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Histone demethylase JARID1C promotes breast cancer metastasis cells via down regulating BRMS1 expression

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ABSTRACT

Metastasis is the leading cause of death in breast cancer patients. However, until now, the mechanisms of breast cancer metastasis remain elusive. Epigenetic switch, including histone methylation or demethylation, which can either activates or represses transcription. The JARID1C is a histone demethylase that promotes cancer cell growth and is involved in transcriptional regulation and chromatin remodeling, cause X-linked mental retardation. But the pathogenic breadth and mechanistic aspects of this effect relative to breast cancer have not been defined. In this study, we aimed to investigate the role of JARID1C in breast cancer. In clinical breast cancer samples, we found that JARID1C expression was significantly upregulated in cancer lesions compared with paired normal breast tissues and its expression level is positively correlated with metastasis. Silencing JARID1C in breast cancer cells could inhibit cell migration and invasion. Moreover, we also found that the expression of BRMS1 was modulated by JARID1C. Silencing of JARID1C dramatically increased BRMS1 expression both at mRNA and protein level. Mechanistically, we found JARID1C exerts its function through modulation of H3K4me3 at the *BRMS1* gene promoter, which was associated with inactive *BRMS1* transcription. *BRMS1* knockdown reversed shJARID1C-induced migration inhibition. Further, BRMS1 expression in human breast cancer is negatively correlated with JARID1C expression. Our results, for the first time, portray a pivotal role of JARID1C in regulating metastatic behaviors of breast cancer cells.

1. Introduction

Breast cancer is the most frequently diagnosed cancer among females [1]. Despite the advances that have been made in the treatment of breast cancer, metastasis is the leading cause of death in breast cancer patients [1,2]. Metastasis is a multistep process that results from genetic alterations, including the activation of oncogenes and the loss of function of tumor suppressors in breast cancer [2]. Despite a considerable amount of research, very few stable biomarkers have been identified for risk assessment or predication of clinical outcome in breast cancer metastasis and further investigations are necessary.

The methylation of the lysine on position 4 of histone 3 is one of the more versatile and widespread histone modifications [3]. Histone posttranslational modifications affecting this residue have been implicated, for the most part, in finely tuning gene expression on promoters [4]. JARID1C belongs to the JARID1 subfamily of JmjC containing proteins, together with JARID1A, JARID1C and JARID1D [5]. The JmjC domain of JARID1C represents its catalytic moiety that specifically demethylates di- and trimethylated lysine 4 on histone 3 in a Fe (II) and α-ketoglutarate-dependent manner [6]. The JmjN domain and the C5HC2 zinc finger are both important for assisting JARID1C catalytic activity [7]. JARID1C contains also a BRIGHT domain and an AT-rich domain interacting domain that binds DNA [8]. JARID1C resides on the X chromosome [9]. It is expressed in multiple human tissues, has a paralogue on the Y chromosome and is highly conserved across evolution [9]. JARID1C missense, frameshift and nonsense mutations have been linked to X-linked mental retardation (XLMR) [5]. JARID1C exerts a prominent role in neuronal development and function [10].

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A line of recent reports indicated that positive nuclear JARID1C staining was significantly associated with a reduced prostate-specific antigen relapse-free survival [11]. JARID1C knockdown resulted in growth retardation of prostate cancer cells *in vitro* and induced regulation of several proliferation-associated genes [12]. Moreover, overexpression of JARID1C is an independent new predictive marker for therapy failure as determined by biochemical recurrence in patients after prostatectomy [12]. However, the contribution of JARID1C in breast cancer tumorigenesis is still unknown.

In this study, we found that JARID1C expression was significantly upregulated in cancer lesions compared with paired normal breast tissues and its expression level is positively correlated with metastasis. By silencing JARID1C in breast cancer cells could inhibit cell migration and invasion. Moreover, we also found that silencing of JARID1C dramatically increased BRMS1 expression both at mRNA and protein level. Mechanistically, we found JARID1C exerts its function through modulation of H3K4me3 at the BRMS1 gene promoter, which was associated with inactive BRMS1 transcription. BRMS1 knockdown reversed shJARID1C-induced migration inhibition. Further, BRMS1 expression in human breast cancer is negatively correlated with JARID1C expression. Our results, for the first time, portray a pivotal role of JARID1C in regulating metastatic behaviors of breast cancer cells.

2. Materials and methods

2.1. Samples and antibodies

Human normal breast tissues and breast cancer tissue samples were obtained from patients who underwent surgery therapeutic procedures at Department of pathology, Qilu Hospital, Shandong University. The characteristics of 23 patients were shown in Supplemental Table 1. All experiments were approved by the ethics committee of Qilu Hospital and informed consent was obtained from all patients prior to specimen collection. Mouse monoclonal JARID1C, BRMS1, Twist1, and β-actin antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA).

2.2. Cell culture

The human breast cancer cell lines, MDA-MB-468, MDA-MB-231, BT549, HCC1806, HCC1954, SK-BR-3, BT474, BT-20, AU565, T47D, non-tumorigenic human breast epithelial cell line, MCF10A, were purchased from the American Type Culture Collection (Manassas, VA, USA), where they were characterized by DNA-fingerprinting and isozyme detection. Breast cancer cells were grown in RPMI 1640 medium supplemented with 10% FBS. MCF10A cells were grown in 50% DMEM, 50% F-12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin, 10 μg/ml insulin, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant human epidermal growth factor, and 1 mM CaCl2. All the cell lines were grown at 37 °C in a 5% CO2/95% air atmosphere and were revived every 3–4 months.

2.3. Plasmid construction and transfection

For JARID1C RNA interference, the control (pSuper) and pSuper-sh JARID1C plasmids were purchased from OligoEngine Biotechnology (Seattle, USA) and was used to transfect the MDA-MB-231 and BT549 cells to establish the JARID1C knockdown cell line. The transfection efficiency of shJARID1C was confirmed by western blot and quantitative reversetranscription PCR (qRT-PCR) analyses.

2.4. Western blot assay

Equal amounts of protein were separated using SDS polyacrylamide gels and were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted overnight at 4 °C with primary antibodies, followed by their respective secondary antibodies. β-actin was used as the loading control.

2.5. Quantitative reverse-transcription PCR

RNA was extracted using TRIzol reagent, according to the manufacturer's recommended protocol (Invitrogen). qRT-PCR was performed using Applied Biosystems (Foster City, CA, USA) StepOne and StepOne Plus Real-Time PCR Systems. GAPDH was used as a loading control. The experiments were repeated a minimum of three times to confirm the results.

2.6. Cell invasion and motility assay

Invasion of cells was measured in Matrigel (BD, Franklin Lakes, NJ, USA) -coated Transwell inserts (6.5 mm, Costar, Manassas, VA, USA) containing polycarbonate filters with 8-μm pores as detailed previously [13]. The inserts were coated with 50 μl of 1 mg/ml Matrigel matrix according to the manufacturer's recommendations. 2 × 105 cells in 200 μl of serum-free medium were plated in the upper chamber, whereas 600 μl of medium with 10% fatal bovine serum were added to lower well. After 24 h incubation, cells that migrated to the lower surface of the membrane were fixed and stained. For each membrane, five random fields were counted at ×10 magnification. The mean was calculated and data were presented as mean ± s.d. from three independent experiments done in triplicate. Motility assays were similar to Matrigel invasion assay except that the Transwell insert was not coated with Matrigel.

2.7. Gene expression profiling

Total RNA quality and quantity were determined using Agilent 2100 Bioanalyzer and NanoDrop ND-1000. Affymetrix HU U133 plus 2.0 arrays were used according to manufacturer's protocol. The data were initially normalized by robust multiarray average (RMA) normalization algorithms in expression console software (Affymetrix). Genes on GeneChip were globally normalized and scaled to a signal intensity of 500. Fold changes were calculated by comparing transcripts between JARID1C silencing and its control samples. Significantly altered genes between JARID1C silencing and its control samples were listed. The genes were filtered by scatter plots and the genes up- and down-regulated ≥5-fold was considered in preparing the gene list. Clustering analysis was done using gene list by Gene Cluster v3.0 software, and heat maps were visualized using Java TreeView v1.1.4r3 software. Gene set enrichment analysis was carried out using ConceptGen (http://conceptgen.ncibi.org/core/conceptGen/index.jsp). Gene sets were either obtained from the ConceptGen or from published gene signatures.

2.8. Chromatin immunoprecipitation (ChIP)-qPCR

Chromatin immunoprecipitation kit (Cat. 17-371) was purchased from Millipore and ChIP experiments were carried out essentially as described [13]. Immunoprecipitated DNA was analyzed on the ABI PRISM 7900HT sequence detection system. The primers used for detection of promoters after ChIP are available upon request.

2.9. Statistical analysis

Experimental data are shown as mean ± standard deviation (S.D.). The results from different treatment groups were compared using a two-tailed Student's t-test. Differences were considered to be statistically significant at a value of P less than 0.05. Statistical analysis was done with SPSS/Win11.0 software (SPSS, Inc., Chicago, Illinois, USA).

3. Results

3.1. JARID1C is highly expressed in breast cancers, especially in distant metastasis cancer tissues

To investigate whether JARID1C might be involved in breast cancer, the protein expression level of JARID1C in breast cancer tissues and its matched normal adjacent tissues was determined by western blot in 23 samples. As compared with normal tissues, breast cancer specimens showed overexpression of JARID1C (Fig. 1A and B). We then analyzed JARID1C expression in breast cancer tissues without or with distant metastasis; we found that JARID1C protein overexpression was significantly correlated with distant metastasis in breast cancer tissues (Fig. 1C). The mRNA level of JARID1C in these tissue samples were also analyzed by qRT-PCR. The mRNA level of JARID1C was upregulated in breast cancer samples as compared with the normal adjacent tissues samples (Supplemental Fig. 1A). Furthermore, we found that JARID1C mRNA expression was also significantly correlated with distant metastasis in breast cancer tissues (Supplemental Fig. 1B). To further reconnoiter the clinical implication of JARID1C in tumorigenesis and metastasis, we investigated JARID1C expression in a series of human mammary epithelial cells and tumor cell lines. As shown in Fig. 1D, E and Supplemental Fig. 1C, JARID1C was highly expressed in breast cancer cells, esp. in invasive cancer cells compared with normal mammary epithelial cells. These results indicate that the level of JARID1C expression in breast carcinomas correlates with clinical progression and metastasis.

3.2. Silencing of JARID1C inhibits migratory and invasive capacities of breast cancer cells

In order to test the oncogenic activity of JARID1C in breast cancer, we retrovirally established stable silencing of JARID1C in MDA-MB-231 and BT549 cell lines. The levels of JARID1C in these resultant cell lines were verified by western blot (Fig. 2A and B). We next assessed whether JARID1C could affect the ability of breast cancer cells to migrate and invade using a transwell assay. Both MDA-MB-231-shJARID1Cs and BT549-shJARID1Cs cells had significantly slower migratory capacity compared to their control cells (Fig. 2C and D). Moreover, MDA-MB-231-shJARID1Cs and BT549-shJARID1Cs cells showed a lower degree of invasion through Matrigel. These results indicated that silencing of JARID1C significantly inhibited the invasion and migration of breast cancer cells and JARID1C may promote migratory and invasive behaviors in breast cancers.

3.3. JARID1C regulates BRMS1 expression in breast cancer

To better understand the mechanisms by which JARID1C engaged in breast cancer cell migration and invasion, we performed gene expression profiling on MDA-MB-231-shJARID1C #3 and its control cells. Microarray analyses identified a list of genes significantly differentially expressed after silencing of JARID1C expression including upregulation of *BRMS1* (Fig. 3A and Supplemental Table 2). Furthermore, gene set enrichment analysis indicated that *BRMS1* related gene signatures were significantly changed in JARID1C silenced breast cancer cells (Fig. 3B). These data also led us to hypothesize that JARID1C exerts these functions possibly via BRMS1. To test this, we first determined whether BRMS1 is a downstream target of JARID1C in breast cancer cells. Expression of BRMS1 in the cells with silencing of JARID1C expression was further evaluated by western blot and qRT-PCR. Silencing JARID1C in MDA-MB-231 and BT549 cells dramatically increased BRMS1 protein (Fig. 3C and D) and mRNA levels (Fig. 3E and F). Suggesting the regulation of BRMS1 expression by JARID1C is at transcriptional level.

It has been shown that BRMS1 can inhibit Twist1 expression in tumor cells [14]. So we next examined the role of shJARID1C-mediated up regulation of BRMS1 in the Twist1 expression. Of note, silencing JARID1C in MDA-MB-231 and BT549 cells robustly suppressed expression of Twist1 both in protein (Supplemental Fig. 2A and Supplemental 2B) and mRNA (Supplemental Fig. 2C and Supplemental Fig. 2D) levels. To recognize any clinical correlation of JARID1C and BRMS1, we analyzed BRMS1 expression in the same human breast cancer tissues by qRT-PCR. Highly negative correlation between JARID1C and BRMS1 expression was drawn (Supplemental Fig. 3).

3.4. Silencing JARID1C alters BRMS1 expression through decreases H3K4me3

We then explored how JARID1C regulates BRMS1 expression at the transcriptional level. JARID1s are frequently involved in chromatin regulation and histone modifications that play important roles tumor progression. To determine whether JARID1C activity was associated with specific histone modifications in breast cancer cells, histone modification patterns were measured after silencing of JARID1C expression. Among histone H3K4 and H3K9, we found that only H3K4me3 was affected by changes in JARID1C expression (Fig. 4A and B). Silencing of JARID1C increased H3K4me3.

Because H3K4me3 is associated with active transcription, we tested whether JARID1C expression was correlated with the H3K4me3 modification at the BRMS1 gene promoter in breast cancer cells. Quantitative chromatin immunoprecipitation (qChIP) assays were performed in MDA-MB-231-shJARID1C #3 and BT549-shJARID1C #3 cells. Antibodies against H3K4me3 and H3k9me3 were used to pull down the chromatin complex, and two pairs of primers against the BRMS1 gene promoter region (a: −816 bp to −603 bp and b: −365 bp to −138 bp) were used to assess the occupancy of the BRMS1 gene promoter (Fig. 4C). More occupancy of those two BRMS1 gene promoter regions by H3K4me3 was detected in both MDA-MB-231-shJARID1C #3 and BT549-shJARID1C #3 cells (Fig. 4D and F). The occupancy of chromatin repressors such as methylated H3K9 at the BRMS1 gene promoter was not changed by silencing JARID1C expression (Fig. 4E and G). These results clearly indicate that shJARID1C induces transcriptional activation of BRMS1 expression through regulating trimethylation of H3K4 and enriching H3K4me3 to the BRMS1 gene promoter. We next tested the effect of specific blockage of BRMS1 on the migrative function of shJARID1C. As shown in Supplemental Fig. 4A and Supplemental Fig. 4B, treatment with a specific BRMS1 siRNAs considerably increased the abilities of these shJARID1C-transduced cells to induce migration changes. These data suggested that JARID1C promoted the migration of breast cancer cells by simultaneously BRMS1 expression.

4. Discussion

Breast cancer belongs to common and aggressive human malignancies and is one of the leading causes of death by tumor

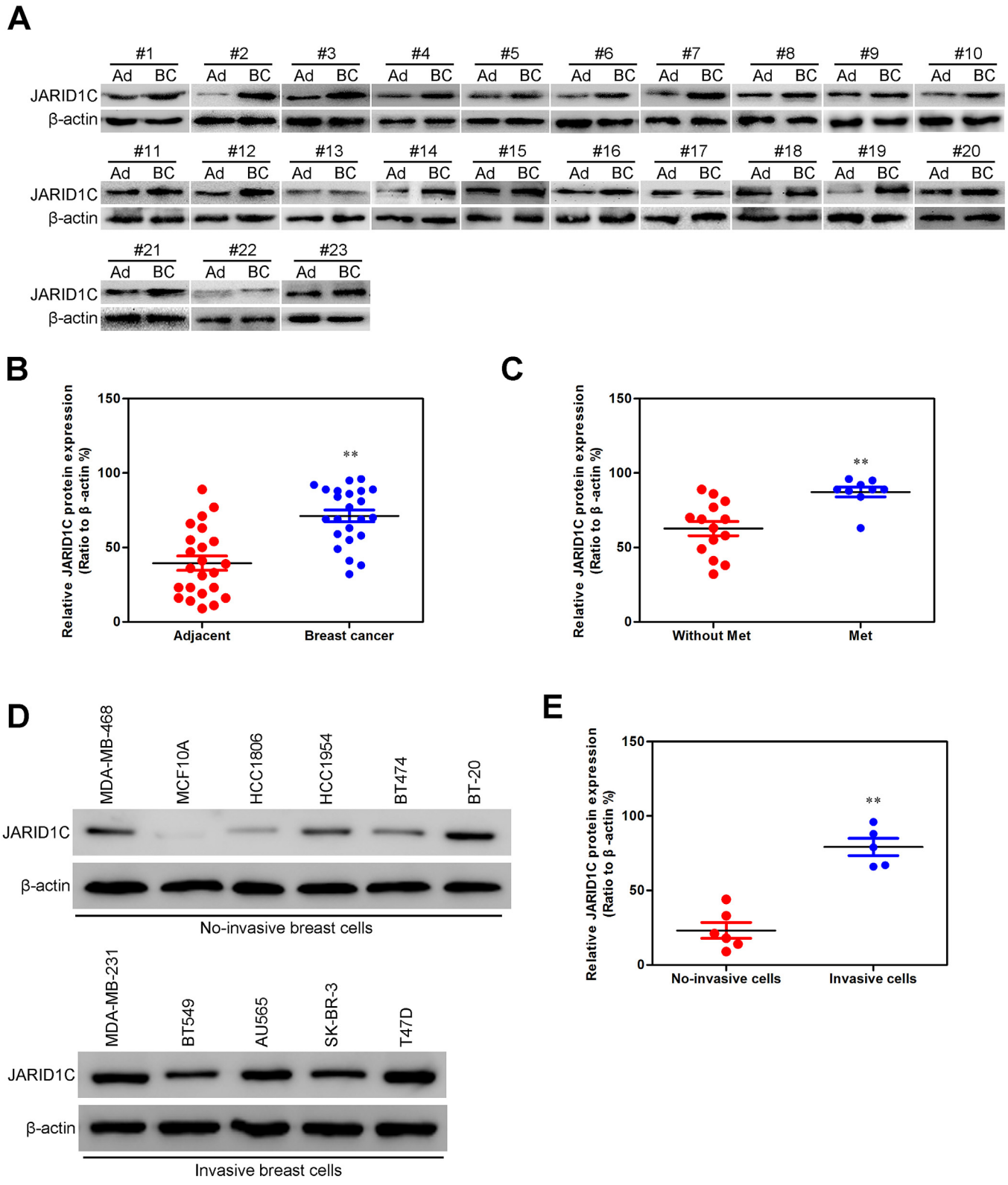

Fig. 1. JARID1C is highly expressed in breast cancers, especially in distant metastasis cancer tissues. A: The expression of JARID1C protein was measured by western blot in breast cancer tissues and its matched normal adjacent tissues. B: Comparison of the relative expression of JARID1C protein in adjacent tissues and breast cancer tissues. C: Comparison of the relative expression of JARID1C protein in not metastasis and metastatic breast cancer tissues. D: The expression of JARID1C protein was measured by western blot in non-tumorigenic human breast epithelial cell line (MCF10A), no-invasive cell lines (MDA-MB-468, HCC1806, HCC1954, BT474, and BT-20), and invasive cell lines (MDA-MB-231, SK-BR-3, BT549, AU565, and T47D). E: Comparison of the relative expression of JARID1C protein in no-invasive cells and invasive cells. **, $P < 0.01$ is based on the Student t test. All results are from three independent experiments. Error bars, SD.

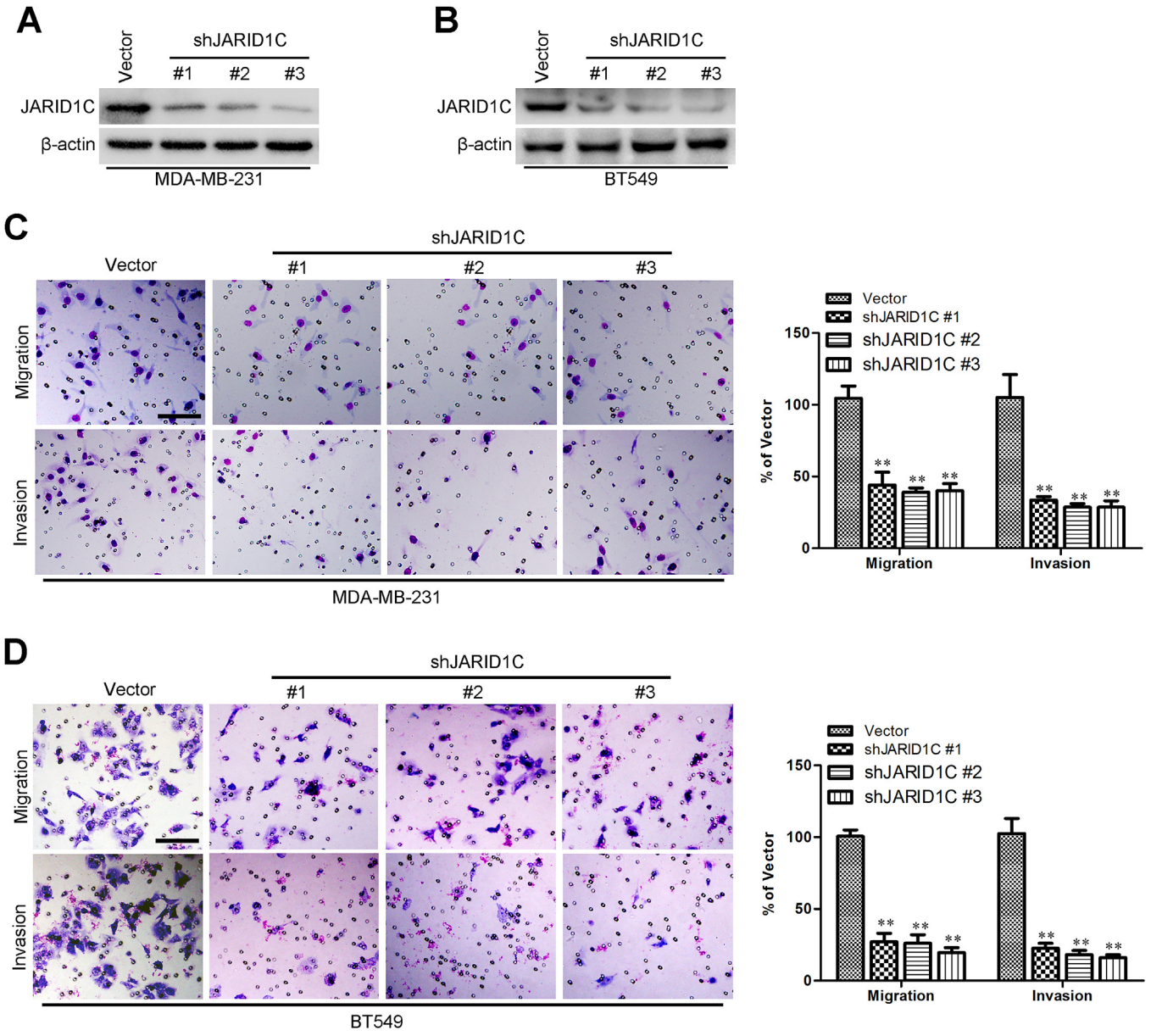

Fig. 2. Silencing of JARID1C inhibits migratory and invasive capacities of breast cancer cells. A: The level of JARID1C in silencing of JARID1C MDA-MB-231 cell line was verified by western blot. B: The level of JARID1C in silencing of JARID1C BT549 cell line was verified by western blot. C: MDA-MB-231-shJARID1Cs, and its control vector cells were subjected to Transwell migration (top), and Matrigel invasion assays (bottom), quantification of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls. D: BT549-shJARID1Cs, and its control vector cells were subjected to Transwell migration (top), and Matrigel invasion assays (bottom), quantification of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls. **, $P < 0.01$ is based on the Student t test. All results are from three independent experiments. Error bars, SD. Scale bar, C and D = 100 μm.

worldwide [1]. The development of breast cancer is a complex multistep process associated with numerous genetic alterations, downregulation of tumor suppressor genes, upregulation of oncogenes and early hematogeneous dissemination of tumor cells [12]. Accordingly, the elucidation of the molecular mechanisms in breast cancer has been the subject of extensive research over the past decade. The high mortality rate of breast cancer is caused by frequent tumor metastasis, postsurgical recurrence, and late detection at advanced stages [2]. However, good diagnostic markers, drug targets and therapeutic strategies are still insufficient for successful treatment of breast cancer.

It has become evident that epigenetic gene regulation plays a crucial role in breast cancer initiation and progression [15]. Epigenetic refers to all heritable changes that do not depend on modifications of DNA primary sequence [3]. Recently, several histone demethylases have been identified that catalyze the removal of methylation from histone H3 lysine residues [3]. Previous studies had found that JARID1s as histone demethylases that can catalyze the removal of methyl groups from the H3 lysine residue [4]. Members of the JARID1 family have distinct biological functions; they can be crucial in the expression and repression of oncogenes and tumor suppressor genes and can themselves serve as both [10]. Loss of JARID1A inhibits tumorigenesis in a mouse model. In addition, JARID1A is overexpressed in breast cancer and PCa [3]. Another JARID1 family protein, JARID1C, has mostly been studied in the context of mental retardation, but it has been linked to clear cell renal cell carcinomas [16]. As part of the Cancer Genome Project, an extensive analysis of clear cell renal cell carcinoma led to the

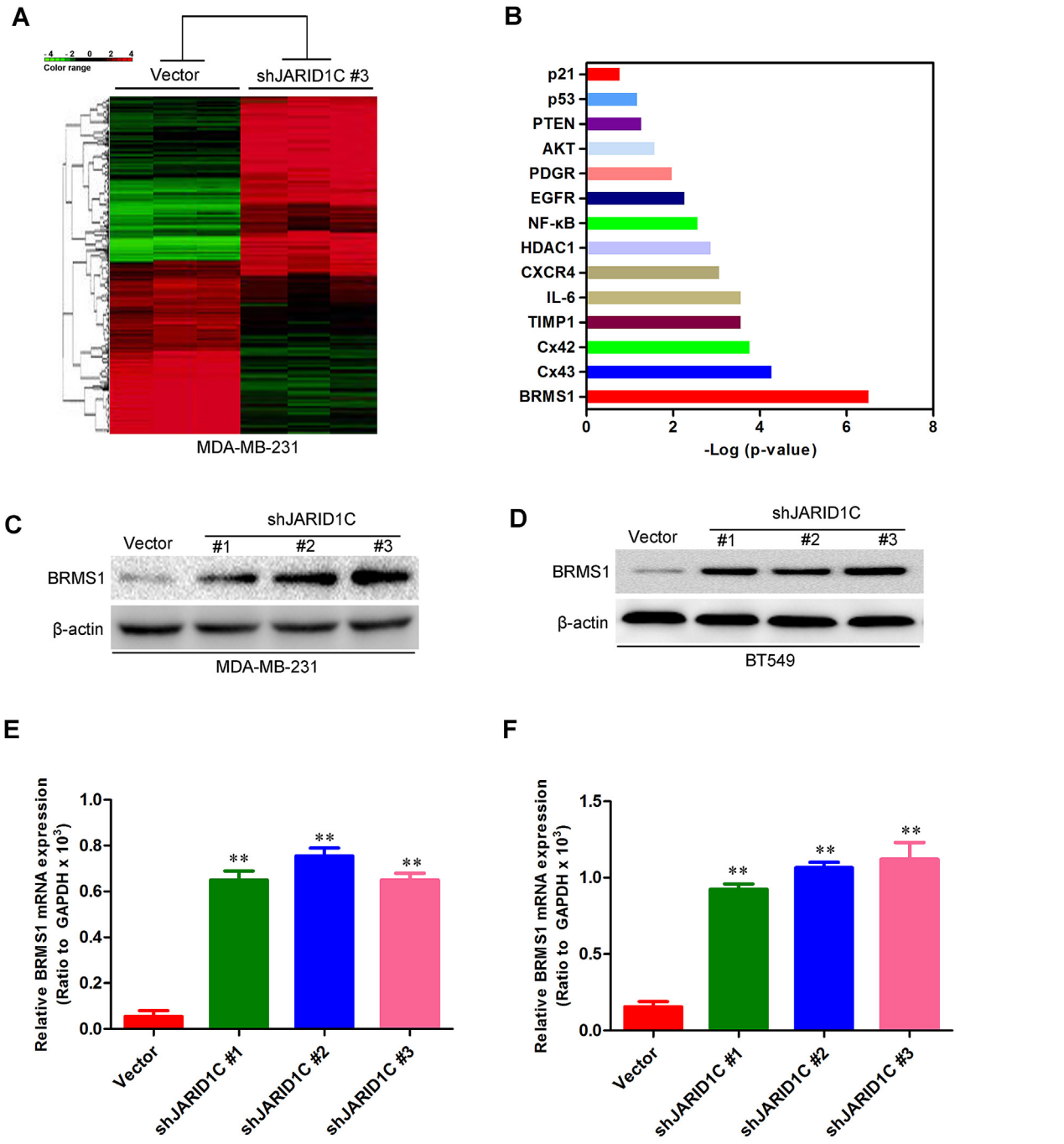

Fig. 3. JARID1C regulates BRMS1 expression in breast cancer cells. A: Supervised hierarchical clustering of the genes differentially expressed after JARID1C silencing in MDA-MB-231 cells. B: Gene set enrichment analysis was carried out using ConceptGen. C: Protein level of BRMS1 was measured in MDA-MB-231 cells with JARID1C silencing by western blot assay. D: Protein level of BRMS1 was measured in BT549 cells with JARID1C silencing by western blot assay. E: mRNA level of BRMS1 was measured in MDA-MB-231 cells with JARID1C silencing by qRT-PCR assay. F: mRNA level of BRMS1 was measured in BT549 cells with JARID1C silencing by qRT-PCR assay. **, $P < 0.01$ is based on the Student t test. All results are from three independent experiments. Error bars, SD.

discovery that 3% of clear cell renal cell carcinoma tumors contain truncation mutations in JARID1C [12]. However, its role and underlying mechanisms in breast cancer still unknown.

To our knowledge, this is the first research to show that JARID1C plays an important part in breast cancer. We found that as compared with normal tissues, breast cancer specimens showed overexpression of JARID1C and its overexpression were significantly correlated with distant metastasis in breast cancer tissues. By silencing JARID1C in breast cancer cells, we found that shJARID1C could inhibit cell migration and invasion. Moreover, we also found that the expression of BRMS1 was modulated by JARID1C. Silencing of JARID1C dramatically increased BRMS1 expression both at mRNA and protein level. Mechanistically, we found JARID1C exerts its function through modulation of H3K4me3 at the BRMS1 gene promoter, which was associated with inactive BRMS1 transcription. BRMS1 knockdown reversed shJARID1C-induced migration inhibition. Further, BRMS1 expression in human breast cancer is negatively correlated with JARID1C expression. These results lead us to propose a model for JARID1C regulation of metastasis through transcriptional regulation of BRMS1 in breast cancer.

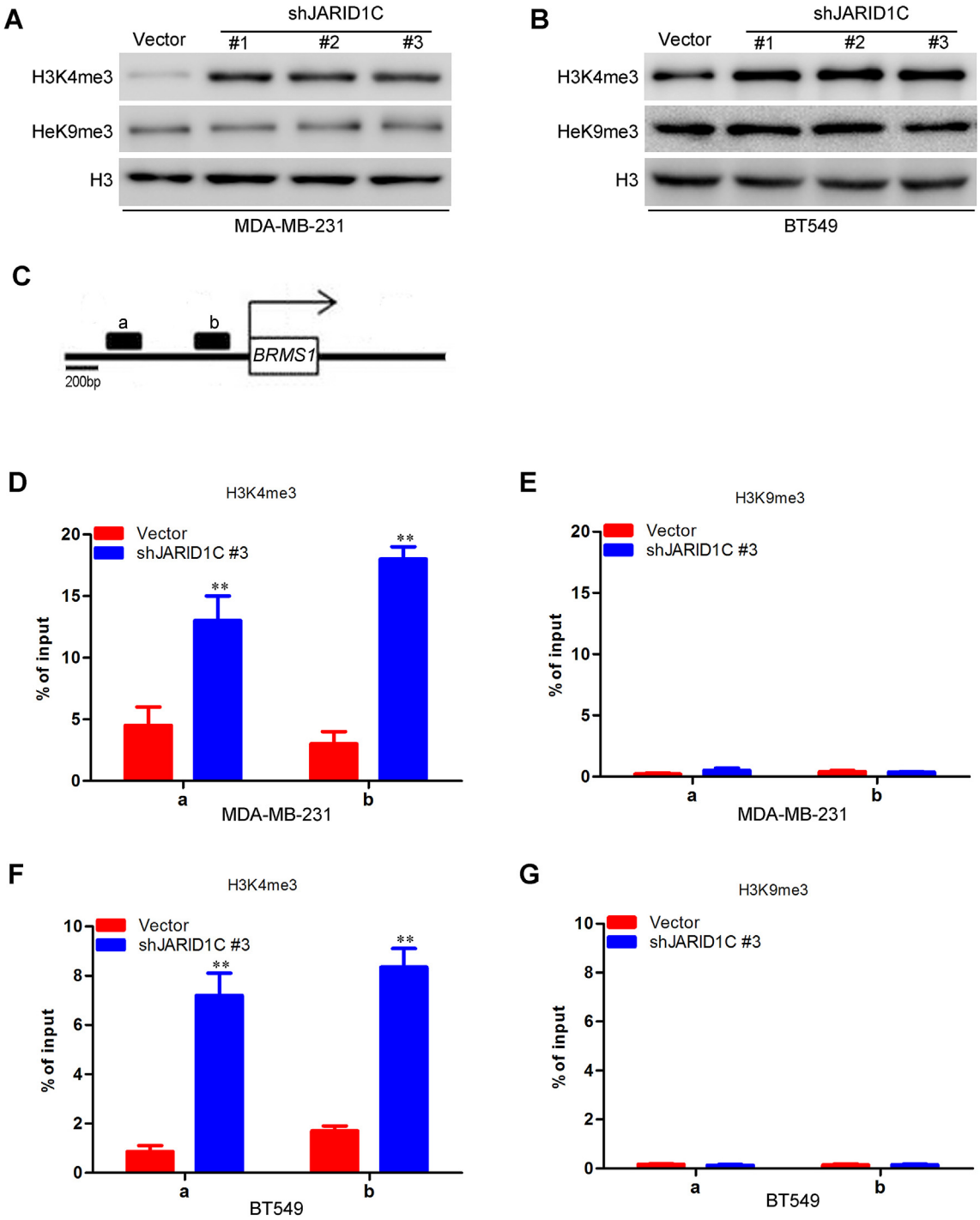

Fig. 4. Silencing JARID1C alters BRMS1 expression through decreases H3K4me3. A: The abundance of H3K4me3 and H3K9me3 was assessed in MDA-MB-231 cells with JARID1C silencing by western blot using whole-cell lysate; total H3 was used as a loading control. B: The abundance of H3K4me3 and H3K9me3 was assessed in BT549 cells with JARID1V silencing by western blot using whole-cell lysate; total H3 was used as a loading control. C: Schematic presentation of two regions relative to the BRMS1 transcriptional start site used as primers to test histone occupied abundance. D: ChIP-qPCR was performed to assess H3K4me3 occupancy in MDA-MB-231-shJARID1C or its control cells. E: ChIP-qPCR was performed to assess H3K9me3 occupancy in MDA-MB-231-shJARID1C or its control cells. F: ChIP-qPCR was performed to assess H3K4me3 occupancy in BT549-shJARID1C or its control cells. G: ChIP-qPCR was performed to assess H3K9me3 occupancy in BT549-shJARID1C or its control cells. "Percentage of input" indicates the ratio of DNA fragment of each promoter region bound by H3K4me3 or H3K9me3 to the total amount of input DNA fragment without H3K4me3 or H3K9me3 antibody pull-down. **, $P < 0.01$ is based on the Student *t* test. All results are from three independent experiments. Error bars, SD.

The putative role of JARID1C as an oncogene in cancer development is supported by the observations that JARID1C is highly expressed in prostate cancer and other malignant tumors relative to normal tissues [12]. Consistent with this research, in the present study we showed that a novel function of JARID1C in breast cell migration and invasion. Breast cancer cells with silencing expression of JARID1C displayed effects on migration and invasion *in vitro*. All of these characteristics induced by shJARID1C *in vitro* maybe culminated to increased numbers of distant metastases *in vivo*. These empirical findings provide a mechanistic framework to

explain the clinical observations that breast cancer patients with high levels of JARID1C in tissue samples have more chance of distant metastasis.

The roles of several transcription factors as migration regulators have been extensively reported [2]. In our effort to elucidate the mechanism how JARID1C modulates migration in breast cancer cells, we identified BRMS1 as an effective mediator of JARID1C-induced migration. BRMS1 is one of the most frequently mutated tumor suppressors in human cancer including breast cancer [14,17]. Mechanistically, BRMS1 reduces cell motility through a variety of pathways, and Twist1 is one important target of BRMS1 [14,18]. The mechanistic connection between JARID1C and BRMS1 was previously unknown. In this study, we showed that modulation of JARID1C expression altered the methylation status of H3K4 at the *BRMS1* gene promoter, which in turn transcriptionally controlled expression of BRMS1. However, we did not detect any influence of JARID1C expression on the methylation status of H3K9. Thus, we conclude that JARID1C transcriptionally inactivates BRMS1 expression through H3K4 demethylation and reduces H3K4me3 to the BRMS1 gene promoter, resulting in increase in migration and invasion.

In summary, we provide the first evidence that JARID1C is overexpressed in breast cancer, especially in the breast cancer with distant metastasis. And silencing JARID1C inhibits breast cancer cells migration and invasion. These found may provide an optimal therapeutic option to manipulate JARID1C levels in breast cancer patients' clinical practice.

Competing interests

The authors have no competing interests to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.049.

Transparency document

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