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Highly expressed microRNA-940 promotes early abortion by regulating placenta implantation by targeting ZNF672

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Abstract. – OBJECTIVE: Our study aims to explore whether microRNA-940 could participate in early abortion by inhibiting placenta implantation and its underlying mechanism.

PATIENTS AND METHODS: Expressions of microRNA-940 and ZNF672 in the placental villi of 6 early abortion pregnancies and 6 normal pregnancies were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Expressions of microRNA-940 and ZNF672 in trophoblast cells (BeWo, JEG3, Wish and HTR-8) were also detected. Cell counting kit-8 (CCK-8) assay was performed to detect the effect of microRNA-940 on the proliferation of trophoblast cells after being transfected with microRNA-940 inhibitor or mimics, respectively. Dual-Luciferase reporter gene assay and RNA binding protein immunoprecipitation (RIP) assay were conducted to demonstrate the binding condition of microRNA-940 to ZNF672.

RESULTS: MicroRNA-940 was highly expressed in placental villi of early abortion pregnancies, whereas ZNF672 was lowly expressed. HTR-8 cells expressed the highest level and BeWo cells expressed the lowest level of microRNA-940. After transfection of microRNA-940 inhibitor in HTR-8 cells, the proliferative capacity was remarkably increased. On the contrary, the transfection of microRNA-940 mimic downregulated the proliferation of BeWo cells. Dual-Luciferase reporter gene assay demonstrated that microRNA-940 targets ZNF672. RIP results further indicated that microRNA-940 binds to ZNF672.

CONCLUSIONS: MicroRNA-940 is highly expressed in the placental villi of early abortion pregnancies and promotes the occurrence of early abortion by inhibiting the proliferation of trophoblast cells by targeting ZNF672.

Key Words:
MicroRNA-940, Early abortion, Placental implantation, ZNF672.

Introduction

Early abortion is the absence of gestational sac in the intrauterine pregnancy or no primordial cardiac pulsation in the gestation sac within 12 weeks of gestation[1]. It is an abortion that occurs without external intervention, mainly including unrecognized and well-defined histological abortions. Among them, the incidence of early abortion with defined histology accounts for 10%-15%, whereas the incidence of recurrent miscarriage is 0.5%-3%[2]. Spontaneous abortion is a way of self-selection for the survival of the fittest, which greatly reduces the birth of malformed children. Although there are many possible causes of miscarriage, such as embryonic chromosome abnormalities, maternal endocrine diseases, structural abnormalities of the reproductive system, environmental factors and immune factors[3], the molecular mechanism leading to early abortion is not yet fully understood.

Pregnancy is the process of embryonic, fetal development and maturation in the womb. It is an extremely complex and delicate physiological process regulated by various factors. Trophoblast cells are derived from extra-embryonic trophoblasts and exert an essential role in maintaining normal pregnancy. Normal trophoblast cells have certain biological characteristics similar to tho-

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se of tumor cells, such as rapid proliferation and strong invasive ability. However, the invasion of trophoblastic cells has been more precisely regulated in time and space. Excessive apoptosis or infiltration of trophoblast cells during this process would lead to abnormal development of the blastocysts or embryos. Failure of normal implantation of the embryos results in embryonic abortion, followed by spontaneous abortion. The normal pregnancy relies on the normal differentiation, proliferation and infiltration of chorionic trophoblast cells, endometrial membrane detachment and construction of the uteroplacental vascular network.

MicroRNAs (miRNAs) were first discovered by Victor Ambros in 1992. MiRNAs are non-encoding, single-stranded small RNAs composed of 18-25 small nucleotide molecules. They participate in cell development, proliferation, metabolism and cell differentiation by controlling the expressions of target genes[4-8]. It has been reported that miRNAs are differentially expressed in villi of spontaneous abortion in the first trimester of pregnancy. By regulating the corresponding target genes, the altered biological functions of trophoblast cells lead to the occurrence of early abortions. Relative studies have found that microRNA-940 is differentially expressed in lung cancer[9], gastric cancer[10], prostate[11], and liver cancer[12]. Because trophoblastic cells and tumor cells have similar biological behaviors, we therefore hypothesized whether microRNA-940 could affect the function of trophoblasts and participate in the occurrence of early abortions. Our research aims to provide new directions in exploring the mechanism of early abortion.

Patients and Methods

Patients

12 pregnancies diagnosed as early abortion in the Xinchang People Hospital from April 2016 to September 2017 were selected. Among them, 6 cases were threatened abortion, 6 were induced abortion. In the same period, 6 normal pregnancies were enrolled as the controls. This investigation was approved by the Xinchang People Hospital Ethics Committee and all pregnancies signed informed consent. Early abortion diagnosis criteria were: history of menopause less than 12 weeks, or accompanied by a corresponding response to early pregnancy. Pregnancies experienced symptoms of a small amount of vaginal bleeding, backache, lower abdominal bulge and lower abdominal pain. For clinical examinations, early abortion pregnancies should be inconsistent with one of the following diagnosis: 1. Blood or urine HCG (human chorionic gonadotropin) was positive; 2. The cervix was not opened and the uterus was soft. The fetal membrane was not broken and the size of the uterus was consistent with the gestational weeks; 3. B-ultrasonic examination indicated prompt intrauterine pregnancy. Gestational sac, germ or original fetal heartbeat was observed. The uterine size was inconsistent with gestational weeks.

Sample Collection

Villus tissues in each group were collected and washed with saline. Villus tissue was labeled and preserved in liquid nitrogen. On the next day, tissue samples were stored in -80°C for the following experiments.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted in 50-100 mg tissues using 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). Homogenate was preserved in a 1.5 mL Eppendorf (EP) tube and incubated with 0.2 mL of chloroform for 2-3 min. After centrifugation at 12000 rpm/min for 15 min, the supernatant was collected and incubated with 0.5 ml of isopropanol for 10 min. Centrifugation at 12000 rpm/min for 15 min was performed again, followed by RNA precipitate cleaned with 75% ethanol and air dried.

RNA concentration was detected using a spectrometer and those samples with A260/A280 ratio of 1.8-2.0 were selected for the following qRT-PCR reaction. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using the 2-ΔCt method. Primers used in the study were as follows: MicroRNA-940, F: 5′-GCATCGTTCCTTCAAGCCGATCT-3′, R: 5′-TGGGTGAGTCGTTCGG-3′; ZNF672, F: 5’-CAGCGGGGAAGCCTTACTC-3’, R: 5’-CTCACCGCATTCTAGGCAAC-3’.

Cell Culture and Transfection

Human trophoblast cells (BeWo, JEG3, Wish and HTR-8) were cultured in Dulbecco’s Mo-

dified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA). Cells were incubated in a 5% CO2 incubator at 37°C and transfected with the corresponding plasmids when the confluence was up to 50%, following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The culture medium was replaced 6 h later. Plasmid sequences were: MicroRNA-940 mimics, 5'-UCAGUGCAUGACAGAACUUGG-3', R: 5'-AAGUUCUGUCAUGCACUGAUU-3'; MicroRNA-940 inhibitor, 5'-CCAAGUUCUGUCAUGCACUGA-3'.

CCK-8 (Cell Counting Kit-8) Assay

Transfected cells were seeded into 96-well plates at a density of 2×103/μL. 10 μL of the Cell Counting kit-8 solution (CCK-8; Dojindo, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Bioinformatics Prediction

The target gene of microRNA-940 was predicted by TargetScan, mirBase and mirDB, respectively. The most tumor-related gene with the highest miRNA binding score was selected for the following functional experiments.

Dual-Luciferase Reporter Gene Assay

3'UTR of ZNF672 was downloaded from NCBI for constructing wild-type ZNF672 (ZNF672 wt) and mutant-type ZNF672 (ZNF672 mut). HTR-8 cells were seeded in the 96-well plates and cotransfected with 50 pmol/L microRNA-940 mimics or negative control and 80 ng ZNF672 wt or ZNF672 mut, respectively. Cells were lysed and incubated for 15 min at room temperature, followed by detection of Firefly-Luc/Renilla Luc.

RNA Binding Protein Immunoprecipitation (RIP)

Cells were washed and cross-linked with 0.01% formaldehyde for 15 min. After centrifugation and cell lysis, extracted cells were incubated with RNA protein immunoprecipitation (RIP) buffer containing protein A/G magnetic beads coated with anti-Ago2 or negative control anti-IgG antibody. After overnight incubation at 4°C, cells were incubated with Protein A Agarose for 1 h at 4°C, followed by the isolation of RNA. The microRNA-940 level was then detected by qRT-PCR.

Statistical Analysis

SPSS (Statistical Product and Service Solutions) 19.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$) and compared using the t-test. $p<0.05$ was considered statistically significant.

Results

MicroRNA-940 was Highly Expressed in Placental Villi of Early Abortion Pregnancies

MicroRNA-940 expression was higher in early abortion pregnancies compared with that of normal pregnancies ($p<0.001$, Figure 1A). Furthermore, microRNA-940 expression was higher in the placental villi of spontaneous abortion pregnancies than that of induced abortion pregnancies (Figure 1B). The target genes of microRNA-940 were predicted by bioinformatics and ZNF672 was finally screened out. ZNF672 expression in the placental villi was examined. It was found that the mRNA expression of ZNF672 in the threatened abortion pregnancies was remarkably lower than that of the normal pregnancies (Figure 1C). Besides, the mRNA expression of ZNF672 in spontaneous abortion pregnancies was lower than that of induced abortion pregnancies (Figure 1D). These results indicated that microRNA-940 may be involved in the occurrence of early abortion.

MicroRNA-940 Inhibited Proliferation of Trophoblast Cells

To explore the effect of microRNA-940 on early abortion, the expressions of microRNA-940 and ZNF672 in trophoblast cells (BeWo, JEG3, Wish and HTR-8) were detected. HTR-8 cells expressed the highest level and BeWo cells expressed the lowest level of microRNA-940 (Figure 2A, 2B). The transfection efficacies of microRNA-940 mimic and inhibitor were verified by qRT-PCR (Figure 2C, 2D). After transfection of microRNA-940 inhibitor in HTR-8 cells, the proliferative capacity was remarkably increased (Figure 2E). On the contrary, the transfection of microRNA-940 mimic downregulated the proliferation of BeWo cells (Figure 2F).

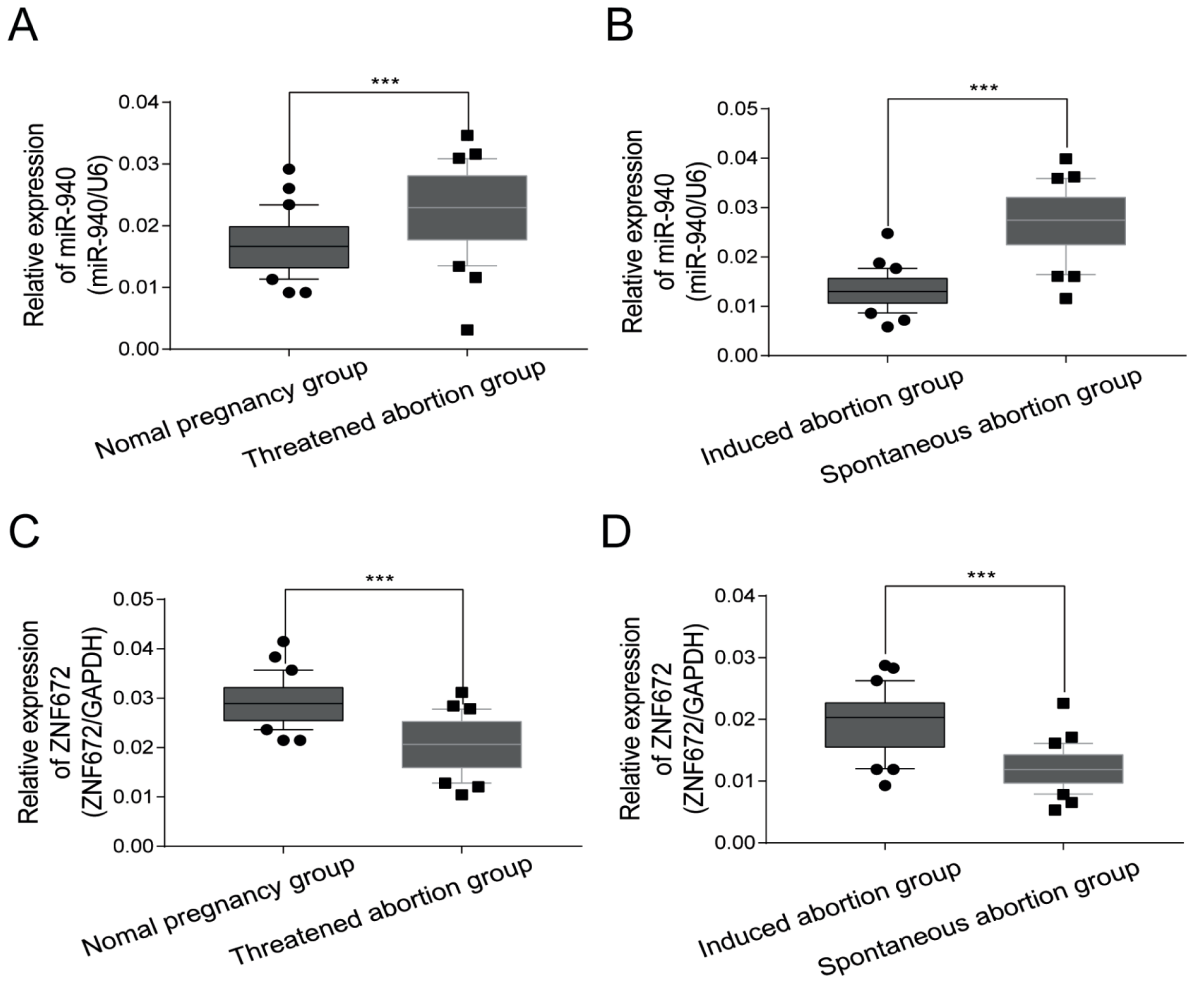

Figure 1. MicroRNA-940 was highly expressed in placental villi of early abortion pregnancies. ***A,*** MicroRNA-940 expression was higher in early abortion pregnancies compared with that of normal pregnancies. ***B,*** MicroRNA-940 expression was higher in the placental villi of spontaneous abortion pregnancies than that of induced abortion pregnancies. ***C,*** The mRNA expression of ZNF672 in the threatened abortion pregnancies was remarkably lower than that of the normal pregnancies. ***D,*** The mRNA expression of ZNF672 in spontaneous abortion pregnancies was lower than that of induced abortion pregnancies.

MicroRNA-940 Promoted Early Abortion by Regulating Target Gene ZNF672

To further verify whether microRNA-940 can bind to ZNF672, ZNF672 wt and ZNF672 mut were first constructed (Figure 3A). The correlation analysis showed that microRNA-940 was negatively correlated with ZNF672 expression (Figure 3B). Dual-Luciferase reporter gene assay indicated that the Luciferase activity of ZNF672 wt group was reduced after microRNA-940 mimic transfection in HTR-8 cells. However, no significant difference in the Luciferase activity was found in the ZNF672 mut group (Figure 3C). Similar results were obtained in BeWo cells (Figure 3D). RIP assay was then conducted to verify the binding condition of microRNA-940 and ZNF672. The data indicated that the expression of Ago2-bound microRNA-940 was remarkably higher than that of IgG-bound microRNA-940 (Figure 3E, 3F), indicating that microRNA-940 participates in the formation of RISC complexes.

Discussion

Spontaneous abortion is the most common complication of human pregnancy. Early abortion is defined as abortion occurring within 12 weeks of pregnancy. The causes of spontaneous abortion are

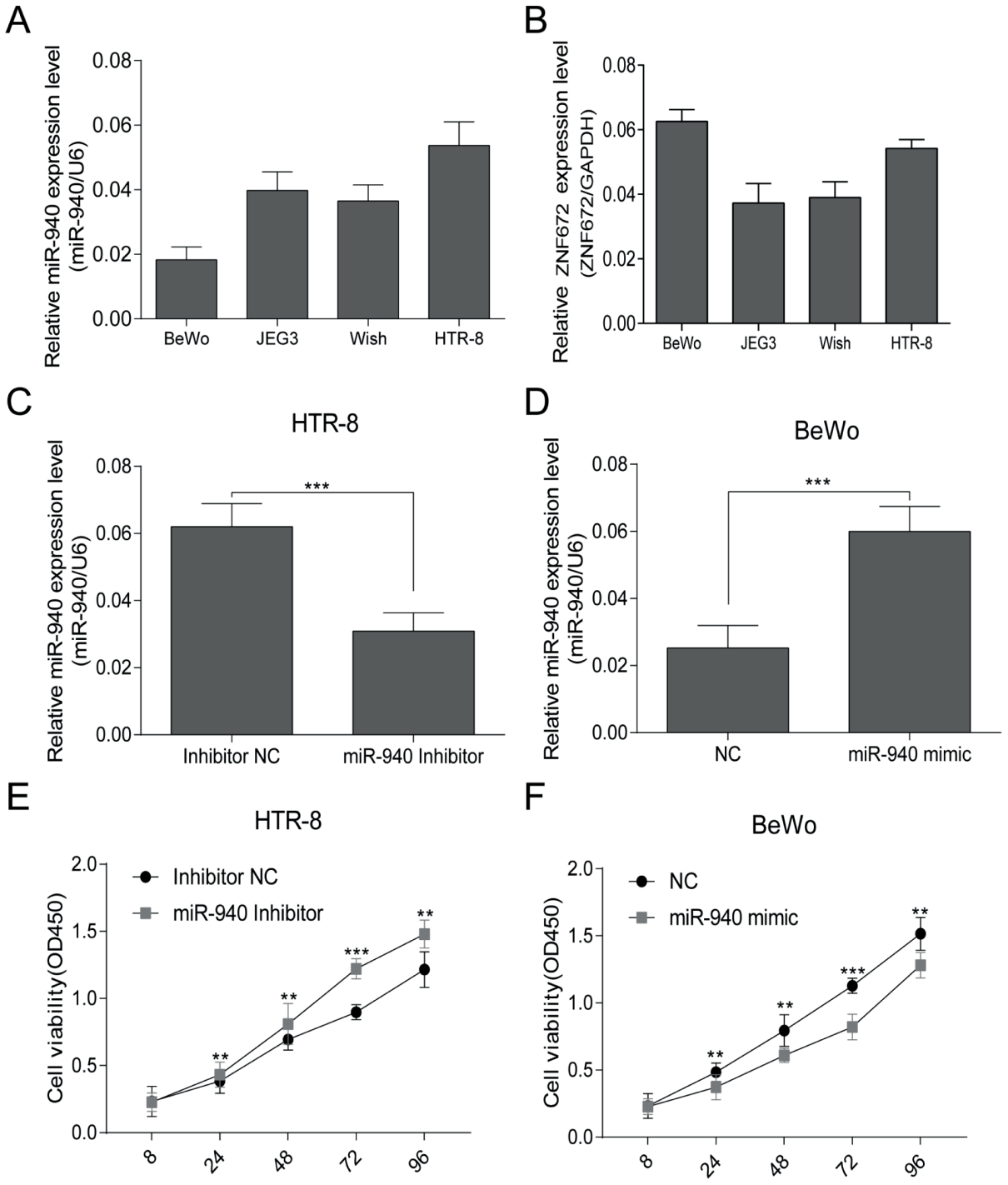

Figure 2. MicroRNA-940 inhibited proliferation of trophoblast cells. ***A-B***, HTR-8 cells expressed the highest level and BeWo cells expressed the lowest level of microRNA-940. ***C-D***, Transfection efficacies of microRNA-940 mimic and inhibitor were verified by qRT-PCR. ***E***, After transfection of microRNA-940 inhibitor in HTR-8 cells, the proliferative capacity was remarkably increased. ***F,*** Transfection of microRNA-940 mimic downregulated the proliferation of BeWo cells.

complicated and involve genetic and immune factors, endocrine disorders, etc. Placental trophoblast cells are closely related to early abortion[13].

Placental trophoblast cells exert a vital role in the implantation of human blastocysts in early pregnancy and the regulation of fetal growth

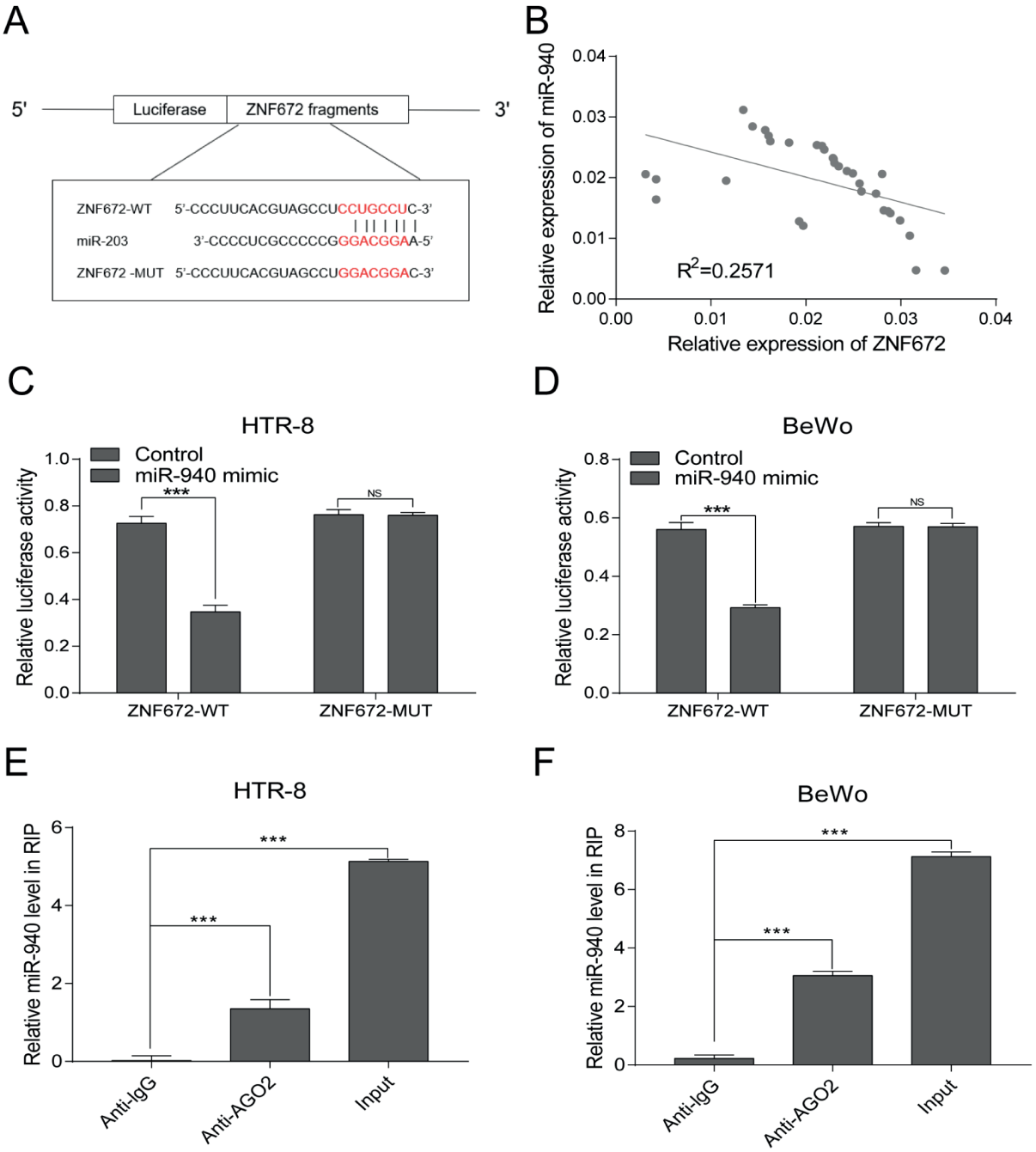

Figure 3. MicroRNA-940 promoted early abortion by regulating target gene ZNF672 ***A,*** Construction of ZNF672 wt and ZNF672 mut. ***B,*** Correlation analysis showed that microRNA-940 was negatively correlated to ZNF672 expression. ***C,*** Dual-Luciferase reporter gene assay indicated that the Luciferase activity of the ZNF672 wt group was reduced after microRNA-940 mimic transfection in HTR-8 cells. ***D,*** No significant difference in the Luciferase activity was found in the ZNF672 mut group. ***E-F***, RIP assay indicated that the expression of Ago2-bound microRNA-940 was remarkably higher than that of IgG-bound microRNA-940.

in late pregnancy[14]. The proliferation and apoptosis of placental trophoblasts are important for maintaining normal development and homeostasis[15]. The proliferation and apoptosis of placental trophoblast cells participate in the development, maturation and aging of the placenta. The abnormal proliferative capacity of trophoblast cells in early pregnancy may lead to pathological pre-

gnancy, such as miscarriage, hydatidiform mole, etc. It may also lead to preeclampsia and fetal growth restriction (FGR) in late pregnancy[16].

MiRNAs are non-coding, single-stranded, small-molecule RNAs. They inhibit the target mRNA by complementary pairing with 3'UTR of mRNA. Previous studies[17] showed that miRNA expression is associated with many types of tumors. About 50% of miRNAs are located on the genome in tumor-associated fragile sites, suggesting that miRNAs exert a crucial role in the midterm of tumorigenesis[18]. The proliferation behavior of placental trophoblast cells is undoubtedly similar to that of malignant tumors. We believed that the proliferation behavior of trophoblast cells may be regulated by some certain miRNAs. There are some miRNAs only expressing in the placenta, including miR-512-3p, miR-517a, miR-517b, miR-518b, miR-519a, miR-1185, miR-1283 and miR-1323[19]. These certain miRNAs are placenta-specific miRNAs. We found that microRNA-940 is highly expressed in placental villi of early abortion pregnancies, which could inhibit the proliferation of trophoblast cells.

Some studies[20-24] have showed that NF-κB, Wnt/β-catenin, ZNF24, GSK3β and MIEN1 are the target genes of microRNA-940. They participate in the migration, metastasis and proliferation of pancreatic and gastric cancer. In the present work, ZNF762 was screened out to be the target gene of microRNA-940 by online prediction. Dual-Luciferase reporter gene and RIP assay proved that microRNA-940 can promote early abortion by regulating ZNF672.

Conclusions

We detected that was microRNA-940 is highly expressed in the placental villi of early abortion pregnancies. MicroRNA-940 promoted the occurrence of early abortion by inhibiting the proliferation of trophoblast cells by targeting ZNF672.

Conflict of interest

The authors declare no conflicts of interest.

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