Int J Clin Exp Pathol 2019;12(5):1609-1617
www.ijcep.com /ISSN:1936-2625/IJCEP0086322

Original Article

Long noncoding RNA PCA3 contributes to the progression of choriocarcinoma by acting as a ceRNA against miR-106b

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Received October 3, 2018; Accepted December 13, 2018; Epub May 1, 2019; Published May 15, 2019

Abstract: Background: Choriocarcinoma is the most aggressive gestational trophoblastic disease, with massive local trophoblast invasion and vascular percolation, resulting in multiple organ metastases. Recent evidence has shown that long noncoding RNAs (lncRNAs) play an important role in tumor progression. This study aimed to investigate the expression and role of lncRNA PCA3 in the progression of choriocarcinoma. Methods: First, the expression of lncRNA PCA3 in choriocarcinoma cells was detected using quantitative real-time PCR (qRT-PCR). Then functional assays such as cell proliferation assay, wound healing assay, and invasion assay were conducted to determine the role of PCA3. In addition, the specific molecular mechanism was studied using western blot, luciferase assay, and rescue experiment. Results: We demonstrated that the expression of PCA3 is significantly higher in choriocarcinoma cells in contrast to normal human chorionic trophoblast cells. Furthermore, PCA3 could promote cell proliferation, migration and invasion in gestational choriocarcinoma cells and facilitated epithelial to mesenchymal transition (EMT) in vitro. In addition, PAC3 could directly bind to miR-106b and effectively liberate the expression of its endogenous target matrix metallopeptidase 2 (MMP2). Conclusion: Our results suggest that PCA3 contributes to the progression of choriocarcinoma by acting as a ceRNA against miR-106b.

Keywords: PCA3, choriocarcinoma, ceRNA, miR-106b

Introduction

Choriocarcinoma is a malignant tumor, the vast majority of which is associated with pregnancy, secondary to hydatidiform mole, abortion, or normal delivery [1]. It is a highly malignant form of gestational trophoblastic neoplasia (GTN) with a high mortality rate [2], which develops in approximately one in every 50,000 pregnancies [3]. Choriocarcinoma is associated with early vascular invasion, and spreads hematogenously to, most commonly, lung, vagina, brain and liver, which can cause destruction of the organs and lead to hemorrhage and necrosis [4]. Despite well-established chemotherapy, about 25% of choriocarcinoma patients showed incomplete response, or relapsed due to tumor remission [5, 6]. Therefore, exploring the key molecular mechanisms involved in choriocarcinoma progression would have a significant impact for choriocarcinoma therapy.

Long noncoding RNAs (lncRNAs) are defined as a class of transcript greater than 200 nucleotides, which have no protein encoding ability, and show less sequence conservation in related species [7]. Recently, increasing evidence has suggested that lncRNAs are biologically functional and have been implicated in the initiation, progression, and metastasis of various types of cancers, and a number of lncRNAs have been clarified [8-10].

The PCA3 gene is located on chromosome 9q21-22, in antisense orientation within intron 6 of the Prune homolog 2 gene (PRUNE2 or BMCC1) [11, 12], which has been shown to be significantly upregulated in prostate cancer [13, 14]. Research has also showed that PCA3 enhances the ability of cell proliferation, invasion, and migration of prostate cancer and it is currently used as a diagnostic tool and management strategy for prostate cancer [15, 16]. Liu et al. also found PCA3 expressed highly in epithelial ovarian cancer and it may coordinate EOC tumorigenesis [17]. The role of lncRNA PCA3 in the progression of choriocarcinoma is

still unknown. This study aimed to investigate the specific molecular mechanism of lncRNA PCA3 in choriocarcinoma. Assembling evidence indicates that lncRNAs act as molecular sponges for microRNAs (miRNAs) through miRNA response elements (MREs), thereby suppressing the expression of the miRNAs' target, respectively. This is proposed as a competitive endogenous RNA (ceRNA) hypothesis. LncRNAs are involved in ceRNA networks and mRNA-miRNA-lncRNA crosstalk, and play an important role in human diseases through this regulatory network [18]. For example, lncRNA PCA3 can up-regulate PRKD3 expression by competitive miR-1261 sponging, thus further promoting invasion and migration of prostate cancer [19]. LncRNA n335586 promoted cell migration, invasion, and EMT through n335586/miR-924/CKMT1A axis in hepatocellular carcinoma [20]. Shi et al. reported that lncRNA MALAT1 might be a pro-oncogene that promoted the proliferation of choriocarcinoma by miR-218-mediated Fbxw8 regulation [21].

In the study, we demonstrated that lncRNA PAC3 is significantly upregulated in choriocarcinoma cells. Through sponging miR-106b, PCA3 promotes the expression of MMP2 thus facilitating the proliferation, migration, invasion and EMT of choriocarcinoma cells. Our research may provide novel understanding into lncRNA function in choriocarcinoma progression.

Materials and methods

Human choriocarcinoma cell lines

The human chorionic trophoblast cell HTR-8 and the choriocarcinoma cell lines JAR, BeWo and JEG-3 were from. HTR-8 cells and JAR cells were incubated in Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal bovine serum (FBS); BeWo cells and JEG-3 cells were supplemented in RPMI-1640 medium with 10% FBS. All cells were supplemented with 1% PS (100 mg/ml streptomycin sulfate and 100 UI/ml penicillin sodium) and maintained at 37°C and 5% CO_2.

Cell transfection

The JAR cells were digested in the logarithmic growth phase and subcultured into a sterile culture plate; when the cells were 80%-90% confluent, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) was applied for transfection following the manufacturer's instructions.

RNA isolation and qRT-PCR

Total RNA from choriocarcinoma cell lines were extracted by TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. NanoDrop-2000 spectrophotometer was used to confirm the quantity and quality of the extracted RNA. RNA were reversely transcribed by M-MLV (Promega, Madison, WI, USA), and then Premix ExTaq II Kit (Takara) was used to perform qPCR. The relative fold changes of candidate genes in the transcripts were analyzed with $2^{-\Delta\Delta Ct}$ method.

Dual-luciferase reporter assay

PCR assays were performed to amplify the sequence which contains the predicted wild-type or mutant miR-106b binding sites of PCA3 and then we subcloned it into a pGL3-basic vector (Promega Corporation, Madison, WI, USA) which generated the reporter vectors pGL3-PCA3-WT (PCA3-WT) and pGL3-PCA3-MUT (PCA3-MUT). For luciferase reporter assay, JAR cells were cultured in a 24 well plate and co-transfected with 0.15 μg PCA3-WT or PCA3-MUT together with miR-106b or miR-scrambled control and 0.05 μg of the internal control vector containing renilla luciferase, pRL-TK (Promega) using Lipofectamine 2000 transfection reagent (Invitrogen). JAR cells were lysed and the luciferase activities were monitored 48 h after post-transfection by the Dual Luciferase Reporter Assay System (Promega, WI, USA). Renilla luciferase activity was used for the standardization.

Cell proliferation assay

MTT assay was conducted to detect cell proliferation in vitro. Briefly, after transfection, the density of JAR cells in a 96-well plate was 5.0×10^3 cells/well, and all groups were incubated for 24, 48 hours, respectively. MTT was added to each well (5 mg/mL, Sigma, USA) and the culture was incubated for 4-6 hours at 37°C. Cell viability was measured at 490 nm by the MTT Cell Proliferation Kit (Cayman Chemical). For more details, refer to Ref [21].

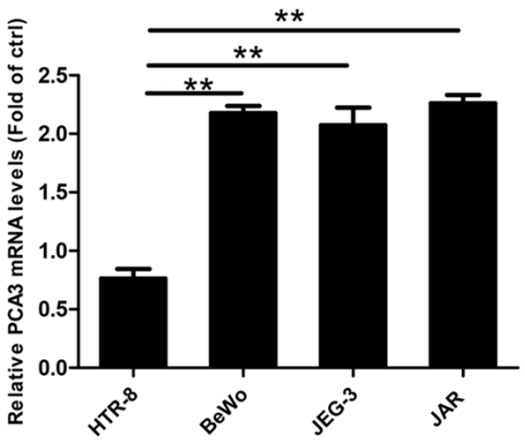

Figure 1. The relative expression of lncRNA PCA3 in choriocarcinoma cells. The relative mRNA expression levels of PCA3 in BeWo, JEG-3, JAR and HTR-8 cell lines. The data are the mean ± SD of three independent experiments. (***P < 0.001).

Wound healing assay

The JAR cells were incubated in six-well plates with normal cell growth medium. When the cells reached 85%, we scraped the cell layer with a 50 μl sterile pipette tip, then washed it with PBS for three times, and then cultured for 48 hours with 1% FBS medium. Cells were incubated with mitomycin C (10 μg/ml) for 1 hour at 37°C since cell proliferation may affect the analysis of the scratch assays. Microscope was used to obtain images of the tablets at different time points (0, 48 hours).

Cell invasion assays

Transwell chamber inserts with a 8 μm pore with Matrigel were used to observe the invasive ability of choriocarcinoma cells. 5×10^4 JAR cells were seeded into the upper compartment each insert, while the bottom of the insert was incubated with 500 μl DMEM with 20% FBS. Last, the cells were counted. Details of the method are provided in Ref [21].

Western blot

Cultured or transfected cells were harvested and lysed using extraction buffer with 1 mM PMSF for 30 min on ice. The protein lysates were separated in SDS-PAGE separation gel and transferred to NC for 90 minutes at 300 mA. After blocking with 5% BSA for 2 h, the primary antibodies were added and incubated with membranes at 4°C overnight, then incubated appropriate secondary antibodies. The GAPDH antibody was used as a control.

Statistical analysis

All data were from 3 independent experiments and presented as means ± standard deviation and processed using GraphPad Prism Software. Student's t test or analysis of variance (ANOVA) was used for comparison between groups, and the *P* value less than 0.05 was considered significant.

Results

LncRNA PCA3 is highly expressed in choriocarcinoma cell lines

To explore the expression profiles of PCA3 in choriocarcinoma, qRT-PCR analysis was performed in choriocarcinoma cell lines. The results showed that PCA3 were markedly overexpressed in BeWo cells, JEG-3 cells and JAR cells compared with in HTR-8 cells (**Figure 1**).

LncRNA PCA3 enhances the proliferation, migration and invasion of choriocarcinoma cells

Due to the significant increase in the expression level of PCA3 in choriocarcinoma cells, we conjectured that it may participate in the progression of choriocarcinoma. To further study the function of PCA3 in choriocarcinoma, pWPXL-PCA3 and si-PCA3 were constructed and the effectiveness was verified using qRT-PCR (**Figure 2A**). To assess the effect of PCA3 on the malignant behaviors of choriocarcinoma cells, MTT assays were executed to calculate whether PCA3 affected cell viability. JAR cells transfected with pWPXL-PCA3 showed a considerable increase in cell viability compared to the controls. In contrast, JAR cells transfected with si-PCA3 demonstrated reduced cell viability (**Figure 2B**).

Then, wound healing test was performed. As shown in **Figure 2C**, **2D**, when PCA3 was overexpressed, the migration ability of JAR cells were significantly increased, while PCA3 knockdown decreased the cell migration ability of JAR cells. Next, we performed transwell assays to estimate the invasive capacity of JAR cells. The results showed that pWPXL-PCA3 enhanced the invasion ability of choriocarcinoma cells, but si-PCA3 inhibited the invasion ability of choriocarcinoma cells, which was consistent with the

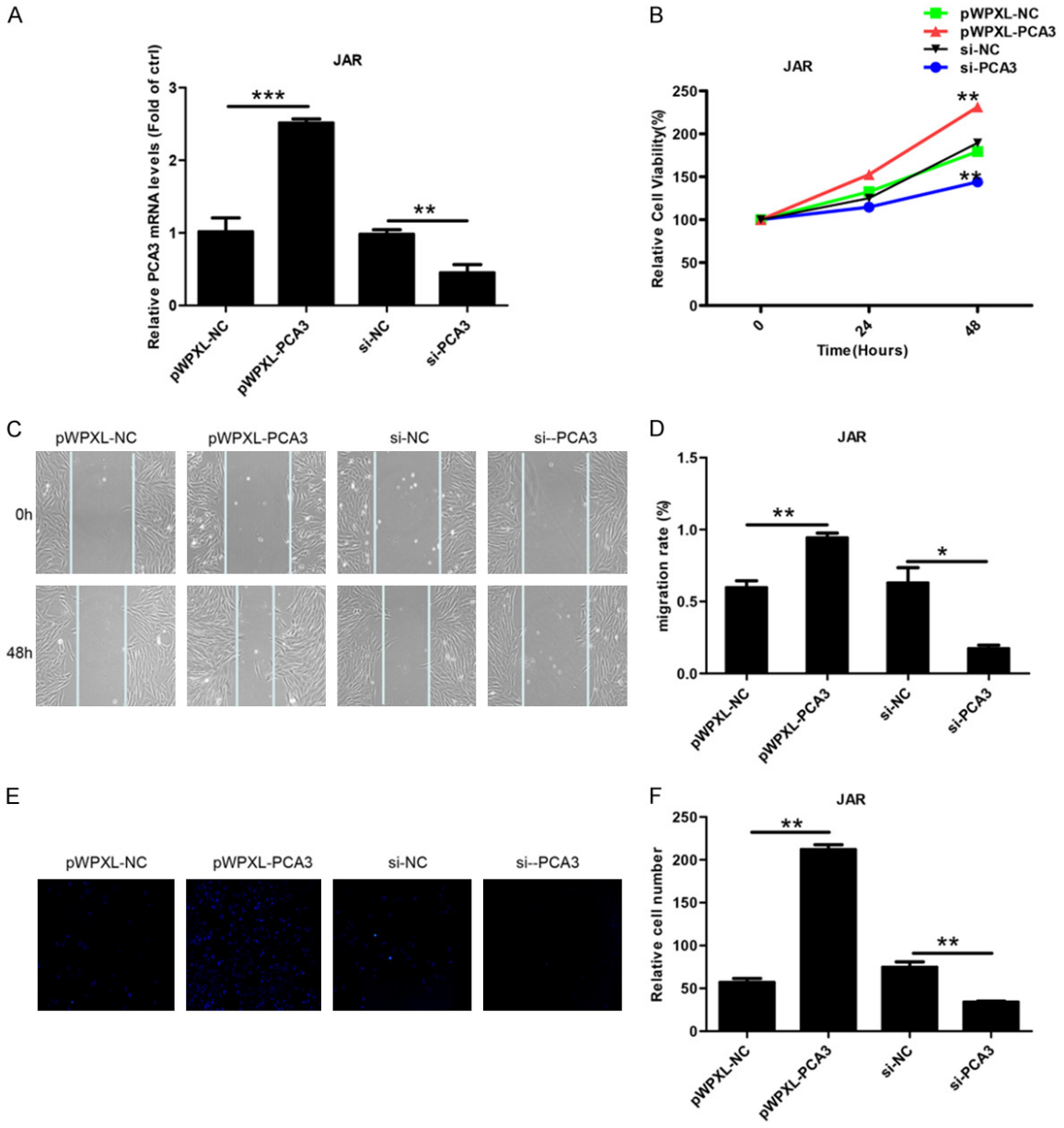

Figure 2. LncRNA PCA3 promotes the proliferation, migration, and invasion of choriocarcinoma cells. A. RT-qPCR assay was performed after transfection with either pWPXL-PCA3 or si-PCA3. B. Cell viability was measured using MTT assay following transfection with corresponding plasmids. C, D. Relative cell mobility was detected through wound healing assays after transfection with either pWPXL-PCA3 or si-PCA3. E, F. Transwell invasion assay was executed twenty-four hours post-transfection, using a transwell system with 8 μm pores in polycarbonate membranes. Representative views are shown. All photomicrographs were taken at 200 × magnification. The data are the mean ± SD of three independent experiments. (*P < 0.5; **P < 0.01; ***P < 0.001).

results of the wound healing assay (**Figure 2E**, **2F**).

LncRNA PCA3 promotes the EMT progression of choriocarcinoma cells

It has been well known that EMT plays a key role in cancer development, and confers cancer cell transfer and invasion capabilities. Therefore, we examined the expression of EMT-related molecular markers. As shown in **Figure 3A**, **3B**, the upregulation of PCA3 reduced the protein levels of E-cadherin and cytokeratin, but increased vimentin level in JAR cells. The opposite influences were observed in cells transfected with si-PCA3.

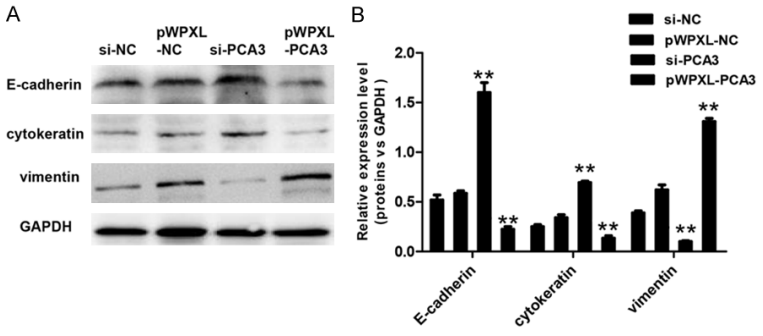

Figure 3. LncRNA PCA3 promotes the EMT progression of choriocarcinoma cells. A, B. Western blot assays were carried out to assess the protein levels of EMT-associated molecules. GAPDH was used as a loading control. The data are the mean ± SD of three independent experiments. (**P < 0.5; ***P < 0.001).

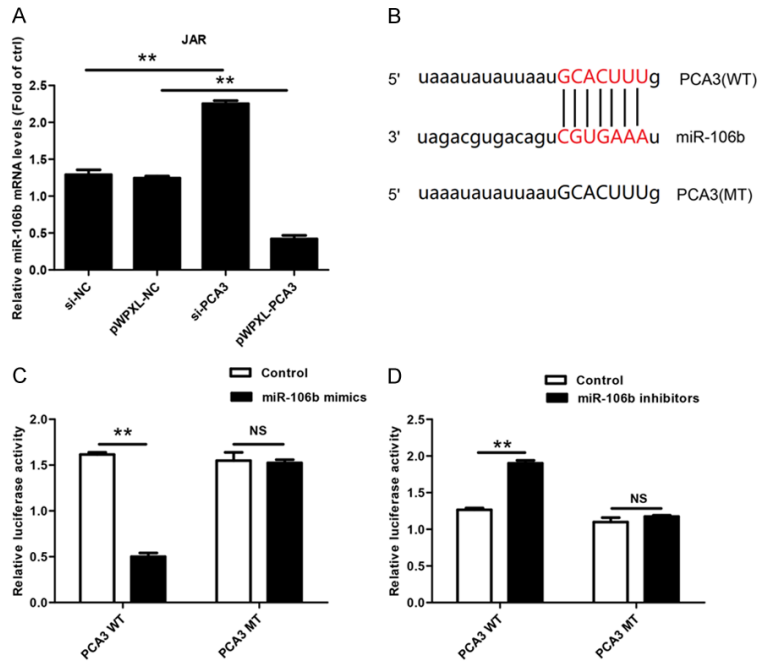

Figure 4. LncRNA PCA3 down-regulates the expression of miR-106b by targeting it. A. The mRNA level of miR-106b in JAR cells was measured using RT-qPCR following transfection with pWPXL-PCA3 and PCA3 inhibitor plasmids, respectively. B. Sequence alignment and schematic diagram of miR-106b with the wild-type (top) and mutated (bottom) 3'UTR of PCA3. C, D. The Luciferase activity of the reporter vectors was detected in JAR cells after 48 h of cotransfection of wild type or mutant PCA3-3'UTR with miR-106b respectively; pRL-TK was used for normalization. The data are the mean ± SD of three independent experiments. (***P < 0.001; ns, not significant).

LncRNA PCA3 regulates the expression of miR-106b by directly interacting with it

To explore the relationship between PCA3 and miR-106b in choriocarcinoma, RT-qPCR assay was performed. As shown in **Figure 4A**, the mRNA levels of miR-106b were markedly decreased after PCA3 overexpressed, and increased when PCA3 was knocked down in JAR cells. These results suggest that PCA3 inhibits the expression of miR-106b in JAR cells.

Numerous Evidence has revealed that lncRNAs can act as a competing endogenous RNA by binding to specific microRNAs. We conducted dual-luciferase reporter assay to confirm whether PCA3 plays its role in such a way. We subcloned the pGL3-promoter vector containing wild-type (WT) or mutant(WT) miR-106b putative binding sites in PCA3 (**Figure 4B**). The result of Dual-luciferase assays suggested that the luciferase activities were significantly decreased after we co-transfected cells with mimics PCA3-WT and miR-106b but not the PCA3-MUT (**Figure 4C**, **4D**). These data demonstrate that PCA3 regulates the expression of miR-106b through directly biding it.

LncRNA PCA3 regulates the progression of choriocarcinoma through regulation of MMP2

In conclusion, PCA3 accelerated cell viability, growth, migration/invasion and EMT in choriocarcinoma cells which indicated that PCA3 may function as an oncogene in choriocarcinoma.

Previous studies have shown that MMP-2 is a target of miR-106b [22]. To identify whether lncRNA PCA3 exerted its function through MMP2 in choriocarcinoma cells, rescue experi-

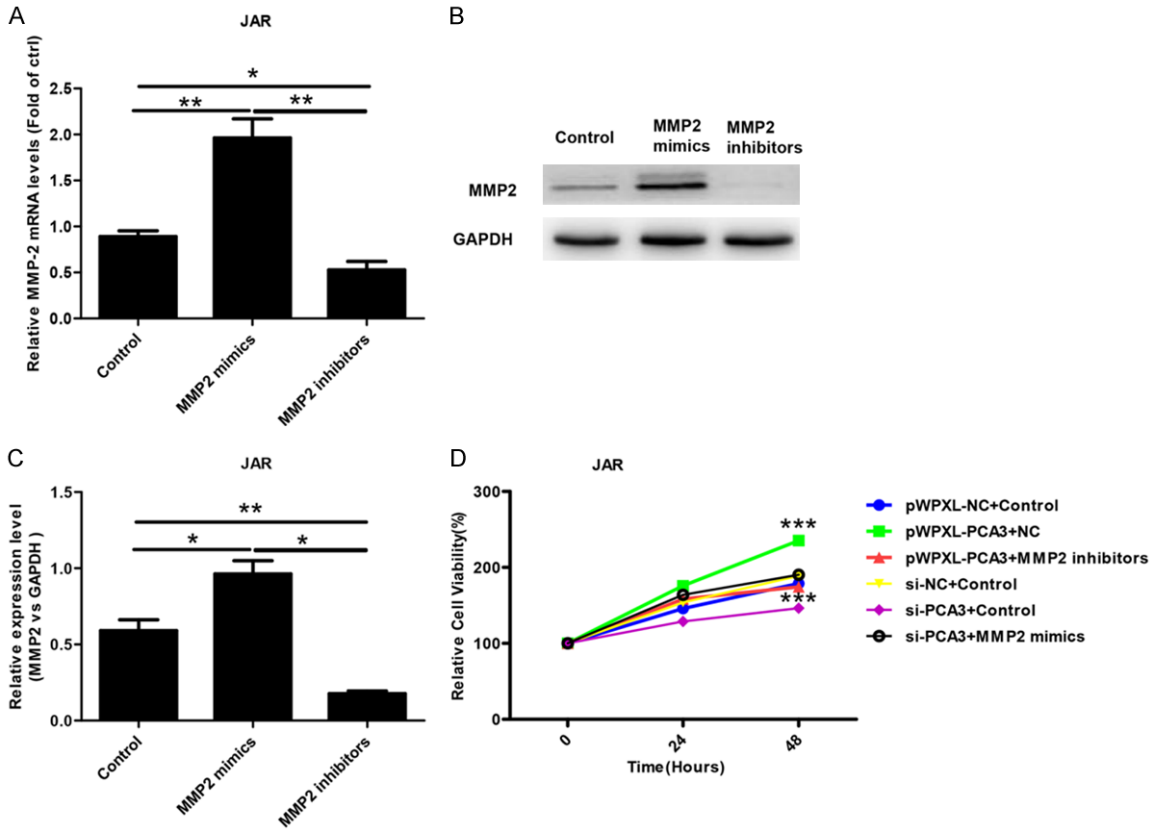

Figure 5. LncRNA PCA3 regulates the progression of choriocarcinoma through regulation of MMP2. A. The mRNA levels of MMP2 were determined by RT-qPCR in JAR cells. B, C. The protein levels of MMP2 were determined by western blot assays in JAR cells. D. MTT assays were used to test the viability and proliferation of co-transfection with pWPXL-PCA3/si-PCA3 and MMP2 mimics/MMP2 inhibitors.

ments were conducted. First, MMP2 mimics and inhibitors were constructed and the effectiveness was verified using qRT-PCR and western blot (**Figure 5A-C**). Then we knocked down MMP2 in PCA3-overexpressed JAR cells. Knockdown of MMP2 attenuated the decrease in proliferation of choriocarcinoma cells caused by PCA3 overexpression. In contrast, MMP2 overexpression resulted in the opposite effect (**Figure 5D**). Our data strongly suggest that lncRNA PCA3 acts as a sponge for miR-106b to up-regulate the expression of its target, MMP2, thereby promoting progression in choriocarcinoma cells.

Discussion

PCA3 is a well-characterized lncRNA that has been approved as a new biomarker with fairly high specificity and sensitivity in prostate cancer. One study found that the diagnostic accuracy of the first prostate biopsy in Chinese patients with a PSA level of 4.0-10.0 ng/mL was moderately improved by using PCA3 detection [12]. In addition, increased PCA3 mRNA levels were correlated with PCa independently of PSA levels [23]. Lin et al. also found that PCA3 elevation in peripheral blood is associated with PCa, and the detection of PCA3 may significantly reduce adverse screening outcomes, which indicates that its expression in peripheral blood had a promising clinical application value in the early diagnosis of PCa [21]. Furthermore, lncRNA PCA3 could also be a novel therapeutic target of ovarian carcinoma [9]. However, no function was reported in choriocarcinoma. In this study, we demonstrated that PCA3 is significantly upregulated in human choriocarcinoma tissues and choriocarcinoma cell lines. Gain and loss of function assays indicated that PCA3 significantly enhanced proliferation, migration, invasion and EMT of choriocarcinoma cells. Therefore, these findings revealed that PCA3 may act as an oncogene in choriocarcinoma.

Recent evidence shows that there is a novel regulatory mechanism between lncRNAs and miRNAs. LncRNAs may bind to miRNAs by acting as endogenous miRNA sponges to regulate the miRNA expression and their function and thus de-repress the expression of its target [24]. Inspired by this, we speculated that lncRNA PCA3 may act in the same way. Based on bioinformatics prediction, we focused on miR-106b. RT-qPCR assay, western blot assay, and luciferase reporter assay showed that PCA3 could directly interact with miR-106 and suppress its expression.

Previous work has shown that miR-106b had different effects in different cancers. In thyroid cancer and ovarian epithelial carcinoma [25-28], it may act as a tumor suppressive factor, while in the serum of renal cell carcinoma and gastric cancer [29-32], miR-106 was up-regulated and had an opposite function. Li also found that miR-106b could inhibit the invasion and proliferation of JAR and JEG3 cells through targeting MMP-2, and had a relationship with the pathogenesis of pre-eclampsia [33]. In the present study, we confirmed that lncRNA PCA3 regulates the progression of choriocarcinoma through regulation of MMP2 by acting as a ceRNA against miR-106, which may be valuable for the development of novel diagnostic and treatment approaches for choriocarcinoma.

Conclusion

Our study demonstrated that PCA3 was upregulated in choriocarcinoma cell lines, and its overexpression enhanced cell proliferation, migration, invasion, and EMT. Mechanistic experiments elucidated that PCA3 contributes to the progression of choriocarcinoma by acting as a ceRNA against miR-106b, which indicates PCA3 may act as a biomarker in choriocarcinoma and provides anunderstanding of the pathogenesis and development of choriocarcinoma.

Disclosure of conflict of interest

None.

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