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Original Article

MicroRNA-320a inhibits proliferation and invasion of breast cancer cells by targeting RAB11A

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Abstract: MicroRNAs (miRNAs) are deregulated in many types of cancer including breast cancer (BC). miR-320a dysregulation has been associated with different malignancies although its prognostic significance remains unclear. Here, we examined the role of miR-320a in BC and explored the underlying mechanisms. Our results showed that miR-320a was significantly downregulated in BC cell lines and tissues, and its ectopic expression inhibited cell proliferation, migration, and invasion *in vitro* and tumor growth in a mouse xenograft model. We identified Rab11a as a direct target of miR-320a and showed that its expression was upregulated in tumor samples and inversely correlated with the expression of miR-320a. In BC cells, the downregulation of Rab11a through miR-320a was concomitant with the inactivation of Akt. Overexpression of Rab11a abrogated miR-320a-induced inhibition of BC growth and invasion. These results suggest that miR-320a may act as a tumor suppressor in BC through a mechanism involving the modulation of Rab11a expression and the activation of the Akt signaling pathway. miR-320a may therefore serve as a biomarker for BC, and the modulation of its expression may represent a novel therapeutic strategy in BC treatment.

Keywords: miR-320a, RAB11A, breast cancer

Introduction

Breast cancer (BC) is the most common cancer in women and the leading cause of cancer-related death, with an estimated 1.38 million new cases and 458,000 deaths worldwide in 2008 [1]. BC is a heterogeneous disease that has been classified into four subtypes, namely luminal A, luminal B, basal-like, and ErbB2$^+$ [2]. Despite advances in the early detection of BC, approximately 5% to 10% of women diagnosed with BC have metastatic disease on presentation, of which one-fifth survive 5 years [3], underscoring the need to identify biomarkers and targets for this disease.

MicroRNAs (miRNAs) are small (22-nucleotide) noncoding RNAs that regulate gene expression by specific binding to the 3'-untranslated region (3'-UTR) of target mRNAs, modulating expression by translational repression or mRNA destabilization [4]. miRNAs are differentially expressed in various cells and tissues, and are therefore important biomarkers and therapeutic targets in several diseases [5]. Deregulation of miRNAs is associated with various diseases including cancer, and tumor-associated miRNAs can function as tumor suppressors or oncogenes [6]. In BC, many miRNAs have been implicated as oncomiRs, which exert their oncogenic activity by targeting tumor-suppressor genes and activating oncogenic transcription factors, or tumor suppressor miRNAs, which are expressed at low levels in cancer cells and suppress oncogene expression [2]. miR-320a belongs to the miR-320 family, which includes

miR-320a-e and has been shown to play both tumor suppressor and oncogenic roles in different malignancies [7].

Rab proteins are 20-25 kDa proteins that belong to the Ras superfamily of small G-proteins and are regulators of membrane trafficking through their involvement in vesicle formation and budding, motility, tethering and fusion [8]. Rab11a plays a role in the recycling of a wide range of receptors to the cell surface, and is involved in many cellular processes including phagocytosis and cell migration [9]. Previously, Rab11a was shown to be overexpressed in the majority of BC tumors [10], suggesting that Rab11a is important in human BC development.

In the present study, we showed that miR-320a was downregulated in BC cell lines and tissues, and its ectopic expression suppressed cell proliferation and invasion by inducing cell cycle arrest and apoptosis, and inhibited tumor growth in a mouse model. Rab11a was identified as a target gene of miR-320a and shown to mediate its tumor suppressor effect via the modulation of associated proliferative signaling pathways. Our results provide evidence supporting the tumor suppressor role of miR-320a and suggest a potential mechanism underlying its suppressive function in BC.

Materials and methods

Cell culture and tissue samples

Primary BC tissues and adjacent non-tumor tissues were obtained from 30 patients who were treated at Zhongshan Hospital. Written informed consent was obtained from each patient, and the study was approved by the Ethics Committee of Zhongshan Hospital. The human breast epithelial cell line HBL-100 and the BC cell lines MCF-7, MDA-MB-231, BT-474, and T-47D were cultured in RPMI-1640 (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 100 U/ml of penicillin and streptomycin (GIBCO) at 37°C and 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissue samples and cell lines using the miRVANA Kit (Ambion, Carlsbad, MA) according to the manufacturer's instructions. cDNA was synthesized using The SuperScript III reverse transcription kit (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (qPCR) was performed using the SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) on Applied Biosystems 7500 Fast Real-time PCR System. U6 small nuclear RNA or β-actin was used as an internal control. The relative expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method [11].

Lentivirus production and infection

Lentiviral vectors that overexpressed miR-320a were constructed using pCDH-CMV-MCS-EF1-coGFP (pCDH-CMV-miR-320a). A lentiviral vector that expressed GFP alone served as a control. Virus particles were harvested 48 h after co-transfection of pCDH-CMV-miR-320a and the packaging plasmids psPAX2 and pMD into 293T cells using Lipofectamine 2000 (Invitrogen). BC cells were infected with the harvested recombinant lentivirus and maintained in RPMI-1640.

Plasmid construction and transfection

The wild-type (WT) 3'-UTRs of Rab11a containing two putative miR-320a binding sites were isolated by PCR. Mutant (MUT) Rab11a 3'-UTRs were generated by point Mutation using the Quick-Change Site-Directed mutagenesis kit (Stratagene, La Jolla, CA). WT and MUT Rab11a 3'-UTRs were cloned into the pGL3 vector (Promega, Madison, WI). Rab11a coding sequences lacking the 3'-UTR were cloned into the pcDNA 3.1 vector. Plasmid transfections were performed using Lipofectamine 2000.

Cell proliferation, cell cycle, and apoptosis analyses

Cells were seeded into 96-well plates at a density of 5000 cells/well and cultured for the indicated times, followed by incubation with 10% CCK-8 reagent (Beyotime Institute of Biotechnology, Jiangsu, China) at 37°C following the manufacturer's instructions. Results are expressed as absorbance at 490 nm. For assessment of apoptosis, cells were treated as indicated and stained using an Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA) with Annexin V-FITC and propidium iodide (PI) and examined by flow cytometry using a FACSCalibur instrument (Becton Di-

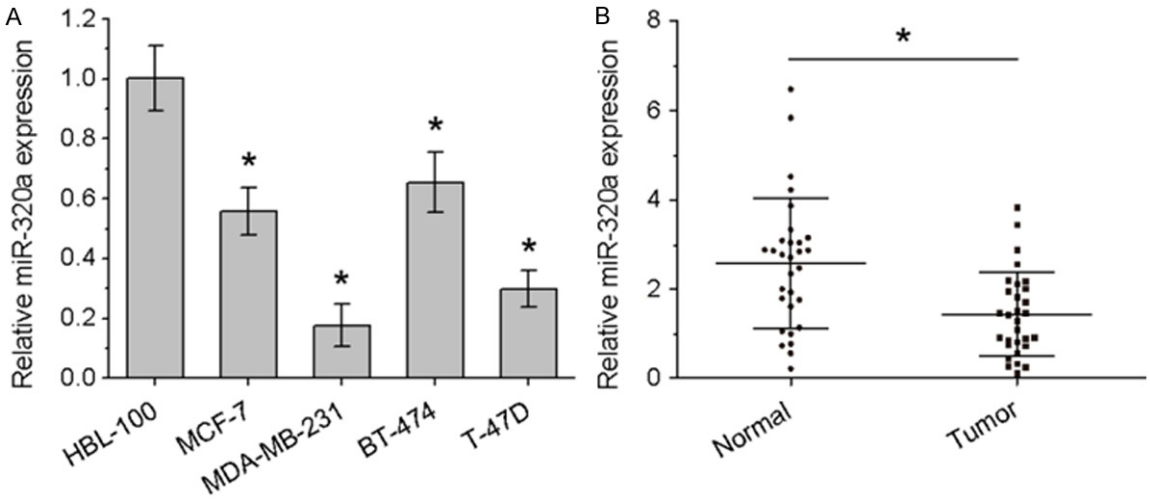

Figure 1. Expression of miR-320a in human breast cancer cell lines and clinical samples. A: miR-320a expression in breast cancer MCF-7, MDA-MB-231, BT-474 and T-47D cells was detected by qPCR. B: qPCR of miR-320a in breast cancer tissues and their adjacent noncancerous tissues. Each sample was repeated three times and normalized to U6. $^{*}P < 0.05$.

ckinson, CA, USA). Untreated cells served as negative controls. To examine cell cycle distribution, cells treated as indicated were collected by trypsinization, fixed in 70% ethanol, washed in PBS, resuspended in 200 ml of PBS containing 1 mg/ml RNase, 0.05% Triton X-100 and 50 mg/ml PI (Sigma, St. Louis, MO), and incubated for 30 min at 37°C in the dark. Immediately after incubation, cells were analyzed using a FACSCalibur instrument.

Caspase activity assay

Cells were treated as indicated, plated into 96-well plates, and caspase 3/7 activity was assayed using the Apo-ONE homogeneous caspase 3/7 assay (Promega) according to the manufacturer's instructions. Briefly, cells were lysed with lysis buffer containing the caspase substrate Z-DEVD-R100 and incubated at room temperature for 1-2 h. Caspase activity was measured with a fluorescence microplate reader at excitation/emission wavelengths of 485/535 nm [12].

Bromodeoxyuridine labeling and immunofluorescence

Cells were grown on coverslips, incubated with bromodeoxyuridine (BrdU, Invitrogen) for 1 h, and labeled with an anti-BrdU antibody according to the manufacturer's instructions. Grayscale images were acquired using a laser scanning microscope (Axioskop 2 plus, Carl Zeiss, Jena Germany).

Transwell migration and invasion assays

Cell invasion was examined using Matrigel-coated BD Falcon 8 μm pore inserts (BD Biosciences, CA, USA). Cells treated as indicated were serum-starved, harvested, resuspended, and added at a density of 1×10^5 cells/well to the upper chamber of Matrigel-coated inserts in 12-well plates and incubated at 37°C for 24 h. The lower chamber contained RPMI-1640 with 10% FBS. Cells on the upper chamber were removed with cotton swabs, and cells invaded to the lower chamber were fixed with paraformaldehyde, counterstained with crystal violet (Sigma), and counted under an inverted microscope. Cell migration was examined using a similar method without Matrigel coating.

Animal models

To establish a xenograft tumor model, MDA-MB-231 cells stably expressing miR-320a or miR-control were subcutaneously injected into the flanks of male BALBc nude mice (n=5 per group, 3-4 weeks of age). Tumors were measured using calipers at 7, 14, 21 and 28 days after implantation and tumor volumes were calculated using the formula volume (mm^3) = ($width^2 \times length$)/2. After 28 days, mice were sacrificed under anesthesia and tumors were

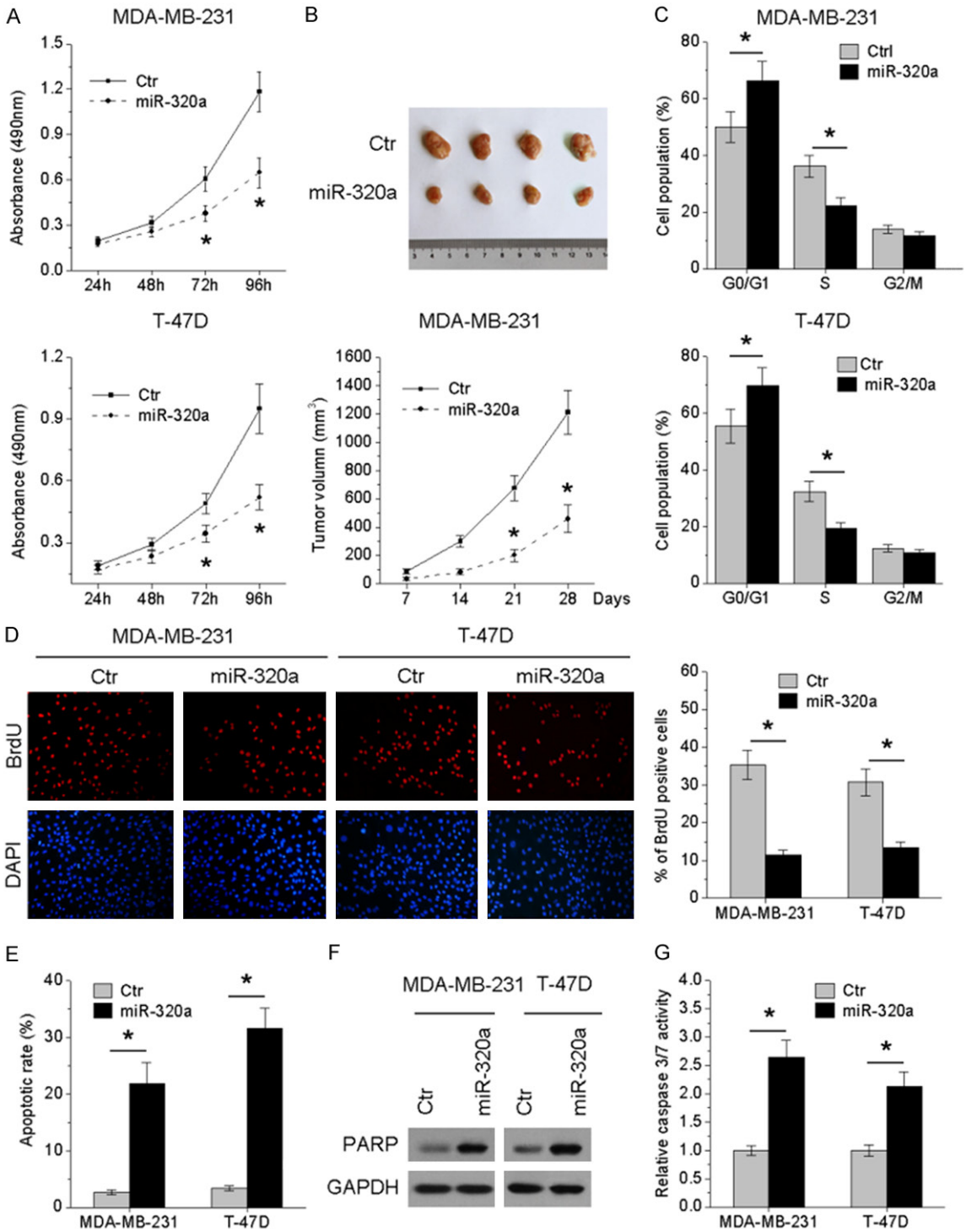

Figure 2. miR-320a suppresses proliferation and tumor growth of breast cancer cells. A: MDA-MB-231 and T-47D cells transduced with lentivirus coding for miR-320a or miR-Ctr were used to perform CCK-8 assays. B: MDA-MB-231 cells stably expressing miR-320a or miR-Ctr were subcutaneously injected into nude mice. Tumor growth was measured on the indicated days. C: Cell cycle distribution of MDA-MB-231- and T-47D-miR-320a cells or controls. D: Representative photos and quantification of BrdU-positive cells. E: Apoptosis assay by flow cytometry. F: Western blot analysis of cleaved PARP in transfected cells. G: Caspase 3/7 activity in transfected cells was validated by caspase activity assay. Experiments were repeated three times. $^{*}P < 0.05$.

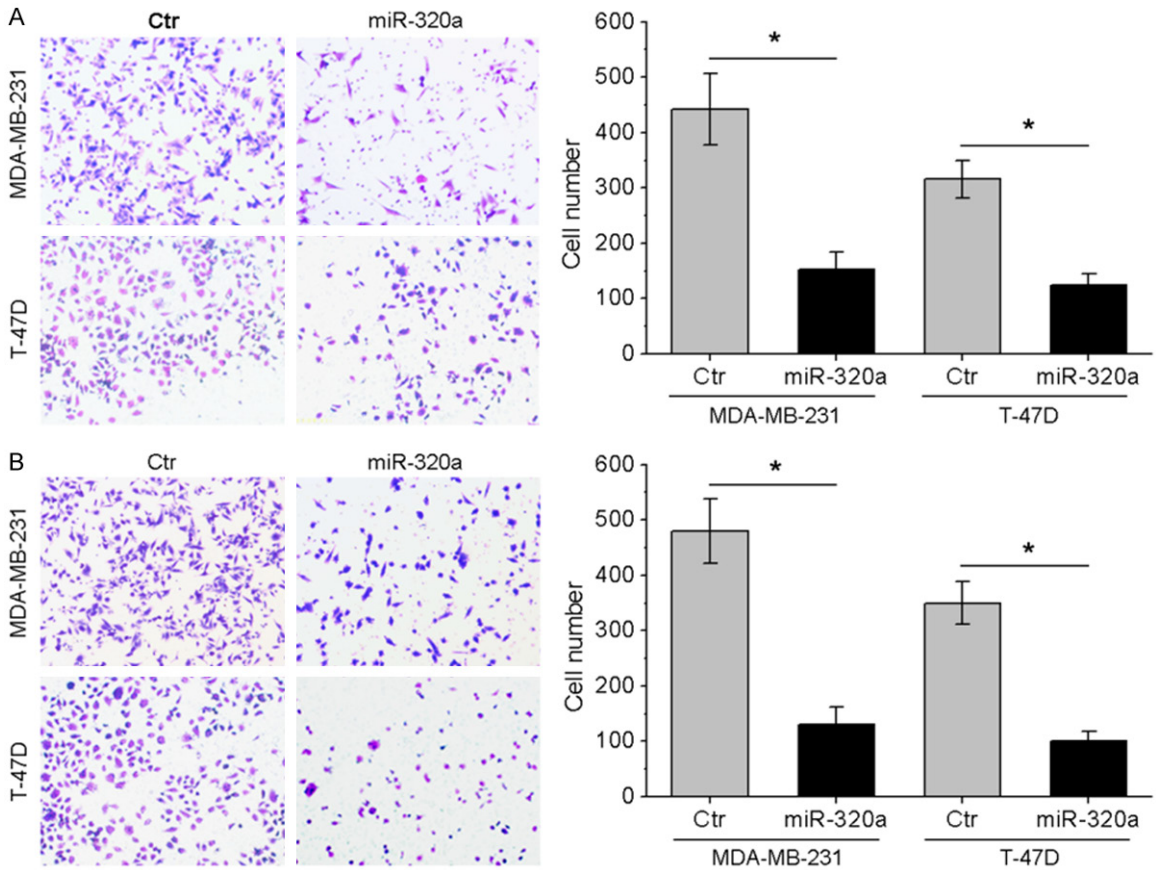

Figure 3. miR-320a represses breast cancer cell migration and invasion. Transwell migration (A) and Matrigel invasion assays (B) were performed to determine breast cancer cell migratory and invasive abilities. Each sample was repeated three times. *P < 0.05.

excised. All animal studies were performed following protocols approved by The Animal Care and Use Committee of Fudan University, China.

Western blot analysis

Protein was extracted, separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). Membranes were blocked with 5% nonfat dry milk in TBST, and incubated with the following primary antibodies: anti-Rab11a antibody (1:900, Abcam, Cambridge, UK), anti-p-AKT (Ser473) (1:1000, Cell Signaling Technology, Danvers, MA), anti-AKT (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were added and bands were visualized using the Supersignal West Pico ECL chemiluminescence kit (Pierce). GAPDH was used as the loading control.

Luciferase assay

WT or MUT Rab11a 3'-UTR reporter constructs and the control pRL-TK vector coding for Renilla luciferase were co-transfected with miR-320a mimics into MDA-MB-231 or T-47D cells using Lipofectamine 2000 (Invitrogen). After 48 h, cells were harvested, lysed, and luciferase activity was assayed using the Dual-Glo luciferase assay kit (Promega). Firefly luciferase values were normalized to Renilla activity, and the relative ratios were reported.

Statistical analysis

SPSS 16.0 software was used for statistical analysis. Data were expressed as the mean ± SD. Comparisons between two independent groups were analyzed by Student's *t*-test. Spearman's correlation analyses were used to identify the correlation between miR-320a and Rab11a. *P* values < 0.05 were considered statistically significant.

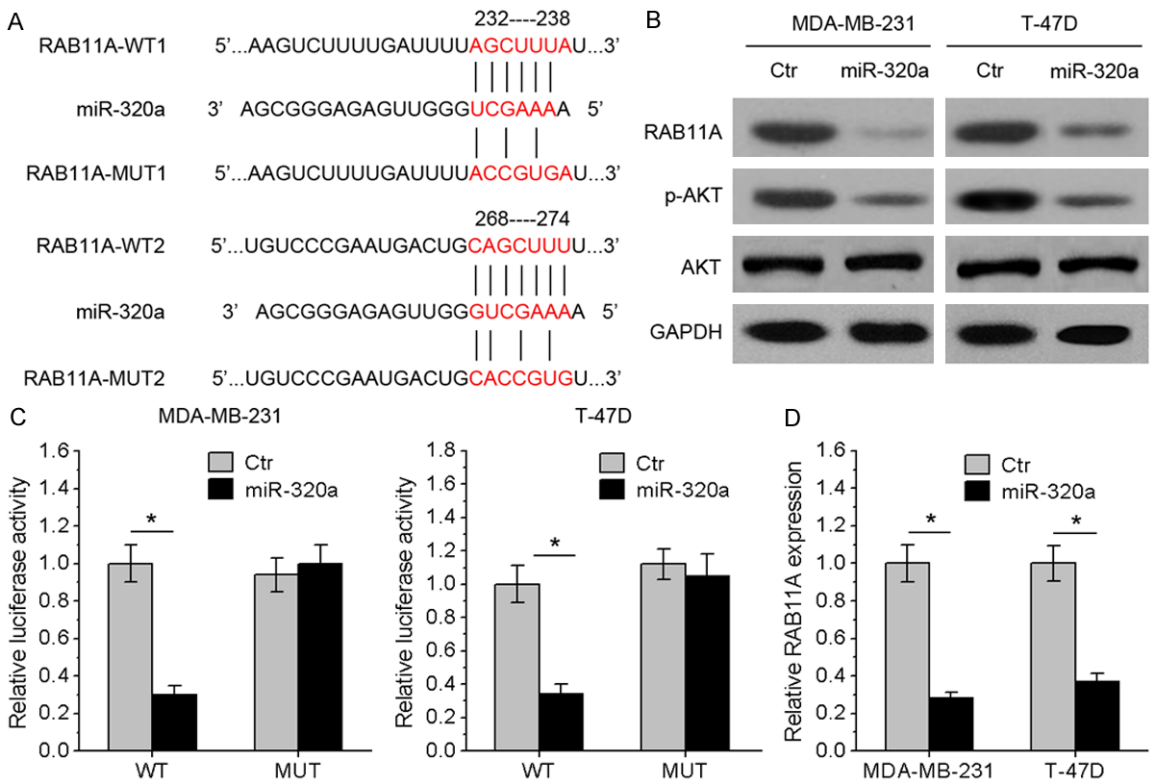

Figure 4. miR-320a directly targeted Rab11a in breast cancer cells. A: Schematic representation of two miR-320a putative binding sites in the 3'-UTR of Rab11a mRNA, and the mutations introduced into the Rab11a 3'-UTR regions. B: Rab11a protein expression and Akt activity were determined by Western blotting. C: Wild-type (WT) or mutated (MUT) Rab11a reporter constructs were cotransfected into MDA-MB-231- and T-47D-miR-320a cells or controls. The relative luciferase activities were measured. D: Downregulation of Rab11a mRNA expression by miR-320a was analyzed using qPCR. Experiments were repeated three times. $^{*}P < 0.05$.

Results

Expression of miR-320a in human BC cell lines and clinical samples

The expression of miR-320a was examined in the BC cell lines MCF-7, MDA-MB-231, BT-474 and T-47D and compared to that in the breast epithelial cell line HBL-100 by qPCR (**Figure 1A**). The results showed that miR-320a expression was significantly down-regulated in BC cells compared to normal breast epithelial cells, with the highest degree of downregulation observed in MDA-MB-231 and T-47D cells. Assessment of miR-320a levels in 30 BC tissue specimens and adjacent noncancerous tissues showed significantly lower levels of miR-320a in tumor than in non-tumor tissues (**Figure 1B**).

miR-320a suppresses breast cancer cell proliferation and tumor growth

To examine the role of miR-320a in BC tumorigenesis, the effect of miR-320a over-expression on cell proliferation and tumor growth was assessed in BC cells and in a mouse xenograft tumor model. Transfection of MDA-MB-231 and T-47D BC cells, which showed the lowest endogenous miR-320a expression, with lentivirus encoding miR-320a, significantly inhibited cell proliferation in a time-dependent manner (**Figure 2A**). In a mouse xenograft model, tumors grew at a significantly slower rate in mice injected with cells overexpressing miR-320a than in those injected with control miR transfected cells (**Figure 2B**). To analyze the mechanism underlying the effect of miR-320a on the inhibition of cell proliferation, MDA-MB-231 and T-47D cell cycle distribution wasexamined, which showed that ectopic expression of miR-320a resulted in an approximately 15% increase in the percentage of cells in the G0/G1 phase of the cycle, concomitant with a decrease in the S phase population in both cell lines, indicating that miR-320a caused cell cycle arrest in the G0/G1 phase (**Figure 2C**). The miR-320a induced inhibition of DNA

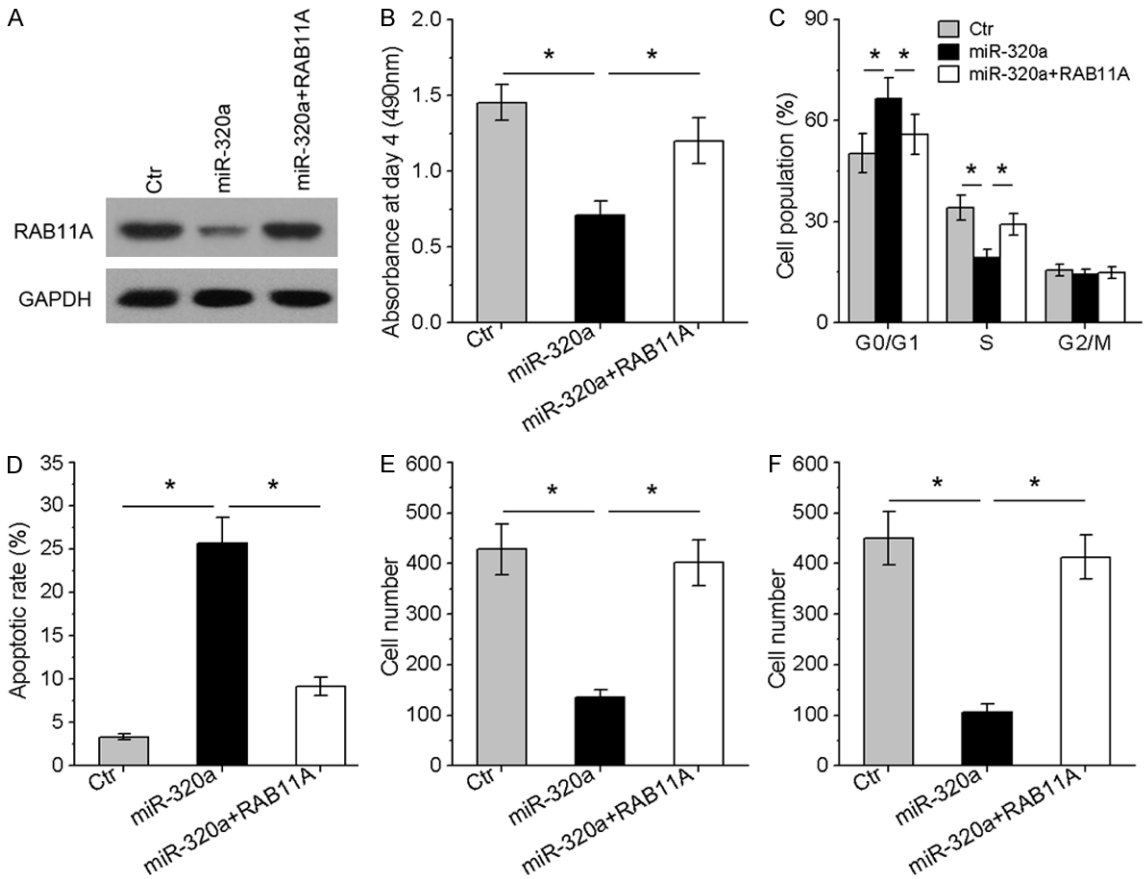

Figure 5. Re-expression of RAB11A abrogates miR-320a-induced inhibition of BC growth and metastasis. (A) Western blot analysis of RAB11A protein expression in MDA-MB-231 cells expressing miR-320a or miR-320a plus RAB11A. Reintroduction of RAB11A reversed the effects of miR-320a on cell viability (B) growth (C) apoptosis (D) migration (E) and invasion (F). Each sample was repeated three times. $^{*}P < 0.05$.

synthesis was analyzed by assessing BrdU incorporation, which showed a 30% and 25% reduction in BrdU-positive cells in MDA-MB-231 and T-47D cells, respectively, in response to miR-320a overexpression (**Figure 2D**). Analysis of apoptosis showed that miR-320a increased the rate of apoptosis by approximately 10- and 14-fold in MDA-MB-231 and T-47D cells, respectively (**Figure 2E**). Western blot analysis showed that miR-320a increased the levels of cleaved PARP and caused approximately 2-3-fold increases in caspase 3/7 activity in both cell lines, consistent with the induction of apoptosis (**Figure 2F** and **2G**). Taken together, these results indicate that miR-320a inhibits BC cell proliferation by inducing apoptosis and cell cycle arrest.

miR-320a represses breast cancer cell migration and invasion

Transwell migration and invasion assays showed that ectopic expression of miR-320a significantly decreased the number of migrating and invading cells in MDA-MB-231 and T-47D cells, causing an approximately 3-fold decrease in migration and 4.5-5-fold decrease in invasive ability (**Figure 3A** and **3B**).

miR-320a directly targets RAB11A in breast cancer cells

Bioinformatics databases (TargetScan and miRanda) identified Rab11a as a potential target of miR-320a, and two putative binding sites for miR-320a in the 3'-UTR of Rab11a were detected (**Figure 4A**). Based on recent evidence showing that Rab11a is necessary for Akt activation in BC [13], we examined the effect of miR-320a overexpression on the levels of Rab11a and Akt activation by western blotting. The results showed that miR-320a over-expression downregulated Rab11a and phospho-Akt in MDA-MB-231 and T-47D cells (**Figure 4B**). To investigate the possible interaction between miR-320a and Rab11a, mutations were intro-

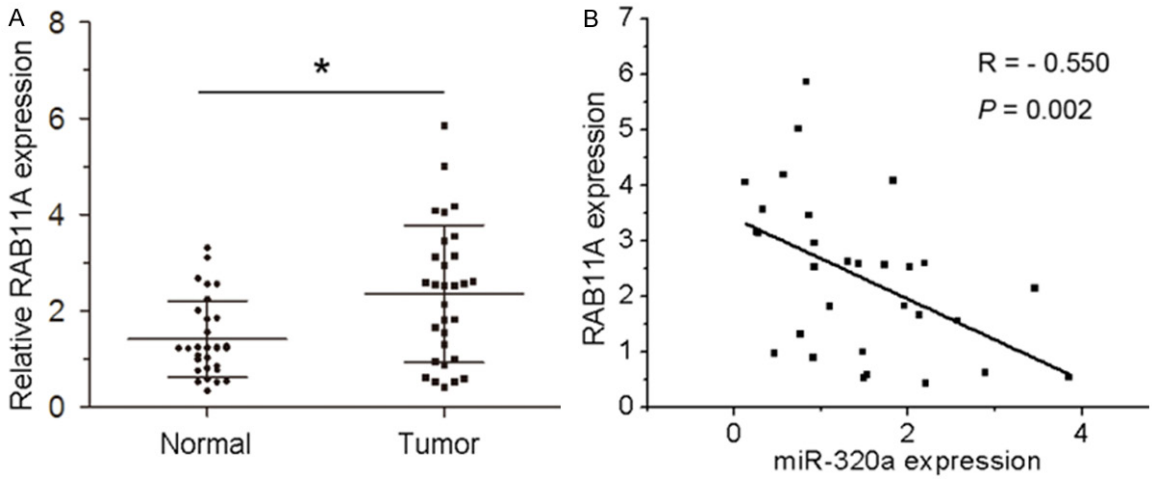

Figure 6. Correlation between miR-320a and RAB11A expression in BC tissues. A: qPCR analysis of miR-320a expression in 30 pairs of BC tissues and adjacent normal tissues. B: Spearman's correlation scatter plot of miR-320a and RAB11A expression in BC tissues. Each sample was repeated three times. *P < 0.05.

duced into two putative miR-320a binding sites in the Rab11a 3'-UTR, and luciferase reporter constructs generated with the WT and MUT 3'-UTRs of Rab11a were co-transfected into MDA-MB-231 and T-47D cells with the miR-320a mimics or miR-ctr vectors. Luciferase assays showed that ectopic expression of miR-320a significantly decreased the luciferase activity of the WT but not that of the MUT Rab11a 3'-UTR in both cell lines (**Figure 4C**). Moreover, qPCR analysis showed that ectopic expression of miR-320a significantly downregulated Rab11a expression in both cell lines (**Figure 4D**). Collectively, these results suggest that Rab11a is a target of miR-320a.

Re-expression of RAB11A abrogates miR-320a-induced inhibition of BC growth and metastasis

To determine whether the effects of miR-320a on BC cell growth and invasion were mediated by its interaction with and modulation of Rab11a, MDA-MB-231 cells were transfected with lentivirus encoding miR-320a in the presence or absence of a Rab11a overexpressing vector. The results of western blotting showed that Rab11a overexpression restored its levels downregulated by miR-320a (**Figure 5A**). Further analyses showed that Rab11a overexpression significantly restored cell viability, migration and invasion inhibited by miR-320a (**Figure 5B**, **5E** and **5F**). Rab11a also significantly reversed the miR-320a induced cell cycle arrest at G0/G1 and apoptosis (**Figure 5C** and **5D**). Taken together, these results indicate that the effects of miR-320a in BC cells are mediated by its modulation of Rab11a expression.

Correlation between miR-320a and RAB11A expression in BC tissues

Rab11a expression level in in 30 paired tumor and adjacent non-tumor tissues was examined by qPCR. The results showed that Rab11a expression was significantly higher in tumor tissues than that in adjacent non-tumor tissues (**Figure 6A**). Correlation analysis of the expression of miR-320a and Rab11a in the same BC tissue specimens showed a significant inverse correlation between miR-320a and Rab11a (**Figure 6B**).

Discussion

The critical role of miRNAs in tumorigenesis via the modulation of various targets has promoted intensive research into miRNAs as biomarkers and miRNA-based therapeutic strategies for the treatment of cancer [14]. In BC, several aberrantly expressed miRNAs have been identified and correlated with specific pathological features. Oncogenic and pro-metastatic miRNAs including miRs-10b, -21, -155, -373 and -520c and tumor- and metastasis-suppressive miRNAs such as miRs-125b, -205, -17-92, -206, -200, -146b, -126, -335, and -31 have been

described [2], and different miRNA signatures have been reported as biomarkers for early diagnosis, as predictors of response to treatment, and as prognostic indicators in BC [15, 16]. In the present study, we examined the role of miR-320a in BC and explored the underlying mechanisms. Our results showed that miR-320a is downregulated in BC cell lines and tissues, suggesting its potential as a biomarker for the detection of BC.

The dysregulation of miR-320a in cancer was reported previously, and several putative targets have been identified. miR-320a is associated with metastasis in colorectal cancer and suppresses cell migration and invasion [17, 18]. miR-320a is downregulated in nasopharyngeal carcinoma, and its overexpression suppresses cell growth and migration *in vitro* and *in vivo*, suggesting its tumor suppressor role [19]. However, in hepatocellular carcinoma, miR-320a was shown to have an oncogenic role by downregulating the tumor suppressor GNAI1, suggesting that the function of miR-320a is context dependent. miR-320a was suggested as a prognostic biomarker for invasive BC and shown to play a role in chemoresistance through the targeting of two factors essential for chemoresistance, including transient receptor potential channel C5 (TRPC5) and nuclear factor of activated T-cells isoform C3 (NFATC3) [7, 20]. The therapeutic potential of miR-320a was suggested in a recent study in which miR-320a sensitized tamoxifen-resistant BC cells by targeting cAMP-regulated phosphoprotein and estrogen-related receptor gamma and their downstream effectors c-Myc and cyclin D1 [21]. In the present study, ectopic expression of miR-320a inhibited cell proliferation, invasion and migration, induced apoptosis, and promoted cell cycle arrest at G0/G1 phase *in vitro*, and suppressed tumor growth *in vivo*, supporting a tumor suppressor role for miR-320a in BC.

We identified Rab11a as a direct target of miR-320a in BC cells, and showed that the tumor suppressor activity of miR-320a is mediated by the modulation of Rab11a expression. The Rab11 family, which includes Rab11a, Rab11b and Rab25, has been implicated in a number of disorders, many of which are associated with the hijacking of the membrane trafficking machinery by intracellular pathogens and viruses [9]. However, Rab proteins have also been implicated in cancer. Rab25 is overexpressed in 67-80% of breast and ovarian cancers and has been shown to determine the aggressiveness of these malignancies [22]. The involvement of the Rab11 family in cancer is mediated by Rab11-family interacting proteins (Rab11-FIPs), which modulate Rab11a dependent vesicle recycling [23]. Rab11-FIP1C, also known as Rab-coupling protein (RCP), plays a role in endocytic sorting of the epidermal growth factor receptor EGFR [24]. The RCP/FIP1C gene is located in a chromosomal region that is frequently amplified in BC [25]. RCP is critical for invasive cancer cell migration through interaction with α5β1, a cell-surface integrin, and RCP expression is increased in invasive breast tumors [26, 27]. These results indicate that Rab11a is involved in breast tumorigenesis and support our findings implicating Rab11a in tumorigenesis as a target of miR-320a. Furthermore, we showed that overexpression of miR-320a suppressed the activation of Akt concomitant with the downregulation of Rab11a. Rab11a was previously shown to be necessary for Akt activation by the phosphatidylinositol 4-kinase PI4KIIIβ in BC, suggesting that the oncogenic role of PI4KIIIβ is mediated by Rab11a [13]. These results support our current findings and suggest a potential mechanism for the tumor suppressor role of miR-320a mediated by the downregulation of Rab11a and the inhibition of phospho-Akt expression.

In conclusion, the present study showed that miR-320a is downregulated in BC cells and tissues, and its inhibitory effects on cell proliferation, migration, invasion, and tumor growth are mediated by the down-regulation of its target Rab11a via a mechanism involving the inactivation of Akt signaling. The present results elucidate a potential mechanism underlying the tumor-suppressor role of miR-320a, and indicate that miR-320a could be a useful marker and potential therapeutic target in BC.

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Disclosure of conflict of interest

None.

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