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LncRNA BCRT1 promotes breast cancer progression by targeting miR-1303/PTBP3 axis

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Abstract

Background: Long noncoding RNAs (lncRNAs) play crucial roles in tumor progression and are aberrantly expressed in various cancers. However, the functional roles of lncRNAs in breast cancer remain largely unknown.

Methods: Based on public databases and integrating bioinformatics analyses, the overexpression of lncRNA BCRT1 in breast cancer tissues was detected and further validated in a cohort of breast cancer tissues. The effects of lncRNA BCRT1 on proliferation, migration, invasion and macrophage polarization were determined by in vitro and in vivo experiments. Luciferase reporter assay and RNA immunoprecipitation (RIP) were carried out to reveal the interaction between lncRNA BCRT1, miR-1303, and PTBP3. Chromatin immunoprecipitation (ChIP) and RT-PCR were used to evaluate the regulatory effect of hypoxia-inducible factor-1α (HIF-1α) on lncRNA BCRT1.

Results: LncRNA BCRT1 was significantly upregulated in breast cancer tissues, which was correlated with poor prognosis in breast cancer patients. LncRNA BCRT1 knockdown remarkably suppressed tumor growth and metastasis in vitro and in vivo. Mechanistically, lncRNA BCRT1 could competitively bind with miR-1303 to prevent the degradation of its target gene PTBP3, which acts as a tumor-promoter in breast cancer. LncRNA BCRT1 overexpression could promote M2 polarization of macrophages, mediated by exosomes, which further accelerated breast cancer progression. Furthermore, lncRNA BCRT1 was upregulated in response to hypoxia, which was attributed to the binding of HIF-1α to HREs in the lncRNA BCRT1 promoter.

Conclusions: Collectively, these results reveal a novel HIF-1α/lncRNA BCRT1/miR-1303/PTBP3 pathway for breast cancer progression and suggest that lncRNA BCRT1 might be a potential biomarker and therapeutic target for breast cancer.

Keywords: LncRNA BCRT1, miR-1303, PTBP3, Progression, Breast cancer

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Background

Breast cancer is one of the most common malignancies among women worldwide. Despite advances in diagnosis and combined treatments, the prognosis of breast cancer patients remains unsatisfactory [1, 2]. Metastasis is one of the leading causes of cancer-related death [3], which greatly hinders treatment success. Therefore, a more comprehensive understanding of the mechanism of progression and metastasis is important for improving the prognosis of breast cancer patients.

Recently, long noncoding RNAs (lncRNAs) have been found to be involved in a variety of physiological and pathological processes [4, 5], especially in cancers [6]. LncRNAs are transcripts with more than 200 nucleotides that have no protein-coding potential [7]. Despite the lack of cross-species conservation [8], researchers in our laboratory and others have demonstrated that lncRNAs are frequently dysregulated in cancers and are involved in the progression and metastasis of multiple malignancies [9, 10]. LncRNA ANCR was found to mediate the degradation of EZH2 and thus attenuate the metastatic ability of breast cancer [11]. Moreover, lncRNA AGAP2-AS1 was found to be upregulated in breast cancer and was associated with trastuzumab resistance [12]. However, the clinical significance and biological mechanisms of the vast majority of lncRNAs in the regulation of breast cancer remain largely unknown.

Several studies have suggested that lncRNAs may function as competing endogenous RNAs (ceRNAs), regulating the biological functions or expression of microRNAs. For instance, lncRNA LINC00963 promotes tumorigenesis and radioresistance by acting as a ceRNA for miR-324-3p in breast cancer cells [13]. LncRNA NONHSAT101069 acted as a ceRNA by effectively sponging miR-129-5p, thereby modulating the repression of Twist1 and promoting epirubicin resistance, migration, and invasion of breast cancer cells [14]. Previous studies have revealed that hypoxia, a major hallmark of the tumor microenvironment, is associated with the progression and metastasis of many solid tumors. HIF-1α is an extensively studied hypoxia-inducible factor (HIF) that mediates the cellular response to hypoxia through transactivation of downstream target genes [15]. Under normoxic conditions, HIF-1α is subjected to proteasome degradation, whereas, hypoxic conditions protect HIF-1α from degradation, allowing HIF-1α translocation into the nucleus to initiate gene expression [16]. Recently, the roles of hypoxic conditions in regulating lncRNA expression have received extensive attention, and various hypoxia-responsive lncRNAs have been reported to play important roles in tumorigenesis and tumor progression [17]. However, more investigations should be carried out on the mechanism of hypoxia in mediating aberrant lncRNA expression as well as the functions of lncRNAs in breast cancer.

In the present study, we analyzed public microarrays to screen lncRNAs that are differentially expressed in breast cancer. LncRNA BCRT1 (breast cancer-related transcript 1), which was significantly overexpressed in breast cancer tissues and associated with poor prognosis of breast cancer patients, was selected for further investigation. LncRNA BCRT1 functioned as a tumor promoter by competitively binding with miR-1303 to protect PTBP3 from degradation and thus promoted the growth and progression of breast cancer cells both in vitro and in vivo. Moreover, lncRNA BCRT1 could be transferred to macrophages via exosomes, promoting M2 polarization and enhancing its effect on tumor progression. Further study revealed that lncRNA BCRT1 was induced by hypoxia via HIF-1α-dependent transcriptional regulation, which consequently facilitated hypoxia-induced EMT. Our results provide novel insight into the metastatic mechanism of breast cancer and a promising therapeutic target for breast cancer treatment.

Methods

Patients and specimens

Human breast cancer tissues and corresponding normal tissues were obtained from patients admitted to Qilu Hospital from January 2004 to December 2011. All participants provided written informed consent, and the research was approved by the Ethical Committee on Scientific Research of Shandong University Qilu Hospital.

RNA sequencing analysis

Breast cancer gene expression data were downloaded from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) dataset GSE112848. The data analysis was performed with R software using the DEGseq package. The threshold set for significant differences was $\log_2|\text{fold change}| \geq 1$ and P-value < 0.05.

Cell culture and reagents

All cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were cultured according to the manufacturer's instructions. MCF10A cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, USA) containing 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 0.5 μg/ml hydrocortisone. MCF-7, MDA-MB-231, MDA-MB-468, and HEK293T cells were cultured with Dulbecco's modified Eagle's medium. T47D and THP1 cells were cultured with RPMI 1640 medium. The above media contained 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Invitrogen, USA). The medium for T47D cells also contained 10 μg/ml insulin. All cells were cultured in a 5% CO2-humidified incubator at 37 °C.

RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, USA). Complementary DNA (cDNA) was synthesized using a PrimeScript RT reagent kit (TaKaRa, Japan). For miRNAs, reverse transcription was carried out using the PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Japan). RT-PCR was performed using SYBR Premix Ex Taq I. Primers used in the study are listed in Additional file 1: Table S1. β-Actin was used as an internal control for mRNA. U6 was used as an internal control for miRNA. Relative RNA abundances were calculated by the standard $2^{-\Delta\Delta Ct}$ method.

Subcellular fractionation

Nuclear and cytoplasmic separation was performed using the PARIS Kit (Life Technologies, USA) according to the manufacturer's instructions.

Fluorescence in situ hybridization (FISH)

The FISH assay was performed in MDA-MB-231 cells according to the specifications of the manufacturers. The Cy3-labeled lncRNA BCRT1 probes used in our study were designed and synthesized by GenePharma (Shanghai, China). Briefly, the prepared cells were fixed with 4% paraformaldehyde for 30 min. After permeabilization, the cells were incubated with specific probes at 37 °C overnight. The cell nuclei were stained with DAPI (Sigma-Aldrich, USA). The staining results were observed using a fluorescence microscope (Nikon, Japan).

Plasmid construction and transfection

The full-length lncRNA BCRT1 cDNA was cloned into pcDNA3.1 (Invitrogen, USA). The primers used for vector construction are showed in Additional file 1: Table S1. The lncRNA BCRT1 plasmid and corresponding empty vector were transfected into breast cancer cells using Lipofectamine 2000 reagent (Invitrogen, USA). G418 (2 mg/ml) was used to generate stably transfected cells. For PTBP3 knockdown, the pLKO.1 plasmid was used as a negative control. The 3′UTR sequences of lncRNA BCRT1 and PTBP3 with wild-type or mutant miR-1303 binding sites were cloned into the pmirGLO vector (Invitrogen, USA). Different fragments of the lncRNA BCRT1 promoter were cloned into the pmirGLO vector. The negative control, lncRNA BCRT1 siRNAs, and miR-1303 mimics (GenePharma, China) were transfected using Lipofectamine 2000.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Transfected cells were seeded at a density of 5000 cells/well in 96-well plates. After incubation, 20 μl of 5 mg/ml MTT was added to each well and incubated for another 4 h. Then, the supernatants were carefully removed, and 100 μl DMSO was added to each well. The proliferation curves were determined by calculating the relative value of absorbance measured at 570 nm on a microplate reader (Bio-Rad, USA).

Colony formation assay

Transfected cells were counted and seeded at 500 cells per 6 cm plate. After 10–14 days, cell colonies were washed with PBS, fixed with ethanol for 20 min and stained with crystal violet for 20 min. The colonies were imaged and counted.

EdU incorporation assay

Transfected cells were seeded into 96-well plates at a density of 1×10^4 cells/well. The EdU incorporation assay kit (RiboBio, China) was used to evaluate cell proliferation. A fluorescence microscope (Nikon, Japan) was used to obtain images.

Cell apoptosis assay

EDTA-free trypsin was used to collect cells, and the cells were resuspended in 500 μl of binding buffer. After incubation with 5 μl Annexin V-FITC and 5 μl PI (BD Biosciences, USA) for 15 min in the dark, the cells were examined on a FACSCalibur (BD, Biosciences, USA) within 1 h.

Transwell assay

Transwell assays were performed using Transwell chambers (pore size 8 μm; Costar Corporation, USA) with or without matrigel (BD Biosciences, USA). A total of 1×10^5 cells were added to the upper insert. The lower chamber contained 700 μl medium with 20% FBS as a chemoattractant. After incubation for 24–48 h, the cells on the lower surface were fixed with ethanol and stained with 0.2% crystal violet. The relative cell number was calculated.

Tube formation assay

Seventy-five microliters of Matrigel (BD Biosciences, USA) was pipetted into each well of a 48-well plate and allowed to solidify for more than 1 h at 37 °C. HUVECs were suspended in the indicated conditioned medium and seeded onto the gel. After 4–6 h of incubation, a bright-field microscope was used to observe the tubular structures and acquire images. Tube formation was quantified by measuring the total length of the tubes using ImageJ software.

Western blot assay

Cell proteins were extracted and separated by 10% SDS-PAGE gels and transferred to 0.22 μm PVDF membranes (Millipore, USA). The membranes were blocked with 5% skim milk powder and incubated with specific antibodies at 4 °C overnight. The membranes were then incubated

with the appropriate secondary antibodies, and an ECL detection system (Bio-Rad, USA) was used to detect the protein bands. β-Actin was used as a control. The primary antibodies and secondary antibodies used are described in Additional file 2: Table S2.

Tumor xenograft model

MDA-MB-231 cells (1×10^7 cells) with or without lncRNA BCRT1 overexpression were suspended in 200 μl PBS and subcutaneously injected into each flank of 4–6-week-old BALB/c nu/nu female mice. The mice were sacrificed after 30 days, and the maximum (L) and minimum (W) length and weight of the tumors were measured. Tumor volume was calculated as $½LW^2$. To evaluate the influence of lncRNA BCRT1 on metastasis, 5×10^5 cells were injected into the lateral tail veins of nude female mice (five mice per group). After 4 weeks, the mice were euthanized, and the lungs were collected to evaluate the number of pulmonary metastatic lesions. Hematoxylin and eosin (H&E) staining was performed for tissue morphology evaluation. The animal experiments were approved by the Shandong University Animal Care and Use Committee.

Immunohistochemical (IHC) analysis

The paraffin-embedded sections were dewaxed in xylene and rehydrated in alcohol. Endogenous peroxidase was blocked by 3% H_2O_2, and microwave heating was performed for antigen retrieval. After blocking nonspecific antigen binding with 5% BSA at 37 °C for 1 h, the sections were incubated with a specific primary antibody against Ki67, PTBP3 or CD31 (1100 dilution, Abcam, USA) at 4 °C overnight. After incubating with the corresponding secondary antibodies at 37 °C for 1 h, the sections were stained with diaminobenzidine and counterstained with hematoxylin. Representative images were taken using an Olympus light microscope.

Luciferase assay

The wild-type or mutant lncRNA BCRT1 or 3'UTR of PTBP3 was amplified and cloned into pmirGLO vector separately. Then, HEK293T cells were plated on a 96-well plate and cotransfected with wild-type or mutant luciferase plasmids and miR-1303 or control miRNA. The lncRNA BCRT1 promoter segment was cloned into the pGL3-basic vector. The pGL3-BCRT1 and pRL-TK vectors were cotransfected with si-NC or si-HIF1α. A Dual-Luciferase Reporter Assay System (Promega, USA) was used to measure the luciferase activity.

RNA immunoprecipitation (RIP) assay

A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was used to determine the relationship between lncRNA BCRT1 and miR-1303. Antibodies used for the RIP assay included anti-AGO2 and control IgG (Millipore, USA), and the coprecipitated RNAs were used for cDNA synthesis and evaluated by qRT-PCR.

Isolation and characterization of exosomes

Exosomes were isolated from the supernatant of MDA-MB-231 cells that had been cultured in DMEM containing 10% exosome-depleted FBS for 48 h via a polyethylene glycol-based method as previously described [18]. Briefly, the culture medium was centrifuged at 500×g for 5 min, and the supernatant was further centrifuged at 2000×g for 30 min. Then, 2× PEG solution was added to the supernatant and gently mixed. The mixture was stored at 4 °C for more than 12 h and then centrifuged at 10,000×g for 1 h at 4 °C to collect the exosomes. The supernatant was removed, and the exosome pellet was resuspended in 0.2 μm-filtered PBS.

Exosome uptake assay

PKH26, a fluorescent dye (Sigma-Aldrich, USA), was used to label exosomes obtained from conditioned medium [19]. After incubation with the recipient cells for 24 h, fluorescence microscopy was used for imaging.

Kaplan-Meier plotter tool analysis

The Kaplan-Meier Plotter tool (http://kmplot.com/analysis/) was used to determine the association between PTBP3 and the prognosis of breast cancer patients.

Chick chorioallantoic membranes (CAM)

The fertilized chicken eggs were cultured at 37 °C in an 80% humidified atmosphere for 7 days. Then, a square window was cut on the shell to expose the CAM and was covered with a gelatin sponge (0.3 cm × 0.3 cm × 0.3 cm) containing PBS or the indicated conditioned medium (CM). Next, tape was used to cover the window for further incubation. After 2 days, the CAM were visualized under a stereoscope.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using a ChIP kit (CST, USA) following the manufacturer's instructions. Briefly, cells were crosslinked with formaldehyde and sonicated to an average length of 200–1000 bp. Immunoprecipitation was conducted with an anti-HIF-1α antibody (Abcam, UK) or IgG control. Precipitated DNA was amplified by RT-PCR. Primer sequences are provided in Supplementary Table S4.

Elisa

The TGFβ concentration in the cell culture medium was measured by ELISA using the Quantikine human TGFβ

ELISA kit (R&D Systems, USA) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± S.D. of three independent experiments and analyzed by the SPSS software program (version 17.0). Student's t-test was used for two-group comparisons. Kaplan–Meier survival analysis was performed for survival rate calculation. Cox proportional hazards model multivariate analyses were used to evaluate the significance of lncRNA BCRT1 expression and clinicopathological features on overall survival. $P < 0.05$ was considered statistically significant.

Results

LncRNA BCRT1 expression is upregulated in breast cancer and associated with poor prognosis

To identify important lncRNAs that potentially participate in breast cancer progression, we analyzed the lncRNA expression profiles using public databases (GSE112848 and a TCGA dataset) (Fig. 1a-b). In the present study, we mainly focused on the upregulated lncRNAs given that these lncRNAs might serve as therapeutic targets or prognostic biomarkers. Among them, lncRNA BCRT1 (breast cancer related transcript 1), which was one of the prominently upregulated lncRNAs in breast cancer tissues, was chosen for further evaluation. LncRNA BCRT1 is located on 10q25.1 in humans and is composed of 3 exons with a full length of 1013 nt (Additional file 3: Figure S1a). The sequence of full-length lncRNA BCRT1 and its secondary structure based on minimum free energy (MFE) are shown in Additional file 3: Figure S1b and c, respectively. Moreover, using the Open Reading Frame (ORF) Finder and conserved domain database, we found that lncRNA BCRT1 had little potential to code proteins, which was in accordance with the results of five different online metrics (Additional file 3: Figure S1d-f). In addition, we failed to identify a valid Kozak consensus sequence in lncRNA BCRT1, further supporting the notion that lncRNA BCRT1 had no protein coding potential [20].

Compared with that in normal breast epithelial cells (MCF10A), the expression of lncRNA BCRT1 in four breast cancer cell lines was significantly higher (Fig. 1c). Moreover, we further investigated the lncRNA BCRT1 expression levels in 18 paired breast cancer tissues and normal breast tissues using real-time PCR analysis, and the results revealed that lncRNA BCRT1 was significantly overexpressed in breast cancer tissues compared with adjacent normal tissues (Fig. 1d). The association between the clinicopathological characteristics of breast cancer patients and lncRNA BCRT1 expression level is summarized in Additional file 4: Table S3. LncRNA BCRT1 was overexpressed in breast cancer tissues with distant metastasis (Fig. 1d), and higher lncRNA BCRT1 expression levels were correlated with significantly shorter disease-free survival (DFS) and overall survival (OS) (Fig. 1e). Univariate (Additional file 5: Table S4) and multivariate (Additional file 6: Table S5) analyses further showed that lncRNA BCRT1 expression was a major prognostic factor for breast cancer patients. The results of nuclear/cytoplasmic RNA fractionation from the subcellular distribution assay confirmed that lncRNA BCRT1 was mainly located in the cytoplasm (Fig. 1f), which was further confirmed by the fluorescence in situ hybridization (FISH) analysis (Fig. 1g). Collectively, these findings revealed that lncRNA BCRT1 was upregulated in breast cancer and that high expression of lncRNA BCRT1 was associated with poor outcomes in breast cancer.

LncRNA BCRT1 promotes cell proliferation and tumor growth in breast cancer

To determine the biological function of lncRNA BCRT1 in breast cancer cells, short interference siRNAs against human lncRNA BCRT1 (si-BCRT1) were applied to knock down lncRNA BCRT1, and the knockdown efficiency was confirmed by RT-PCR (Fig. 2a and Additional file 7: Figure S2a). After lncRNA BCRT1 knockdown, the proliferation, colony-formation abilities, and DNA synthesis activities of breast cancer cells were significantly decreased (Fig. 2b-d and Additional file 7: Figure S2b). The results of flow cytometry revealed that lncRNA BCRT1 knockdown obviously increased the total apoptosis rate in breast cancer cells (Fig. 2e). On the other hand, when lncRNA BCRT1 was overexpressed by transfection with the pcDNA3.1 plasmid containing the lncRNA BCRT1 sequence, the proliferation and colony formation of breast cancer cells was significantly increased (Fig. 2f-g and Additional file 7: Figure S2c-f). Furthermore, a subcutaneous xenograft model was used to validate the biological function of lncRNA BCRT1 in vivo. Consistent with the results in vitro, lncRNA BCRT1 overexpression significantly increased tumor weight and tumor volume compared with those in the control group (Fig. 2h-i). Moreover, immunohistochemistry (IHC) assays confirmed that lncRNA BCRT1 overexpression caused increased Ki67 expression (Fig. 2j), indicating enhanced cell proliferation. Our findings indicated that lncRNA BCRT1 could promote breast cancer cell proliferation both in vitro and in vivo.

LncRNA BCRT1 promotes cell mobility and tumor metastasis in breast cancer

We then investigated the role of lncRNA BCRT1 in the motility of breast cancer cells. The results showed that lncRNA BCRT1 knockdown significantly impaired the migration and invasion of breast cancer cells, whereas lncRNA BCRT1 overexpression led to increased cell mobility (Fig. 3a-b and Additional file 7: Figure S2g-h).

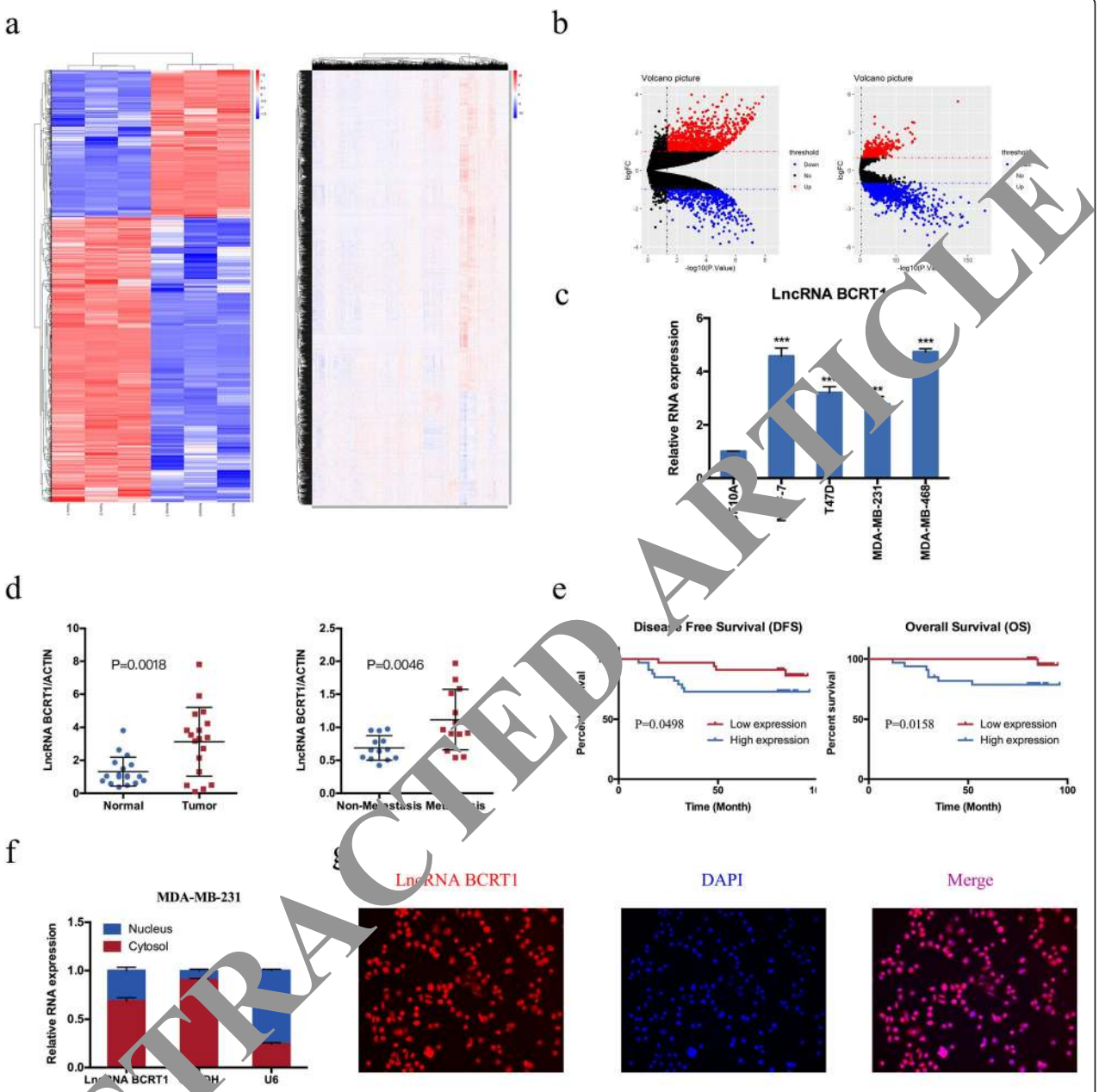

Fig. 1 LncRNA BCRT1 upregulation is associated with advanced progression and poor prognosis in breast cancer. **a** Heat maps showing the top differentially expressed lncRNAs in breast cancer samples compared to normal tissues (left, GSE112848; right, TCGA). The red shades represent high expression and green shades represent low expression. **b** Volcano plots showing the expression profiles of lncRNAs. **c-d** RT-PCR analysis was used to detect the expression of lncRNA BCRT1 in cell lines and tissues. Actin was the internal control. **e** Kaplan–Meier analysis showed the association between lncRNA BCRT1 expression and disease-free survival or overall survival of breast cancer patients ($n = 68$). **f** The expression level of lncRNA BCRT1 in the subcellular fractions of MDA-MB-231 cells was detected by qRT-PCR. U6 and GAPDH were used as nuclear and cytoplasmic markers, respectively. **g** The location of lncRNA BCRT1 (red) in MDA-MB-231 cells was determined by FISH assay. DAPI-stained nuclei are blue. (**$P < 0.01$ and ***$P < 0.001$)

Moreover, we used breast cancer-conditioned medium to stimulate angiogenesis in HUVECs to evaluate angiogenesis activity in vitro [21]. The results showed that the relative length of tubes was decreased in the si-BCRT1 group compared with the si-NC group. On the other hand, lncRNA BCRT1 overexpression led to a significantly elevated tube length (Fig. 3c). Given that epithelial-mesenchymal transition (EMT) is one of the major mechanisms for cancer metastasis, we further evaluated the effect of lncRNA BCRT1 on EMT-related markers. Western blot analysis showed that lncRNA BCRT1 knockdown could increase the expression of epithelial markers (E-cadherin) and decrease the

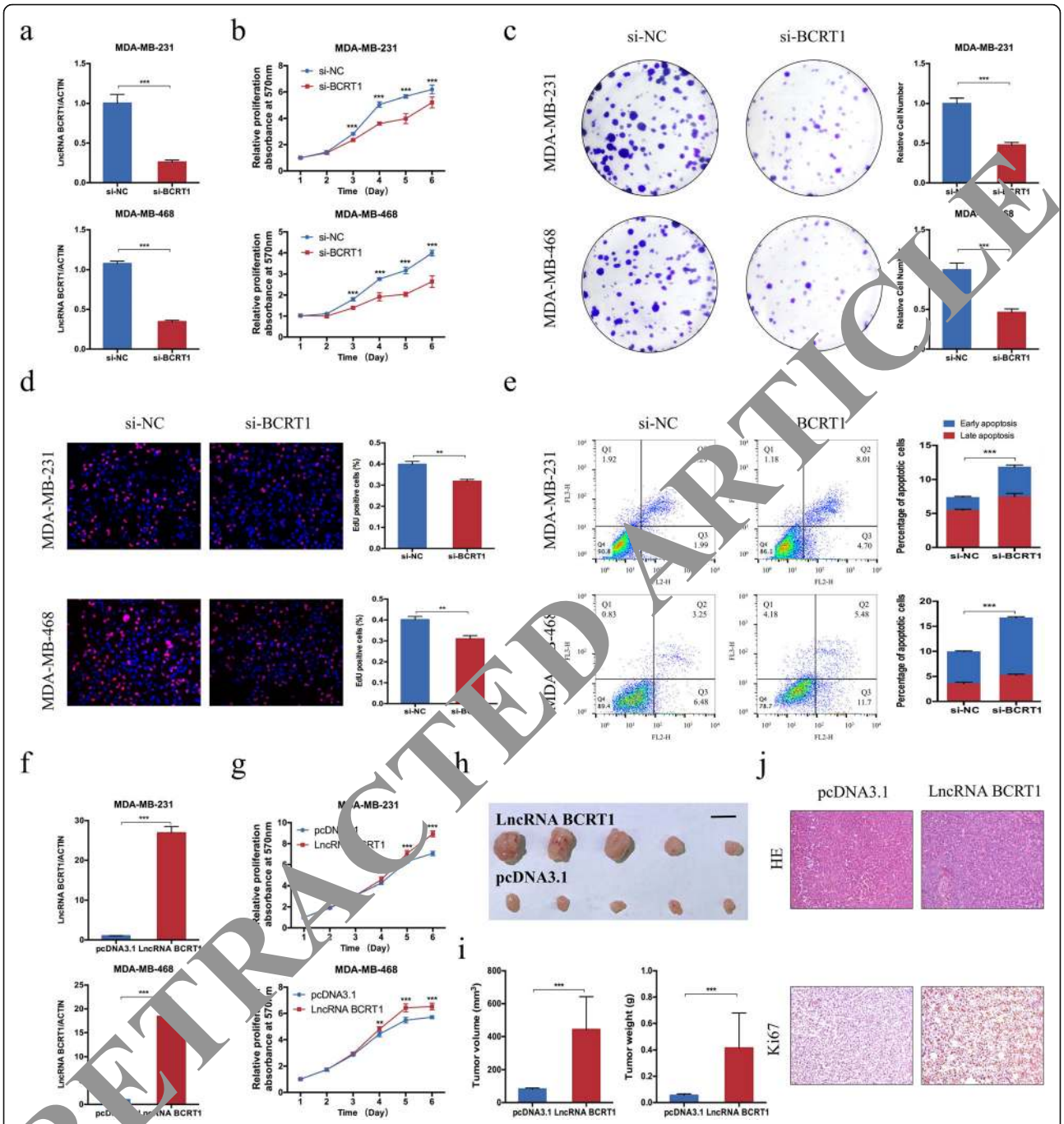

Fig. 2 LncRNA BCRT1 knockdown inhibited breast cancer cell proliferation in vitro and in vivo. **a** The expression levels of lncRNA BCRT1 in MDA-MB-231 and MDA-MB-468 cells after transfection with si-NC or si-BCRT1 were detected by RT-PCR. **b-c** The effects of lncRNA BCRT1 knockdown on the proliferation of MDA-MB-231 and MDA-MB-468 cells were examined by MTT assay (**b**) and colony formation assays (**c**). Experiments were performed in triplicate. **d** EdU assays were used to detect the proliferation rate of MDA-MB-231 and MDA-MB-468 cells after lncRNA BCRT1 knockdown. Columns are the average of three independent experiments. **e** Flow cytometry was performed to determine the effect of lncRNA BCRT1 on apoptosis by flow cytometry analysis. **f** RT-PCR was used to determine the efficiency of the lncRNA BCRT1-overexpressing vector. **g** MTT assay indicated an increased proliferative ability of MDA-MB-231 and MDA-MB-468 cells after lncRNA BCRT1 overexpression. **h** MDA-MB-231 cells were stably transfected with the lncRNA BCRT1-overexpressing vector or control vector and injected subcutaneously into nude mice. Compared with the vector group, lncRNA BCRT1 overexpression promoted tumor growth. **i** Tumor volume and weight were significantly increased in the lncRNA BCRT1-overexpressing group. **j** Representative images of H&E and Ki67 staining in the tumor. Immunohistochemical staining revealed that lncRNA BCRT1 overexpression led to increased expression of Ki67. (**$P < 0.01$ and ***$P < 0.001$)

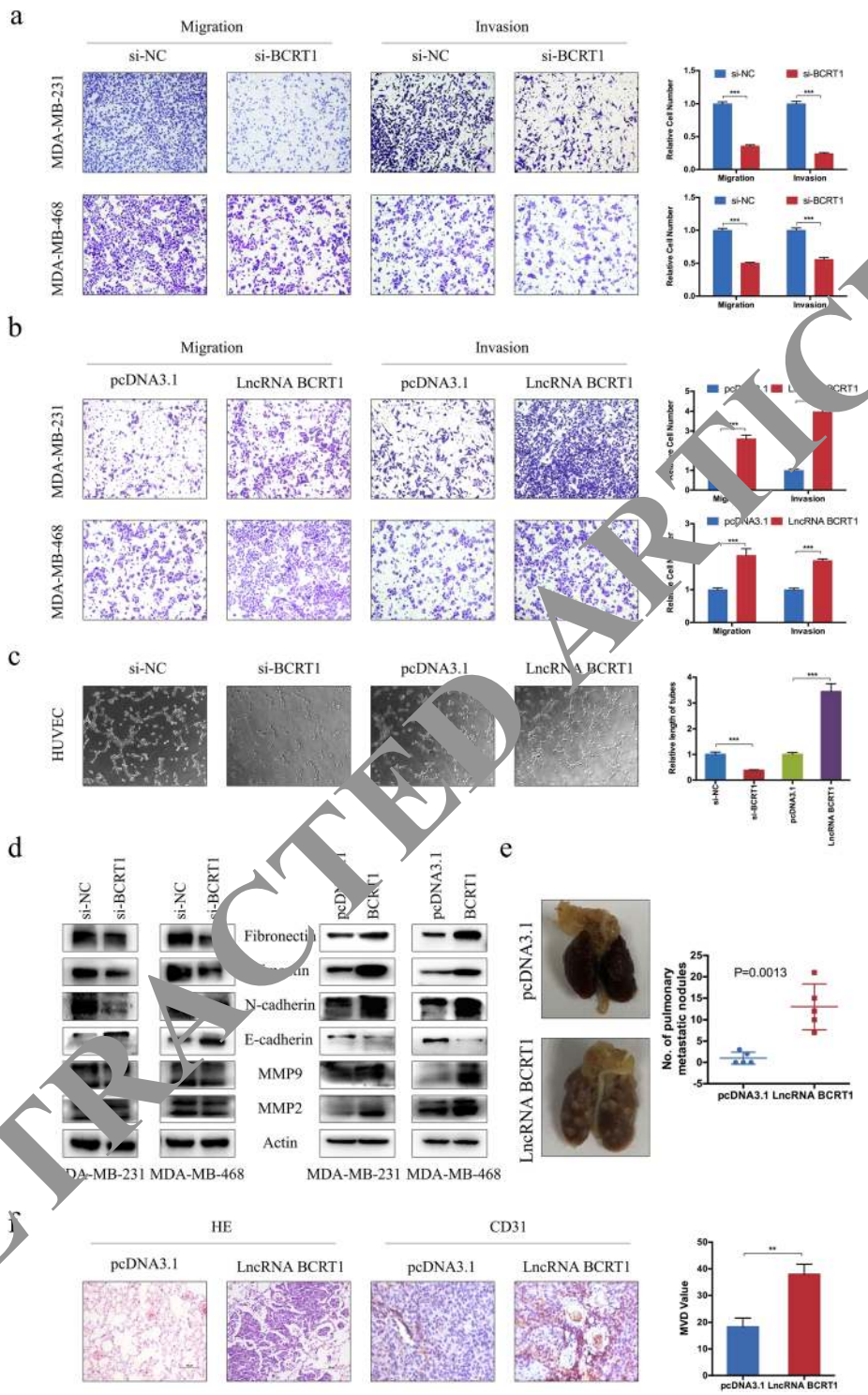

Fig. 3 (See legend on next page.)

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Fig. 3 LncRNA BCRT1 knockdown inhibited breast cancer cell metastasis in vitro and in vivo. **a** Transwell migration and invasion assays were used to evaluate the motility of MDA-MB-231 and MDA-MB-468 cells transfected with si-NC or si-BCRT1. Columns are the average of three independent experiments. **b** LncRNA BCRT1 overexpression led to increased migration and invasion of MDA-MB-231 and MDA-MB-468 cells. **c** Tube formation in HUVECs was inhibited by conditioned medium from MDA-MB-231 cells transfected with si-BCRT1 and was promoted by that from MDA-MB-231 cells transfected with the lncRNA BCRT1-overexpressing vector. **d** EMT-related proteins were detected by western blot in MDA-MB-231 and MDA-MB-468 cells after knockdown or overexpression of lncRNA BCRT1. **e** Stably transfected MDA-MB-231 cells were injected into the tail veins of nude mice ($n = 5$). Representative images of lungs and H&E staining of lungs isolated from mice. LncRNA BCRT1 overexpression resulted in an increased number of lung metastatic colonies. **f** Representative immunohistochemistry staining of CD31 in the indicated xenografts. The corresponding statistical plots are presented in the lower panel. (**$P < 0.01$ and ***$P < 0.001$)

expression of mesenchymal markers (such as Fibronectin, N-cadherin, and Vimentin) (Fig. 3d), indicating that lncRNA BCRT1 could regulate the EMT process to modulate breast cancer progression. To confirm these findings in vivo, we injected breast cancer cells through the tail vein to establish a pulmonary metastasis model in nude mice. Two of the five mice (2/5) injected with breast cancer cells in the control group and all five mice (5/5) injected with breast cancer cells in the lncRNA BCRT1-overexpressing group showed metastatic foci in their lungs after 4 weeks (Fig. 3e). Then, all mice were sacrificed, and their lungs were subjected to hematoxylin and eosin (H&E) staining. The results revealed that lncRNA BCRT1 overexpression remarkably increased the volume and number of lung metastatic lesions compared with those in the control group (Fig. 3f). Similarly, vascular density was increased in the lncRNA BCRT1-overexpressing group (Fig. 3f). Taken together, these data show that lncRNA BCRT1 promotes tumor metastasis in breast cancer cells.

LncRNA BCRT1 functions as a miR-1303 sponge in breast cancer cells

Recently, many lncRNAs have been reported to function as competing endogenous RNAs (ceRNAs), in modulating the expression and biological functions of miRNAs [22, 23]. Since lncRNA BCRT1 was distributed predominantly in the cell cytoplasm, we hypothesized that lncRNA BCRT1 might act as a miRNA sponge to prevent miRNAs from binding with their target mRNAs. Through the RegRNA database, we identified miR-1303 as a potential target of lncRNA BCRT1 (Fig. 4a). To validate the binding potential, a luciferase reporter assay was performed. Overexpression of miR-1303 significantly reduced the luciferase activity of the pmirGLO-BCRT1-wt vector but failed to decrease that of the mutant vector (Fig. 4b). The AGO2 immunoprecipitation assay showed that the AGO2 antibody was able to pull down both endogenous lncRNA BCRT1 and miR-1303 (Fig. 4c), further validating their binding potential. Moreover, lncRNA BCRT1 knockdown promoted miR-1303 expression (Fig. 4d), whereas lncRNA BCRT1 overexpression inhibited miR-1303 expression (Additional file 8: Figure S3a). Our above data supported the hypothesis that miR-1303 is an inhibitory target of lncRNA BCRT1 in breast cancer. A negative association between lncRNA BCRT1 and miR-1303 was also detected in xenograft tumors (Additional file 8: Figure S3b).

Then, we examined the role of miR-1303 in breast cancer. Higher expression of miR-1303 was correlated with better overall survival of breast cancer patients according to the LinkedOmics database [24] (Additional file 8: Figure S3c), indicating that miR-1303 acted as a tumor suppressor in breast cancer. The transfection efficiency of miR-1303 mimics was determined by RT-PCR (Fig. 4e and Additional file 8: Figure S3d), and miR-1303 overexpression led to a decreased proliferation rate and increased apoptotic rate of breast cancer cells (Fig. 4f-g, and Additional file 8: Figure S3e-f). Moreover, miR-1303 overexpression decreased cell migration and invasion (Fig. 4h and Additional file 8: Figure S3g-h). Importantly, rescue experiments further validated the functional relationship between lncRNA BCRT1 and miR-1303 (Fig. 3i-k). Moreover, lncRNA BCRT1 expression was decreased after miR-1303 overexpression in breast cancer cells (Additional file 8: Figure S3g), indicating a reciprocal suppression between them. Overall, we chose miR-1303 as an inhibitory target of lncRNA BCRT1 for further investigation in breast cancer.

LncRNA BCRT1 upregulates PTBP3 expression via inhibition of miR-1303

Using the miRDB, miRWalk, miRPathDB, and TargetScan databases, we found that PTBP3 was a potential target of miR-1303 (Fig. 5a). Additionally, we found that the expression of PTBP3 was elevated in breast cancer tissues compared to normal tissues using the TCGA and GEO databases (Fig. 5b), and high PTBP3 expression was associated with poor prognosis of breast cancer patients (Additional file 9: Figure S4). Furthermore, we found that the expression of PTBP3 was positively associated with the expression of lncRNA BCRT1 in breast cancer cells (Fig. 5c). Therefore, PTBP3 was selected as a putative target of miR-1303 for further observation. Luciferase assays showed that overexpression of miR-1303 decreased the luciferase activity of the wild-type PTBP3 reporter but not the mutant reporter (Fig. 5d), indicating that PTBP3 was the direct target of miR-1303. Furthermore, the mRNA and protein levels of PTBP3 were reduced by miR-1303 overexpression (Fig. 5e) or

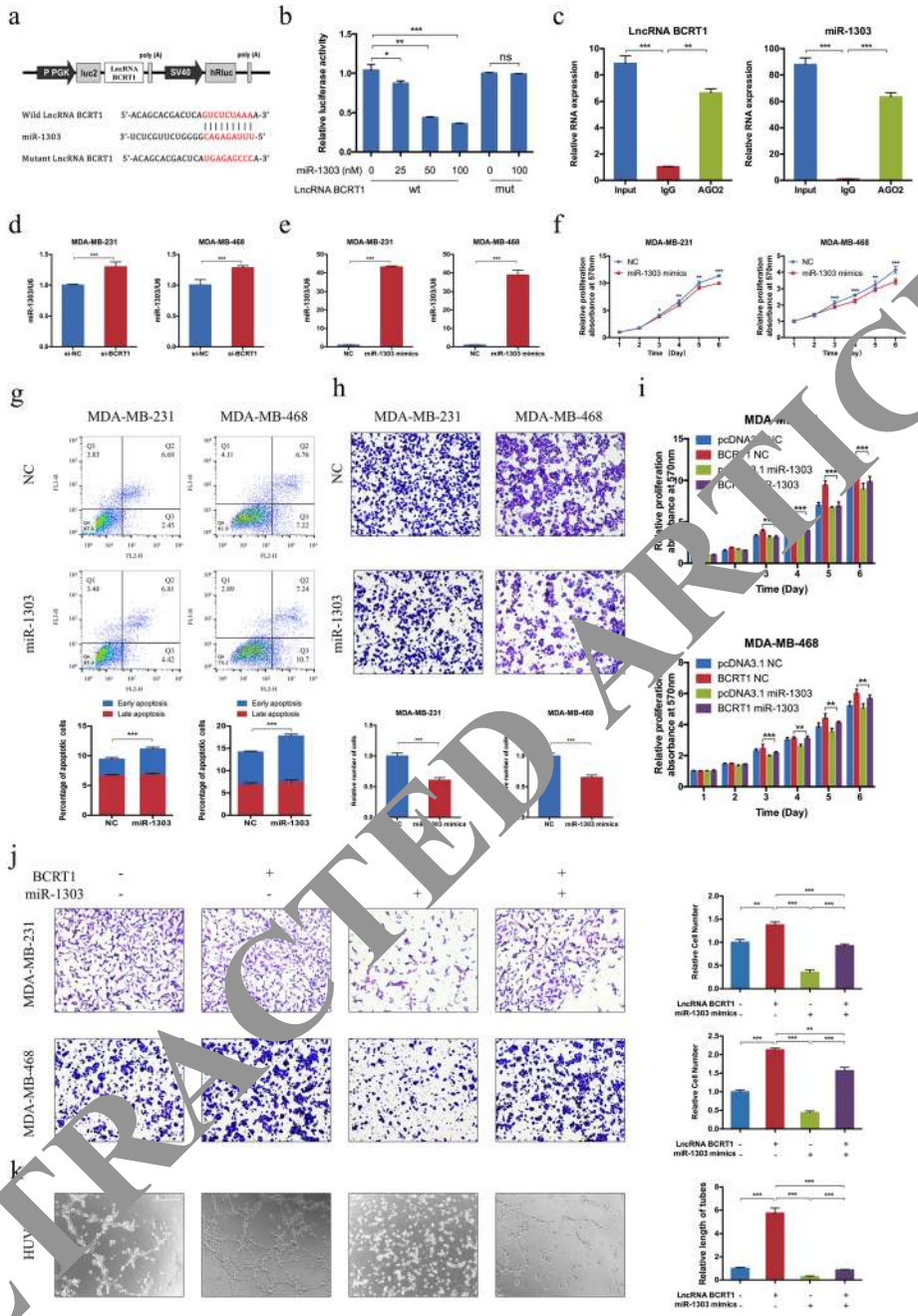

Fig. lncRNA BCRT1 acts as a sponge of miR-1303 in breast cancer. **a** Schematic diagram representing the predicted binding sites for miR-1303 and lncRNA BCRT1 and mutant sequences of the potential miR-1303 binding sites. **b** Luciferase assays in HEK293T cells cotransfected with wild-type or mutant lncRNA BCRT1 and miR-1303 or NC. The data are shown as the means ± SD of triplicate samples. **c** Anti-AGO2 RIP was performed in HEK293T cells, followed by RT-PCR to detect the expression of lncRNA BCRT1 or miR-1303 associated with AGO2. **d** RT-PCR was used to detect the effect of lncRNA BCRT1 knockdown on the expression of miR-1303 in breast cancer cells. **e** The overexpression of miR-1303 in breast cancer cells was validated by RT-PCR. **f** The proliferation of breast cancer cells transfected with NC or miR-1303 was measured by MTT assay. **g** MDA-MB-231 and MDA-MB-468 cells were transfected with miR-1303 mimics or NC, and the apoptotic rates were determined by FACS analysis. Representative results are shown, and data are presented as the mean ± SD. **h** Transwell assays were used to measure the migration of breast cancer cells transfected with miR-1303 mimics or NC. **i** The effects of lncRNA BCRT1 and miR-1303 cotransfection on cell proliferation were measured by MTT assay. **j** Transwell assay was used to determine the migration of breast cancer cells cotransfected with lncRNA BCRT1 and miR-1303. **k** Overexpression of miR-1303 inhibited the effect of conditioned medium from lncRNA BCRT1-overexpressing cells on the tube formation of HUVECs. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

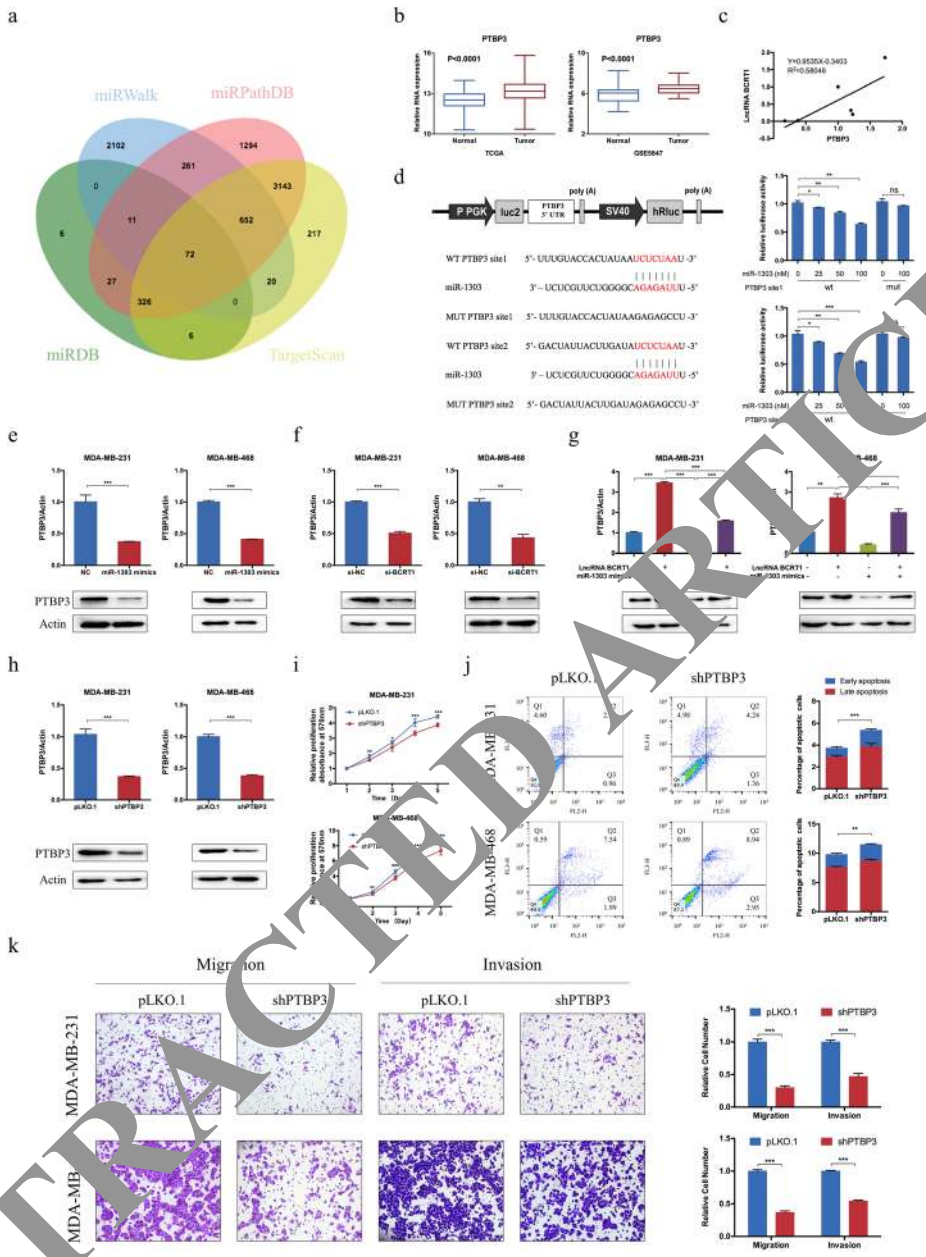

Fig. 5 LncRNA BCRT1 promoted breast cancer cell proliferation and progression by protecting PTBP3 from miR-1303-induced degradation. **a** Schematic illustration showing the overlapping target genes of miR-1303 predicted by miRDB, miRWalk, miRPathDB, and TargetScan. **b** The expression of PTBP3 was increased in breast cancer tissues compared to normal tissues based on the TCGA and GEO databases. **c** RT-PCR revealed a positive correlation between lncRNA BCRT1 expression and PTBP3 expression in breast cancer cells. **d** The upper schematic diagram illustrates the construction of the luciferase reporter plasmids. The lower panel shows the predicted and the mutated binding sites of miR-1303 in the 3'UTR of PTBP3. The statistical graphs on the right show the luciferase activity in HEK293T cells with or without miR-1303 overexpression and transfected with the WT or MUT luciferase plasmids. **e** RT-PCR and western blot assays revealed the effect of miR-1303 on PTBP3 expression. **f** RT-PCR and western blot assays showed that lncRNA BCRT1 knockdown repressed the expression of PTBP3. **g** RT-PCR and western blot assays were used to determine the PTBP3 expression level in MDA-MB-231 cells cotransfected with pcDNA3.1-BCRT1 and miR-1303 mimics. **h** RT-PCR was used to detect the efficiency of PTBP3 knockdown in breast cancer cells. **i** MTT assay was performed to examine the proliferation ability after PTBP3 knockdown. **j** PTBP3 knockdown led to increased cell apoptosis. **k** Transwell assays revealed that PTBP3 knockdown inhibited the migration and invasion abilities of breast cancer cells. (*P < 0.05, **P < 0.01, and ***P < 0.001)

lncRNA BCRT1 knockdown (Fig. 5f). In the rescue experiments, overexpression of miR-1303 could partly counteract the corresponding increases in PTBP3 expression induced by lncRNA BCRT1 overexpression in breast cancer cells (Fig. 5g). In addition, lncRNA BCRT1 overexpression also led to increased expression of PTBP3 in xenograft tumors (Additional file 10: Figure S5a-b). Previous studies reported that PTBP3 acted as a tumor promoter in various cancers, such as gastric cancer [25], hepatocellular carcinoma [26], and colorectal cancer [27]. However, the role of PTBP3 in breast cancer has not been fully elucidated. PTBP3 knockdown resulted in significantly inhibited cell proliferation and increased cell apoptosis (Fig. 5h-j). Moreover, Transwell assays showed that PTBP3 knockdown led to attenuated migration and invasion of breast cancer cells (Fig. 5k). These data suggested that PTBP3 acted as a tumor promoter in breast cancer, and lncRNA BCRT1 played significant roles in regulating PTBP3 expression by regulating miR-1303.

Exosomal lncRNA BCRT1 promotes M2 phenotype polarization and enhances macrophage-induced tumor progression

Previous studies have reported that tumor-associated macrophages (TAMs), which are considered to have an M2-like phenotype, are the most abundant immune-related cells in the tumor microenvironment (TME) and participate in tumor development by mediating angiogenesis, metastasis, and immune escape [28–30]. To investigate whether lncRNA BCRT1 contributes to M2 polarization, we evaluated the expression of lncRNA BCRT1, M1 markers, and M2 markers in unpolarized macrophages, LPS/INF-γ-induced M1 macrophages, and IL-4/IL-13-induced M2 macrophages. The results revealed that the expression levels of M1-associated genes (CD80, MCP-1, iNOS, and IL-6) were significantly upregulated in M1 macrophages, whereas those of M2-associated genes, including CD206 and MRC-2, were significantly upregulated in M2 macrophages (Fig. 6a), indicating the successful polarization of monocytes. Moreover, lncRNA BCRT1 expression was elevated in M2 macrophages compared to M1 macrophages (Fig. 6b), indicating a potential role of lncRNA BCRT1 in macrophage polarization. After PMA treatment for 24 h, THP-1 cells were transfected with si-NC or si-BCRT1, and then IL-4 and IL-13 were added for 24 h to induce the M2 phenotype. The results showed that M1 markers were significantly increased, while M2 markers were remarkable decreased in the si-BCRT1 group (Fig. 6c). Accordingly, lncRNA BCRT1 overexpression led to the opposite results (Fig. 6d). Moreover, the supernatant from lncRNA BCRT1-overexpressing MDA-MB-231 cells caused an elevated expression of M2 markers compared to that from control MDA-MB-231 cells (Fig. 6e). Then, we attempted to investigate the mechanism mediating the communication between breast cancer cells and macrophages. Various studies have reported that lncRNAs can be transferred by exosomes to modulate the tumor microenvironment [31]. To investigate whether lncRNA BCRT1 can be packed into exosomes, we extracted exosomes from the cultured supernatants of breast cancer cells and used western blotting to detect the expression of exosome-related proteins, such as CD63, HSP70, and HSP90 (Fig. 6f). LncRNA BCRT1 overexpression in MDA-MB-231 cells led to increased levels of lncRNA BCRT1 in the secreted exosomes, whereas lncRNA BCRT1 knockdown produced the opposite results (Fig. 6g), indicating the existence of lncRNA BCRT1 in exosomes. We labeled MDA-MB-231 cell-derived exosomes with PKH26 and incubated them with macrophages to examine exosome incorporation and confirmed that the labeled exosomal RNAs could be internalized by macrophages (Fig. 6h). Then, we cocultured unpolarized macrophages with exosomes isolated from lncRNA BCRT1-overexpressing or control MDA-MB-231 cells. The expression of lncRNA BCRT1 and M2 phenotype markers (CD206 and MRC-2) was significantly increased in the lncRNA BCRT1-overexpressing group compared to the control group (Fig. 6i), indicating that exosomal lncRNA BCRT1 promoted M2 polarization. Then, we investigated the role of lncRNA BCRT1 in modulating the behaviors of macrophages. As expected, supernatants from lncRNA BCRT1-overexpressing cells led to increased migration ability of macrophages and showed enhanced chemotaxis (Additional file 11: Figure S6a-b). Moreover, supernatant or exosomes from lncRNA BCRT1-overexpressing cells promoted the expression and secretion of TGF-β compared with the control groups (Additional file 11: Figure S6c-e). To further investigate whether lncRNA BCRT1-educated M2 phenotype macrophages have the characteristic function of tumor promotion, we treated macrophages with exosomes or supernatants isolated from lncRNA BCRT1-overexpressing or control cells. Then, the conditioned medium of educated macrophages was collected and used to treat breast cancer cells or HUVECs. The results showed that macrophages treated with exosomes or supernatants isolated from lncRNA BCRT1-overexpressing groups significantly promoted cell migration and angiogenesis (Fig. 6j-k). Moreover, a chick chorioallantoic membrane (CAM) assay revealed that chick embryos injected with conditioned medium of educated macrophages treated with exosomes or supernatants isolated from lncRNA BCRT1-overexpressing groups had an increase in new vessel density (Fig. 6l). Taken together, these results suggested that lncRNA BCRT1 could be transferred through exosomes, thus promoting M2 phenotype polarization and enhancing its tumor promoting function.

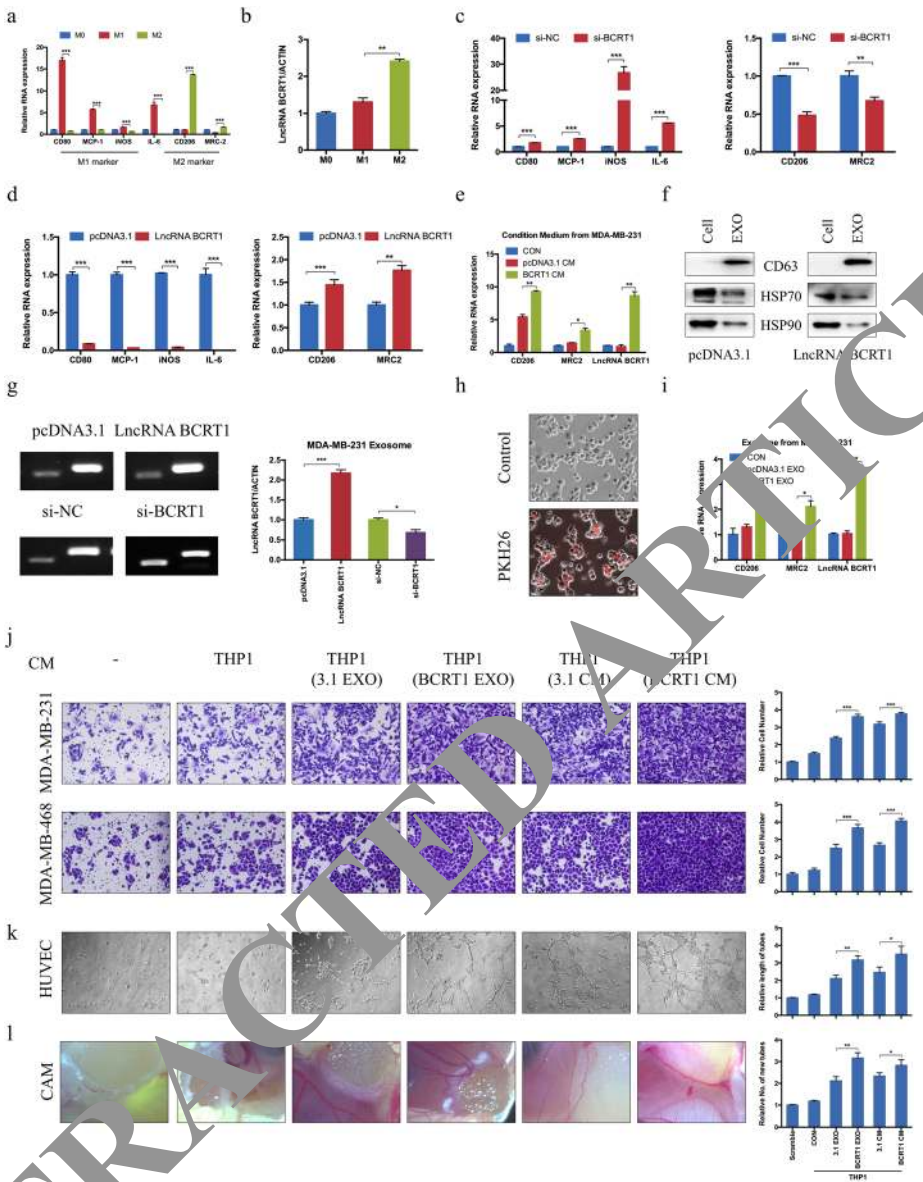

Fig. 6 LncRNA BCRT1 could be secreted by breast cancer cells and promoted M2 polarization. **a** RT-PCR was used to detect the expression of M1 markers and M2 markers after LPS/INF-γ or IL-4/IL-13 treatment. **b** The expression of lncRNA BCRT1 was elevated in M2 macrophages. **c** M1 markers (CD80, MCP-1, iNOS and IL-6) were significantly increased, while M2 markers (CD206 and MRC-2) were remarkably decreased in the lncRNA BCRT1 knockdown group. **d** LncRNA BCRT1 overexpression led to decreased expression of M1 markers and increased expression of M2 markers. **e** Conditioned medium derived from lncRNA BCRT1-overexpressing cells further increased the expression of M2 markers and lncRNA BCRT1 in macrophages. **f** Western blotting analysis of the exosomal markers CD63, Hsp70 and Hsp90 in exosomes derived from breast cancer cells with or without lncRNA BCRT1 overexpression. **g** Agarose gel electrophoresis and RT-PCR assays were used to detect the expression of lncRNA BCRT1 in exosomes. **h** Representative microscopy showing the uptake of PKH26-labeled exosomes (red fluorescent dye) derived from MDA-MB-231 cells by recipient macrophages. **i** The expression of M2 markers and lncRNA BCRT1 in macrophages was detected after culture with the indicated exosomes. **j** Cell migration was increased after cultured with lncRNA BCRT1-overexpressing exosomes or conditioned media. **k-l** Tube formation or CAM assays were used to evaluate the angiogenesis ability after culture with lncRNA BCRT1-overexpressing exosomes or conditioned media. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

LncRNA BCRT1 is transcriptionally regulated by HIF-1α under hypoxic conditions

Hypoxia is one of the major intratumor characteristics in various cancers, and several studies have revealed that the hypoxic microenvironment of cancers might be responsible for the aberrant expression of some lncRNAs [32, 33]. To investigate whether lncRNA BCRT1 is a hypoxia-sensitive lncRNA, breast cancer cells were treated with hypoxia or normoxia for 48 h. The results showed that the expression of lncRNA

BCRT1 was clearly elevated along with the increase in HIF-1α expression (Fig. 7a-b). HIF-1α knockdown dramatically decreased HIF-1α and lncRNA BCRT1 expression under both normoxic and hypoxic conditions (Fig. 7c-e). Moreover, knockdown of HIF-1α substantially attenuated hypoxia-induced lncRNA BCRT1 upregulation (Fig. 7c-e). To elucidate the potential mechanism of hypoxia-induced upregulation of lncRNA BCRT1, we analyzed the JASPAR database [34], and two putative HIF-1α response elements (HREs) in the lncRNA BCRT1 promoter were identified (Fig. 7f-g). To determine whether HIF-1α regulates the expression of lncRNA BCRT1 through these HREs, we constructed two luciferase reporter vectors containing the full-length lncRNA BCRT1 promoter (HRE1 and HRE2) and a truncated fragment (HRE2). As expected, hypoxia treatment significantly increased the luciferase activity in cells transfected with the full-length lncRNA BCRT1 promoter vector compared with the control cells, whereas the lack of HRE1 impaired the luciferase activity, which suggested that HRE1 was crucial for lncRNA BCRT1 transcription (Fig. 7h). In addition, HIF-1α knockdown reversed the luciferase activity induced by hypoxia treatment (Fig. 7 h), suggesting that hypoxia promoted lncRNA BCRT1 transcription through HIF-1α by binding to HRE1 in its promoter region. We performed chromatin immunoprecipitation (ChIP) assays with a HIF-1α antibody to further confirm the binding of HIF-1α with the two predicted HREs in the lncRNA BCRT1 promoter (Fig. 7i), and the results confirmed that HRE1 in the lncRNA BCRT1 promoter was the major region mediating HIF-1α-induced transcriptional regulation. Using the ChIPBase database, we found that the expression of PTBP3 was positively associated with HIF-1α expression (Fig. 7j). Moreover, hypoxia treatment led to elevated expression of PTBP3 at the mRNA and protein levels (Fig. 7k), while HIF-1α knockdown attenuated this effect (Fig. 7l). These results suggested that hypoxia transcriptionally regulated lncRNA BCRT1 expression by HIF-1α through direct binding with HRE1 on its promoter.

LncRNA BCRT1 mediates hypoxia-induced malignant properties of breast cancer cells

Hypoxia is a hallmark of the tumor microenvironment and is associated with proliferation, metastasis, and drug resistance in various solid tumors [35]. Therefore, we first investigated whether lncRNA BCRT1 was involved in hypoxia-induced cell proliferation. Hypoxia treatment led to increased expression of lncRNA BCRT1 and PTBP3, in accordance with enhanced cell proliferation (Fig. 8a-c, Additional file 12: Figure S7a-c). Moreover, HIF-1α or lncRNA BCRT1 knockdown attenuated the effects induced by hypoxia, whereas lncRNA BCRT1 overexpression partly reversed the inhibitory effect of HIF-1α knockdown (Fig. 8a-c, Additional file 12: Figure S7a-c). Previous studies revealed a close association between hypoxia and EMT; therefore, the role of lncRNA BCRT1 in hypoxia-induced EMT was further investigated. After treatment with hypoxia, MDA-MB-231 cells demonstrated a more fibroblast-like morphology and elevated migration ability, which was dramatically reversed by knockdown of HIF-1α or lncRNA BCRT1 (Fig. 8d-f, Additional file 12: Figure S7d-e). Moreover, the siHIF-1α-repressed EMT profile under hypoxic conditions was obviously rescued by overexpression of lncRNA BCRT1 (Fig. 8d-f, Additional file 12: Figure S7d-e). These results indicated that lncRNA BCRT1 might participate in hypoxia-induced biological functions in breast cancer cells.

Discussion

The therapeutic methods available to breast cancer patients with metastatic lesions are complicated, but their clinical outcomes is far from satisfactory. It is of great importance to comprehensively understand the molecular mechanisms involved in breast cancer metastasis and identify novel prognostic predictors. Recently, aberrant expression of lncRNAs has been reported in various cancers [9, 36], and lncRNAs have been shown to play important roles in tumor progression. Increasing studies have focused on the functions and regulation of lncRNAs to discover novel targets for the diagnosis and treatment of cancers. In this study, we determined that the uncharacterized lncRNA BCRT1 was significantly increased in breast cancer tissues compared to normal tissues, and high lncRNA BCRT1 expression was associated with poor prognosis of breast cancer patients. Functional studies revealed that lncRNA BCRT1 could promote the proliferation and mobility of breast cancer cells in vitro and in vivo, indicating a tumor-promoter role in breast cancer. Although several dysregulated lncRNAs have also been identified, more studies are needed to elucidate their function.

The biological function of lncRNAs is largely dependent on their subcellular localization. Accumulated evidence has shown that lncRNAs located in the cytoplasm could participate in gene regulation at the posttranscriptional level, including by acting as ceRNAs and protecting the target mRNAs from repression [37, 38]. By using cell cytoplasmic/nuclear fractionation and RNA FISH assays, we found that lncRNA BCRT1 was preferentially localized in the cytoplasm, indicating its potential for functioning as a miRNA sponge. Subsequently, bioinformatics analysis indicated that there existed binding sites of miR-1303 in the lncRNA BCRT1 sequence, which was further validated by luciferase reporter assay and RIP assay. Moreover, the expression of lncRNA BCRT1 was negatively associated with miR-1303, and a significant reciprocal repression feedback loop present in breast cancer cells. Importantly, miR-1303

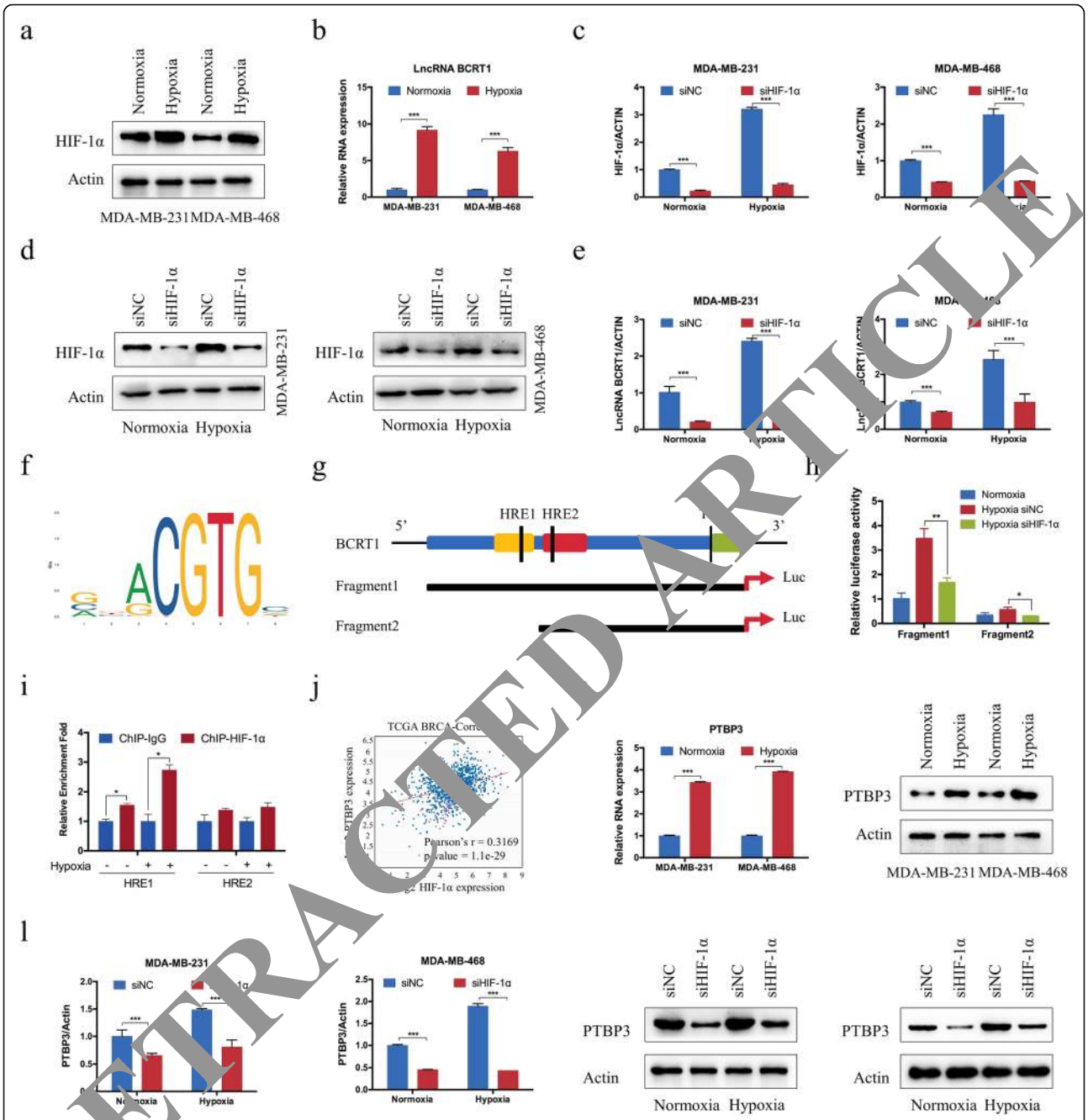

Fig lncRN CR1 was transcriptionally regulated by HIF-1α during hypoxia. **a-b** The expression levels of HIF-1α protein (a) or lncRNA BCRT1 (RNA b) in MDA-MB-231 and MDA-MB-468 cells were measured after culture under normoxia or hypoxia for 48 h by western blot or RT-PCR. **c-d** ... cy of HIF-1α knockdown was detected using RT-PCR and western blot. **e** HIF-1α knockdown inhibited the expression of lncRNA BCR... in MDA-MB-231 and MDA-MB-468 cells under normoxia or hypoxia. **f** The recognition motif of HIF-1α from the JASPAR database. **g** Schematic illustration of the proximal region of the lncRNA BCRT1 promoter and the putative hypoxia responsive elements (HREs). **h** MDA-MB-231 cells were transfected with a lncRNA BCRT1 promoter-containing pGL3 reporter vector and further treated with hypoxia or hypoxia combined with siHIF-1α. After 48 h, Luciferase activity was measured with the dual-luciferase reporter assay system. **i** ChIP assays with anti-HIF-1α antibody were performed to verify the binding between HIF-1α and the HREs of the lncRNA BCRT1 promoter under normoxia and hypoxia. **j** PTBP3 and HIF-1α expression from the TCGA breast cancer dataset was analyzed by the starBase database. **k** The mRNA and protein expression of PTBP3 was elevated under hypoxic conditions. **l** After HIF-1α knockdown, the expression of PTBP3 was evaluated by RT-PCR and western blot in MDA-MB-231 and MDA-MB-468 cells under normoxia or hypoxia. (*P < 0.05, **P < 0.01, and ***P < 0.001)

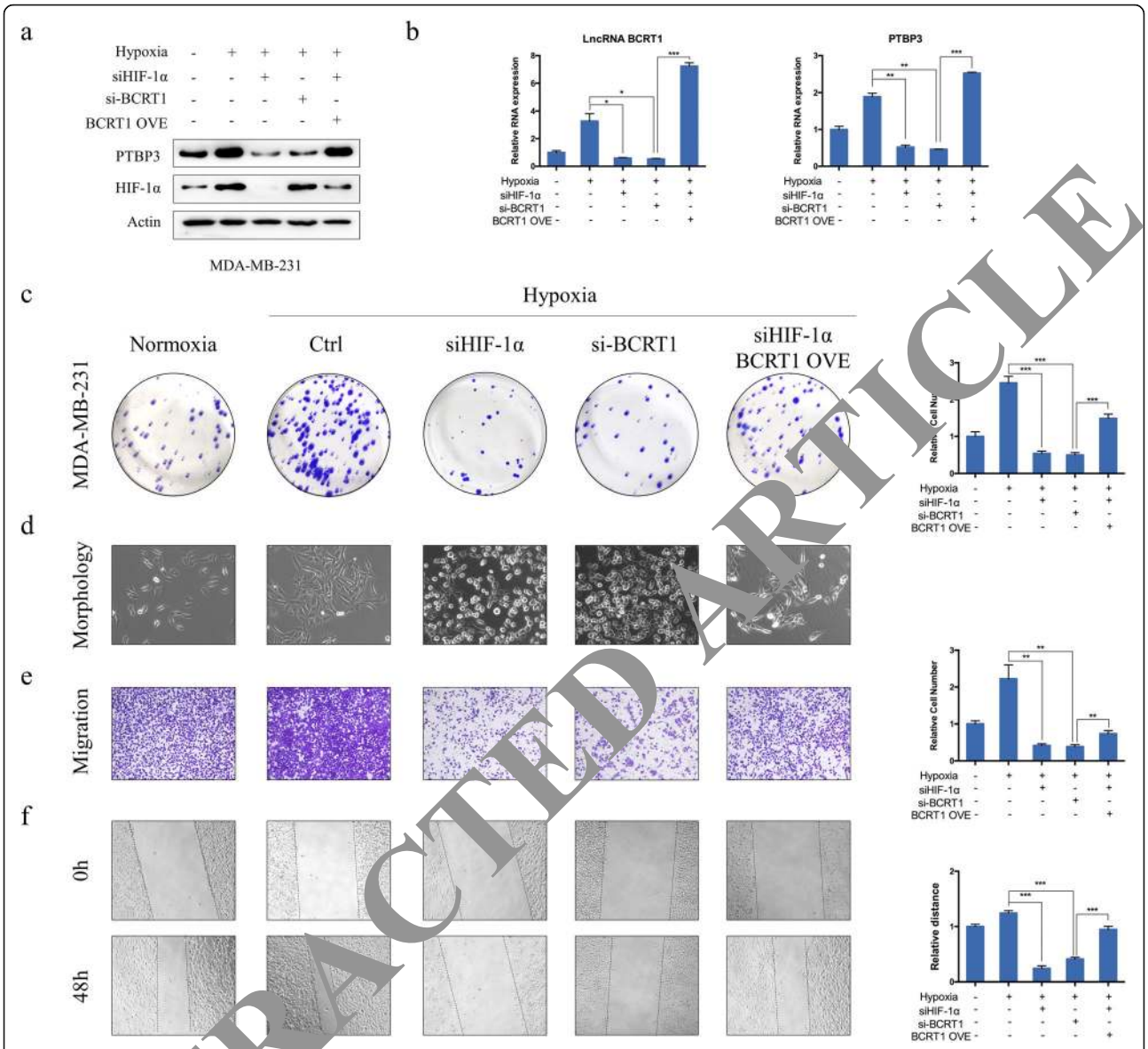

Fig. 8 LncRNA BCRT1 is essential for HIF-1α-mediated hypoxia-induced malignant properties. MDA-MB-231 cells were treated with normoxia, hypoxia, a combination of siHIF-1α and hypoxia, a combination of si-BCRT1 and hypoxia, or hypoxia and cotransfection with siHIF-1α and the pcDNA3.1-BCRT1 plasmid. **a** The expression of HIF-1α and PTBP3 was assessed by western blot. **b** The expression of lncRNA BCRT1 and PTBP3 was evaluated by RT-PCR. **c** Colony formation assay was used to evaluated the proliferation of MDA-MB-231 cells. **d** Monolayer morphology of MDA-MB-231 cells were photographed. **e-f** Transwell assay and wound healing assay were used to analyze the migration ability of MDA-MB-231 cells. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

acts as a tumor suppressor in breast cancer, and miR-1303 overexpression partially reversed lncRNA BCRT1 overexpression-mediated promotion of proliferation, migration, invasion, and angiogenesis of breast cancer cells. Together, our results revealed that lncRNA BCRT1 could serve as a ceRNA by sponging miR-1303 in breast cancer.

Polypyrimidine tract-binding protein 3 (PTBP3), an essential RNA-binding protein with roles in RNA alternative splicing (AS) [39], plays an important role in regulating gene expression and affects the biological behavior of various cancers. PTBP3 was found to be upregulated in gastric cancer compared with normal gastric mucosa [40], and high PTBP3 expression was correlated with poor prognosis and higher lymph node metastasis in gastric cancer patients. Further study revealed that PTBP3 was positively associated with metastasis of gastric cancer by regulating CAV1 through alternative splicing [25]. Moreover, a prooncogenic role for PTBP3 has also been discovered in hepatocellular carcinoma mediated by regulation of the splicing balance of NEAT1 and

pre-miR-612 [26]. In addition, previous studies reported that PTBP3 knockdown led to increased apoptosis and cell cycle arrest, either through regulation of p53 signaling [41] or through HDAC6-mediated inhibition of the phosphorylation of Akt and thymidylate synthase (TYMS) expression [42]. However, the physiological roles or molecular functions of PTBP3 in breast cancer remain largely unclear, except one study that reported that PTBP3 promoted cell proliferation, migration, and invasion of breast cancer cells by preventing ZEB1 mRNA degradation [43]. However, the regulatory mechanism involved in the expression and function of PTBP3 in breast cancer has not been fully elucidated. In the current study, we identified PTBP3 as a target protein of the lncRNA BCRT1/miR-1303 axis on the basis of the following observations. Through bioinformatic prediction and dual-luciferase reporter assays, PTBP3 was demonstrated to be a direct target gene of miR-1303 in breast cancer cells. Moreover, lncRNA BCRT1 overexpression led to increased expression of PTBP3, which could be partially reversed by miR-1303 overexpression, indicating a lncRNA BRCT1/miR-1303/PTBP3 axis in breast cancer. We also revealed a significant positive relationship between the expression of lncRNA BCRT1 and PTBP3 in breast cancer cells. Furthermore, we revealed that PTBP3 was increased in breast cancer tissues and that PTBP3 knockdown clearly inhibited the proliferation, migration and invasion of breast cancer cells. Hence, we further demonstrated the oncogenic role of PTBP3 and provided evidence for the posttranscriptional regulation of PTBP3 by a lncRNA in breast cancer.

Recently, considerable attention has been focused on the significance of the tumor environment in tumor progression, a complex community that includes cancer cells, cancer-associated fibroblasts (CAFs), and immune inflammatory cells [44]. The interaction between cancer cells and TAMs, one of the most abundant immune cells in various solid cancers, was correlated with tumor progression, drug resistance, and poor prognosis in cancer patients [45]. Based on their biological properties, macrophages are generally categorized into two major phenotypes, pro-inflammatory (M1) and anti-inflammatory (M2) macrophages. Many studies have demonstrated that TAMs are considered M2-like macrophages that are closely associated with cancer progression. Our results showed that lncRNA BCRT1 was increased in M2-like macrophages compared to M1-like macrophages and unpolarized macrophages. Moreover, lncRNA BCRT1 overexpression remarkably promoted the expression of markers of M2-like macrophages, whereas lncRNA BCRT1 knockdown produced the opposite results, indicating a promoting role of lncRNA BCRT1 in M2 polarization. The conditioned medium of breast cancer cells could influence the polarization of macrophages, indicating the existence of a transfer mediator. Exosomes, 30–100 nm vesicles, can be secreted by cancer cells and influence tumor progression or drug resistance by modulating other cells in the microenvironment via intercellular communication [46]. Various exosomal lncRNAs have been reported to participate in intercellular communication and are associated with the diagnosis and prognosis of cancer [47]. Our results revealed that breast cancer cell-derived exosomes could promote M2 polarization and enhance its tumor-promoting function by transmitting lncRNA BCRT1. Nevertheless, the correlation between exosomal lncRNA BCRT1 expression and the diagnostic or prognostic values in breast cancer still needs further investigation.

Hypoxia is a common phenomenon in various cancers and is associated with cancer progression. Several lncRNAs have been reported to be regulated by hypoxia via HIF-1α-mediated transcriptional regulation. These hypoxia-sensitive lncRNAs, such as lncRNA PVT1 [48], lncRNA HITT [49] and LncRNA-MTA2TR [50], participate in tumorigenesis and tumor metastasis. In our study, we identified lncRNA BCRT1 as a hypoxia-sensitive lncRNA. Using the JASPAR database, we predicted two potential HREs in the promoter of lncRNA BCRT1. Moreover, the expression of lncRNA BCRT1 was increased during hypoxia, which could be repressed by HIF-1α knockdown. Furthermore, ChIP and dual-luciferase reporter assays verified the regulatory effect of HIF-1α on lncRNA BCRT1 transcription in response to hypoxia. Notably, our results showed that hypoxia led to increased expression of PTBP3 and lncRNA BCRT1 knockdown repressed hypoxia-induced PTBP3, while lncRNA BCRT1 overexpression partially reversed the inhibition of PTBP3 expression by HIF-1α knockdown. These results indicated a novel indirect pathway for hypoxia-induced PTBP3 expression that was stimulated by increased lncRNA BCRT1 levels. Moreover, our results revealed that lncRNA BCRT1 knockdown could suppress hypoxia-induced proliferation and migration, whereas lncRNA BCRT1 overexpression could rescue these effects, which was repressed by HIF-1α knockdown under hypoxic conditions. Therefore, our study provided novel evidence supporting lncRNA as a link between hypoxia and cancer progression.

Conclusions

In summary, we identified hypoxia-responsive lncRNA BCRT1 as a tumor-promoter in breast cancer, and the higher expression of lncRNA BCRT1 was associated with tumor metastasis and poor prognosis. LncRNA BCRT1 acted as a sponge for miR-1303 to attenuate its repressive effect on PTBP3 and promoted M2 polarization through exosome-mediated transfer. Our results provide a better understanding of the role of lncRNAs in breast cancer progression and a potential therapeutic target and prognostic predictor against this malignancy.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12943-020-01206-5.

Additional file 1: Table S1. Primers used for RT-PCR and vector construction.

Additional file 2: Table S2. Antibodies used in the experiments.

Additional file 3: Figure S1. The sequence, secondary structure and coding capacity of lncRNA BCRT1. a Schematic diagram showing the genomic locus of lncRNA BCRT1 in humans. Pink rectangles represent exons. b The sequence of lncRNA BCRT1. c The secondary structure of lncRNA BCRT1 from AnnoLnc (http://annolnc.cbi.pku.edu.cn/). d Putative ORFs of lncRNA BCRT1 were predicted by the ORF Finder. e The amino acid sequences of the putative proteins. f The coding potential of lncRNA BCRT1 was measured by 5 different metrics and the results showed that lncRNA BCRT1 had no coding potential.

Additional file 4: Table S3. Correlation between LncRNA BCRT1 expression and clinicopathological features in breast cancer patients.

Additional file 5: Table S4. Univariate analysis of overall survival in breast cancer patients ($n = 68$).

Additional file 6: Table S5. Multivariate analysis of overall survival in breast cancer patients (n = 68).

Additional file 7: Figure S2. LncRNA BCRT1 regulates proliferation and migration of breast cancer cells in vitro. a The efficiency of lncRNA BCRT1 knockdown in MCF-7 cells was validated with RT-PCR. b MTT assays showed the reduced proliferation of MCF-7 cells transfected with si-BCRT1. c Colony formation assay showed the decreased proliferation of MDA-MB-231 and MDA-MB-468 cells after lncRNA BCRT1 knockdown. d The proliferation rate of MCF-7 cells was evaluated after lncRNA BCRT1 overexpression. e-f MTT assay and colony formation assay were used to evaluate proliferation rate after lncRNA BCRT1 overexpression in MCF-7 cells. g-h Transwell assays demonstrated that lncRNA BCRT1 knockdown inhibited whereas lncRNA BCRT1 overexpression promoted cell migration and invasion abilities in MCF-7 cells. (**$P < 0.01$, ***$P < 0.001$, Student's t test).

Additional file 8: Figure S3. LncRNA BCRT1 and miR-1303 could mutually regulate each other and miR-1303 overexpression inhibited cell proliferation and metastasis in vitro. a RT-PCR was used to validate the change of miR-1303 levels after lncRNA BCRT1 overexpression in MDA-MB-231 and MDA-MB-468 cells. b LncRNA BCRT1 expression was increased in tumor tissues from lncRNA BCRT1-overexpressing group compared to control group. MiR-1303 expression in tumor tissues from lncRNA BCRT1-overexpressing group was lower than those in control group. c Overexpression of miR-1303 was associated with better overall survival of breast cancer patients according to the LinkedOmics databases. d The efficiency of of miR-1303 overexpression in MCF-7 cells was validated by RT-PCR. e MTT assays showed the reduced proliferation in miR-1303-overexpresing MCF-7 cells. f Transwell migration assays demonstrated that miR-1303 overexpression inhibited cell migration. Columns are the average of three independent experiments. g LncRNA BCRT1 expression was decreased in MDA-MB-231 and MDA-MB-468 cells transfected with miR-1303 mimic (**$P < 0.01$, ***$P < 0.001$, Student's t test).

Additional file 9: Figure S4. PTBP3 was associated with poor prognosis of breast cancer patients. a-c Higher PTBP3 expression was associated with poor overall survival, disease-free survival, and distant metastasis free survival of breast cancer patients according to the data from TCGA and GEO.

Additional file 10: Figure S5. PTBP3 was positively regulated by lncRNA BCRT1 in vivo. a RT-PCR and western blot were used to detect the expression of PTBP3 in xenograft tumors. b IHC assay showed that PTBP3 expression was increased in lncRNA BCRT1-overexpressing xenograft tumors. (***P < 0.001, Student's t test).

Additional file 11: Figure S6. LncRNA BCRT1 promoted the function of macrophages. a-b Conditioned medium from lncRNA BCRT1-overexpressing cells promoted the migration of macrophages and had enhanced chemotaxis. c-e RT-PCR, western blot and ELISA were used to detect the expression of TGFβ in macrophages after indicated treatment. (**P < 0.01, ***P < 0.001, Student's t test).

Additional file 12: Figure S7. LncRNA BCRT1 is essential for HIF-1α-mediated hypoxia-induced malignant properties. MDA-MB-468 cells were treated with normoxia, hypoxia, a combination of siHIF-1α and hypoxia, a combination of si-BCRT1 and hypoxia, or hypoxia further and cotransfection with siHIF-1α and the pcDNA3.1-BCRT1 plasmid. a The expression of HIF-1α and PTBP3 was evaluated by western blot. b RT-PCR was used to detect the expression of lncRNA BCRT1 and PTBP3. c Proliferation of MDA-MB-468 cells was assessed by colony formation assay. d-f Transwell assay and wound healing assay were applied to analyze the migration ability of MDA-MB-468 cells. (*$P < 0.05$, **P < 0.01, and ***P < 0.001).

Abbreviations

LncRNAs: Long non-coding RNAs; GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; ceRNA: competing endogenous RNA; NC: Negative control; EMT: Epithelial–mesenchymal transition; RIP assay: RNA immunoprecipitation assay; ChIP: Chromatin immunoprecipitation

Acknowledgements

Not applicable.

Authors' contributions

YRL and QFY conceived the study; YRL, XJS, YML, HWZ, YL, and DWH performed the experiments; BC, WJZ, NZ, TTM, LJW, and XYL collected clinical samples; YJW, FZY, and DL analyzed the data; YRL and XJS wrote the paper; YRL and CXY revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

This project was approved by the Ethical Committee on Scientific Research of Shandong University Qilu Hospital.

Consent for publication

All human tissue samples were obtained with written informed consent from all subjects.

Competing interests

The authors declare that they have no competing interests.

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