

Knockdown of DNA/RNA-binding protein KIN17 promotes apoptosis of triple-negative breast cancer cells

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Abstract. Effective therapy for breast cancer has been extensively studied worldwide, particularly for triple-negative breast cancer and drug-resistant subtypes. DNA/RNA-binding protein KIN17 (kin17) has been reported to be significantly upregulated in breast cancer cells, and is proposed to serve a role in the regulation of cell proliferation. The present study further investigated the association of kin17-knockdown with breast cancer cell apoptosis. Cell Counting kit-8, flow cytometry, TUNEL assay and caspase 3/7 analysis were performed on MDA-MB-231 cells to determine the association between kin17 and breast cancer cell apoptosis. In addition, western blot analysis was performed to investigate the mechanism of kin17 in the apoptosis of MDA-MB-231 cells. The results revealed that knockdown of kin17 inhibited proliferation and promoted apoptosis of MDA-MB-231 cells, and suggested a poly (adenosine diphosphate-ribose) polymerase-related mechanism behind the apoptosis of the cells. These findings suggested that kin17 could become a novel target for breast cancer therapy.

Introduction

Breast cancer is the most common type of cancer and the second leading cause of cancer-related mortality in women globally (1). Although the mortality rates have recently

decreased due to the advancement in detection techniques and treatment (2), the efficacy of available breast cancer therapies remain unsatisfactory. For instance, triple-negative breast cancer (TNBC) and drug resistance pose a therapeutic challenge. Currently, there are no specific targets for TNBC (3). Thus, investigation into novel biomarkers that can act as drug targets is critical (4-6).

DNA/RNA-binding protein KIN17 (kin17) is a constitutively expressed protein in mammalian cells that is generally expressed at low levels in human tissues and organs (7). Studies have shown that kin17 is closely correlated with DNA replication, mRNA processing and cell cycle regulation (8,9). Kin17 was previously revealed to be upregulated in various tumors, including breast cancer, colorectal carcinoma, hepatocellular carcinoma and non-small cell lung cancer (10-13). Specifically, Zeng *et al* (10) demonstrated that kin17 is essential for the proliferation of breast cancer. Therefore, the present study aimed to comprehensively investigate the role of kin17 in breast cancer.

In the present study, the influence of kin17 knockdown in the proliferation and apoptosis of MDA-MB-231 cells, the representative cell line of TNBC which is negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), was investigated.

Materials and methods

Cell culture and transfection. Human breast cancer MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% antibiotic cocktail (60 µg/ml penicillin and 100 µg/ml streptomycin). The cultures were maintained in 5% CO₂ and 95% humidity at 37°C. Cells were seeded in 6-well plates at a density of 1x10⁵ cells/well and transfected with lentiviral vector against kin17 (MDA-MB-231^{KD} cells) or NC vector (MDA-MB-231^{NC} cells) (Shanghai GeneChem Co., Ltd., Shanghai, China) using opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) and

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polybrene (Shanghai GeneChem Co., Ltd., Shanghai, China) according to the manufacturer's protocol. The volume of lentiviral vector against kin17 and NC vector were 3.5 and 4.6 μ l, respectively, which were calculated according to the manufacturer's formula and the concentration of polybrene was 5 μ g/ml as recommended by the manufacturer. MDA-MB-231 cells without transfection with vector (Mock MDA-MB-231 cells) were used as a blank control. The transfected cells were cultured in the suspension supplemented with 1.5 μ g/ml of puromycin on the pre-medium in 5% CO₂ and 95% humidity at 37°C and the subsequent experiments were conducted when the cells containing the fluorescent vector reached 90% and the knockdown of kin17 was verified by western blot analysis prior to any other experiments.

Cytotoxicity assays. Cytotoxicity was measured in 96-well plates using a Cell Counting kit-8 (CCK-8) assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to manufacturer's protocols. MDA-MB-231^{NC} and MDA-MB-231^{KD} cells were seeded at a density of 2,000 cells/well and allowed to grow for 24 h. Absorbance was measured at 450 nm using amicroplate reader (Thermo Fisher Scientific, Inc.) following incubation for 24, 48, 72, 96 and 120 h.

Clone formation test. The clone formation test was performed to measure cell proliferation. MDA-MB-231^{NC} and MDA-MB-231^{KD} cells were cultured in 6-well plates at a density of 200 cells/well for 2 weeks. Cell clones were immobilized with methanol and stained with crystal violet at room temperature for 15-20 min. Colonies containing >50 cells were counted using CKX41 Inverted MicroScope (OLYMPUS Corporation, Tokyo, Japan) at x10 magnification and ImageQuant TL7.0 Image Analysis software was applied to image analysis (GE Healthcare Life Sciences, Little Chalfont, UK).

Flow cytometry. MDA-MB-231^{NC} and MDA-MB-231^{KD} cells were digested with EDTA-free trypsin and harvested by centrifugation at 400 x g for 5 min at room temperature. The collected cells were washed twice using PBS (Gibco; Thermo Fisher Scientific, Inc.). The final pellet was resuspended in binding buffer and stained with Annexin V-APC and propidium iodide (PI) (Shanghai GeneChem Co.) for 15 min in the dark at room temperature prior to the apoptosis analysis. For cell cycle analysis, the final pellet was fixed in 70% cold ethanol overnight at 4°C and treated with RNaseA for 30 min in a water bath at 37°C. PI staining was then performed according to the manufacturer's protocol. Flow cytometry analysis (BD LSRFortessa™; BD Biosciences, San Jose, CA, USA) was used for the determination of the level of apoptosis and distribution of the cell cycle. The fluorescence lifetime, intensity and other optical data were collected from 10,000 cells using Annexin V-APC or PI for apoptosis and cell cycle analysis, respectively, using BD FACSDiva Software v8.0.1 (BD Biosciences).

TUNEL assay. TUNEL assay was performed using *In Situ* Cell Death Detection kit, TMR Red (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. Positive and negative controls were assessed in parallel, and

the experiment was performed in triplicate. Briefly, cells were cultured in 96-well plates at a volume of 100 μ l/well and fixed with 4% paraformaldehyde at pH7.4 at room temperature for 60 min. Cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). PBS was then used to wash the cells. For the experimental and positive control groups, the prepared cells were incubated with DNase I at room temperature for 10 min and 50 μ l TUNEL reaction mixture was added following the incubation. The negative control group was treated with Label solution (50 μ l) only, without incubation with DNase I. The cells were then incubated with the mixture in a humidified atmosphere for 60 min at 37°C in the dark. DAPI was added prior to the analysis with a fluorescence microscope. Three high-power fields were randomly evaluated from each sample at x100 magnification.

Caspase 3/7 assay. MDA-MB-231^{KD} and MDA-MB-231^{NC} cells were harvested and resuspended in binding buffer. The suspension was added to 96-well plates in 100- μ l volumes to give a concentration of 1x10⁴ cells/well. Caspase-Glo® 3/7 assay (Promega Corporation, Madison, WI, USA) was later added to detect apoptotic cells according to the manufacturer's protocol. A blank control was set up in parallel to eliminate background interference. Absorbance was measured at 405 nm using amicroplate reader (Thermo Fisher Scientific, Inc.) following incubation for 2 h at 37°C. The activity of Caspase 3/7 in the MDA-MB-231^{KD} group was calculated compared with the NC group, and therefore the mean value of NC group was always 100%.

Western blot analysis. Protein was extracted using Cell Total Protein Extraction kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The MDA-MB-231^{KD}, MDA-MB-231^{NC} and mock MDA-MB-231 cells were digested by radioimmunoprecipitation lysis buffer on ice and then collected in Eppendorf tubes. The suspension was centrifuged at 20.8x10³ x g at 4°C for 20 min to collect the supernatant. Protein concentration was determined by Bradford protein quantitation assay (Nanjing KeyGen Biotech Co., Ltd.), and 5XSDS was added to 0.25% volume of the collected supernatant. The mixture was heated in boiling water for 10 min. Following this, 50 μ g protein from the MDA-MB-231^{KD} and MDA-MB-231^{NC} cells was loaded on to 12% SDS-PAGE gels. The separated proteins were transferred to polyvinylidene difluoride membranes (Merck KGaA) and blocked with 5% non-fat milk suspended in tris-buffered saline with 0.1% Tween-20 buffer at room temperature for 1 h. The samples were incubated with anti-kin17 (dilution, 1:500; cat. no. sc-32769; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-PARP (dilution, 1:1,000; cat. no. sc-8007; Santa Cruz Biotechnology, Inc.), anti-cleaved PARP (dilution, 1:1,000; cat no. #5625; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-GADPH (dilution, 1:500; cat no. sc-47778; Santa Cruz Biotechnology, Inc.) monoclonal primary antibodies overnight at 4°C. Following the incubation, the samples were washed with PBS and incubated with horseradish peroxidase-conjugated secondary antibodies (donkey anti-mouse IgG, dilution, 1:2,000, cat. no. sc-2318; Santa Cruz Biotechnology and goat anti-rabbit IgG, dilution, 1:2,000, cat. no. #7074; Santa Cruz Biotechnology) at room temperature

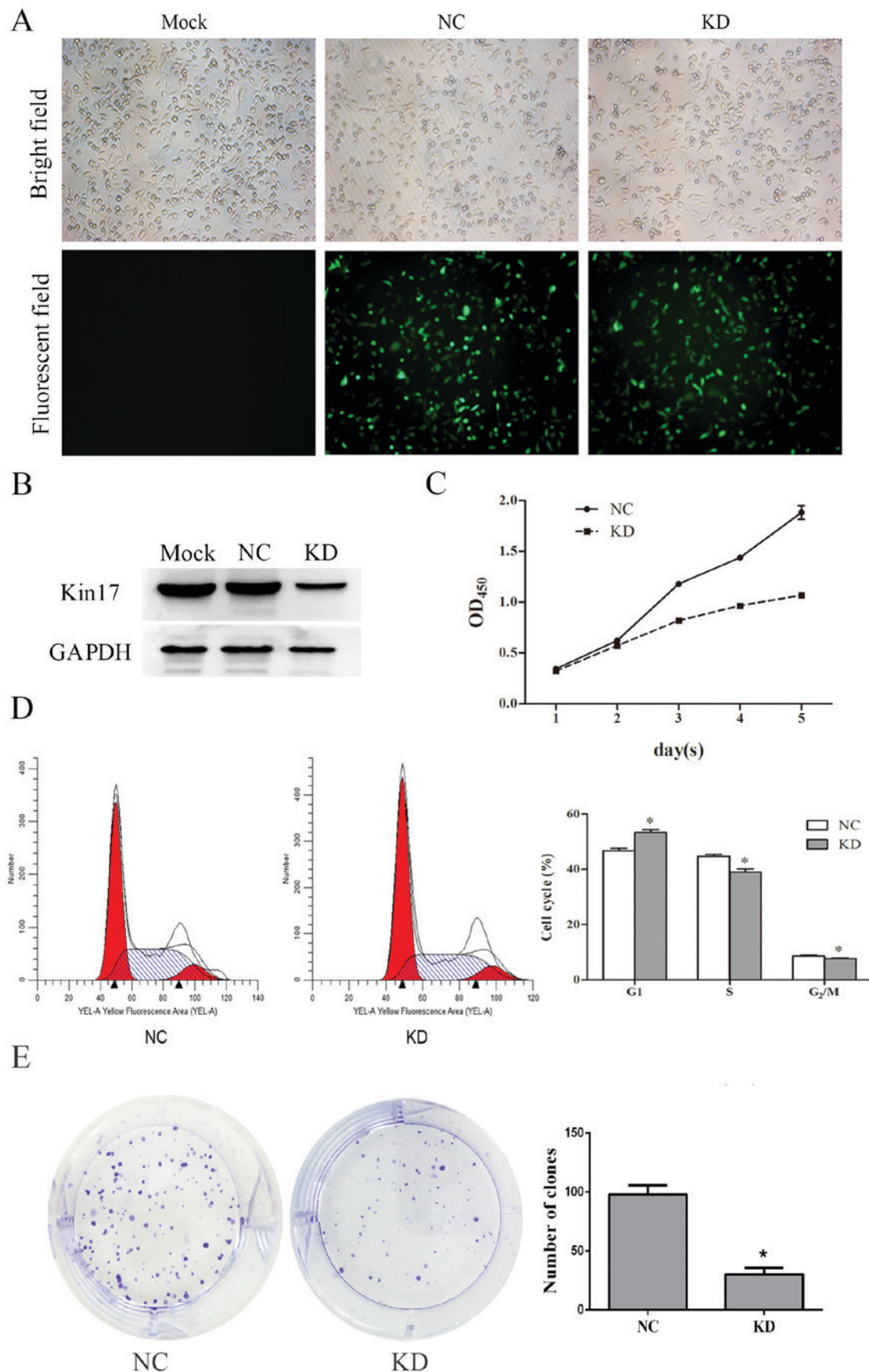


Figure 1. Kin17-knockdown inhibits the proliferation of MDA-MB-231 cells. (A) Morphological features and fluorescence-indicated transfection of mock MDA-MB-231, MDA-MB-231^{NC} and MDA-MB-231^{KD} cells (x100 magnification). (B) Protein level of kin17 in MDA-MB-231^{KD} cells was decreased followed kin17-knockdown. (C) CCK8 assay at 1, 2, 3, 4 and 5 days after seeding revealed differences in proliferation between the NC and KD groups. (D) The cell cycle profiles of MDA-MB-231^{KD} and MDA-MB-231^{NC} cells indicated the effect of kin17 knockdown, which blocked the cell cycle in the G1 phase. (E) Clone formation test revealed a difference in proliferation between the KD and NC groups. *P<0.05 vs. NC. KD, knockdown; NC, negative control; OD, optical density; kin17, DNA/RNA-binding protein KIN17.

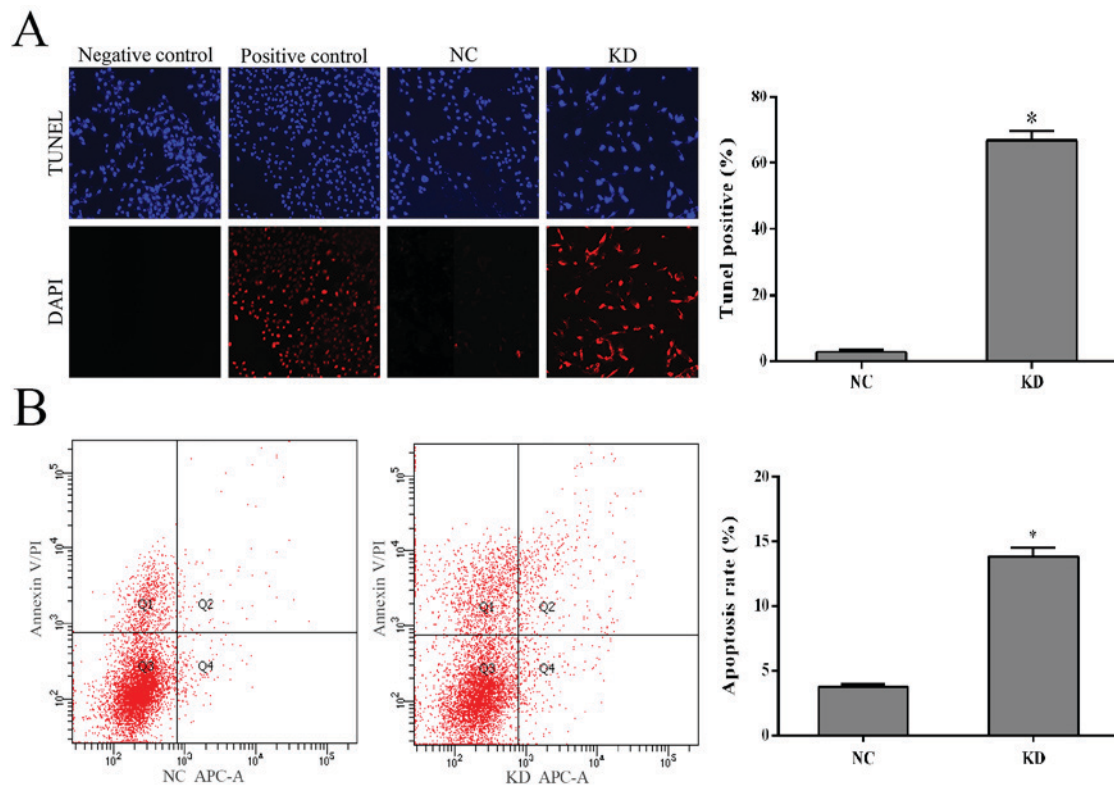


Figure 2. Knockdown of kin17 promotes the apoptosis of MDA-MB-231 cells. (A) Representative micrographs of TUNEL-positive cells are shown, and cumulative data of TUNEL levels for each group were analyzed. (B) Flow cytometry was used to assess the number of apoptotic cells and the sum of early and late apoptosis cells was calculated. Representative dot plots with APC fluorescence area (APC-A) as the horizontal ordinate and cumulative data shown. * $P < 0.05$ vs. NC. KD, knockdown; NC, negative control; kin17, DNA/RNA-binding protein KIN17.

for 1 h. Membranes were visualized with an enhanced chemiluminescence system (Fdbio Science, Hangzhou, China) and observed by QuantityOne v4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data analysis was performed with GraphPad Prism v6.0 and SPSS v19.0. Data were presented as mean \pm standard deviation. Continuity correction and Student's *t* test was used to assess variance among groups. All statistical tests and corresponding *P*-values reported were for two-sided tests, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Knockdown of kin17 reduces MDA-MB-231 cell proliferation. To investigate the role of kin17 in breast cancer, MDA-MB-231 cells were transfected with lentiviral vector against kin17 to knockdown its expression (Fig. 1A). Western blot assays confirmed that the protein levels of kin17 in the MDA-MB-231^{KD} cells were markedly decreased compared with the levels in the MDA-MB-231^{NC} and mock MDA-MB-231 cells (Fig. 1B).

CCK8 assay and cell cycle analysis were performed to assess the effects of kin17-knockdown on the proliferation of breast cancer cells. Notably, MDA-MB-231^{KD} cell proliferation was significantly reduced compared with that of MDA-MB-231^{NC} cells ($P < 0.05$; Fig. 1C), indicating that kin17-knockdown altered the proliferation of breast cancer cells. Cell cycle

analysis revealed that the ratios of cells in the S and G2/M phases were lower in the knockdown group compared with the NC group, while the percentage of G1 cells was higher in the knockdown group (Fig. 1D). The clone formation test was also performed to assess the effects of kin17 on proliferation, which revealed that the number of clones was significantly less in the knockdown group than in the NC group ($P < 0.05$; Fig. 1E).

Knockdown of kin17 enhances apoptosis of MDA-MB-231 cells. To assess the role of kin17 in breast cancer cell apoptosis, endogenous kin17 was silenced in MDA-MB-231 cells with a lentivirus vector against kin17. The kin17-knockdown group revealed significantly higher apoptosis rates compared with the NC group ($P < 0.05$) (Fig. 2A). TUNEL assay also indicated that kin17-knockdown enhanced apoptosis in the MDA-MB-231 cells compared with that in the control cells ($P < 0.05$; Fig. 2B).

Caspase 3/7 may be involved in kin17-knockdown-induced apoptosis. Caspase 3/7 were detected following 3 days of culture. Notably, caspase activity was higher in the kin17-knockdown group than in the kin17 negative control group ($P < 0.05$; Fig. 3).

Kin17-knockdown may induce apoptosis of MDA-MB-231 cells via poly (adenosine diphosphate-ribose) polymerase (PARP). The effect of kin17-knockdown on expression levels of PARP and cleaved PARP in MDA-MB-231 cells was investigated. The expression levels of PARP were markedly

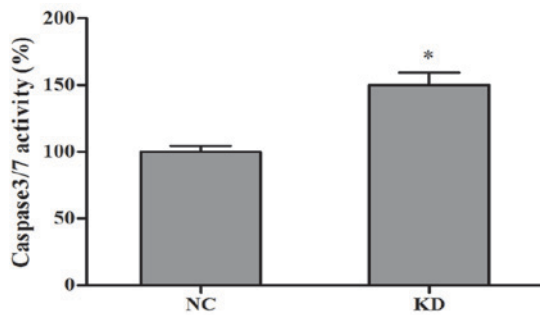


Figure 3. Caspase 3/7 activity of the kin17^{KD} group was normalized to the kin17^{NC} group, the average value of which was set as 100%. *P<0.05 vs. NC. KD, knockdown; NC, negative control; kin17, DNA/RNA-binding protein KIN17.

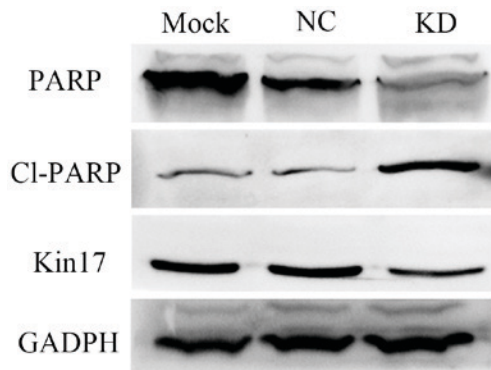


Figure 4. Knockdown of kin17 following the transfection of lentiviral vector affected the level of PARP and cleaved PARP in the MDA-MB-231 cells. PARP, poly (adenosine diphosphate-ribose) polymerase; CI-PARP, cleaved PARP; KD, knockdown; NC, negative control; kin17, DNA/RNA-binding protein KIN17.

decreased following kin17 knockdown, while the level of cleaved PARP was simultaneously increased compared with mock and negative control cells (Fig. 4).

Discussion

TNBC is a subtype of breast cancer that does not express ER, PR and HER2; it accounts for 15% of all breast cancer cases (14). Patients with TNBC always demonstrate higher rates of mortality and metastasis compared with other subtypes due to the absence of effective target therapies (15,16). Efforts to identify effective targets for TNBC have thus far yielded unsatisfactory results.

To investigate the potential targets for TNBC, the role of kin17 in a TNBC cell line was investigated. Kin17, a constitutive gene with extremely low expression in the majority of human tissues, has been reported to be markedly overexpressed in multiple tumors including breast cancer (10-13). In the present study, a lentiviral vector was used to stably knockdown kin17. Kin17-knockdown was revealed to significantly suppress the proliferation of MDA-MB-231 cells and prevent G1 to S phase transition, corroborating the findings of Zeng *et al* (10). Kin17 was also revealed to facilitate the apoptosis of MDA-MB-231 cells, as investigated with flow cytometry and TUNEL assays. Higher caspase 3/7 activity in the knockdown group compared with

the NC group also suggested that caspase 3 and 7, which are wellknown to induce cell apoptosis (17,18), may be involved in the kin17-mediated apoptosis of MDA-MB-231 cells. The underlying mechanism, however, requires further investigation.

Hormonal therapy has been considered as the first-line therapy for breast cancer since its emergence in 1973 (19,20). However, its application is limited, as only 20-30% of the patients exhibit a HER-2-positive subtype (21). PARPs are a family of enzymes that are involved in numerous processes, including DNA damage repair (22). PARP inhibitor, designed for germline breast cancer susceptibility protein-mutated TNBC, was revealed to be effective for the treatment of TNBC in a recent breast cancer trial (23,24). In the present study, the level of PARP was revealed to be significantly decreased by western blot analysis following knockdown of kin17, indicating that kin17 may enhance apoptosis of MDA-MB-231 cells by affecting the function of PARP. A previous study also reported that kin17 upregulation was significantly associated with p53 mutation and increased cyclin D1 and extracellular signal related kinase 1/2 expression in breast cancer cells, including TNBC cells (10). Thus, kin17 could become a novel target for the personalized treatment of TNBC and other breast cancer subtypes.

Frequent adverse events and the increasing risk of endometrial diseases, including endometrial cancer and endometrial polyps, have not only been observed in TNBC, but also in other types of breast cancer due to existing treatment methods (25,26). Development of targets for evaluation or treatment is necessary for the improvement of breast cancer therapy. Since it is unclear how kin17 alters proliferation and apoptosis of breast cancer cells, future investigations should focus on determining underlying mechanisms that could facilitate the use of kin17 a target for breast cancer.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TZ, XG and KW conceived and designed the experiments. XG, YZ and MZ performed the experiments. XG, ZL and HW analyzed the data. XG and TZ wrote the paper.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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