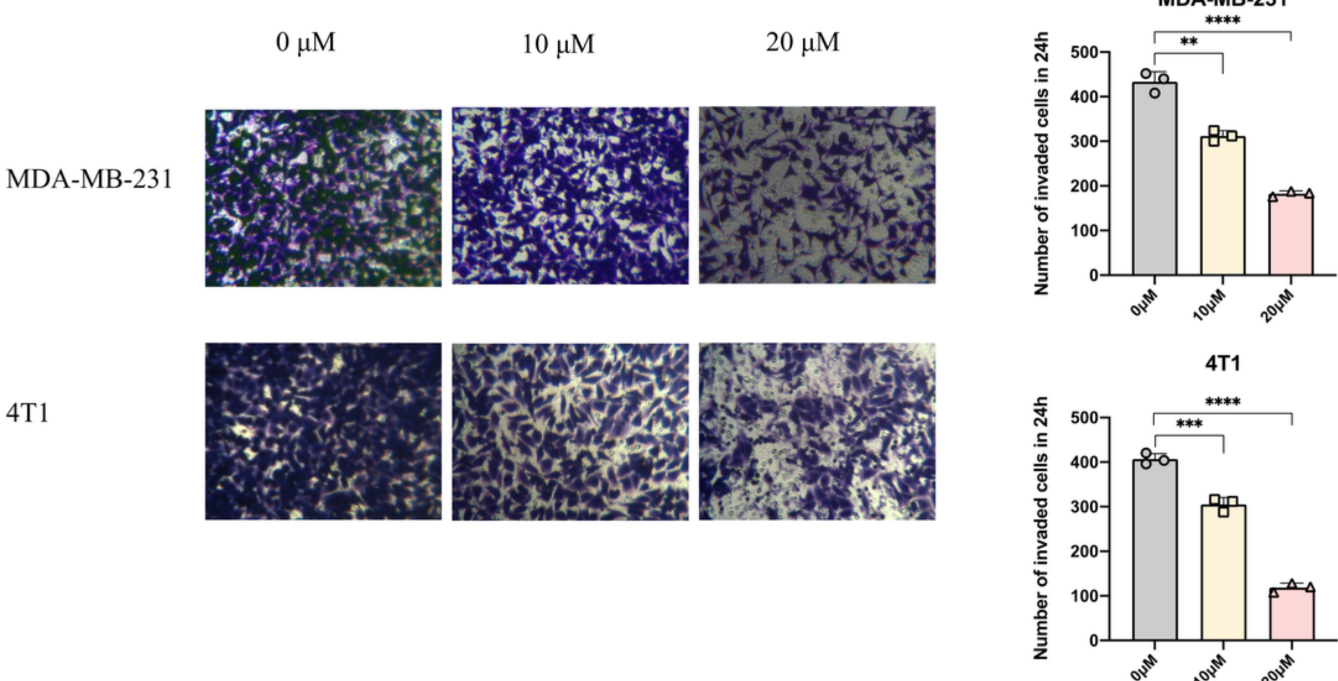


Figure 2

Effect of ezetimibe on the invasive ability of MDA-MB-231 and 4T1 cells. Transwell invasion assays (with Matrigel) were performed after MDA-MB-231 and 4T1 cells were treated with different concentrations of ezetimibe (0, 10, 20 μmol/L) for 24 h. Cell images at 0 h and 24 h were taken under a light microscope at a magnification of × 100 in three random fields for each experimental setting. The results shown are representative of three independent experiments, and the results are expressed as the mean ± standard deviation (mean ± SD): * P < 0.05, * * P < 0.01, * * * P < 0.001, * * * * P < 0.0001.

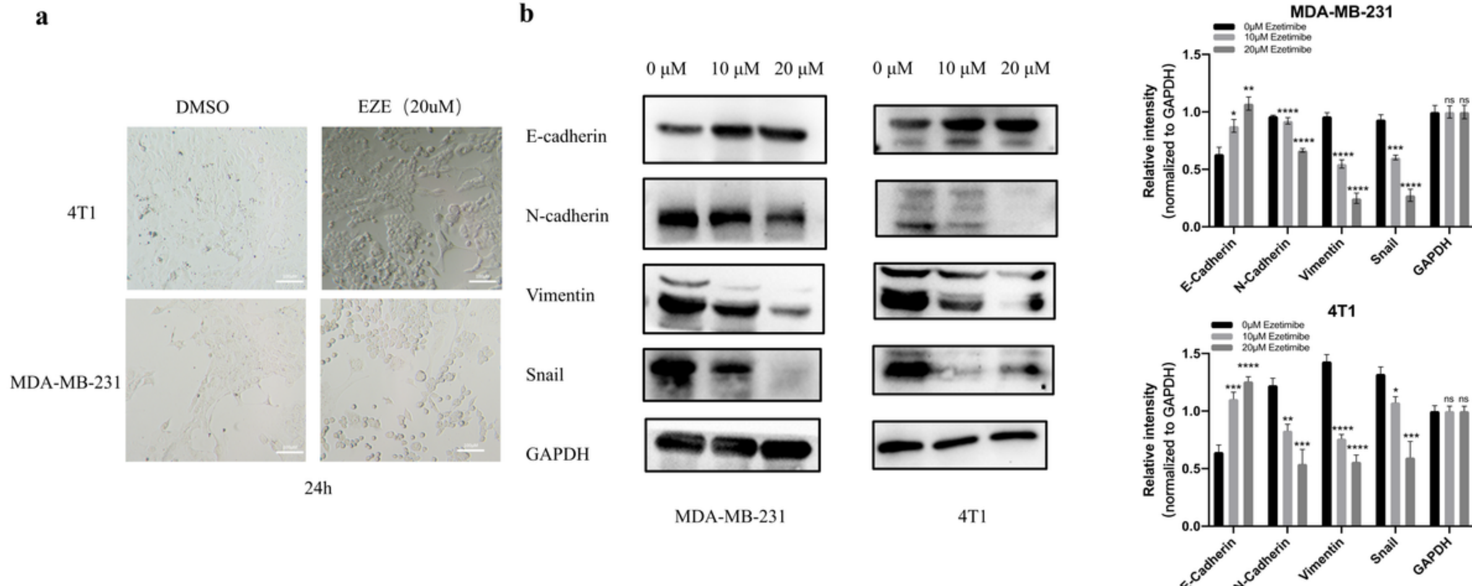


Figure 3

Effect of ezetimibe on the morphology and EMT-related markers in MDA-MB-231 and 4T1 cells. (a) MDA-MB-231 and 4T1 cells were treated with 20 μmol/L ezetimibe for 24 hours, and then cell morphology was observed and photographed by microscopy at a magnification of × 100. (b) MDA-MB-231 and 4T1 cells were treated with different concentrations of ezetimibe (0, 10, 20 μmol/L) for 24 hours, followed by Western blot assay to detect the expression of E-cadherin, N-cadherin, vimentin and snail protein, the blots cropped from different parts of the same gel and the grayscale values of each protein band in the cells were analyzed. The results shown in the figures are representative of three independent experiments.

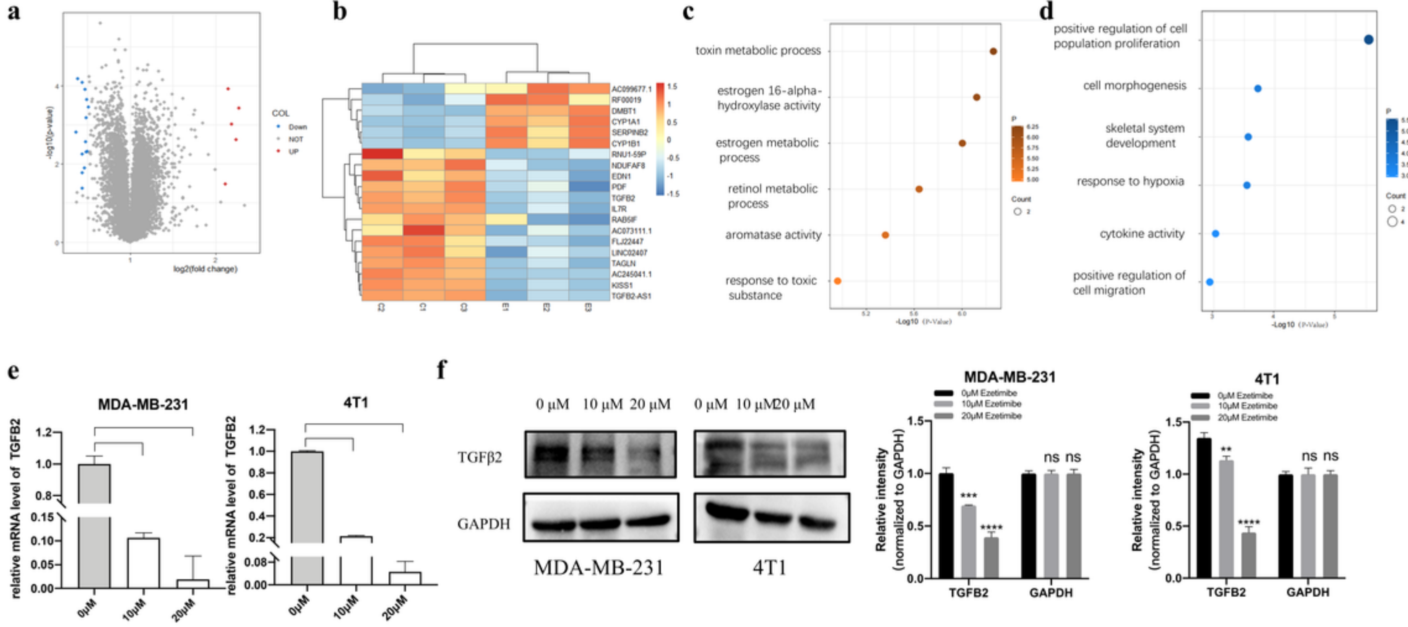


Figure 4

Effect of ezetimibe on the transcriptome of MDA-MB-231 cells and the mRNA and protein levels of TGFβ2 in MDA-MB-231 and 4T1 cells. (a) Transcriptome sequencing was performed in MDA-MB-231 cells treated with 20 μM ezetimibe for 48 h. Differentially expressed genes between ezetimibe-treated cells and control cells were then plotted as a volcano plot. (b) Differentially expressed genes between MDA-MB-231 cells treated with ezetimibe and control cells were selected based on the gene's fold change value using |log2-fold change| ≥ 1, upregulation fold change > 2, and downregulation fold change< 0.52 as the filtering criterion. (C)(D) The enriched pathways were extracted and merged based on Gene Ontology, and distribution plots were made according to the enrichment Q-value of each pathway. (c) Pathway enrichment analysis of upregulated genes in (b) is shown. (d) Pathway enrichment analysis of downregulated genes in (b) is shown. (e) qRT-PCR experiments were performed to determine TGFβ2 mRNA in MDA-MB-231 and 4T1 cells after treatment with ezetimibe (0, 10, 20 μmol/L) for 24 h. (f) MDA-MB-231 and 4T1 cells were treated with different concentrations of ezetimibe (0, 10, 20 μmol/L) for 24 h, and then the expression of TGFβ2 was determined using Western blotting, the blots cropped from different parts of the same gel and the grayscale values of each band were analyzed according to the experimental results. The results shown are representative of three independent experiments and are expressed as the means ± standard deviations (means ± SDs): * P < 0.05, * * P < 0.01, * * * P < 0.001, * * * * P < 0.0001.

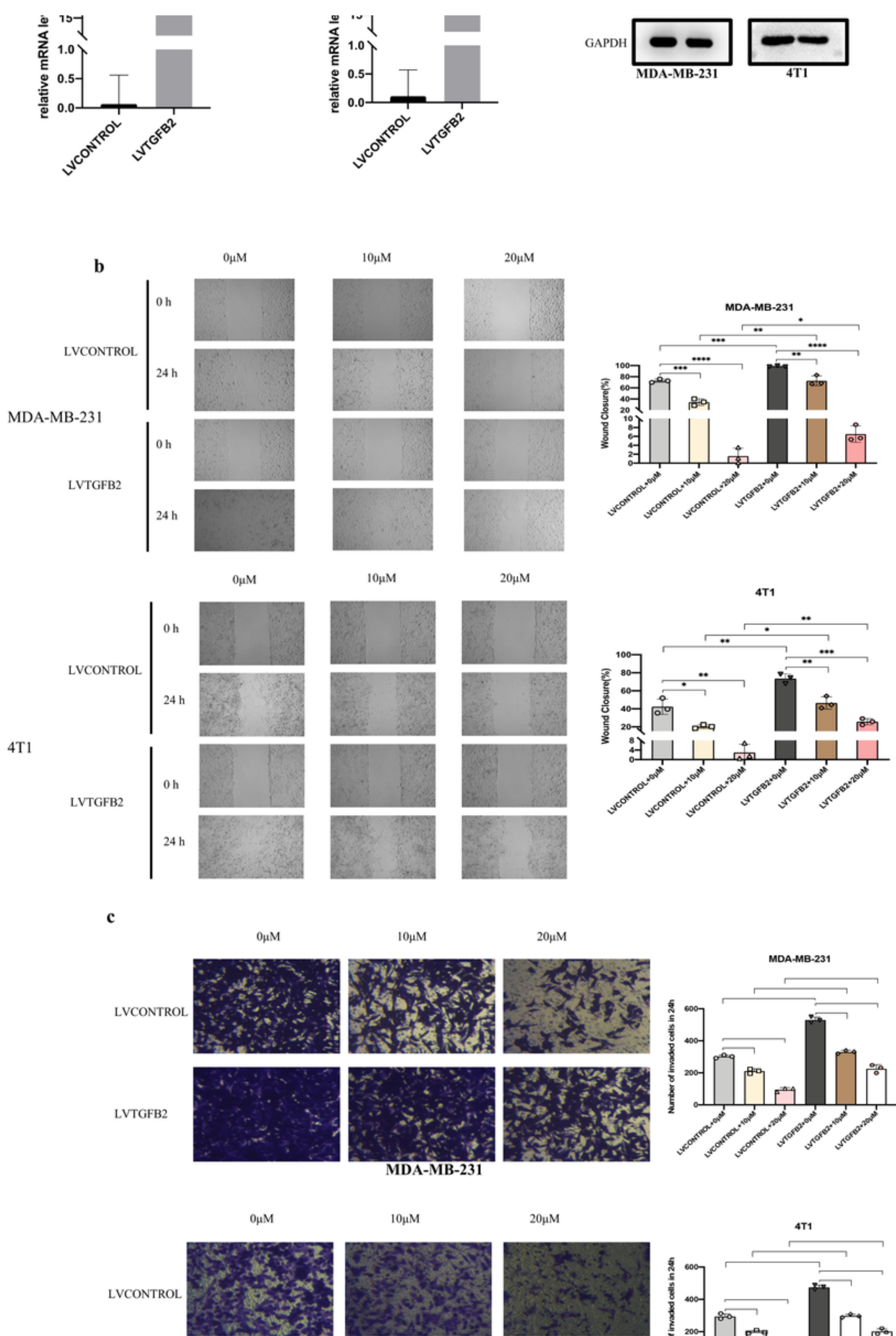


Figure 5

Effect of TGFβ2 overexpression on the ability of ezetimibe to inhibit cell migration and invasion in MDA-MB-231 and 4T1 cells. (a) Detection of TGFβ2 in TGFβ2-overexpressing MDA-MB-231 and 4T1 cells and control cells using qRT-PCR and Western blotting the blots cropped from different parts of the same gel. (b) TGFβ2-overexpressing MDA-MB-231 and 4T1 cells and control cells were treated with different concentrations of ezetimibe (0, 10, 20 μmol/L) for 24 h, and then the cell healing rate was detected using

a scratch healing assay. Cell images at 0 h and 24 h were taken under a light microscope at a magnification of × 40 in three random fields for each experimental setting. (c) TGFβ2-overexpressing MDA-MB-231 and 4T1 cells and control cells were treated with different concentrations of ezetimibe (0, 10, 20 μmol/L) for 24 h, and then a transwell invasion assay (with Matrigel) was used to detect the number of cells that crossed the Matrigel. Cell images at 0 h and 24 h were taken under a light microscope at a magnification of × 100 in three random fields for each experimental setting. The results shown are representative of three independent experiments and are expressed as the means ± standard deviations (means ± SDs): * $P < 0.05$, * * $P < 0.01$, * * * $P < 0.001$, * * * * $P < 0.0001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalData.pdf
- TableS1.Geneexpressiontablefortranscriptomesequencing.xls
- TableS2.Differentialgenes.xlsx