RBM3 upregulates *ARPC2* by binding the 3'UTR and contributes to breast cancer progression

PING CHEN[1*], XIAOLI YUE[1*], HONGBING XIONG[1*], XIAOHONG LU[2] and ZHENLING JI[1,3]

[1]Zhongda Hospital Lishui Branch, Southeast University, Nanjing Lishui People's Hospital, Nanjing, Jiangsu 211200; [2]Medical School, Xiamen University, Xiamen, Fujian 361005; [3]Zhongda Hospital, Southeast University, Nanjing, Jiangsu 210000, P.R. China

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Abstract. Breast cancer is one of the most common types of cancers which results in a high mortality rate for patients worldwide. In this study, we performed systematical experiments including tissue analysis (immunohistochemistry etc.) and cell functional experiments (cell counting assay, MTT assay, cell colony formation, cell migration assay, cell invasion assay etc.). We demonstrated that the expression level of RNA binding motif protein 3 (RBM3) was higher in human breast cancer tissues compared with adjacent non-tumor tissues. A high level of RBM3 was associated with worse post-operative relapse-free survival (RFS) and overall survival (OS) rates in patients with breast cancer. Among the patients with breast cancer, the expression of RBM3 was associated with patient lymph node metastasis and a high tumor grade. The knockdown of RBM3 markedly decreased the proliferation and metastasis of human breast cancer cells. In downstream pathway analysis, actin related protein 2/3 complex subunit 2 (ARPC2) was determined to be positively regulated by RBM3 through a post-transcriptional 3'UTR-binding manner. ARPC2 also played an oncogenic role and mediated the promoting role of RBM3 in the proliferation and metastasis of human breast cancer cells. Thus, on the whole, the findings of this study demonstrate that RBM3 acts as an oncogene in human breast cancer cells and that the functional depletion of RBM3 may be considered as a potential method for breast cancer therapy.

Introduction

Breast cancer is one of the most frequently diagnosed types of cancer and the second major cause of cancer-related mortality among women worldwide (1,2). It has been reported that 1 in 8 women may suffer from breast cancer in her lifetime (3,4). Surgery, chemotherapy, radiotherapy, endocrine therapy and targeted therapy present the main treatment strategies for breast cancer. However, patients with breast cancer always suffer from tumor recurrence and treatment resistance (5,6). Although numerous studies have focused on tumor suppressors and oncogenes for breast cancer and have attempted to unveil the mechanisms responsible tumorigenesis, development and metastasis, the accurate underlying molecular mechanisms remain to be fully understood. Further studies of the underlying molecular mechanisms of breast cancer are desirable, and may aid in the development of novel clinical diagnostic and therapeutic methods.

RNA binding motif protein 3 (RBM3), a member of the highly conserved family of RNABPs, is a glycine rich protein and is expressed in various human tissues (7-10). RBM3 protein contains a RNA-recognition motif (RRM), through which RBM3 protein can bind to both DNA and RNA (11,12). RBM3 protein interacts with the untranslated regions (UTRs) of mRNAs, resulting in either the stabilization or destabilization of the mRNA (11,13). However, in fact, the exact genes that can be regulated by RBM3 are limited. RBM3 contributes to the response of cellular stressors, such as degenerative and hypoxic conditions (7,14). Moreover, RBM3 has been documented to promote cell proliferation and erythropoietic differentiation (15,16). Recent studies have demonstrated that RBM3 plays a promoting role in human colorectal cancer (17,18) and prostate cancer (15). However, RBM3 has been identified to be a tumor suppressor in human ovarian cancer (11), urothelial bladder cancer (19), malignant melanoma (8), and esophageal and gastric adenocarcinoma (20). These data suggest RBM3 plays differential roles in various human cancers and exhibits tissue specificity. To the best of our knowledge, to date, the role of RBM3 in human breast cancer remains unclear.

In the present study, we identified the tumor-promoting role of RBM3 in human breast cancer. The overexpression of RBM3 was observed in human breast cancer tissues compared with adjacent non-tumor tissues. A high level of RBM3 was found to be associated with both a low relapse-free survival (RFS) and overall survival (OS) rates of breast cancer patients. In patients with breast cancer, the expression

Correspondence to: Dr Zhenling Ji, Zhongda Hospital Lishui Branch, Southeast University, Nanjing Lishui People's Hospital, 86 Chongwen Road, Nanjing, Jiangsu 211200, P.R. China
E-mail: zlji@vip.sina.com

*Contributed equally

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of RBM3 was associated with patient lymph node metastasis and a high tumor grade. RBM3 was also found to promote the proliferation and metastasis of human breast cancer cells. Moreover, RBM3 was determined to regulate the expression of actin related protein 2/3 complex subunit 2 (*ARPC2*; a member of actin-related proteins that forms the Arp2/3 complex). *ARPC2* was also found to act as an oncogene in breast cancer cells and to mediate the promoting role of RBM3 in the proliferation and metastasis of breast cancer cells. Therefore, RBM3 acts as an oncogene in human breast cancer cells and the functional depletion of RBM3 may prove to be considered a potential therapeutic strategy for breast cancer.

Materials and methods

Tissue samples and patients. A total of 103 human breast cancer tissues and 103 adjacent non-tumor tissues were collected from patients with breast cancer who underwent surgery at Zhongda Hospital (Nanjing, China) between 2010 and 2013. All these patients with breast cancer were females. These tissues did not contain tissues from patients who had received special therapies prior to surgery and patients with other diseases. These patients with breast cancer were followed-up for >60 months and their RFS and OS were documented. We also collected the clinicopathological parameters of these patients with breast cancer. Experiments related to the use of human tissues were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and local approval (from the Institutional Review Boards of Southeast University) was obtained prior to the commencement of this study. Informed consent form was signed by each patient.

Immunohistochemistry. The protein levels of RBM3 and ARPC2 in the neutral buffered formalin-fixed (room temperature for 24 h) and paraffin-embedded tissue sections (4 μm) were examined by immunohistochemistry using an Ultra Sensitive-SP kit (Maixin-Bio, Fuzhou, China) as previously described (21,22). RBM3 rabbit polyclonal antibody (1:100, 14363-1-AP; Proteintech Group, Rosemont, IL, USA) and ARPC2 rabbit polyclonal antibody (1:200, 15058-1-AP; Proteintech Group) were used. The sections stained by immunohistochemistry were evaluated by two senior pathologists using an Olympus microscope (Olympus, Tokyo, Japan) independently. Positive signals of RBM3 or ARPC2 ≥20% were designated as having a high expression of RBM3 or ARPC2 and positive signals <20% were designated as having a low expression of RBM3 or ARPC2, respectively.

Cell lines and cell culture. Human normal breast cells (MCF10A and HMEC) and breast cancer cells (MCF7, T47D, MDA-MB-468, BT474, MDA-MB-231 and BT549) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MCF10A, MCF7, T47D, MDA-MB-231 and BT549 cells were cultured using RPMI-1640 medium containing 10% FBS. The HMEC cells were cultured using DMEM:F12 (1:1) medium containing 10% FBS. The BT474 cells were cultured using DMEM medium containing 10% FBS. The MDA-MB-468 cells were cultured using L-15 medium containing 10% FBS. All these cell lines were cultured using T25 plates at 5% CO_2 and 37˚C in a humidified atmosphere as recommended by ATCC.

Western blot analysis. Protein levels of RBM3 and ARPC2 in both fresh human tissues and cell lines were detected by western blot analysis, which was carried essentially as described in previous studies (21,22). Briefly, 30 μg proteins were resolved by 10% SDS-PAGE and then transferred onto nitrocellulose (NC) membranes (Roche, USA). Membrane blocking was carried out at room temperature for 1.5 h with 1% (w/v) BSA. The membranes were then incubated with RBM3 rabbit polyclonal antibody (1:1,000, 14363-1-AP; Proteintech Group), ARPC2 rabbit polyclonal antibody (1:1,000, 15058-1-AP; Proteintech Group) or β-actin mouse monoclonal antibody (1:5,000, A1978, Sigma, St. Louis, MO, USA) overnight at 4˚C, respectively. The membranes were then incubated with secondary antibody (1:50,000; Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1.5 h. The protein bands were examined by a chemiluminescence system (EMD Millipore, Billerica, MA, USA). We used IPP6.0 software to analyze the densitometry of blots according to standard methods. β-actin was detected as a control.

Cell transfection. The shRNA plasmids, including si-RBM3#1, si-RBM3#2, si-ARPC2#1, si-ARPC2#2 and si-NC were designed and synthesized by GenePharma (Shanghai, China). The mammalian expression plasmid pIRESneo3 (Invitrogen/Thermo Fisher Scientific) was used for the construction of the *ARPC2* overexpression plasmid, and the Vec plasmid was used as a control. In this study, we used Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific) for transfection as previously described (21,22). The sequences used in this study were as follows: si-RBM3#1, 5'-GGAGG GCUCAACUUUAACATT-3'; si-RBM3#2, 5'-GGACGUUC CAGAGACUAUATT-3'; si-ARPC2#1, 5'-CATTGTGCATC AAGCTGGCTT-3'; si-ARPC2#2, 5'-CACAGGTCCTCTTTA GCCATT-3'; siNC, 5'-UUCUCCGAACGUGUCACGUTT-3'. The subsequent experiments were carried out 48 h later; the transfection efficiency was determined by western blot analysis, as described above.

Cellular proliferation assays. The cellular proliferation assays performed in this study included a cell counting assay, MTT assay and cell colony formation assay, which were carried out as described in previous studies (21,23).

For cell counting assay, the cells were plated into 6-well plates. The total cell number was counted every day for 5 days and cell growth curves were created for cell proliferation analysis. For MTT assay, the cells were plated into 96-well plates (2,000 cells per well). MTT evaluation was performed 96 h later. Briefly, 100 μl 5 mg/ml MTT (A100793, Sangon Biotech, Shanghai, China) was added into 1 ml cell culture medium; the cell culture medium in 96-well plates was then changed into the upper medium (normal medium containing MTT) and the cells were incubated in an incubator with 5% CO_2 at 37˚C for 1.5 h, discarding the MTT medium and adding 200 μl DMSO per well into the 96-well plates.

This was followed by mixing for uniformity and detection of the absorbance at OD570. The absorbance at OD570 was measured using a Multiskan FC spectrometer (Thermo Fisher Scientific). For cell colony formation assay, cells were plated into 6-well plates (1,000 per well). Cell colony formation was calculated 10-15 days later. Briefly, this was carried out by the addition of 200 μl 4% formaldehyde per well into the 6-well plates and incubation for 20 min. The cells were then washed with PBS and 100 μl 0.1% crystal violet (A100528, Sangon Biotech) were added per well into the 6-well plates followed by incubation for 20 min at room temperature and washing with water; images were captured using an Canon camera 1500D (Canon, Tokyo, Japan) (magnification, x200) and the cell colony numbers were counted (by the researcher according to the images).

Cellular metastasis assays. Cellular metastasis assays used in this study included cell migration assay and cell invasion assay which were carried out in 24-well 8-μm pore Transwell chambers (Corning Inc., Corning, NY, USA). For cell invasion assay, the upper chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA). The upper chambers uncoated for cell migration assay. The upper chambers were seeded with $2x10^4$ cells in medium containing 0.1% FBS. The lower chambers were added with medium with 5% FBS. For the cell migration assay, MCF7 cells were examined after 24 h, and MDA-MB-231 cells were examined after 12 h. For the cell invasion assay, MCF7 cells were examined after 48 h, and MDA-MB-231 cells were examined after 24 h. The lower chambers (for both cell migration assay and cell invasion assaay) were stained with crystal violet (A100528, Sangon Biotech) for 20 min. Images were captured using an Olympus microscope (20X objective; Olympus) (magnification, x200) and the cell numbers were counted (by the researcher according to the images).

Reverse transcription-quantitative PCR (RT-qPCR). The mRNA levels of human epidermal growth factor receptor 2 (*HER2*), *MYC, ARPC2*, signal transducer and activator of transcription 3 (*STAT3*), phosphatase and tensin homolog (*PTEN*), FOS, cyclin D1 (*CCND1*), epidermal growth factor receptor (*EGFR*), progesterone receptor (*PGR*) and trefoil factor 1 (*TFF1*) were examined by RT-qPCR, which was performed as previously described (21,23). *GAPDH* was used as a control. Briefly, an RT kit (PrimeScript™ RT reagent kit, DRR037A; Takara, Dalian, China) was used for the RT process, and the conditions were 37˚C for 15 min and 85˚C for 5 sec. A qPCR kit [SYBR Premix Ex Taq™ II (Perfect Real Time), DRR081A, Takara] was used for the qPCR process; the conditions were as follows: 95˚C for 10 min, 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec, dissolution curve analysis at 95˚C for 15 sec, 60˚C for 30 sec and 95˚C for 15 sec. The primers used in this study were as follows: *HER2* forward, 5'-GTCTGGACGTGCCAGTGTG-3' and reverse, 5'-CATCTGGGAACTCAAGCAGG-3'; *MYC* forward, 5'-CTGGTGCTCCATGAGGAGAC-3' and reverse, 5'-GCACCTCTTGAGGACCAGTG-3'; *ARPC2* forward, 5'-GACGACGATGTGGTCATTGG-3' and reverse, 5'-CAATGTTGTCACCCACAGCG-3'; *STAT3* forward, 5'-CAGTTCTCCTCCACCACCAAG-3' and reverse, 5'-GGTCAATGATATTGTCCAGCCAG-3'; *PTEN* forward, 5'-ATCAAGAGGGATAAAACACCATG-3' and reverse, 5'-ATCTGACACAATGTCCTATTGCC-3'; *FOS* forward, 5'-GCAGACCGAGATTGCCAAC-3' and reverse, 5'-GATCAAGGGAAGCCACAGAC-3'; *CCND1* forward, 5'-CGTGGCCTCTAAGATGAAGG-3' and reverse, CTGGCATTTTGGAGAGGAAG; *EGFR* forward, 5'-CAGCTTCTTGCAGCGATACAG-3' and reverse, 5'-CTGGTAGTGTGGGTCTCTGCTG-3'; *PGR* forward, 5'-CTGACACCTCCAGTTCTTTGC-3' and reverse, 5'-TCTCCATCCTAGACCAAACACC-3'; *TFF1* forward, 5'-AGCAGAGAGGAGGCAATGG-3' and reverse, 5'-GGATAGAAGCACCAGGGGAC-3'; and *GAPDH* forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, GGCATGGACTGTGGTCATGAG-3'. The RT-qPCR results were quantified using $2^{-\Delta\Delta Cq}$ method (24).

Luciferase reporter assay. Luciferase reporter assay was performed in this study using a Dual Luciferase Reporter Assay System (Promega Corp., Madison, WI, USA) as previously described (21,23). Luciferase reporter plasmids (Promega Corp.) containing the *ARPC2* wild-type 3'untranslated region (UTR) was constructed. *ARPC2*-3'UTR: CTTGGGAATAAGAGGAGGAAGCGGCTGGCAACTGAAGGCTGGAACACTTGCTACTGGATAATCGTAGCTTTTAATGTTGCGCCTCTTCAGGTTCTTAAGGGATTCTCCGTTTTGGTTCCATTTTGTACACGTTTGGAAAATAATCTGCAGAAACGAGCTGTGCTTGCAAAGACTTCATAGTTCCCAAGAATTAAAAAAAAAAAAAAAAGAATTCCACTTGATCAACTTAATTCCTTTTCTTTATCTTCCCTCCCTCACTTCCCTTTTCTCCCACCCTCTTTTCCAAGCTGTTTCGCTTTGCAATATATTACTGGTAATGAGTTGCAGGATAATGCAGTCATAACTTGTTTTCTCCTAAGTATTTGAGTTCAAAACTCCTGTATCTAAAGAAATACGGTTGGGGTCATTAATAAAGAAAATCTTTCTATCTTACATGAGAA.

Co-transfection with siRNAs and luciferase reporter plasmid (*ARPC2*-3'UTR) was carried out in $1x10^6$ cells at 6-well plates using Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific), according to the manufacturer's instructions. After 48 h cells were washed with PBS for twice and lysed using lysis buffer (Promega Corp.). A total of 20 μl cell extract and 100 μl luciferase assay reagent were mixed together at room temperature. The Firefly luciferase activity of this mixture was then quantified using a Dual Luciferase Reporter Assay System (Promega Corp.).

mRNA decay assay. mRNA decay assay was carried out in this study to analyze the interaction between RBM3 protein and ARPC2 mRNA. The cells were treated with actinomycin D (10 μg/ml) for 0, 2, 4, 6 and 8 h, and the mRNA levels of *ARPC2* were examined by RT-qPCR. *GAPDH* was detected as a control.

Ribonucleoprotein (RNP) immunoprecipitation (IP) RT-PCR (RNP-IP RT-PCR). In this study, the binding between RBM3 protein and *ARPC2* mRNA were analyzed using RNP-IP RT-PCR, which was carried out essentially as previously described (25). Fifty million cells were collected per sample, and lysates were used for IP for 12 h at 4˚C in the presence of excess (50 μg) IP antibody (IgG, anti-RBM3). RNA in IP materials was used in RT followed by PCR and qPCR analysis to detect the presence of *ARPC2* and *GAPDH*

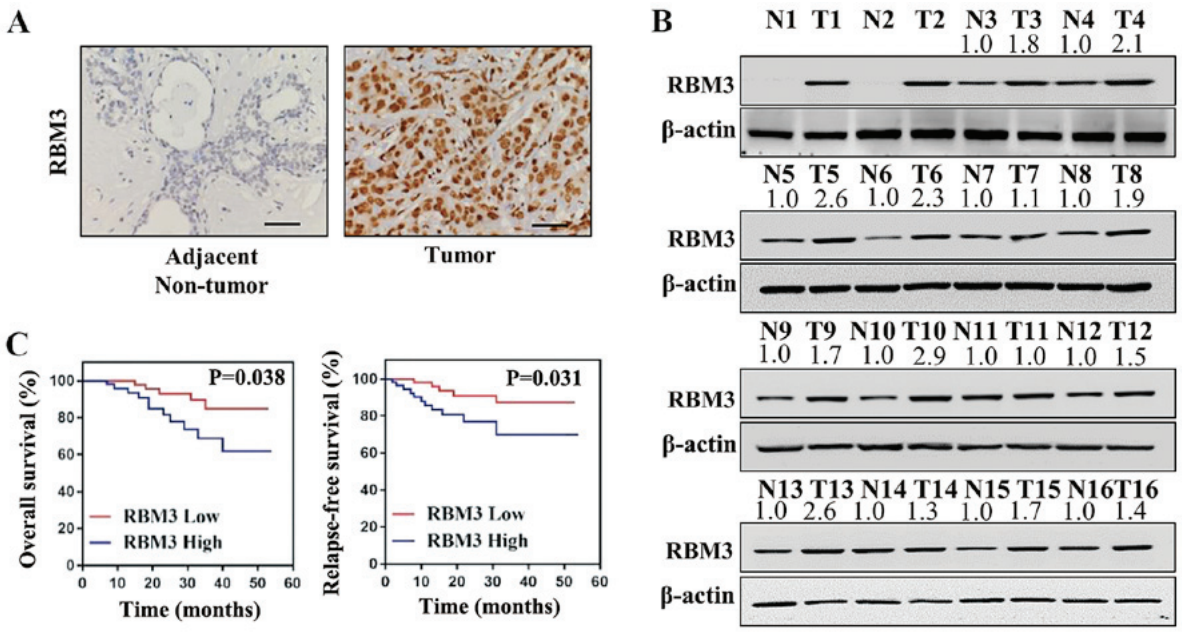

Figure 1. Expression of RBM3 in human tissues and the association of RBM3 expression with the survival rates of patients with breast cancer. (A) Protein levels of RBM3 in breast cancer tissues and adjacent non-tumor tissues were detected by immunohistochemistry. Representative images at x400 magnification are presented; scale bars, 50 μm. (B) Protein levels of RBM3 in 16 pairs of breast cancer fresh tissues and adjacent non-tumor fresh issues were examined by western blot analysis. β-actin expression was also detected as a control. (C) According to the examination of RBM3 protein levels in breast cancer tissues by immunohistochemistry, Kaplan-Meier curves were created to analyzed the overall survival (OS) and relapse-free survival (RFS) rates between the RBM3 expression high group (n=55) and the RBM3 low expression group (n=48) of patients with breast cancer. RBM3, RNA binding motif protein 3.

mRNAs. Anti-RBM3 antibody was used to capture the RBM3 protein-ARPC2 mRNA complex, and the ARPC2 mRNA levels were detected by RT-qPCR. IgG was used as a control. The mRNA levels of the negative control genes, HER2 and MYC, were also detected. The RBM3 rabbit polyclonal antibody (1:100, 14363-1-AP, Proteintech Group), HER2 rabbit polyclonal antibody (1:100, 18299-1-AP, Proteintech Group) and IgG antibody (1:100, 16402-1-AP, Proteintech Group) were used in this experiment.

Biotin pulldown assay. In this study, biotin pulldown assay was performed to examine the binding region of RBM3 protein to ARPC2 mRNA. Different biotinylated transcript regions were synthesized using the MAXIScript T7 kit (Invitrogen/Thermo Fisher Scientific). The protein-mRNA binding complexes were isolated using Streptavidin-coupled Dynabeads (Invitrogen/Thermo Fisher Scientific). Western blot analysis was performed to detect the proteins in the protein-mRNA binding complexes.

Statistical analyses. All the data shown in the figures represent the average of 3 independently repeated experiments. For cellular proliferation and metastasis assays, RT-qPCR and luciferase reporter assay, one-way analysis of variance followed by the Bonferroni or Tamhane post hoc tests were used. Kaplan-Meier curves were created for the analyses of patient RFS and OS, and the log-rank test was also used. We have re-valuated the correlation between RBM3 and ARPC2 using GraphPad Prism 7.03 software. The association between the patient clinicopathological parameters and RBM3 expression was analyzed using Pearson's Chi-square (χ^2) test. The correlation between RBM3 and ARPC2 expression was determined using the Spearman's rank correlation test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of RBM3 in human tissues from patients with breast cancer. Breast cancer tissues and adjacent non-tumor tissues were collected from patients and the protein levels of RBM3 were examined by immunohistochemistry. As shown in Fig. 1A, the protein levels of RBM3 were markedly higher in the human breast cancer tissues compared with the adjacent non-tumor tissues. For further analysis, the expression levels of RBM3 in 16 pairs of tumor tissues and adjacent non-tumor tissues were detected by western blot analysis. Concordantly, the protein levels of RBM3 were markedly higher in the breast cancer tissues compared with the adjacent non-tumor tissues (Fig. 1B). Thus, RBM3 was found to be overexpressed in the breast cancer tissues compared with the normal adjacent non-tumor tissues.

Association of RBM3 expression with survival rates and clinicopathological parameters of patients with breast cancer. The patients with breast cancer were followed-up for >60 months and the association of RBM3 expression with the patient survival rates was analyzed. As shown by the Kaplan-Meier curves, both the OS rate (P=0.038) and RFS rate (P=0.031) were markedly lower in the patients with breast cancer with a high level of RBM3 expression compared with the patients with a low level of RBM3 (Fig. 1C). For further analysis, the association of RBM3 expression with the clinicopathological parameters of these breast cancer patients was examined. As

Table I. Association of RBM3 expression with the clinicopathological parameters of patients with breast cancer.

Parameter	No.	RBM3 expression [n (%)]		P-value	χ^2
		Low	High		
Age (years)					
≤50	53	25 (47.2)	28 (52.8)	0.851	0.363
>50	50	23 (46.0)	27 (50.0)		
Tumor size (cm)					
≤2	30	19 (63.3)	11 (36.6)	0.396	0.508
>2	73	45 (61.6)	28 (38.4)		
Lymph node metastasis					
No	46	30 (65.2)	16 (34.8)	0.031	4.035
Yes	57	28 (49.1)	29 (50.9)		
Tumor grade					
I-II	64	44 (68.8)	20 (31.2)	0.054	6.116
III	39	11 (28.2)	28 (71.8)		
Tumor stage					
I-II	75	51 (68.0)	24(32.0)	0.034	0.011
III-IV	28	19 (67.9)	9 (32.1)		

shown in Table I, the expression of RMB3 in these breast cancer tissues was positively associated with lymph node metastasis (P=0.015) and tumor grade (P=0.005). However, no significant association was observed between RMB3 expression and the age, tumor size or tumor stage of these patients with breast cancer (P>0.05). Therefore, a high expression level of RBM3 was associated with poor a prognosis, lymph node metastasis and a high tumor grade in the patients with breast cancer.

RBM3 promotes the proliferation and metastasis of human breast cancer cells. We then examined the expression levels of RBM3 in human normal breast cells (MCF10A and HMEC) and breast cancer cells (MCF7, T47D, MDA-MB-468, BT474, MDA-MB-231 and BT549) by western blot analysis. As shown in Fig. 2A, the protein levels of RBM3 were markedly higher in the 6 breast cancer cells than in the 2 normal breast cells. The MCF7 and MDA-MB-231 were selected for further functional analyses. As shown in Fig. 2B, the stable knockdown of RBM3 led to a marked decrease in the protein levels of RBM3. As determined by cell counting assay, transfection with both si-RBM3#1 and si-RBM3#2 significantly decreased the cell total number over a period of 5 days in both the MCF7 and MDA-MB-231 cells (Fig. 2C). Concordantly, the viability of both the MCF7 and MDA-MB-231 cells in which RBM3 was silenced decreased significantly, as determined by MTT assay (Fig. 2D). Moreover, the knockdown of RBM3 markedly decreased cell colony formation in both the MCF7 and MDA-MB-231 cells (all P<0.01; Fig. 2E and F). To investigate the role of RBM3 in the metastasis of human breast cancer cells, cell migration assay and invasion assay were carried out. As shown in Fig. 2G-J, both migration and invasion decreased significantly in both the MCF7 and MDA-MB-231 cells in which RBM3 was silenced (all P<0.01). Thus, these findings indicated that the depletion of RBM3 suppressed both the proliferation and metastasis of human breast cancer cells. Therefore, RBM3 plays a promoting role, contributing to the proliferation and metastasis of human breast cancer cells.

ARPC2 is regulated by RBM3 in human breast cancer cells. To identify the downstream mechanisms of action of RBM3 in human breast cancer cells, we selected several candidate genes (including *HER2, MYC, ARPC2, STAT3, PTEN, FOS, CCND1, EGFR, PGR* and *TFF1*) and performed RT-qPCR assay in the MCF7 cells. Among these genes, the mRNA levels of *ARPC2* decreased markedly in the MCF7 si-RBM3#1 and MCF7 si-RBM3#2 cells compared with the MCF7 si-NC cells. However, the mRNA levels of other candidate genes demonstrated no significant changes (Fig. 3A). To confirm the results of RT-qPCR, the protein levels of RBM3 and ARPC2 were examined in the MCF7 and MDA-MB-231 cells. Concordantly, the protein levels of RBM3 decreased markedly following transfection with si-RBM3, and the protein levels of ARPC2 also decreased markedly in both the RBM3-silenced MCF7 and MDA-MB-231 cells (Fig. 3B). Therefore, *ARPC2* was proven to be regulated by RBM3 in human breast cancer cells.

For further analysis regarding the mechanisms of the regulation of *ARPC2* by RBM3, mRNA decay assay, luciferase reporter assay, RNP IP assay and biotin pulldown assay were performed. As shown in Fig. 3C, the mRNA decay rate of *ARPC2* increased markedly in both the RBM3-silenced MCF7 and MDA-MB-231 cells. Moreover, the silencing of RBM3 significantly decreased the level of *ARPC2* 3'UTR luciferase reporter activity in both the MCF7 and MDA-MB-231 cells (Fig. 3D). As shown by RNP IP assay, anti-RBM3 antibody significantly enriched the *ARPC2* mRNA compared with the control IgG group (Fig. 3E). No

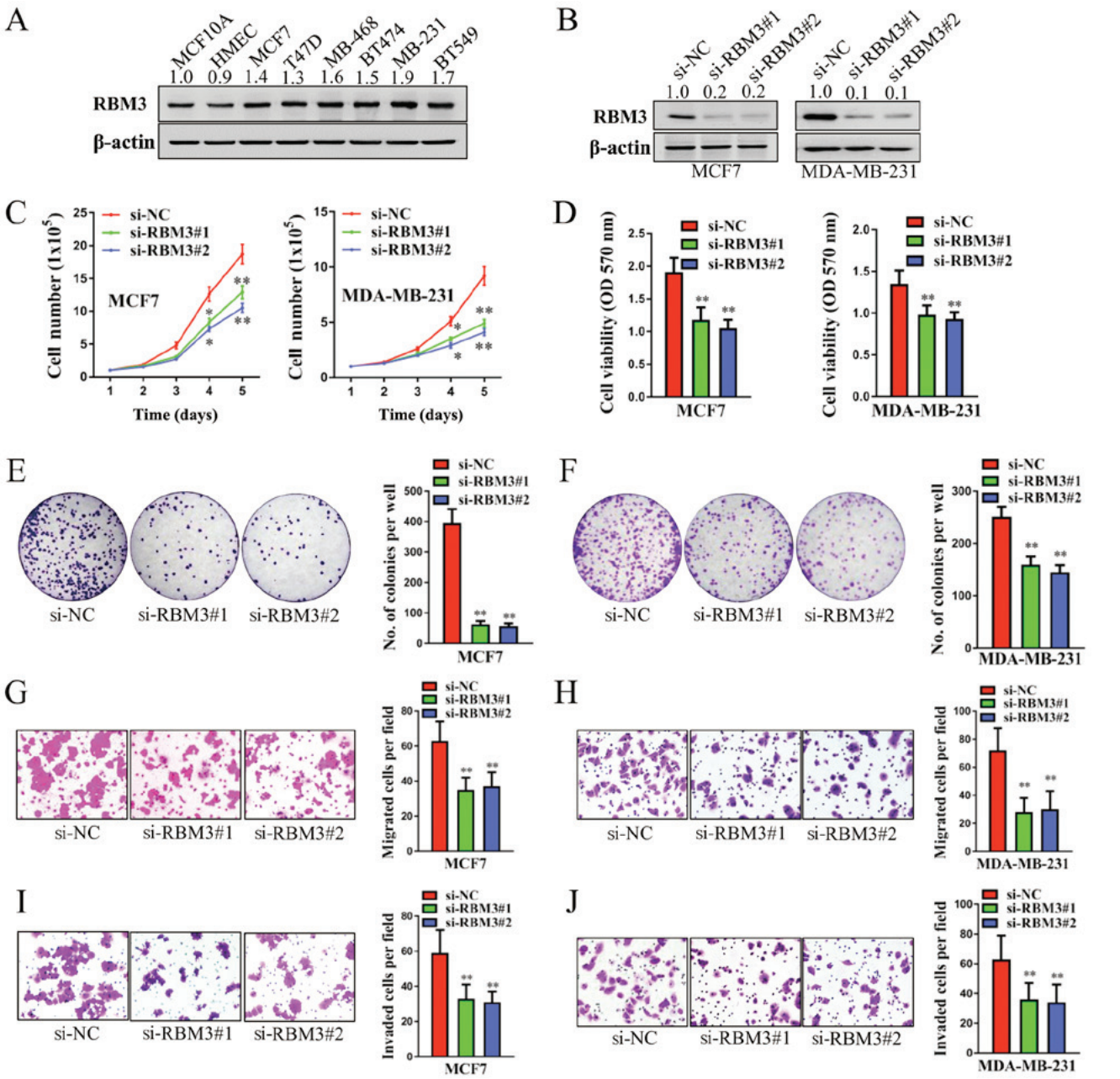

Figure 2. RBM3 promotes the proliferation and metastasis of human breast cancer cells. (A) Protein levels of RBM3 in parental MCF10A, HMEC, MCF7, T47D, MDA-MB-468, BT474, MDA-MB-231 and BT549 cells were examined by western blot analysis. (B) Protein levels of RBM3 in MCF7 and MDA-MB-231 cells following the stable knockdown of RBM3 were detected by western blot analysis. β-actin expression was detected as a control. (C) Cell counting assay ($1.0x10^5$ cells per well were plated into 6-well plates and the total cell number were counted every day for 5 days); (D) MTT assay (2,000 cells per well were plated into 96-well plates and MTT evaluation was performed 96 h later); (E and F) cell colony formation assay (1,000 cells per well were plated into 6-well plates and cell colony formation was calculated 10-15 days later); (G and H) cell migration assay ($1.0x10^5$ cells per well were plated and cell migration was examined 24 h later for MCF7 cells and 12 h later for MDA-MB-231 cells)' and (I and J) cell invasion assay ($1x10^5$ cells per well were plated and cell invasion was examined 48 h later for MCF7 cells and 24 h later for MDA-MB-231 cells) were performed in MCF7 and MDA-MB-231 cells following the stable knockdown of RBM3. *P<0.05 and **P<0.01 compared to the negative control (si-NC) group. RBM3, RNA binding motif protein 3.

significant change was observed in the negative control genes *HER2*. Moreover, no significant differences were observed in the total level of *ARPC2* and *HER2* mRNA between the anti-RBM3 group and IgG group (Fig. 3F). In addition, biotin pulldown assay revealed that RBM3 directly bound to the 3'UTR region of ARPC2, whereas it did not bind to other regions of *ARPC2* mRNA necessarily (Fig. 3G). Thus, *ARPC2* is regulated by RBM2 in a post-transcriptional 3'UTR-binding manner.

ARPC2 promotes the proliferation and metastasis of human breast cancer cells. Following the procedures described above, we continued to examine the protein levels of ARPC2 in human normal breast cells (MCF10A and HMEC) and breast cancer cells (MCF7, T47D, MDA-MB-468, BT474, MDA-MB-231 and BT549). Similar to RBM3, the protein levels of ARPC2 were markedly higher in the 6 breast cancer cells compared with the 2 normal breast cells (Fig. 4A). Transfection with si-*ARPC2*#1 or si-ARPC2#2 markedly decreased the protein

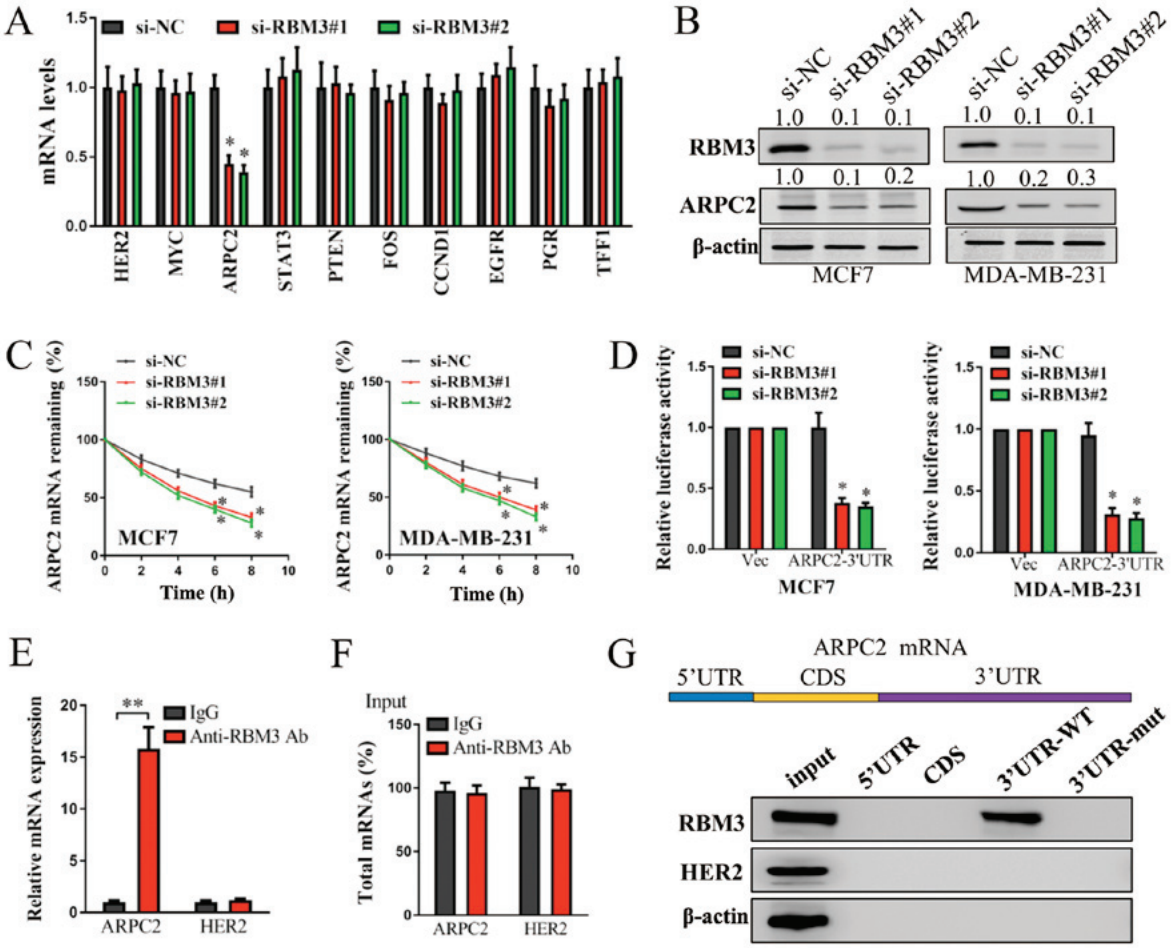

Figure 3. ARPC2 is regulated by RBM3 in human breast cancer cells. (A) mRNA levels of *HER2, MYC, ARPC2, STAT3, PTEN, FOS, CCND1, EGFR, PGR* and *TFF1* were detected in MCF7 cells following the stable knockdown of RBM3 by RT-qPCR. *GAPDH* expression was detected as a control. (B) Protein levels of RBM3 and ARPC2 in MCF7 and MDA-MB-231 cells following the stable knockdown of RBM3 were detected by western blot analysis. β-actin was detected as control. (C) MCF7 and MDA-MB-231 cells following the stable knockdown of RBM3 were treated with actinomycin D (10 μg/ml) for 0, 2, 4, 6 and 8 h, and the mRNA levels of *ARPC2* were examined by RT-qPCR. *GAPDH* expression was detected as a control. (D) Luciferase reporter activities were examined in MCF7 and MDA-MB-231 cells following the stable knockdown of RBM3. (E) Ribonucleoprotein (RNP) immunoprecipitation (IP) assay was carried out in MCF7 cells. mRNA levels of *ARPC2* and negative control genes *HER2* were captured using anti-RBM3 antibody or control IgG and were examined by RT-qPCR. (F) Total input mRNAs of *ARPC2* and negative control genes *HER2* were also examined. (G) Biotinylated RNA pulldown assay was performed in MCF7 cells. Cell lysates were incubated with biotinylated different regions of *ARPC2* mRNA. The interactions between different mRNA regions of *ARPC2* and RBM3 protein were examined by western blot analysis. Protein levels of RBM3, HER2 and β-actin in input cell lysates were examined as controls. *P<0.05 and **P<0.01 compared to the negative control (si-NC) group. RBM3, RNA binding motif protein 3; ARPC2, actin related protein 2/3 complex subunit 2.

levels of ARPC2 in both the MCF7 and MDA-MB-231 cells (Fig. 4B). The silencing of *ARPC2* significantly decreased the total cell number over a period 5 days (Fig. 4C), cell viability (as examined by MTT assay) (Fig. 4D), cell colony formation (Fig. 4E and F), cell migration (Fig. 4G and H) and cell invasion (Fig. 4I and J) in both the MCF7 and MDA-MB-231 cells. Therefore, *ARPC2* also promotes the proliferation and metastasis of human breast cancer cells.

RBM3 promotes the proliferation and metastasis of human breast cancer cells through ARPC2. To determine whether *ARPC2* mediates the promoting effects of RBM3 on the proliferation and metastasis of human breast cancer cells, we re-introduced *ARPC2* without its 3'UTR. The restoration of *ARPC2* in the MCF7-si-NC and MCF7-siRBM3 cells was confirmed by western blot analysis (Fig. 5A). Concordant with the results obtained in our above-mentioned analyses, the enforced expression of ARPC2 significantly increased the total cell number, cell viability (as examined by MTT assay), cell colony formation, cell migration and cell migration in the MCF7 cells (Fig. 5B-F). The silencing of RBM3 markedly decreased the total cell number, cell viability, cell colony formation, cell migration and cell migration in MCF7 cells. However, these decreases were abated by the enforced expression of ARPC2 (Fig. 5B-F). Therefore, ARPC2 mediates the promoting effects of RBM3 on the proliferation and metastasis of human breast cancer cells.

Expression of ARPC2 in tissues from patients with breast cancer and the association of ARPC2 with patient survival rates. For further analysis, the protein levels of ARPC2 in tissues form patients with breast cancer were examined. As shown in Fig. 5G, the breast cancer tissues exhibited higher levels of ARPC2 compared with the adjacent non-tumor tissues, as determined by immunohistochemistry. Moreover, the association between ARPC2 expression and the patient

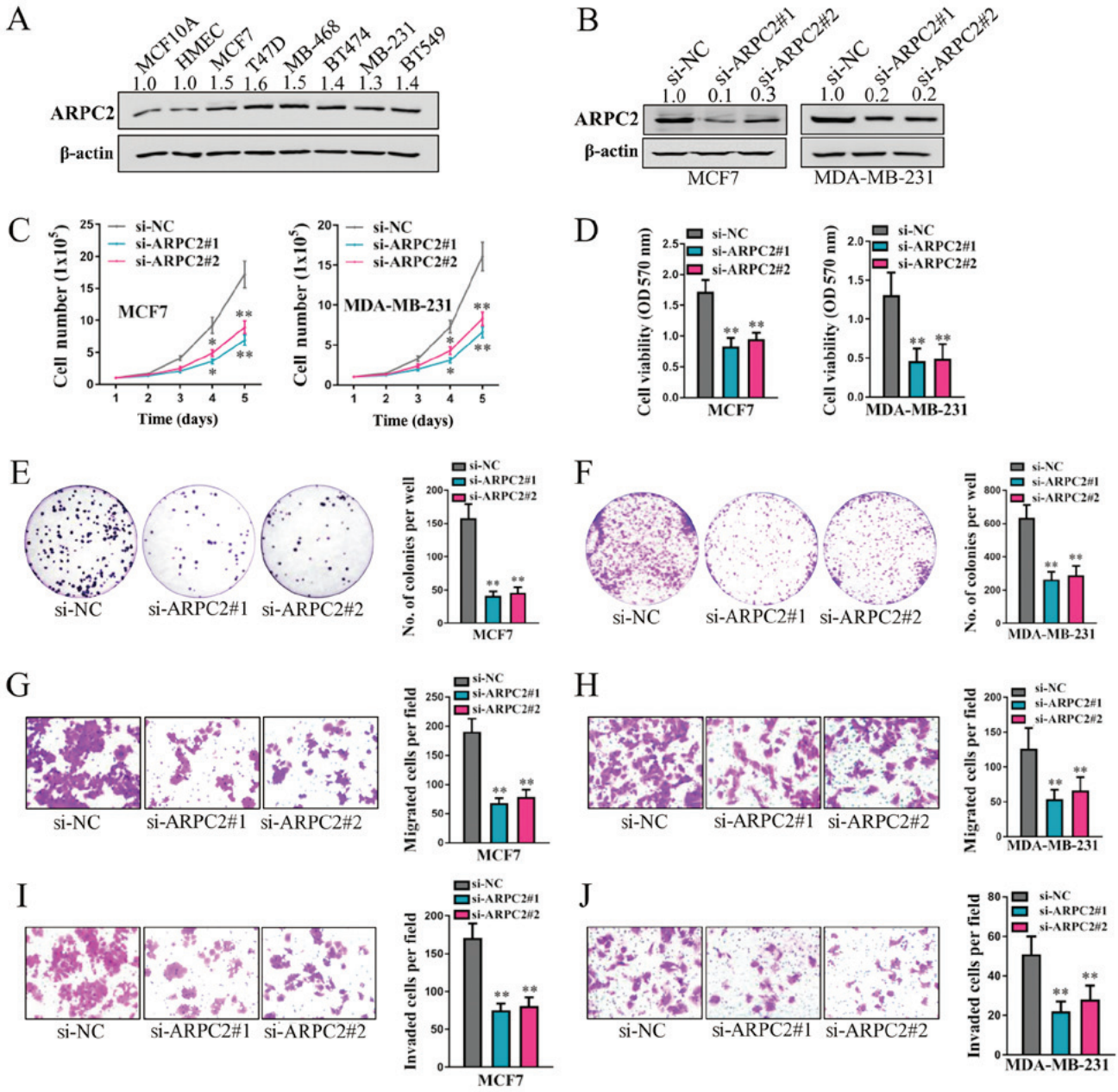

Figure 4. ARPC2 promotes the proliferation and metastasis of human breast cancer cells. (A) Protein levels of ARPC2 in parental MCF10A, HMEC, MCF7, T47D, MDA-MB-468, BT474, MDA-MB-231 and BT549 cells were examined by western blot analysis. (B) Protein levels of ARPC2 in MCF7 and MDA-MB-231 cells following the stable knockdown of ARPC2 were detected by western blot analysis. β-actin expression was detected as a control. (C) Cell counting assay ($1.0x10^5$ cells per well were plated into 6-well plates and cell total number were counted every day for 5 days). (D) MTT assay (2,000 cells per well were plated into 96-well plates and MTT evaluation was performed 96 h later); (E and F) cell colony formation assay (1,000 cells per well were plated into 6-well plates and cell colony formation was calculated 10-15 days later); (G and H) cell migration assay ($1x10^5$ cells per well were plated and cell migration was examined 24 h later for MCF7 cells and 12 h later for MDA-MB-231 cells); and (I and J) cell invasion assay ($1x10^5$ cells per well were plated and cell invasion was examined 48 h later for MCF7 cells and 24 h later for MDA-MB-231 cells) were performed in MCF7 and MDA-MB-231 cells following the stable knockdown of ARPC2. *P<0.05 and **P<0.01 compared to the negative control (si-NC) group. RBM3, RNA binding motif protein 3; ARPC2, actin related protein 2/3 complex subunit 2.

survival rates in the breast cancer patients was also analyzed by the Kaplan-Meier method. The patients with breast cancer with a high level of ARPC2 exhibited both a lower OS rate (P=0.001) and RFS rate (P=0.004) compared with the patients with a low level of ARPC2 (Fig. 5H and I). Hence, the breast cancer tissues overexpressed ARPC2 compared with normal breast tissues, and a high level of ARPC2 was associated with poor prognosis in patients with breast cancer. Moreover, the correlation between RBM3 and ARPC2 expression was analyzed. As shown in Fig. 5J, a positive correlation was observed between the expression levels of RBM3 and ARPC2 in the breast cancer tissues.

Discussion

In this study, we systematically examined the promoting role of RBM3 in human breast cancer cells and tissues. A total of 60 breast cancer tissues and adjacent non-tumor tissues were

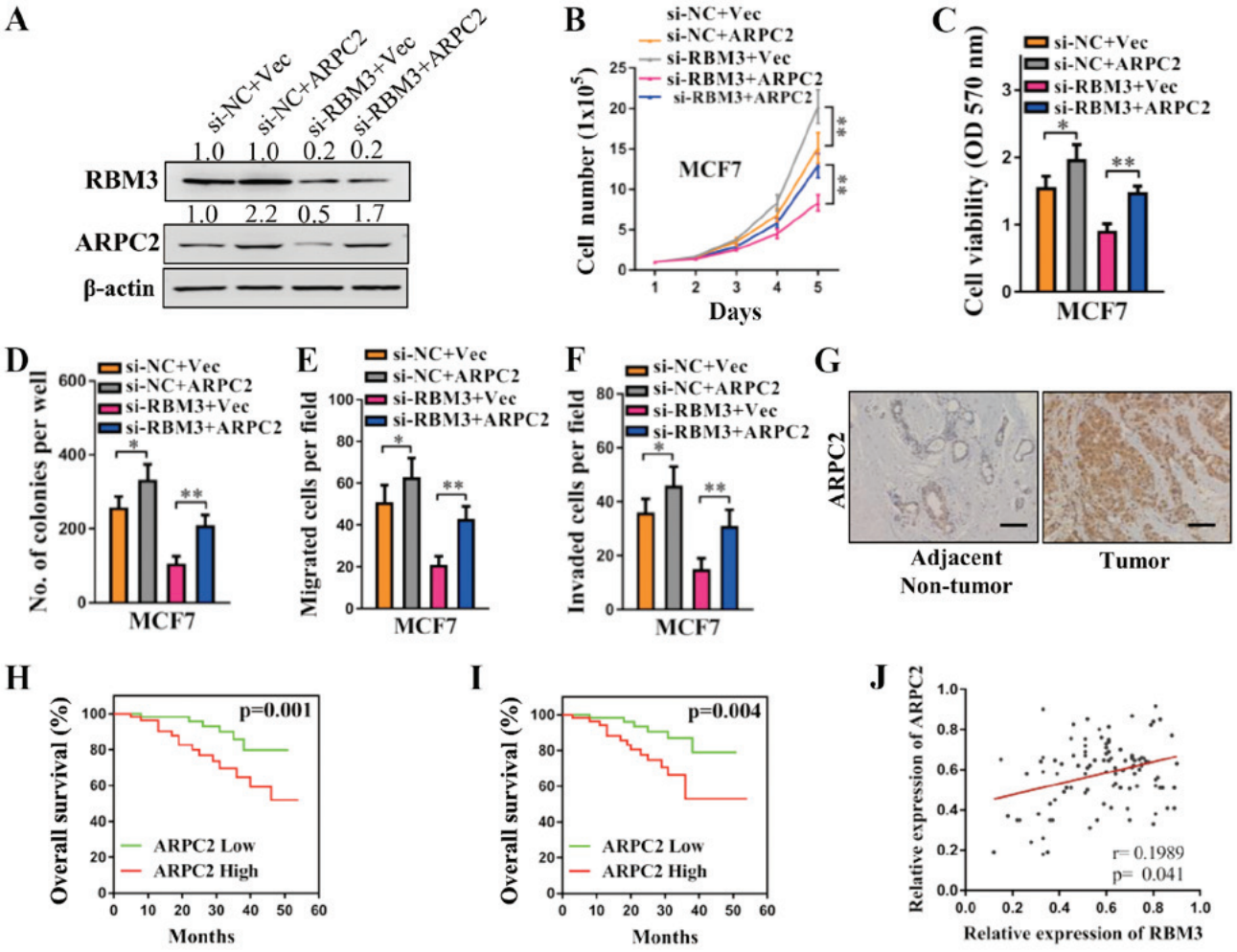

Figure 5. RBM3 promotes the proliferation and metastasis of human breast cancer cells through ARPC2. (A) Protein levels of RBM3 and ARPC2 were examined in MCF7 cells following the stable knockdown of RBM3 and the stable overexpression of ARPC2 by western blot analysis. β-actin expression was detected as a control. (B) Cell total number assay ($1.0x10^5$ cells per well were plated into 6-well plates and cell total number were counted every day for 5 days); (C) MTT assay (2,000 cells per well were plated into 96-well plates and MTT evaluation was performed 96 h later); and (D) cell colony formation assay (1,000 cells per well were plated into 6-well plates and cell colony formation was calculated 10-15 days later) were carried out to evaluate cell proliferation. (E) Cell migration assay ($1x10^5$ cells per well were plated and cell migration was examined 24 h later); and (F) cell invasion assay ($1x10^5$ cells per well were planted and cell invasion was examined 48 h later) were carried out to evaluate cell migration and invasion. (G) Protein levels of ARPC2 in breast cancer tissues and adjacent non-tumor tissues were detected by immunohistochemistry. Representative images at x400 magnification are presented; scale bars, 50 μm. (H and I) According to the immunohistochemistry results, Kaplan-Meier curves were created to analyze the OS and RFS rates between the ARPC2 high expression group (n=56) and the ARPC2 low expression group (n=51) of patients with breast cancer. (J) The correlation between RBM3 and ARPC2 expression was analyzed by Spearman rank correlation analysis. *P<0.05 and **P<0.01. RBM3, RNA binding motif protein 3; ARPC2, actin related protein 2/3 complex subunit 2.

collected, and RBM3 was found to be overexpressed in the tumor tissues compared with the normal tissues. Patients with a high level of RBM3 had markedly poorer OS (P=0.038) and RFS (P=0.031) rates compared with the patients with a low level of RBM3. The expression level of RBM3 was positively associated with patient lymph node metastasis and tumor grade, but exhibited no significant association with patient age, tumor size or tumor stage. Compared with normal breast cells, RBM3 exhibited a higher expression level in breast cancer cells. The silencing of RBM3 with siRNAs markedly decreased cell proliferation (as detected by cell counting assay, MTT assay and cell colony formation assay) and cell metastasis (as detected by cell migration assay and invasion assay) in the MCF7 and MDA-MB-231 cells. Concordantly, it has been documented that RBM3 plays a tumor-promoting role through the prevention of mitotic catastrophe and increasing stem cell behaviors via the regulation of β-catenin in human colorectal cancer cells (17,18). Sakurai *et al* reported that RBM3 promoted the development of colitis-associated cancer (26). Zeng *et al* reported that RBM3 interfering with CD44 variant splicing enhanced the stem-like properties of human prostate cancer cells and acted as a tumor promoter (15). In the study by Karnevi *et al*, RBM3 was found to promote periampullary adenocarcinoma, including pancreatic cancer and to be associated with a poor prognosis of patients (27). However, a high level of RBM3 has also been reported to be associated with an improved survival and the decreased expression of RBM3 has been shown to be associated with tumor progression and a poor prognosis of patients with intestinal-type gastric cancer (28), testicular non-seminomatous germ cell cancer (29), esophageal and gastric adenocarcinoma (20), epithelial ovarian cancer (11),

urothelial bladder cancer (14) and malignant melanoma (8). These studies demonstrate the tissue specificity of RBM3 in different types of human cancer.

In this study, for the downstream pathway analysis, ARPC2 was determined to be positively regulated by RBM3. ARPC2 is a member of actin-related proteins forming the ARP2/3 complex, which contributes to the generation of the branched actin filament network responsible for pushing forward the leading edge of motile eukaryotic cells (30,31). The ARPC2 complex contributes to cell growth, actin nucleation and endocytosis (32). In the present study, we determined that RBM3 positively regulated the expression of ARPC2 by RT-qPCR and western blot analysis. Furthermore, RBM3 was identified to interact with the mRNA of *ARPC2* and to regulate the expression of ARPC2 through a post-transcriptional pathway by using mRNA decay assay and luciferase reporter assay. Moreover, we identified the interacted region between RBM3 protein and *ARPC2* mRNA is the 3'UTR region of *ARPC2* by using RNP-IP RT-PCR assay and biotin pulldown assay. In breast cancer cells, ARPC2 was found to promote both cell proliferation and metastasis. Combinatorial cell functional experiments revealed that ARPC2 mediated the promoting role of RBM3 in human breast cancer cells. As reported previously, the silencing of the ARP2/3 complex decreased the migration of pancreatic cancer cells (33). Zhang *et al* reported that ARPC2 promoted the proliferation and invasion of the human gastric cancer cell line, MKN-28; ARPC2 exhibited a higher expression in gastric cancer tissues than in normal gastric tissues; ARPC2 was significantly associated with a large tumor size, lymph node invasion, and a high tumor stage of gastric cancer patients; ARPC2-positive patients exhibited lower RFS and OS rates compared with ARPC2-negative patients (34). These data all support our present results. In addition, we determined that the protein levels of ARPC2 were markedly higher in breast cancer tissues compared with adjacent non-tumor tissues. Patients with a high level of ARPC2 exhibited markedly poorer OS (P=0.001) and RFS (P=0.004) rates compared with patients with a low level of ARPC2. Therefore, ARPC2 is also an important oncogene in human breast cancer cells. The RBM3-ARPC2 pathway plays an important role in human breast cancer.

In conclusion, this study systematically examined the oncogenic role of RBM3 in human breast cancer cells. A high expression level of RBM3 was found to be associated with worse outcomes of breast cancer patients. In the downstream pathway analysis, RBM3 was found to regulate ARPC2 in a positive manner, which also played an oncogenic role in human breast cancer cells. Furthermore, the regulatory effects were mediated by post-transcriptional 3'UTR binding. In addition, ARPC2 mediated the promoting role of RBM3 in the proliferation and metastasis of breast cancer cells. A high expression level of ARPC2 was also associated with worse survival rates of breast cancer patients. Thus, in this study, it was identified that RBM3 was associated with ARPC2 and were both tumor promoters, and thus may be used as biomarkers for breast cancer therapy. It was also identified that RBM3 correlates with ARPC2 in breast cancer and both promote the tumor progression. The findings of this study may aid in the identification and use of novel biomarkers for breast cancer therapy in the future.

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Availability of data and materials

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

Authors' contributions

PC, XY and HX maintained all of the cell cultures and ran qRT-PCR and western blots. PC designed and performed shRNA experiment. XY and XL helped with Clinical tissue samples collection. PC and ZJ conceived the study and drafted statistical methods. PC and HX wrote the manuscript. ZJ provided funding for the experiments performed in the manuscript. All authors have read and approved the manuscript for publication.

Ethics approval and consent to participate

Experiments related to the use of human tissues were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and local approval (from the Institutional Review Boards of Southeast University) was obtained prior to the commencement of this study. Informed consent form was signed by each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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