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Original Article

MicroRNA-199a-3p promotes drug sensitivity in triple negative breast cancer by down-regulation of *BRCA1*

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Abstract: MiR-199a-3p was previously predicted to target tumor suppressor gene *BRCA1*, which has been linked to cancer onset and therapeutic response. In this study, the effects of miR-199a-3p-mediated *BRCA1* dysfunction on triple-negative breast cancer (TNBC) progression and chemosensitivity were assessed. The association between miR-199a-3p and *BRCA1* expression was examined in TNBC tumors and verified with luciferase reporter and protein assays. Tumorigenic functions of miR-199a-3p in TNBC cells were investigated by cell proliferation, clonogenic and migration assays. The sensitivities to chemotherapeutic drugs were tested with cisplatin and PARP inhibitor (veliparib) treatments. Mouse xenograft model was used to examine the effects of miR-199a-3p on tumor growth and drug response *in vivo*. MiR-199a-3p was shown to directly target *BRCA1* in TNBC cells, resulting its downregulation and reduced luciferase reporter activity mediated by *BRCA1* 3'-UTR. Ectopic miR-199a-3p in TNBC cells exerted inhibitory effects on cell proliferation, migration and xenograft tumor growth. Moreover, miR-199a-3p was shown to reverse cisplatin-resistance and sensitize TNBC cells to veliparib, which might be due to repressed DNA repair ability and induced cell apoptosis. Our results demonstrated the tumor suppressive effects of miR-199a-3p on TNBC and induction on chemotherapeutic sensitivities, which were correlated with *BRCA1* gene dysfunction. These findings may provide insights into the potential prognostic and therapeutic values of miR-199a-3p in patients with TNBC.

Keywords: MicroRNA, *BRCA1*, triple negative breast cancer, chemoresistance

Introduction

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer with the characteristics of early onset, poor clinical outcomes and higher rates of metastasis and recurrence [1, 2]. Due to the lack of hormonal receptor expression, traditional chemotherapy and targeted therapy remain the primary option of systemic treatment for TNBC. However, incomplete pathological response and chemoresistant relapse are common among TNBC patients. GeparSixto study reported the pathological complete response (pCR) rate of 36.9% and a 3-year survival rate of 76.1% for TNBC patients having standard chemotherapy [3]. The onset and treatment sensitivity of TNBC have been associated with the tumor suppressor genes, *BRCA1* and *BRCA2*.

BRCA1/2 are high-penetrance genes for susceptibility to the hereditary breast and ovarian cancer syndrome (HBOC) [4]. Both genes are key components of the homologous recombination (HR) DNA repair pathway and maintenance of genomic stability by exerting multiple functions in cell cycle checkpoints arrest, apoptosis, chromatin remodeling and transcriptional regulation [5, 6]. Mutations in *BRCA1/2*, particularly *BRCA1*, are linked to elevated risks of developing TNBC [7] and ovarian cancers [8]. However, *BRCA1/2* mutation carriers were also found to have better therapeutic response to platinum-based agents [9, 10] and poly-ADP ribose polymerase (PARP) inhibitors [11], due to the impaired HR DNA repair capability and thus accumulated DNA lesions in *BRCA*-deficient cells [12]. Recent clinical studies have demonstrated that DNA-damaging platinum agents

improved the outcomes of neoadjuvant chemotherapy regimens in TNBC patients with *BRCA1/2* mutations [13].

BRCA1 mutation carriers account for a minority (10-20%) of TNBCs and are less common in sporadic breast cancer. However, aside from germline or somatic mutations, *BRCA1* dysfunction or the "BRCAness" phenotype could be attributed to epigenetic regulation such as promoter methylation [14], and microRNA-mediated gene silencing [15, 16]. MicroRNAs (miRNAs) are post-transcriptional regulators that might act as tumor-suppressors or oncomirs in carcinogenesis, and were thus studied as biomarkers for disease progression and therapeutic sensitivity. For instance, tumor-specific expressions of miR-7 and miR-340 have shown prognostic values for neoadjuvant chemotherapy in breast cancer [17]. Recent studies have also identified several *BRCA1*-targeting miRNAs including miR-9, miR-182 and miR-638, which have been demonstrated to enhance drug sensitivities to platinum-based agents and PARP inhibitors in breast and ovarian cancer cells [15, 16, 18]. Identification of the subgroup of TNBC patients with wild-type but miRNA-mediated dysfunctional *BRCA1* might provide great benefits to improve prognosis and therapeutic efficiency.

In our previous study, we have identified miR-199a-5p as a potential TNBC-associated biomarker, which showed reduced expressions in TNBC as compared with non-TNBC and healthy subjects [19]. We further demonstrated the tumor suppressive roles of miR-199a-5p in regulating epithelial-mesenchymal transition process in TNBC cells [20]. On the other hand, its counterpart miR-199a-3p has been predicted as one of the potential *BRCA1*-targeting miRNAs [15]. MiR-199a-3p has been shown to be downregulated in different cancer cell types including breast [21], ovarian [22], prostate [23], hepatocellular carcinoma [24] and renal cancers [25]. Recent studies have reported miR-199a-3p as a tumor suppressor to regulate cancer cell proliferation, metastasis, drug resistance and cancer stemness [26, 27]. However, its functions in TNBC progression and chemo-therapeutic sensitivity are not fully understood.

In this study, we test the hypothesis that miR-199a-3p is a target of *BRCA1* in TNBC by confirming its expression in TNBC tumor tissues and by sequence-specific reporter and protein assays. miR-199a-3p may exert inhibitory functions in TNBC cell growth and progression, at least partially, by inducing *BRCA1* dysfunction, and whether miR-199a-3p-mediated *BRCA1*-dysfunction associates with the chemosensitivity and therefore confers drug-resistance in TNBC were also explored.

Materials and methods

Patients

32 TNBC patients with no germline *BRCA1/2* mutations were recruited with written informed consents from Queen Mary Hospital and Tung Wah Hospital through the Hong Kong Hereditary and High-Risk Breast Cancer Programme. Blood, tumor specimens and clinical information were collected. Germline *BRCA1/2* mutation status was tested as described previously [28]. This study was approved by the Institutional Review Board of The University of Hong Kong and Hospital Authority, Hong Kong West Cluster (UW 15-441).

Cells, drugs and reagents

Human TNBC cell lines, MDA-MB-231 and MDA-MB-468, carrying the wild-type *BRCA1* were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 5% CO_2 atmosphere. A cisplatin-resistant subline of MDA-MB-231 cells (hereby termed as cisR) was developed by chronic culture with cisplatin-supplemented medium. Cisplatin was obtained from Sigma-Aldrich (Saint Louis, MO, USA). PARP inhibitor, veliparib, was obtained from Selleckchem (Houston, TX, USA). *BRCA1* silencing RNA (siRNA) and miR-199a-3p and -5p mimics were purchased from Qiagen (Hilden, Germany). Mouse anti-human BRCA1 antibody (ab16780) and Alexa Fluor 488-conjugated anti-mouse IgG antibody (ab150113) were purchased from Abcam (Cambridge, MA, USA). Mouse anti-β-actin (8H10D10) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA).

Table 1. Primer sequences used for quantitative RT-PCR and plasmid constructions

Primers	Primer nucleotide sequence (5'-3')
For quantitative RT-PCR	
ACTB-F	AGAGCTACGAGCTGCCTGAC
ACTB-R	AGCACTGTGTTGGCGTACAG
BRCA1-F	AGGAACCTGTCTCCACAAAGT
BRCA1-R	TTTGGCACGGTTTCTGTAGC
U6B	ACGCAAATTCGTGAAGCGTT
hsa-miR-199a-3p	ACAGTAGTCTGCACATTGGTTA
For cloning of luciferase reporter constructs	
BRCA1-3UTR-F	TGACTGGCTAGCGGACACCTACCTGATACCCCA
BRCA1-3UTR-R	TGACTGGTCGACTCAAGTCTTCACTGCCCTTGC
BRCA1-3UTR-R-WT	TTAGTAGTCGACACAGTAGAAGGACTG
BRCA1-3UTR-R-Mut	TTAGTAGTCGACAACTGGAAAGGACTGAAGAGTG
For cloning of short-hairpin RNA expression plasmids	
BRCA1-shRNA-F	GATCCGCAGGAAATGGCTGAACTAGAACTTCCTGTCATTCTAGTTCAGCCATTTCCTGTTTTTTGGAAA
BRCA1-shRNA-R	AGCTTTTCCAAAAAACAGGAAATGGCTGAACTAGAATGACAGGAAGTTCTAGTTCAGCCATTTCCTGCG
miR-199a-3p shRNA-F	GATCCGTAACCAATGTGCAGACTACTGTCTTCCTGTCAACAGTAGTCTGCACATTGGTTATTTTTTGGAAA
miR-199a-3p shRNA-R	AGCTTTTCCAAAAAATAACCAATGTGCAGACTACTGTTGACAGGAAGACAGTAGTCTGCACATTGGTTACG

F: forward primer, R: reverse primer.

Drug sensitivity assay

Drug sensitivities of TNBC cells upon miR-199a-3p induction or *BRCA1* silencing were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays (Sigma-Aldrich). Cells were transfected with miR-199a-3p mimic (50 nM), *BRCA1* siRNA (20 nM) or negative control using Lipofectamine 3000 (Thermo Fisher Scientific) for 72 h. Transfected cells were seeded onto 96-well plates at a density of 5,000 cells per well and cultured for 24 h. Cells were treated with indicated concentrations of cisplatin (72 h) or veliparib (5 days), and cell viability was assayed with MTT incubation for 2 h. Absorbance at 570 nm was measured using Multiskan FC Microplate Photometer (Thermo Fisher Scientific).

RNA extraction and quantitative RT-PCR

Total RNAs were isolated from homogenized tissue and cell samples using miRNeasy Mini Kit (Qiagen). For detection of miRNAs, RNA was reverse transcribed using the miScript PCR System (Qiagen), and U6 snRNA was used as internal control. For detection of *BRCA1*, RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and β-actin (ACTB) was used as internal control. Quantitative PCR was performed using LightCycler 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany) with the corresponding gene primers (**Table 1**), and relative gene expressions were calculated using the $2^{-\Delta\Delta Ct}$ method.

Dual-luciferase reporter assay

BRCA1 3'-untranslated region (UTR) fragments containing the wild type (WT) or mismatched (Mut) miR-199a-3p seed binding sequences were PCR amplified (**Table 1**) from human genomic DNA, and cloned into pmirGLO (Promega, Madison, WI, USA) luciferase reporter vector. The reporter constructs were co-transfected with miR-199a-3p, -5p mimics or negative control into MDA-MB-231 cells. Cells were lysed after 72 h transfection, Firefly and *Renilla* luciferase activities were measured using Dual-Glo Luciferase Assay System (Promega).

Western blot

Cells were harvested in cell lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail (Thermo Fisher Scientific). Protein samples were resolved by SDS/PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). After blocking with 5% bovine serum albumin (BSA), the membranes were

incubated with anti-BRCA1 (1:500) or anti-ACTB (1:2000) antibodies overnight at 4°C. Proteins were blotted with horseradish peroxidase-conjugated secondary antibodies and were visualized using the Amersham Enhanced Chemiluminescence Detection Reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunofluorescence microscopy

Cells cultured on coverslips were fixed with 4% paraformaldehyde, followed by permeabilization with 0.3% Triton X-100, blocked with 3% BSA, and incubated with anti-BRCA1 (1:200) antibody at 4°C overnight. After washing, coverslips were incubated with Alexa Fluor 488-conjugated secondary antibody (1:200) at room temperature for 1 h, and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) for 10 min. Images were acquired under immunofluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan).

Clonogenic and wound healing assays

For clonogenic assay, transfected cells were seeded onto 6-well plates at a density of 500 cells per well and cultured in complete medium for 7 days. Cell colonies were fixed with methanol and stained with 0.5% crystal violet. For wound healing assay, cells were seeded onto 6-well plates at 90% confluency for 24 h. Line scratches were made through the cell layer using pipette tips, and images were acquired at 0, 10 and 72 h under microscope. Cell colonies and scratch areas were analyzed using ImageJ software version 1.50i (National Institutes of Health).

Host cell reactivation assay

Firefly luciferase (*luc2*) and *Renilla* luciferase genes (*Rluc*) were cloned into pcDNA3.1(+) expression vector (Thermo Fisher Scientific) as reporter constructs. 2 μg plasmid pcDNA3.1/*luc2* in Tris/EDTA buffer was treated with or without cisplatin (1 μM) at 37°C for 6 h, and harvested with ethanol precipitation. Cisplatin-damaged or undamaged pcDNA3.1/*luc2* was co-transfected with pcDNA3.1/*Rluc* as internal control, together with miR-199a-3p mimic, *BRCA1* siRNA or negative control into cells. Cells were lysed 72 h after transfection, and relative luciferase activities were measured using Dual-Glo Luciferase Assay System.

Flow cytometry analysis of apoptosis and cell cycle arrest

Cell apoptosis was assessed using FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Briefly, cells were stained with 5 μl each of FITC Annexin V and propidium iodide (PI, Thermo Fisher Scientific) in 100 μl of 1× Binding Buffer at room temperature for 15 min. For cell cycle analysis, cells were fixed with cold 70% ethanol overnight, and then stained with 20 μg/ml of PI and 0.2 mg/ml of RNase A (Thermo Fisher Scientific) for 30 min. Stained cells were counted using BD FACSCalibur (BD Biosciences) flow cytometer and analyzed with FlowJo software (FlowJo LLC, Ashland, OR, USA).

Stable cell lines and in vivo xenograft study

MiR-199a-3p and *BRCA1* short-hairpin RNA (shRNA) expression plasmids were constructed by cloning the annealed oligonucleotides (**Table 1**) into pSilencer 2.1-U6 neo vector (Thermo Fisher Scientific). Cells were transfected with shRNA expression plasmids and stable cell clones were isolated from G418 selection (Thermo Fisher Scientific). The stable cells were subcutaneously injected into mammary fat pads of 5-week-old female NOD-SCID mice. Tumor volumes were measured weekly and calculated with the formula "pi/6 × length × width^2". When tumor volumes reached 100 mm^3, mice were randomly divided into groups with or without cisplatin administration. Mice were injected intraperitoneally with PBS-diluted cisplatin (5 mg per kg weekly) for 4 weeks before being sacrificed. All animal experiments were carried out in accordance with regulations from the Committee on the Use of Live Animals in Teaching and Research (CULATR; 4409-17) of The University of Hong Kong.

Statistical analysis

GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. All experiments were performed in triplicates and repeated in at least three independent experiments, data was expressed as the mean ± standard error of mean (SEM). Statistical analyses were performed using two-

Table 2. Clinical characteristics of triple negative breast cancer patients

Mean age at diagnosis	60.06 y
Age range	30-83 y
Age at breast cancer diagnosis	n (total 32)
Below 40	3
40-49	2
50-59	13
60 or above	14
Bilateral cases	2
Metastasis cases	4
Histology (Tumors)	
Invasive ductal carcinoma	29
Metaplastic	1
Mixed	2
Stage	
1	9
2	17
3	5
4	1
Personal history of ovarian cancer	2
Family history of breast cancer (first- and second-degree relatives)	5
5-year survival	
Yes	6
No	5
Not available	21

tailed Student's t-test or analysis of variance (ANOVA) unless indicated otherwise. P<0.05 was considered statistically significant.

Results

Correlation of miR-199a-3p and BRCA1 in TNBC tumors

The underexpression of miR-199a-3p has been previously reported in various cancer types including breast cancer [21], however its expression profile in TNBC tumors remained inadequate. We hence tested the expression of miR-199a-3p in TNBC tumor and adjacent non-tumor tissues. The clinic-pathological information of 32 TNBC patients with no germline *BRCA1/2* mutation was summarized in **Table 2**. Quantitative RT-PCR results showed that miR-199a-3p expression was downregulated in TNBC tumors as compared with the adjacent non-tumor tissues (**Figure 1A**). Furthermore, to assess the correlation between miR-199a-3p and *BRCA1*, their intrinsic levels in the tumor specimens were compared. Spearman correlation test of *BRCA1* and miR-199a-3p expressions resulted in a significantly inverse correlation (P=0.034), suggesting a causal relationship (**Figure 1B**).

MiR-199a-3p targets BRCA1 in TNBC

MiR-199a-3p was *in silico* predicted as one of the potential *BRCA1*-targeting miRNAs using algorithms including DIANA-microT-CDS (http://www.microrna.gr/microT-CDS) and RNA22 (https://cm.jefferson.edu/rna22/Interactive/). We identified a potential 7-mer binding seed sequence from the *BRCA1* 3'-UTR, locating at +99 to +105 bp downstream of the stop codon (**Figure 1C**). To confirm whether *BRCA1* is a direct target of miR-199a-3p, luciferase reporter assays were performed to assess the interaction between *BRCA1* 3'-UTR and miR-199a-3p. Results showed that ectopic miR-199a-3p, but not its counterpart miR-199a-5p, significantly reduced the relative luciferase activity mediated by *BRCA1* 3'-UTR WT-sequence in MDA-MB-231 cells (**Figure 1D**). No significant differences were seen in the empty nor the Mut-sequence construct groups.

Next, we directly analyzed BRCA1 protein levels upon miR-199a-3p overexpression. Western blot results showed that BRCA1 proteins were downregulated upon miR-199a-3p mimic transfection, in parallel with the *BRCA1* siRNA positive controls, in MDA-MB-231, MDA-MB-231/cisR and MDA-MB-468 cells (**Figures 1E** and S1A). Similarly, ectopic miR-199a-3p suppressed the immunofluorescent signal intensity against BRCA1 protein (**Figure 1F**). Taken together, these data suggested that *BRCA1* is a direct target of miR-199a-3p in TNBC.

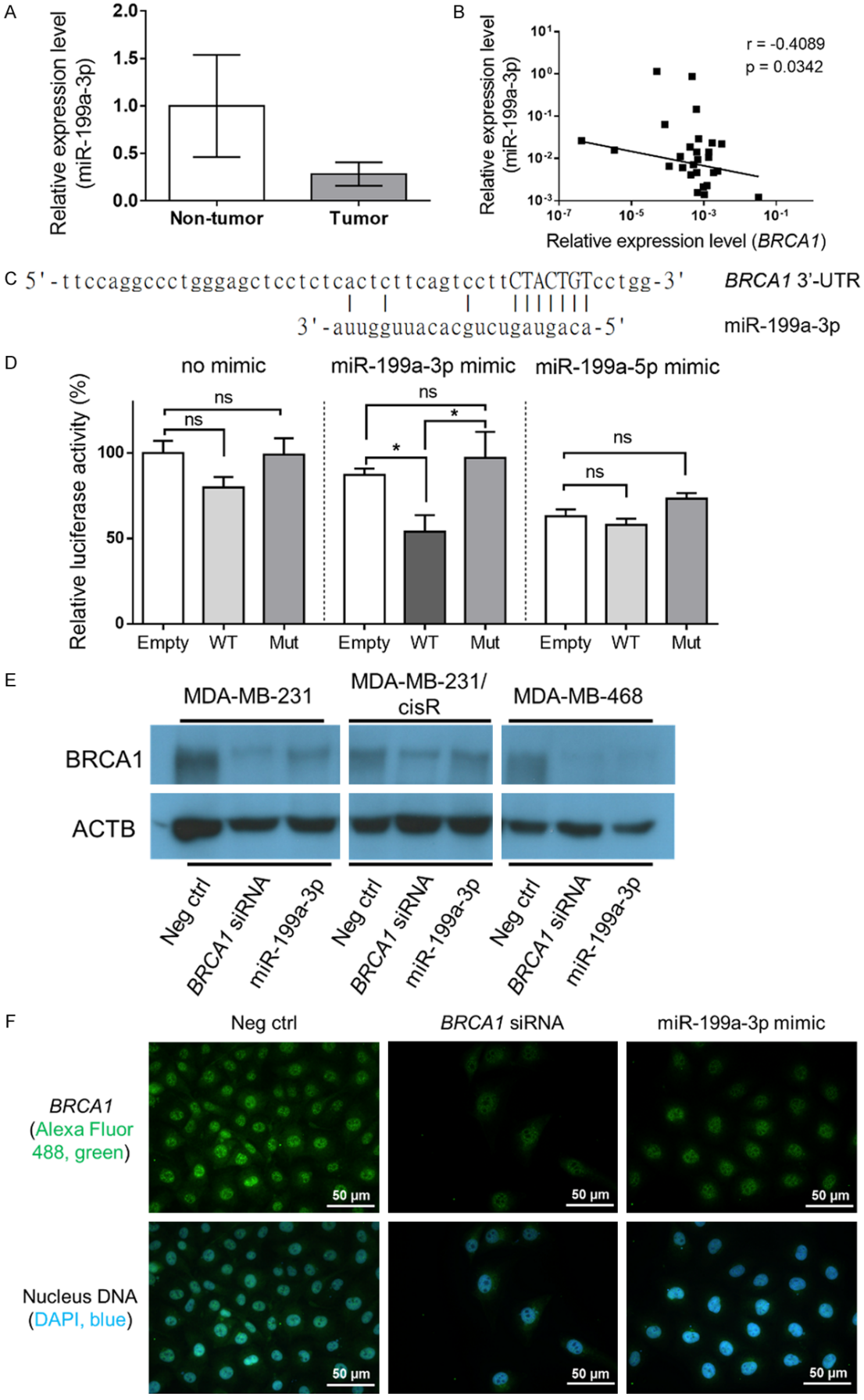
A
Relative expression level (miR-199a-3p)
2.0
1.5
1.0
0.5
0.0
Non-tumor
Tumor
B
Relative expression level (miR-199a-3p)
r = -0.4089
p = 0.0342
Relative expression level (BRCA1)
C
5'-ttccaggccctgggagctcctctcactcttcagtccttCTACTGTcctgg-3'
BRCA1 3'-UTR
3'-auugguuacacgucugaugaca-5'
miR-199a-3p
D
no mimic
miR-199a-3p mimic
miR-199a-5p mimic
Relative luciferase activity (%)
ns
*
Empty
WT
Mut
E
MDA-MB-231
MDA-MB-231/ cisR
MDA-MB-468
BRCA1
ACTB
Neg ctrl
BRCA1 siRNA
miR-199a-3p
F
Neg ctrl
BRCA1 siRNA
miR-199a-3p mimic
BRCA1 (Alexa Fluor 488, green)
Nucleus DNA (DAPI, blue)
50 μm

Figure 1. MiR-199a-3p targets *BRCA1* in TNBC. A. Relative expression of miR-199a-3p in TNBC tumor and adjacent non-tumor tissues from 32 non-*BRCA1/2* mutation carriers. B. A scatter plot showing the negative correlation between *BRCA1* and miR-199a-3p expressions in TNBC tumors from 32 non-*BRCA* mutation carriers, analyzed using Spearman correlation test. C. The predicted miR-199a-3p binding site on human *BRCA1* 3'-UTR, with the microRNA binding seed sequence shown in upper case letters. D. Dual-luciferase reporter assays showing the relative luciferase activity mediated by *BRCA1* 3'-UTR after co-transfection with miR-199a-3p or -5p mimic or negative control in MDA-MB-231 cells. Negative control: empty pmirGLO vector; WT/Mut: pmirGLO vector inserted with the wild-type or mismatched *BRCA1* 3'-UTR sequences, respectively. E. Representative images of Western blot analysis of BRCA1 protein expression in MDA-MB-231, cisR and MDA-MB-468 cells transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control. F. Representative images (at 200× magnification) of immunofluorescent staining of BRCA1 protein (green) in MDA-MB-231 cells transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control. *P<0.05, ns: not significant.

MiR-199a-3p suppresses cell proliferation, clonogenic ability and migration

We next investigated the tumorigenic functionalities of miR-199a-3p in TNBC. Using the highly metastatic MDA-MB-231 and MDA-MB-231/cisR and the less aggressive MDA-MB-468 cells, we evaluated the miR-199a-3p-mediated *BRCA1*-dysfunction on cell proliferation and aggressiveness. As shown by MTT assays, ectopic expression of miR-199a-3p and silenced *BRCA1* resulted in significant suppression in cell proliferation (**Figure 2A**). The clonogenic assays also showed marked reduction of clonal capacities in cells transfected with miR-199a-3p mimic or *BRCA1* siRNA, as compared to the mock transfection groups (**Figure 2B** and **2C**). Furthermore, miR-199a-3p significantly reduced cell migration rates in TNBC cells, as shown by the larger scratch areas in wound healing assays (**Figure 2D** and **2E**). Collectively, these results demonstrated that miR-199a-3p exerted inhibitory effects on cell growth and migration *in vitro*.

MiR-199a-3p sensitizes cells to chemotherapeutic drugs

As *BRCA1* plays a critical role in the HR pathway of DNA repair, which greatly contributes to chemotherapeutic sensitivity, it led our interest to investigate whether miR-199a-3p-induced *BRCA1* dysfunction could disrupt DNA repair and sensitize TNBC cells to different classes of chemotherapeutic drugs. First, a cisplatin-resistant subline of MDA-MB-231 (cisR) was developed, which was characterized by a higher intrinsic *BRCA1* expression as compared with the parental MDA-MB-231 (**Figure 3A** and **3B**). Short-term cisplatin treatments (72 h) in MDA-MB-231, MDA-MB-231/cisR and MDA-MB-468 cells were demonstrated to induce *BRCA1* expressions (**Figures 3C** and S1B), concurrent with reduced miR-199a-3p (**Figure 3D**), which might be the induced HR pathway in response to DNA damage. Subsequently, cytotoxicity MTT assays showed that restoration of miR-199a-3p level and *BRCA1* silencing significantly sensitized both parental and resistant MDA-MB-231 cells to cisplatin (**Figure 4A**). Similar inductive effects on chemosensitivity were also observed in MDA-MB-468 cells.

As *BRCA1/2* mutation-related tumors have been shown to confer hypersensitivity to PARP inhibitors in epithelial ovarian cancer [29] and TNBC [30], we also examined the effects of miR-199a-3p-induced *BRCA1* dysfunction on TNBC cell sensitivity towards PARP inhibitor, veliparib. As shown in **Figure 4B**, MDA-MB-231, MDA-MB-231/cisR and MDA-MB-468 cells transfected with miR-199a-3p mimic significantly increased their sensitivities to veliparib, whilst *BRCA1* silencing conferred hypersensitivity. These results suggested that miR-199a-3p might enhance TNBC sensitivity to chemotherapeutic drugs, at least partially, through the dysfunction of *BRCA1*.

MiR-199a-3p suppresses DNA repair, induces cell cycle arrest and apoptosis

Since drug resistance to DNA-damaging agents is highly associated with the regulation of cell cycle arrest, DNA repair and apoptosis, these functions in response to cisplatin and miR-199a-3p were examined. DNA repair function was investigated using host cell reactivation assays with cisplatin-damaged luciferase reporter plasmids (**Figure 5A**). Results showed that pre-treatments of cells with cisplatin (5 μM for 72 h) resulted in a significantly higher reactivated luciferase activity in MDA-MB-231/cisR, suggesting an increased DNA repair activity possibly due to the cisplatin-induced *BRCA1*

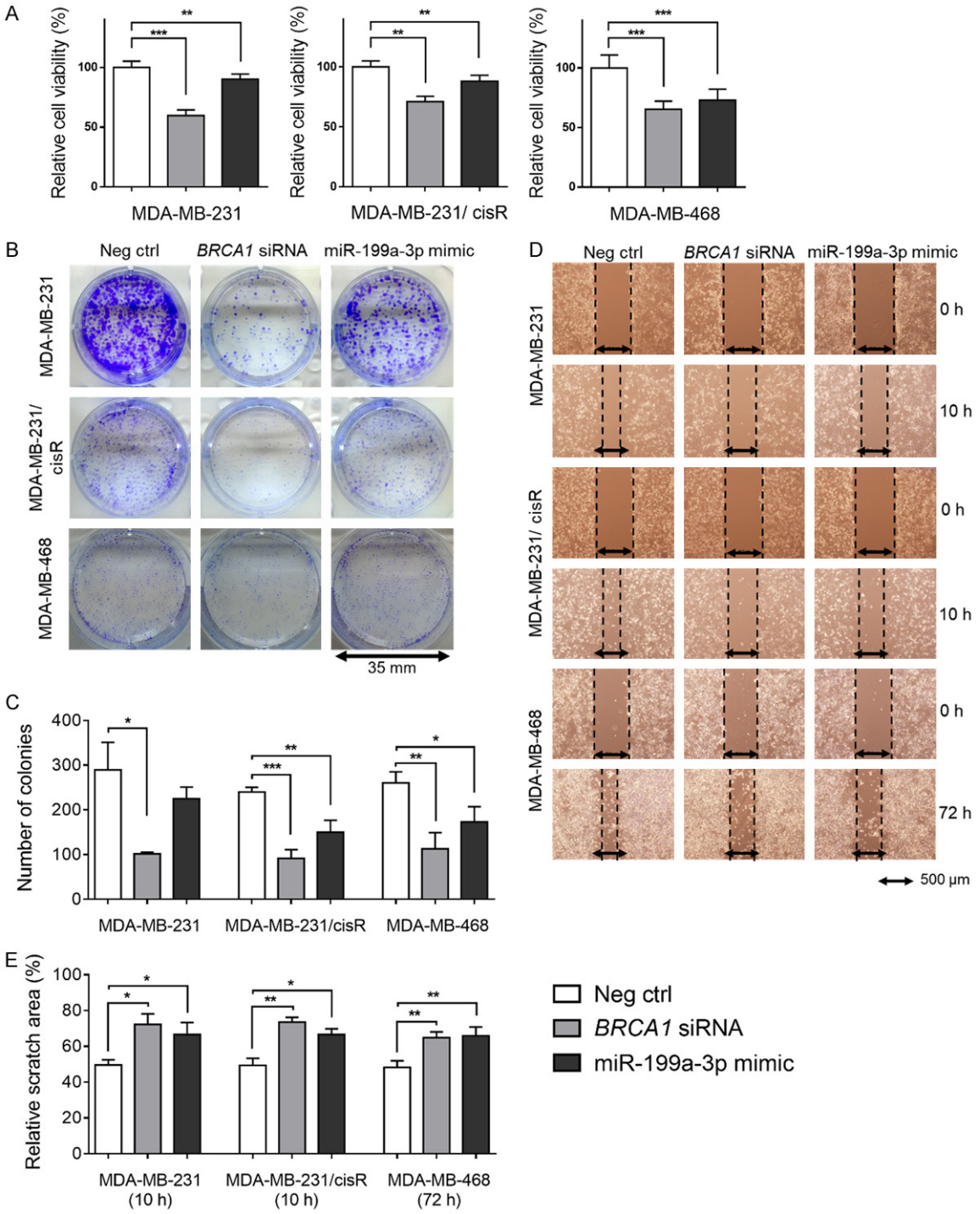

Figure 2. MiR-199a-3p suppresses cell proliferation, clonogenic ability and migration. (A) MTT assays showing the relative cell viability of MDA-MB-231, cisR and MDA-MB-468 cells transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control for 72 h. (B) Representative images of clonogenic assays and (C) numbers of colony formation in different TNBC cells transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control. (D) Representative images of wound healing assays showing the migration of different TNBC cells transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control at 0, 10 or 72 h after cell monolayer scratching, at 40× magnification. (E) Relative cell scratch areas of different TNBC cells at 10 or 72 h after cell monolayer scratching, as determined using ImageJ software v1.50i. *P<0.05, **P<0.01, ***P<0.001.

expression. On the other hand, ectopic miR-199a-3p and *BRCA1* siRNA significantly reduced the luciferase activities in both MDA-MB-231 and MDA-MB-231/cisR cells, suggest-

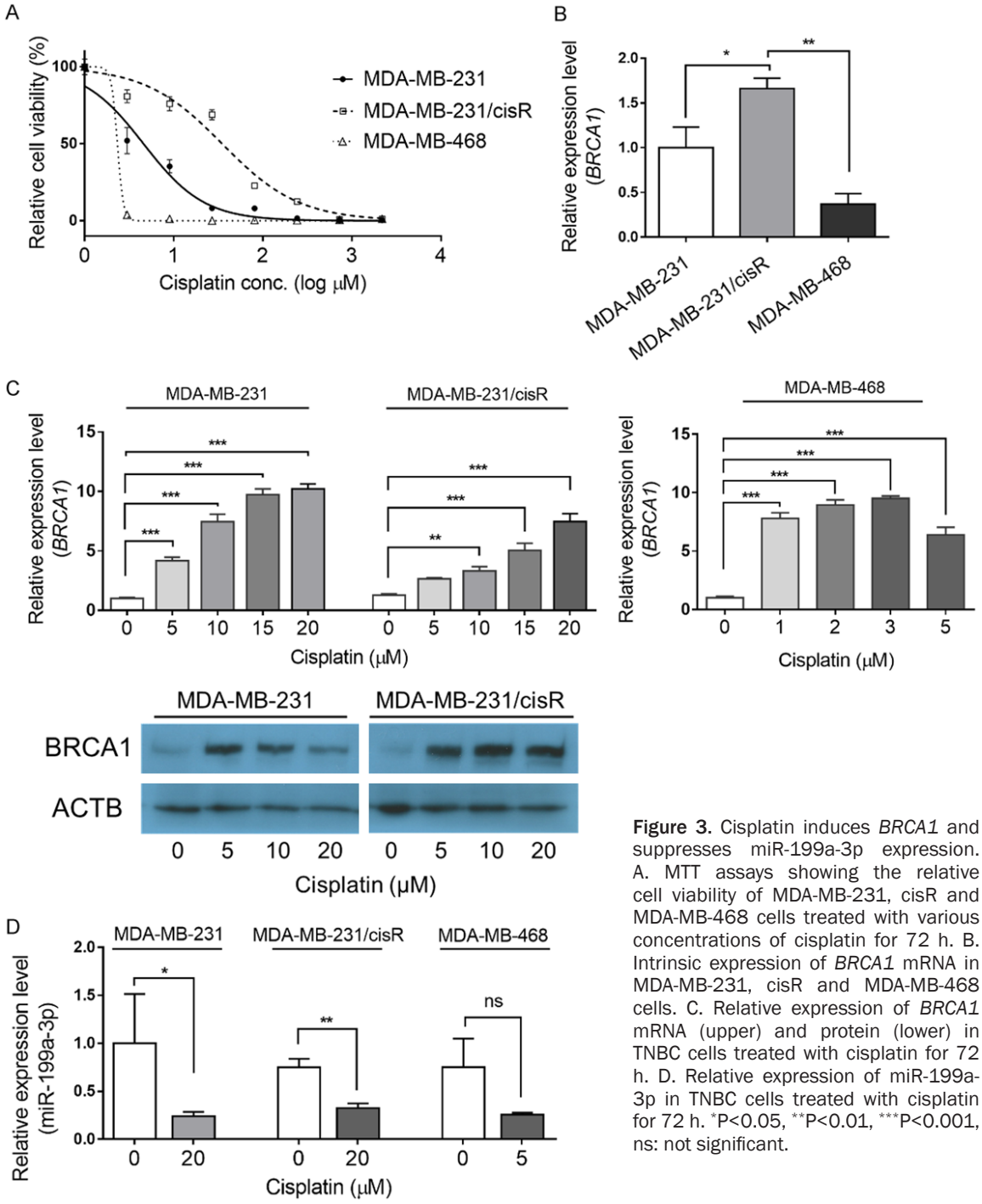

Figure 3. Cisplatin induces *BRCA1* and suppresses miR-199a-3p expression. A. MTT assays showing the relative cell viability of MDA-MB-231, cisR and MDA-MB-468 cells treated with various concentrations of cisplatin for 72 h. B. Intrinsic expression of *BRCA1* mRNA in MDA-MB-231, cisR and MDA-MB-468 cells. C. Relative expression of *BRCA1* mRNA (upper) and protein (lower) in TNBC cells treated with cisplatin for 72 h. D. Relative expression of miR-199a-3p in TNBC cells treated with cisplatin for 72 h. *P<0.05, **P<0.01, ***P<0.001, ns: not significant.

ing a suppressed DNA repair activity from *BRCA1* dysfunction.

Cisplatin was known to cause DNA cross-linking, which induced the G2/M phase arrest and HR repair pathway for resolution [31]. Cell cycle analysis (Figure S2) showed that cisplatin significantly increased the sub-G1 cell population and caused G2/M phase arrest in MDA-MB-231 and MDA-MB-468, whilst conversely, the effect was not as significant as in the resistant MDA-MB-231/cisR cells (**Figure 5B**). Neither ectopic miR-199a-3p nor *BRCA1* silencing alone caused significant alternation in cell cycle phases. However, it was found that miR-199a-3p combined with cisplatin treat-

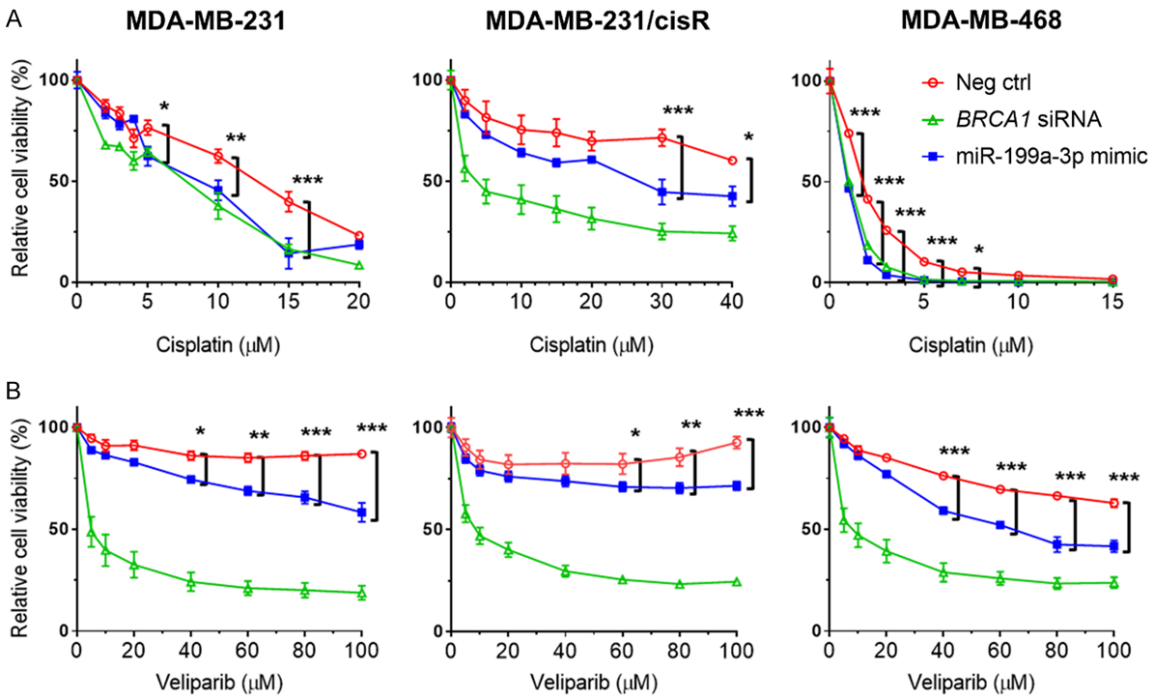

Figure 4. MiR-199a-3p sensitizes TNBC cells to chemotherapeutic drugs. MTT assays showing the relative cell viability of MDA-MB-231 (left), cisR (middle) and MDA-MB-468 cells (right) transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control, and treated with indicated concentrations of (A) cisplatin for 72 h or (B) veliparib for 5 days. *P<0.05, **P<0.01, ***P<0.001.

ment had an additive effect on G2/M phase arrest in both parental and MDA-MB-231/cisR cells.

We next examined the effects of miR-199a-3p overexpression and *BRCA1* silencing on cell apoptosis by Annexin V apoptosis assays. Results showed that cisplatin caused slight increase in apoptotic cell populations in both MDA-MB-231 and MDA-MB-468, but not in MDA-MB-231/cisR cells with higher cisplatin tolerance (**Figure 5C**). Ectopic miR-199a-3p showed little inductive effects on cell apoptosis, but it showed an additive effect with cisplatin in MDA-MB-231 and MDA-MB-468 cells.

MiR-199a-3p suppresses TNBC xenograft growth and induced in vivo chemo-sensitivity

To verify the tumor suppressive effects of miR-199a-3p-mediated *BRCA1* dysfunction *in vivo*, we developed stable cell lines with miR-199a-3p overexpression and *BRCA1*-shRNA silencing from the MDA-MB-231/cisR cells, which were designated as cisR/miR-199a-3p and cisR/*BRCA1*-shRNA, respectively. 5×10^5 and 1×10^6 cells were injected into mammary fat pads of NOD-SCID mice to investigate tumor growth and drug sensitivities. MDA-MB-231/cisR xenografts were found to have a larger tumor growth rate than those of MDA-MB-231 at week 5 (**Figure 6A**). On the other hand, cisR/miR-199a-3p and cisR/*BRCA1*-shRNA xenografts had reduced tumor sizes when compared with MDA-MB-231/cisR-bearing mice.

Lastly, cisplatin was demonstrated to significantly reduce the tumor sizes of all cell groups after 4 weeks of drug treatments (**Figure 6B**). Notably, MDA-MB-231/cisR tumors showed the least degree of size reduction among different groups, whilst cisR/*BRCA1*-shRNA and cisR/miR-199a-3p tumors were more sensitized to cisplatin treatments, resulting in significantly greater reduction in tumor volumes (**Figure 6C**).

Discussion

BRCA1 mutation carriers were associated with higher risks of developing the basal-like or triple-negative subtype of breast tumors [32]. The underlying mechanisms remain uncertain.

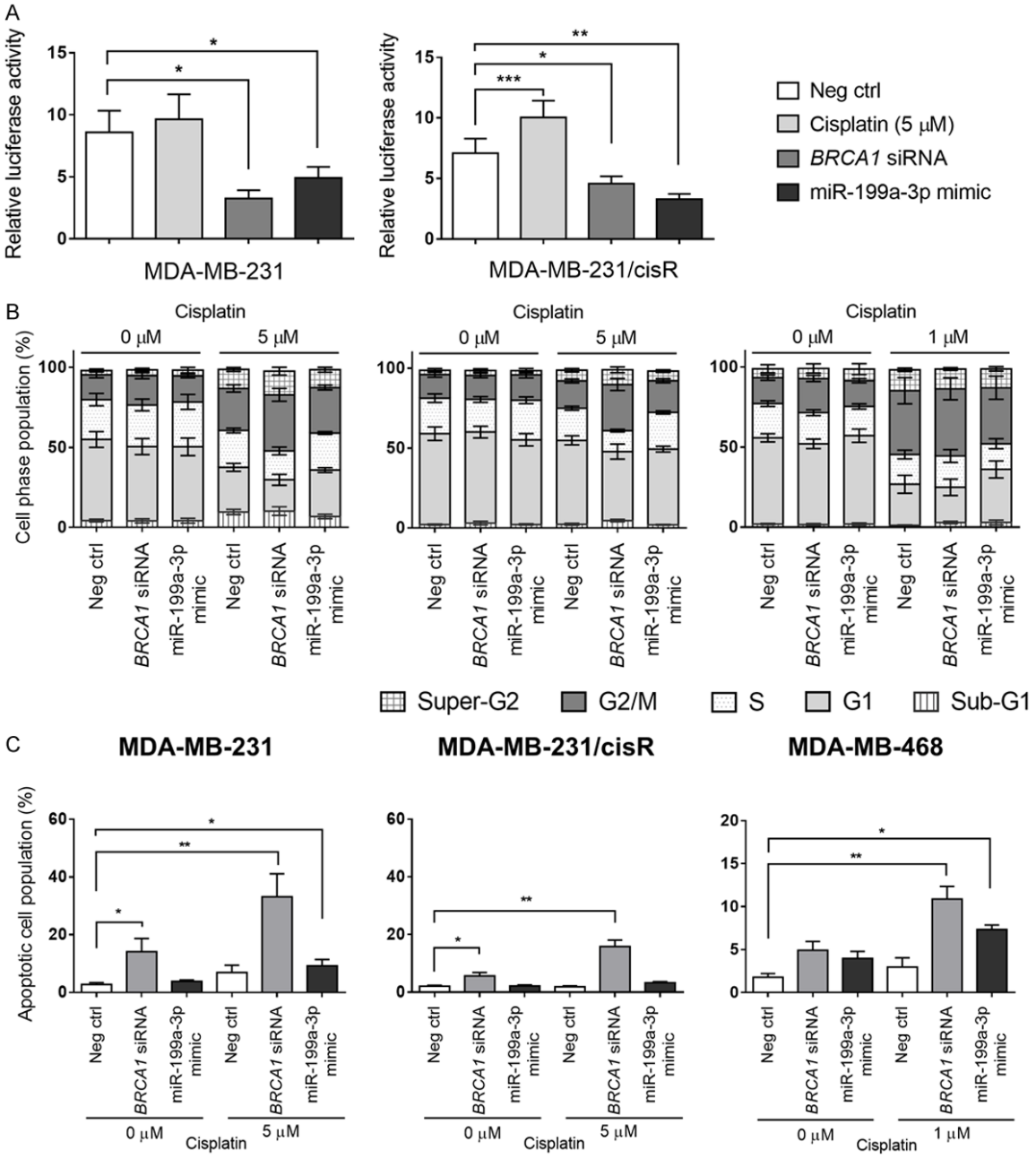

Figure 5. MiR-199a-3p suppresses DNA repair, induces cell cycle arrest and apoptosis. (A) Relative DNA repair capability of MDA-MB-231 (left) and cisR cells (right) on the damaged luciferase reporter plasmids pre-treated with cisplatin in host cell reactivation assays. (B) Cell cycle analysis and (C) Annexin V apoptosis analysis of MDA-MB-231, cisR and MDA-MB-468 cells transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control and treated with 1 or 5 μM cisplatin for 72 h. *P<0.05, **P<0.01, ***P<0.001.

However, it has been suggested that *BRCA1* modulates the mammary progenitor cell differentiation through other cell fate regulator such as *SNAI2*, and controls the mammary epithelial lineage commitment between secretory luminal cells and contractile basal-like cells [33-35]. Aside from germline mutations, *BRCA1* dysfunction has been attributed to different miRNA suppression. For instance, Moskwa *et al.* first reported that miR-182 downregulated *BRCA1* expression in breast cancer cells, resulting in impaired HR-mediated DNA repair and hypersensitivity to *PARP1* inhibitors [18]. Subsequently, miR-9 was identified from a

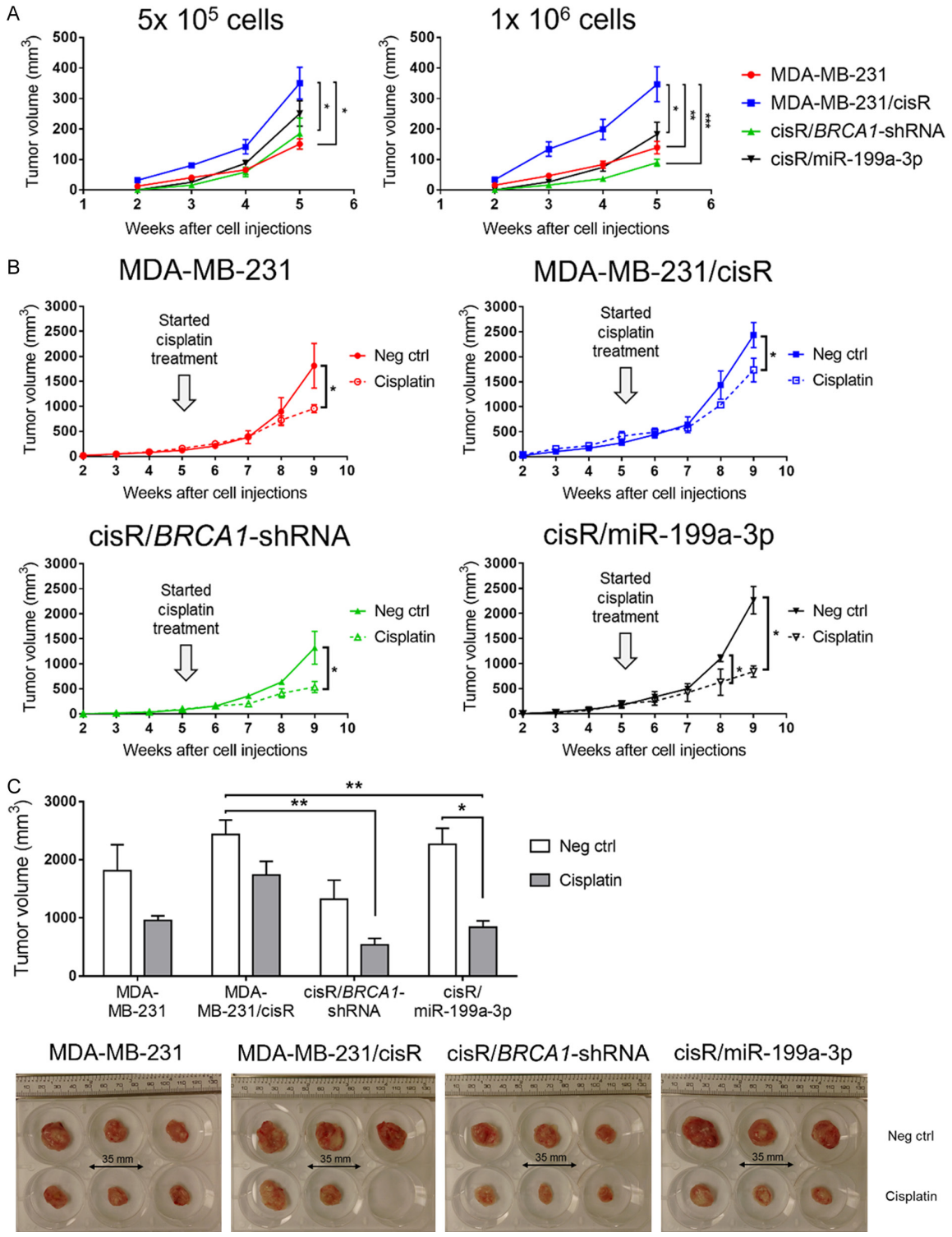

Figure 6. MiR-199a-3p suppresses TNBC xenograft growth and induced *in vivo* chemo-sensitivity. A. Tumor growth curves showing the estimated volumes of NOD-SCID mice xenografts developed from 5×10^5 cells (left) and 1×10^6 cells (right) of MDA-MB-231, MDA-MB-231/cisR, cisR/*BRCA1*-shRNA and cisR/miR-199a-3p cells. B. Tumor growth curves showing the estimated volumes of different xenografts in NOD-SCID mice with weekly cisplatin administration. C. The measured tumor volumes (upper) and images (lower) of different xenografts in NOD-SCID mice after 4 weeks of cisplatin treatment. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

reverse miRNA library screening, showing an inverse correlation with *BRCA1* expression in serous ovarian cancer tissues. Patients with higher miR-9 levels were found to have better progression-free survival and therapeutic response to platinum-based chemotherapy [15]. Moreover, miR-638 expression was downregulated in the early pre-invasive stages of breast cancers [36], and it enhanced TNBC chemotherapy sensitivity by targeting *BRCA1* and impairing DNA repair [16]. These studies demonstrated the potentials of miRNA-mediated *BRCA1* dysfunction in TNBC.

miR-199a-5p has previously been identified as a TNBC-associated biomarker [19], and its miRNA counterpart, miR-199a-3p, had been predicted to target *BRCA1* by multiple *in silico* algorithms [15]. Our study showed that miR-199a-3p was negatively correlated with *BRCA1* levels in TNBC tumors (**Figure 1B**), and ectopic miR-199a-3p repressed the luciferase reporter activity mediated by *BRCA1* 3'-UTR (**Figure 1E**), suggesting miR-199a-3p is a direct target of *BRCA1*. MiR-199a-3p downregulation has been widely reported in various cancer types, including breast, ovarian, hepatocellular, renal, colorectal, prostate, osteosarcoma and thyroid cancers [23, 24, 27, 37-41]. Similar observation was also seen in our cohort of TNBC tumors (**Figure 1A**). However, it did not reach statistical significance possibly due to the limited sample size. MiR-199a-3p exhibited tumor suppressor function by inhibiting multiple pathways, including mTOR/c-Met and Jagged1-Notch signaling in hepatocarcinoma [24, 37] and renal cancer [42], the PAK4/MEK/ERK pathway in breast cancer [43], and the stem cell marker CD44 in prostate cancer and osteosarcoma [26, 44]. In consistent with other studies, our results showed that overexpression of miR-199a-3p inhibited TNBC cell proliferation, aggressiveness and xenograft growth (**Figures 2** and **6A**).

BRCA1 has been well documented for its functions in maintaining genomic stability, regulating cell cycle checkpoint arrest and HR DNA repair pathway. HR deficiency caused by *BRCA1* dysfunction was shown to enhance chemosensitivity in breast and ovarian cancers. We also demonstrated that miR-199a-3p significantly sensitized cells to cisplatin both *in vitro* and *in vivo* (**Figures 4** and **6C**), concurrent with the impaired DNA repair ability and induction of cell apoptosis (**Figure 5**). All these findings suggested that the action of miR-199a-3p on tumor inhibition and drug sensitization was at least partially correlated with *BRCA1* dysfunction in breast cancer.

With the advances in personalized medicine and genetic testing technologies, *BRCA1/2* mutation screening has become a common practice for high-risk HBOC patients for deciding therapeutic options. For instance, new emerging class of PARP inhibitors (olaparib, rucaparib and niraparib) have been approved for the treatments of germline *BRCA*-mutated advanced ovarian cancers, and were recently applied to advanced metastatic breast cancers, showing a significantly longer progression-free survival (median: 7.0 months vs. 4.2 months) over standard therapy [45]. Although *BRCA1/2* represent the top prevalence genes for HBOC, majority (about 80%) of the TNBC patients do not harbor germline mutation. Findings from the Phase II clinical trial of platinum monotherapy for metastatic TNBC (TBCRC009) demonstrated an improved platinum sensitivity in patients with germline *BRCA* mutations and non-carriers with *BRCA*-associated genomic instability signature [13]. Hence, further investigation is needed to determine whether the expression levels of miR-199a-3p and other *BRCA1*-targeting miRNAs can be used as circulating biomarker to identify patients with *BRCA* dysfunction, as well as predicting treatment outcomes. This might facilitate the development of specific chemotherapy and identification of TNBC patients who may benefit from DNA-damaging agents.

In conclusion, we demonstrated for the first time that miR-199a-3p directly targeted *BRCA1* and exerted tumor suppressive functions in TNBC. MiR-199a-3p enhanced cell sensitivities to DNA-damaging agent (cisplatin) and PARP inhibitor (veliparib), which correlated with its inhibitory effects on host cell DNA repair and induction of cell apoptosis. Our results provide a better understanding on miRNA-induced *BRCA1* dysfunction in TNBC progression, and new insights for clinical significance to those non-*BRCA1* mutation carriers with *BRCA* dysfunction.

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Disclosure of conflict of interest

None.

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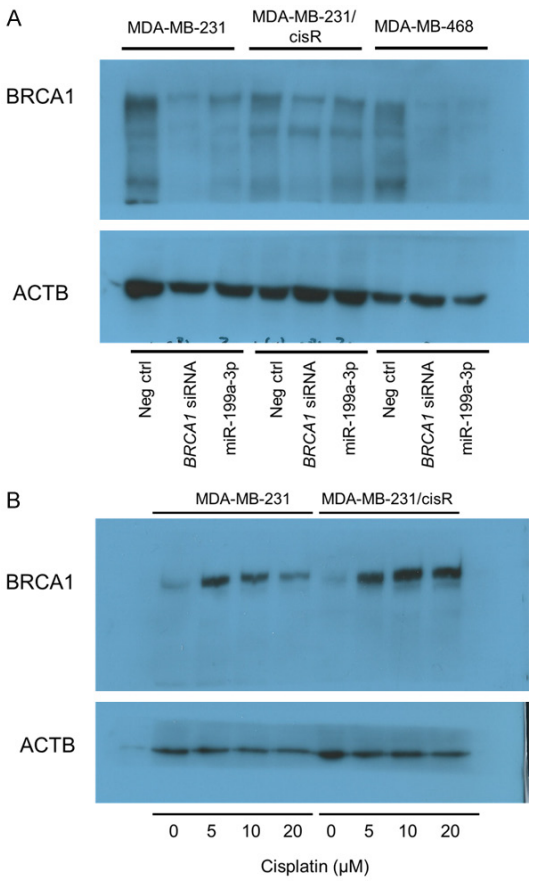

Figure S1. Western-blot analyses showing BRCA1 protein levels in (A) MDA-MB-231, cisR and MDA-MB-468 cells transfected with miR-199a-3p mimic, *BRCA1* siRNA or negative control, and (B) in TNBC cells treated with indicated concentrations of cisplatin for 72 h.

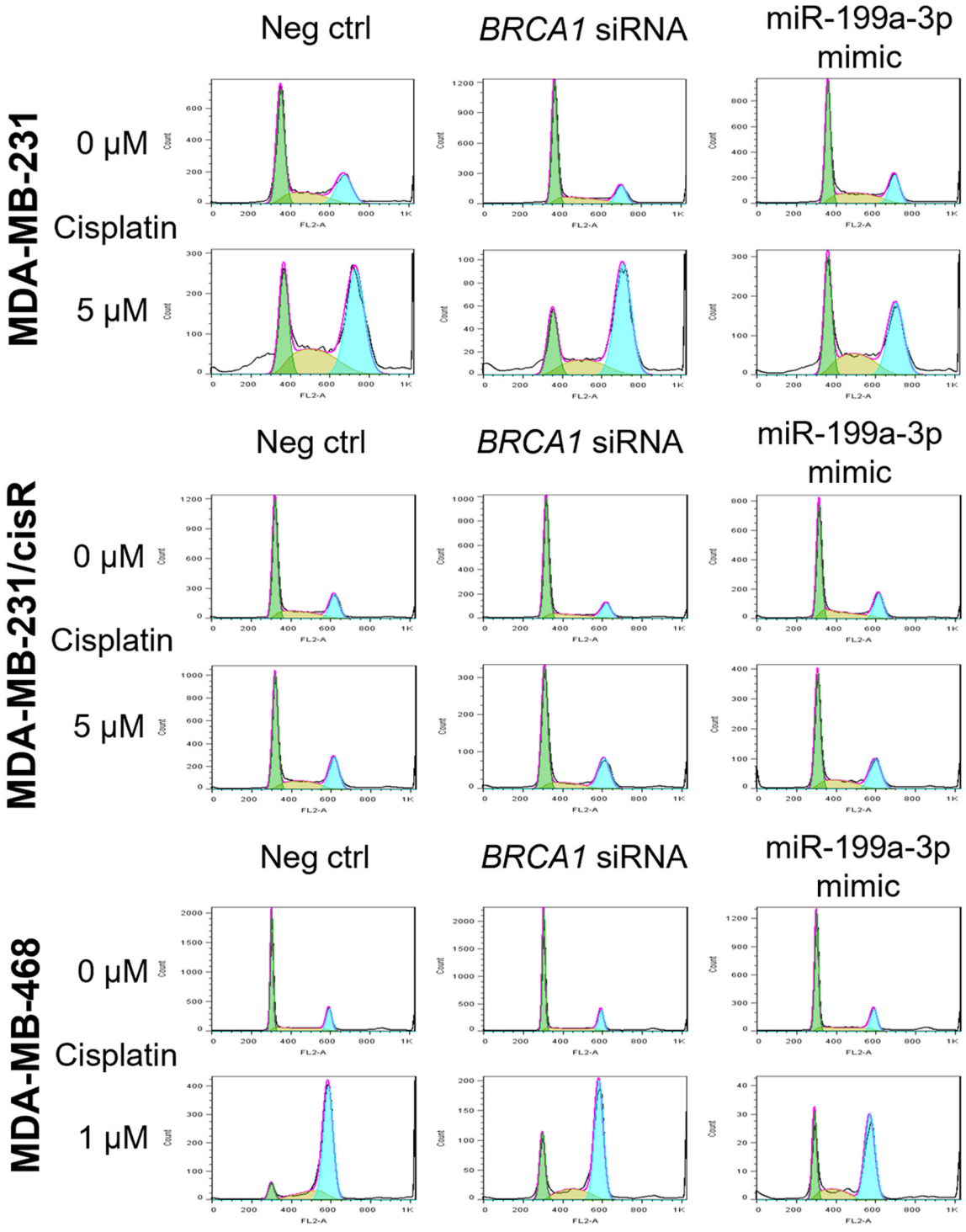

Figure S2. MiR-199a-3p and cisplatin treatments induce cell cycle arrest. Flow cytometry analyses showing the cell cycle phase distributions of MDA-MB-231, cisR and MDA-MB-468 cells transfected with miR-199a-3p mimic, *BRCA1* siRNA, or negative control and treated with 1 or 5 μM cisplatin for 72 h.