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LncRNA APPAT regulated miR-328a/Pkp1 signal pathway to participate in breast cancer

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Abstract. – OBJECTIVE: Recent studies indicated long non-coding RNA (lncRNA) is involved in the development of breast cancer, which is the pathological basis of breast cancer. Here, we reported the molecular mechanisms by which lncRNA APPAT regulated in the progression of breast cancer.

MATERIALS AND METHODS: QPCR was used to inspect the expression of lncRNA APPAT and miR-328a in breast cancer cell lines. Luciferase reporter assay confirmed the direct target effect of APPAT and miR-328a. Western blot was used to check Pkp1 protein expression in breast cancer cell lines.

RESULTS: The expressions of lncRNA APPAT, Pkp1 protein levels and miR-328a were commonly expressed in breast cancer cells. The inhibition of lncRNA APPAT expression repressed cell proliferation, migration and invasion in breast cancer and reverse results were found after lncRNA APPAT overexpressing. Mechanistically, the binding targets of lncRNA APPAT *vs.* miR-328a and Pkp1 *vs.* miR-328a were checked in breast cancer. Meanwhile, miR-328a silencing enhanced the proliferation, migration and invasion of breast cancer cells. Moreover, the effect caused by Pkp1 silencing on cell proliferation, migration and invasion was reversed by miR-328a inhibitor in MCF-7 and BT594 cells. Additionally, Pkp1 knockout reversed the effect of cell proliferation, migration and invasion triggered by APPAT elevated. Taken together, these results showed miR-328a as a downstream target of lncRNA APPAT linking lncRNA APPAT to Pkp1.

CONCLUSIONS: LncRNA APPAT regulated the proliferation, migration, invasion of breast cancer by regulating miR-328a/Pkp1 signaling pathway, providing a novel possible strategy for the treatment of breast cancer.

Key Words:
Pkp1, Breast cancer, MiR-328a, APPAT, Proliferation, Migration, Invasion.

Introduction

Breast cancer is the most common diagnostic cancer among women all over the world[1,2]. Migration and invasion play an important role in the treatment of breast cancer[3,4]. They can not only provide local control, reduce local recurrence of tumor, but also reduce the mortality of patients[5]. However, cell migration and invasion are the main reasons for failure of breast cancer treatment. Therefore, the study of the molecular mechanism of breast cancer metastasis and invasion will help to find more effective treatment strategies.

Noncoding RNA is a kind of widely existing genes involving many biological functions[6-12]. LncRNA plays an important role in the biological process of tumor[13-16] proliferation, invasion, migratory and metastasis[17-20]. So, DSCAM-AS1 mediated tumor progression and tamoxifen resistance by interacting with mechanism proteins, which provides clues for further understanding of the potential clinical significance of lncRNA in breast cancer. Tetila interferes with the healing process of diabetic skin wound mainly through genome homing signal[21]. Findings emphasize the importance of lncRNA in the detection of tumor mediated T cell acid, suggesting that engineering lncRNA in adoptive T cells may provide a new anti-tumor immunotherapy[11]. Furthermore, linc00052 can inhibit Stat3 to repress the metastasis and invasion of cervical cancer cells, which means that linc00052 may be a new type of cervical cancer suppressor[19]. LNC CRCMSL can stabilize the anti-metastasis effect of HMGB2 by interacting with lncRNA protein in the cytoplasm, indicating that targeting LNC CRCMSL may be an opportunity to treat metastatic CRC[18].

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In addition, Casc19 sponge miRNA-130b-3p regulated ZBR2 as a ceRNA, accelerating the progress of NSCLC via regulating the proliferation, migration and invasion of tumor cells[16]. In the present study, we identified lncRNA APPAT as an upstream target of miR-328a and was required for migratory and invasion in breast cancer cells, which provided a new perspective for the treatment of breast cancer.

Materials and Methods

Cell Culture and Treatment

MCF-10A cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1) medium supplemented with 5% horse serum (HyClone, South Logan, UT, USA), 100 ng/ml cholera toxin (Sigma-Aldrich, St. Louis, MO, USA), 20 ng/ml epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA). MCF-7, SKBR3 and T47D cells were preserved in DMEM containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). All other cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% FBS.

Lentiviral Construction and Transduction

Gene Pharmaceutical Company (Shanghai, China) constructed the expression vector of lncRNA APPAT lentivirus. To produce a stable and highly expressed clone of lncRNA APPAT, cells were infected by a lentivirus vector expressing lncRNA APPAT or an empty lentivirus vector. Sh-lncRNA APPAT lentivirus vector was constructed by gene Chemistry (Shanghai, China) and used to inhibit the expression of lncRNA APPAT. Negative control lentivirus vector containing non silent short hairpin RNA (shRNA) was used.

Transfection

Gene Pharma (Shanghai) Co., Ltd. designed and synthesized Pkp1-si, miR-328a-mimics, miR-328a inhibitors and corresponding negative controls. According to the instructions, breast cells were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 4 hours and then replaced with normal medium. After 48 hours of treatment, the transfection efficiency was detected by QPCR or Western blot.

Luciferase Reporter Assay

HEK293T cells were co-transfected with 50 ng miR-328a or 90ng APPAT (Pkp1) 3'-UTR-WT or APPAT (Pkp1) 3'-UTR-mut control plasmids by liposome 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of treatment, the cells were collected and tested according to the instructions of Dual-Luciferase assay kit (Promega, Madison, WI, USA). MiR-328a, control, plasmid, Luciferase structure, APPAT (Pkp1) 3'- UTR or 3' -UTR-mut were purchased from Gene Pharmacy Co., Ltd. (Shanghai, China).

RNA Pull-Down

Biotinylated miRNA-328a or antisense RNA was transcribed *in vitro* with biotin RNA marker mixture (Roche Diagnostics, Basel, Switzerland and T7 RNA polymerase), treated with RNase free DNase I (Roche Diagnostics, Basel, Switzerland) and purified with Rnaeasy small Kit (Qiagen, Hilden, Germany). One milligram of protein was then mixed with 40 pmol of biotinylated RNA and incubated at room temperature. The proteins were separated by twelve alkyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver. The specific bands were excised and analyzed.

RNA Immunoprecipitation

RIP experiments were conducted according to the manufacturer's Magna RIP RNA binding protein immunoprecipitation Kit (Millipore, Billerica, MA, USA). The cells were dissolved with radioimmunoprecipitation assay RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After incubation with primary antibody or anti rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) for 30 minutes, the lysates were immunoprecipitated by magnetic beads and rotated overnight at 4°C. RNA was purified from the magnetic bead binding RNA protein complex and analyzed by QPCR.

Cell Counting Kit-8 Assays

The transfected breast cancer cells were inoculated into 96 well plate medium. Then, according to the reagent instructions, 10 μL CCK-8 (CCK-8, Ck04, Dojindo Molecular Technologies, Kumamoto, Japan) solution was added into 96 well plate and cultured for 3 hours. Then, the absorbance was measured at 450 nm with a micro reader (9200, BioRad Laboratories, Hercules, CA, USA).

Transwell Assay

Breast cancer cells from 200 μl serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) were inoculated into the upper cavity, and the medium containing 10% fetal bovine serum (FBS) was added to the lower cavity (BD Biosciences, Franklin Lakes, NJ, USA). After incubation at 37°C for 48 hours, the cells on the upper side of the membrane were removed with cotton swabs. The cells fixed on the lower surface were fixed with 100% precooled methanol and counted with 0.1% crystal violet solution for counting.

Isolation of RNA and Real Time PCR

RNA was extracted from the samples with TRIzol reagent (Cwbio, Beijing, China). DNA was synthesized according to the instructions of PrimeScript RT Kit (perfect real time) (TaKaRa, Otsu, Shiga, Japan). PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). GAPDH expressed as internal control. Relevant primers were listed in Table I.

Western Blot Analysis

The 10% SDS-PAGE gel was separated from the cells and transferred to the immobilon (polyvinylidene difluoride) PVDF membrane (Merck KGaA, Darmstadt, Germany). The membrane was sealed with 5% skimmed milk and then shaken overnight at 4°C with primary antibodies against Pkp1 (1:1000, Cell Signaling Technology, Danvers, MA, USA) and β-actin (1:1000, Cell Signaling Technology, Danvers, MA, USA). The membrane was incubated with the second antibody binding to horseradish peroxidase (HRP; Goat anti rabbit IgG, Proteintech, Rosemont, IL, USA). Next, it was prepared by enhanced chemiluminescence (ECL) detection kit (Cwbio, China, Beijing), and the results were detected by gel imaging system.

Statistical Analysis

The results were showed as mean±SD. All statistical analyses were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Comparisons among three groups were performed by the one-way analysis of variance (ANOVA) followed by the Tukey's test. $p < 0.05$ was considered to have statistical significance.

Results

LncRNA APPAT Upregulation Distinctly Promoted Breast Cancer Cells Proliferation, Migration and Invasion

To investigate the potential involvement of lncRNA APPAT in breast cancer cells, we determined the lncRNA APPAT expression level in several breast cancer cell lines. Consistent with our prediction, lncRNA APPAT was commonly expressed in breast cancer cells (Figure 1A). Meanwhile, overexpression of lncRNA APPAT sharply increased cell survival (Figure 1B-1C), migration (Figure 1D) and invasion (Figure 1E) in MDA-MB-231 and BT549. Conversely, downregulation of lncRNA APPAT (Figure 1F) reduced cell survival (Figure 1G-1H), migration (Figure 1I) and invasion (Figure 1J) in MCF-7 and BT474 cells. All in all, these results showed that overexpression of lncRNA APPAT significantly increased breast cancer cells proliferation, migration and invasion.

Table I. Sequences used for QPCR.

Name	Sequences
APPAT-F	5'-GCAGAGGCAGGTCACCAAC-3'
APPAT-R	5'-CAGGATGATTCGAGACCAGGA-3'
miR-328a sense	5'-ACGGAAGGGCAGAGAGGGCCAG-3'
miR-328a anti-sense	5'-TGCCAGAAGGAGCACTTAGG-3'
U6 sense	5'-CTCGCT TCGGCAGCACA-3'
U6 anti-sense	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH-F	5'-GATTCCACCCATGHCCAAATTC-3'
GAPDH-R	5'-CTGGAAGATGGTGATGGGATT-3'
Pkp1-F	5'-TTAGTGTTTTATATAGGGGATTTGT-3'
Pkp1-R	5'-ACTCCCTACAACTACTCCTAACACT-3'

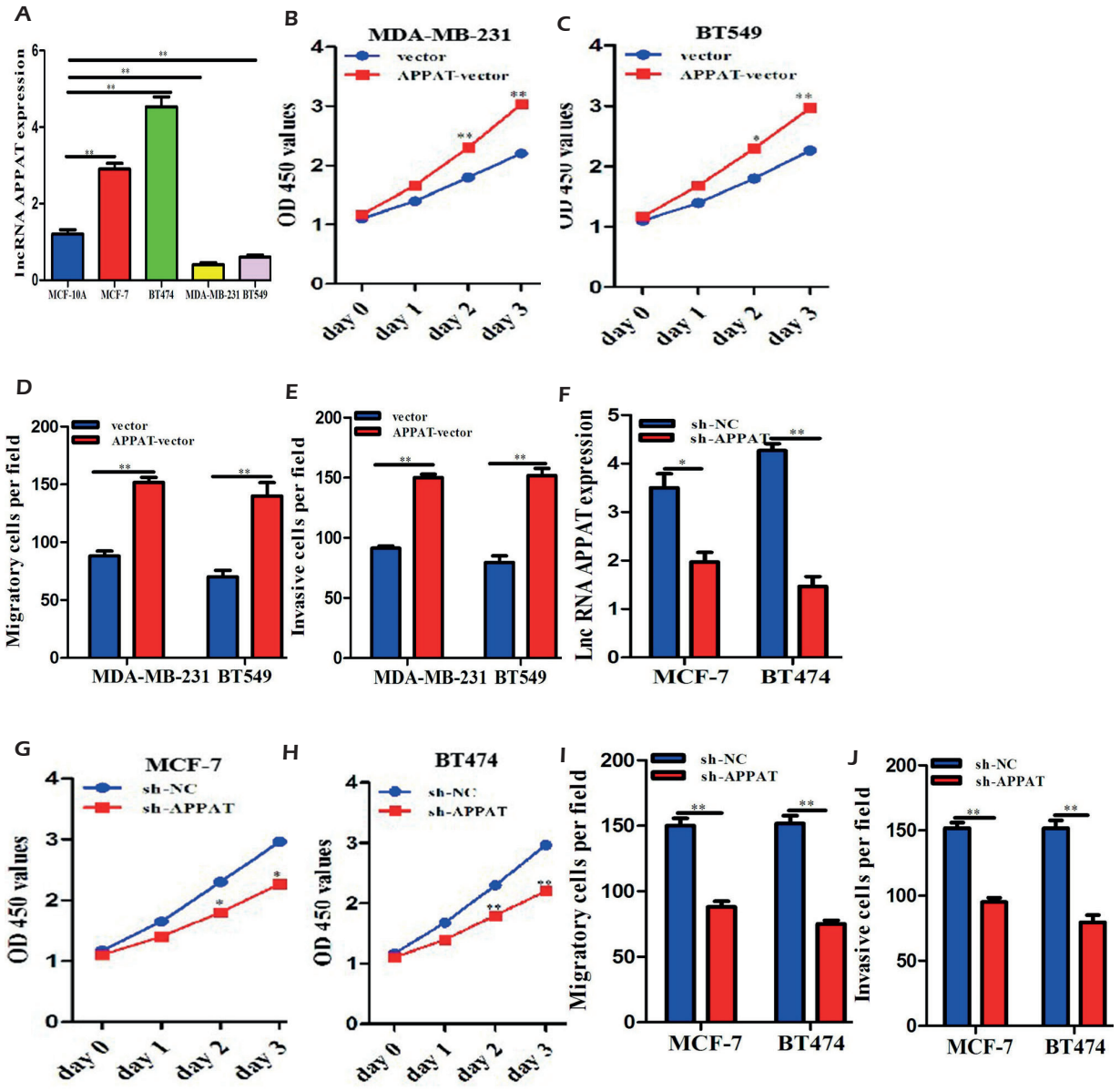

Figure 1. LncRNA APPAT upregulation distinctly promoted breast cancer cells proliferation, migration and invasion. **A**, The expression of lncRNA APPAT was detected by QPCR. **B**, The expression of lncRNA APPAT in MDA-MB-231 and BT549 cells was detected by QPCR. **C-D**, The cell proliferation on MDA-MB-231 and BT549 cells after infection with lentivirus expressing lncRNA APPAT was measured by CCK-8 assay. **E**, The cell migration of MDA-MB-231 and BT549 cells after infection with lentivirus expressing lncRNA APPAT was measured using transwell assay. **F**, The cell invasion of MDA-MB-231 and BT549 cells after infection with lentivirus expressing lncRNA APPAT was measured by transwell assay. **G**, The expression of lncRNA APPAT in MCF-7 and BT474 cells was detected by QPCR. **H-I**, The cell proliferation on MCF-7 and BT474 cells after transfection with sh-lncRNA APPAT was measured by CCK-8 assay. **J**, The cell migration and invasion of MCF-7 and BT474 cells after infection with transfection with sh-lncRNA APPAT was measured using transwell assay. The data are expressed as mean ± SD. n=3. *p <0.05.

MiR-328a Was a Direct Target of LncRNA APPAT

To investigate miRNA mechanism regulated by APPAT in the occurrence and development of breast cancer, we found that miR-328a was a possible candidate target combining predictive analysis and its expression level in several breast cancer cell lines was tested (Figure 2A). We further confirmed that miR-328a expression was higher in MCF-7, BT474 with low

APPAT levels (Figure 2B) and lower expression cell lines in MDA-MB-231, BT549 with high APPAT levels (Figure 2C). Additionally, bioinformatics analysis was used to predict the potential targeting miR-328a of APPAT. As a result, miR-328a was a potential regulator of APPAT (Figure 2D). Meanwhile, the double luciferase analysis showed that the miR-328a mimic significantly reduced the luciferase activity of APPAT-WT reporter in HEK293T cells (Figure 2E). More importantly, RNA immunoprecipitation (RIP) using anti-AGO2 antibodies was used to further test APPAT regulated miR-328a in an AGO2-dependent manner (Figure 2F). Taken together, these results indicated the direct binding between APPAT and miR-328a.

MiR-328a Overexpression Clearly Inhibited Breast Cancer Cells Proliferation, Migration and Invasion

To evaluate the biological functions of miR-328a in breast cancer progression, we inhibited its expression in MDA-MB-231 and BT549 cells (Figure 3A). Conversely, overexpression of miR-328a was detected in MCF-7 and BT474 cells (Figure 3B). Interestingly, miR-328a inhibition increased the proliferation (Figure 3C-3D), migration (Figure 3E) and invasion (Figure 3F) of MDA-MB-231 and

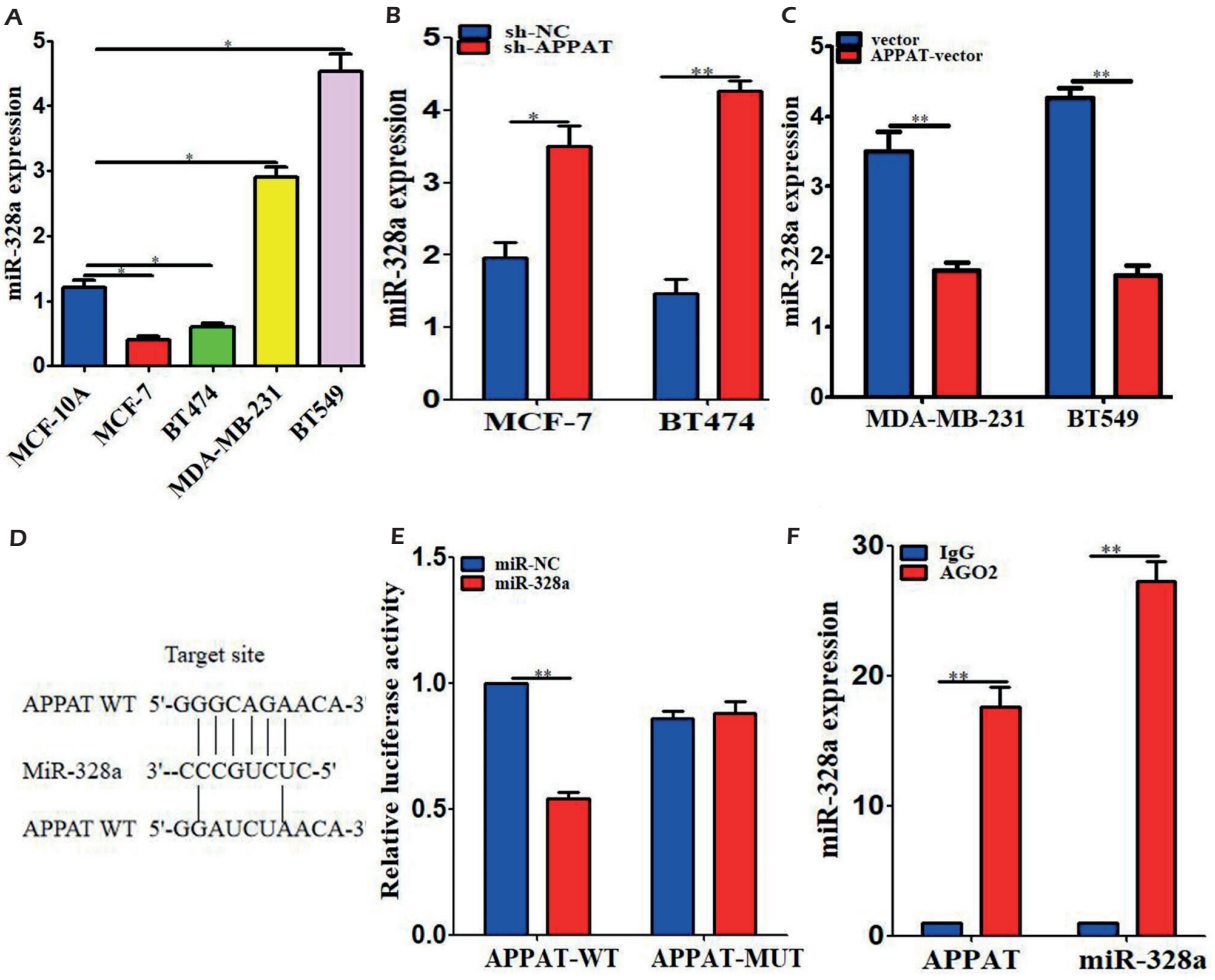

Figure 2. MiR-328a was a direct target of LncRNA APPAT. **A**, The expression of miR-328a was detected by QPCR. **B**, The expression of miR-328a was detected by QPCR in MCF-7 and BT474 cells after transfection with sh-lncRNA APPAT. **C**, The expression of miR-328a was detected by QPCR in MDA-MB-231 and BT549 cells after infection with lentivirus expressing lncRNA APPAT. **D**, The predicted miR-328a binding sites in APPAT. **E**, Effect of miR-328a on the activity of lncRNA APPAT reporter luciferase was checked by luciferase assay. **F**, The levels of lncRNA APPAT and miR-328a were detected by QPCR and presented as fold enrichment of AGO2 relative to input. The data are expressed as mean ± SD. n=3. *p <0.05.

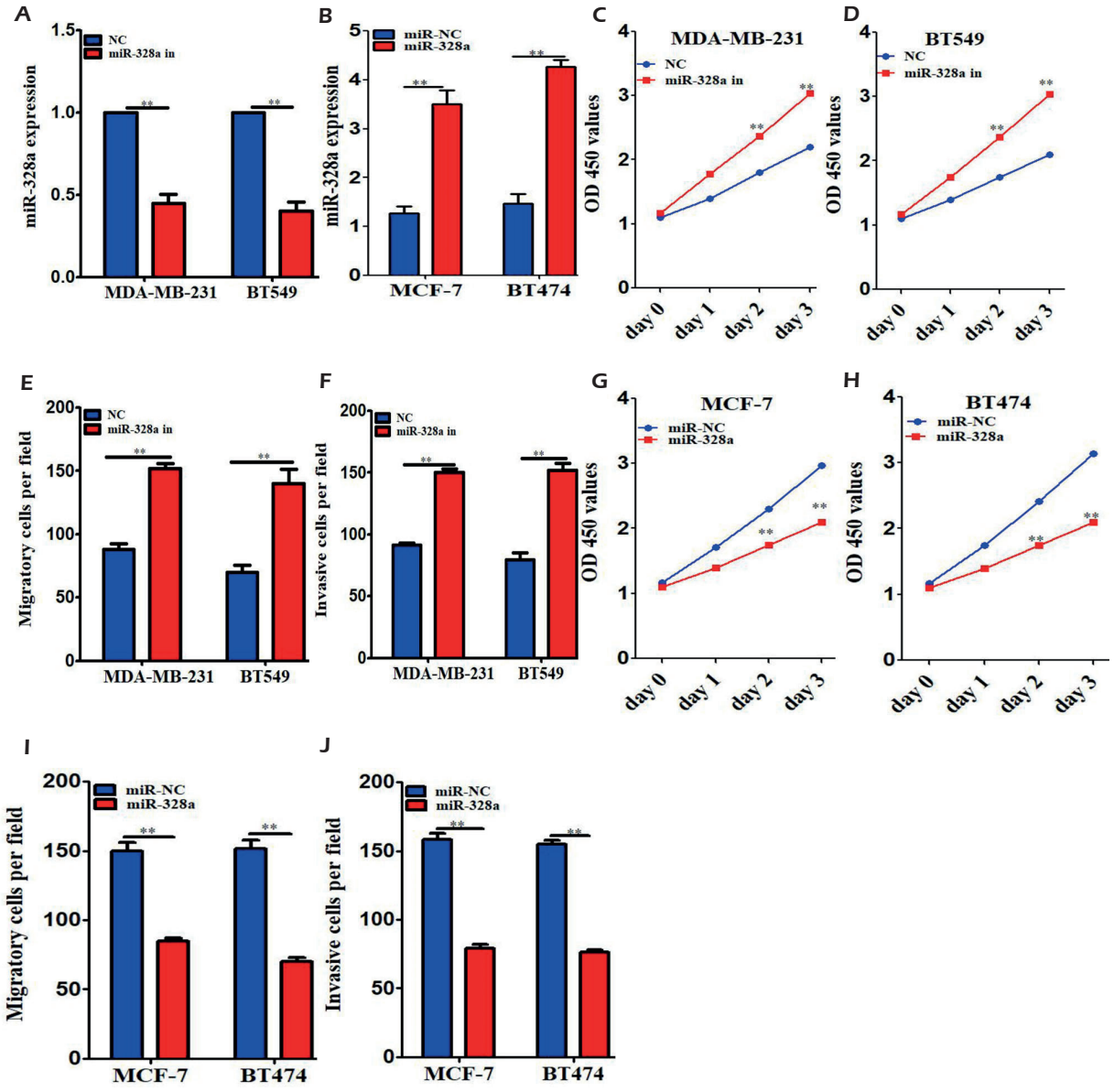

Figure 3. MicroRNA-328a overexpression clearly inhibited breast cancer cells proliferation, migration and invasion. **A**, MiRNA-328a expression was investigated after miRNA-328a inhibitor treatment in MDA-MB-231 and BT549 cells by QPCR. **B**, MiRNA-328a expression was examined after miRNA-328a mimic treatment in MCF-7 and BT474 cells by QPCR. **C-D**, The cell proliferation on MDA-MB-231 and BT549 cells after miRNA-328a inhibitor treatment was measured by CCK-8 assay. **E-F**, Migration and invasion of MDA-MB-231 and BT549 cells after miRNA-328a inhibitor treatment were measured using transwell assay. **G-H**, The cell proliferation on MCF-7 and BT474 cells after miRNA-328a mimic treatment was measured by CCK-8 assay. **I-J**, Migration and invasion of MCF-7 and BT474 cells after miRNA-328a mimic treatment were measured using transwell assay. The data are expressed as mean ± SD. n=3. *p <0.05.

BT549 cells after miR-328a inhibitor treatment and reverse results were found after treated with miR-328a mimic in MCF-7 and BT474 cells (Figure 3G-3J). On the whole, these results indicated that silencing of miR-328a enhanced the proliferation, migration and invasion of breast cancer cells.

MiR-328a Interacted with Pkp1

MiRNA plays an important regulatory role by interacting with proteins[22-24]. Pkp1 is a member of armadillo protein family and it can mediate the interaction of desmosomer cadherin with desmosomer and keratin intermediate filaments[25-29]. To evaluate

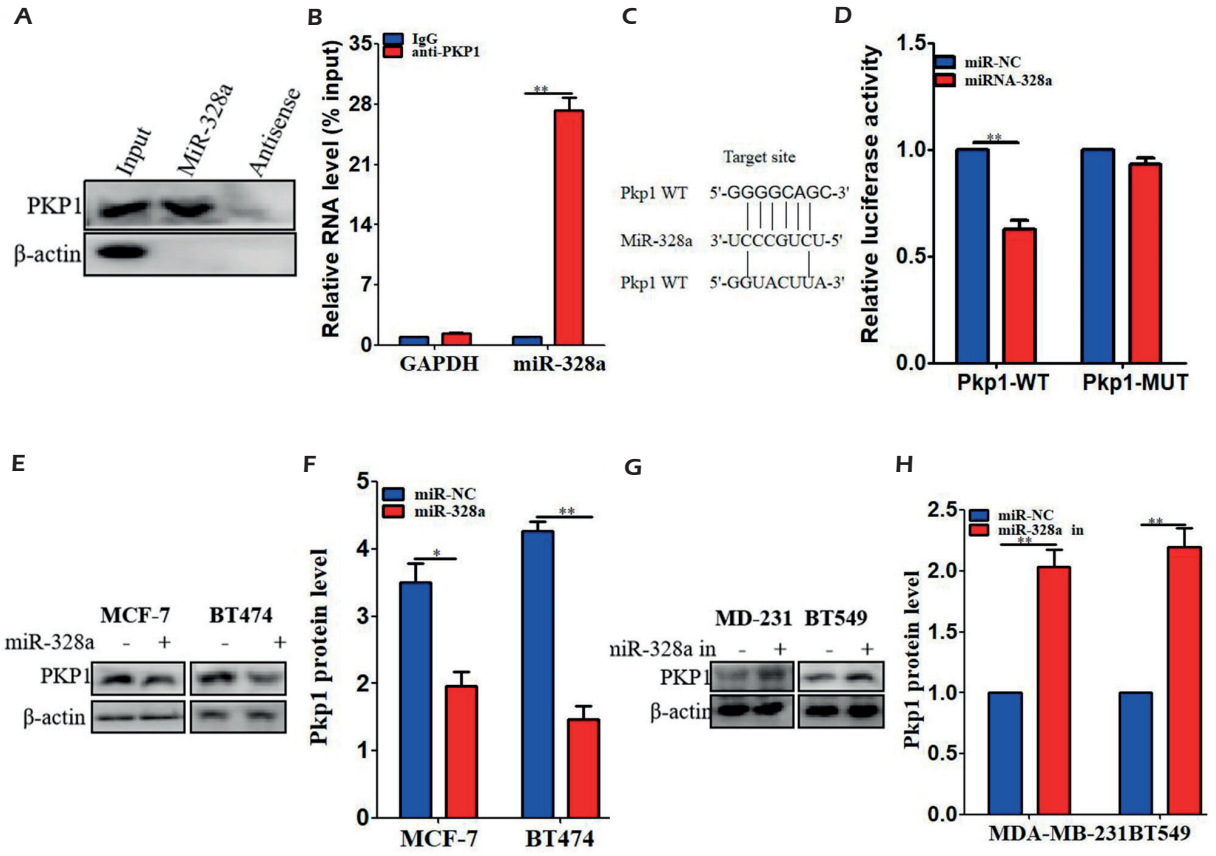

Figure 4. MiR-328a interacted with Pkp1. **A**, Western blotting analysis showing the interaction of miR-328a with Pkp1. **B**, Enrichment of RNA immunoprecipitation was determined as the amount of RNA associated with immunoprecipitation of Pkp1 relative to the input control. **C**, Target site of miR-328a in the Pkp1 sequence. **D**, Relative luciferase activity after co-transfection with wild type or mutant Pkp1 vectors and miR-328a mimic. **E-H**, Pkp1 protein level was checked by Western blotting after miR-328a mimic or inhibitor treatment. The data are expressed as mean ± SD. n=3. **p* <0.05.

whether miR-328a acted via this mechanism, we performed RNA pull-down assays to conform our prediction that Pkp1 may interact with miR-328a (Figure 4A). Additionally, RIP experiment using anti-Pkp1 antibodies showed enrichment of miR-328a using the Pkp1 antibodies (Figure 4B). Importantly, bioinformatics analysis and the double luciferase report confirmed that Pkp1 was a direct target of miR-328a (Figure 4C-4D). Moreover, overexpression of miR-328a significantly reduced Pkp1 protein level in MCF-7 and BT474 cells (Figure 4E) and reverse results were found after miR-328a downregulation (Figure 4F) in MDA-MB-231 and BT549 cells. Altogether, these results suggested a specific interaction between Pkp1 and miR-328a.

MiR-328a Functioned Through Its Interaction with Pkp1

To further investigate the function of miR-328a, we searched its downstream targets by performing QPCR. Results showed Pkp1 was commonly expressed in breast cancer cells (Figure 5A) and the knockout efficiency of Pkp1 was detected by Western blotting (Figure 5B). In addition, we observed that the reduction in proliferation, migration and invasion caused by inhibition of Pkp1 was reversed by miR-328a inhibitor in MCF-7 and BT474 cells (Figure 5C-5F). In general, these results stated that the effect of miR-328a on cell proliferation, migration and invasion was at least partly dependent on Pkp1.

APPAT Enhanced Breast Cancer Cells Proliferation, Migration and invasion by Upregulation of Pkp1

We sought to gain further mechanistic insight into the regulatory role of APPAT in Pkp1 expression. The overexpression of APPAT elevated Pkp1 protein levels (Figure 6A) in MDA-MD-231 and BT549 cells, and opposite results

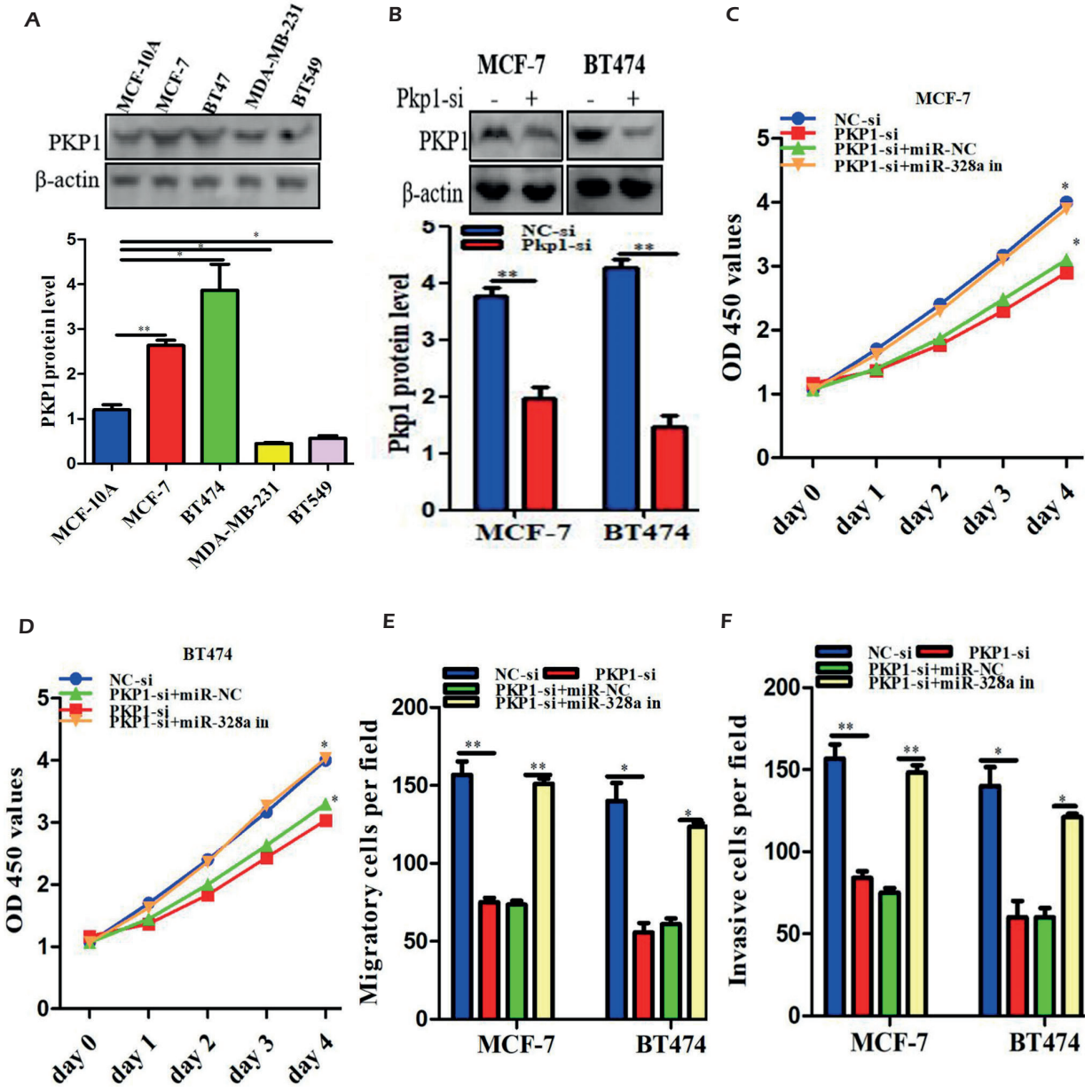

Figure 5. MiR-328a functioned through its interaction with Pkp1. **A**, Pkp1 protein level was tested by western blotting. **B**, Pkp1 protein level was detected by western blot after Pkp1-si treatment in MCF-7 and BT474 cells. **C-D**, The cell proliferation on MCF-7 and BT474 cells was measured by CCK-8 assay. **E-F**, Migration and invasion of MCF-7 and BT474 cells were investigated using transwell assay. The data are expressed as mean ± SD. n=3. **p* <0.05.

were found (Figure 6B) in MCF-7 and BT474 cells. In addition, Pkp1 knockout blocked the increase induced by APPAT overexpression on cells proliferation (Figure 6C-D), migration (Figure 6E) and invasion (Figure 6F). Importantly, Pkp1 knockout promoted the decrease of cells proliferation (Figure 6G-6H), migration (Figure 6I) and invasion (Figure 6J) induced by APPAT downregulation. Taken together, these evidences demonstrated that lncRNA APPAT enhanced the proliferation, migration and invasion of breast cancer cells by regulating the miR-328a/Pkp1 signaling pathway, playing a critical role in progression of breast cancer.

Discussion

The results of the present study indicated that the expression of lncRNA APPAT led to widespread alterations in breast cancer cells. We identified miR-328a as a downstream target of lncRNA APPAT and lncRNA APPAT was required for cell proliferation, migration and invasion in breast cancer. Mechanistically, lncRNA APPAT directly interacted with miR-328a to take party in breast cancer progression. These findings revealed that the lncRNA APPAT/miR-328a signaling axis played a critical role in regulating the cell proliferation, migration and invasion of breast cancer cells. The abnormal expression of lncRNA was related to many kinds of cancer and plays a key role in many kinds of cancer biological processes[13-20]. However, the effect of lncRNA APPAT in

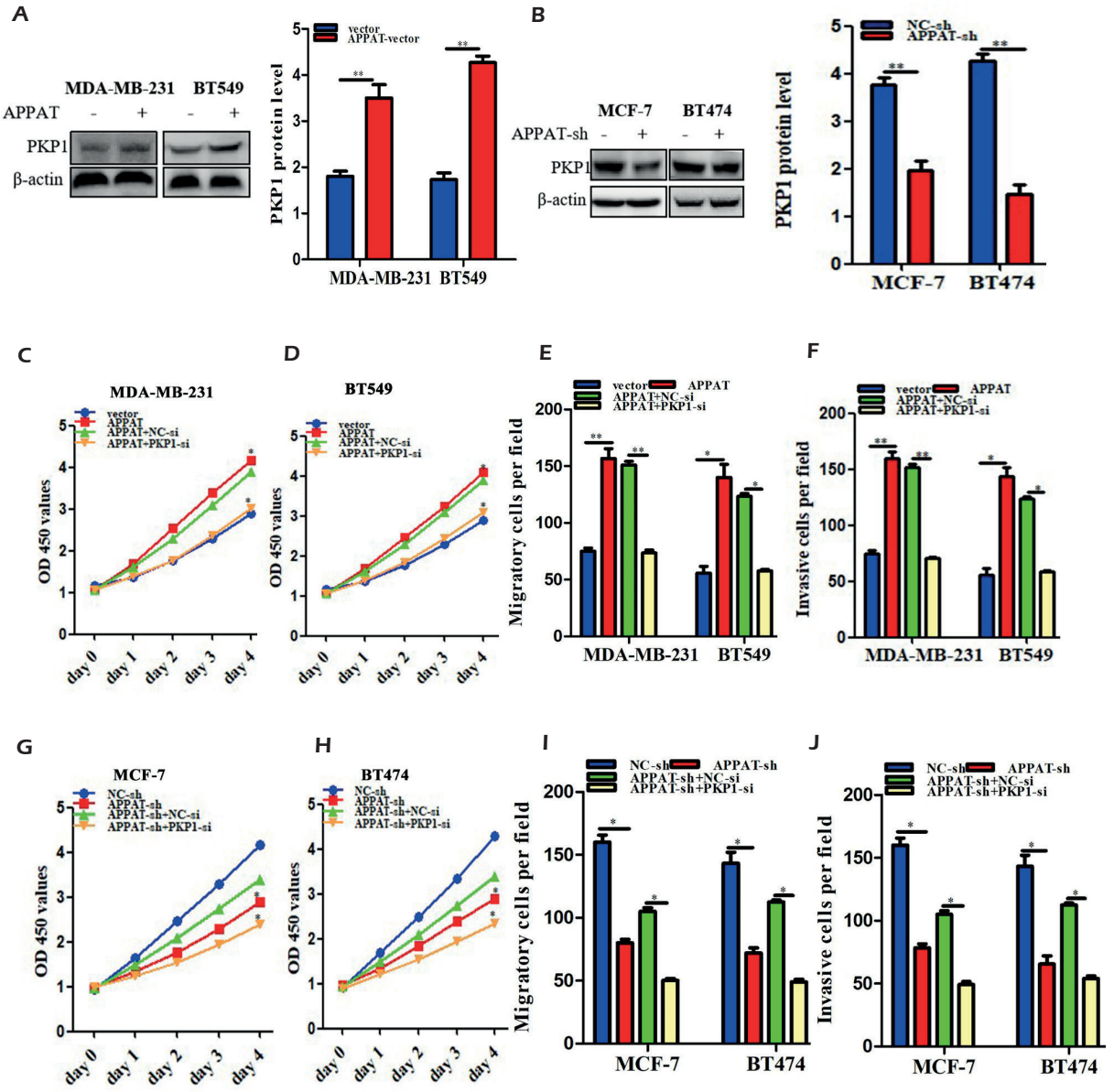

Figure 6. APPAT enhanced breast cancer cells proliferation, migration and invasion by upregulation of Pkp1. **A-B**, The protein level of Pkp1 was measured after APPAT-overexpressing or APPAT-si treatment by western blotting in MDA-MB-231 and BT549 cells or MCF-7 and BT474 cells. **C-D**, The cell proliferation of MDA-MB-231 and BT549 cells was checked by CCK-8 assay. **E-F**, Cell migration and invasion of MDA-MB-231 and BT549 cells were tested using transwell assay. **G-H**, The cell proliferation of MCF-7 and BT474 cells was checked by CCK-8 assay. **I-J**, Cell migration and invasion of MCF-7 and BT474 cells were tested by transwell assay. The data are expressed as mean ± SD. n=3. **p* <0.05.

breast cancer was largely unknown and whether lncRNA APPAT could regulate cell proliferation, migration and invasion of breast cancer through miR-328a has not been previously investigated.

In our study, luciferase analysis and bioinformatics analysis showed that miR-328a was the target of lncRNA APPAT. Meanwhile, miR-328a expression was higher in MCF-7, BT474 with low APPAT levels and lower expression cell lines in MDA-MB-231, BT549 with high APPAT levels. MiR-328a inhibition increased the proliferation, migration and invasion of MDA-MB-231 and BT549 cells after miR-328a inhibitor treatment and reverse results were found after treated with miR-328a mimic in MCF-7 and BT474 cells. Our studies revealed that miR-328a was an important component of the lncRNA APPAT regulatory network and highlighted the important regulatory relationships between miRNAs and lncRNAs. These results suggested that lncRNA APPAT as a ceRNA regulated miR-328a to promote the proliferation, invasion and metastasis of breast cancer.

MiRNAs interact with specific proteins[28,29]. In this study, we performed RNA pull-down accompanied to identify miR-328a-interacting proteins. Notably, Pkp1 was confirmed as a specific binding protein for miR-328a. Meanwhile, bioinformatics analysis and luciferase report assay showed that Pkp1 was a target of miR-328a. Additionally, miR-328a mimic reduced Pkp1 protein level, while miR-328a inhibitor upregulated the Pkp1 protein level. More importantly, the downregulation of Pkp1 reversed the proliferation, migration and invasion of induced by miR-328a inhibitor. These results indicated that the effect of miR-328a on cell proliferation, migration and invasion in breast cancer was at least partly mediated by Pkp1. Therefore, we reasoned that miR-328a interacted with Pkp1 to regulate proliferation, migration and invasion of breast cancer.

Our study also found overexpression of lncRNA APPAT increased Pkp1 protein level, while inhibition of lncRNA APPAT decreased the Pkp1 protein level. In addition, Pkp1 downregulation enhanced the decrease of proliferation, migration and invasion induced by lncRNA APPAT knockout in MCF-7 and BT474 cells. Conversely, downregulation of Pkp1 reversed the increase on the proliferation, migration and invasion of MDA-MB-231 and BT 549 cells induced by lncRNA APPAT overexpression. On the whole, these results indicated that the effect of lncRNA APPAT on cell proliferation, migration and invasion in breast cancer cells was at least partly mediated by regulating miR-328a/Pkp1.

Conclusions

To the best of our knowledge, this was the first time that lncRNA APPAT was involved in the development of breast cancer and the regulatory relationship between lncRNA APPAT with miR-328a and miR-328a with Pkp1 was clearly proposed. It was the first time that miR-328a and Pkp1 played important role in breast cancer. Our results suggested that the lncRNA APPAT/miR-328a/Pkp1 signaling axis was a potential target to inhibit the response of breast cancer to proliferation, migration and invasion.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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