miR-338-3p suppresses progression of gastric cancer through PTEN-AKT signaling pathways by targeting P-REX2a

Running Title: miR-338-3p targets P-Rex2a pathway in GC

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Abstract:
Aberrant expression of miRNAs correlates with various cancers. Although miR-338-3p has been implicated in hepatocellular carcinoma, its role is unknown in gastric cancer. To determine whether and how miR-338-3p influences gastric cancer progression, we report that miR-338-3p is down-regulated in both tissues and cell lines of gastric cancer. Forced expression of miR-338-3p could inhibit cell proliferation, clonogenicity, and induce G1/S arrest, apoptosis in BGC-823 cells. Furthermore, P-Rex2a is identified as a direct target of miR-338-3p, and silencing P-Rex2a results in the same trend with the effects of miR-338-3p in BGC-823 cells. Both enforcement of the miR-338-3p and silence of P-Rex2a could reduce the expression of P-Rex2a which results in activation of PTEN, leading to a decline in phosphorylation of AKT. Also, enforcement of miR-338-3p could remarkably inhibit the tumorigenicity of BGC-823 cells in the nude mouse xenograft model. These results demonstrate that miR-338-3p could affect gastric cancer progression through PTEN-AKT signaling pathways by targeting in P-Rex2a in gastric cancer cells, which provides that miR-338-3p might be a novel potential strategy for gastric cancer treatment.

Implications: We demonstrated that miR-338-3p may be a novel tumor-suppressor that blocks the growth of gastric cancer cells through PTEN/PI3K signalling pathways by targeting P-Rex2a firstly.

Keywords: miR-338-3p; gastric cancer; P-Rex2a; PTEN-AKT signaling pathways
Introduction

Gastric cancer (GC) is the fourth most common human malignant disease and the second most frequent cause of cancer-related death worldwide. Almost two-thirds of cases occurred in developing countries, of which 42% occurred in China (1). Currently, treatment for gastric cancer may include surgery, chemotherapy, radiation therapy or multimodality therapy (2). Unfortunately, gastric cancer is difficult to cure because the disease is usually advanced before the diagnosis. Thus, new treatment approaches such as genome therapy (3), photodynamic therapy (4, 5) and therapeutic miRNA (6) worth being further studied.

MicroRNAs (miRNAs) are small, non-coding RNAs of 19-24 nucleotides in length, which could bind at the 3’-untranslated region of potentially hundreds of target genes imperfect complementarity, resulting in degradation of target mRNAs and inhibition of translation (7). miRNAs dysfunction has been found in variety of human diseases such as cancer. Recent evidences have strongly supported that miRNAs played a part in crucial cellular processes, including development, differentiation, apoptosis and proliferation (8). miRNA-135a contributes to breast cancer cell migration and invasion by targeting HOXA10 (9), while miR-10b promotes cell migration and invasion in breast cancer cells by targeting HOXD10 (10). Besides, miRNA-223 leads to promotion of gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3 (11).

In our recent work, miR-338-3p showes a strongly down expression in gastric
cancer patients which indicates its potential role as a tumor suppressor in cancer progression. The previous studies proved that miR-338 is downregulated in hepatocellular carcinoma (12) and malignant melanoma (13). In addition, miR-338-3p could suppress progression of cancer cell by targeting smoothened in both liver cancer (14) and colorectal carcinoma (15). Recently, Hepatitis B virus X protein (HBx) was found associated with the down-regulated expression of miR-338-3p, which could inhibits proliferation by regulating CyclinD1 in liver cancer (16, 17). However, its role in gastric cancer is unclear until now. Meanwhile, we found phosphatidylinositol 3,4,5-trisphosphate RAC exchanger 2a (P-REX2a) had an over-expression in gastric cancer compared to normal tissues. By using gene target prediction databases (miRanda and TargetScan), we found that P-REX2a might be a potential target of miR-338-3p. P-REX2a is a guanine nucleotide exchange factor (GEF) for the RAC guanosine triphosphatase (GTPase), acting as a PTEN-interacting protein, which should activate the PI3K pathway by antagonizing PTEN in cancer cells (18). For the first time, we tested the hypothesis that miR-338-3p should suppress gastric cancer cell growth by targeting P-REX2a.

Materials and Methods

Human tissue samples and cell lines

Tissues were obtained from patients who had undergone surgical gastric resection at the First Affiliated Hospital of Xi’an Jiaotong University (Informed consent was obtained from each patient and was approved by the Institute Research
Ethics Committee at Cancer Center, Xi’an Jiaotong University). The immortalized gastric epithelial cell lines (GES-1) and human gastric cancer cell lines, BGC-823 and AGS, were maintained in the Key Laboratory of Environment and Genes Related to Diseases at Xi’an Jiaotong University College of Medicine. These cells were cultivated in DMEM medium (PAA, Austria) supplemented with 10% fetal bovine serum (PAA, Austria) at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Real-time reverse transcription (qRT) polymerase chain reaction (PCR)

Total RNA was extracted from the cells and tissues with TRIzol reagent (Invitrogen, USA). PrimeScript® RT reagent Kit and SYBR® Premix Ex Taq™ II kit were purchased from TAKARA (Dalian, China) for the detection of mature miR-338-3p expression and mRNA expression. The primer for miRNA was purchased from AuGCT Corporation as shown in supplementary table 2. According to the manufacturer’s instructions, the 2^-ΔΔCt method was used in qRT-PCR with the RNU6B (U6) gene as a control for miRNAs, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a control for mRNAs. All reactions were performed in triplicate using the IQ-5 Real-Time PCR System (Bio-Rad, USA).

Expression vector construction

The miR-338-3p expression vector (pre-miR-338-3p) and control vector were constructed with synthetic oligonucleotides and cloned in between the ECORI and HindIII sites of the pcDNA6.2-GW/EmGFP vector (Invitrogen, USA). The 3’untranslated region (3’-UTR) of human P-REX2a mRNA was constructed by synthetic oligonucleotides and cloned in between the SacI and XhoI sites of the
pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA). The inhibitor of miR-338-3p and small interfering RNA (siRNA) targeting P-REX2a were purchased from Bio-technology Company (GenePharma, China). All the vector sequence information were shown in supplementary table 2.

**Bioinformatic analysis**

The information of human miR-338-3p was registered and obtained in miRBase (http://www.mirBase.org/). Prediction of miRNA targets was acquired from three publicly available programs: TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/) and PicTar (http://pictar.mdc-berlin.de/).

**Cell proliferation assay**

The BGC-823 cells (5000 cells/well) were seeded into 96-well plates with 100μl of DMEM and incubated for 24 h. Thereafter, 0.2μg of the DNA vectors or siRNAs were transfected respectively and the cells were further cultivated for additional 1–3 days. Cell viability was assessed using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay FLUOstar OPTIMA (BMG). Each experiment contained three replicates and was repeated at least twice. The data were summarized as mean ± s.d.

**Cell apoptosis analysis**

Cell apoptosis analysis was performed with Annexin-V FITC Apoptosis Detection Kit (Invitrogen, USA) according to the manufacturer’s instructions. The cells were seeded into 12-well plates at a density of 1×10^6 cells/well in triplicate and transfected with DNA vectors or siRNAs for 48h, and then examined by using a flow
cytometer (Becton, USA), and the apoptosis populations were determined by ModFit software.

**Cell cycle analysis**

The BGC-823 cells at $1 \times 10^6$ cells/well were cultured in 12-well plates in triplicate and transfected with DNA vectors or siRNAs for 48h. The cells were harvested by trypsinization and washed in PBS and fixed in ice-cold ethanol overnight at 4 °C. The cells were then washed in PBS and incubated in 1ml staining solution (20ug/ml propidium iodide and 10U/ml RNaseA) for 30 min at room temperature. And cell cycle distributions were assayed by fluorescence-activated cell sorting based on flow cytometer.

**Dual luciferase assay**

PmirGLO-P-Rex2a-3’UTR vector was co-transfected with miR-338-3p into HEK293 cell lines, pmirGLO-vector as their control. Then, the cells were harvested and lysed for luciferase assays 24 hours after transfection. Dual-Luciferase Reporter Assay System (Promega) was utilized to measure the reporter activity according to the manufacturer's protocol.

**Colony formation assay**

Stably transfected cells were seeded at a density of 10,000 per 6-well plate, incubated for two weeks, and then were stained with 0.5% crystal violet for 30 minutes. Excess dye was rinsed off twice with PBS. The number of colonies was counted by computer software.

**Western Blot**
Total protein was extracted by using a RIPA cell lysis buffer (Wolsen, China) from cells harvested 72h after transfection, separated in 10% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, USA). After that, mouse polyclonal anti-P-Rex2a (Antibody dilutions:1ug/ml), rabbit mAb anti-PTEN (Antibody dilutions:1:1000), rabbit mAb anti-Akt (Antibody dilutions:1:1000), rabbit mAb anti-Phospho-Akt (Ser473) (Antibody dilutions:1:2000), rabbit mAb anti-CCND1/p-CCND1 (Antibody dilutions:1:1000), rabbit mAb anti-CDK2 (Antibody dilutions:1:500), rabbit mAb anti-Bax (Antibody dilutions:1:1000), rabbit mAb anti-Bcl2 (Antibody dilutions:1:1000), and mouse monoclonal anti-GAPDH antibody (Antibody dilutions:1:2000) (Cell Signaling Technology, USA) were employed for detection in ECL chemiluminescent detection system (Pierce, USA). The blots were scanned and the band density was measured on the Quantity One imaging software.

**Immunohistochemistry**

The human tumor tissues was made into paraffin sections and pretreated with microwave, blocked, and incubated in the primary antibodies (P-Rex2a; CST). Then the sections were incubated with secondary antibody conjugated with horseradish peroxidase (ZSBIO) and developed with ABC (ZSBIO) and dianiminobenzidine reagent (BOSTER). Finally, Digital images were taken with a Leica Photo Microscope (Leica, Germany).

**In vivo tumor xenograft model**

Six-week-old male nude mice (BALB/c-nude) were used to examine
tumorigenicity. BGC-823 stable cells overexpressing miR-338-3p or vector control cells were established by lentivirus vector and resuspension solution of 1x10^6 were injected into the nude mice. Tumor size was measured every three days. For end-point experiments, the bioluminescence images \textit{in vivo} were obtained by the system of photobiology (Xenogen, USA).

\textbf{Statistics}

Each experiment was repeated at least three times independently. The data were presented as mean ± SD and analyzed using PASW Statistics 18 software (IBM, USA). Differences or correlations between two groups were calculated with Student’s \(t\) , chi-square tests or Pearson’s correlation. \(P\leq0.05\) was considered significant.

\textbf{Results}

\textbf{Aberrant miR-338-3p expression in human gastric cancer}

To validate the expression of miR-338-3p in gastric cancer, we analyzed the expression in 53 pairs of gastric cancer tissue and matched adjacent normal tissue samples by using Real-time PCR. Compared with their normal tissues, significant downregulation of miR-338-3p was observed in 73.6\% (39/53) of gastric cancer samples (Fig 1a). Furthermore, analysis of miR-338-3p expression in three gastric cancer cell lines (GES-1, BGC-823 and AGS), revealed that miR-338-3p was down-regulated in tumor cell lines as well (Fig 1b). These data suggested that miR-338-3p might act as a tumor suppressor in gastric cancer.

\textbf{P-Rex2a is a direct target of miR-338-3p}
We searched three bioinformatic databases (TargetScan, PicTar, and miRanda) to identify a large number of potential target genes of miR-338-3p. Among these candidates, P-Rex2a was selected for the further analysis. A binding site of miR-338-3p was observed in the 3’-UTR of P-Rex2a mRNA. Moreover, the seed sequences were exactly conserved after comparing the human sequence with different species (Fig. 2a). To validate the hypothesis that P-Rex2a might be a target of miR-338-3p, a dual luciferase reporter system containing wild type 3’UTR of P-Rex2a was employed. HEK-293 cells were co-transfected with pre-miR-338-3p and reporter plasmid (P-Rex2a_UTR) or pmirGLO-control vector. As a result, pre-miR-338-3p/P-Rex2a_UTR transfected cells showed a significant reduction (~40%) of luciferase activity (Fig. 2b), indicating that miR-338-3p may suppress gene expression through its binding sequences at the 3’UTR of P-Rex2a. Meanwhile, the result showed that P-Rex2a protein has an over-expression in gastric cancer samples compared with their normal tissues control (Fig. 2c, d). Furthermore, a reduction of P-Rex2a mRNA (Fig. 2e) and protein (Fig. 2f) expression level were observed in BGC-823 cells transfected with pre-miR-338-3p compared to control vector transfected cells. These results indicate that miR-338-3p directly recognizes the 3’-UTR of P-Rex2a mRNA and inhibits P-Rex2a translation.

**Overexpression of miR-338-3p induces G1/S arrest and decreases gastric cancer cell growth by suppressing the Akt signaling pathway**

Result of qRT-PCR showed that the expression of miR-338-3p had a more than ten-fold increase in the pre-miR-338-3p transfected cells compared to the control
vector transfected cells (Supplementary Figure S1). In order to examine the role of miR-338-3p in GC cell growth, MTT assays, cell cycle analysis and colony formation assay were adopted. The results showed that the transient overexpression of miR-338-3p led to the inhibition of the growth of BGC-823 at 24, 48 and 72 hours after transfection (Figure 3a), resulted in a marked accumulation of the G1-population and a reduction of the S-phase population in gastric cancer cell line BGC-823 (Figure 3b), and reduced colony formation (Figure 3c) in comparison with control vector transfected cells. These finds suggested that miR-338-3p can arrest cell cycle at G1/S transition phase and suppress cell proliferation in vitro.

Pre-miR-338-3p and control vector transfected cells were injected subcutaneously into each side of posterior flanks of the nude mouse. The mice were followed for observation of xenograft growth for 4 weeks. The tumors injected with control-vector cell displayed obviously from the bioluminescence images obtained by using the system of photobiology, whereas pre-miR-338-3p cells almost showed non-tumors (Fig. 3d). These data indicate that miR-338-3p could remarkably inhibit the tumorigenicity of BGC-823 cells in the nude mouse xenograft model.

To further investigate the possible molecular mechanisms of miR-338-3p-induced cell proliferation repression, we detected protein expression level of P-Rex2a and its downstream pathways regulators by western blotting after transfection with pre-miR-338-3p and control vector. Our results show that miR-338-3p can reduce the expression of P-Rex2a protein and the phosphorylation of p-AKT at serine 473, whereas the protein expression of PTEN and total AKT has not
any change (Figure 3e). Moreover, we found that miR-338-3p could inhibit cell proliferation at G1/S phase (Fig3b), which pushed us to investigate the underlying mechanisms of cell cycle regulation. As a consequence, both of the miR-338-3p and si-P-Rex2a could reduce the expression of cyclin D and cdk2 (Figure 3e), whatsmore they also induced a clear decrease of cyclin D1 at a phosphorylation site at Thr286 (Figure 3e). These results demonstrate that miR-338-3p affects gastric cancer cell growth, proliferation and cell cycle by controlling P-Rex2a/PTEN/AKT pathways.

Inhibition of miR-338-3p contributes to tumorigenicity of BGC-823 cells

Loss-of-function studies were also performed by employing anti-miR-338-3p oligonucleotides to silence miR-338-3p expression. Unexpectedly, the inhibitory effect is moderate may because the low expression of endogenous miR-338-3p in BGC-823 cells, but it rather showed that miR-338-3p could contributes to the tumorigenicity of BGC-823 cells. As shown in Fig 4, transfection with inhibitor of miR-338-3p promoted cell clone formation and induced a G1/S transition in BGC-823 cells. Furthermore, knockdown of miR-338-3p increased expression of P-Rex2a, CCND1/p-CCND1 and CDK2 tested by western blot analysis. Together with the above experiments, these suggested an essential contribution of endogenous miR-338-3p to the anti-carcinogenesis of BGC-823 cells in gastric cancer progression.

miR-338-3p could induce apoptosis in BGC-823 cells

During the experiment in vitro, we found that overexpression of miR-338-3p also could induce cell apoptosis. Compared with the cells transfected with an control
vector, pre-miR-338-3p transfected cells displayed higher apoptic rates at an early phase, whereas miR-338-3p inhibitor exhibited no significantly difference as the cells transfected with negative control (Figure 5a, b). The data shows that miR-338-3p induces apoptosis in human gastric cancer cells \textit{in vitro}.

Deregulation of apoptosis is an important event in cancer as it allows the genetically unstable cells to survive and accumulate further mutations that eventually lead to tumourigenesis. We also focused on the apoptosis of gastric cancer cells and examined whether miR-338-3p modified the downstream targets of PI3K related to apoptosis. As a result, suppression of p-AKT could promote apoptosis by accelerating proapoptotic proteins Bax and inactivating antiapoptotic proteins Bcl-2 (Fig 5c).

\textbf{Silence of P-Rex2a could suppress gastric cancer cell growth and induces G1/S arrest, cell apoptosis similarly to miR-338-3p}

As we showed above, over-expression of miR-338-3p affects cell growth, proliferation, cycle and apoptosis in gastric cancer cells BGC-823. We also validated P-Rex2a as a direct target of miR-338-3p. Therefore, we silenced P-Rex2a expression by RNAi to confirm that P-Rex2a is involved in the antitumor effects of miR-338-3p. From mRNA and protein expression level, P-Rex2a can be specific knockdown by siRNA (Figure 6a). Moreover, silence of P-Rex2a resulted in suppressed cell growth, proliferation and induced G1/S arrest, cell apoptosis (Figure 6b,c,d,e), which followed the same trend as miR-338-3p in BGC-823 cells.

Furthermore, this was confirmed by an analysis of protein expression in AKT pathway. As shown in Fig. 6E, the expression of P-Rex2a protein and the
phosphorylation of p-AKT at serine 473 were suppressed by si-P-Rex2a, whereas the protein expression of PTEN and total AKT has not any change. For a cell cycle regulation, si-P-Rex2a could reduce the expression of cyclin D and cdk2, also induce a clear decrease of cyclin D1 at a phosphorylation site at Thr286. Moreover, siRNA could promote apoptosis by activating proapoptotic proteins Bax and inactivating antiapoptotic proteins Bcl-2. Based on these findings, we concluded that miR-338-3p could regulate gastric cancer cell progression by directly targeting the P-Rex2a through the PTEN/Akt signalling pathways.

**Discussion**

Gastric cancer (GC) remains as one of common cancer types and still be a leading cause of cancer-related death (19), and its genesis is considered to be predominantly due to genetic and environmental factors (20). Furthermore, gastric carcinogenesis is multifactorial process associated with multiple genetic and epigenetic events. During the past years, dysregulation of miRNAs has been relative to control cell proliferation (21), metastasis (22) and cell cycle (23) in GC. The miR-338 gene, located on chromosome 17q25 within eighth intron of the apoptosis-associated tyrosine kinase (AATK) gene, produces two mature forms (miR-338-3p and miR-338-5p) (24), and AATK kinase activity plays an essential role in promoting neurite extension in developing neurons (25). Our studies showed that miR-338-3p was frequently down-regulated in both GC tissues and cell lines, which indicates that miR-338-3p might be a novel tumor suppresser miRNA.
Furthermore, for the first time, Our miR-338-3p target analysis identified P-REX2a as a direct target of miR-338-3p. P-Rex2a is a guanine nucleotide exchange factor (GEF) for the RAC guanosine triphosphatase (GTPase) (26), which could encodes a protein inhibiting phosphatase activity against PIP3 in vitro (18). In our study, we found P-REX2a has an over-expression in gastric cancer compared to normal tissues, which shown an inverse correlation between P-Rex2a expression and the miR-338-3p expression in GC tissues. These results implied that miR-338-3p may affect the progression of gastric cancer by targeting P-Rex2a. Further bio-information analysis showed that there was a miR-338-3p binding site at 4898-4917nt of the P-Rex2a 3' UTR. The dual-luciferase reporter assays demonstrated that miR-338-3p targeted directly to P-Rex2a by recognizing the 3'-UTR of P-Rex2a mRNA and inhibited P-Rex2a translation.

The manipulation of P-Rex2 expression could affected AKT phosphorylation in a PTEN-dependent manner by bind to PTEN in cells (18). PTEN (phosphatase and tensin homolog on chromosome 10) gene is frequently lost in cancers, and germline PTEN mutations are linked to inherited cancer predisposition syndromes (27). Biochemically, PTEN is a phosphatase that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3), the lipid product of the class I phosphoinositide 3-kinases (PI3K) (28). The phosphatidylinositol 3' kinase (PI3K) pathway is one of the most potent pro-survival pathways in cancer (29). Inactivation of PTEN leads to accumulation of PIP3 and, as a consequence, increases activity of the kinase AKT, which contributes to oncogenesis in numerous other cancers including glioblastoma.
(30), prostate (31) and liver cancers (32). From existent literature, we found that AKT phosphorylation is essential for progression from G0/G1 to S-phase (33). And in our research, P-Rex2a has been inhibited by the overexpression of miR-338-3p and siRNA, which resulted in activation of PTEN and tend to a decline in phosphorylation of AKT. Then we turned to study the effect on AKT downstream target genes Cyclin D1 and CDK2 (34), which are key transcriptional factors in the G0–G1 phase. The results showed that there was a reduction on expression level of CDK2 as well as the phosphorylation of the cyclin D1 at threonine 286 in the cells transfected by miR-338-3p. Besides, we also provide evidence that miR-338-3p induced PI3K-Akt pathway plays an important role in the regulation of the Bcl-2 and Bax family as previous shown (35).

In addition, RNAi-mediated knockdown or overexpression of P-Rex2 was also able to control xenograft tumour formation in vivo (36). The evidences of cancer xenograft models in our study was already shown, but studies to data showed only changes in tumor size when cells were transfected by miR-338-3p and control vector, and then injected to produce the tumor model. Such studies were also done in prostate cancer (37), hepatocellular carcinoma (38) and pancreatic cancer (39). These results also confirmed the tumor-suppressive role of miR-338-3p in gastric cancer.

In conclusion, we investigated the role of miR-338-3p, its target gene P-Rex2a firstly and their potential implications in the processes of GC. These data suggest that miR-338-3p may be a novel tumor-suppressor that blocks the growth of gastric cancer cells through PTEN/PI3K signalling pathways by targeting P-Rex2a. Our findings
provide that miR-338-3p could be a useful tool in the future control of gastric cancer.

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Figure legends

Figure 1. miR-338-3p is downregulated in gastric cancer tissues and cell lines. (A) Quantitative real-time PCR (qRT-PCR) was performed to examine miR-338-3p expression in 53 paired human gastric cancer and adjacent normal tissues. The expression of miR-338-3p was normalized to U6 with respect to specimen N1. (B) qRT–PCR analysis of miR-338-3p expression in normal gastric and gastric cancer cells and normalized against an endogenous control (U6 RNA). Data were analyzed using a \( \Delta \Delta C_t \) approach. One-way analysis of variance was adopted to compare miR-338-3p expression and all data are shown as mean±s.d. for three independent experiments (*P<0.05 Student’s t-test).

Figure 2. P-Rex2a is experimentally validated as a direct target of miR-338-3p in gastric cancer cells. (A) miR-338-3p is highly conserved across species and binding sites within seed region sequence in the 3’-UTR of human P-Rex2a. (B) Luciferase assay in HEK293 cells. pLUC-P-Rex2a_WT vector was cotransfected with control or pre-miR-338-3p vector. Relative repression of luciferase expression was standardized to \( \beta \)-gal signal. Luciferase activity in pLUC-P-Rex2a_WT group denoted a statistically significant decrease following ectopic expression of miR-338-3p. (C/D) Expression of P-Rex2a determined by Western Blot and Immunohistochemical in GC tissues and their corresponding normal tissues. (E) P-Rex2a mRNA (left) and protein (right) expression level measured by qRT-PCR and Western Blot 48 h after
transfection with pre-miR-338-3p vector. GAPDH was employed as a housekeeping control. Statistical differences were analyzed with the Wilcoxon signed-rank test (*P<0.05, Student’s t-test).

**Figure 3. Overexpression of miR-338-3p affect gastric cancer progression in vitro and vivo.** (A) The effects of miR-338-3p on BGC-823 cell proliferation were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24, 48 and 72 h after transfection with pre-miR-338-3p or control vector, respectively. (B) Cell cycle detected in BGC-823 cells 48 h after transfection by Propidium-iodide staining flow cytometry. Histogram represented the percentage of cells in G0–G1, S and G2–M cell-cycle phases. (C) Representative results of colony formation and anchorage independent growth of BGC-823 cells in soft agar after transfection with pre-miR-338-3p or control vector, respectively. (D) Small animal imaging analysis to calculate the tumor volume *in situ* during the tumor development at the fourth week. Upper portion indicated the flanks injected with control-vector cells (right flank) and miR-338-3p-transfected cells (left flank) in nude mice. Lower portion showed the morphology of mice injected with control-vector cells (right flank). (E) Expression analysis for P-Rex2a/AKT signaling pathway regulation proteins in BGC-823 cells at 48h after transfection with pre-miR-338-3p or control vector by western blot. GAPDH was employed as a housekeeping control. Statistical differences were analyzed with the Wilcoxon signed-rank test (*P<0.05, Student’s t-test).
**Figure 4 Inhibition of miR-338-3p contributes to tumorigenicity of BGC-823 cells.**  
(A) The effects of miR-338-3p on BGC-823 cell proliferation were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24, 48 and 72 h after transfection with inhibitor of miR-338-3p or NC control, respectively. (B) Cell cycle was determined in BGC-823 cells 48 h after transfection by Propidium-iodide staining flow cytometry. Histogram represented the percentage of cells in G0–G1, S and G2–M cell-cycle phases. (C) Representative results of colony formation and anchorage independent growth of cells in soft agar after transfection with inhibitor of miR-338-3p or NC control, respectively. (D) Expression analysis for P-Rex2a/AKT signaling pathway regulation proteins in BGC-823 cells at 48h after transfection with inhibitor of miR-338-3p or NC control by Western Blot. GAPDH was employed as a housekeeping control.

**Figure 5. miR-338-3p could induce apoptosis in BGC-823 cells.** (A/B) Cell apoptosis were detected by Annexin-V/propidium iodide combined labeling flow cytometry in BGC-823 cells 48 h after transfection with pre-miR-338-3p or miR-338-3p inhibitor, with control or N.C vector, respectively. Apoptotic evaluation was calculated by the percentage of apoptotic cell number in total cell number. (*P<0.05, Student’s t-test). (B) Expression of cell apoptosis related protein in the downstream of P-Rex2a/AKT signaling pathway were determined in BGC-823 cells at 48h after transfection by Western Blot. GAPDH was employed as a housekeeping
control.

Figure 6 Silence of P-Rex2a could suppress gastric cancer cell growth and induces G1/S arrest, cell apoptosis in accordance with miR-338-3p. (A) qRT–PCR (upper portion) and Western Blot analysis (lower portion) were performed to determine the expression level of P-Rex2a after transfection with P-Rex2a siRNA. (B–E) MTT assay/Cell cycle/Cell apoptosis/Clone formation were performed to determine the impact of BGC-823 cells treated with siRNA of P-Rex2a. (F) Expression analysis for P-Rex2a/AKT signaling pathway regulation proteins in BGC-823 cells at 48h after transfected with control siRNA or P-Rex2a siRNA by Western Blot. GAPDH was employed as a housekeeping control. Statistical differences were analyzed with the Wilcoxon signed-rank test (*P<0.05, **P<0.01, Student’s t-test).
Figure 1

A. Expression level of miR-338-3p

- Normal: n=53
- Cancer: n=53

B. Expression level of miR-338-3p

- GES-1
- AGS
- BGC-823

** p < 0.01
* p < 0.05
Figure 2
Figure 3
Figure 4
Figure 5

A.

Control vector  
miR-338-3p

Cell apoptosis rate (%)

Early  Late

B.

N.C  Inhibitor

Cell apoptosis rate (%)

Early  Late

C.

Bcl-2  
Bax  
GAPDH

control vector  miR338-3p  N.C  Inhibitor
Figure 6
Molecular Cancer Research

miR-338-3p suppresses progression of gastric cancer through PTEN-AKT signaling pathways by targeting P-REX2a

Bo Guo, Liying Liu, Jiayi Yao, et al.

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