

Original Article

Long noncoding RNA IncBRM facilitates the proliferation, migration and invasion of ovarian cancer cells via upregulation of Sox4

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Abstract: Ovarian cancer (OC) is one of the most fatal gynecological cancer in women worldwide. Long noncoding RNA (lncRNA) IncBRM was found to be associated with the progression and prognosis of hepatocellular carcinoma (HCC). However, the expression level, clinical significance and functions in OC tumorigenesis and progression remain unclear. Our present research demonstrated that IncBRM expression was significantly increased in OC tissues. Upregulation of IncBRM expression was correlated with histological grade, FIGO stages, lymph node metastasis and poor prognosis of patients with OC. Functional assays showed that IncBRM positively regulated cell proliferation, migration and invasion in OC. Moreover, IncBRM upregulated Sox4 by competitively binding miR-204. Together, IncBRM functions as an oncogene in OC and can be a promising therapeutic target for OC treatment.

Keywords: IncBRM, Sox4, miR-204

Introduction

Ovarian cancer (OC), the most fatal gynecological cancer, is a common cause of cancer-related deaths in women worldwide [1, 2]. Despite developments in surgery and chemotherapy, the overall 5-year survival rate in OC patients remains below 30% [3]. Tumor invasion, metastasis and chemoresistance may play critical roles in the poor prognosis associated with OC [4]. Thus, it is necessary to reveal the molecular mechanisms underlying OC progression in order to develop novel methods for better diagnosis and treatment.

Long noncoding RNAs (lncRNAs) have recently emerged as central regulators of gene expression in different biological field, including tumor growth and development, apoptosis, proliferation, differentiation, and cell autophagy [5, 6]. Many lncRNAs have been reported to play crucial roles in OC tumorigenesis and progression via interaction with protein, microRNA or mRNA. For example, lncRNA HOTAIR enhances cell proliferation, migration and invasion in OC through the regulation of PIK3R3 [7]. lncRNA HOST2 was proven to regulate biological behaviors of OC mediated by microRNA let-7b [8]. However,

only a handful of lncRNAs have been functionally identified in human OC. Identification of OC-related lncRNA is necessary to understanding the progression and molecular mechanisms in order to establish better OC treatment methods. lncRNA for association with Brahma (IncBRM), located on chromosome 5 between the *ACTBL2* and *PLK2* genes, was originally found to be associated with the progression and prognosis of hepatocellular carcinoma (HCC). lncBRM is highly expressed in liver cancer stem cells (CSCs) and HCC tumours. lncBRM promotes the self-renewal maintenance of liver CSCs and tumor initiation through activation of YAP1 expression in a KLF4-dependent manner [9]. However, little is known about the role of IncBRM in OC tumorigenesis and progression. The objective of our study was to detect the biological functions and molecular mechanisms of IncBRM in OC in order to seek a potential therapeutic target for OC patients.

Materials and methods

Cell culture

Five human OC cell lines (A2780, TOV112D, HO-8910, OVCAR-3, and SKOV3) and a normal

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ovarian cell line (ISOE80) were obtained from the Chinese Type Culture Collection, Chinese Academy of Sciences, and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin sodium, and 100 mg/mL streptomycin sulfate, at 37°C in a humidified air atmosphere containing 5% CO₂.

Tissue samples

A total of 80 patients underwent resection of the primary OC were involved in this study at the Cangzhou Central Hospital between 2012 and 2016. The study was approved by the Research Ethics Committee of Cangzhou Central Hospital, and written informed consent was obtained from all patients. Tumor samples and normal tissues were rapidly frozen in liquid nitrogen and kept at -80°C until used. The tumor stage and grade was consistent with the International Federation of Gynecology and Obstetrics (FIGO) guidelines. Complete follow-up information was obtained for each patient until 2017. The survival time of each patient was counted from the day of the first operation to death or the last day of follow-up.

Construction of OC cells with stable overexpression or knockdown of IncBRM

The lentiviral system was used for ectopic or knockdown expression of IncBRM. shRNA against scramble or IncBRM was cloned into pLKO.1 vector. The target sequences of IncBRM were as follow: sh1: AAGGGATGACGC-TGTGTTT, sh2: CCCTGAGAAGTCATTTGAC. Lentiviral particles expressing above shRNAs were produced in 293T cells. Full-length IncBRM was cloned into pLV vector. Lentiviral particles expressing empty vector or full-length IncBRM were produced in 293T cells. Cells were infected with above lentiviral particles for 24 h and selected by 1 µg/ml puromycin for 5 days.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues and cell lines using the TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The expression levels of NBAT-1 were detected by qRT-PCR using the SYBR[®] Green Master Mix (Takara BIO INC, Otsu, Japan), with GAPDH as an internal control. The

primers are as follows: IncBRM-F: GAGGAGA-GAAGTCACTGAAATGG, IncBRM-R: CTCTTCAA-AGCAGACCCTCTAC; GAPDH-F: CACCCACTCCT-CCACCTTTG-3; GAPDH-R: CCACCACCCTGTTG-CTGTAG-; Sox4-F: CAGCGACAAGATCCCTTTCA; Sox4-R: GCCGGACTTCACCTTCTTC. The comparative Ct method was used for the quantification of the transcripts.

siRNA transfection

siRNAs against Sox4 were purchased from RiboBio company. The target sequence against Sox4 is as follow: GCGACAAGATCCCTTTTCAT. Sox4 siRNAs were transfected into cells using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was determined by the Cell Counting Kit-8 (CCK-8) assay. 2×10³ cells were seeded in 96-well plates. Each well was incubated with WST-8 solution (Dojindo) for 90 mins, and the absorbance was measured at 450 nm using a spectrophotometer.

Migration and invasion assays

For cell migration and invasion assays, 24-well Transwell chambers with 8 µm pore size polycarbonate membrane were used (Corning, NY, USA). Cells were planted on the top side of the membrane precoated with Matrigel (BD bioscience, NJ, USA; without Matrigel for cell migration assay) and incubated for 24 h. Cells inside the upper chamber were obliterated with cotton swabs, while cells on the lower membrane surface were fixed and then stained with 0.5% crystal violet solution. Five fields were counted randomly in each well.

Western blot assay

Western blot assays were performed using the following primary antibodies: anti-Sox4 (1:1000; Abcam, MA, USA) and anti-GAPDH (1:10000; Abcam, MA, USA). Cells were lysed with RIPA lysis buffer (Beyotime, Beijing, China) containing 1% protease inhibitors (phenylmethanesulfonyl fluoride, PMSF); cell protein lysates were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were

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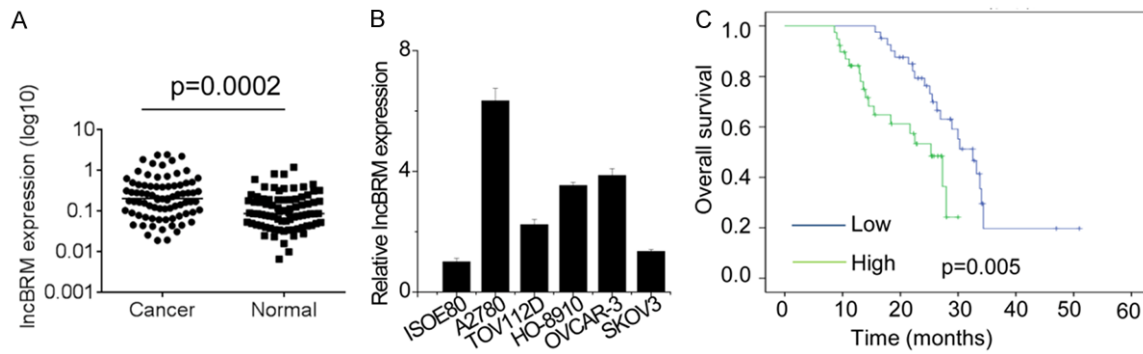


Figure 1. IncBRM is upregulated in OC tissues and associated with the prognosis of OC patients. A. The IncBRM expression levels of 80 pairs of OC tissues and matched normal tissues were detected by using qRT-PCR. B. The relative expression levels of IncBRM in different cell lines were examined by qRT-PCR. C. Kaplan-Meier survival curve and log-rank test were used to evaluate the association of IncBRM expression with overall survival rate. Patients were segregated into IncBRM-high group and IncBRM-low according to the median of IncBRM expression in OC tissues.

incubated with specific antibodies, and visualized by the enhanced chemiluminescence (ECL) system (Roche).

RNA immunoprecipitation (RIP)

Anti-MS2 RIP and anti-AGO2 RIP was performed as previously describe by using the EZ-magna RNA Immunoprecipitation Kit (Millipore) [10]. Briefly, cells were collected and lysed using RIP lysis buffer. Whole-cell extracts were incubated with RIP buffer containing magnetic beads conjugated to human anti-GFP (Abcam) or anti-AGO2 (Millipore) antibody or the control IgG. Proteinase K was added to the samples to digest the protein, and the immunoprecipitated RNA isolated. Purified RNA was used for qRT-PCR analysis.

Luciferase reporter assay

The Sox4 3' untranslated region (3'UTR) and a mutant Sox4 3'UTR (mutant in miR-204 binding site, Sox4-mut) cloned to luciferase reporter plasmid, pmirGLO. These reporter vectors were cotransfected with miR-NC (negative control) or miR-204 mimics into cells using Turbofect (Thermo) according to the manufacturer's instructions. The relative luciferase activity was normalized to Renilla luciferase activity 48 hours after transfection. The luciferase activity was measured using a Dual-luciferase Reporter Gene assay system (Promega).

Statistical analysis

All statistical analyses in our experiment were performed using the SPSS version 20.0 soft-

ware system (IBM, Armonk, NY, USA). Data are shown as mean \pm standard error of mean (SEM). The differences between groups were analyzed by the Student's t-test or χ^2 test. The Kaplan-Meier method was performed for patients' overall survival analysis. All experiments were performed in triplicate. $P < 0.05$ was considered statistically significant difference.

Results

IncBRM is upregulated in OC tissues and associated with the prognosis of OC patients

To investigate the role of IncBRM in OC, we examined the expression levels of IncBRM in OC tissues by performing qRT-PCR experiments. As shown in **Figure 1A**, the expression of IncBRM in OC tissues was significantly higher than that in adjacent normal ovarian tissues. Furthermore, the expression levels of IncBRM in normal ovarian cells and OC cell lines were determined, and the results showed that the expression levels of IncBRM in OC cells including A2780, TOV112D, HO-8910, OVCAR-3 and SKOV3, were significantly upregulated when compared to that in normal ovarian cell line, ISOE80 (**Figure 1B**). In addition, the association between the IncBRM expression in OC tissues and clinicopathological features was analyzed. There is no association between the IncBRM expression and age ($P=1.000$), histological type ($P=0.260$), and ascites ($P=0.648$); the IncBRM expression in OC tissues were positively correlated with histological grade ($P=0.004$), FIGO stages ($P=0.002$) and lymph node metas-

Table 1. Relationship between IncBRM expression and clinicopathologic parameters of OC patients

Characteristics	IncBRM		P value
	High	Low	
Age (years)			
≤50	17	17	1.000
>50	23	23	
Histological type			
Serous	25	20	0.260
Other	15	20	
Histological grade			
G1	12	25	0.004
G2-G3	28	15	
FIGO stage			
I-II	14	28	0.002
III-IV	26	12	
Lymph node metastasis			
Yes	24	15	0.044
No	16	25	
Ascites			
Yes	25	23	0.648
No	15	17	

tasis (P=0.044) (**Table 1**). These results suggest that upregulation of IncBRM may be related to the OC progression.

Next, we determined the association of IncBRM and prognosis of OC patients by performing Kaplan-Meier analysis. The results showed that patients with higher IncBRM expression had a significantly poorer prognosis compared to patients with lower IncBRM expression (**Figure 1C**). These results suggest that IncBRM may function as an oncogene in OC progression.

IncBRM promotes OC cell proliferation, migration and invasion

To determine the functional role of IncBRM in OC cells, we constructed stable cells with knockdown of IncBRM in A2780 (**Figure 2A**). The cell proliferation of OC cells was examined by CCK-8 assay, and the result showed that the cell growth of A2780 cells with IncBRM knockdown was significantly repressed when compared to that transfected with scramble shRNA (**Figure 2B**). Furthermore, we examined the effect of IncBRM on the cell migration and invasion in OC cells. Using a transwell assay, we found that the migratory and invasive ability of

the A2780 cells was dramatically inhibited following IncBRM silencing (**Figure 2C**). In contrast, we constructed stable cells with overexpression of IncBRM in SKOV3 cells (**Figure 2D**), and found that IncBRM significantly promotes cell proliferation, migration and invasion (**Figure 2E and 2F**). Together, these results indicated that IncBRM facilitates the cell proliferation, migration and invasion of OC cells.

IncBRM increases Sox4 expression

To reveal the exact mechanism by which IncBRM regulate malignant phenotypes in OC cells, we analyzed the transcriptome sequencing profiles to identify the target genes regulated by IncBRM from a previous study. Among these genes, Sox4 arose our interest for its remarkable expression fold change upon IncBRM knockdown and its relationship with cancer progression [11, 12]. We found that upregulation of IncBRM markedly increased both Sox4 mRNA and protein level (**Figure 3A**), while silence of IncBRM significantly decreased Sox4 expression (**Figure 3B**). To determine whether IncBRM promotes the progression of OC cells in a Sox4-dependent manner, rescue experiments were performed. Downregulation of Sox4 almost abolished the promotion of proliferation, migration and invasion induced by IncBRM overexpression (**Figure 3C and 3D**). In contrast, upregulation of Sox4 rescued the proliferation, migration and invasion reduced by IncBRM knockdown (**Figure 3E and 3F**).

To further confirm the pathological correlation between IncBRM and Sox4 in OC tissue samples, we detected the Sox4 expression in the same set of 80 OC tissues. We found a significant increase of Sox4 expression (**Figure 3G**) and a positive correlation between IncBRM and Sox4 expression in OC tissues ($r^2 = 0.4781$, $p < 0.0001$; **Figure 3H**). Together, these results demonstrate that IncBRM exerts its function via regulating IncBRM expression.

IncBRM functions as a competitive endogenous RNA of Sox4 through competitively interaction with miR-204

Next, we determined the mechanism by which IncBRM regulates Sox4 expression. We first performed cellular fractionation to analyze the subcellular localization of IncBRM. The result showed that IncBRM mainly located in cyto-

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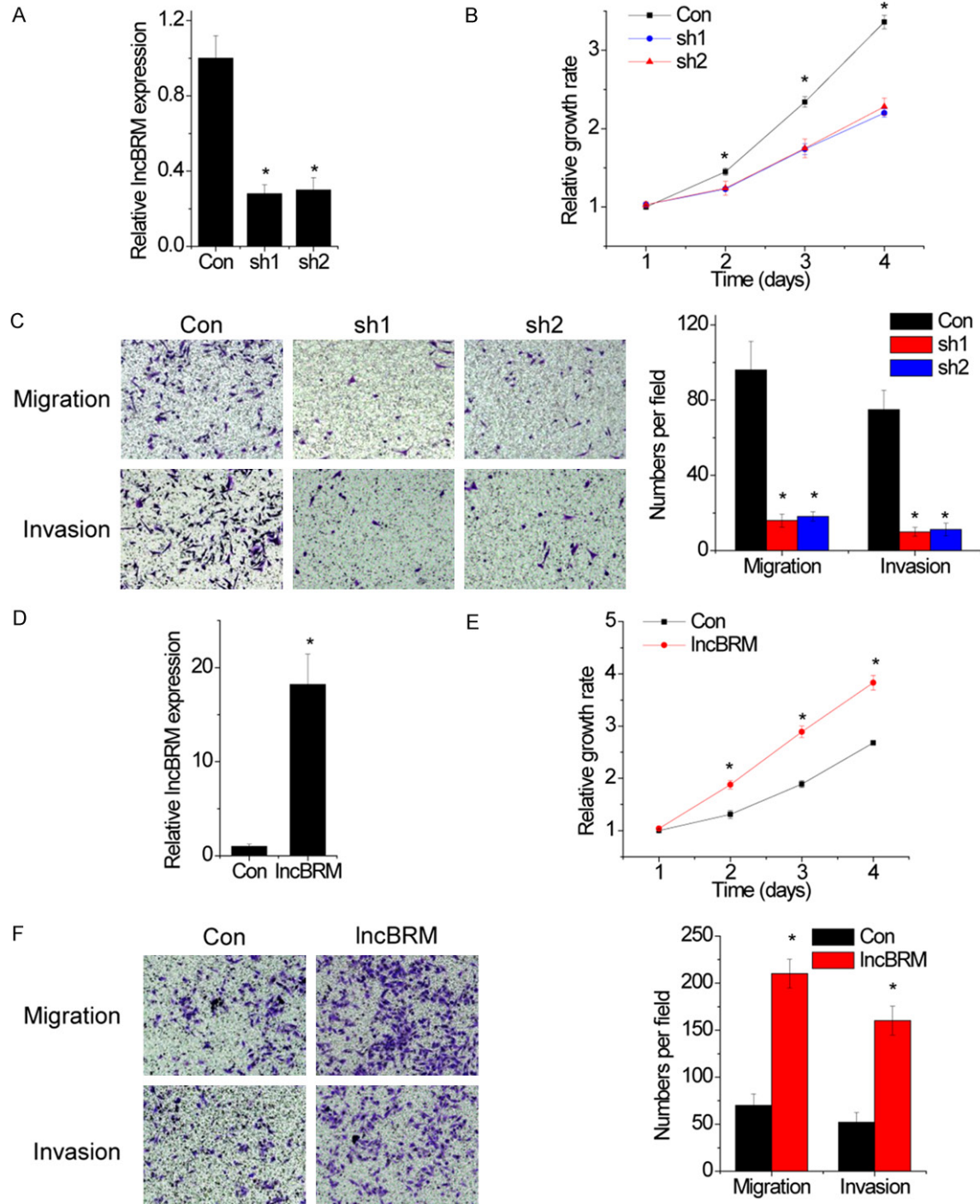


Figure 2. lncBRM promotes OC cell proliferation, migration and invasion. **A.** The relative expression level of lncBRM in control and lncBRM knockdown A2780 cells was determined by qRT-PCR. **B.** Growth curves for A2780 cells after transfection with lncBRM shRNA or the negative control were determined by CCK-8 assay. **C.** The migration and invasive ability after knockdown of lncBRM in A2780 cells was assessed using transwell assays. **D.** The relative expression level of lncBRM in control and lncBRM-overexpressing SKOV3 cells was determined by qRT-PCR. **E.** Growth curves for SKOV3 cells after upregulation of lncBRM were determined by CCK-8 assay. **F.** The migration and invasive ability after upregulation of lncBRM in SKOV3 cells was assessed using transwell assays. Data are shown as mean \pm SD; * p <0.05.

plasm (**Figure 4A**), suggesting a potential role of lncBRM in post-transcriptional regulation.

Interactions between lncRNAs and miRNAs, provide an additional layer of control in gene

lncBRM upregulates Sox4

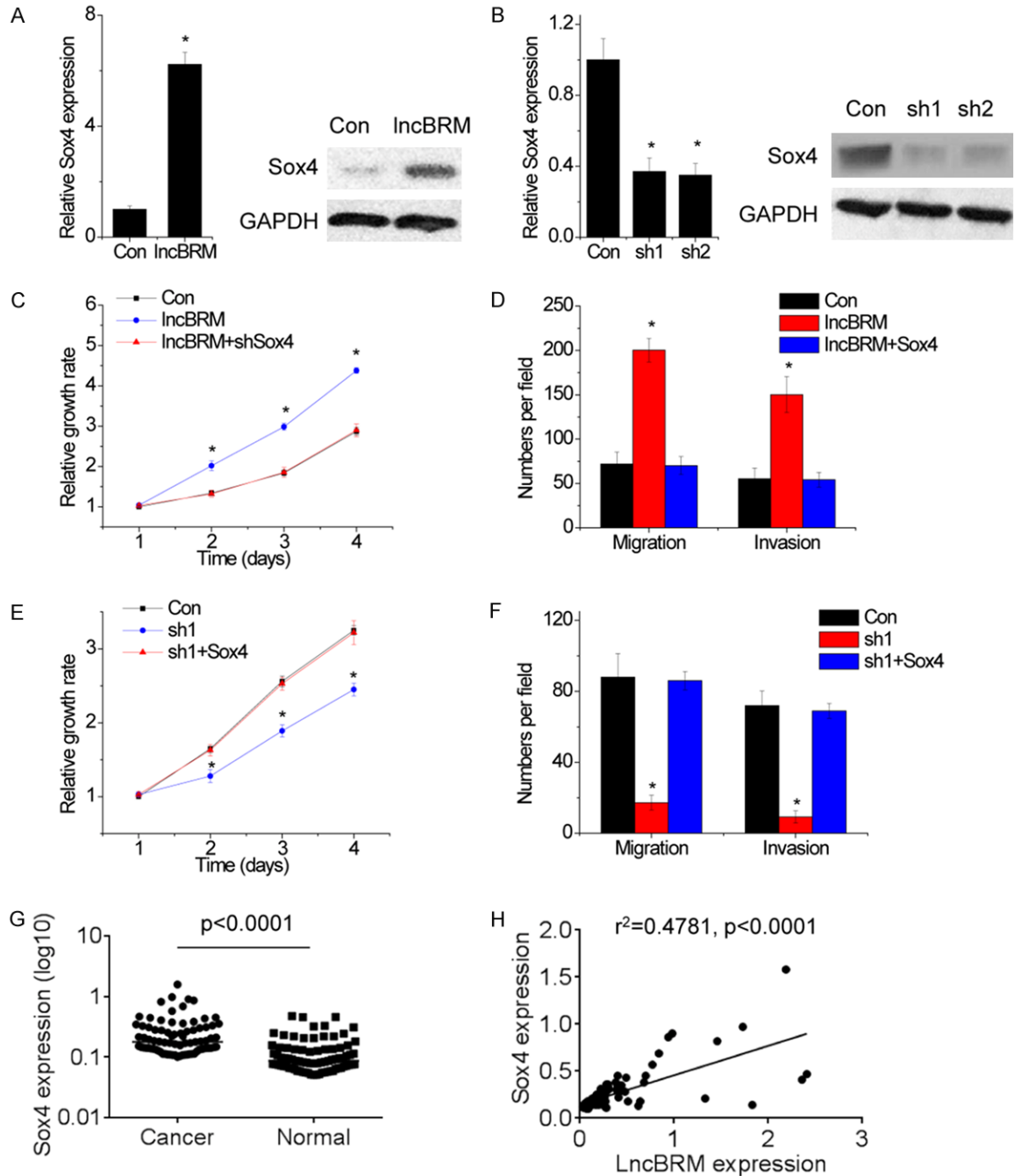


Figure 3. lncBRM increases Sox4 expression. A. The effect of lncBRM overexpression on Sox4 mRNA and protein level in SKOV3 cells. B. The effect of lncBRM knockdown on Sox4 mRNA and protein level in A2780 cells. C. Downregulation of Sox4 abolished the proliferation increased by lncBRM overexpression. D. Downregulation of Sox4 abolished the migration and invasion increased by lncBRM overexpression. E. Upregulation of Sox4 rescued the proliferation reduced by lncBRM knockdown. F. Upregulation of Sox4 rescued the migration and invasion reduced by lncBRM knockdown. G. The expression of Sox4 in 80 pairs of OC tissues and matched normal tissues was examined by qRT-PCR. H. The correlation between lncBRM and Sox4 expression in OC tissues. Data are shown as mean \pm SD; * $p < 0.05$.

regulation [13]. Using Targetscan and Micro-inspector software, we found a set of miRNAs that potentially bind to lncBRM. Among these

miRNA candidates, we found that miR-204 may directly bind to lncBRM. In addition, it has been reported that miR-204 exerts tumor suppres-

lncBRM upregulates Sox4

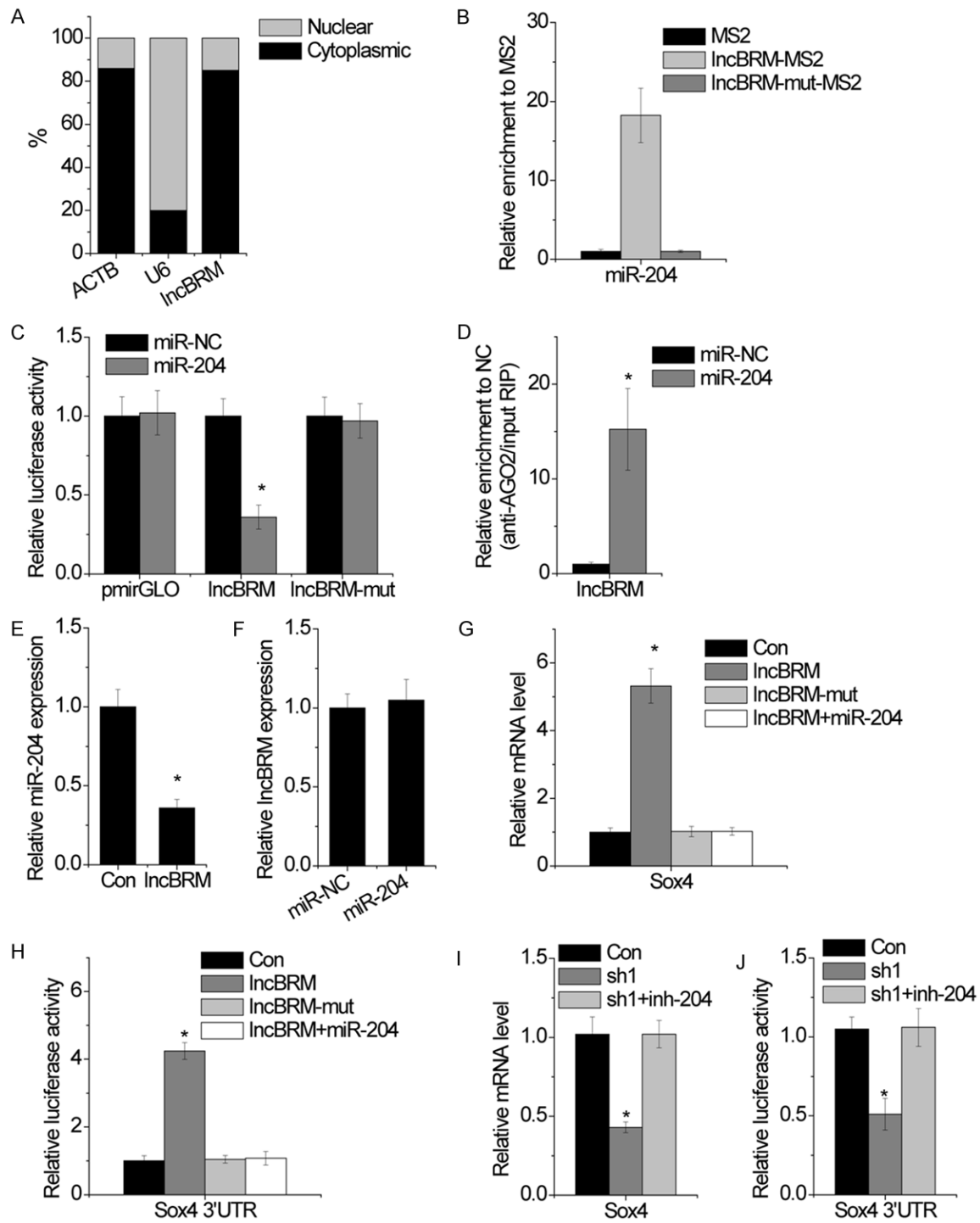


Figure 4. lncBRM functions as a competitive endogenous RNA of Sox4 through competitive interaction with miR-204. A. The subcellular location of lncBRM in OC cells. B. The interaction between lncBRM and miR-204 was determined by RIP assay. C. Luciferase activity in cells cotransfected with miR-204 and luciferase reporters containing nothing, lncBRM or lncBRM-mut. Data are presented as the relative ratio of firefly luciferase activity to renilla luciferase activity. D. Anti-AGO2 RIP was performed in cells transfected with miR-204 mimic, followed by qRT-PCR to detect lncBRM associated with AGO2. E. The expression of miR-204 in control and lncBRM-overexpressing cells was detected by qRT-PCR. F. The expression of lncBRM in control and miR-204-overexpressing cells was detected by qRT-PCR. G. The mRNA levels of Sox4 in stable cell clones transfected with or without miR-204 mimics. H. Luciferase activity in stable cell clones transfected with luciferase reporters containing Sox4 3'UTR. I. miR-204 inhibitor (inh-204) rescued the suppression of Sox4 expression mediated by lncBRM knockdown. J. miR-204 inhibitor (inh-204) rescued the luciferase activity of Sox4 3'UTR decreased by lncBRM knockdown. Data are shown as mean \pm SD; * p <0.05.

sive function through negatively regulating Sox4 expression in cancer [14]. To confirm the direct association between lncBRM and miR-204, we performed an RIP assay to pull down endogenous miRNAs interacting with lncBRM and followed by qRT-PCR detection. We found that the amount of lncBRM pull-down in SKOV3 cells was significantly higher for miR-204 compared to the empty vector (MS2), negative control IgG and lncBRM-mut (mutant in miR-204 binding site) (**Figure 4B**). For further confirmation, we constructed luciferase reporters containing the full length of lncBRM, which contains wild-type (WT) or mutated miR-200s binding sites (lncBRM-mut). We found that overexpression of miR-204 reduced the luciferase activities of the WT reporter vector but not empty vector or mutant reporter vector (**Figure 4C**). To investigate whether lncBRM was regulated by miR-204 in an AGO2-dependent manner, we performed anti-AGO2 RIP in A2780 cells transfected with miR-204 mimics. Endogenous lncBRM pull-down by AGO2 was specifically enriched in miR-204-overexpressing cells (**Figure 4D**), indicating that miR-204 is a lncBRM-targeting microRNAs. We further clarified the regulatory relationship between lncBRM and miR-204. Overexpression of lncBRM significantly inhibited miR-204 expression (**Figure 4E**), whereas overexpression of miR-204 did not affect lncBRM expression (**Figure 4F**). These data indicated that miR-204 directly binds to lncBRM, but didn't degrade lncBRM.

We suspected that lncBRM regulated Sox4 expression through competitively interaction with miR-204. To confirm this hypothesis, we transiently transfected with miR-204 into lncBRM- and lncBRM-mut-overexpressing SKOV3 cells. Ectopic expression of wild-type lncBRM, but not lncBRM-mut, significantly upregulated Sox4 expression. Upregulation of miR-204 attenuated Sox4 expression increased by lncBRM (**Figure 4G**). Moreover, we examined whether lncBRM increased Sox4 expression through the regulation of Sox4 3'UTR. The luciferase reporter vector containing Sox4 3'UTR was transfected into the wild-type or mutant lncBRM overexpressed SKOV3 cells with or without miR-204. Similarly, overexpression of wild-type lncBRM, but not lncBRM-mut, increased the luciferase activity of Sox4 3'UTR, and miR-204 abolished the effect of lncBRM on the luciferase activity of Sox4 3'UTR (**Figure**

4H). On the contrary, inhibition of miR-204 overcame the suppression of Sox4 by lncBRM knockdown (**Figure 4I**). And the miR-204 silence rescued the inhibition of the luciferase activity of Sox4 3'UTR mediated by lncBRM knockdown (**Figure 4J**). Taken together, lncBRM functions as a competitive endogenous (ceRNA) of Sox4, and competed with Sox4 mRNA for the common miR-204.

Discussion

Our present research demonstrated that lncBRM expression was significantly increased in OC tissues. Upregulation of lncBRM expression was correlated with histological grade, FIGO stages, lymph node metastasis and poor prognosis of patients with OC. Functional assays showed that lncBRM positively regulated cell proliferation, migration and invasion in OC through upregulation of Sox4. Together, lncBRM functions as an oncogene in OC and can be a promising therapeutic target for OC treatment.

Emerging evidences have reported that lncRNAs play critical roles in OC tumorigenesis and progression. For example, lncRNA TUG1 expression was upregulated in OC tissues and OC cells, and TUG1 expression was positively correlated with tumor grade and FIGO stage. TUG1 promoted OC proliferation and metastasis through affecting epithelial-mesenchymal transition (EMT) [15]. lncRNA NBAT-1 was significantly decreased in OC and closely related with FIGO stage, tumor size and prognosis of patients with OC. NBAT-1 functioned as a tumor suppressor in OC, which suppressed proliferation, migration and invasion via targeting the ERK1/2 and AKT signaling pathways [16]. However, only a handful of lncRNA has been functionally identified in OC progression. In our study, gain- and loss-of-function assays demonstrated that lncBRM facilitates cell proliferation, migration and invasion in OC through functioning as a ceRNA of Sox4. lncBRM completed with Sox4 mRNA for common miR-204 which was a critical tumor suppressive microRNA in various cancers [17-19]. RIP and luciferase assays demonstrated that lncBRM physically associated with miR-204 and decreased the binding level of miR-204 to Sox4 mRNA 3'UTR, and subsequently inhibited the degradation of Sox4 mRNA induced by miR-204. Our study is

the first to demonstrate that lncBRM functions as a ceRNA to regulate cancer progression.

Sox4, the member of the Sry-related highmobility group box (Sox) family of transcription factors, is as a master mediator in tumorigenicity and cancer stemness [20]. Sox4 is up-regulated in various cancers, including colorectal cancer, prostate cancer, esophagus cancer and OC [21-24]. miR-363, miR-204 and miR-187 could negatively regulate Sox4 expression [25, 26]. A recent study demonstrated that Sox4 was regulated by lncRNA UCA1 via competitively binding miR-204. In our present study, our data also showed that overexpression of lncBRM increased Sox4 expression, while knockdown of lncBRM reduced Sox4 expression. Moreover, we observed a positive correlation between lncBRM and Sox4 expression in OC tissues, suggesting that Sox4 is a bona fide target of lncBRM.

In conclusion, this study demonstrates that the overexpression of lncBRM in OC is a strong indicator of poor clinical outcome. lncBRM promotes OC cells proliferation, migration and invasion through upregulation of Sox4. Thus, lncBRM may be a candidate biomarker for OC prognosis and a target for new therapies.

Disclosure of conflict of interest

None.

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