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LncRNA RHPN1-AS1 promoted cell proliferation, invasion and migration in cervical cancer via the modulation of miR-299–3p/FGF2 axis

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ABSTRACT

Aims: This study aims to determine the biological function and underlying mechanisms of lncRNA RHPN1 antisense RNA1 (RHPN1-AS1) in cervical cancer cell proliferation, invasion and migration.

Main methods: Gene expression was analysed by quantitative real-time PCR; protein levels were determined by western blot assay; *in vitro* functional assays determined the cervical cancer cell progression; *in vivo* tumor growth of cervical cancer cell was determined in nude mice xenograft models.

Key findings: The results showed that RHPN1-AS1 was up-regulated in cervical cancer tissues and cell lines. *In vitro* functional assays demonstrated that RHPN1-AS1 overexpression promoted SiHa cell proliferation, invasion and migration; while RHPN1-AS1 knockdown showed the opposite effects. *In vivo* study showed that RHPN1-AS1 knockdown suppressed tumor growth in the nude mice. Further investigation showed that miR-299–3p was targeted and inversely regulated by RHPN1-AS1. In addition, miR-299–3p targeted the 3' untranslated region of fibroblast growth factor 2 (FGF2) to suppress its expression. The rescue experiments showed that the enhanced effects of RHPN1-AS1 overexpression on cell proliferation, growth, invasion and migration in SiHa cells were significantly attenuated by miR-299–3p overexpression or FGF2 inhibition. On the other hand, knockdown of miR-299–3p and overexpression of FGF2 both significantly increased cell proliferation, growth, invasion and migration in SiHa cells transfected with RHPN1-AS1 siRNA.

Significance: In conclusion, our results revealed that RHPN1-AS1 promoted cervical cancer progression via targeting miR-299–3p/FGF2 axis. Our data suggested that RHPN1-AS1/miR-299–3p/FGF2 axis may be a promising target for cervical cancer treatment.

1. Introduction

Cervical cancer is one of the leading causes of female cancer-associated deaths around the world. There are about 0.5 million new cases diagnosed and around 0.3 million deaths annually [1]. Cervical cancer is curable by surgical resection if it is diagnosed at an early stage [2,3]. However, many of the patients with cervical cancer had advanced or metastatic disease stage, which renders the patients with poor prognosis [4,5]. At present, surgery, chemotherapy and radiotherapy are the main treatments for cervical cancer, but clinical outcomes are far from satisfactory [6]. Consequently, exploration of the novel molecular mechanisms underlying cervical cancer progression is crucial for finding new therapeutic strategies for cervical cancer.

Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNAs without protein-coding capacity, and lncRNAs are more than 200 nucleotides in length and exert their biological functions via epigenetic, transcription or post-transcriptional mechanisms [7–9]. Up to date, lncRNAs have been demonstrated to regulate many cellular processes such as cell proliferation, metastasis, differentiation and metabolism, which contribute to the progression of various diseases including malignant tumors [10]. As such, many lncRNAs, to date, have been identified for their functional roles in the pathophysiology of cervical cancer. Mao et al., identified a 15-lncRNA signature for the improvement in cervical cancer prognosis [11]. LncRNA LNMICC was found to be up-regulated in cervical cancer tissues and LNMICC promoted nodal metastasis of cervical cancer via reprogramming fatty acid metabolism

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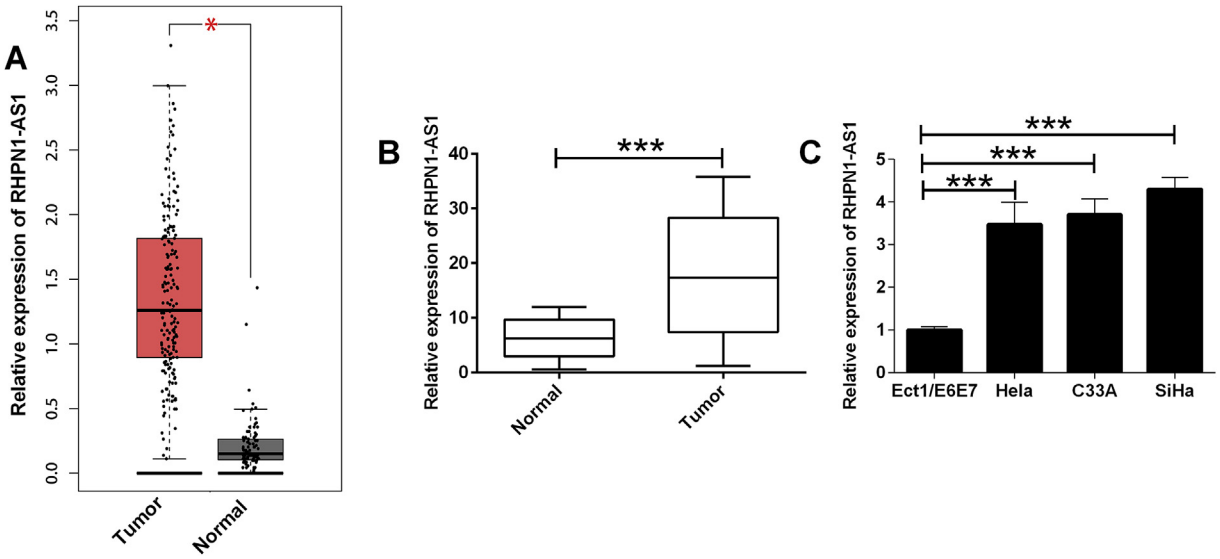

Fig. 1. Expression levels of RHPN1-AS1 in clinical tissues and cell lines. (A) GEPIA database showed that RHPN1-AS1 expression was up-regulated in cervical cancer tissues (n = 174) compared with normal cervical tissues (n = 91). (B) qRT-PCR detection showed that RHPN1-AS1 expression was up-regulated in cervical cancer tissues (n = 60) compared with normal cervical tissues (n = 60). (C) qRT-PCR analysis showed increased expression of RHPN1-AS1 in cervical cancer cell lines compared with normal cervical cells (n = 3). *P < 0.05 and ***P < 0.001.

[12]. Zheng et al., revealed that lncRNA HOTAIR promoted HeLa cell migration and invasion by regulating megakaryoblastic leukaemia 1 via inhibiting miR-206 [13]. Rui et al., showed that lncRNA C50rf66-AS1 promoted cervical cancer cell proliferation via regulating miR-637/ring finger protein 1 axis [14]. Recently, the lncRNA RHPN1-AS1 has been studied in several types of malignant tumors including uveal melanoma, lung cancer and breast cancer [15–17]. So far, the expression of RHPN1 antisense RNA1 (RHPN1-AS1) has not been reported in cervical cancer, not to mention the mechanisms underlying RHPN1-AS1 regulated cervical cancer progression.

In this study, we firstly used the data mining to identify the up-regulation of RHPN1-AS1 in cervical cancer tissues and confirmed the up-regulation of RHPN1-AS1 in the collected clinical cervical cancer tissues. Our *in vitro* functional assays further deciphered the oncogenic role of RHPN1-AS1 in regulating cervical cancer progression, and further exploration of underlying mechanisms showed that RHPN1-AS1-mediated effects on cervical cancer progression may involve in miR-299–3p/FGF2 axis.

2. Materials and methods

2.1. Clinical sample collection

A total of 60 cervical cancer tissues and the normal adjacent cervical tissues were obtained from cervical cancer patients, who underwent the surgical resection at Northwest Women and Children's Hospital between January 2016 and July 2017. All the tissues were immediately frozen in liquid nitrogen after the collecting from patients with surgical resection. Each patient signed the written informed consent, and the study was approved by the Ethics Committee of Northwest Women and Children's Hospital.

2.2. Cell culture, nucleotides and cell transfections

The normal cervical cell line (Ect1/E6E7) and the cervical cancer cell lines (HeLa, C33A and SiHa) were all purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, USA). The cells were kept in a sterilized incubator with 5% CO_2 at 37 °C.

The RHPN1-AS1 overexpressing vector (pcDNA3.1-RHPN1-AS1), fibroblast growth factor 2 (FGF2) overexpressing vector (pcDNA3.1-FGF2), along with the negative control (pcDNA3.1) were purchased from GenePharma (Shanghai, China). The mimics and inhibitors of miR-299–3p, along with their corresponding negative controls (mimics NC and inhibitors NC) were purchased from RiboBio (Guangzhou, China). The siRNAs for RHPN1-AS1 and FGF2, along with their negative controls (si-NC) were purchased from RiboBio. The transfections with plasmids, miRNAs or siRNAs were performed using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendation. At 24 h after transections, cells were collected and processed for further *in vitro* investigations.

2.3. Data mining

The data of RHPN1-AS1 expression in cervical cancer tissues were obtained from the Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/), a bioinformatics database on TCGA and GETx.

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA from cells or tissues were extracted using Trizol reagent (Invitrogen, Carlsbad, USA). For the detection of lncRNA and mRNA, reverse transcription was carried out using PrimeScript RT Master Mix kit (Takara, Dalian, China). The real-time PCR was performed on an ABI7900 system (Applied Biosystems, Foster City, USA) using SYBR Green Master Mix kit (Takara). For the analysis of miR-299–3p, miRNA specific cDNA was synthesized using the stem-loop primers and the TaqMan MicroRNA reverse Transcription Kit (Applied Biosystems) followed by running real-time PCR on an ABI7900 system (Applied Biosystems) using TaqMan MicroRNA Assays Kit (Applied Biosystems). GAPDH was used as the internal control for lncRNA and mRNA; while RNU6B was used as the internal control for miR-299–3p. Relative expression of the respective genes was calculated with the comparative threshold cycle method.

2.5. Western blot assay

Proteins from cells or samples were extracted using RIPA lysis buffer

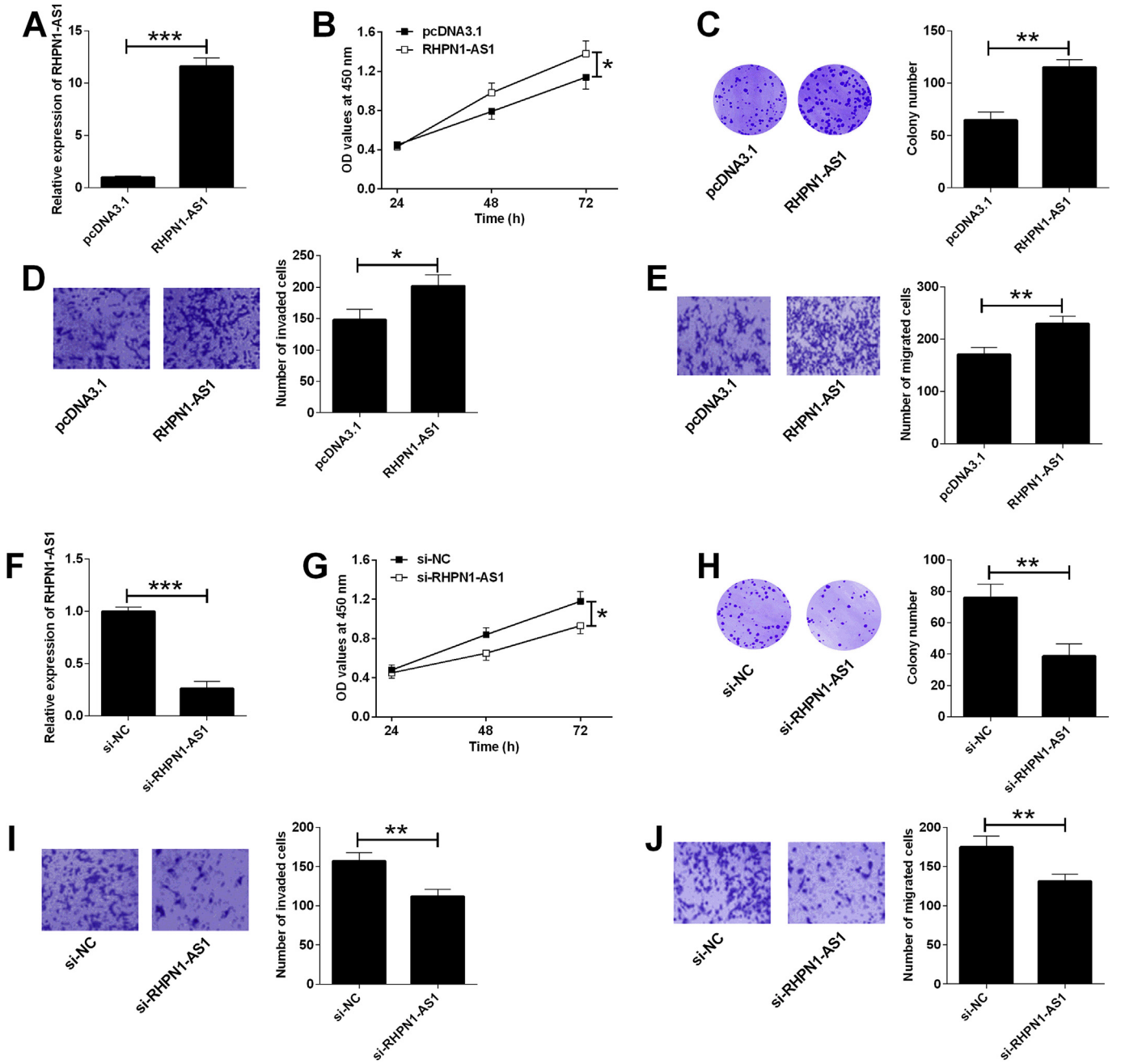

Fig. 2. Effects of RHPN1-AS1 overexpression/knockdown on cervical cancer cell proliferation, growth, invasion and migration. (A) qRT-PCR analysis showed that RHPN1-AS1 was up-regulated in SiHa cells with pcDNA3.1-RHPN1-AS1 transfection compared with pcDNA3.1 transfection. (B) Cell proliferation, (C) cell growth, (D) cell invasion and (E) migration were assessed with CCK-8, colony formation, Transwell invasion and migration assays, respectively, in SiHa cells with pcDNA3.1 or pcDNA3.1-RHPN1-AS1 transfection. (F) qRT-PCR analysis showed that RHPN1-AS1 was down-regulated in SiHa cells with si-RHPN1-AS1 transfection compared with si-NC transfection. (G) Cell proliferation, (H) cell growth, (I) cell invasion and (J) migration were assessed with CCK-8, colony formation, Transwell invasion and migration assays, respectively, in SiHa cells with si-NC or si-RHPN1-AS1 transfection. N = 3. *P < 0.05, **P < 0.01 and ***P < 0.001.

(Sigma, St. Louis, USA), and concentrations of the protein samples were measured using a BCA assay kit (Bio-Rad, Hercules, USA). Thirty micrograms of proteins were then resolved by 10% SDS-PAGE and then transferred to the PVDF membranes. The transferred membranes were then incubated with tris-buffered saline containing 0.1% Tween-20 (TBST) with 5% non-fat milk at room temperature for 1 h. After that, the PVDF membranes were again incubated with respective primary antibodies overnight at 4 °C. After washing with TBST for 3 times, the PVDF membranes were incubated with horse-radish peroxidase conjugated secondary antibodies for 2 h at room temperature. After that, the bands of the bound proteins were visualized using enhanced chemiluminescence kit (Thermo Fisher Scientific) according to the instructions of the manufacturer. Primary antibodies for FGF2 (ab208687; 1:1000) and β-actin (ab227387; 1:2000) were purchased from Abcam (Cambridge, USA).

2.6. Cell counting Kit-8 (CCK-8) assay

SiHa cell proliferation was assessed by the CCK-8 assay (Beyotime, Beijing, China) according to the protocol of the manufacturer. Briefly, SiHa cells after different treatments were plated onto the 96-well plates at a density of 1×10^5 cells/well. After culturing for the indicated time

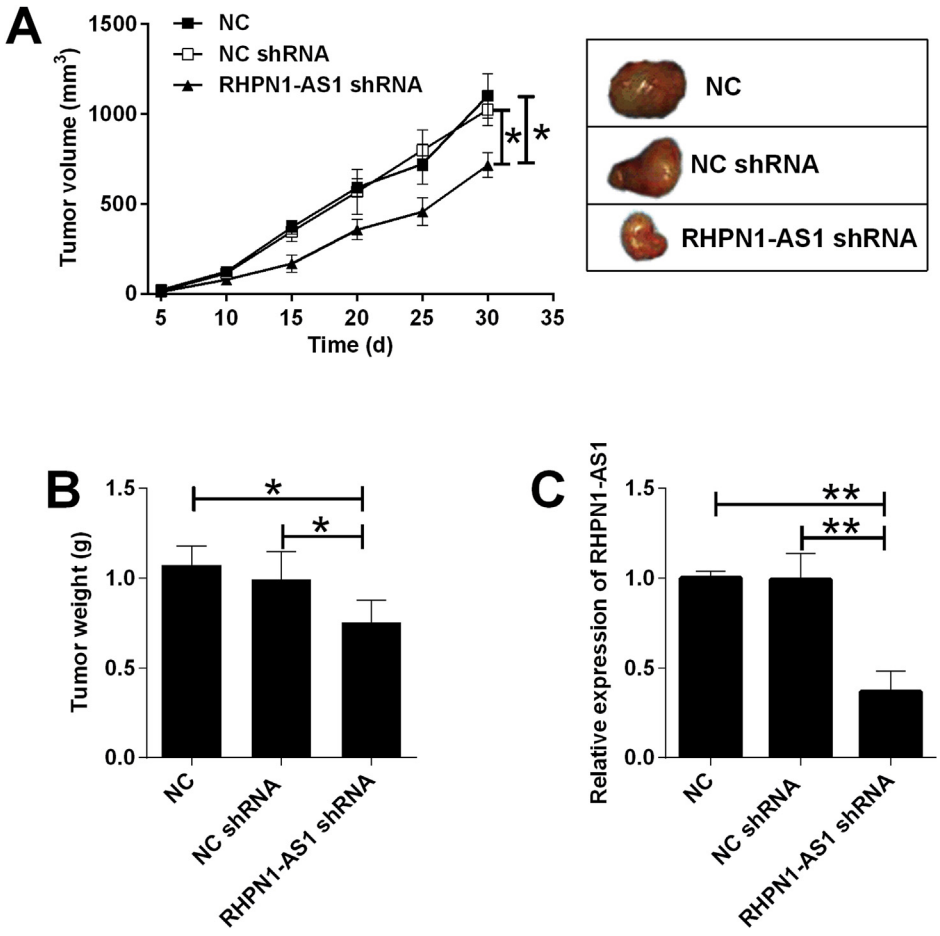

Fig. 3. RHPN1-AS1 knockdown inhibited *in vivo* tumor growth. (A) Tumor volume in the nude mice was measured every 5 days for 30 days in NC (cells without any treatment), NC shRNA and RHPN1-AS1 shRNA groups. (B) Tumor weight was measured in the excised tumors from NC, NC shRNA and RHPN1-AS1 shRNA groups. (C) qRT-PCR detection of RHPN1-AS1 expression in excised tumors from NC, NC shRNA and RHPN1-AS1 shRNA groups. N = 5. *P < 0.05, **P < 0.01 and ***P < 0.001.

points, cells were incubated with CCK-8 solution for 2 h at 37 °C. The optical density (OD) values were detected by measuring the absorbance at 450 nm on a microplate reader (Molecular Devices, San Jose, USA). The cell proliferation curves were plotted using the OD values at the indicated time points.

2.7. Colony formation assay

SiHa cells after receiving different treatments were collected by trypsinization and were plated onto the 6-well plates a density of 1000 cells/well. After culturing for 14 days, cells were fixed with 10% formaldehyde and were then stained with 0.1% crystal violet for 10 min. The number of colonies was counted under a light microscope (Olympus, Tokyo, Japan).

2.8. Transwell invasion and migration assays

SiHa cell invasion and migration were evaluated using Matrigel Tranwell and Tranwell with 8-μm pore size filter inserts (Corning Costar, New York, USA), respectively. Briefly, SiHa cells after different treatments were re-suspended in the FBS-free RMPI-1640 medium and were plated on transwell inserts of the upper chamber; while the lower chamber was filled with RMPI-1640 medium containing 20% FBS. After a further culture for 24 h, invaded and migrated cells were fixed with 10% formaldehyde, and stained with 0.1% crystal violet for 10 min. The number of invaded or migrated SiHa cells were quantified by randomly selecting five fields under a light microscope (Olympus).

2.9. In vivo tumor growth assay

All the animal studies were approved under the Institutional Animal Care and Use Committee of Northwest Women and Children's Hospital. The 4-5 week-old female nude mice were purchased from Experimental Animal Center of Xi'an Jiaotong University. The lentiviral viruses overexpressing RHPN1-AS1 shRNA, miR-299–3p mimics, NC shRNA or mimics NC were obtained from Genechem (Shanghai, China), and were infected into SiHa cells to generate the stable expressing cell lines. For the *in vivo* tumor growth assay, animals were randomly divided into different treatment groups (n = 5 for each group), and animals were received subcutaneous injection of untreated SiHa cells, SiHa cells expressing NC shRNA, RHPN1-AS1 shRNA, mimics NC or miR-299–3p mimics. Tumor size in the mice was measured every 5 days for 30 days. At the end of the experiment, animals were killed by cervical dislocation, and tumors were collected for further analysis.

2.10. Luciferase reporter assay

RHPN1-AS1 fragment and FGF2 3′ untranslated region (UTR) fragment containing the predicted binding sequences for miR-299–3p was amplified from genomic DNA using PCR and the amplified fragments were cloned into the pMIR-Report plasmids (Promega, Madison, USA) to generate reporter vector pMIR-RHPN1-AS1-wild type (WT) and pMIR-FGF2 3′UTR-WT, respectively. The corresponding mutated fragments for the binding sites were generated using a Site-Directed Mutagenesis Kit (Agilent, Santa Clara, USA) and were then used for constructing pMIR-RHPN1-AS1-mutant (MUT) and pMIR-FGF2 3′UTR –MUT. For the luciferase reporter assay, the corresponding reporter vectors were co-transfected with different miRNAs into SiHa cells using Lipofecatmine RNAiMAX reagent (Invitrogen). At 48 h following transfection, firefly and Renilla luciferase activities were assessed with the Dual-Luciferase Reporter Assay System (Promega).

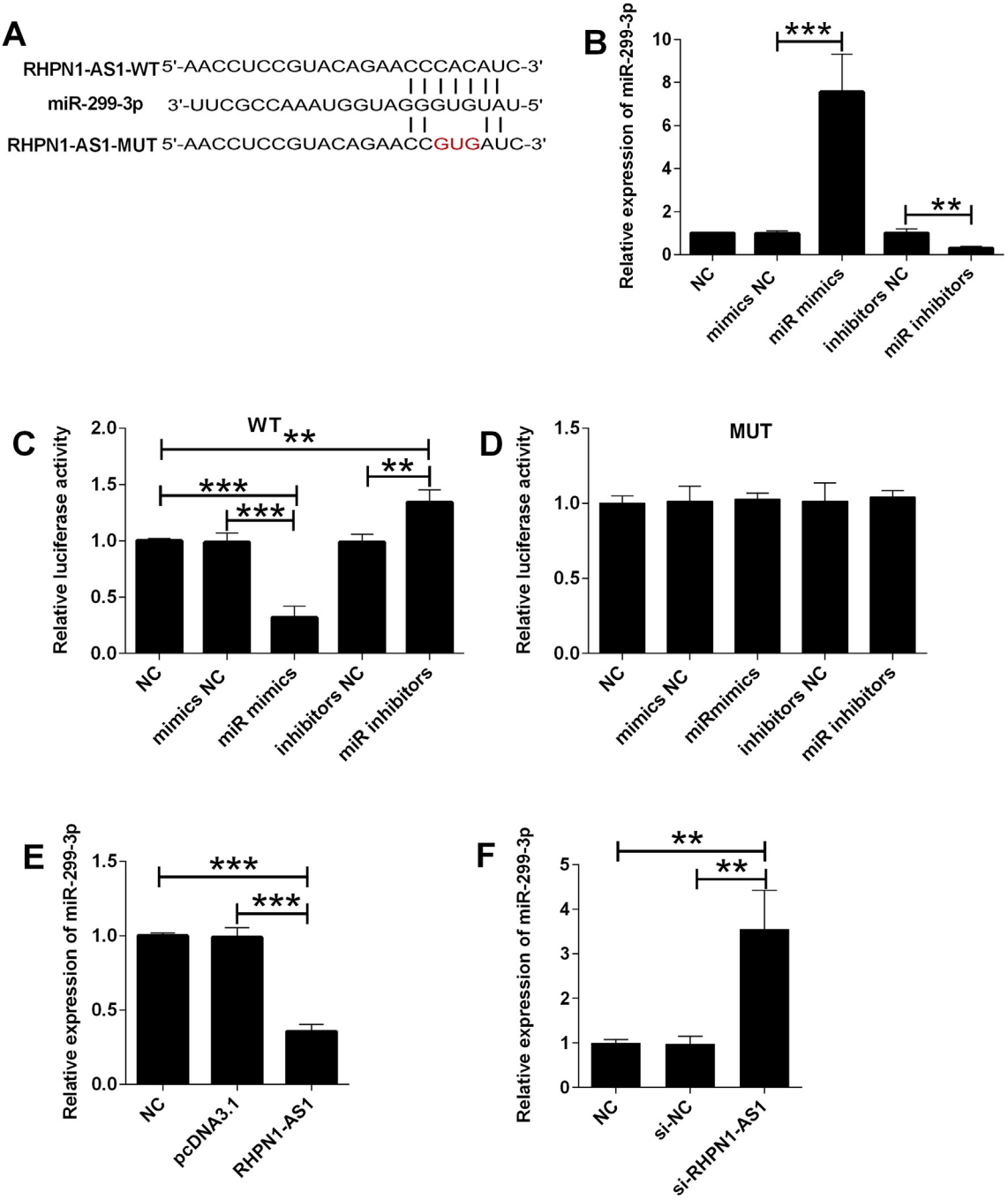

Fig. 4. RHPN1-AS1 suppressed miR-299–3p expression in cervical cancer cells. (A) StarBase V2.0 predicted complementary sequences between miR-299–3p and RHPN1-AS1. (B) qRT-PCR analysis of miR-299–3p expression in untreated SiHa cells (NC) or SiHa cells with mimics NC, miR mimics, inhibitors NC, or miR inhibitors transfection. (C) Luciferase reporter assay detected the luciferase activity of reporter vector containing RHPN1-AS1-WT in untreated SiHa cells or SiHa cells with mimics NC, miR mimics, inhibitors NC, or miR inhibitors transfection. (D) Luciferase reporter assay detected the luciferase activity of reporter vector containing RHPN1-AS1-MUT in untreated SiHa cells or SiHa cells with mimics NC, miR mimics, inhibitors NC, or miR inhibitors transfection. (E) qRT-PCR analysis showed that miR-299–3p was down-regulated in untreated SiHa cells or SiHa cells with pcDNA3.1-RHPN1-AS1 transfection compared with pcDNA3.1 transfection. (F) qRT-PCR analysis showed that miR-299–3p was up-regulated in SiHa cells with si-RHPN1-AS1 transfection compared with untreated SiHa cells or SiHa cells with si-NC transfection. N = 3. **P < 0.01 and ***P < 0.001.

2.11. Statistical analysis

All the experiments were performed at least three times and the experimental data were presented as mean ± standard deviation. Statistical analysis for different data (continuous data) sets was analysed by Student's t-test or One-way ANOVA with Dunnett's post-hoc test. Categorical data was analysed using Chi-square test. All the data analysis was performed using GraphPad Prism V5.0 (GraphPad Software, La Jolla, USA). The P value for statistical significance was set at < 0.05.

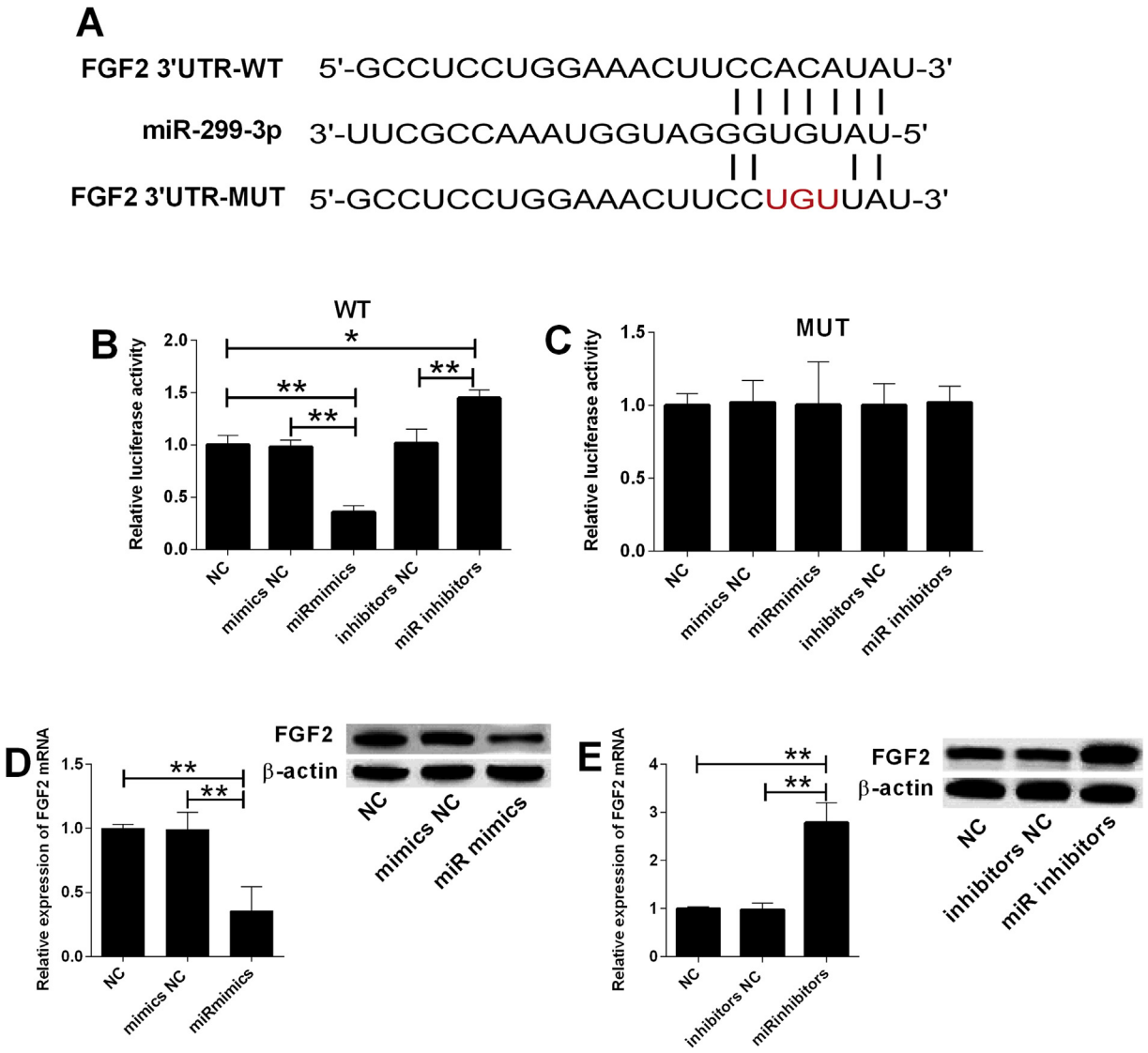

Fig. 5. MiR-299–3p suppressed FGF2 expression in cervical cancer cells. (A) TargetScan V7.2 predicted complementary sequences between miR-299–3p and FGF2 3′UTR. (B) Luciferase reporter assay detected the luciferase activity of reporter vector containing FGF2 3′UTR-WT in untreated SiHa cells (NC) or SiHa cells with mimics NC, miR mimics, inhibitors NC, or miR inhibitors transfection. (C) Luciferase reporter assay detected the luciferase activity of reporter vector containing FGF2 3′UTR-MUT in untreated SiHa cells or SiHa cells with mimics NC, miR mimics, inhibitors NC, or miR inhibitors transfection. (D) qRT-PCR and western blot analysis showed that FGF2 was down-regulated in SiHa cells with miR mimics transfection compared with untreated SiHa cells or SiHa cells with mimics NC transfection. (E) qRT-PCR and western blot analysis showed that FGF2 was up-regulated in SiHa cells with inhibitors NC transfection compared with untreated SiHa cells or SiHa cells with inhibitors NC transfection. N = 3. **P < 0.01 and ***P < 0.001.

3. Results

3.1. Expression levels of RHPN1-AS1 in cervical cancer tissues and cervical cancer cell lines

We first analysed the data from GEPIA database, and the results showed that RHPN1-AS1 expression was significantly up-regulated in cervical cancer tissues compared with normal cervical tissues (Fig. 1A). To further confirm the data mining results, we collected the clinical samples for verification. The qRT-PCR results showed that RHPN1-AS1 expression was significantly up-regulated in 60 cervical cancerous tissues compared with the corresponding adjacent normal tissues (Fig. 1B). The determination of RHPN1-AS1 expression in the normal cervical cell line (Ect1/E6E7) and the cervical cancer cell lines (HeLa, C33A and SiHa) revealed that RHPN1-AS1 was markedly up-regulated in these cervical cancer cell lines compared with Ect1/E6E7 cells (Fig. 1C).

3.2. Effects of RHPN1-AS1 up-regulation or down-regulation on in vitro cervical cancer progression

Firstly, we looked at the effects of RHPN1-AS1 overexpression on SiHa cell proliferation, growth, invasion and migration. The transient overexpression of RHPN1-AS1 was observed in SiHa cells being transfected with pcDNA3.1-RHPN1-AS1 compared with that being transfected with pcDNA3.1 (Fig. 2A). The CCK-8 assay results showed that the increased expression of RHPN1-AS1 in SiHa cells effectively enhanced cell proliferation as indicated by the increased OD values at 450 nm in the pcDNA3.1-RHPN1-AS1 group (Fig. 2B). Consistently, RHPN1-AS1 up-regulation increased the number of colonies in SiHa cells (Fig. 2C). Furthermore, Transwell invasion and migration assays were carried out to determine the metastatic potentials of SiHa cells after plasmid transfection, and SiHa cell invasive and migratory abilities were significantly enhanced by RHPN1-AS1 overexpression (Fig. 2D and E). On the other hand, the effects of RHPN1-AS1

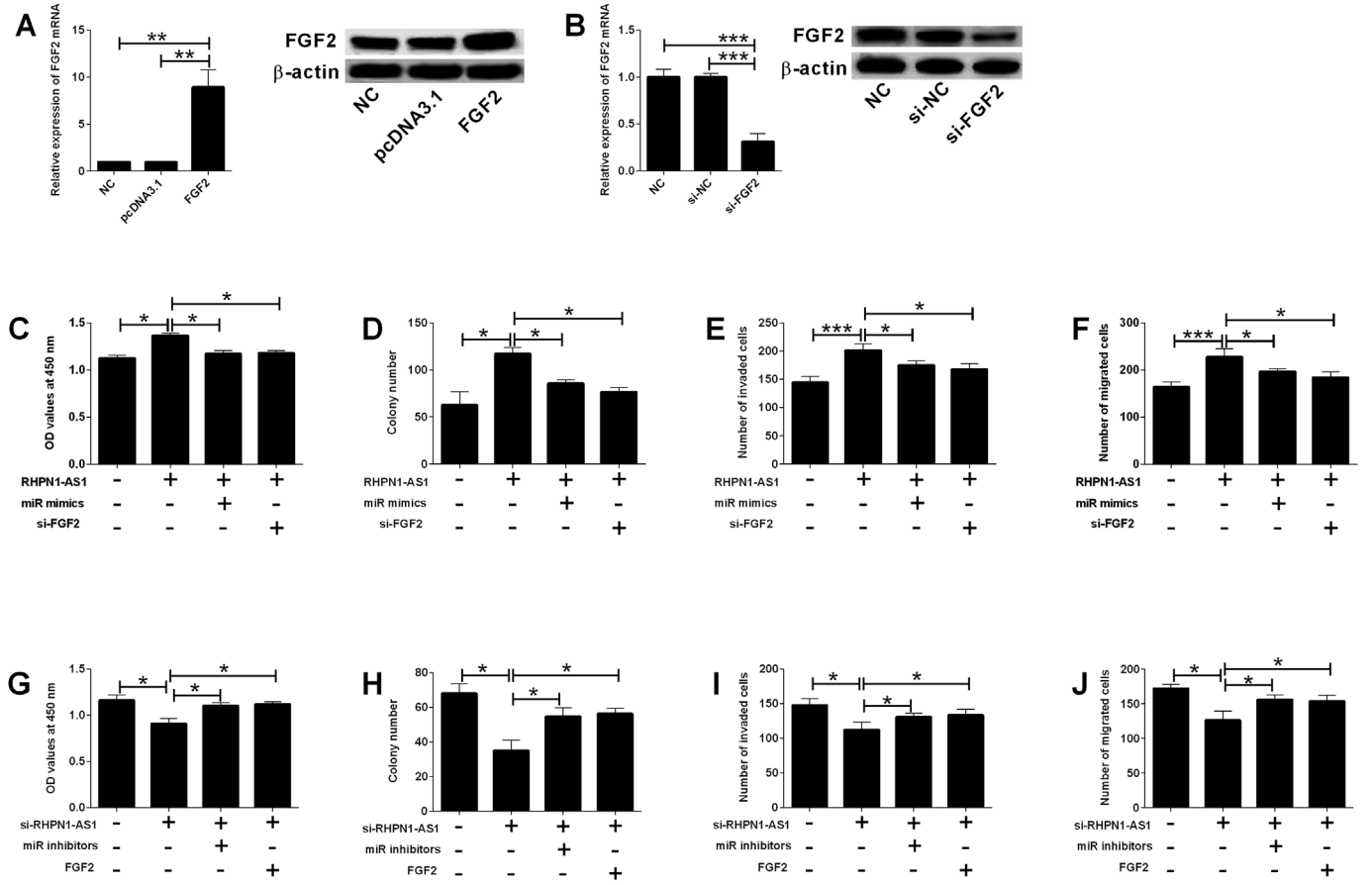

Fig. 6. RHPN1-AS1 regulated cervical cancer cell proliferation, growth, invasion and migration via targeting miR-299–3p/FGF2 axis. (A) qRT-PCR analysis showed that FGF2 was up-regulated in SiHa cells with pcDNA3.1-FGF2 transfection compared with untreated SiHa cells (NC) or SiHa cells with pcDNA3.1 transfection. (B) qRT-PCR analysis showed that FGF2 was down-regulated in SiHa cells with si-FGF2 transfection compared with untreated SiHa cells (NC) or SiHa cells with si-NC transfection. (C) Cell proliferation, (D) cell growth, (E) cell invasion and (F) migration were assessed with CCK-8, colony formation, Transwell invasion and migration assays, respectively, in SiHa cells co-transfected with pcDNA3.1-RHPN1-AS1 + miR mimics, pcDNA3.1-RHPN1-AS1 + si-FGF2 or the respective controls. (G) Cell proliferation, (H) cell growth, (I) cell invasion and (J) migration were assessed with CCK-8, colony formation, Transwell invasion and migration assays, respectively, in SiHa cells co-transfected with si-RHPN1-AS1 + miR inhibitors, si-RHPN1-AS1 + pcDNA3.1-FGF2 or their respectively controls. N = 3. *P < 0.05, **P < 0.01 and ***P < 0.001.

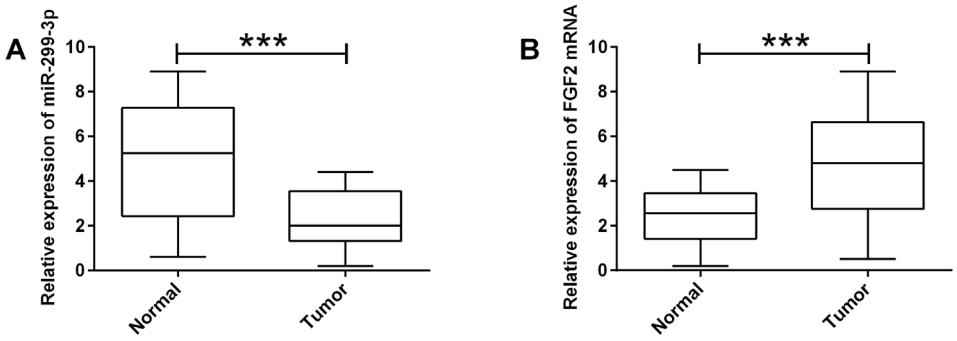

Fig. 7. Expression levels of miR-299–3p and FGF2 in clinical tissues. (A) qRT-PCR detection showed that miR-299–3p expression was down-regulated in cervical cancer tissues (n = 60) compared with normal cervical tissues (n = 60). (B) qRT-PCR detection showed that FGF2 expression was up-regulated cervical cancer tissues (n = 60) compared with normal cervical tissues (n = 60). ***P < 0.001.

knockdown on SiHa cell progression was also determined. Expectedly, the RHPN1-AS1 siRNA (si-RHPN1-AS1) transfection markedly reduced RHPN1-AS1 expression in SiHa cells compared with si-NC transfection group (Fig. 3F). Further *in vitro* functional assays demonstrated that RHPN1-AS1 knockdown effectively suppressed cell proliferation, growth, invasion and migration in SiHa cells (Fig. 2G–J).

3.3. Effects of RHPN1-AS1 down-regulation on in vivo tumor growth of SiHa cells

The *in vivo* effects of RHPN1-AS1 down-regulation on tumor growth of SiHa cells were evaluated using a xenograft nude mice model. As shown in Fig. 3A, the tumor growth in the RHPN1-AS1 shRNA group showed much slower growth rates than that in the NC (cells without any treatment) and NC shRNA group. The examination of the excised tumor showed that tumor weight was significantly reduced in RHPN1-AS1 shRNA group compared with NC and NC shRNA group (Fig. 3B), and the expression of RHPN1-AS1 was down-regulated in RHPN1-AS1 group compared with NC and NC shRNA group (Fig. 3C).

3.4. RHPN1-AS1 targeted miR-299–3p and suppressed its expression in SiHa cells

The StarBase V2.0 database predicted the potential binding sites between RHPN1-AS1 and miR-299–3p (Fig. 4A). To confirm this potential binding, we generated the luciferase reporter vector containing RHPN1-AS1-WT or RHPN1-AS1-MUT (Fig. 4A). The SiHa cells were treated with miR-299–3p mimics (miR mimics) or miR-299–3p inhibitors (miR inhibitors) to, respectively, up-regulated or down-regulated miR-299–3p expression (Fig. 4B), and up-regulation of miR-299–3p suppressed the luciferase activity of the reporter vector with RHPN1-AS1-WT; while down-regulation of miR-299–3p increased luciferase activity for the WT vector (Fig. 4C). Moreover, the luciferase activity of the MUT vector in SiHa cells was not affected by miR-299–3p overexpression or knockdown (Fig. 4D). To confirm the findings from luciferase reporter assay, we performed qRT-PCR to observe if RHPN1-AS1 could inversely regulated miR-299–3p expression in SiHa cells. RHPN1-AS1 overexpression caused a significant down-regulation of miR-299–3p in SiHa cells (Fig. 4E); while RHPN1-AS1 siRNA treatment induced an up-regulation of miR-299–3p in SiHa cells (Fig. 4F).

3.5. MiR-299–3p targeted FGF2 3′UTR and down-regulated FGF2 expression in SiHa cells

The TargetScan database predicted the potential binding sites between miR-299–3p and FGF2 3′UTR (Fig. 5A). To confirm this potential binding, we generated the luciferase reporter vector containing FGF2 3′UTR-WT or FGF2 3′UTR-MUT (Fig. 5A). Up-regulation of miR-299–3p suppressed the luciferase activity of the reporter vector with FGF2 3′UTR-WT; while down-regulation of miR-299–3p increased luciferase activity for the WT vector (Fig. 5B). Luciferase activity of the MUT vector in SiHa cells was not affected by miR-299–3p overexpression or knockdown (Fig. 5C). Furthermore, qRT-PCR and western blot assays showed that miR-299–3p mimics transfection down-regulated, while miR-299–3p inhibitor transfection up-regulated the expression of FGF2 in SiHa cells (Fig. 5D and E).

3.6. RHPN1-AS1 regulated cervical cancer cell proliferation, growth, invasion and migration via targeting miR-299–3p/FGF2 axis

The overexpression of miR-299–3p showed the inhibitory effects on the cervical cancer cell proliferation, growth, invasion and migration as revealed by CCK-8, colony formation, transwell invasion and migration assays, respectively (Supplementary Fig. S1), and suppressed the *in vivo* tumor growth of the nude mice (Supplementary Fig. S1). Thus, the rescue experiments were performed to confirm the involvement of miR-299–3p/FGF2 axis in RHPN1-AS1-mediated actions in SiHa cells. The up-regulation and down-regulation of FGF2 in SiHa cells were achieved via transfection with FGF2 overexpressing vector and FGF2 siRNA, respectively. (Fig. 6A and B). The *in vitro* functional assays revealed that the enhanced effects of RHPN1-AS1 overexpression on cell proliferation, growth, invasion and migration in SiHa cells were significantly attenuated by miR-299–3p overexpression or FGF2 inhibition (Fig. 6C–F). On the other hand, Knockdown of miR-299–3p and overexpression of FGF2 both significantly increased cell proliferation, growth, invasion and migration in SiHa cells transfected with RHPN1-AS1 siRNA (Fig. 6G–J).

3.7. Expression levels of miR-299–3p and FGF2 in clinical tissues

Furthermore, the expression levels of miR-299–3p and FGF2 were confirmed in the cervical cancer tissues. As shown in Fig. 7A, miR-299–3p expression as determined by qRT-PCR was significantly down-regulated in 60 cervical cancer tissues compared with normal adjacent cervical tissues (Fig. 7A); while FGF2 mRNA expression level was significantly higher in the cancerous tissues than that in normal adjacent tissues (Fig. 7B).

4. Discussion

Recently, various lncRNAs have been demonstrated to have regulatory functions in cervical cancer progression [7], suggesting the important role in the pathophysiology of cervical cancer. In this regard, further exploration of novel lncRNAs in cervical cancer progression may provide us with a better strategy for cervical cancer treatment. Here, we showed the up-regulation of RHPN1-AS1 in cervical cancer tissues compared with the normal cervical tissues and the up-regulation of RHPN1-AS1 in cervical cancer cell lines. *In vitro* functional assays showed that RHPN1-AS1 overexpression promoted cervical cancer cell proliferation, growth, invasion and migration; while RHPN1-AS1 knockdown exerted tumor-suppressive actions in SiHa cells. *In vivo* animal studies showed that RHPN1-AS1 knockdown suppressed tumor growth of SiHa cells. Mechanistically, further *in vitro* studies revealed that RHPN1-AS1 regulated cervical cancer progression via modulation FGF2 expression by acting as a sponge for miR-299–3p.

RHPN1-AS1 gene is located at the chromosome 8q24.3 and its functional role has been not widely documented. The role of RHPN1-AS1 in cancer was first reported by Lu et al., showing that RHPN1-AS1 was highly expressed in uveal melanoma cancerous tissues and cell lines, and RHPN1-AS1 functioned as an oncogene to promote uveal melanoma progression [16]. In addition, RHPN1-AS1 showed higher expression levels in human breast cancer tissues and cell line MCF-7; further mechanistic investigations showed that RHPN1-AS1 promoted tumorigenesis by regulating p53 expression via mouse double minute 2 homolog gene [17]. Importantly, bioinformatics analysis revealed that RHPN1-S1 were significantly correlated with the pathogenesis, development and metastasis of cancers [18]. Consistently, our study first identified the up-regulation of RHPN1-AS1 using data mining, which was further confirmed in clinical samples. More importantly, overexpression of RHPN1-AS1 promoted SiHa cell proliferation, invasion and migration *in vitro*; while RHPN1-AS1 knockdown inhibited SiHa cell proliferation, invasion and migration *in vitro* and tumor growth of SiHa cells *in vivo*. Collectively, these data suggested the oncogenic role of RHPN1-AS1 in cervical cancer.

The mechanisms for lncRNAs acting as competing endogenous RNAs (ceRNAs) for miRNAs have been reported in various studies. Not surprisingly, many lncRNAs such as HOTAIR, MALAT1, XIST and so on have been demonstrated as ceRNAs in regulating cervical cancer progression [19–21]. In this study, we predicted miR-299–3p as a target of RHPN1-AS1 using StarBase V2.0, where the interaction between miR-299–3p and RHPN1-AS1 was confirmed by luciferase reporter assay. MiR-299–3p has been found to be dysregulated in several types of cancers including colon cancer, liver cancer and thyroid cancer. Among these types of cancers, miR-299–3p commonly acted as a tumor suppressor. MiR-299–3p could suppress colon carcinoma progression via targeting vascular endothelial growth factor A [22]. MiR-299–3p also functioned as a tumor suppressor in liver cancer via modulation of Sirtuin 5 [23]. MiR-299–3p was also found to be down-regulated in thyroid cancer and inhibited thyroid cancer progression via targeting Soc-2 suppressor of clear homolog gene [24]. In our study, we found that miR-299–3p was inversely regulated by RHPN1-AS1. Furthermore, miR-299–3p overexpression or inhibition counteracted the effects of RHPN1-AS1 up-regulation or down-regulation on the cancer cell progression. All in all, our data implied that RHPN1-AS1 regulated cervical cancer cell proliferation via sponging miR-299–3p.

FGF2 is a member of the fibroblast growth factors family, and FGF2 could bind to receptor tyrosine kinases FGFR1–FGFR4 to exert its functional roles including modulation of the morphogenic and mitogenic pathways [25–27]. In our study, using TargetScan tool, FGF2 was predicted to be a target of miR-299–3p, which was confirmed by luciferase reporter assay. More importantly, FGF2 was negatively regulated by miR-299–3p in SiHa cells. The role of FGF2 has been deciphered in

various types of cancers including cervical cancer. FGF2 expression could be induced by chemoradiation in patients with cervical cancer. FGF2 could be upregulated by HPV16 E6/7 stimulation, which contributed to the progression of cervical carcinogenesis [28]. Zhang et al., showed that FGF2 was up-regulated in cervical carcinoma, and FGF2 treatment enhanced HeLa cell proliferation [29]. In addition, activation of FGF2 could trigger cancer stem cell-like properties in cervical cancer [30]. Our rescue experiments showed that inhibition of FGF2 attenuated the enhanced effects of RHPN1-AS1 overexpression on cervical cancer cell proliferation; while overexpression of FGF2 promoted cervical cancer cell progression in SiHa cells with RHPN1-AS1 knockdown. Consistently, miR-299–3p down-regulated and FGF2 was up-regulated in cervical cancer tissues. Collectively, these results may suggest the involvement of miR-299–3p/FGF2 in the RHPN1-AS1 mediated effects.

The investigation of clinical cervical cancer tissues was limited to qRT-PCR determination of RHPN1-AS1 and miR-299–3p expression, and further experiments such as RNA fluorescence *in situ* hybridization and Northern blotting should be performed to verify the expression pattern in RHPN1-AS1 and miR-299–3p in clinical samples. RHPN1-AS1 may exert the effects on cervical cancer cells via other signalling pathways, and future directions may use high-throughput techniques to explore more signalling pathways that affected by RHPN1-AS1. Whether RHPN1-AS1 has a prognostic role in patients with cervical cancer still require further follow-up study.

In conclusion, the present study showed that up-regulation of RHPN1-AS1 in cervical cancer tissues and cell lines, and the *in vitro* and *in vivo* results revealed that RHPN1-AS1 promoted cervical cancer progression via targeting miR-299–3p/FGF2 axis. Our data suggested that RHPN1-AS1/miR-299–3p/FGF2 axis may be a promising target for cervical cancer treatment.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116856.

References

[1] P. Tsikouras, S. Zervoudis, B. Manav, E. Tomara, G. Iatrakis, C. Romanidis, et al., Cervical cancer: screening, diagnosis and staging, J. BUON: Off. J. Balkan Union Oncol. 21 (2016) 320–325.
[2] S.Y. Brucker, U.A. Ulrich, Surgical treatment of early-stage cervical cancer, Oncol. Res. Treat. 39 (2016) 508–514.
[3] S.P. Somashekhar, K.R. Ashwin, Management of early stage cervical cancer, Rev. Recent Clin. Trials 10 (2015) 302–308.
[4] A. Duenas-Gonzalez, S. Campbell, Global strategies for the treatment of early-stage and advanced cervical cancer, Curr. Opin. Obstet. Gynecol. 28 (2016) 11–17.
[5] R.N. Eskander, K.S. Tewari, Immunotherapy: an evolving paradigm in the treatment of advanced cervical cancer, Clin. Ther. 37 (2015) 20–38.
[6] K.S. Pfaendler, K.S. Tewari, Changing paradigms in the systemic treatment of advanced cervical cancer, Am. J. Obstet. Gynecol. 214 (2016) 22–30.
[7] H. Aalijahan, S. Ghorbian, Long non-coding RNAs and cervical cancer, Exp. Mol. Pathol. 106 (2019) 7–16.
[8] E.S. Hosseini, M. Meryet-Figuiere, H. Sabzalipoor, H.H. Kashani, H. Nikzad, Z. Asemi, Dysregulated expression of long noncoding RNAs in gynecologic cancers, Mol. Cancer 16 (2017) 107.
[9] D. Shi, C. Zhang, X. Liu, Long noncoding RNAs in cervical cancer, J. Cancer Res. Ther. 14 (2018) 745–753.
[10] M. Zhao, Y. Qiu, B. Yang, L. Sun, K. Hei, X. Du, et al., Long non-coding RNAs involved in gynecological cancer, Int. J. Gynecol. Cancer : Off. J. Int. Gynecol. Cancer Soc. 24 (2014) 1140–1145.
[11] X. Mao, X. Qin, L. Li, J. Zhou, M. Zhou, X. Li, et al., A 15-long non-coding RNA signature to improve prognosis prediction of cervical squamous cell carcinoma, Gynecol. Oncol. 149 (2018) 181–187.
[12] C. Shang, W. Wang, Y. Liao, Y. Chen, T. Liu, Q. Du, et al., LNMICC promotes nodal metastasis of cervical cancer by reprogramming fatty acid metabolism, Cancer Res. 78 (2018) 877–890.
[13] P. Zheng, Z. Yin, Y. Wu, Y. Xu, Y. Luo, T.C. Zhang, LncRNA HOTAIR promotes cell migration and invasion by regulating MKL1 via inhibition miR206 expression in HeLa cells, Cell Commun. Signal. : CCS 16 (2018) 5.
[14] X. Rui, Y. Xu, X. Jiang, W. Ye, Y. Huang, J. Jiang, Long non-coding RNA C5orf66-AS1 promotes cell proliferation in cervical cancer by targeting miR-637/RING1 axis, Cell Death Dis. 9 (2018) 1175.
[15] X. Li, X. Zhang, C. Yang, S. Cui, Q. Shen, S. Xu, The lncRNA RHPN1-AS1 down-regulation promotes gefitinib resistance by targeting miR-299-3p/TNFSF12 pathway in NSCLC, Cell Cycle 17 (2018) 1772–1783.
[16] L. Lu, X. Yu, L. Zhang, X. Ding, H. Pan, X. Wen, et al., The long non-coding RNA RHPN1-AS1 promotes uveal melanoma progression, Int. J. Mol. Sci. 18 (2017).
[17] P. Zhu, Y. Li, P. Li, Y. Zhang, X. Wang, c-Myc induced the regulation of long non-coding RNA RHPN1-AS1 on breast cancer cell proliferation via inhibiting P53, Mol. Genet. Genom.: MGG 295 (5) (2019) 1219–1229.
[18] C. Xu, Y. Ping, H. Zhao, S. Ning, P. Xia, W. Wang, et al., LncNetP, a systematical lncRNA prioritization approach based on ceRNA and disease phenotype association assumptions, Oncotarget 8 (2017) 114603–114612.
[19] X. Chen, D. Xiong, L. Ye, K. Wang, L. Huang, S. Mei, et al., Up-regulated lncRNA XIST contributes to progression of cervical cancer via regulating miR-140-5p and ORC1, Cancer Cell Int. 19 (2019) 45.
[20] X. Guo, H. Xiao, S. Guo, J. Li, Y. Wang, J. Chen, et al., Long noncoding RNA HOTAIR knockdown inhibits autophagy and epithelial-mesenchymal transition through the Wnt signaling pathway in radioresistant human cervical cancer HeLa cells, J. Cell. Physiol. 234 (2019) 3478–3489.
[21] F. Shen, H. Zheng, L. Zhou, W. Li, X. Xu, Overexpression of MALAT1 contributes to cervical cancer progression by acting as a sponge of miR-429, J. Cell. Physiol. 234 (2019) 11219–11226.
[22] J.Y. Wang, J.B. Jiang, Y. Li, Y.L. Wang, Y. Dai, MicroRNA-299-3p suppresses proliferation and invasion by targeting VEGFA in human colon carcinoma, Biomed. pharmacother. = Biomedecine & pharmacotherapie 93 (2017) 1047–1054.
[23] S. Dang, J. Zhou, Z. Wang, K. Wang, S. Dai, S. He, MiR-299-3p functions as a tumor suppressor via targeting Sirtuin 5 in hepatocellular carcinoma, Biomed. pharmacother. = Biomedecine & pharmacotherapie 106 (2018) 966–975.
[24] X. Chen, M. Qi, Q. Yang, J.Y. Li, MiR-299-3p functions as a tumor suppressor in thyroid cancer by regulating SHOC2, Eur. Rev. Med. Pharmacol. Sci. 23 (2019) 232–240.
[25] H. Goto, Y. Nishioka, Fibrocytes: a novel stromal cells to regulate resistance to anti-angiogenic therapy and cancer progression, Int. J. Mol. Sci. 19 (2017).
[26] M. Katoh, Cancer genomics and genetics of FGFR2 (Review), Int. J. Oncol. 33 (2008) 233–237.
[27] J.P. Steringer, W. Nickel, The molecular mechanism underlying unconventional secretion of Fibroblast Growth Factor 2 from tumour cells, Biol. Cell 109 (2017) 375–380.
[28] Y.M. Cheng, C.Y. Chou, Y.C. Hsu, M.J. Chen, Influence of HPV16 E6/7 on the expression of FGF2 and FGFR type B in cervical carcinogenesis, Reprod. Sci. 19 (2012) 580–586.
[29] Q.H. Zhang, P. Xu, Y.X. Lu, H.T. Dou, Acidic and basic fibroblast growth factor expression levels in cervical cancer and their effects on tumor cell proliferation, Genet. Mol. Res. : GMR 15 (2016).
[30] K.H. Song, H. Cho, S. Kim, H.J. Lee, S.J. Oh, S.R. Woo, et al., API5 confers cancer stem cell-like properties through the FGF2-NANOG axis, Oncogenesis 6 (2017) e285.