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Original Article

Dicer initiates *HER2* overexpression in breast cancer, expanding the indications for trastuzumab

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Abstract: Purpose: Trastuzumab, a monoclonal antibody, is widely employed for targeting human epidermal growth factor receptor 2 (*HER2*) in patients with advanced breast cancer (BC). However, the application of trastuzumab is limited, since only 25%-30% of BC patients exhibit a high *HER2* expression level. This study aimed to expand the therapeutic scope of trastuzumab in BC treatment. Methods: Gain-of-function studies were performed to evaluate the effects of various treatments on breast cancer cell proliferation, migration, invasion and apoptosis. Additionally, the cooperative effects of calcitriol and trastuzumab were investigated using xenograft models. Results: *Dicer* overexpression was observed to induce *HER2* overexpression in triple-negative breast cancer (TNBC) MDA-MB-231 cells while simultaneously suppressing their proliferative, migratory, and invasive properties. Calcitriol-induced *Dicer* expression was found to drive TNBC cells into a *HER2*-overexpressed state, rendering them suitable for trastuzumab treatment. This induction also inhibited the proliferation, invasion, and migration of BC cells. Moreover, calcitriol-mediated *HER2* overexpression through the upregulation of *Dicer* expression enhanced trastuzumab's efficacy in suppressing TNBC cell proliferation and migration. In xenograft models, this combination accelerated trastuzumab-induced inhibition of TNBC tumor growth. Conclusion: The findings of this study demonstrate that calcitriol may expand the therapeutic applications of trastuzumab for TNBC patients by initiating *HER2* overexpression. Consequently, *Dicer* may broaden the clinical application of *HER2* antibody therapy for TNBC patients who were previously not candidates for anti-*HER2* antibody treatment.

Keywords: Breast cancer, *HER2*, *Dicer*, calcitriol, TNBC

Introduction

Breast cancer (BC) remains the most prevalent cancer and the leading cause of cancer-related mortality among women globally [1]. In China, BC is recorded as the most common cancer and the fifth leading cause of cancer-related death in women [2]. Despite advancements in comprehensive treatment options, BC continues to exhibit a high mortality rate [3]. Based on gene expression profiling and immunohistochemical (IHC) analysis, BC can be categorized into several molecular subtypes: Luminal A, Luminal B, human epidermal growth factor receptor 2 (*HER2*) positive/estrogen receptor (ER) negative, ordinary-like subtype, and basal-like subtype [4, 5]. These distinct molecular subtypes determine the therapeutic approach prescribed to patients. Anti-*HER2* treatment is typically recommended for most HER2-positive BC patients [6, 7]. Triple-negative breast cancer (TNBC) presents significant therapeutic challenges, as its name suggests, characterized by the absence of ER, progesterone receptor (PR), and *HER2* expression [8]. TNBC patients generally face a poor prognosis, with a younger age and a lower five-year survival rate [8].

The combination of anti-*HER2* antibody therapy and chemotherapy as the first-line treatment has demonstrated significant improvements in survival rates for BC patients with *HER2* amplification. This consolidated therapy has achieved a 37% relative improvement in overall survival (OS) and an increase in the 10-year OS rate from 75.2% to 84% [9].

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HER2, a proto-oncogene located on the long arm of chromosome 17, encodes a 185 kDa plasma membrane-bound tyrosine kinase receptor [10, 11]. *HER2* promotes cell proliferation and suppresses apoptosis. Approximately 25%-30% of BC patients exhibit *HER2* overexpression, which is associated with a high level of malignancy and poor prognosis [10, 12]. Trastuzumab, a cultured monoclonal antibody targeting *HER2*, inhibits *HER2*-mediated signal transduction and is prescribed for BC and gastric cancer patients with a high *HER2* expression level [13, 14]. However, the application of trastuzumab is limited due to the relatively low percentage of BC patients exhibiting *HER2* amplification [12, 15].

MicroRNAs (miRNAs) are small non-coding RNAs, 18-25 nucleotides in length, that bind to the 3'-untranslated region (3'UTR) of target mRNAs to modulate gene expression [16, 17]. The cytoplasmic RNase III endonuclease *Dicer* plays a crucial role in miRNA biogenesis by cleaving long double-stranded RNA and the loop of primary miRNA into short interfering RNA (siRNA) and ~22 bp double-stranded miRNA [18, 19]. *Dicer* regulates cell proliferation and apoptosis, and its expression has been associated with a favorable prognoses in BC, acting both as a tumor suppressor and an inducer of *HER2* overexpression [20]. Given that *Dicer* has been shown to up-regulate *HER2* expression without increasing the invasiveness of gastric cancer cells [21], this study aimed to evaluate the relationship between *Dicer* and *HER2* in TNBC and its potential impact on BC treatment strategies.

Materials and methods

BC tissue specimens

Tissue samples were obtained from 63 BC at the Breast Center in the Fourth Hospital of Hebei Medical University between January 2013 and March 2016. Histopathologic assessment was done for all BC patients. The hospital's Human Tissue Research Committee approved all procedures, and written consent was obtained from participating patients.

Immunohistochemical (IHC) staining

IHC staining for *Dicer* was performed following established protocols [22]. BC tissues were fixed in 10% formalin at room temperature for 24 hours and subsequently embedded in paraffin. The tissues were then sectioned into 4-μm thick slices and immunostained with an anti-*Dicer* antibody (cat no. ab82539; dilution rate 1:100, Abcam, Cambridge, UK) overnight at 4°C. Following this, the sections were treated with a biotinylated secondary anti-mouse IgG antibody (cat no. ab5887; dilution rate 1:200, Abcam, Cambridge, UK) for 60 minutes at room temperature. After incubation with HRP-conjugated streptavidin, the sections were stained with 3, 3'-diaminobenzidine after HRP-conjugated streptavidin incubation. *Dicer* quantitation using HSCORE was performed by two pathologists blinded to clinical information [23]. *HER2* staining was performed using the fully-automated BenchMark ULTRA platform with an anti-*HER2*/neu (4B5) antibody (cat no. 5999570001; no dilution, Ventana Medical Systems, Inc., Tucson, Arizona, USA). *HER2* expression was evaluated on a scale of 0 to 3+ by two pathologists as previously described [24]. Patients with an IHC score of 2+ underwent further testing for *HER2* amplification by fluorescent in situ hybridization (FISH). FISH for *HER2* was carried out using the PathVysion *HER2* DNA Probe Kit (Vysis/Abbott Molecular Inc., Chicago, IL) according to the manufacturer's instructions.

Cell lines and transfection

The human BC cell line MDA-MB-231, obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), was cultured in DMEM high-glucose medium (GibcoTMLife Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS; GibcoTMLife Technologies, NY, USA) at 37°C and 5% CO_2. Trastuzumab (10 μg/mL) (Herceptin®, Roche pharma, South San Francisco, CA) was added to the medium for subsequent analysis.

Green fluorescent protein (GFP)-labeled *Dicer*-overexpressing lentivirus (pCMV-*Dicer*) and control lentivirus (pCMV-NC) were acquired from GeneCopoeia Corp (FulenGen, Guangzhou, China). Successful viral infection was confirmed by GFP signal 72 hours post-transfection. Polyclonal populations of stable-transduced cells over-expressing *Dicer* were selected using 0.3 μg/mL puromycin and verified by western blotting.

Silencer siRNAs targeting Dicer and negative control siRNA were chemically synthesized (Zsgentech, Tianjin, China). The siRNA sequences are presented in Table S1. Transfection was performed using Lipofectamine 2000 (Invitrogen, San Diego, CA) in accordance with the instructions of the manufacturer.

Western blotting analysis

Total protein from MDA-MB-231 cells was extracted using RIPA buffer (Roche, Basel, Switzerland) and subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, CH). The membranes were blocked with 5% skim milk and incubated with primary antibodies against *Dicer* (cat no. ab82539; dilution rate 1:1000; Abcam, Cambridge, UK), *HER2* (cat no. ab242195; dilution rate 1:1000; Abcam, Cambridge, UK), and β-actin (cat no. ab8226; dilution rate 1:10,000; Abcam, Cambridge, UK), followed by overnight incubation at 4°C. After incubation with secondary anti-mouse antibody (cat no. 31430; dilution rate 1:5000; Thermo Fisher, New York, NY) for 1 h at room temperature, protein bands were visualized using an enhanced chemiluminescence reagent (Thermo Fisher, New York, NY). The protein bands were analyzed using a FluorChem® HD2 protein imaging system (Alpha InnoTec, San Leandro, CA).

Cell proliferation assay

Cell proliferation was assessed using the Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan). Following the manufacturer's protocol, approximately 1×10^3 cells were seeded in each well of a 96-well plates (six copy wells for each experimental condition) with 100 μL medium. At various time points (0, 24, 48, and 72 h), 10 μL of CCK-8 reagent was added to each well and incubated for 2 h. The absorbance at 450 nm was then measured using a microplate reader (BIOTEK, Winooski, VT) to quantify cell proliferation.

Wound healing assay

BC cells were cultured in 6-well plates until a confluent mono-layer was formed. A 'wound' was created in the mono-layer using a 200 μL pipette tip. The cells were then washed twice with PBS and supplemented with fresh culture medium containing 2% FBS. Images of the wound area were captured at 0 and 24 h using an inverted microscope (Nikon, Tokyo, Japan). The rate of cell migration into the wound was determined by comparing the width of the scratch (wound gap) at 24 h to the initial width of the same scratch, as previously described [25].

Invasion assay

The invasive capacity of BC cells was evaluated using 24-well transwell chambers with 8 μm pore size (Corning, New York, USA), pre-coated with 1 mg/mL BD Matrigel (BD Biosciences, NJ, USA) [26]. A suspension of 1×10^5 cells in 200 μL of serum-free medium was added to the upper chamber of each transwell. The lower chamber was filled with 500 μL of medium containing 10% FBS. The invasion assay was conducted for 24 h at 37°C in a 5% CO_2 atmosphere. Cells that had invaded through the Matrigel-coated membrane were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The stained cells were imaged and quantified using an inverted microscope, with five random fields analyzed for each membrane (amplification, ×200).

Cell apoptosis assay

The PE Annexin V Apoptosis Detection Kit (BD Biosciences Pharmingen, San Diego, CA) was employed to assess cell apoptosis through flow cytometry. The staining procedure was done in accordance with the manufacturer's guidelines [27]. A total of 1×10^5 cells were harvested using 0.5% trypsin-EDTA (GibcoTMLife Technologies, NY, USA) and subsequently washed twice with PBS (4°C). The cells were then resuspended in 500 μL of binding buffer and stained with 5 μL of Annexin V and 5 μL 7-amino-actinomycin D for 15 minutes in darkness at ambient temperature. Following this, 400 μL of binding buffer was introduced. Flow cytometric analysis was conducted using a FACS Aria II flow cytometer (BD Biosciences, San Diego, CA).

Xenograft tumor model

Twenty 4-week-old female nude BALB/c mice were procured from Charles River Laboratories [Beijing, China; permission no. SCXK (Jing)

Table 1. Correlation between *Dicer* level and *HER2* level

HER2 level	*Dicer* level		χ^2	*P* value
	High	Low		
High	13	11	5.216	0.022
Low	10	29		

Notes: *HER2* 2+ while FISH analysis was positive and 3+ were defined as high expression. *HER2* 0, 1+ and 2+ while FISH analysis was negative were defined as low expression. *HER2*, human epidermal growth factor receptor 2; FISH, fluorescent in situ hybridization.

20160006]. The housing and treatment of these nude mice were carried out in strict adherence to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tumor xenografts were established through subcutaneous injection of 5×10^6 MDA-MB-231 cells into the flank region of the mice. Eight days post-injection, the mice were randomly allocated into four groups (five mice per group): MDA-MB-231 (group 1), MDA-MB-231 plus trastuzumab (group 2), MDA-MB-231 plus calcitriol (group 3), and MDA-MB-231 plus trastuzumab and calcitriol (group 4). Group 1 received intraperitoneal injections of 10 mL/kg saline and 0.2 mL DMSO as the negative control. Group 2 was administered intraperitoneal injections of 10 mg/kg trastuzumab (1 mg/mL) and 0.2 mL DMSO. Group 3 was received intraperitoneal injections of 10 mL/kg saline and 4 μg calcitriol in 0.2 mL DMSO. Group 4 was given intraperitoneal injections of 10 mg/kg trastuzumab (1 mg/mL) and 4 μg calcitriol in 0.2 mL DMSO. All intraperitoneal injections were administered at 4-day intervals, with concurrent measurements of tumor growth and weight. Tumor volume was calculated using the formula: Volume = Length × $(\text{Width})^2/2$ [28]. On day 32, the mice were euthanized by cervical dislocation, and the tumors were excised.

miRNA microarray analysis

Total RNA was extracted from tumor cells collected from groups 1 and 3 (three samples for each group), and subsequently analyzed using microarray technology at Cnkingbio Biotechnology Corporation (Beijing, China) employing the FlashTag Biotin HSR RNA Labeling Kit (Afymetrix, Santa Clara, CA). Labeling and hybridization procedures were executed in accordance with the manufacturer's instructions. The microarray for miRNAs was manufactured and processed as previously described [29]. Microarray scanning was performed on a GeneChip Scanner 3000 (Afymetrix, Santa Clara, CA), and data analysis was conducted using GeneChip Command Console software (Afymetrix, Santa Clara, CA).

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (version 21.0; SPSS Company, Chicago, IL). Data were expressed as mean ± standard deviations (SD) for individual experiments. The χ^2 test was employed to determine the relationship between the expression levels of Dicer and *HER2*. Cell proliferation, migration, invasion, apoptosis, and tumor growth assays were estimated by ANOVA. Statistical significance was established at $P\leq0.05$.

Results

Dicer upregulates HER2 expression in BC

The expression of *HER2* and *Dicer* in BC tissue was assessed using semi-quantitative strategies by IHC examination. A positive correlation between *Dicer* expression and *HER2* expression was observed in BC tissue (**Table 1** and **Figure 1**, $P=0.022$). The successful transfection of *Dicer* into MDA-MB-231 cells was confirmed through western blot (**Figure 2A**). Notably, *HER2* expression was significantly upregulated following *Dicer* overexpression, as illustrated in **Figure 2A**. These findings suggest that *Dicer* plays a role in up-regulating *HER2* expression in both BC tissue and cells.

Dicer inhibits proliferation, migration, and invasion of BC cells

The proliferative capacity of MDA-MB-231 cells over-expressing *Dicer* was evaluated using the CCK assay. A significant decrease in proliferation was observed in MDA-MB-231 cells transfected with pCMV-*Dicer* compared to those transfected with pCMV-NC between 48 to 72 hours (**Figure 2B**, $P<0.05$). The wound healing assay was employed to assess the effect of *Dicer* on cell migration. Results demonstrated that *Dicer* overexpression significantly suppressed the migration of MDA-MB-231 cells

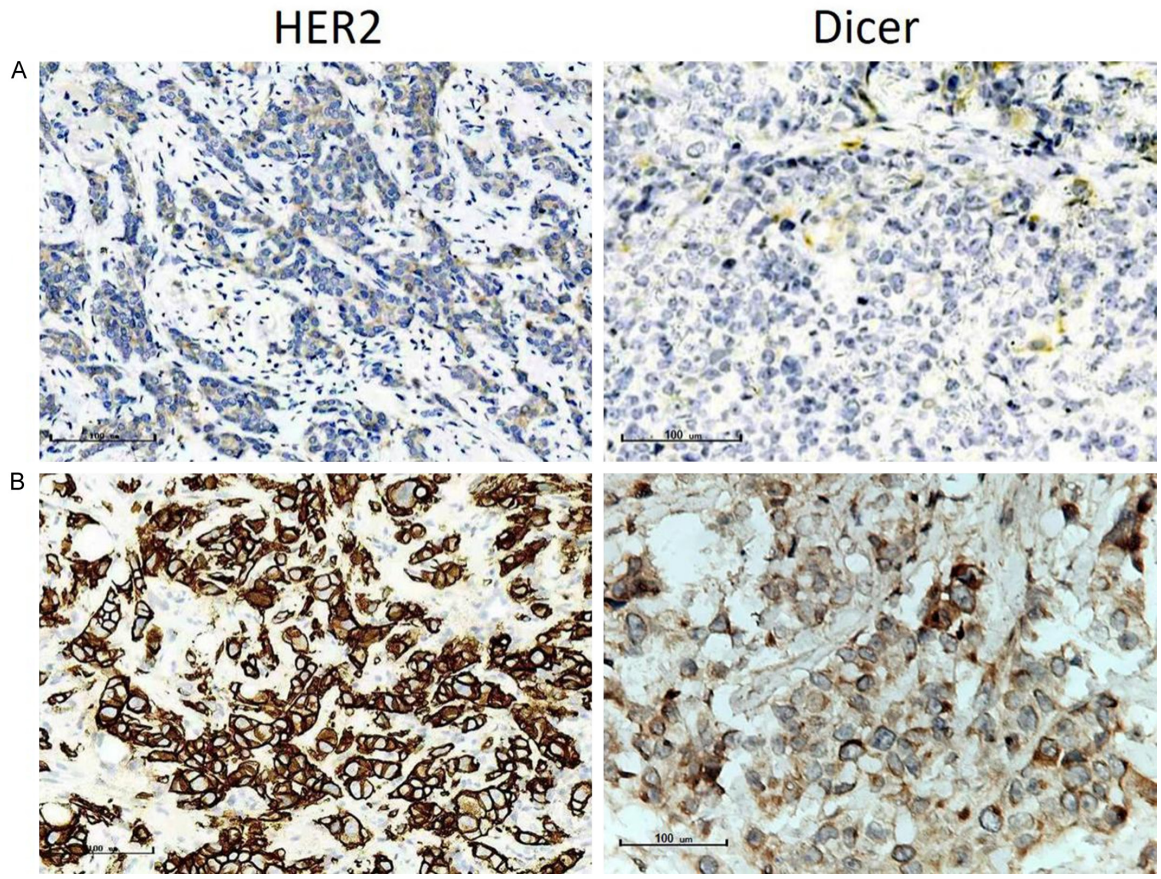

Figure 1. Immunostaining of *Dicer* and *HER2* in BC tissues. A. A *HER2* negative BC patient with low *Dicer* level; B. A *HER2* positive BC patient with high *Dicer* level. Original magnification, ×200. *HER2*, human epidermal growth factor receptor 2; BC, breast cancer.

(**Figure 2C** and **2D**, *P*<0.001). Furthermore, the invasiveness of pCMV-*Dicer* cells was significantly reduced compared to pCMV-NC cells (**Figure 2E** and **2F**, *P*<0.001). To elucidate the mechanism underlying *Dicer*-induced inhibition of BC cell proliferation, the Annexin V-PE/7-AAD apoptosis assay was conducted. However, no significant difference was observed between pCMV-NC cells and pCMV-*Dicer* cells (**Figure 2G** and **2H**, *P*=0.705). These results collectively indicate that *Dicer* suppresses the proliferation, migration, and invasion of BC cells while inducing *HER2* overexpression, without rendering BC cells more malignant.

Trastuzumab enhances Dicer-induced inhibition

Given that *Dicer* induces *HER2* overexpression, the possible sensitization of the TNBC cell line MDA-MB-231 to anti-*HER2* antibody treatment was investigated. The efficacy of trastuzumab in the presence of *Dicer* was evaluated by comparing the effects of 10 μg/mL trastuzumab on pCMV-NC cells and pCMV-*Dicer* cells. Monotreatment with trastuzumab inhibited cell proliferation between 48 to 72 hours (**Figure 3A**, *P*<0.01), migration (**Figure 3B** and **3C**, *P*=0.042), and invasion (**Figure 3D** and **3E**, *P*=0.001) of pCMV-NC transfected MDA-MB-231 cells. When pCMV-*Dicer* cells were incubated with trastuzumab, an enhanced suppression of BC cell proliferation was observed from 24 to 72 hours (**Figure 3A**, *P*<0.05), along with increased inhibition of migration (**Figure 3B** and **3C**, *P*<0.001), and invasion (**Figure 3D** and **3E**, *P*<0.001). Moreover, a more pronounced suppression of proliferation from 24 to 72 hours (**Figure 3A**, *P*<0.01), migration (**Figure 3B** and **3C**, *P*<0.001), and invasion (**Figure 3D** and **3E**, *P*<0.001) was observed when comparing pCMV-NC and pCMV-*Dicer* plus trastuzumab treatments. These data suggest that trastuzumab enhances *Dicer*-induced suppression by

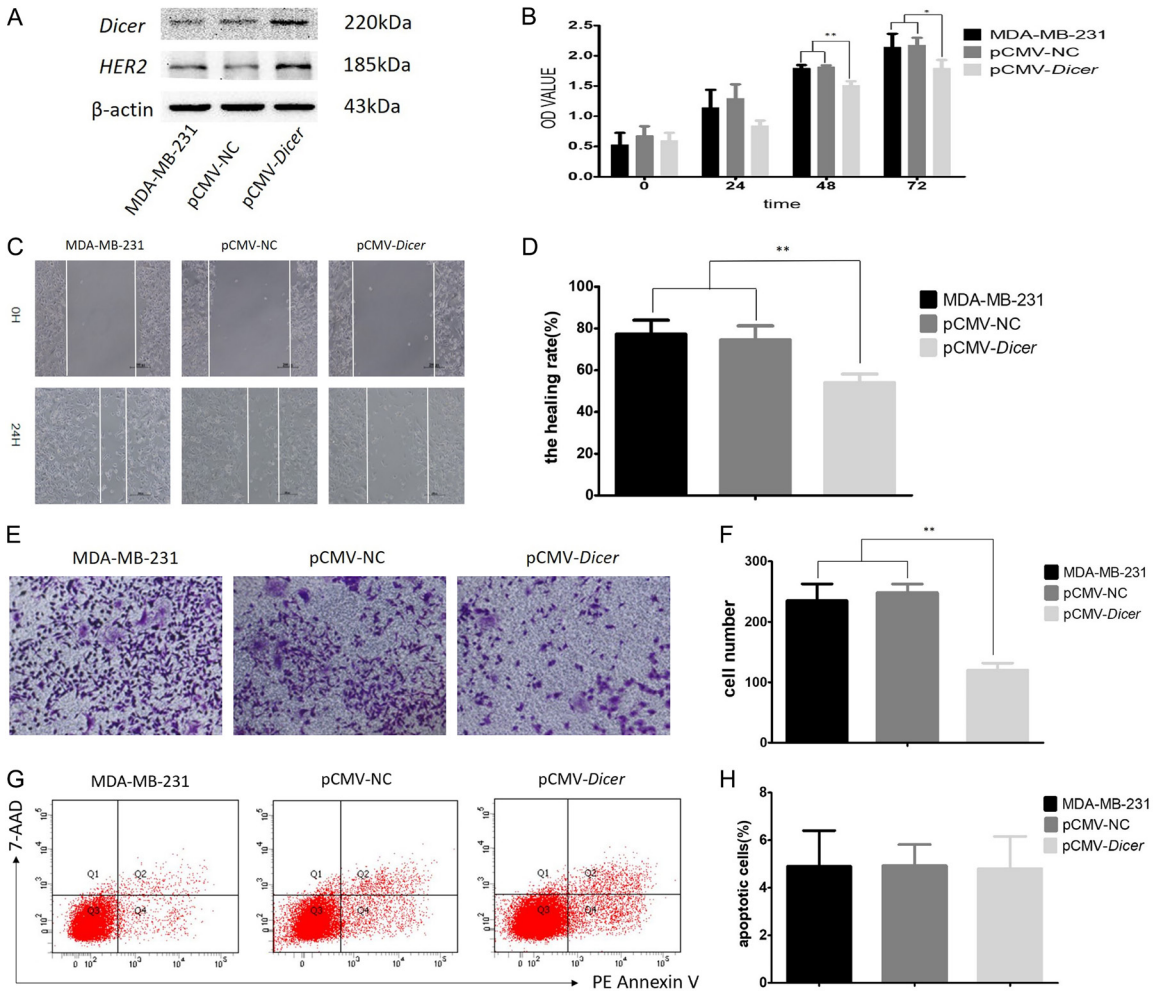

Figure 2. Overexpression of *Dicer* inhibits the proliferation, migration, and invasion of MDA-MB-231 cells. (A) Western blot of *Dicer* and *HER2* in MDA-MB-231 cells; (B) The proliferation measured by CCK-8 assay; (C) Migration measured by wound healing assay; (D) Quantification of results of (C); (E) The invasion measurement by transwell assay; (F) Quantification of results of (E); (G) Apoptosis measurement by flow cytometry; (H) Quantification of results of (G). $^{*}P<0.05$, $^{**}P<0.01$. *HER2*, human epidermal growth factor receptor 2; BC, breast cancer; CCK-8, Cell Counting Kit-8.

targeting the over-expressed *HER2* induced by *Dicer* in TNBC cells.

Calcitriol enhances trastuzumab-related inhibition in BC cells through Dicer expression induction

Since calcitriol induced *Dicer* expression in the cervical cancer cell line SiHa and gastric cancer cells [21, 30], the induction of *Dicer* expression by calcitriol in TNBC cells to determine its potential to induce *HER2* overexpression. As illustrated in **Figure 4A**, a dose-dependent increase in *Dicer* expression levels was observed following a 72-hour incubation with calcitriol at concentrations of 5, 10, 15, or 20 mmol/L. Notably, HER2 up-regulation coincided with the increase in *Dicer* expression. Mono-treatment with calcitriol resulted in significant reductions in cell proliferation between 48 to 72 hours (**Figure 4B**, $P<0.01$), migration (**Figure 4C** and **4D**, $P<0.001$), and invasion (**Figure 4E** and **4F**, $P<0.001$) in MDA-MB-231 cells. These findings suggest that calcitriol suppresses BC cell proliferation, migration, and invasion, presumably through *Dicer* induction. Combined treatment with trastuzumab and calcitriol exhibited additive inhibition of cell proliferation between 48 to 72 hours (**Figure 4B**, $P<0.05$) and migration (**Figure 4C** and **4D**, $P<0.001$) compared to calcitriol mono-therapy. A dramatic decrease in proliferation between 48 to 72 hours (**Figure 4B**, $P<0.01$), migration (**Figure 4C** and **4D**, $P<0.001$), and invasion (**Figure 4E** and

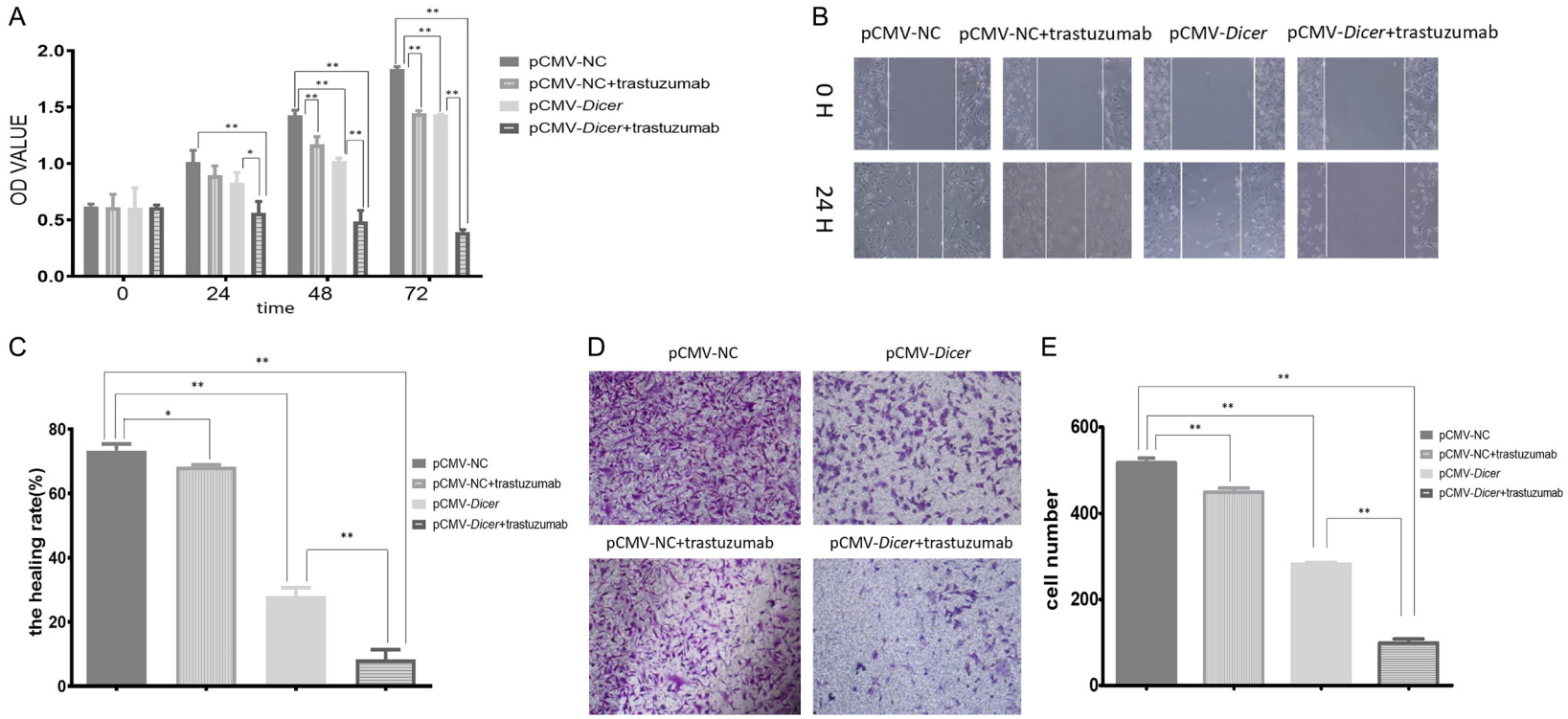

Figure 3. Trastuzumab enhances the *Dicer* related inhibition for MDA-MB-231 cells. (A) Proliferation measurement by CCK-8 assay; (B) Migration measurement by wound healing assay; (C) Quantification of results of (B); (D) Invasion measured by transwell assay; (E) Quantification of results of (D). $^{*}P<0.05$, $^{**}P<0.01$. BC, breast cancer; CCK-8, Cell Counting Kit-8.

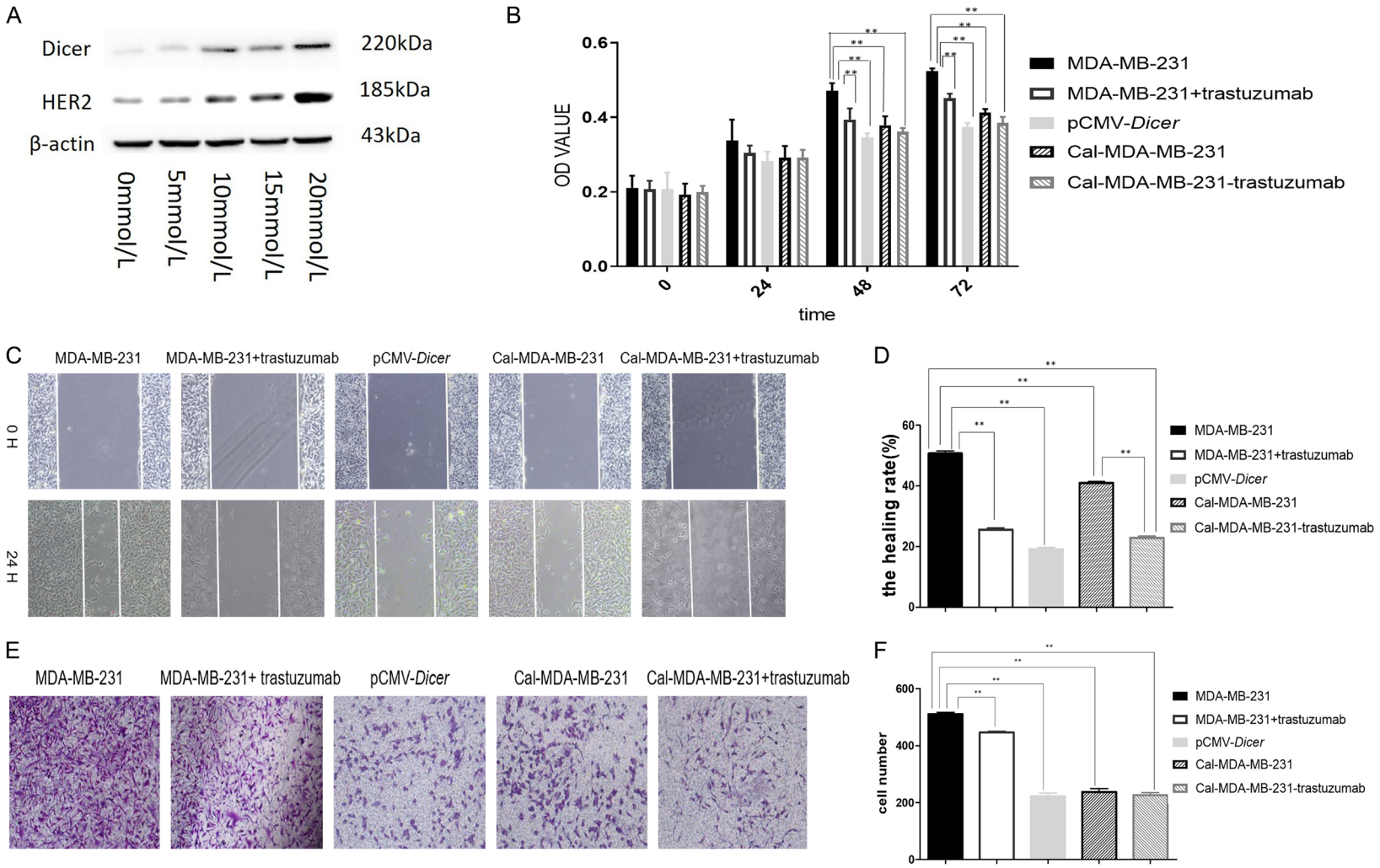

Figure 4. Calcitriol could induce *HER2* expression and synergy with Trastuzumab for BC inhibition. (A) Western blot analysis of *Dicer* and *HER2* expression upon calcitriol induction in BC cells; (B) Proliferation measured by CCK-8 assay; (C) Migration measured by wound healing assay; (D) Quantification of results of (C); (E) Invasion measured by transwell assay; (F) Quantification of results of (E). $^{*}P<0.05$, $^{**}P<0.01$. *HER2*, human epidermal growth factor receptor 2; BC, breast cancer; CCK-8, Cell Counting Kit-8.

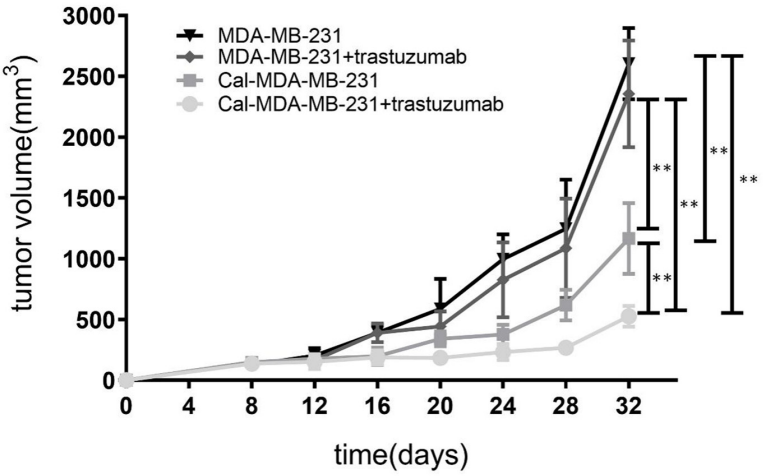

Figure 5. Effects of Calcitriol and Trastuzumab on TNBC cell growth *in vivo* (n=5 for each group). **P<0.01. TNBC, triple-negative breast cancer.

4F, *P*<0.001) was observed in MDA-MB-231 cells treated with calcitriol plus trastuzumab compared to untreated cells. These results demonstrate that calcitriol can sensitize the TNBC cell line MDA-MB-231 to trastuzumab treatment through *HER2* overexpression induction.

Calcitriol counteracts Dicer knockdown-induced growth promotion in BC cells

Three siRNAs targeting Dicer were transfected into MDA-MB-231 cells. Western blot analysis revealed that *Dicer* and *HER2* levels decreased significantly in siRNA3-Dicer transfected cells compared to others (Figure S1A). Consequently, the siRNA3-Dicer construct was selected for subsequent analysis. Comparison of BC cells infected with siRNA3-*Dicer* or siRNA-NC revealed significantly increased cell proliferation between 48 to 72 hours when *Dicer* was knocked down (Figure S1B, *P*<0.01). Migration (Figure S1C and S1D, *P*=0.038), and invasion capacity (Figure S1E and S1F, *P*<0.001) were also increased in *Dicer* knockdown cells.

The addition of 20 mmol/L calcitriol to si-231 cells resulted in increased *Dicer* and *HER2* levels (Figure S1A). Subsequent gain-of-function experiments demonstrated significantly reduced proliferation between 48 to 72 hours (Figure S1B, *P*<0.01), as well as migration (Figure S1C and S1D, *P*<0.01), and invasion (Figure S1E and S1F, *P*<0.01) in si-231 plus calcitriol cells compared to si-231 and 231-NC cells. Furthermore, the combination of trastuzumab and calcitriol led to enhanced inhibition of proliferation between 48 to 72 hours (Figure S1B, *P*<0.01), migration (Figure S1C and S1D, *P*< 0.01), and invasion (Figure S1E and S1F, *P*<0.01) in si-231 cells compared to other groups. The ability of calcitriol to counteract the growth advantage induced by *Dicer* knockdown in BC cells suggests that calcitriol may enhance trastuzumab efficacy through *HER2* expression induction.

Calcitriol and trastuzumab synergistically inhibit BC growth in vivo

The effect of calcitriol and trastuzumab on TNBC was evaluated *in vivo*. No significant difference in tumor volumes was observed between MDA-MB-231 xenografts and those treated with trastuzumab (**Figure 5**, *P*=0.215). However, the growth of MDA-MB-231 xenografts treated with calcitriol was significantly reduced compared to untreated xenografts (**Figure 5**, *P*<0.001). Moreover, the tumor volume of MDA-MB-231 xenografts treated with a combination of calcitriol and trastuzumab was significantly smaller compared to xenografts in the three other groups (**Figure 5**, *P*<0.01). These data provide evidence that calcitriol and trastuzumab synergistically inhibit TNBC xenograft growth.

Discussion

Triple negative breast cancer (TNBC) is characterized by a poor prognosis, exhibiting high rates of recurrence, metastasis, and mortality. TNBC typically manifests at a younger age and is histologically classified as grade III [31]. Due to the absence of ER, PR, and *HER2* expression, TNBC patients are ineligible for corresponding targeted therapies [32]. Although TNBC demonstrates a higher pathologic complete reaction (pCR) to neoadjuvant chemotherapy, patients who fail to achieve pCR experience significantly worse survival outcomes [33]. Regrettably, TNBC patients achieve a

median survival time of only 8 years after enduring the side effects of anthracycline-taxane combination adjuvant chemotherapy [34]. The poor prognosis and limited treatment options render TNBC a formidable clinical challenge. According to the 2018 ASCO/CAP BC *HER2* testing guidelines [35], TNBC is characterized by low *HER2* expression, and patients with low *HER2* expression do not benefit from trastuzumab treatment [36].

Trastuzumab has been used as a first-line treatment for BC with favorable outcomes. However, its application is restricted to only 25%-35% of BC patients who exhibit *HER2* overexpression [11, 22, 37]. In an effort to expand trastuzumab's utility, we sought to identify an inducer capable of stimulating *HER2* overexpression in *HER2*-negative BC cells without exacerbating their aggressiveness, thereby enhancing their susceptibility to trastuzumab treatment. The investigations revealed that *Dicer* inhibited the TNBC cell line MDA-MB-231 and induced *HER2* overexpression, possibly sensitizing TNBC cells to trastuzumab targeting. As gene transfection to elevate *Dicer* level in BC tumors is clinically unfeasible, we explored the effects of calcitriol, a known *Dicer* inducer. Our findings demonstrate that calcitriol could induce Dicer overexpression and subsequent *HER2* overexpression, rendering TNBC cells sensitive to trastuzumab inhibition. Calcitriol enhanced trastuzumab-induced inhibition of cell proliferation and migration through the induction of *Dicer* and *HER2* overexpression in BC cells. These results suggest that calcitriol can improve the efficacy of anti-*HER2* antibody treatment by inducing *HER2* overexpression in TNBC cells. *In vivo* studies have shown that a calcitriol dose of 0.4 μg does not induce hypercalcemia in mice [38], and we found this dose sufficient to accelerate trastuzumab-induced inhibition of BC xenografts. However, appropriate calcitriol dosing for TNBC patients requires further investigation in subsequent clinical trials. Additionally, future studies are warranted to determine the optimal dosage combination of trastuzumab and calcitriol to achieve maximum therapeutic efficacy.

As an RNase III endonuclease involved in miRNA processing, altered *Dicer* expression may modify miRNA expression profiles, leading to both *HER2* overexpression and changes in the BC cell cycle. To elucidate the mechanism by which calcitriol promotes *Dicer* expression and induces *HER2* overexpression in TNBC cells, we conducted miRNA microarray analysis. This analysis identified candidate miRNAs affecting *HER2* expression following calcitriol treatment. A total of 12 miRNAs were identified with a fold change ≥200 upon calcitriol treatment (Figure S2; Table S2). Among the aberrantly expressed miRNAs, calcitriol-induced down-regulation of miR-145-5p has been shown to be negatively associated with *HER2* expression through the mitogen-activated protein kinase signaling pathway [39-41]. Conversely, calcitriol-induced up-regulation of miR-130b-3p was positively correlated with *HER2* expression through inhibition of PIEZO2, which is negatively associated with *HER2* [42]. In summary, calcitriol down-regulated miR-145-5p and up-regulated miR-130b-3p to promote *HER2* overexpression.

Conclusion

The *Dicer*-inducing agent calcitriol has been demonstrated to enhance the therapeutic efficacy of trastuzumab for TNBC patients who were previously unresponsive to anti-*HER2* targeted therapy. This enhancement is achieved through the induction of *HER2* overexpression, possibly expanding the application of trastuzumab to a broader patient population.

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Disclosure of conflict of interest

None.

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Table S1. The siRNA sequences

Name	*Sequence (5'-3')*
siRNA1-*Dicer*	GCUCGAAAUCUUACGCAAAUAdTdT
siRNA2-*Dicer*	CCACACAUCUUCAAGACUUAAdTdT
siRNA2-*Dicer*	GCUGGCUGUAAAGUACGACUAdTdT
siRNA-negative control	UUCUCCGAACGUGUCACGUTT

Table S2. Differentially expressed miRNAs in Calcitriol treated group compared to control group

miRNA	*p-Value*	*Fold change*	*Regulation*
hsa-miR-31-5p	2.10951E-13	-6251.558	Down
hsa-miR-199a-3p	3.2003E-13	-617.374	Down
hsa-miR-199b-3p	3.2003E-13	-617.374	Down
hsa-miR-145-5p	3.04009E-12	-342.509	Down
hsa-miR-625-5p	3.49017E-12	263.197	Up
hsa-miR-424-3p	5.4559E-12	261.379	Up
hsa-miR-31-3p	2.35431E-11	-235.568	Down
hsa-miR-328-3p	4.12605E-11	-213.783	Down
hsa-miR-4521	3.56659E-10	284.05	Up
hsa-miR-6872-5p	7.98357E-10	-501.463	Down
hsa-miR-129-2-3p	9.56361E-10	-206.5	Down
hsa-miR-130b-3p	1.14105E-08	212.306	Up

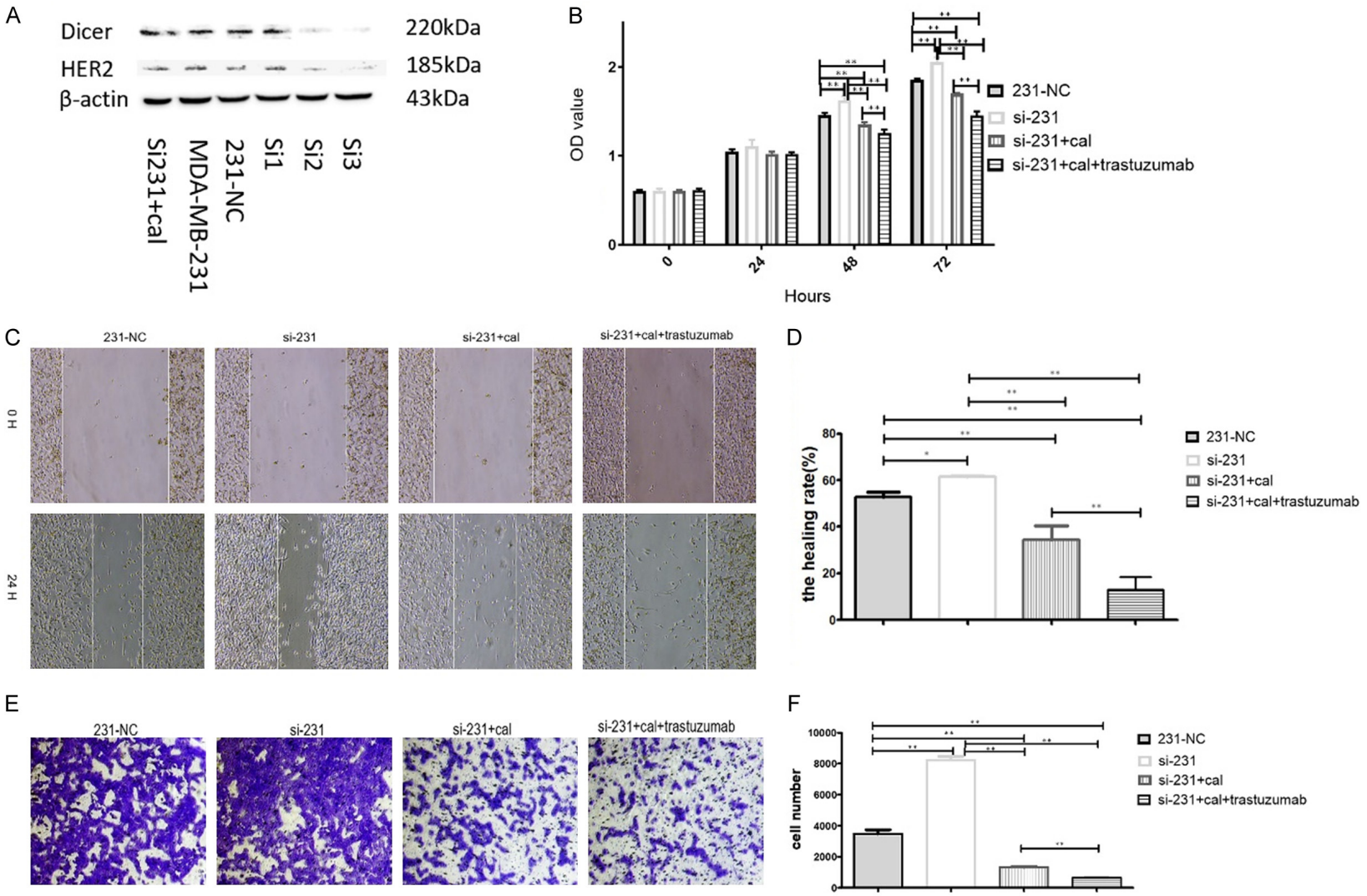

Figure S1. Calcitriol counteracts the growth promotion induced by *Dicer* knockdown in MDA-MB-231 cell. (A) Western Blot analysis of *Dicer* and *HER2* expression in BC cells; (B) The proliferation measurement by CCK-8 assay in MDA-MB-231 cell; (C) The migration measurement by wound healing assay in MDA-MB-231 cell; (D) Quantification of results of (C); (E) The invasion measurement by transwell assay in MDA-MB-231 cell; (F) Quantification of results of (E). $^{*}P<0.05$, $^{**}P<0.01$. *HER2*, human epidermal growth factor receptor 2; BC, breast cancer; CCK-8, cell counting kit8.

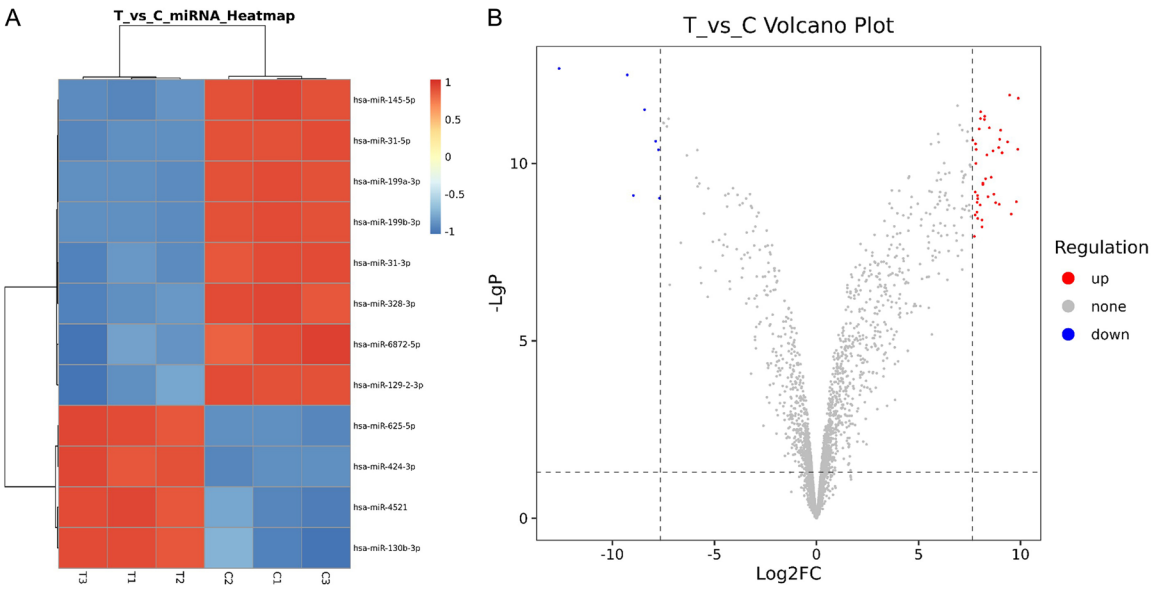

Figure S2. miRNA expression profile differences between the Calcitriol treated group and the control group. A. Hierarchical Cluster analysis of differentially expressed miRNAs between the Calcitriol treated group and the control group. B. Volcano plot shows significant differences between the Calcitriol treated group and the control group. T, Calcitriol treated group; C, Calcitriol untreated group.