MicroRNA-937 inhibits the malignant phenotypes of breast cancer by directly targeting and downregulating forkhead box Q1

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Purpose: Numerous microRNAs (miRNAs) are aberrantly expressed in breast cancer, and the dysregulation of miRNAs may affect the aggressiveness of this cancer. Aberrant expression of miRNA-937 (miR-937) in gastric and lung cancers has been reported, which plays tumor-suppressive or oncogenic roles in carcinogenesis including cancer progression. Our purpose was to investigate the involvement of miR-937 in breast cancer progression.

Patients and methods: The expression profile of miR-937 in breast cancer was assessed by reverse-transcription quantitative PCR. Biological effects of miR-937 upregulation on the malignant characteristics of breast cancer cells were determined in a series of functional experiments. The direct target of miR-937 in breast cancer cells was also identified.

Results: Herein, the expression levels of miR-937 were notably lower in breast cancer, and its underexpression was significantly correlated with lymph node metastasis and TNM stage. Patients with breast cancer underexpressing miR-937 showed shorter overall survival than did patients with breast cancer overexpressing miR-937. Proliferation, migration, and invasiveness of breast cancer cells were evidently suppressed by miR-937 upregulation. In addition, ectopic miR-937 expression hindered breast cancer tumor growth in vivo. Forkhead box Q1 (FOXQ1) mRNA was found to be a direct target of miR-937 in breast cancer. FOXQ1 turned out to be overexpressed in breast cancer tissues, and its overexpression negatively correlated with miR-937 expression. Moreover, silencing of FOXQ1 recapitulated the tumor-suppressive effects of miR-937 overexpression on breast cancer cells. Notably, FOXQ1 restoration abrogated the miR-937-mediated suppression of proliferation, migration, and invasiveness of breast cancer cells.

Conclusion: These results collectively revealed that miR-937 acts as a tumor suppressor in breast cancer and restrains cancer progression by directly targeting FOXQ1 mRNA. These data suggest that targeting of the novel miR-937–FOXQ1 axis is an attractive therapeutic method against breast cancer.

Keywords: breast cancer, microRNA-937, forkhead box Q1

Introduction
Breast cancer, a highly heterogeneous disease, is the most commonly diagnosed type of malignant tumor and the top cause of cancer-related mortalities among females worldwide. Currently, surgical resection in combination with hormonal therapy, chemoradiotherapy, and biological therapy remains the major therapeutic strategy for patients with breast cancer. Despite considerable progress in the diagnosis and therapy, the treatment outcomes among patients with breast cancer diagnosed at an advanced stage are still unsatisfactory. Multiple risk factors,
including the lifestyle, environment, heredity, and reproductive parameters, are linked to the initiation and progression of breast cancer; however, detailed mechanisms are not completely clarified. Hence, an improved understanding of the pathogenesis of breast cancer may be useful for the identification of promising therapeutic approaches and for improvement of the clinical outcomes of breast cancer.

MicroRNAs (miRNAs) are a group of noncoding single-stranded short RNA molecules consisting of 19–25 nucleotides and serve as powerful regulators of gene expression. An miRNA can recognize and directly bind to a complementary site(s) in the 3′-UTR of a target mRNA, resulting in transcription suppression and/or degradation of the targeted mRNA. In recent years, considerable evidence uncovered the involvement of miRNAs in a wide array of biological events, eg, tumorigenesis including tumor progression. MiRNAs differentially expressed in almost all types of human malignant tumors have been widely reported, including breast cancer. For instance, miR-433, miR-577, and miR-644 are clearly downregulated in breast cancer and play tumor-suppressive actions of miR-937 on breast cancer progression were investigated in this study.

Materials and methods

Human tissue samples

In total, breast cancer tissue samples and corresponding normal adjacent tissue (NAT) samples were collected from 47 patients who underwent surgical resection in the Weihai Central Hospital. NATs were obtained 2 cm away from breast cancer tissues. Patients who received radiotherapy or chemotherapy were excluded from the study. After the resection, all tissue specimens were snap-frozen in liquid nitrogen and then stored at −80°C. The Ethics Committee of Weihai Central Hospital approved this study. The study was performed in accordance with the Declaration of Helsinki, and written informed consent was provided by all the participants.

Cell culture conditions

A total of four human breast cancer cell lines, MDA-MB-231, MCF-7, BT-474 and SKBR3, as well as a normal human breast epithelial cell line MCF-10A were bought from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% of heat-inactivated FBS(HI-FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% of a penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA) was utilized for the cell culture. All cells were grown at 37°C in a humidified cell incubator supplied with 5% CO2.

Transfection assays

Cells were seeded in 6-well plates 24 hrs before transfection. To restore miR-937 expression, agomir-937 (Shanghai GenePharma Co., Ltd; Shanghai, China) was transfected into the cells, with agomir-NC as a control in a separate group of cells. Small interfering (si)RNA targeting FOXQ1 mRNA (si-FOXQ1; Guangzhou RiboBio Co., Ltd; Guangzhou, China) was applied to knock down endogenous FOXQ1 expression. Negative control siRNA (si-NC) served as the control for the si-FOXQ1 transfection. FOXQ1 overexpression plasmid pcDNA3.1-FOXQ1 (pc-FOXQ1) was chemically synthesized by GeneCopoeia Co., Ltd. (Guangzhou, China) and was transfected into the cells to increase endogenous FOXQ1 expression. All transient transfection procedures were conducted using Lipofectamine™ 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Reverse-transcription quantitative PCR (RT-qPCR)

Total RNA from tissues or cells was extracted with the High Purity Total RNA Extraction Kit (Biotek Corporation, Beijing, China). A One Step PrimeScript™ RT-PCR Kit (Takara Biotechnology Co., Ltd., Dalian, China) was applied to measure the expression level of
miR-937. To measure FOXQ1 mRNA expression, complementary DNA (cDNA) synthesis was performed with the PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.). The resulting cDNA product was subjected to qPCR with SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.). U6 small nuclear RNA and GAPDH served as the internal controls for calculating the expression levels of miR-937 and FOXQ1, respectively. All data were analyzed by the $2^{-\Delta\Delta Cq}$ method.\(^{22}\)

**The Cell Counting Kit-8 kit (CCK-8) assay**
Transfected cells were incubated at 37°C for 24 hrs, and then collected for the preparation of a cell suspension. A total of 100 µL of a cell suspension containing 2,000 cells was inoculated into each well of a 96-well plate. Each group contained three replicate wells. Cellular proliferation was evaluated at four time points: 0, 1, 2, and 3 days after the inoculation. Cells were treated with 10 µL of the CCK-8 solution (Dojindo, Tokyo, Japan) prior to the additional 2 hrs of incubation. Finally, the absorbance of each well at a 450 nm wavelength was read on an EnSpire™ 2300 Multilabel Reader (PerkinElmer, Inc., Waltham, MA, USA).

**Transwell migration and invasion assays**
Transfected cells were harvested, centrifuged, and then resuspended in DMEM without HI-FBS. In total, 200 µL of a cell suspension containing $10^5$ cells were added into an upper compartment of Corning Costar Transwell 24-well plates (Corning Incorporated, Corning, NY, USA), while the lower compartments were covered with 600 µL of DMEM that was supplemented with 10% of HI-FBS. Following incubation for 24 hrs, nonmigratory cells were carefully removed with a cotton swab, whereas the migratory cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet (Sigma-Aldrich). The capacity for migration was assessed by counting the migratory cells in five randomly selected visual fields per plate in images captured by means of an Olympus light microscope (Olympus IX83; Olympus Corporation, Tokyo, Japan). The experimental procedures of the Transwell invasion assay were similar to those of the migration assay, except that the plates were precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

**A tumorigenicity assay in nude mice**
BALB/c nude mice (4–6 weeks old) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences. Agomir-937-transfected or agomir-NC-transfected cells were harvested after 24 hrs of incubation and subcutaneously injected into the flank of nude mice. Starting at 12 days after the injection, tumor width and length were examined every 4 days. The following formula, Volume=1/2 (length×width$^2$), was used to calculate the volumes of tumor xenografts. All nude mice were euthanized by cervical dislocation at the end of the experiment. Each tumor xenograft was resected and stored for subsequent analysis. The animal experiment was approved by the Ethics Committee of Weihai Central Hospital and conducted in accordance with the Animal Protection Law of the People’s Republic of China 2009 for experimental animals.

**miRNA target prediction**
The potential targets of miR-937 were predicted by means of software tools TargetScan (http://www.targetscan.org/vert_71/) and microRNA (http://www.microrna.org/microrna/home.do).

**A luciferase reporter assay**
Fragments of the 3′-UTR of FOXQ1 containing the wild-type (WT) or mutant (MUT) miR-937-binding site were amplified by Shanghai GenePharma Co., Ltd., and inserted into the pMIR-REPORT™ Luciferase plasmid (Promega Corporation, Madison, WI, USA) to generate WT and MUT luciferase reporter plasmids, respectively. Cells seeded in 24-well plates were cotransfected with either the WT or MUT luciferase reporter plasmid and either agomir-937 or agomir-NC by means of Lipofectamine™ 2000. At 48 hrs post-transfection, the cells were harvested for the detection of luciferase activity using a Dual-Luciferase® Reporter Assay System (Promega Corporation). Renilla luciferase activity was measured as a normalization control.

**Western blot analysis**
The isolation of total protein was carried out with the ProteoPrep® Total Extraction Sample Kit (Sigma-Aldrich; EMD Millipore, Billerica, MA, USA). The concentration of total protein was quantified via the Bicinchoninic Acid Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein were loaded and separated by SDS-PAGE in a 10% gel and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Prior to incubation with primary antibodies overnight at
4°C, the membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% of Tween 20 (TBST) at room temperature for 2 hrs. After three washes with TBST, a goat anti-rabbit IgG antibody (1:5,000 dilution; horseradish peroxidase-conjugated secondary antibody; cat. # ab6721; Abcam, Cambridge, UK) was incubated with the membranes at room temperature for 1 hr. Finally, the bands were detected using the Enhanced Chemiluminescence Detection Reagent (Pierce Biotechnology, Inc., Rockford, IL, USA).

Rabbit anti-human FOXQ1 (ab51340) and anti-human GAPDH (ab181602) antibodies were purchased from Abcam and employed at 1:1,000 dilution.

Statistical analysis
All data from at least three independent experiments were expressed as the mean ± SD. The association between miR-937 and the clinical features of the patients with breast cancer was examined by the χ² test. Spearman’s correlation analysis was applied to determine the expression correlation between miR-937 and FOXQ1 mRNA in breast cancer tissue samples. The comparison of means between groups was carried out by two-tailed Student’s t-test and one-way analysis of variance followed by Tukey’s post hoc test. The overall survival rates were calculated by the Kaplan–Meier method and analyzed using a log-rank test. Statistical Package for Social Sciences version 19.0 (IBM SPSS, Inc., Armonk, NY, USA) was used for all statistical analyses. Data with P<0.05 were considered statistically significant.

Results
miR-937 expression is low in breast cancer
To investigate the expression profile of miR-937 in breast cancer, its expression in 47 pairs of breast cancer tissue samples and NAT samples was measured via RT-qPCR. The expression level of miR-937 was

Table 1 The association between miR-937 expression and clinicopathological characteristics in patients with breast cancer

| Characteristics       | miR-937 expression | P-value |
|-----------------------|--------------------|---------|
|                       | Low | High |
| Age (years)           |     |      |
| <50                   | 10  | 7    | 0.547   |
| ≥50                   | 14  | 16   |         |
| Tumor diameter (cm)   |     |      |
| <2                    | 12  | 14   | 0.561   |
| ≥2                    | 12  | 9    |         |
| Lymph node metastasis |     |      |
| Negative              | 8   | 17   | 0.008*  |
| Positive              | 16  | 6    |         |
| TNM stage             |     |      |
| I–II                  | 6   | 16   | 0.003*  |
| III                   | 18  | 7    |         |
| Histology grade       |     |      |
| I–II                  | 11  | 8    | 0.556   |
| III                   | 13  | 15   |         |
| Pathological type     |     |      |
| Invasive ductal carcinoma | 16 | 18  | 0.738   |
| Other                 | 8   | 5    |         |

Note: *P<0.05.
evidently lower in breast cancer tissues than in NAT samples (Figure 1A, \(P<0.05\)). Furthermore, we examined miR-937 expression in a panel of breast cancer cell lines: MDA-MB-231, MCF-7, BT-474, and SKBR3. A human breast epithelial cell line (MCF-10A, noncancerous) served as a control. The data indicated that expression of miR-937 was lower in all the four tested breast cancer cell lines than in MCF-10A cells (Figure 1B, \(P<0.05\)).

**MiR-937 underexpression is correlated with a poor prognosis of patients with breast cancer**

To assess the clinical value of miR-937 in breast cancer, all our patients with breast cancer were distributed between a miR-937-low-expression group (n=24) and miR-937-high-expression group (n=23) based on the median value of miR-937 expression in breast cancer tissues. First, we examined the association between the miR-937 level and clinical parameters in patients with breast cancer. Low miR-937 expression obviously correlated with lymph node metastasis (\(P=0.008\)) and TNM stage (\(P=0.003\)) among the patients with breast cancer (Table 1). Notably, patients with breast cancer underexpressing miR-937 showed shorter overall survival than did the patients with breast cancer overexpressing miR-937 (Figure 1C, \(P=0.006\)). These observations indicated that downregulation of miR-937 may be closely linked with the poor prognosis of patients with breast cancer.

**miR-937 inhibits the proliferation, migration, and invasiveness of breast cancer cells in vitro**

MiR-937 was found to be underexpressed in breast cancer; therefore, we hypothesized that miR-937 may serve as a tumor-suppressive miRNA during breast

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**Figure 2** miR-937 overexpression leads to a significant decrease in the proliferation, migration, and invasion of MDA-MB-231 and MCF-7 cells. (A) MDA-MB-231 and MCF-7 cells were transfected with agomir-937 or agomir-NC. After 48 hrs culture, transfected cells were used for the determination of miR-937 expression through RT-qPCR. **\(P<0.01\)** vs agomir-NC. (B) CCK-8 assay was utilized to measure the proliferative ability in MDA-MB-231 and MCF-7 cells that were transfected with agomir-937 or agomir-NC. \(*P<0.05\) vs agomir-NC. (C and D) Quantification of the migration and invasion of MDA-MB-231 and MCF-7 cells transfected with agomir-937 or agomir-NC was performed using transwell migration and invasion assays. \(*P<0.05\) vs agomir-NC.

**Abbreviations:** CCK-8, Cell Counting Kit-8 kit; RT-qPCR, reverse-transcription quantitative PCR.
cancer progression. To test this hypothesis, MDA-MB-231 and MCF-7 cells were transfected with agomir-937 or agomir-NC, and the transfection efficiency was confirmed via RT-qPCR (Figure 2A, $P<0.05$). The results of the CCK-8 assay revealed that the transfection of agomir-937 obviously suppressed the proliferative capacity of MDA-MB-231 and MCF-7 cells (Figure 2B, $P<0.05$). Next, we conducted Transwell migration and invasion assays to determine the impact of miR-937 upregulation on the migration and invasiveness of breast cancer cells. The findings indicated that miR-937-overexpressing MDA-MB-231 and MCF-7 cells had weaker migratory (Figure 2C, $P<0.05$) and invasive (Figure 2D, $P<0.05$) abilities in comparison with the agomir-NC group. These results implied that miR-937 overexpression inhibited the malignant progression of breast cancer cells in vitro.

**FOXQ1 mRNA is the direct target of miR-937 in breast cancer**

It is widely accepted that miRNAs regulate the biological processes associated with cancer by targeting relevant mRNAs.\(^8\) Hence, bioinformatics analysis was conducted to search for the putative target of miR-937 and elucidate the mechanism underlying the suppressive influence of miR-937 on breast cancer progression. As shown in Figure 3A, a putative 7-mer binding site for miR-937 was found in the 3'-UTR of FOXQ1. FOXQ1 was chosen for validation considering its...
miR-937 is a functional target gene of miR-937 in breast cancer tissues. (A) Relative expression of FOXQ1 mRNA in 47 pairs of breast cancer tissues and NATs was detected by RT-qPCR. *P<0.05 vs NATs. (B) The expression association between miR-937 and FOXQ1 mRNA in breast cancer tissues was examined using Spearman’s correlation analysis. R²=0.3458, P<0.0001. (C and D) The expression levels of FOXQ1 mRNA and protein were lower in miR-937-high-expression group than that in miR-937-low-expression group. *P<0.05 vs miR-937-low-expression group.

**Abbreviations:** NAT, normal adjacent tissue; RT-qPCR, reverse-transcription quantitative PCR.

Figure 4 miR-937 expression is negatively correlated with FOXQ1 levels in breast cancer tissues. (A) Relative expression of FOXQ1 mRNA in 47 pairs of breast cancer tissues and NATs was detected by RT-qPCR. *P<0.05 vs NATs. (B) The expression association between miR-937 and FOXQ1 mRNA in breast cancer tissues was examined using Spearman’s correlation analysis. R²=0.3458, P<0.0001. (C and D) The expression levels of FOXQ1 mRNA and protein were lower in miR-937-high-expression group than that in miR-937-low-expression group. *P<0.05 vs miR-937-low-expression group.

**Abbreviations:** NAT, normal adjacent tissue; RT-qPCR, reverse-transcription quantitative PCR.

Downregulation of FOXQ1 has effects similar to those of miR-937 upregulation in breast cancer cells
Loss-of-function assays were carried out to determine the role of FOXQ1 in breast cancer progression. Si-FOXQ1 was employed for a knockdown of FOXQ1 in MDA-MB-231 and MCF-7 cells. Western blot analysis confirmed that FOXQ1 expression was efficiently silenced in MDA-MB-231 and MCF-7 cells after si-FOXQ1 transfection (Figure 5A; P<0.05). Silencing of FOXQ1 restricted the proliferation (Figure 5B; P<0.05) of MDA-MB-231 and MCF-7 cells. In addition, the downregulation of FOXQ1 suppressed the migration (Figure 5C; P<0.05) and invasiveness (Figure 5D; P<0.05) of MDA-MB-231 and MCF-7 cells. Hence, decreased FOXQ1 expression manifested the actions similar to those caused by miR-937 overexpression in breast cancer cells, thereby suggesting that FOXQ1 mRNA is a direct target of miR-937 in breast cancer cells.

FOXQ1 downregulation mediates the tumor-suppressive roles of miR-937 in breast cancer cells
Next, rescue experiments were conducted to prove that FOXQ1 is a functional target gene of miR-937 in breast cancer cells. To recover FOXQ1 expression, the FOXQ1 overexpression plasmid (pc-FOXQ1) that lacks the FOXQ1 3’-UTR was transfected into miR-937-overexpressing MDA-MB-231 and MCF-7 cells. miR-937 overexpression-mediated downregulation of FOXQ1 was reversed in MDA-MB-231 and MCF-7 cells after cotransfection with pc-FOXQ1 (Figure 6A, P<0.05). Furthermore, the inhibitory actions of miR-937 on the proliferation (Figure 6B, P<0.05), migration (Figure 6C,
miR-937 inhibits tumor growth in vivo

To better examine the influence of miR-937 on the in vivo growth of breast cancer, MCF-7 cells transfected with agomir-937 or agomir-NC were implanted into nude mice. Consistently with the results observed in vitro, the xenografts derived from the agomir-937-transfected cell group had a smaller tumor volume (Figure 7A and B, \( P<0.05 \)) and weight (Figure 7C, \( P<0.05 \)) as compared with those in the agomir-NC group of nude mice. Next, miR-937 expression in the xenografts was determined by RT-qPCR. The expression level of miR-937 was notably higher in the agomir-937-transfected group than in the agomir-NC-transfected group (Figure 7D, \( P<0.05 \)). Meanwhile, Western blot analysis was carried out to measure FOXQ1 protein expression in the tumor xenografts. This expression was lower in the agomir-937 group (Figure 7E, \( P<0.05 \)). Collectively, these observations illustrated that miR-937 can effectively inhibit the tumor growth of breast cancer cells in vivo.

Discussion

Numerous miRNAs are differentially expressed in breast cancer, and their expression alterations may affect the initiation and progression of breast cancer.\(^{27-29}\) Hence, a comprehensive understanding of the detailed roles of miRNAs in breast cancer might facilitate the identification of novel targets for the treatment of this malignant tumor. In this study, we measured miR-937 expression in breast cancer tissues and cell lines. In addition, the clinical significance of miR-937 in patients with breast cancer was examined. Furthermore, we investigated the biological influence of miR-937 overexpression on breast cancer progression and explored the mechanisms...
underlying the tumor-suppressive activity of miR-937 in breast cancer cells in vitro and in vivo.

MiR-937 is downregulated in gastric cancer tissues and cell lines. On the contrary, miR-937 expression is high in lung cancer. These inconsistent observations prompted us to evaluate the expression pattern of miR-937 in breast cancer. Herein, the results revealed that miR-937 expression is low in breast cancer tissues and cell lines. The low miR-937 expression was obviously correlated with lymph node metastasis and TNM stage among the patients with breast cancer. Patients with breast cancer underexpressing miR-937 showed shorter overall survival than did the patients with breast cancer overexpressing miR-937. These findings suggest that miR-937 may be a diagnostic and/or prognostic biomarker of breast cancer.

MiR-937 has been identified as a tumor suppressor in gastric cancer. In particular, resumption of miR-937 expression suppressed gastric cancer cell viability, colony formation, migration, and invasion but induced apoptosis in vitro. In addition, miR-937 overexpression impaired epithelial–mesenchymal transition of gastric cancer cells. On the contrary, miR-937 plays an oncogenic part in the progression of lung cancer by promoting anchorage-dependent and -independent growth. Nonetheless, the specific roles of miR-937 in breast cancer have remained largely unclear. In this study, miR-937 was found to have a tumor-suppressive effect on the malignant characteristics of breast cancer, ie, to inhibit breast cancer cell proliferation, migration, and invasion.

**Figure 6** FOXQ1 is involved in miR-937-regulated proliferation, migration, and invasion inhibition in MDA-MB-231 and MCF-7 cells. (A) Agomir-937, along with pcDNA3.1 or pc-FOXQ1, was co-transfected into MDA-MB-231 and MCF-7 cells. Western blot analysis was applied to detect FOXQ1 protein expression after 72 hrs transfection. *P*<0.05 vs Agomir-NC. #P<0.05 vs Agomir-937+pc-FOXQ1. (B–D) CCK-8 and transwell migration and invasion assays were applied to determine the proliferation, migration, and invasion of MDA-MB-231 and MCF-7 cells treated as above described. *P*<0.05 vs Agomir-NC. #P<0.05 vs Agomir-937+pc-FOXQ1.

Abbreviation: CCK-8, Cell Counting Kit-8 kit.
tumor cell initiation, proliferation, epithelial–mesenchymal transition, and metastasis. Our current study revealed that miR-937-retarded breast cancer progression in vitro and in vivo, and these inhibitory activities were mediated by downregulation of FOXQ1. These findings suggest that resumption of miR-937 expression, which results in FOXQ1 silencing, might be a promising therapeutic method for the management of breast cancer.

**Conclusion**

MiR-937 expression was found to be low in breast cancer, and this underexpression is closely related to a poor prognosis. MiR-937 plays a tumor-suppressive part in the malignant progression of breast cancer by directly targeting FOXQ1 mRNA, thereby downregulating FOXQ1. These observations provide novel insight into the breast cancer pathogenesis, prompting us to identify effective therapeutic targets in breast cancer in the near future.

**Abbreviation list**

CCK-8, Cell Counting Kit-8; miRNAs, microRNAs; MUT, mutant; RT-qPCR, reverse-transcription quantitative PCR; qPCR, quantitative PCR; siRNA, small interfering; TBST, Tris-buffered saline containing 0.1% of Tween 20; WT, wild-type.

**Ethics approval and informed consent**

The Ethics Committee of Weihai Central Hospital approved this study, and written informed consent was provided by all the participants.

**Disclosure**

The authors report no conflicts of interest in this work.

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