Received: 24 May 2022 | Revised: 18 September 2022 | Accepted: 6 October 2022

DOI: 10.1111/cas.15623

ORIGINAL ARTICLE

Cancer Science WILEY

BMI-1 promotes breast cancer proliferation and metastasis through different mechanisms in different subtypes

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Funding information
"333" Talent Project of Jiangsu, Grant/Award Number: BRA2017057; Gusu Health Talent Project of Suzhou, Grant/Award Number: GSWS2020056; Suzhou Health and Family Planning Commission, Grant/Award Number: SKJYD2021104

Abstract

Breast cancer is among the most common malignant cancers in women. B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) is a transcriptional repressor that has been shown to be involved in tumorigenesis, the cell cycle, and stem cell maintenance. In our study, increased expression of BMI-1 was found in both human triple negative breast cancer and luminal A-type breast cancer tissues compared with adjacent tissues. We also found that knockdown of BMI-1 significantly suppressed cell proliferation and migration in vitro and in vivo. Further mechanistic research demonstrated that BMI-1 directly bound to the promoter region of CDKN2D/BRCA1 and inhibited its transcription in MCF-7/MDA-MB-231. More importantly, we discovered that knockdown of CDKN2D/BRCA1 could promote cell proliferation and migration after repression by PTC-209. Our results reveal that BMI-1 transcriptionally suppressed *BRCA1* in TNBC cell lines whereas, in luminal A cell lines, *CDKN2D* was the target gene. This provides a reference for the precise treatment of different types of breast cancer in clinical practice.

KEYWORDS
BMI-1, BRCA1, breast cancer, CDKN2D, histone modification

Abbreviations: BMI-1, B-cell-specific Moloney murine leukemia virus integration site 1; BRCA1, breast cancer susceptibility gene 1.; CDKN2D, cyclin-dependent kinase inhibitor 2D; PRC1, polycomb repressive complex 1; TNBC, triple breast cancer.

Jin-yan Liu, Yan-nan Jiang, and Hai Huang contributed equally to this work.

1 | INTRODUCTION

Recently, breast cancer has received increasing amounts of attention owing to its high morbidity and mortality.[1] There is increasing evidence that it is a heterogeneous disease, with different molecular subtypes potentially having different etiologies, clinical presentations, and outcomes. Clinically, based on histology and immunohistochemical detection of expression of key proteins—estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and proliferation marker Ki-67—breast cancer can be divided into five subtypes: TNBC, HER2-enriched (nonluminal), luminal B-like HER2$^+$, luminal A-type, and luminal B type.[2] Therapy for breast cancer consists of local and systemic treatments. Endocrine therapy is the primary systemic therapy for luminal A-type breast cancer and can considerably reduce recurrence and mortality. For TNBC, chemotherapy is essential.[3] However, acquired resistance is a major challenge. Thus, new molecular markers and novel therapeutic targets for breast cancer need to be identified as early as possible.

B-lymphoma Moloney murine leukemiavirus insertion region-1 was first identified as a c-Myc cooperating oncogene that promotes lymphomagenesis in mice.[4,5] Subsequently, BMI-1 was found to be a transcriptional repressor belonging to the mouse polycomb protein (PcG) family.[6,7] An epigenetic gene silencing factor, PcG regulates gene activity at the chromatin level by forming at least two multimeric complexes, namely PRC1 and PRC2. Many studies have shown that BMI-1 is frequently upregulated in various human cancer types, and is associated with poor prognosis in solid tumors including lung cancer,[8] medulloblastoma,[9] neuroblastoma,[10] hepatocellular carcinoma,[11] nasopharyngeal carcinoma,[12] and prostate cancer.[13] BMI-1 is highly expressed in breast cancer tissues and cells and is associated with migration, invasion, and chemotherapy tolerance.[14–16] However, the specific mechanism of the oncogenic role of BMI-1 in breast cancer is still not well understood.

In this study, we provide evidence that the tumor-promoting effects driven by BMI-1 differ in terms of their underlying mechanisms in breast cancer cell lines of different types. We suggest that BMI-1 promotes proliferation and migration via transcriptional inhibition of CDKN2D in luminal A-type breast cancer but via transcriptional inhibition of BRCA1 in TNBC. These results provide a laboratory basis for different treatment options for different molecular types of breast cancer in clinical practice.

2 | MATERIALS AND METHODS

2.1 | Collection of tumor samples

Human breast cancer and adjacent tissues were obtained from 31 patients (Table S1) who underwent surgery at the Suzhou Municipal Hospital of Nanjing Medical University between November 2020 and July 2021. Signed informed consent regarding this study was provided by all participants, and ethical approval for the study was received from the Suzhou Municipal Hospital. The approval number is KL901273. Participant information was fully protected.

2.2 | RNA extraction and real-time quantitative PCR (qRT-PCR) assays

The RNA isolater Total RNA Extraction Reagent (Vazyme, Nanjing, Jiangsu, China) was used to extract total RNA as previously described.[17] RNA was reverse transcribed into cDNA using a HiScript III RT SuperMix qPCR kit (R323-01, Vazyme, Nanjing, China). Then, qRT-PCR was carried out using SYBR qPCR SuperMix Plus (Novoprotein Scientific Inc., Shanghai, China) on an Applied Biosystems 7500 Real-Time PCR system three times. Results were normalized to 18S RNA expression and calculated using the $2^{-\Delta\Delta CT}$ method. The primers used in this study are presented in Table S2.

2.3 | Immunofluorescence

As previously described,[18–21] the paraffin-embedded tissues were dewaxed and hydrated, then antigen retrieval was performed by boiling samples in 10 mM sodium citrate buffer (pH 6.0). The sections were blocked with 1% BSA in PBS for 2 h and then incubated with primary antibody and secondary antibody successively. Finally, images were captured on a Zeiss laser confocal microscope (LSM 810, Carl Zeiss, Oberkochen, Germany) and quantified with Zeiss software. The antibodies used in this experiment were as follows: rabbit anti-BMI-1 antibody (Proteintech Group Inc., 1:100), mouse anti-Ki67 antibody (Abcam, 1:100).

2.4 | Cell culture and treatments

Human breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468, and T47D) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured at 37 °C and 5% CO_2 in a humidified incubator.

PTC-209 was obtained from Selleck (Shanghai, China) and dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at a stock concentration of 10 mM as previously described.[22] Cells were treated with diverse concentrations of PTC-209 for 48 h and then harvested for further analyses.

siRNAs (GenePharma, Shanghai, China) targeting CDKN2D, BRCA1 and negative control were transfected into cells with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells were harvested for analysis at 48 h post-transfection. The target sequences recognized by the siRNAs were as follows:

si-BMI-1-5′-CCAGAUUGAUGUCAUGUAUTT-3′,
si-CDKN2D-5′-CAAUCCAUCUGGCAGUUCA-3′,
si-BRCA1-5′-GCUAGAAAUCUGUUGCUAU-3′,
si-NC-5′-UUCUCCGAACGUGUCACGU-3′.

2.5 | Western blotting

Western blotting was performed as previously described.[17,22] Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk in TBS-Tween for 1 h, then reacted overnight with anti-BMI-1 rabbit antibody (1:1000 dilution; Proteintech Group Inc.) and anti-tubulin mouse antibody (1:3000 dilution; Beyotime Institute of Biotechnology, Nantong, China) at 4°C. This was followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, band signals were visualized using an enhanced chemiluminescent substrate and quantified by Image-Pro Plus (Media Cybernetics, San Diego, CA, USA).

2.6 | Cell proliferation assay

Cell viability was assessed every 24 h by using a Cell Counting Kit-8 kit (CCK8; Beyotime Institute of Biotechnology, Nantong, China) at an optical density of 450 nm with a microplate reader (Bio-Rad Model 680, Richmond, CA, USA).

In the colony formation assay, the same cells were inoculated in six-well plates (800 cells/well) for 2 weeks. During this period, the medium was changed every 5 days. After 14 days, the cells were washed with PBS, fixed with methanol, and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Nantong, China).

2.7 | Cell migration assays

Cell migration capacity was evaluated via transwell assays using 24-well transwell chambers with 8-μm pore size purchased from Corning (USA). At 48 h after treatment, 4.5×10^4 cells were seeded into the upper chamber with 300 μl serum-free medium, and 700 μl complete medium was added to the lower chamber. After 48 h, cells on the lower membrane surface were fixed and stained. Five randomly selected views were imaged and counted in each well.

2.8 | In vivo assays

Four-week-old female athymic BALB/c nude mice were housed in maximum barrier facilities with individually ventilated cages, sterilized food, and water at the Animal Center of Nanjing Medical University. Human breast cancer cells (MDA-MB-231) were treated with 20 μM PTC-209 or an equal volume of DMSO for 48 h, then harvested, washed, and re-suspended. Each mouse was subcutaneously injected in the flanks on the left and right sides with the control and experimental treatment, respectively ($n = 7$). After 14 days, the mice were sacrificed, and subcutaneous tumors were removed, measured, and fixed in formalin. The study protocol was approved by the Animal Ethical and Welfare Committee of Nanjing Medical University.

2.9 | ChIP-qPCR

ChIP assays were carried out using a ChIP Assay Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Cross-linked chromatin was sonicated into fragments and then immunoprecipitated using an anti-BMI-1 antibody. Approximately 10% of the starting material were used as the input. Immunoprecipitated DNA was analyzed by qPCR. The primers used for ChIP-qPCR were follows:

GAPDH-F: 5′-TGGCATTGCCCTCAACGAC-3′
GAPDH-R: 5′-TTTTCTGAGCCAGCCACCAGAG-3′
BRCA1-F: 5′-GTGATGCTCTGGGGTACTGG-3′
BRCA1-R: 5′-CCTTACGCCTCTCAGGTTCC-3′
CDKN2D-F: 5′-GCGGAACGCCGTAAGGT-3′
CDKN2D-R: 5′-CGAACGCAACTGATTTGTTTTCTT-3′

2.10 | Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0. Dates are presented as mean ± standard deviation. Student's *t*-test was used for two-group comparisons. Comparisons between three or more groups were analyzed using one-way ANOVA followed by Dunnett's test. A p-value <0.05 was considered to indicate statistical significance.

3 | RESULTS

3.1 | BMI-1 expression is significantly upregulated in human breast cancer tissues

To investigate the clinical significance of BMI-1, we analyzed BMI-1 mRNA levels in normal breast and adenocarcinoma tissues that were extracted from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. We found that BMI-1 expression was significantly increased in tumor tissues compared with normal tissues (Figure 1A). Subsequently, 31 paired human breast cancer tissues and the corresponding adjacent tissues were examined using qRT-PCR (Figure 1B). As a result, BMI-1 was indeed overexpressed in breast cancer tissues. Next, the protein expression of BMI-1 was tested in human TNBC and luminal A-type breast cancer by immunofluorescence. The results showed that BMI-1 expression was upregulated in both TNBC and luminal A-type breast cancer (Figure 1C–F). In summary, similar to the findings of previous studies,[15,16,23] our results showed that BMI-1 expression was significantly upregulated in human breast cancer tissues.

3.2 | Repression of BMI-1 decreases breast cancer cell proliferation and migration in vitro

BMI-1 is overexpressed in several breast cancer cell lines including MCF7, MDA-MB-231, SKBR-3, and ZR-75-1.[15] In this study,

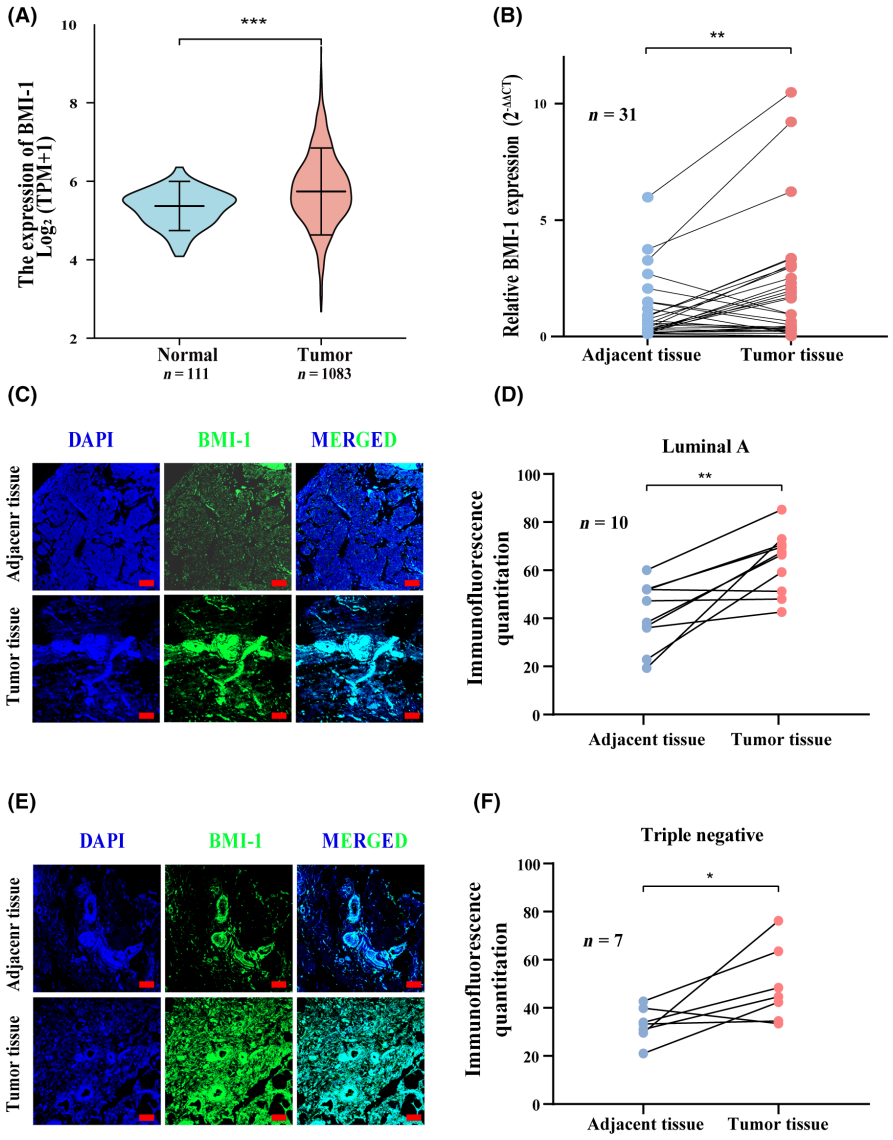


FIGURE 1 BMI-1 expression was upregulated in human breast cancer tissues. (A) Differential BMI-1 expression between normal ($n = 111$) and breast cancer tissues ($n = 1083$) was determined based on TCGA and GTEx data. (B) Relative *BMI-1* mRNA levels were examined by qRT-PCR and normalized to 18S RNA expression in 31 paired human breast cancer tissues and adjacent tissues. (C) BMI-1 protein expression in luminal A-type breast cancer ($n = 10$) was detected by immunofluorescence. Immunofluorescence staining for DAPI was used to stain nuclei. Scale bars: 50 μm. (D) Quantification of intensity of immunofluorescence of (C). (E) BMI-1 protein expression in TNBC ($n = 7$) was detected by immunofluorescence. Scale bars: 50 μm. (F) Quantification of intensity of immunofluorescence of (E). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with adjacent tissues for each condition via Student's *t*-test. The data are shown as means ± SD.

western blotting was performed to analyze BMI-1 expression in MDA-MB-231, MCF-7, and MDA-MB-468 cells. The MDA-MB-231 cell line showed the highest levels of expression, whereas MCF-7 showed moderate expression, and MDA-MB-468 showed the lowest expression (Figure 2A,B). Therefore, we used the MDA-MB-231 and MCF-7 cell lines for analysis of the mechanistic and functional roles of BMI-1 in breast cancer. MDA-MB-231 was chosen as a model for TNBC, which is known to have a more aggressive phenotype and limited treatment choices.[24] MCF-7 was used as the model for luminal A-type breast cancer,[25] which has a better prognosis. We first determined the effects of BMI-1 inhibition by western blotting using a BMI-1-specific inhibitor, PTC-209.[26,27] We observed that treatment with PTC-209 for 48 h led to downregulation of BMI-1 in a dose-dependent manner (Figure 2C,D).

To further clarify the biological effects of BMI-1 on breast cancer cells in vitro, the cells were grown in the increasing concentrations of PTC-209 (1–20 μM) for 48 h. CCK8 and colony formation assays in vitro showed that treatment with PTC-209 inhibited proliferation of breast cancer cells in a dose-dependent manner (Figure 2E–H). Cancer cell migration also plays an important part in tumor malignant progression. Transwell assays showed that the numbers of migrating cells were significantly decreased in the PTC-209 treatment group (Figure 2I–J). Subsequently, we also obtained the same experimental results via downregulating the BMI-1 using siRNA (Figure S1).

Collectively, these findings revealed that BMI-1 could promote proliferation and migration of breast cancer cells (MDA-MB-231 and MCF-7) in vitro.

3.3 | Repression of BMI-1 decreases breast cancer cell proliferation in vivo

To further assess the effects of BMI-1 on tumorigenesis in vivo, a subcutaneous xenograft tumor model of human breast cancer in nude mice was established. MDA-MB-231 cells were treated with DMSO or 20 μM PTC-209 and injected into nude mice. The mice were sacrificed after 2 weeks. It was obvious that the control group (injected with DMSO) had larger tumor volumes and weights than

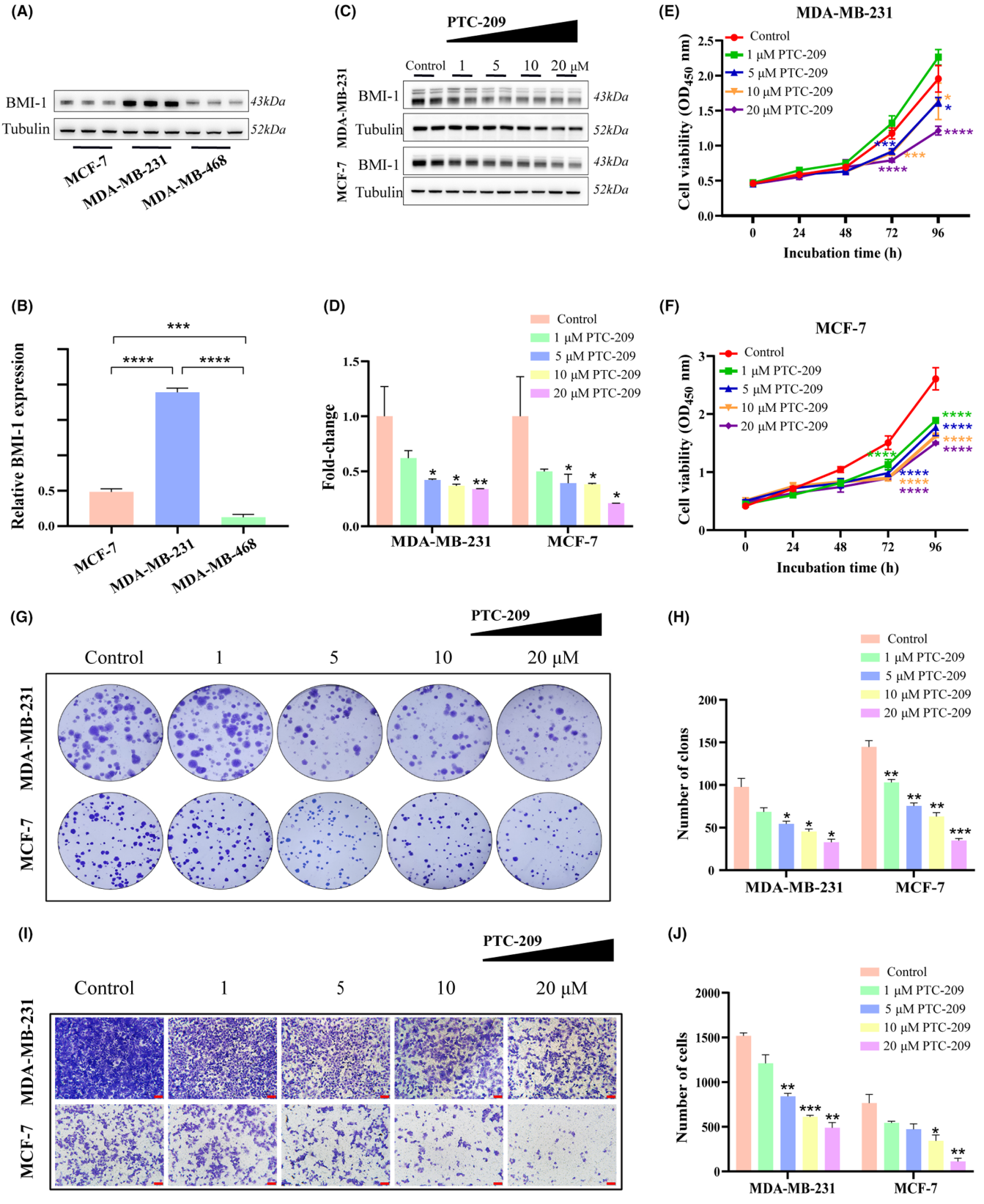


FIGURE 2 Effects of BMI-1 on breast cancer cell proliferation and migration in vitro. (A) BMI-1 expression was examined by western blotting in breast cancer cell lines. (B) Quantification of results from (A). (C) After treatment of MDA-MB-231 and MCF-7 cells with the indicated doses of PTC-209 for 48 h, relative expression levels of BMI-1 were determined by western blot analysis. (D) Quantification of results from (C). (E, F) CCK8 assays ($n = 6$ for each group) were used to determine cell viability. (G) Colony formation assays ($n = 3$) were performed to determine cell proliferation. (H) Quantification of results from (G). (I) Transwell assays ($n = 3$) were carried out to investigate cell migration. Scale bars: 100 μm. (J) Quantification of results from (I). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

the PTC-209 group (Figure 3A–C). Immunofluorescence analysis showed that the expression of Ki-67 was significantly lower in the PTC-209 group (Figure 3D–E). These results indicated that BMI-1 could promote tumor progression in vivo.

3.4 | BMI-1 promotes cell proliferation and migration through different molecular pathways in MDA-MB-231 and MCF-7 cells probably

Previous studies have demonstrated that BMI-1, a major member of PRC1, cooperates with E3 ubiquitin-protein ligase RING2 (RING1B) in the nucleus to monoubiquitinate histone H2A at K119, thereby mediating gene silencing.[28,29] GSEA analysis showed that BMI-1 was significantly correlated with histone ubiquitination in breast cancer (Figure 4A). Next, publicly available ENCODE datasets were used to predict binding sites for the transcription factor BMI-1. Bioinformatic analysis of publicly available BMI-1 ChIP-seq data for the human MCF-7 (ENCODE1, ENCSR966YYJ), K562 (ENCSR782WRO), and GM12878 (ENCSR469WII) cell lines revealed an enriched distribution of BMI-1 binding sites from −1 kb to +1 kb relative to the transcription start sites (TSS) of target genes (Figure 4B). In addition, we performed a visual analysis of the above ChIP-seq data to identify the potential DNA binding loci of BMI-1 in MCF-7 cells (Figure 4C). Gene ontology analysis based on the enriched binding sites of BMI-1 from −1 kb to +1 kb relative to the TSS in MCF-7 cells was also carried out. We found that genes associated with BMI-1 DNA binding sites were highly enriched in Cell Cycle, Signaling by Hedgehog, mRNA Splicing, Cellular responses to stress, Protein processing in endoplasmic reticulum, and Membrane Trafficking. The most meaningful of these terms was Cell Cycle, which has minimum *p*-value and maximum gene count (Figure 4D). Subsequently, we revised the literature to identify tumor suppressor genes involved in the Cell Cycle. qRT-PCR was used to select target genes from these candidate genes. After removal of some candidate target genes without significant amplification, we found that there are 12 out of 45 candidate target genes were expressed more highly in MDA-MB-231 cells treated with 20 μM PTC-209, these included CEP152, TP53BP1, CNTRL, PCM1, CEP164, BRCA1, CDC14A, CENPW, ENSA, LIN54, ABRAXAS1, and HUS1 (Figure 4E). The *BRCA1* gene encodes the tumor suppressor BRCA1, which is linked to breast cancer. Thus, *BRCA1* was selected as the target gene for BMI-1 in MDA-MB-231. By contrast, out of 44 genes, only CDKN2D expression was altered in MCF-7 cells treated with 20 μM PTC-209 compared with the control group (Figure 4F). The above findings indicate that BMI-1 is likely to mediate cell proliferation and migration via distinct target genes in MDA-MB-231 and MCF-7 cells.

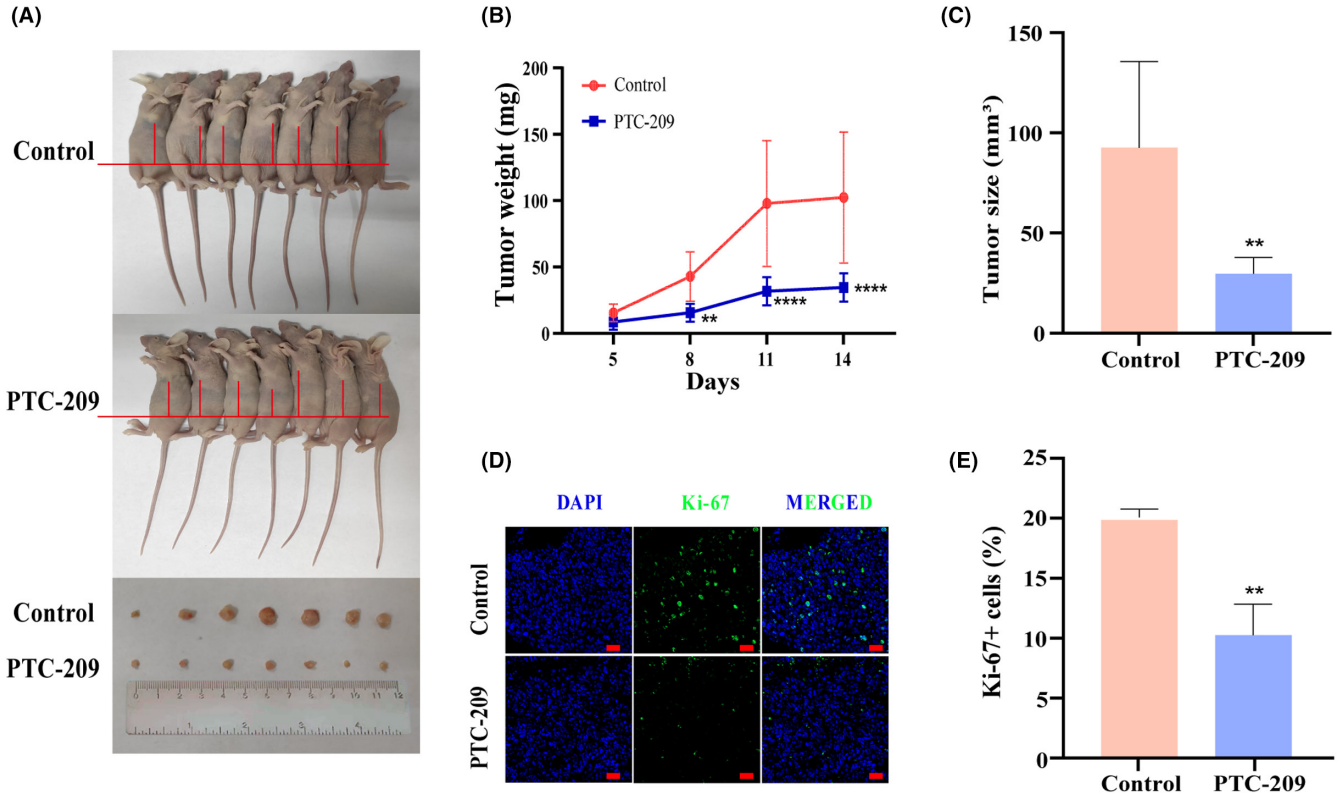


FIGURE 3 Effects of BMI-1 on breast cancer cell proliferation in vivo. MDA-MB-231 cells were treated with DMSO or 20 μM PTC-209 and injected into nude mice ($n = 7$). (A–C) Macroscopic appearance, volume, and weight of subcutaneous tumors in mice. (D) The expression of Ki-67 in the tumor tissues in mice was examined by immunofluorescence assay ($n = 3$). Scale bar: 50 μm. (E) Quantification of results from (D). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

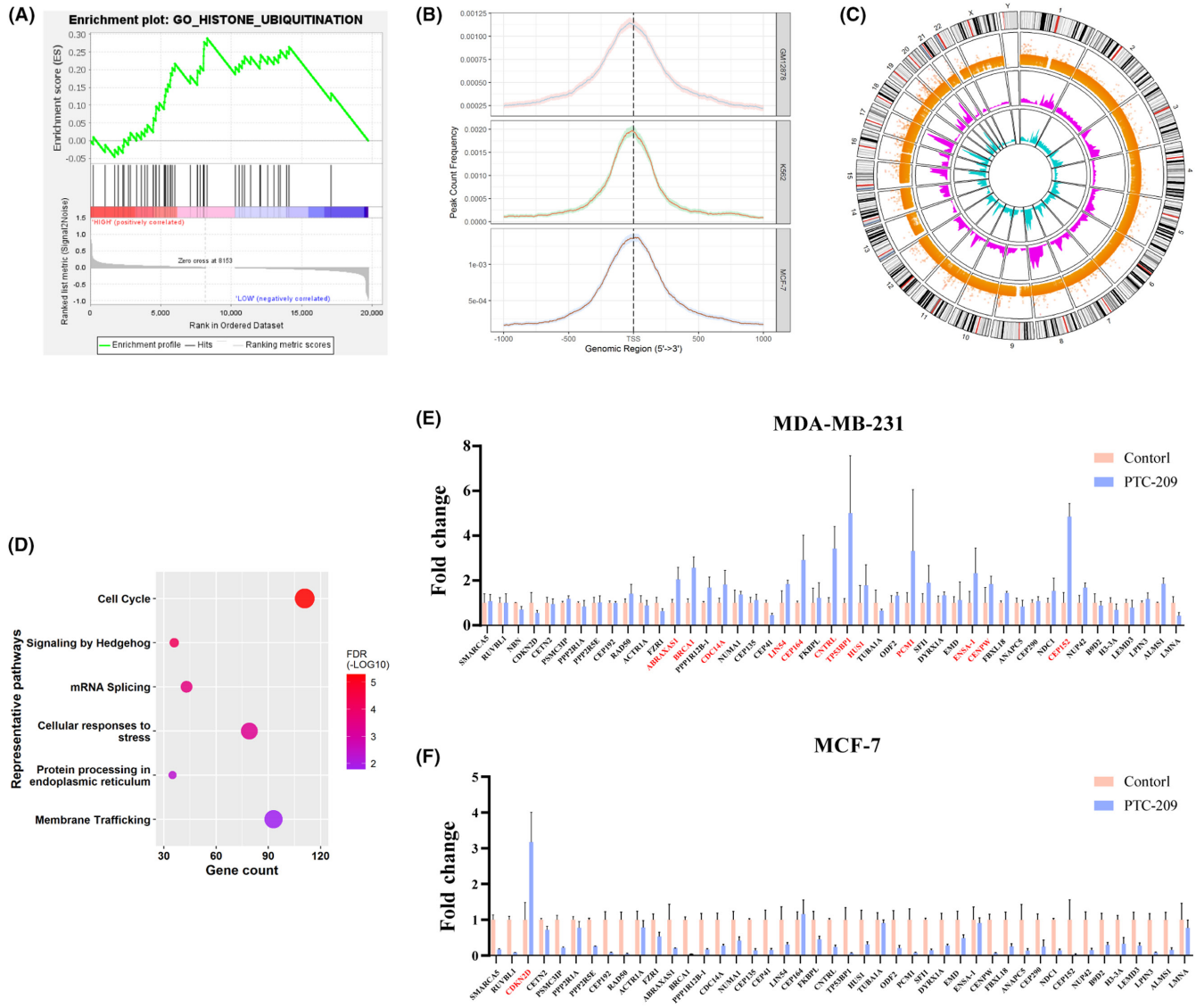


FIGURE 4 Potential molecular mechanisms of BMI-1 in breast cancer cells. (A) GSEA analysis revealed the gene set involved in histone ubiquitination. (B) Distribution of transcription factor-binding loci relative to the TSS in the indicated cell lines. (C) Representative ChIP-seq peaks surrounding *BMI-1* in MCF-7. (D) Gene ontology functional enrichment analysis for predicted target genes of BMI-1 from −1 kb to +1 kb relative to the TSS. (E, F) MDA-MB-231 and MCF-7 were treated with 20 μM PTC-209; the relative expression of genes involved in the cell cycle was detected by qRT-PCR.

3.5 | BMI-1 works through repressing the transcription of CDKN2D in MCF-7 cells and BRCA1 in MDA-MB-231 cells

Bioinformatic analysis of publicly available BMI-1 ChIP-seq data in MCF-7 cells revealed strong BMI-1 binding peaks at the promoter region of *BRCA1* (Figure 5A). ChIP-qPCR assay validated the significant enrichment of BMI-1-bound DNA fragments at BRCA1 in MDA-MB-231 cells (Figure 5B). In addition, after treatment of MDA-MB-231 cells with PTC-209, measurably smaller amounts of H2AK119ub and RING1B were precipitated with BMI-1-bound DNA fragments (Figure 5C,D), and H3K4me3 levels were significantly increased (Figure 5E). These data suggested that BMI-1 represses BRCA1 transcription in MDA-MB-231 cells through assembling PRC1 and binding to the promoter region of *BRCA1* and modulating its chromatin status.

Similarly, we observed an obvious peak in BMI-1 binding at the promoter region of *CDKN2D* in MCF-7 cells (Figure 5F). The ChIP-qPCR assay validated the significant enrichment of BMI-1-bound DNA fragments at CDKN2D in MCF-7 cells (Figure 5G). Similar to the results in MDA-MB-231 cells, after treatment of MCF-7 cells with PTC-209, measurably smaller amounts of H2AK119ub and RING1B were precipitated with BMI-1-bound DNA fragments (Figure 5H,I), and H3K4me3 levels were significantly increased (Figure 5J). Taken together, these results indicated that BMI-1 represses CDKN2D transcription in MCF-7 cells through assembling PRC1, and binding to the promoter region of *CDKN2D* and modulating its chromatin status.

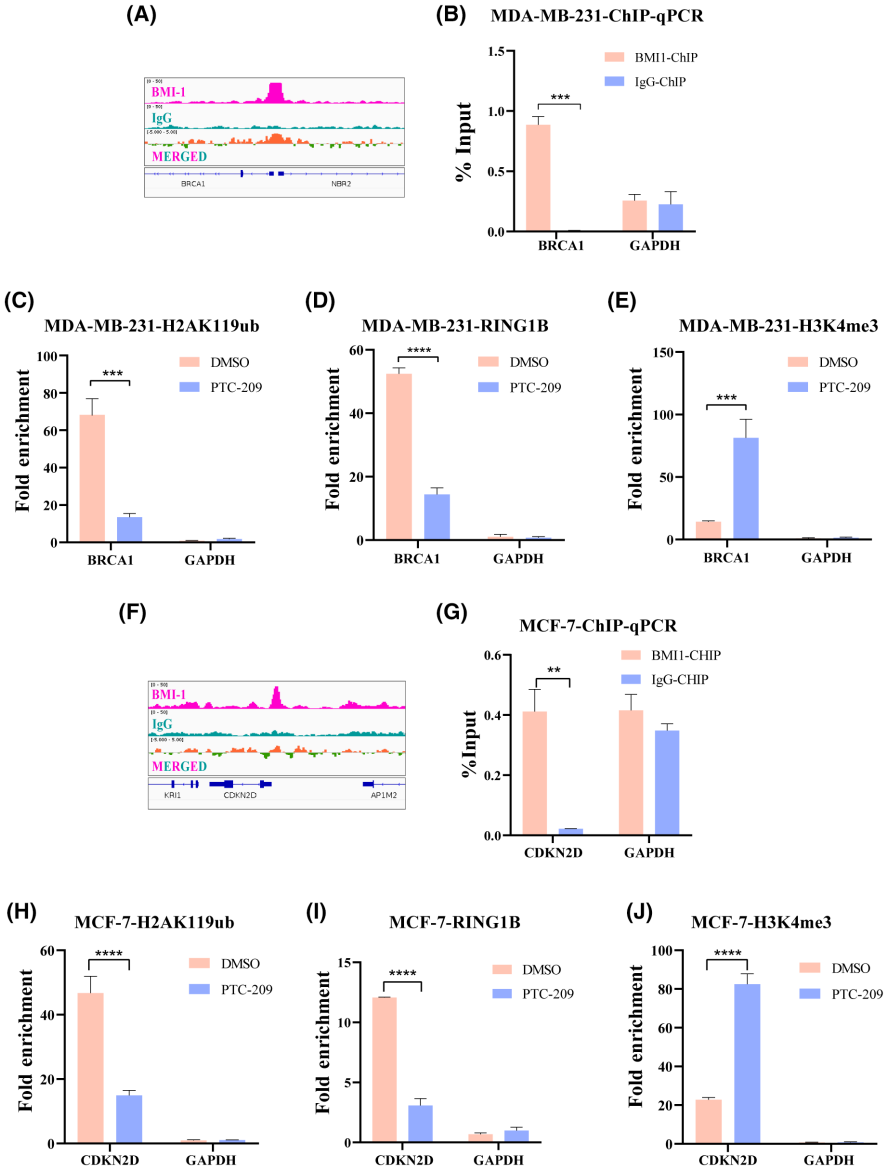


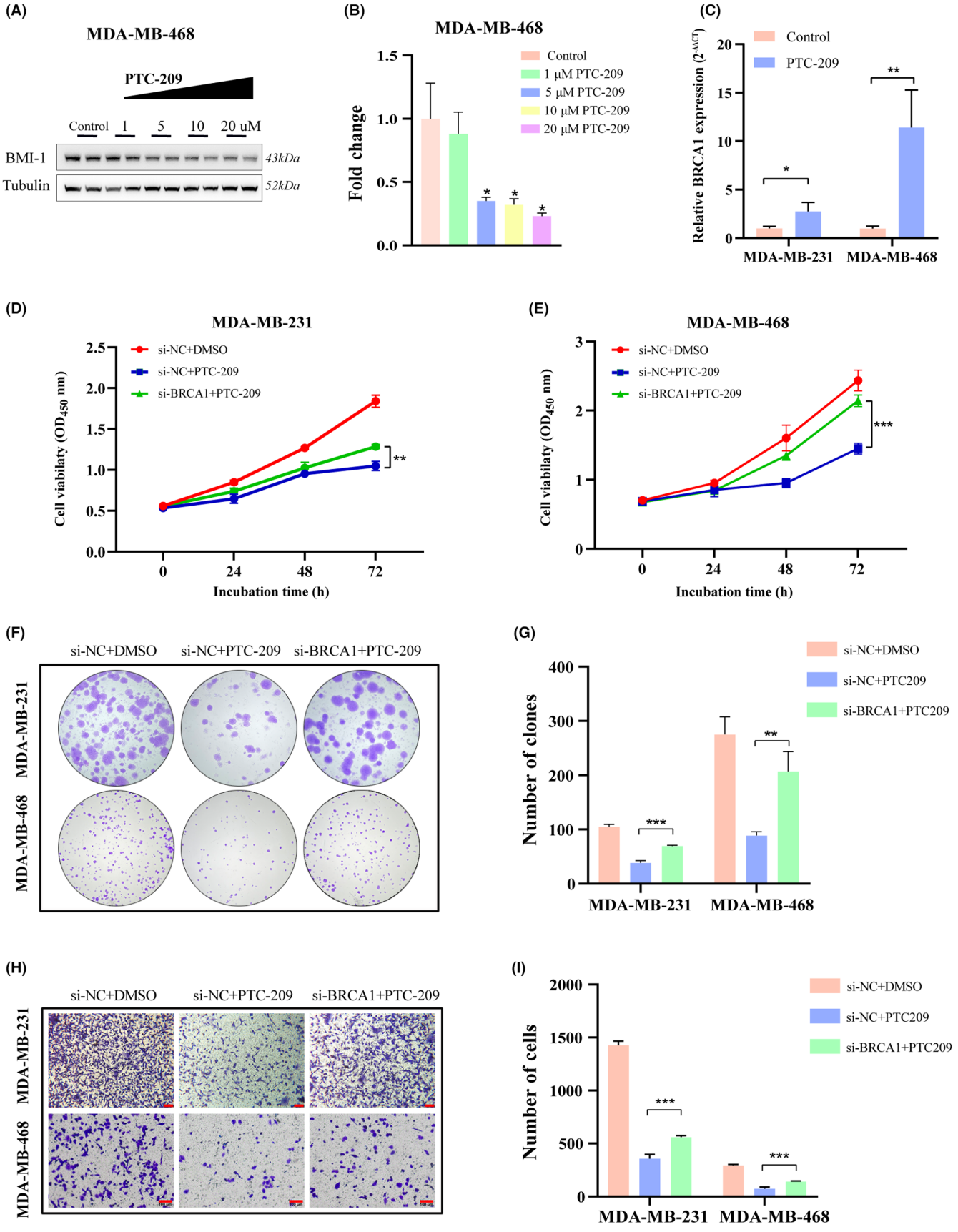
FIGURE 5 BMI-1 represses the transcription of BRCA1 in MDA-MB-231 cell lines and CDKN2D in MCF-7 cells. (A) Representative ChIP-seq peaks surrounding BMI-1 target genes BRCA1. (B) ChIP-qPCR of BMI-1-associated DNA sequences from the putative BMI-1-binding region of the BRCA1 promoter in MDA-MB-231 cells. (C–E) ChIP-qPCR of H2AK119ub-associated (C), RING1B-associated (D), and H3K4me3-associated (E) DNA sequences in the putative BMI-1-binding region of the BRCA1 promoter in DMSO-treated or PTC-209-treated MDA-MB-231 cells ($n = 3$). (F) Representative ChIP-seq peaks surrounding BMI-1 target gene *CDKN2D*. (G) ChIP-qPCR of BMI-1-associated DNA sequences from the putative BMI-1-binding region of the CDKN2D promoter in MCF-7 cells. (H–J) ChIP-qPCR of H2AK119ub-associated (H), RING1B-associated (I), and H3K4me3-associated (J) DNA sequences in the putative BMI-1-binding region of the *CDKN2D* promoter in DMSO-treated or PTC-209-treated MCF-7 cells ($n = 3$). The *GAPDH* gene was used as a negative control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

3.6 | Knockdown of *BRCA1* in TNBC cell lines and *CDKN2D* in luminal A cell lines with PTC-209 treatment restores breast cancer progression

To further clarify whether the repression of *BRCA1* in TNBC cell lines and *CDKN2D* in luminal A cell lines contributed to the pro-tumor effect of BMI-1 in breast cancer cells, MDA-MB-231 and MDA-MB-468 were used as representative cell lines for TNBC, and MCF-7 and T47D for luminal A. Similarly, treatment with PTC-209 led to the downregulation of *BMI-1* in a dose-dependent manner in MDA-MB-468 (Figure 6A,B) and T47D (Figure 7A,B).

First, higher BRCA1 expression levels were found in PTC-209 group in MDA-MB-231 and MDA-MB-468 (Figure 6C). Afterward, we downregulated *BRCA1* via siRNA-mediated gene silencing in MDA-MB-231 and MDA-MB-468 cells. As expected, treatment with si-BRCA1 of PTC-209-treated MDA-MB-231 and MDA-MB-468 significantly increased cell proliferation (Figure 6D–G) and migration (Figure 6H,I) compared with cells treated with PTC-209 alone.

FIGURE 6 Inhibition of BRCA1 restores breast cancer progression in PTC-209-treated MDA-MB-231 and MDA-MB-468 cells in vitro. (A) After treatment of MDA-MB-468 cells with the indicated doses of PTC-209 for 48 h, relative expression levels of BMI-1 were determined by western blot analysis. (B) Quantification of results from (A). (C) Relative BRCA1 expression levels in MDA-MB-231 and MDA-MB-468 treated with 20 μM PTC-209 determined by qRT-PCR ($n = 3$). (D, E) After treatment with si-NC + DMSO, si-NC + 20 μM PTC-209, or si-BRCA1+ 20 μM PTC-209 for 48 h, CCK8 assays were used to determine cell viability ($n = 6$). (F) Colony formation assays were performed to determine cell proliferation ($n = 3$). (G) Quantification of results from (F). (H) Transwell assays were carried out to investigate cell migration ($n = 3$). Scale bar: 100 μm. (I) Quantification of results from (H). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.


(A)
MDA-MB-468
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52kDa
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10 μM PTC-209
20 μM PTC-209
Fold change
(C)
Control
PTC-209
Relative BRCA1 expression (2-ΔΔCT)
MDA-MB-231
MDA-MB-468
(D)
MDA-MB-231
si-NC+DMSO
si-NC+PTC-209
si-BRCA1+PTC-209
Cell viabilaty (OD450 nm)
Incubation time (h)
(E)
MDA-MB-468
si-NC+DMSO
si-NC+PTC-209
si-BRCA1+PTC-209
Cell viabilaty (OD450 nm)
Incubation time (h)
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si-NC+DMSO
si-NC+PTC-209
si-BRCA1+PTC-209
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MDA-MB-468
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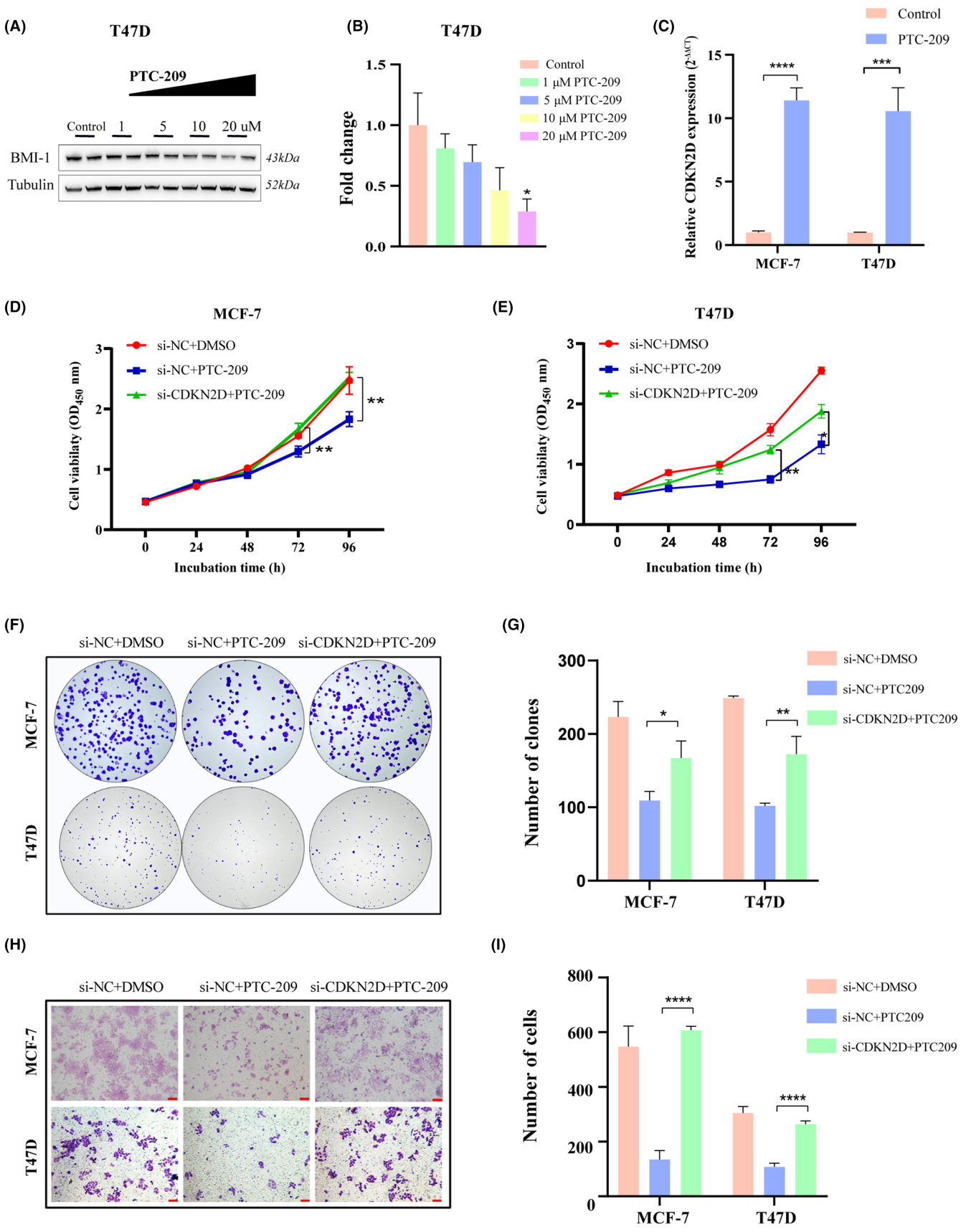
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10 μM PTC-209
20 μM PTC-209
Fold change
(C)
Control
PTC-209
Relative CDKN2D expression (2-ΔΔCT)
MCF-7
T47D
(D)
MCF-7
si-NC+DMSO
si-NC+PTC-209
si-CDKN2D+PTC-209
Cell viability (OD450 nm)
Incubation time (h)
(E)
T47D
si-NC+DMSO
si-NC+PTC-209
si-CDKN2D+PTC-209
Cell viability (OD450 nm)
Incubation time (h)
(F)
si-NC+DMSO
si-NC+PTC-209
si-CDKN2D+PTC-209
MCF-7
T47D
(G)
si-NC+DMSO
si-NC+PTC209
si-CDKN2D+PTC209
Number of clones
MCF-7
T47D
(H)
si-NC+DMSO
si-NC+PTC-209
si-CDKN2D+PTC-209
MCF-7
T47D
(I)
si-NC+DMSO
si-NC+PTC209
si-CDKN2D+PTC209
Number of cells
MCF-7
T47D

FIGURE 7 Inhibition of CDKN2D restores breast cancer progression in PTC-209-treated MCF-7 and T47D cells in vitro. (A) After treatment of T47D cells with the indicated doses of PTC-209 for 48 h, relative BMI-1 levels were determined by western blot analysis. (B) Quantification of results from (A). (C) Relative *CDKN2D* expression levels in MCF-7 and T47D treated with 20 μM PTC-209 determined by qRT-PCR ($n = 3$). (D, E) After treatment of MCF-7 and T47D cells with si-NC + DMSO, si-NC + 20 μM PTC-209, or si-CDKN2D + 20 μM PTC-209 for 48 h, CCK8 assays were used to determine cell viability ($n = 6$). (F) Colony formation assays were performed to determine cell proliferation ($n = 3$). (G) Quantification of results from (F). (H) Transwell assays were carried out to investigate cell migration ($n = 3$). Scale bar: 100 μm. (I) Quantification of results from (H). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

In the same way, higher CDKN2D expression levels were found in PTC-209 group in MCF-7 and T47D (Figure 7C). Treatment of PTC-209-treated MCF-7 and T47D cells with si-CDKN2D significantly increased cell proliferation and migration (Figure 7D–I), compared with cells treated with PTC-209 alone.

In summary, knockdown of *BRCA1* in MDA-MB-231 and MDA-MB-468 as well as *CDKN2D* in MCF-7 and T47D restored breast cancer progression in PTC-209-treated cells.

All the results of this study indicated that BMI-1 exerts a protumor effect via assembling PRC1 to regulate chromatin accessibility and repress transcription of distinct target genes in different breast cancer cell types. In MDA-MB-231 and MDA-MB-468 cells, BMI-1 targeted BRCA1 (Figure 8A), whereas it targeted CDKN2D in MCF-7 and T47D cells (Figure 8B).

4 | DISCUSSION

The present study showed that BMI-1 expression was significantly upregulated in breast cancer tissues compared with adjacent tissues at both the mRNA and protein levels, consistent with the results of a previous study.[30] Loss-of-function assays verified that reducing the expression of BMI-1 could inhibit breast cancer cell proliferation and migration in vitro and in vivo. These findings indicate that BMI-1 acts as an oncogene in breast cancer development.

Extensive research has shown that BMI-1 functions as an oncogene by suppressing the p14ARF/MDM2/p53 and/or p16INK4A/RB transcriptional corepressor 1 signaling pathways. However, accumulating evidence indicates that this locus is not the only crucial BMI-1 target regulating cancer progression.[31–34] Here, using publicly available BMI-1 ChIP-seq data, functional enrichment analysis, and ChIP-qPCR assay, we identified different downstream target genes in TNBC and luminal A-type breast cancer cells.

In TNBC cells, *BRCA1* was selected as a target gene in our study. This gene encodes the tumor suppressor BRCA1, which was first linked to hereditary breast and ovarian cancer in the early 1990s.[35] A series of studies has demonstrated that BRCA1 may exert its tumor-suppressive function through its involvement in DNA repair by distinct pathways, control of cell cycle checkpoints, centrosome amplification, transcriptional activation, and ubiquitin ligation.[36–38] It has been reported that downregulation of wild-type BRCA1 or alterations in BRCA1-related pathway(s) contribute to breast cancer.[39,40] In depth, Hong and colleagues found that BRCA1 modulates aspartate biosynthesis through transcriptional repression of GOT2, which in turn decreases aspartate and alpha ketoglutarate production, leading to slowdown in cell proliferation of breast cancer cells.[41] TNRC9 downregulates BRCA1 expression and promotes breast cancer cell proliferation and migration.[42] Our study also provides further insight into the significance of BRCA1 in breast cancer.

In luminal A-type breast cancer cells, we focused on CDKN2D ($p19^{INK4d}$), which belongs to the INK4 protein family of inhibitors of CDK4. The INK4 family consists of CDKN2A, CDKN2B, CDKN2C, and CDKN2D, which bind to CDK4 and CDK6 and specifically inhibit the pRb kinase activity of cyclin D–CDK4 and cyclin D–CDK6 complexes to regulate the G1–S phase transition.[43,44] The genes of the INK4 family act as tumor suppressors in the pathogenesis of many malignancies, including leukemia,[45] and provide a direct link between tumorigenesis and the loss of negative control of cell cycle progression. Wang and colleagues found that PML/RARα could disrupt both proliferation and differentiation by repressing CDKN2D expression, thereby promoting the pathogenesis of acute promyelocytic leukemia (APL).[46] However, the role of CDKN2D in breast cancer has not been studied extensively. In this study, we showed that BMI-1 might function in MCF-7 by inhibiting CDKN2D, suggesting that CDKN2D could serve as a target gene of BMI-1 in luminal A-type breast cancer.

A possible mechanism of PRC1-mediated repression is the recruitment of certain regulatory factors, or chromatin-modifying activities, into a unique nuclear domain, which results in inhibition of the chromatin remodeling required for the transcriptional process.[47] PRC1 possesses ubiquitin E3 ligase activity that targets H2AK119, and this modification is associated with gene repression.[28] Trimethylation of histone H3 at lysine 4 (H3K4me3) marks the TSS.[48] Thus, H3K4me3 plays an important role in the initiation of transcriptional repression. In the present study, after treatment with PTC-209, measurably smaller amounts of H2AK119ub and RING1B were precipitated with BMI-1-bound DNA fragments, and H3K4me3 levels were significantly increased at the promoter regions of *CDKN2D* and *BRCA1*, which derepressed their expression. A similar finding was reported by Zhou and co-workers who showed that knockdown of BMI-1 resulted in a substantial loss of H2AK119ub and an increase in H3K4me3 levels, and suppressed the expression of cardiogenic genes.[49] Similar results were obtained in our previous study.[50] Overall, repression of the transcription of target genes by BMI-1 not only required the formation of PRC1 but also relies on the presence of H3K4me3.

In summary, we uncovered a novel pathway by which BMI-1 regulates breast cancer progression. In luminal A cells, BMI-1 binds to the promoter region of CDKN2D, thereby recruiting PRC1 proteins to modify its chromatin status and repress its expression.

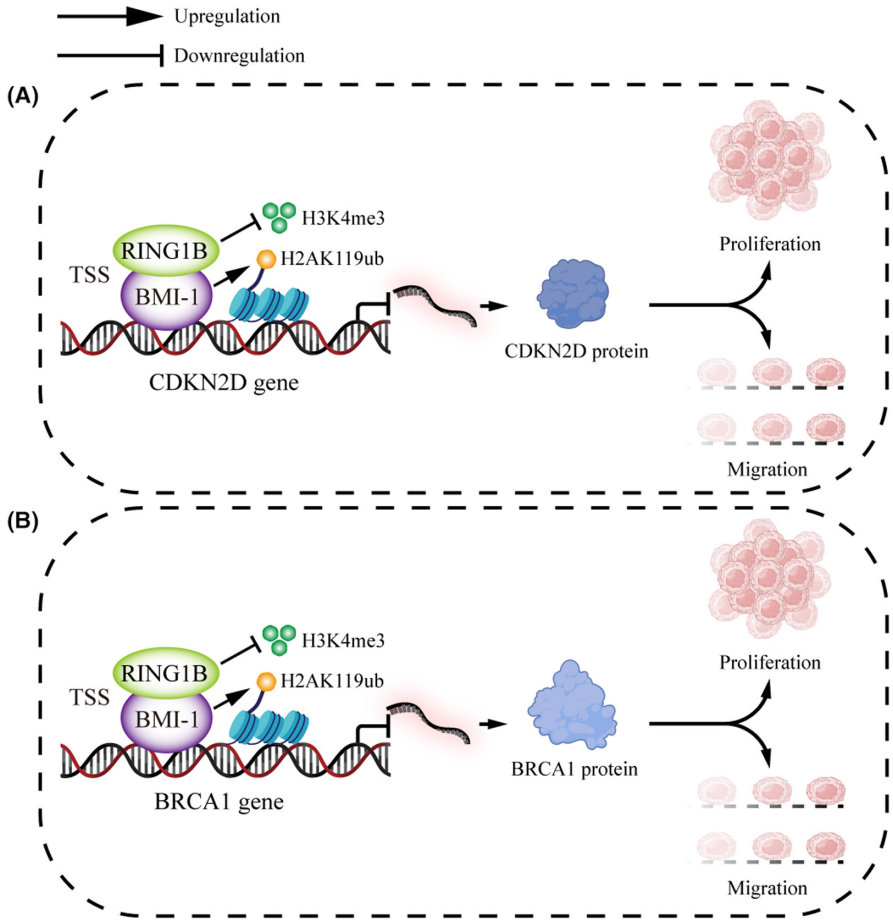

FIGURE 8 Schematic illustration of a working model for the role of BMI-1 in breast cancer cells. (A) MCF-7 and T47D. (B) MDA-MB-231 and MDA-MB-468.

Importantly, knockdown of CDKN2D greatly improved cell proliferation and migration in PTC-209-treated MCF-7 cells (Figure 8A). In TNBC cells, BMI-1 transcriptionally represses BRCA1, whereas knockdown of BRCA1 can restore cell proliferation and migration repressed by PTC-209 (Figure 8B).

The findings reported here shed new light on the distinct mechanisms by which BMI-1 promotes cell proliferation and migration in different breast cancer cell types in vivo and in vitro. Our results demonstrate that the BMI-1/CDKN2D axis in luminal A cells and the BMI-1/BRCA1 signaling pathway in TNBC cells are critical for cell proliferation and migration. These results indicate potential new directions for novel avenues for exploring efficient therapeutic strategies for different molecular types of breast cancer.

AUTHOR CONTRIBUTIONS

JL, CS, YJ, and HH performed most of the experiments. JX, YW, QW, and YZ performed some of the experiments. BZ analyzed the data. CS, WQ, and JS initiated the project and designed the experiments. JL and CS wrote the paper. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

Funding from the Gusu Health Talent Project of Suzhou (GSWS2020056), "333" Talent Project of Jiangsu (BRA2017057) and the Suzhou Health and Family Planning Commission (SKJYD2021104) is gratefully acknowledged. The funder had no involvement in the study design, data analysis, and interpretation of the results. The authors are grateful to all study participants.

FUNDING INFORMATION

Gusu Health Talent Project of Suzhou (GSWS2020056), the "333" Talent Project of Jiangsu (BRA2017057), and the Suzhou Health and Family Planning Commission (SKJYD2021104).

CONFLICT OF INTEREST

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethics Committee approval was obtained from the Institutional Ethics Committee of the Suzhou Municipal Hospital to the commencement of the study, approval number is KL901273. Animal experiments were approved by the Animal Ethical and Welfare Committee of Nanjing Medical University.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Liu J-y, Jiang Y-n, Huang H, et al. BMI-1 promotes breast cancer proliferation and metastasis through different mechanisms in different subtypes. *Cancer Sci*. 2023;114:449-462. doi: 10.1111/cas.15623