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Original Articles

MicroRNA-17 acts as a tumor chemosensitizer by targeting *JAB1/CSN5* in triple-negative breast cancer

Sumei Wang[a,b,c,d,*,1], Do-Youn Oh[d,f,1], Vasiliki Leventaki[g], Elias Drakos[e], Ronghua Zhang[d], Aysegul A. Sahin[i], Erika Resetkova[i], Mary Elizabeth Edgerton[i], Wanyin Wu[a,c,**], Francois X. Claret[d,h,j,***]

[a] Department of Oncology, Guangdong Provincial Hospital of Chinese Medicine, The Second Clinical Medical College of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong Province, PR China
[b] The Postdoctoral Research Station, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong Province, PR China
[c] Guangdong Provincial Key Laboratory of Clinical Research on Traditional Chinese Medicine Syndrome, Guangzhou, Guangdong Province, PR China
[d] Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
[e] Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
[f] Department of Internal Medicine, Seoul National University Hospital, Cancer Research Institute, Seoul National University College of Medicine, Seoul, South Korea
[g] Department of Pathology, Medical College of Wisconsin Milwaukee, Wisconsin, USA
[h] Experimental Therapeutics, The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, Houston, TX, USA
[i] Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
[j] Cancer Biology Programs, The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, Houston, TX, USA

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ABSTRACT

Triple-negative breast cancer (TNBC) is the breast cancer subtype with the poorest prognosis. Evidence indicates that aberrant *JAB1/CSN5* expression is associated with advanced tumor stage and poor prognosis in breast cancer. In this study, we evaluated expression of JAB1 in TNBC and potential mechanisms regulating this expression. We found that miR-17 expression was lower in TNBC than in normal breast tissue, and miR-17 expression in patients with TNBC was associated with a good prognosis. Furthermore, JAB1 expression was regulated by miR-17 in TNBC cells, and mice with miR-17-overexpressing tumors had less tumor growth and lower tumor JAB1 expression than control mice. We also demonstrated that miR-17 suppressed *JAB1*'s oncogenic function, leading to tumor growth inhibition and sensitizing TNBC cells to chemotherapy treatment. JAB1 knockdown in TNBC cells mimicked the effect of miR-17 overexpression and led to significant decreases in cell proliferation, colony formation, and migration, increased p27 expression, and enhanced cisplatin sensitivity. Our findings suggest that miR-17 acts as a tumor suppressor by directly targeting JAB1 in TNBC; this may lead to novel therapeutic targets and strategies for treating TNBC patients.

1. Introduction

Triple-negative breast cancer (TNBC) is defined by the lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) overexpression or amplification. The disease, which accounts for approximately 10%–20% of all breast cancers [1], has the poorest prognosis of all types of breast cancer because of its heterogeneity, lack of specific therapeutic targets, and tendency to develop chemoresistance. TNBC demonstrates a panel of specific molecular alterations, including a high rate of *TP53* mutation, frequent loss of breast cancer 1 (BRCA1) function, phosphatase and tensin homolog (PTEN) loss, and activation of tyrosine kinase fibroblast growth factor

* Corresponding author. Department of Oncology, Guangdong Provincial Hospital of Chinese Medicine, The Second Clinical Medical College of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong Province, PR China.
** Corresponding author. Department of Oncology, Guangdong Provincial Hospital of Chinese Medicine, The Second Clinical Medical College of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong Province, PR China.
*** Corresponding author. Department of Systems Biology, Unit 1058, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA.
E-mail addresses: wangsumei198708@163.com (S. Wang), wwanyin@126.com (W. Wu), fxclaret@mdanderson.org (F.X. Claret).
[1] These authors contributed equally to this work.

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receptor 2 (FGFR2). TNBC can be subdivided into several subtypes on the basis of gene expression profiles [2].

Because TNBC tumors have a defect in the DNA damage repair system, platinum-based cytotoxic agents, such as cisplatin and carboplatin, are initially effective in a subset of patients, but TNBC often recurs and progresses aggressively because of acquired chemoresistance [3,4]. Accordingly, treatment options for advanced TNBC remain limited, and the disease continues to be a major cause of mortality. New targeted agents for TNBC are urgently needed.

The constitutive photomorphogenic-9 signalosome (CSN) complex is critical to cell survival and proliferation because it modulates protein stability, signal transduction, and gene transcription. Among its eight subunits is CSN5 (COPS5), also called c-Jun activation domain-binding protein-1 (JAB1), which is critical in the positive regulation of cellular proliferation. Specifically, it functionally inactivates a number of important tumor suppressors and negative regulatory proteins, including TP53, SMAD family member 4/7, and cyclin-dependent kinase inhibitor 1B (CDKN1B, also known as p27), through subcellular localization, degradation, and deneddylation [5–8]. Aberrant expression of JAB1 has been identified in several tumor types, including non-small cell lung cancer, pancreatic cancer, hepatocellular carcinoma, and colorectal cancer [9–13].

Recent findings demonstrate that *JAB1* is overexpressed in breast cancer; additionally, it enhances pro-survival pathways and confers tamoxifen resistance in estrogen-receptor-α (ERα)-positive breast cancer [14]. Its expression is inversely correlated with p27 expression, especially in node-negative breast cancer [15]. Intriguingly, members of the breast cancer-promoting epidermal growth factor receptor (EGFR) family, including HER2, have been correlated with increased JAB1 expression in tumors [16,17], and JAB1 is associated with the proto-oncogene tyrosine-protein kinase Src/signal transducer and activator of transcription 3 (SRC/STAT3) signaling pathway [18]. It has been shown that JAB1 is a target of EGFR signaling in ERα-negative breast cancer and that its translocation to the nucleus was mediated through the extracellular-signal-regulated kinase (ERK) signaling pathway [17]. Also, HER2 has been found to activate JAB1 expression through the v-akt murine thymoma viral oncogene homolog 1 (AKT)/β-catenin pathway [16]. Thus, JAB1's involvement in breast carcinogenesis is apparently linked to the expression of EGFR and HER2 receptors and may help mediate downstream signaling events contributing to tumor growth progression. However, in the case of TNBC, the role and activity of JAB1 have not been elucidated.

In this study, we tested the hypothesis that JAB1 plays an important role in TNBC. In doing so, we examined expression of microRNAs (miRs), which negatively regulate target genes via post-transcriptional mechanisms by inhibiting translation or degrading messenger RNA (mRNA). Aberrant expression of miRs regulates tumorigenesis by enabling miRs to function as tumor suppressors or oncogenes [19–22]. The function of miRs may vary according to tissue type and may target mRNA and competing endogenous RNA [23,24]. For example, miR-17 functions as a key oncogenic factor by targeting E2F transcription factors (E2Fs), cyclin-dependent kinase inhibitor 1A (CDKN1A, or p21), PTEN, and the apoptosis facilitator BCL2-like 11 (BCL2L11, or BIM) [25]. In contrast, miR-17 may act as a tumor suppressor by preventing the proliferative activity of E2Fs [26,27]. Thus, miR-17 can serve as either a tumor suppressor or an oncogene [28,29].

To test our hypothesis, we evaluated JAB1 expression in tissue samples from patients with TNBC and also examined JAB1's activity in four TNBC cell lines. We report that JAB1 is highly expressed in TNBC and may be a prognostic factor. It is negatively regulated and is directly targeted by miR-17 in TNBC. Furthermore, inhibition of JAB1 by miR-17 results in increased apoptosis and sensitivity to cisplatin. We are the first to report JAB1 as a novel therapeutic target and to give new insights into the role of miR-17 as a tumor suppressor targeting JAB1 and cisplatin response, which could facilitate the development of novel therapeutic strategies in TNBC.

2. Materials and methods

2.1. Cell lines

Human TNBC cell lines MDA-MB-231, MDA-MB-468, BT-549, and HCC1937 were obtained from the ATCC (Manassas, VA) and were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium. The medium was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were incubated at 37 °C in a humidified chamber supplemented with 5% carbon dioxide. Normal mammary cell lines MCF 10F, MCF 10A, and HMEC, also obtained from the American Type Culture Collection, were cultured in mammary epithelial cell growth medium with additives (Lonza/Clonetics Corporation, Walkersville, MD, catalog # CC-3150).

2.2. Chemicals and reagents

Plasmid pcDNA3-miR-17 was provided by Dr. Joshua Mendell (The University of Texas Southwestern Medical Center, Dallas, TX), and miR-17 inhibitor and the reagents for the miR-17 real-time quantitative polymerase chain reaction (qPCR) assay were purchased from Ambion (Austin, TX). Anti-JAB1 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, catalog # sc-13157), and anti-p27 and anti-PARP were purchased from BD Biosciences-Pharmingen (San Jose, CA). Anti-caspase-3 was purchased from Cell Signaling Technology (Danvers, MA), and anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO). The anti-JAB1 and anti-p27 antibodies for immunohistochemical analysis of patients' tissues were purchased from GeneTex (Irvine, CA) and Dako (Carpinteria, CA), respectively. Lentiviral miR expression vectors were purchased from Biosettia (San Diego, CA), and matrigel matrix was purchased from BD Biosciences. The pMIR-REPORT system was a generous gift from Pradeep Chaluvally Raghavan (Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX). Endogenous restriction enzymes *Hin*dIII and *Spe*I were purchased from New England BioLabs (Ipswich, MA). The PureLink Genomic DNA Mini Kit and PureLink Quick Gel Extraction Kit were purchased from Thermo Fisher Scientific (Waltham, MA). The QuikChange Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA), the Rapid DNA Ligation Kit was purchased from Roche (Indianapolis, IN), and the ImProm-II Reverse Transcription System and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI).

2.3. Gene expression in patient tissues

To determine the clinical relevance of *JAB1* expression in patients with breast cancer, we used gene expression profile data from the Genome Institute of Singapore (GIS) cohort. Gene expression data for 448 patients were generated using the U133A and U133B microarray platforms from Affymetrix (Santa Clara, CA). Patients were stratified according to their expression of *JAB1*, and the median expression value was used as the cutoff between the high- and low-expression groups. Patients also were categorized according to their intrinsic cancer subtype (i.e., molecular subtype)—basal-like (TNBC), HER2+, luminal A (ER+), luminal B (ER+/HER2+), or normal-like—as described previously [30]. The prognostic significance of *JAB1* expression was estimated using Kaplan-Meier plots and the log-rank test. Forty patients in the GIS cohort were not included in the Kaplan-Meier plots or log-rank test because data on their overall survival were unavailable. To validate the relationship between *JAB1* gene expression and TNBC prognosis, we used another gene expression dataset that included 386 TNBC patients and was obtained from Vanderbilt University [2].

2.4. Patient tissues and immunohistochemical analysis

A microarray containing breast tissue from 56 female patients with TNBC was obtained from MD Anderson Cancer Center. The characteristics of the patients were as follows: median age, 58 years; tumor stage T1 (51%), T2 (43%), T3 (0%), or T4 (6%); lymph node status negative (72%) or positive (28%); and tumor grade intermediate (13%) or high (87%). All patients had undergone curative surgery and appropriate adjuvant treatments according to MD Anderson's TNBC protocols. We also obtained normal breast tissue samples from 15 women as controls. The p27 and JAB1 expression in each tissue sample was assessed by immunohistochemical analysis, as previously described [11]. Briefly, after the tissue samples were treated with heat to facilitate the retrieval of JAB1 and p27 antigens, the tissue samples were incubated with anti-JAB1 and anti-p27 antibodies. Immunoreactions were detected with an LSAB2 kit (Dako). Expression of JAB1 and p27 was evaluated by counting at least 500 tumor cells in representative high-power fields. If nuclear or cytoplasmic staining was present, tumor cells were considered positive for JAB1 or p27. Cells were considered to have a nuclear staining pattern when the nuclear staining intensity was more intense than the cytoplasmic staining intensity, and a cytoplasmic staining pattern when the cytoplasmic staining intensity was equal to or more intense than the nuclear staining intensity. For each tumor sample, we determined a positivity percentage of tumors, defined as the percentage of JAB1- or p27-positive cells in the sample. The immunohistochemistry results were independently evaluated by two pathologists. This study protocol was approved by MD Anderson's Institutional Review Board.

2.5. Candidate miRs

To identify miRs that might interact with *JAB1* mRNA, we used an alignment matching system (http://www.microrna.org/microrna/) and the candidate miRs miR-106a, miR-106b, miR-17, miR-20a, miR-20b, miR-93, miR-519d, miR-29a, miR-29b, and miR-29c. We also determined alignment using microRNA support vector regression and PhastCons scores [31].

2.6. Real-time qPCR

Total RNA was extracted using TRIzol total RNA isolation reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. We performed reverse transcription of miR and measured the levels of mature miR using a TaqMan miR assay kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The levels were normalized to U6, an internal control, and were measured using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The miR-specific qPCR consisted of 40 cycles (95 °C for 15 s and 60 °C for 60 s) after an initial denaturation step (95 °C for 10 min).

2.7. Western blotting

Cells in the log phase of growth were collected, washed twice in cold phosphate-buffered saline solution, and subjected to lysis as described previously [32]. Proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with anti-JAB1, anti-p27, anti-PARP, and anti-caspase-3. For all immunoblots, we used β-actin as the internal positive control. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies using the Western Lightning Plus (PerkinElmer, Waltham, MA) chemiluminescence reagent. The protein levels were quantified by ImageJ software (National Institutes of Health). The activity of PARP and caspase-3 was measured as a percentage calculated as follows: 100% × Tc/Tt, where Tc is the intensity value of the cleavage bands and Tt is the intensity value of the total bands.

2.8. In situ hybridization

To detect miR-17 levels in the TNBC and control tissue samples, we used a miRCURY locked nucleic acid (LNA) miR array (Exiqon, Denmark), as previously reported [33]. Digoxigenin-labeled, LNA-modified probes for miR-17 and a positive control (U6) were also purchased from Exiqon. The tissue slides were hybridized with the double-digoxigenin-labeled LNA miR probe and were heated in a Ventana Discovery Ultra (Ventana Medical Systems, Inc., Tucson, AZ) for 2 h at 55 °C. Then, digoxigenin was detected using a polyclonal anti-digoxigenin antibody and an alkaline phosphatase-conjugated secondary antibody (Ventana Medical Systems, Inc.) with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyphospate p-toluidine salt (NBT-BCIP) as the substrate. We analyzed the signal intensity during *in situ* hybridization using inForm 1.3 software (Nuance 2.6 System, PerkinElmer). To normalize the miR-17 level, we divided the signal intensity of miR-17 by the signal intensity of U6 for the same area of each sample.

2.9. Plasmids and anti-miR transfection

Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for the transfection of plasmid DNA or anti-miR inhibitor into breast cancer cells. Forty-eight hours after transfection, cells were collected, and the cell lysates and total RNA were prepared for Western blotting and real-time PCR. For the time-dependent experiment, we collected cells after 24, 48, and 72 h of transfection.

2.10. Lentivirus infection, constitutive expression, and stable miR expression

MDA-MB-231 cells were seeded (1.5×10^4 per well) onto a 12-well plate 1 day before transduction with human miR-LV-17 or miR-LV-control. Medium was replaced with new medium containing 6 μg/mL Polybrene (Santa Cruz Biotechnology), and 100 μL of miR-LV-17 or miR-LV-control was added. The samples were centrifuged at 1000*g* for 40 min at room temperature. When the cell confluence was greater than 50%, transduced cells were selected using puromycin at a concentration of 4 μg/mL. Transduction efficiency was measured under a microscope, and a single colony was chosen to determine the differential transduction efficiency of the cells that stably overexpressed miR-17 and those that did not.

2.11. Mouse model

All experimental procedures using mice were performed in accordance with protocols approved by The University of Texas MD Anderson Cancer Center's Institutional Animal Care and Use Committee. Four-to 6-week-old BALB/c athymic nu/nu female mice were subcutaneously injected with 1.0×10^6 MDA-MB-231 cells in 50 μL phosphate-buffered saline solution combined with matrigel; the cells stably overexpressed miR-17 (miR-17-#1 and miR-17-#2) or control miR. When the tumor masses became palpable, the tumors were measured every 3 or 4 days with digital calipers. Tumor volumes (in mm^3) were calculated using the following formula: volume = $(L \times W^2)/2$, where L is length and W is width. Mice were killed by carbon dioxide asphyxiation on day 45 after tumor cell injection, when some of the tumors reached the size limit set by the Institutional Animal Care and Use Committee. Tumors were weighed after resection.

2.12. Vector construction and mutagenesis of JAB1's predicted binding sites

To generate a luciferase reporter assay for the evaluation of miR activity, we used PCR to amplify the 3′ untranslated region (UTR) of *JAB1* mRNA obtained from genomic DNA isolated from MDA-MB-231 cells. Inserts were retrieved with *Hin*dIII and *Spe*I enzymes and

were cloned into the same sites of the luciferase plasmid reporter (pMIR) vector (Applied Biosystems) downstream of the firefly open reading frame. The primers for PCR amplification were as follows:

Sense: 5′ CGCACTAGTACAGTCTCTGAGAAGTACTTTACCTG 3′

Anti-sense: 5′ GGTAAGCTTTTCATTTTAAAGAGCTTTATTACAGG 3′

A QuikChange Site-Directed Mutagenesis Kit was used to generate the mutation in the predicted binding sites. Using PCR, we deleted four nucleotides (CTGT) in the 3′UTR of the wild-type *JAB1* gene from the wild-type *JAB1* gene. Both the wild-type and mutant inserts were confirmed by sequencing (DeWalch Technologies, Houston, TX). The primers for confirming the mutation were as follows:

Forward: 5′ CTTCACAGGATTTATAATTATAGTTATTTCGAGAAATT 3′

Reverse: 5′ AATTTCTCGAAATAACTATAATTATAAATCCTGTGAAG 3′

2.13. Dual luciferase reporter assay

pMIR containing the 3′UTR of *JAB1* were transiently transfected with miR-17 plasmid into MDA-MB-231 cells. Cells were seeded onto 24-well plates. After 24 h, they were co-transfected with pMIR-3′UTR, miR-17 plasmid (200 ng), or miR control and a promoter-*Renilla* luciferase plasmid control using Western Lightning Plus chemiluminescence reagent according to the manufacturer's protocol. After 48 h, the cells were washed and suspended in lysis buffer, and luciferase activity was assayed with a luminometer using the Dual-Luciferase Reporter Assay System. All experiments were performed in triplicate, and the results are presented as the means of these separate experiments.

2.14. Cell proliferation assay

An MTT assay was used to evaluate cell viability, as described previously [34]. Briefly, 48 h after transfection, cells were seeded in 96-well plates (500 cells/well for growth or 2000 cells/well for cisplatin treatment) containing 100 μL RPMI-1640 medium and 10% FBS. After an incubation period of 48 h, the MTT labeling reagent (final concentration, 0.5 mg/mL) was added, and the spectrophotometric absorbance of the samples was read with a microplate reader (enzyme-linked immunosorbent assay) at 570 nm.

2.15. JAB1 knockdown experiments

Transfection was carried out using Lipofectamine 2000 according to the manufacturer's instructions. For the dose-response assay, the cells were treated with cisplatin 48 h after transfection. Cell lysates and RNA were prepared for further analysis with Western blotting and real-time qPCR. For the small-interfering RNA (siRNA) analysis, JAB1 siRNA (si-JAB1) and control siRNA (si-Control) oligonucleotides were cloned into an RNA interference vector (BD Biosciences-Pharmingen) according to the manufacturer's instructions and as described by Kouvaraki and colleagues [11].

2.16. Colony formation assay

Forty-eight hours after transfection, cells were seeded in 6-well plates containing RPMI-1640 medium with 10% FBS for growth analysis. After 10 days, the cells were fixed with methanol and stained with 0.1% crystal violet. Then, an inverted microscope was used to count the number of colonies using the standard definition that a colony consists of 50 or more cells.

2.17. Matrigel invasion assay

Invasion assays were performed in Boyden chambers. Transwell filters (8-μm pore size, 24 wells; BD Biosciences) were coated with 1 mg/mL growth factor-reduced matrigel. Cells in culture medium supplemented with 0.1% FBS were incubated for 48 h against a gradient of 10% FBS. Cells that penetrated the membrane were fixed with cold methanol, and cell nuclei were stained with crystal violet and counted. Triplicate inserts were used for each individual experiment.

2.18. Flow cytometry analysis of cell cycle

Forty-eight hours after transfection, the cells were collected and fixed overnight in 75% cold ethanol at −20 °C. Cells were washed twice in cold phosphate-buffered saline solution, labeled with propidium iodide, and analyzed immediately after staining with a FACScan flow cytometer and FlowJo software (BD Biosciences).

2.19. Statistical analysis

The Fisher exact test was used to determine associations between JAB1 and p27 expression and various clinicopathologic variables. The Spearman test was used to analyze the association between JAB1 and p27. Relapse-free survival was defined as the time from diagnosis to recurrence of breast cancer. A Kaplan-Meier analysis was used to examine the association between JAB1 and p27 expression and survival. The results were analyzed with the Student *t*-test when only two groups were examined or with a one-way analysis of variance when more than two groups were examined. Differences between groups were considered to be statistically significant when $P < 0.05$. All computations were carried out with SPSS 16.0 software.

3. Results

3.1. High JAB1 gene expression in patients with TNBC could be a prognostic factor

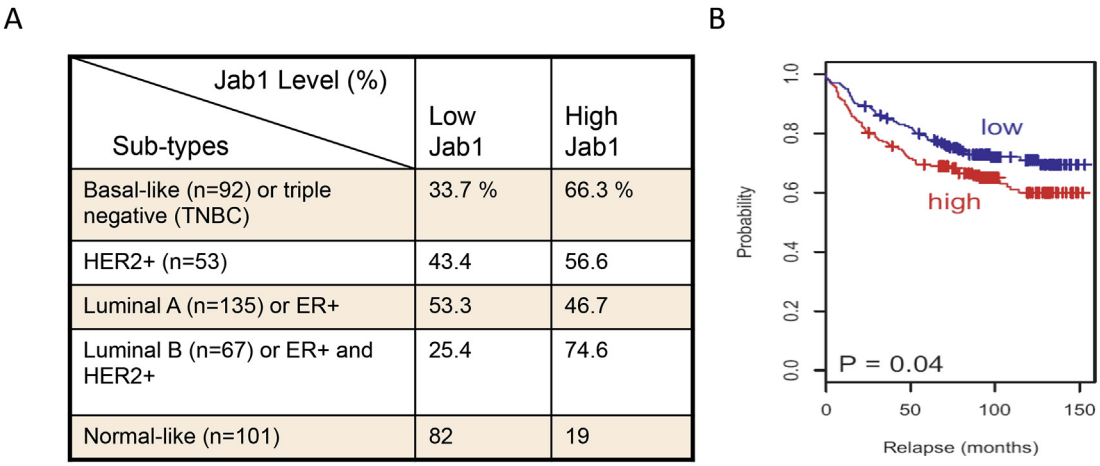
Of the 448 breast cancer patients in the GIS cohort, 92 (20.5%) had the basal-like (TNBC) subtype, 66.3% of whom showed high *JAB1* gene expression; this was a higher percentage than that among patients with the HER2+ subtype (53 patients, 56.6% of whom had high *JAB1* expression) or luminal A (ER+) subtype (135 patients, 46.7% of whom had high *JAB1* expression) (Fig. 1A). Of the 101 patients in the normal breast-like subtype group, 82% had low *JAB1* expression. The high-*JAB1* group had a shorter period of relapse-free survival than the low-*JAB1* group ($p = 0.04$; Fig. 1B). Among the patients with the TNBC subtype, those in the high-*JAB1* group showed a tendency toward a shorter period of relapse-free survival than those in the low-*JAB1* group, although the difference was not statistically significant ($p = 0.7895$; Fig. 1C). A similar trend was seen in the TCGA dataset; TNBC patients in the high-*JAB1* group had a shorter period of survival than those in the low-*JAB1* group ($p = 0.137$) (Fig. 1D). Furthermore, in the Vanderbilt TNBC dataset, *JAB1* gene expression differed across the subtypes of TNBC. Of the six stable subtypes, the basal-like 1 subtype had the highest proportion of tumors with high *JAB1* expression (66.2%), and the mesenchymal stem-like group showed the lowest proportion with high *JAB1* expression (12.0%; Fig. 1E). These results suggest a prognostic role for *JAB1* in TNBC.

3.2. High JAB1 protein expression in patients with TNBC could be a prognostic factor

We then evaluated the protein expression of JAB1 and p27 in tissue samples from patients with TNBC and normal controls. The expression of JAB1 was higher in TNBC tissues than in normal breast tissue ($p = 0.029$), and, inversely, the expression of p27 was much lower ($p < 0.001$; Fig. 2A and B). Normal breast tissues had an entirely nuclear pattern of JAB1 expression, whereas 35% of JAB1-positive TNBC tissues showed the cytoplasmic pattern (Fig. 2C). Additionally, the disease-free survival period of patients with high JAB1 expression was shorter than that of patients with low JAB1 expression ($p = 0.035$;

A

Jab1 Level (%) / Sub-types	Low Jab1	High Jab1
Basal-like (n=92) or triple negative (TNBC)	33.7 %	66.3 %
HER2+ (n=53)	43.4	56.6
Luminal A (n=135) or ER+	53.3	46.7
Luminal B (n=67) or ER+ and HER2+	25.4	74.6
Normal-like (n=101)	82	19



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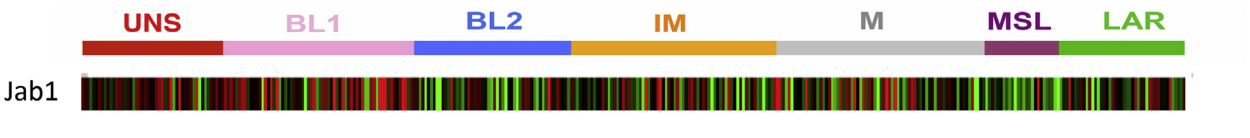

	Jab1-Low	Jab1-High
BL1	22 (33.8%)	43 (66.2%)
BL2	30 (54.5%)	25 (45.5%)
IM	39 (54.2%)	33(45.8%)
M	37 (51.4%)	35 (48.6%)
MSL	22 (88.0%)	3 (12.0%)
LAR	25 (56.8%)	19 (43.2%)
UNS	15 (31.9%)	32 (68.1%)

Fig. 1. *Jab1* gene expression in TNBC tissues and its impact on patient prognosis. **A**. Stratification of patients according to breast cancer subtype and *Jab1* expression level. The median expression value was used as the cutoff for the high- and low-expression groups. **B.** Correlation between patient survival and *JAB1* expression in the Genome Institute of Singapore (GIS) cohort. **C.** Correlation between patients' relapse-free survival duration and *JAB1* expression in the basal-like (TNBC) subtype group of the GIS cohort. **D.** Correlation between patient overall survival and *JAB1* expression in the TCGA dataset. **E.** *Jab1* gene expression attribution from the Vanderbilt triple-negative breast cancer (TNBC) dataset. ER+, estrogen receptor positive; HER2+, HER2 positive; OS, overall survival; 5Y-RFS, 5-year relapse-free survival.

Fig. 2D). Patients with the cytoplasmic pattern of JAB1 showed a shorter disease-free survival period than did those with the nuclear pattern of JAB1 (p = 0.058; Fig. 2E). These results suggest that JAB1 protein expression might be a prognostic factor in TNBC.

3.3. MiR-17 is a potential regulator of JAB1 in TNBC

To identify miRs that might regulate and interact with *JAB1* mRNA, we used an alignment matching system and the candidate miRs miR-

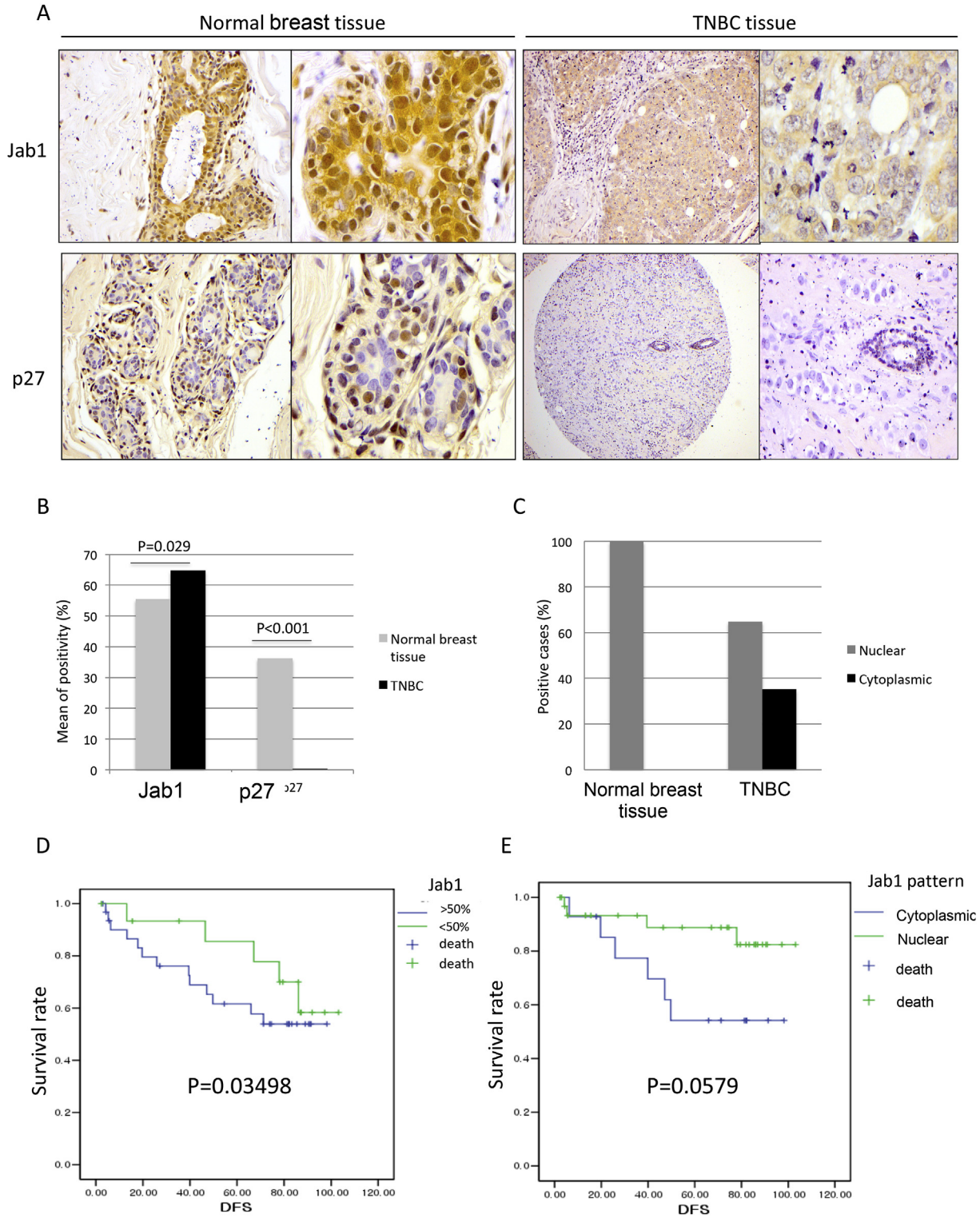

Fig. 2. JAB1 protein expression in TNBC tissue. A, B. Immunohistochemical analysis of JAB1 and p27 expression in TNBC and normal breast tissue. The expression of JAB1 was higher in TNBC than in normal breast tissue ($p = 0.029$) and, inversely, the expression of p27 was much lower in TNBC than in normal breast tissue ($p < 0.001$). The magnification is 200 × (left panel) and 400 × (right panel). **C.** The pattern of JAB1 in normal breast tissue and TNBC tissue. **D.** Comparison of disease-free survival duration between patients with high versus low JAB1 expression ($p = 0.035$). **E.** Comparison of disease-free survival duration between patients with a cytoplasmic versus a nuclear pattern of JAB1 expression ($p = 0.058$). DFS, disease-free survival. TNBC, triple-negative breast cancer.

106a, miR-106b, miR-17, miR-20a, miR-20b, miR-93, miR-519d, miR-29a, miR-29b, and miR-29c. The best alignment was between miR-17 and JAB1 (Fig. 3A). The microRNA support vector regression score for the predicted miR target sites was −0.3126, and the PhastCons score was 0.5835.

We then investigated the expression of both miR-17 and JAB1 in normal mammary and TNBC cell lines. With the use of real-time qPCR, we determined that the level of miR-17 was higher in normal mammary cells (MCF 10F, MCF 10A, and HMEC) than in TNBC cells. In contrast, the level of JAB1 was higher in TNBC cells than in normal mammary

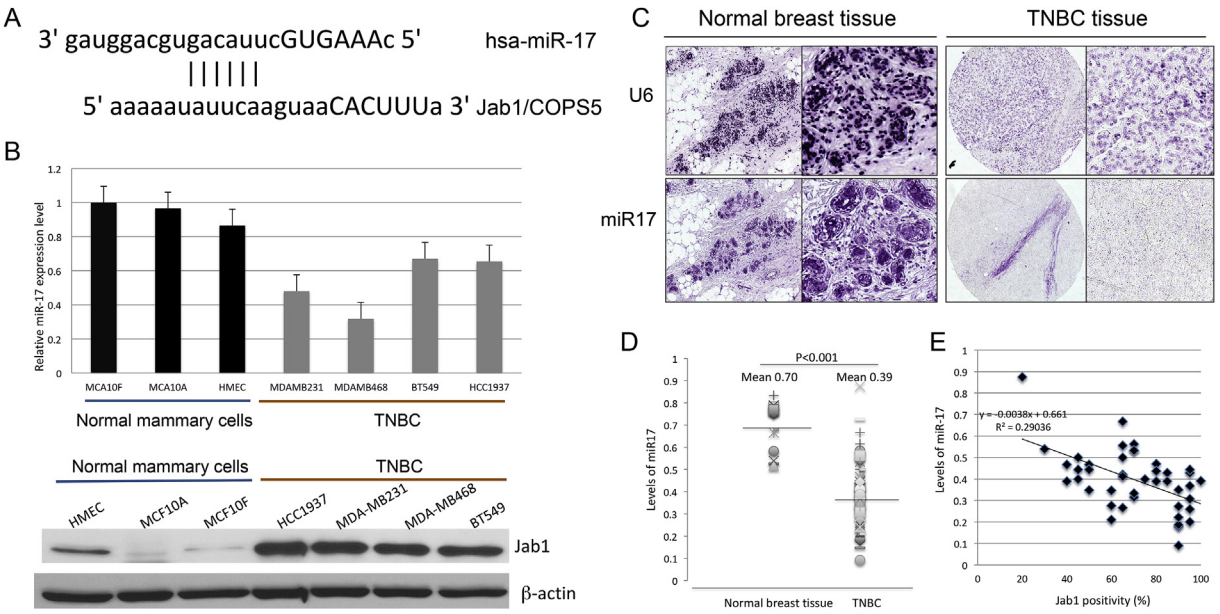

Fig. 3. Correlation between miRNA-17 and JAB1 expression in TNBC. **A.** Alignment of miR-17 and JAB1. **B.** Top panel: Real-time quantitative polymerase chain reaction results for miR-17 expression in normal mammary cells and TNBC cells. Bottom panel: Western blot results for JAB1 expression in normal mammary cells and TNBC cells. **C, D.** *In situ* hybridization results of miR-17 expression, normalized to U6 (an internal control), in normal breast tissues and TNBC tissues. The magnification is 200 × (left panel) and 400 × (right panel). **E.** The inverse correlation of miR-17 levels and the expression of JAB1 in TNBC (R^2 = 0.29). TNBC, triple-negative breast cancer.

cells (Fig. 3B). Next, we investigated the levels of miR-17 in tissue from TNBC patients and normal controls and found that miR-17 levels were significantly lower in TNBC than in normal breast tissue (mean, 0.70 vs. 0.39; $p < 0.001$; Fig. 3C and D). Furthermore, in TNBC tissue, Kaplan-Meier analysis was performed and the results showed that the level of miR-17 and the expression of JAB1 protein were inversely correlated ($R^2 = 0.29$; Fig. 3E), suggesting that miR-17 might be a potential regulator of JBA1 in TNBC.

3.4. JAB1 is negatively regulated by miR-17 in vitro

To determine whether miR-17 functionally regulates the expression of JAB1, we transfected TNBC and normal cells with miR-17 and analyzed the JAB1 protein levels. In all four TNBC cell lines, miR-17 downregulated JAB1 expression in a dose-dependent and time-dependent manner (Fig. 4A). To further examine this negative regulation, we treated the TNBC cells with miR-17 inhibitor and performed immunoblotting for JAB1. With miR-17 inhibitor treatment, JAB1 expression was restored in a dose-dependent and time-dependent manner (Fig. 4B). These results suggest that miR-17 negatively regulated JAB1 expression *in vitro*.

3.5. JAB1 is directly targeted by miR-17 in TNBC

To further confirm miR-17's regulation of JAB1, we established TNBC cells that stably overexpressed miR-17 by infecting MBA-MB-231 cells with lentivirus miR-LV-17, resulting in cell lines miR-17-#1 and miR-17-#2, or miR-LV-control. We then transplanted either the miR-17-#1 cells, the miR-17-#2 cells, or the miR-control cells into nude mice. As shown in Fig. 5A, tumor weights were significantly lower and tumor growth was more inhibited in miR-17-overexpressing mice than in control mice. We then investigated JAB1 protein expression in the mouse tumors and found that JAB1 expression was significantly downregulated in the miR-17-overexpressing groups compared with the control group ($p = 0.003$) (Fig. 5B). These results reveal that JAB1 was also negatively regulated *in vivo*.

To determine whether JAB1 is a direct target of miR-17, we constructed pMIR-Luc-JAB1 3′UTR and transfected cells with the vector and either miR-17 plasmid or miR-control. Luciferase assay results showed that miR-17 plasmid decreased the luciferase activity in cells with pMIR-Luc-JAB1 3′UTR (left panel, Fig. 5C). When we mutated the predicted sites of miR-17 binding on JAB1, the luciferase activity was rescued (right panel, Fig. 5C). These results further show that JAB1 is directly targeted by miR-17 in TNBC.

3.6. JAB1 increases TNBC cell proliferation and invasion, decreases p27, and increases TNBC resistance to cisplatin

To examine the role of JAB1 in TNBC, we knocked down JAB1 in all four TNBC cell lines using siRNA. In all cells, downregulation of JAB1 inhibited cell proliferation (left panel, Fig. 6A). We also conducted a colony formation assay to evaluate the effect of JAB1 on anchorage-independent cell growth. Downregulation of JAB1 reduced the ability of TNBC cells to form colonies (right panel, Fig. 6A) and resulted in the inhibition of cell migration (Fig. 6B). Additionally, the results of cell-cycle analysis showed that si-JAB1-treated samples had a higher proportion of cells in the G1 phase than did si-Control-treated samples (73% vs. 52% in MDA-MB-231; 54% vs. 25% in MDA-MB-468). The si-JAB1-treated samples also had a lower proportion of cells in the S phase (19% vs. 31% in MDA-MB-231; 5% vs. 22% in MDA-MB-468) (Fig. 6C). We next transfected the TNBC cells with si-JAB1 and immunoblotted for p27, a cell-cycle inhibitor. In all four TNBC cell lines, p27 levels were higher after JAB1 inhibition, and the effects were dose-dependent (Fig. 6D). These findings further demonstrate the oncogenic role of *JAB1* in TNBC, including its promotion of cell growth and cell invasion and inhibition of cell-cycle inhibitor p27.

We then investigated whether the inhibition of JAB1 can sensitize TNBC cells to cisplatin. The proliferation of cisplatin-treated cells was lower in cells in which JAB1 was inhibited compared with cells treated with cisplatin alone (top panel, Fig. 6E). In the colony formation assay, TNBC cells co-treated with cisplatin and si-JAB1 had significantly less anchorage-independent growth than did cells treated with cisplatin or si-JAB1 alone (bottom panel, Fig. 6E). In the matrigel invasion assay, TNBC cells co-treated with cisplatin and si-JAB1 had dramatically fewer

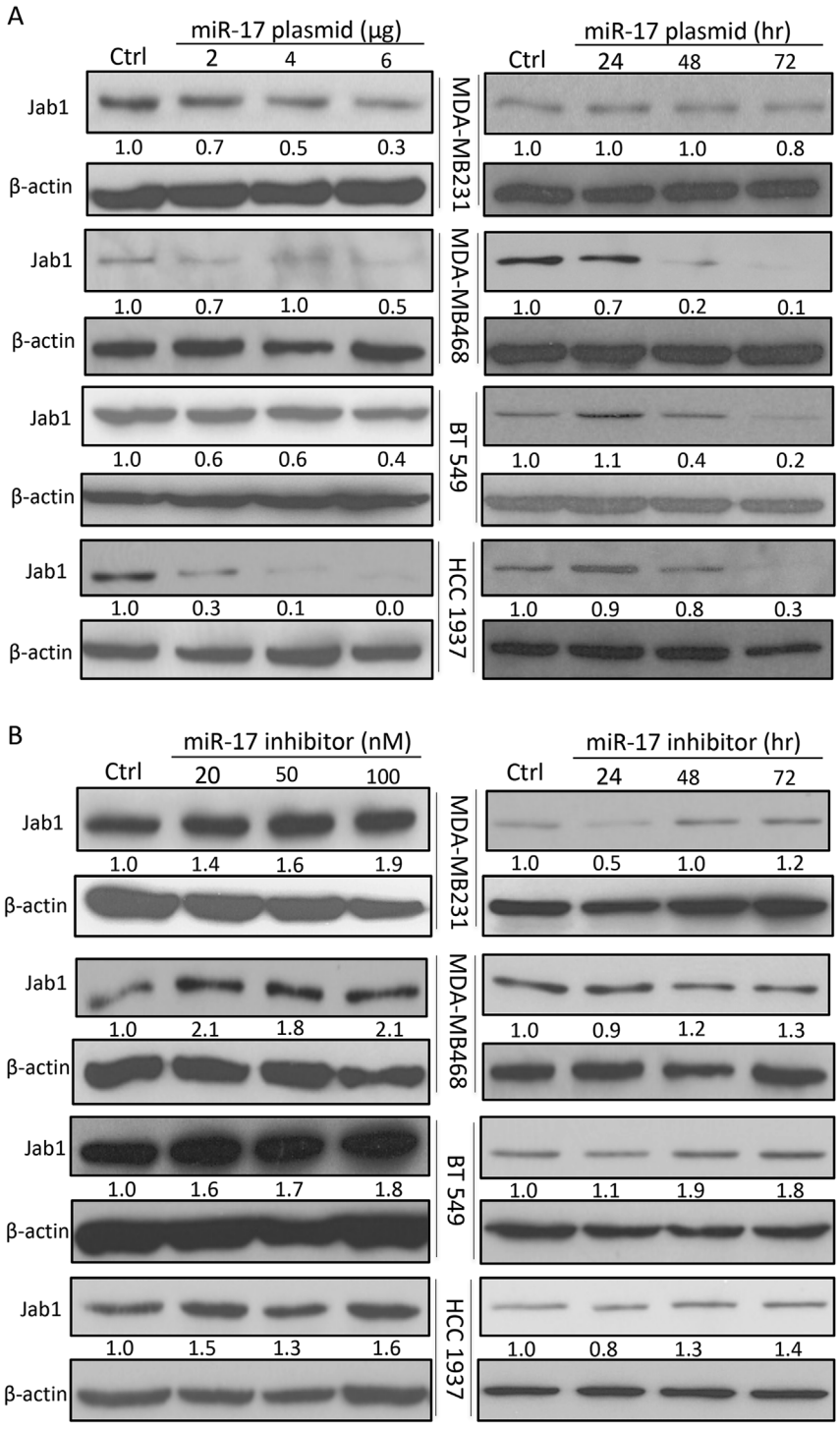

Fig. 4. MicroRNA-17 regulation of JAB1 in TNBC. A. Western blot results for JAB1 expression by time and dose in TNBC cells transfected with miR-17. TNBC cells were transfected with different doses of miR-17 plasmid (2 μg, 4 μg, or 6 μg) for 48 h or miR-17 plasmid (4 μg) for different amounts of time (24 h, 48 h, or 72 h). JAB1 protein levels were quantified using ImageJ software. **B.** Western blot results for JAB1 expression by time and dose in TNBC cells transfected with miR-17 inhibitor. TNBC cells were transfected with different doses of miR-17 inhibitor (20 nM, 50 nM, or 100 nM) for 48 h or miR-17 inhibitor (50 nM) for different amounts of time (24 h, 48 h, or 72 h). TNBC, triple-negative breast cancer.

invasive cells than did cells treated with cisplatin alone (Fig. 6F). JAB1 inhibition also enhanced cisplatin-induced apoptosis, as shown by the increase in caspase-3 cleavage and PARP cleavage in MDA-MB-231 cells treated with siJAB1 compared with those treated with cisplatin alone (Fig. 6G). These results show that JAB1 could be a new therapeutic target for TNBC.

4. Discussion

To the best of our knowledge, we are the first to report the relevance of JAB1 in TNBC, as well as miR-17's role as a tumor suppressor in TNBC through its targeting of JAB1. In this study, we found that *JAB1* gene expression and JAB1 protein levels were high in tissue samples from patients with TNBC, suggesting that JAB1 could be a prognostic factor for the disease. We further showed that JAB1 was negatively regulated by miR-17, both *in vitro* and *in vivo*, and was a direct target of miR-17. Our results also indicated that miR-17 was downregulated in TNBC compared with normal breast tissue. Furthermore, down-regulation of JAB1 led to the inhibition of proliferation and invasion in TNBC cells and sensitized TNBC cells to cisplatin. Our findings show the important role of JAB1 as an oncogene or oncoprotein in TNBC and a novel role of miR-17 as a tumor suppressor targeting JAB1 in TNBC.

To determine the seed pairing for JAB1, we used a computational approach developed to predict miR target sites *in silico*. In previous

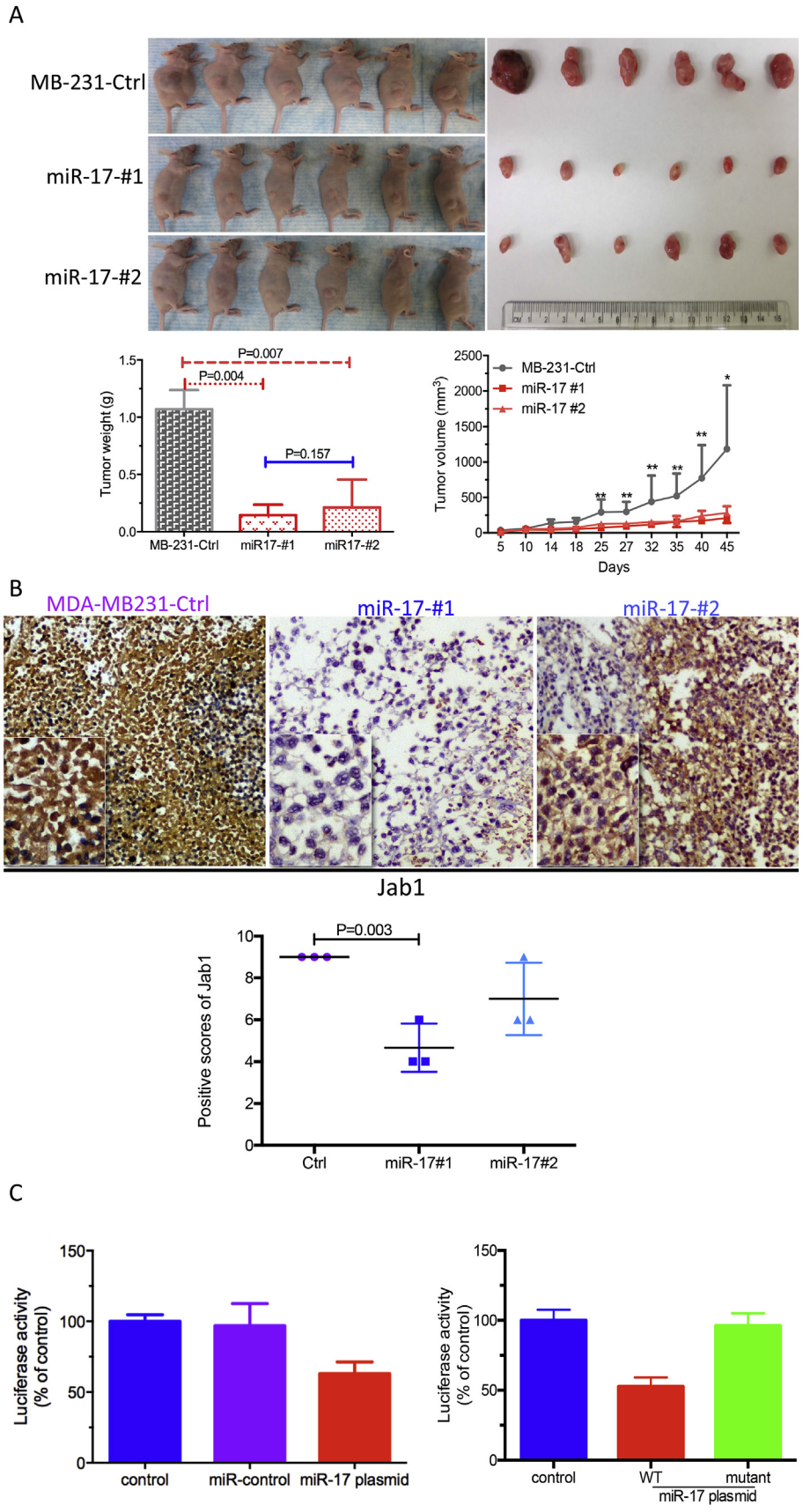

Fig. 5. JAB1 as a direct target of microRNA-17 in TNBC. A. Top panel shows tumors in BALB/c athymic nu/nu mice and after resection. Bottom panel shows that miR-17 overexpression significantly reduced the weight of xenograft tumors and inhibited tumor growth. *, $p < 0.05$; **, $p < 0.01$. **B.** Top panel shows representative pictures of JAB1 expression in the tissues resected from mice injected with cells that overexpressed miR-17 or with control cells. Magnification: 200 × ; inset, 400 × . Bottom panel shows positive scores of JAB1 expression in the tissues resected from the mice (3 mice per group). **C.** Left panel shows luciferase assay results for cells co-transfected with pMIR-Luc-JAB1 3′UTR and either miR-17 plasmid or miR-control. Right panel shows luciferase assay results for cells co-transfected with miR-17 plasmid and either wild-type or mutant pMIR-Luc-JAB1 3′UTR. Data were normalized to the control. TNBC, triple-negative breast cancer.

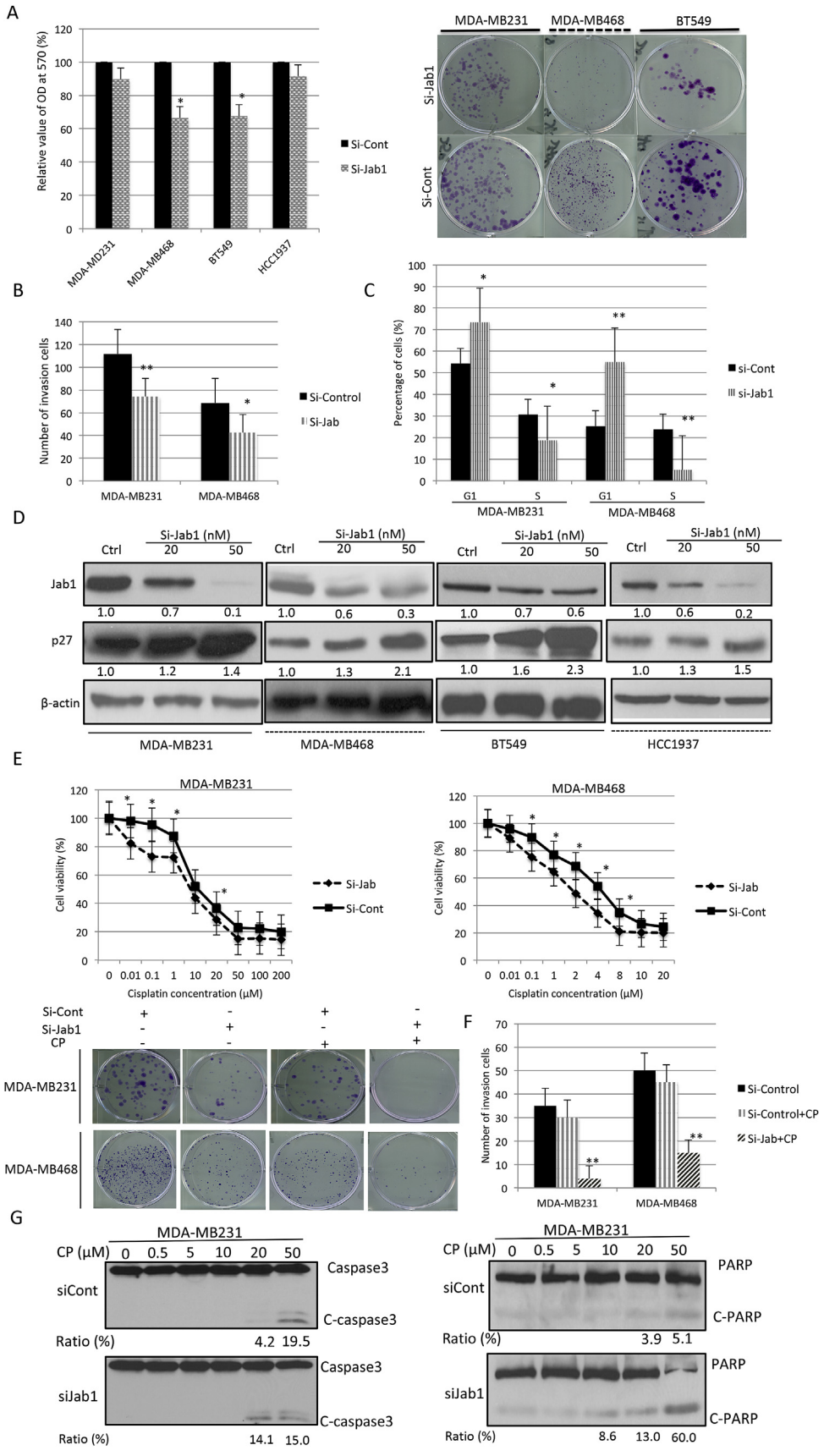

Fig. 6. JAB1 increases TNBC cell proliferation and invasion, decreases p27, and increases TNBC resistance to cisplatin. **A.** MTT assay results. After 48 h of transfection with 20 nM of si-JAB1 or si-Control (si-Cont), an MTT assay was performed for all four TNBC cell lines. Left panel: Data are shown as the relative value of optical density at 570 nm. Right panel: Colony formation assay results showed that MDA-MB-231, MDA-MB-468, and BT-549 TNBC cells treated with si-JAB1 (20 nM) had lower colony-forming ability than cells treated with si-Cont. **B.** Matrigel invasion assay. MDA-MB-231 and MDA-MB-468 cells were treated with 20 nM of si-JAB1 and si-Cont for 48 h and then were transferred to the matrigel invasion chamber. Data represent three independent experiments (mean ± SD). *p < 0.05; **p < 0.01. **C.** Cell-cycle analysis of si-JAB1-treated cells and si-Cont-treated cells. Data represent three independent experiments (mean ± SD). *p < 0.05; **p < 0.01. **D.** p27 immunoblots for TNBC cells treated with si-Cont, 20 nM of si-JAB1, or 50 nM of si-JAB1 for 48 h. JAB1 and p27 protein levels were quantified using ImageJ software. **E.** Upper panel: Dose-response assay for cisplatin. TNBC cells were treated with 20 nM of si-JAB1 or si-Cont for 48 h and then were treated with different doses of cisplatin for 48 h. Cells treated with cisplatin and si-JAB1 showed significantly lower proliferation than those treated with cisplatin and si-Cont. Lower panel: Colony-formation assay. MDA-MB-231 and MDA-MB-468 cells were treated with cisplatin (5 μM and 1 μM, respectively) and/or si-JAB1 (20 nM). Anchorage-independent cell growth was dramatically lower in cells treated with cisplatin and si-JAB1 than in cells treated with cisplatin alone or si-JAB1 alone. **F.** Matrigel invasive assay. Samples treated with cisplatin and si-JAB1 had significantly fewer invasive cells than those treated with cisplatin alone. **G.** Caspase-3 and PARP cleavage. MDA-MB-231-si-JAB1 cells showed more caspase-3 and PARP cleavage than did MDA-MB-231-si-Cont cells when treated with cisplatin. In MDA-MB-468 cells, levels of the intact form of PARP were lower after cisplatin and si-JAB1 combination treatment than after cisplatin and si-Cont treatment, and the effect was dose-dependent. Intact caspase-3 levels were lower in cells co-treated with cisplatin and si-JAB1 than in those co-treated with cisplatin and si-Cont. C, cleaved; CP, cisplatin.

reports, miR-17 was shown to be an oncogenic factor targeting E2Fs, PTEN, and the zinc finger and BTB domain-containing protein 4 (ZBTB4) [35–39]. However, miR-17 may act as a tumor suppressor by preventing the proliferative activity of E2Fs [39,40]. Whether a miR acts as an oncogene or as a tumor suppressor is context-specific [41]. In our study, we showed consistent *in vitro* and *in vivo* negative regulation of JAB1 by miR-17, suggesting that miR-17 might act as a tumor suppressor in TNBC.

JAB1 and p27 were negatively correlated in both TNBC and normal breast tissue. We found that the level of p27 was remarkably lower in TNBC tissue compared with normal breast tissue, which supports previous findings. As an inhibitor of cyclin E-cyclin-dependent kinase 2 (CDK2), p27 plays a critical role in controlling the cell cycle. JAB1's role in the nuclear-to-cytoplasmic export of p27 is important for cytoplasmic accumulation of p27, which in turn results in increased cell motility and pathogenesis [7].

In studies of neoadjuvant therapy for TNBC, treatment with cisplatin or carboplatin achieved higher pathologic complete response rates than did treatment with paclitaxel or docetaxel [42,43]. This finding can be explained, in part, by TNBC's dysfunctional DNA damage repair system. Although platinum agents have been effective in treating TNBCs, the efficacy of cytotoxic chemotherapy in TNBC is still limited. In our study, cisplatin-treated TNBC cells in which JAB1 had been knocked down were less likely to proliferate and invade and more likely to undergo apoptosis compared with TNBC cells treated with cisplatin alone. The p27 levels were also higher in cells co-treated with cisplatin and si-JAB1 compared with those treated with cisplatin alone.

The synergy between cisplatin and JAB1 knockdown can be partially explained by the essential role of JAB1 in DNA repair. We previously showed that JAB1 is essential for efficient DNA repair and that it is mechanistically linked to the maintenance of genome integrity and to cell survival [44]. Co-treatment with a DNA-damaging agent, such as a platinum agent, and JAB1 knockdown strongly affects cell survival; this is especially true in TNBC because of the disease's dysfunctional DNA-repair system. On the basis of these results, JAB1 should be investigated as a potential therapeutic target in TNBC.

Disclosure of potential conflicts of interest

D-Y Oh reports receiving grants from AstraZeneca, Array and Mogam (all outside of this work) and is a consultant/advisory for AstraZeneca, Merck Serono, Bayer, Novartis, Genentech, Abbvie, Halozyme, ASLAN, BMS, ONO (outside this work), No potential conflicts of interest were disclosed by the other authors.

Authors' contributions

Conceptualization and design: S. Wang, D.-Y. Oh, F.X. Claret, and W. Wu.

Methodology: S. Wang, D.-Y. Oh, V. Leventaki, E. Drakos, R. Zhang, A.A. Sahin, E. Resetkova, and M.E. Edgerton.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Sahin, E. Resetkova, M.E. Edgerton.

Writing, review, and/or revision of the manuscript: S. Wang, D.-Y. Oh, F.X. Claret, and W. Wu.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Wang, D.-Y. Oh, F.X. Claret, and W. Wu.

Study supervision: F.X. Claret.

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