miR-98 is involved in missed abortion by targeting *GDF6* and *FAPP2*

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Abstract

Missed abortion (MA) is a common disease in obstetrics and gynecology. More and more studies have focused on the relationship between miRNAs and pregnancy maintenance and its related diseases. The aim of this article is to explore the relationship between miRNA and MA. The expression of *miR-98* were detected by *in situ* hybridization and real-time PCR. Cell proliferation, activity and migration were measured via Edu, MTT, and transwell assays. The target genes of *miR-98* are identified by dual-luciferase activity assay. And the expression levels of target genes were determined by Western blot, real-time PCR and immunohistochemistry. *miR-98* was significantly up-regulated in placental villi from over 35 years old MA patients compared with the age-matched normal pregnant women. Up-regulation of *miR-98* suppressed the proliferation, activity and migration of the human trophoblast HTR-8/SVneo cell *in vitro*. *miR-98* could bind to *GDF6* and *FAPP2* mRNA 3'-UTR and negatively regulate their expression. The downregulation of *miR-98* promoted cell proliferation, then knockdown of *GDF6* or *FAPP2* inhibited *miR-98*-mediated cell proliferation. *GDF6* and *FAPP2* expression in the placental villi from MA patients were decreased compared to normal placental tissues. The expression of *miR-98* in MA had an opposite relationship with the expression of *GDF6* and *FAPP2*. Overexpression of *miR-98* is associated with the occurrence of MA. *miR-98* prevents proliferation, viability and migration of trophoblast cells partially through targeting *GDF6* and *FAPP2*.

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Introduction

Missed abortion (MA) is a special type of spontaneous abortion manifested as that intrauterine embryo stops growing and dies, but embryo and trophoblastic tissue are not spontaneously evacuated from uterine cavity in early pregnancy (Kafil & Arain 2017). In the process of MA, placental tissue closely adheres to the uterine wall, causing difficulty in curettage (Xiao *et al.* 2019). It makes women confronting with the surgery and facing the risk of severe bleeding and complications associated with anesthesia. Previous studies have shown that there are multiple etiologic factors involved in the occurrence of MA, including genetic and uterine abnormalities, endocrine and immunological dysfunctions, infections, nutritional and environmental factors, psychogenetic factors, and endometriosis (Clifford *et al.* 1994, Hatasaka & Gynecology 1994, Bulletti *et al.* 1996). However, the pathogenesis of the disease is currently unclear.

miRNAs are small, endogenous, noncoding single-stranded RNA molecules with 17–22 nucleotides. They degrade target mRNA or inhibit target mRNA translation by binding to 3' untranslated regions (3'-UTR) of target mRNAs. They are highly conservative, tissue-specific and time-sequential and do not have an open reading

frame. Up to 2018, the number of miRNAs annotated in miRBase has grown to 38,589 hairpin precursors and 48,860 mature miRNAs from 271 organisms (miRBase, release 22.1: October 2018). Almost one-third of the protein-coding genes are susceptible to be regulated by miRNAs post-transcriptionally (Lewis *et al.* 2005). By negatively regulating the expression of target genes, miRNAs are involved in the regulation of many central biological processes, such as cells development, proliferation, differentiation and apoptosis (Ni *et al.* 2015), cancer proliferation and metastasis (Long *et al.* 2017). One miRNA can regulate many different genes and simultaneously a gene can be targeted by multiple miRNAs (Reinhart *et al.* 2000, Girard *et al.* 2008). Therefore, miRNA has been used to explore the pathogenesis of many diseases since been found. Recently, there had some reports demonstrate that abnormally expressed miRNAs are associated with pregnancy maintenance and pregnancy-specific diseases (Hromadnikova *et al.* 2012, Li *et al.* 2012, Zhu *et al.* 2016, Wang *et al.* 2015), indicating that miRNAs play an important role in pregnancy.

Our previous study indicates that *miR-98* was differentially expressed in rat uteri during the peri-implantation period and that down-regulation of *miR-98*

in rat uterus during the receptive phase was linked to successful embryo implantation via targeting *Bcl-xl* (Xia *et al.* 2014). The implantation failure is one of major reasons for spontaneous abortion. And another study found that the expression of *miR-98* was abnormal in deciduas of spontaneous miscarriage patients at 6–8 weeks (Wang *et al.* 2015). Therefore, we speculated that *miR-98* may be associated with MA. In this study, we explore the relationship between *miR-98* and MA occurrence and investigate the functional roles of *miR-98* in trophoblast cell. Additionally, we also test the possible molecular mechanisms in which *miR-98* is implicated.

Materials and methods

Consent to participate

All participants gave written informed consent and the study has been approved by Ethics Committee of Research Institute for Family Planning (2011-10).

Tissue samples

Two hundred and sixty samples of human MA placenta tissues and 396 samples of normal placenta tissues were collected from patients at Sixth Hospital of Shijiazhuang (Hebei, China). Both MA group samples and case group samples were taken before 12 weeks' gestation. Most of the possible factors led to spontaneous abortion were excluded, including the couple's chromosomal karyotype abnormality, anomaly of reproductive system, glucose level, T3/T4/TSH and sexual hormones level in maternal blood, immune antibodies (ANA/ACLA) test, virus infection (TORCH) test and the routine semen analysis from the patients' partner. Moreover, the patients who had genetic defects or hereditary diseases associated with spontaneous abortion in their family history were excluded.

Gestational age-matched normal gravidas who voluntarily chose induced abortion were served as controls. All samples were collected with informed consent of the patients. The tissues were frozen immediately in liquid nitrogen and stored at -80°C . This study has been approved by the ethics committee of National Research Institute for Family Planning (No.2011-06). We pair the placenta tissues by age, one case and one control. Hundred and seventy one pairs samples were sent to Beijing Longmida Technology Co., Ltd. to make tissue microarrays, a technology that makes it possible to sample up to 1000 tissue samples on one glass slide. Analysis of qRT-PCR was performed on 40 pairs samples that were 3 months pregnant from age groups $Y < 25$, $Y25-30$, $Y30-35$ and $Y > 35$.

Cells culture and transfection

HTR8/SVneo cells were gifted by Professor Yanling Wang from Institute of Zoology of Chinese Academy of Sciences (Beijing, China). The HTR8/SVneo cells were cultured in RPMI-1640 with 10%(v/v) fetal bovine serum (FBS), 100 IU/mL penicillin and 100 IU/mL streptomycin in 5%(v/v) CO_2 atmosphere at 37°C . *miR-98* mimic, miRNA mimic negative control, *miR-98* inhibitor or miRNA inhibitor negative control (GenePharma,

China) was, respectively, transfected into the HTR8/SVneo cells through Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. The final concentration *miR-98* mimic, miRNA mimic negative control, *miR-98* inhibitor and miRNA inhibitor negative control were 50 nM. Non-treated cells and cells treated with lipofectamine only were served as two additional controls. *miR-98* mimic is a double stranded microRNA molecule designed according to the sequence of mature *miR-98*, which is used to simulate the sequence of endogenous mature miRNA. *miR-98* inhibitor is sequence-specific and chemically modified to specifically target and knockdown individual *miR-98* molecules. The designs of miRNA mimic negative control and miRNA inhibitor negative control are similar with *miR-98* mimic and *miR-98* inhibitor, respectively. However, either miRNA mimic negative control or miRNA inhibitor negative control is a random sequence based on *C. elegans* microRNA sequence that has been extensively tested in human cell lines and tissues and validated to produce no identifiable effects on known miRNA function.

In situ hybridization of miR-98 with DIG-labeled LNA probe

The tissue microarrays were treated with proteinase K (100 $\mu\text{g}/\text{mL}$) for 15 min after deparaffinization by xylol and then refixed in 4%(w/v) paraformaldehyde for 5 min. After rinsing with phosphate buffer saline, the slides were prehybridized with hybridization buffer at 40°C for 1 h and then hybridized with digoxigenin (DIG)-labeled LNA-*miR-98* probe (LNA-*miR-98* sequences: 5'-DIG-aCaaTaCaaCtaCtAcCtCa-3') overnight at 40°C . On day 2, the slides were washed in serial dilutions of saline sodium citrate solutions (4x, 2x and 0.1x) and blocked with blocking reagent at room temperature for 30 min, then incubated in blocking reagent with alkaline phosphatase (AP) labeled anti-DIG-antibody (Roche) overnight at 4°C . Staining was performed using BCIP/NBT Color Development Substrate (Promega). The sections were hybridized with DIG-labeled LNA-scrambled probe (LNA-scrambled sequences: 5'-caTtaAtgTcGgaCaaCtcAat-3') as negative control. Imaging was performed by iScanCoreo Au (Roche). Positive signals were quantitated by Image J software.

Real-time PCR procedure to quantify miR-98 expression and target genes expression

Total RNA was extracted by using TRIzol reagent and dissolved in diethylpyrocarbonate-treated water. The RNA concentration and purity were estimated by NanoDrop 2000 (ThermoFisher Scientific). The *miR-98* expression was determined by TaqMan miRNA RT-Real Time PCR kit (ThermoFisher Scientific). U6snoRNA was used for normalization. Total mRNA was synthesized by 5x All-In-One RT MasterMix kit (Applied Biological Materials Inc). The mRNA level was amplified by 2x SYBR Green qPCR mix (Applied Biological Materials Inc) and the Real-Time PCR primers in Table 1. *Gapdh* gene was used for normalization. Relative miRNA/mRNA expression was calculated by efficiency-corrected $\Delta\Delta\text{Ct}$ method. Each sample in each group was measured in triplicate, and the experiment was repeated for three times at least.

Table 1 Primer sequences.

Gene name	Primer sequence (5'→3')		Application
	Forward	Reverse	
<i>GDF6</i> 3'-UTR	CTAGCTAGCGGTGACTGAAAGTGCAAAGAGG	ACGCGTCGACGCATATCATCCAGGAAAGGTGTAC	PCR
<i>FAPP2</i> 3'-UTR	CTAGCTAGCTCCTGTGTATGCGTGTCTG	ACGCGTCGACTAGCCTTTGGCATCTGGTC	PCR
<i>mut-GDF6</i> 3'-UTR	GAGTTTTTTTTAACTGTTTCTTTATATAATACAAGT	TAAAGCAAACAGTTTAAAAAACTCTCCATTAGACAG	PCR
<i>mut-FAPP2</i> 3'-UTR	TGTCATTAAACAGCCCAATTGCTTTATGTGAAATTGGC	TAAAGCAATTGGGCTGTTAATGACATTCTAGTGAAAT	PCR
<i>GDF6</i>	AATACCCATTCCAGATAGTCAAA	CTAACTCTCAAAGGGCCTACTC	qRT-PCR
<i>FAPP2</i>	CCAAGGTGTGTGTGTATTTC	ACGCGTCGACTAGCCTTTGGCATCTGGTC	qRT-PCR

MTT assay

Effect of *miR-98* on viability of human trophoblasts cells was studied by MTT Assay. HTR8/SVneo cells were seeded in 96-well tissue culture plate (5000 cells per well) and incubated for 48 h after transfecting with *miR-98* mimic, miRNA mimic negative control, *miR-98* inhibitor and miRNA inhibitor negative control, respectively. The final concentration of miRNA mimic, inhibitor or corresponding controls was 50 nM. Non-treated cells and cells treated with lipofectamine only were served as two additional controls. Twenty microliters of MTT (5 mg/mL; Promega Corporation) was added to each well and incubated at 37°C. After 2 h, the MTT was removed and 100 µL of dimethyl sulfoxide was added to each well. The absorbance value of each well was read at 562 nm by SynergyMx microplate Reader (BioTek). Each sample was measured in triplicate, and all these assays were performed at least three times.

Cell migration assay

Effect of *miR-98* on migration of human trophoblasts cells was studied by Transwell Assay. After 48 h of incubation, HTR8/SVneo cells were transfected with *miR-98* mimic, miRNA mimic negative control, *miR-98* inhibitor and miRNA inhibitor negative control, respectively, in a 24-well plate. The final concentration of miRNA mimic, inhibitor or corresponding controls was 50 nM. Non-treated cells and cells treated with lipofectamine only served as two additional controls. Then the cells were trypsinized, washed and suspended in medium RPMI-1640 with 1%(v/v) FBS. A total of 5×10^4 cells were seeded on the top of 8.0 mm pore membrane chamber (Corning CostarCorp.). To the bottom compartment, RPMI-1640 with 5%(v/v) FBS was added as the chemotactic factor. The chamber was incubated for 12 h. Then the non-migrated cells on the top of chamber were carefully removed with a cotton swab. Migrated cells on the bottom of chamber were fixed and stained with 0.1%(w/v) crystal violet solution. The cell migration was quantified by the number of cells passing through the pores from five different fields per sample. All experiments were performed at least three times.

EdU assay

The proliferation of HTR8/SVneo cells was determined by Cell-Light™ EdU Apollo®567 In Vitro Imaging kit (Ribobio) according to the manufacturer's instructions. Cells were transfected with the *miR-98* mimic, miRNA mimic negative control, *miR-98* inhibitor and miRNA inhibitor

negative control, respectively, in a 24-well plate. The final concentration of miRNA mimic, inhibitor or corresponding controls was 50 nM. Non-treated cells and cells treated with lipofectamine only were served as two additional controls. EdU is a thymidine analog which can label cells when DNA replicating (Salic & Mitchison 2008). Cells were cultured in 24-well plates with 1×10^5 cells/well and exposed to 50 µM EdU for 2 h at 37°C, then fixed in 4%(w/v) formaldehyde for 30 min at room temperature and permeabilized in 0.5%(v/v) Triton X-100 for 10 min. Subsequently, the cells were washed with PBS, and each well was incubated with 200 µL 1× Apollo reaction cocktail for 30 min. DNA was stained with 200 µL 1× Hoechst33342 for 30 min and imaged under Confocal Microscope (LSM 710; Zeiss). This experiment was repeated for at least three times.

Plasmid construction

The *GDF6* 3'-UTR and *FAPP2* 3'-UTR sequences were amplified by PCR from human genomic DNA using the primers in Table 1. After being double digested with Sall and NheI, the PCR product was cloned into pmirGLO vector (Invitrogen). The *GDF6* 3'-UTR-mutant and *FAPP2* 3'-UTR-mutant vector were obtained following Fast Mutagenesis System (TransGen Biotech) using the primers in Table 1. Scramble siRNA control, *GDF6* siRNA and *FAPP2* siRNA were synthesized by GenePharma.

Dual-luciferase activity assay

The HTR8/SVneo cells seeded in a 48-well plate were treated with recombinant plasmid and *miR-98* mimic, miRNA mimic negative control, *miR-98* inhibitor and miRNA inhibitor negative control, respectively, through co-transfection with Lipofectamine 2000 Reagent, according to the manufacturer's protocol (siRNA Plasmid Co-Transfection Protocol with Lipofectamine 2000, Invitrogen). The final concentration of miRNA mimic, inhibitor or corresponding controls was 50 nM. The concentration of plasmid DNA was 0.5 ng/µL. After 48 h of transfection, cells were harvested and lysed. The luciferase reporter activities were measured using Dual-Glo® Luciferase Assay System (Promega). Each sample was measured in triplicate, and all experiments were repeated for at least three times.

Western blotting analysis

Cell protein lysates were collected in RIPA Lysis Buffer. Protein concentration was measured by Easy II Protein Quantitative kit

(BCA) (TransGen). Sixty microgram of total protein was separated on 12%(w/v) acrylamide gel by electrophoresis and transferred to polyvinylidene fluoride membrane (Amersham Pharmacia Biotech), probed with antibodies against human GDF6 (1:250, Immunoway), FAPP2 (1:250, Proteintech) and anti- β -actin polyclonal antibody (1:250, Santa Cruz Biotechnology Inc.), respectively, overnight at 4°C. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:10,000, Biodragon) were used to detect primary antibodies. Chemiluminescence reaction was visualized on ECL kit (Millipore). The experiment has been repeated for at least three times.

Immunohistochemistry

The tissue microarrays (6 μ m) were deparaffinized in xylene and rehydrated in descending ethanol series. Antigen retrieval was accomplished through autoclave boiling the slide in 10 mM sodium citrate buffer. Then the slide was incubated with rabbit anti-GDF6 polyclonal antibody (1:100, Immunoway) and rabbit anti-FAPP2 polyclonal antibody (1:100, Proteintech),

respectively, then incubated with HRP-conjugated goat anti-rabbit IgG (1:500, BiodragonTechnology Co.). The antibody stains were developed through diaminobenzidine (ZsbioTechnology Co.) and nuclei were stained with hematoxylin (Sigma-Aldrich). The slide was incubated with fetal bovine serum as negative control. Digital images were obtained from slice scanning device NanoZoomer S210 (Hamamatsu). Positive signal was quantitatively analyzed through ImageJ software with H-score grading system (Budwit-Novotny *et al.* 1986). Random ten-pair samples in each age group (Y<25, Y 25–30, Y30–35, Y>35) were selected for optical density quantitative analysis.

Statistical analysis

The GraphpadPrism 7.0 software was applied for statistical analyses. Data are presented as mean $\pm$ S.E.M. from at least three independent experiments. Two groups comparisons were performed using *t*-test and multiple groups comparisons

Figure 1 qRT-PCR and ISH analysis of missed abortion placental tissues and normal tissues. (A) The relative expression of *miR-98* in individual sample was detected by TaqMan *miRNA* RT-Real Time PCR. U6 *snoRNA* was used for normalization. (B) The relative expression of *miR-98* in individual sample was grouped in 25 years old (Y<25), 25–30 years old (Y25–30), 30–35 years old (Y30–35), over 35 years old (Y>35). (C) ISH shows that *miR-98* was expressed both in MA sample (case group) and normal villi tissues (control group) at early pregnancy and mainly distributed in villi syncytiotrophoblast, cytotrophoblast and villous stroma. Differential expression of *miR-98* in MA placental tissues between different age groups compared to normal tissues. Strong *miR-98* staining signal (blue purple) in MA placental tissues and weak signal in normal placenta tissues. ISH: *in situ* hybridization; CTB: cytotrophoblast; STB: syncytiotrophoblast; VS: villous stroma. The scale bar is 400 μ m. ****P*<0.001, *****P*<0.0001.

using one-way ANOVA. Differences were considered significantly at $P < 0.05$.

Results

miR-98 was up-regulated in the placental villi from MA patients

In order to explore the role of miR-98 in MA, the expression patterns of miR-98 in the placental villi from MA patients and matching normal placental villi were analyzed. Because advanced age is a risk factor for missed abortion (Zhang *et al.* 2011), the placental villi tissues were divided into four groups according to the maternal age, including under 25 years old ($Y < 25$), 25–30 years old (Y_{25-30}), 30–35 years old (Y_{30-35}), over 35 years old ($Y > 35$).

qRT-PCR was used to determine the miR-98 level in the placental villi from MA patients (Fig. 1A and B). We firstly analyzed the miR-98 expression level in a single pair of case and control samples (Fig. 1A). In 38 pairs samples, miR-98 level was up-regulated in 26 pairs samples and down-regulated in 12 pairs samples. However, only 14 pairs of up-regulation of miR-98 levels showed statistically significant differences (at least $P < 0.05$). We further analyzed the percentage of miR-98 up-regulation in different age groups. The results showed that the percentage of up-regulation of miR-98 expression in the placental villi from MA patients was 37.50% (3/8 pair samples) in $Y < 25$ group, 30.00% (3/10 pair samples) in Y_{25-30} group, 10% (1/10 pair samples) in Y_{30-35} group and 70% (7/10 pair samples) in $Y > 35$ group. The

average level of miR-98 in placental villi from MA patients was higher than that in control group in $Y > 35$ group ($P < 0.001$; Fig. 1B).

In situ hybridization (ISH) was performed on placental villi tissues to clarify the distribution of miR-98 (Fig. 1C). It demonstrated that miR-98 was expressed both in MA samples and normal villi tissues at early pregnancy and mainly distributed in villi syncytiotrophoblast, cytotrophoblast and villous stroma. In different age groups, miR-98 staining signals were strong in MA placental tissues and weak in normal placenta tissues.

Figure 2 miR-98 expression level in different cell lines. The relative expression of miR-98 in HTR8/SVneo, HELA, HEK-293T and HEC-1-B was detected by TaqMan miRNA RT-Real Time PCR. The y-axis displays the relative log₂ ratio of miR-98 normalized by U6. ** $P < 0.01$, *** $P < 0.001$.

Figure 3 The effects of miR-98 on proliferation and viability. (A and B) Cells proliferation was determined by Edu assay. Red represents the proliferative cells and blue indicates cell nuclei. HTR8/SVneo cells transfected with miRNA mimic negative control, miR-98 mimic, miRNA inhibitor negative control and miR-98 inhibitor, respectively, were incubated for 48 h. The final concentration of miRNA mimic negative control, miR-98 mimic, miRNA inhibitor negative control and miR-98 inhibitor was 50 nM. Non-treated cells and cells treated with lipofectamine only were served as two additional controls. The scale bar is 50 μ m. (A1 and B1) The cell proliferation was quantified by counting the amount of Edu-positive cells vs total cells from five different fields per sample in randomly selection. (C and D) HTR8/SVneo cells were transfected with the miRNA mimic negative control, miR-98 mimic, miRNA inhibitor negative control and miR-98 inhibitor, respectively. The final concentration was 50 nM. Using non-treated cells and cells treated with lipofectamine only for additional controls. After 48 h, cells viability was determined by MTT assay. The y-axis displays the OD value at A570 nm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4 The effects of *miR-98* on migration of trophoblastic cells. (A) The migration of HTR8/SVneo cells were detected by transwell assay. HTR8/SVneo cells were transfected with miRNA mimic negative control, *miR-98* mimic, miRNA inhibitor negative control and *miR-98* inhibitor, respectively. The final concentration was 50 nM. Non-treated cells and cells treated with lipofectamine only were served as two additional controls. After 48 h, cells from each group were transferred separately to transwell chambers at density of 0.5×10^6 cells/mL. After incubation for 16 h, the cells on top of the transwell chamber were removed and the cells on the bottoms were stained with crystal violet. The scale bar is 1000 μ m. (A1 and A2) The cell migration was quantified by counting the amount of cells passing through the membrane from five different fields per sample at 100 $\times$ in randomly selection. ** $P < 0.01$.

Overexpression of *miR-98* suppresses the proliferation of HTR8/Svneo Cells in vitro

To study the possible effect of *miR-98* in the pathological process of placenta, HTR8/Svneo cells have been selected to be the *in vitro* cell lines model, as it developed from human placenta villus trophoblast cells and expressed *miR-98* modestly according to qRT-PCR result (Fig. 2).

Edu assay was applied to observe the effect of *miR-98* on proliferation of HTR8/Svneo cells (Fig. 3A and B). The relative proliferation rates in HTR8/Svneo cells transfected with *miR-98* inhibitor was significantly higher than that in the miRNA inhibitor negative control group and cells treated with lipofectamine only ($P < 0.05$, $P < 0.01$; Fig. 3B1). Compared with miRNA mimic negative control, the relative proliferation rates in *miR-98* mimic had downward trends, but no statistically significant differences.

Overexpression of *miR-98* suppresses the viability of HTR8/Svneo cells in vitro

MTT assay was used to further assess the effect of *miR-98* on the viability of HTR8/Svneo cells (Fig. 3C and D). The viability in cells transfected with *miR-98* mimic was obviously weaker than that miRNA mimic negative control group ($P < 0.001$). *miR-98* inhibitor significantly

strengthened cell viability as compared with miRNA inhibitor negative control group, non-treated cells and cells treated with lipofectamine only ($P < 0.01$ and $P < 0.001$).

Overexpression of *miR-98* inhibits the migration of HTR8/Svneo cells in vitro

The influence of *miR-98* on HTR8/Svneo cells migration was detected by transwell assay (Fig. 4A). The results displayed that the migration of HTR8/Svneo cells have no difference among non-treated cells, cells treated with lipofectamine-only and miRNA mimic negative control or miRNA inhibitor negative control. The migration of HTR8/Svneo cells transfected with *miR-98* mimic was significantly lower than the miRNA mimic negative control group ($P < 0.01$; Fig. 4A1). Otherwise, the migration of HTR8/Svneo cells was enhanced in *miR-98* inhibitor group ($P < 0.01$; Fig. 4A2). These findings suggest that *miR-98* suppressed the migration of HTR8/Svneo cells.

miR-98 negatively regulates GDF6 and FAPP2 expression by binding to their 3'-UTR

To elucidate the molecular mechanisms of *miR-98* effects in placenta development, computational

Figure 5 The prediction and confirmation of the *miR-98* target gene *GDF6*. (A) *miR-98* has a conservative binding site in *GDF6* 3'-UTR regions between different species. (B) Schematic diagram for constructing *miR-98* binding site into pmirGLO vector. (B1 and B2) Confirmation of *miR-98* target gene *GDF6* through transfecting HTR8/Svneo cells with *miR-98* mimic negative control, *miR-98* mimic, *miR-98* inhibitor negative control and *miR-98* inhibitor, respectively, for dual-luciferase assay. The final concentration of *miR-98* mimic, inhibitor or corresponding controls was 50 nM and the concentration of plasmid DNA was 0.5 µg/µL. (C) Schematic diagram for constructing *miR-98* mutant binding site into pmirGLO vector. (C1) Mutation analysis of the *miR-98* binding site. (D) *GDF6* protein level in HTR8/Svneo cells treated with *miR-98* mimic and *miR-98* inhibitor. The expression of β -ACTIN served as an internal control. The black histogram represents the optical densities of signals quantified by ImageJ and normalized with β -ACTIN intensity. (E1 and E2) *GDF6* expression level in HTR8/Svneo cells treated with *miR-98* mimic and inhibitor. The expression of *GDF6* mRNA in HTR8/Svneo cells were detected by qRT-PCR. *Gapdh* serves as an internal reference. The y-axis displays the relative log2 ratio of *GDF6* normalized by *Gapdh*. * $P < 0.05$, ** $P < 0.01$.

approaches were applied to predicted putative genes for *miR-98*. MiRWalk and Targetscan are two online databases on predicting and publishing miRNAs. By overlapping data between the two databases, we chose *GDF6* and *FAPP2* to study, since *GDF6* related to embryonic development and *FAPP2* abundantly expressed in placenta (Fagerberg et al. 2014, Hwang et al. 2015, Wei et al. 2016). The responsive sites in 3'-UTR of *GDF6* and *FAPP2* are both highly conserved among different species (Figs 5A and 6A). The pairing types of *GDF6* and *FAPP2* responsive element in 3'-UTR to the seed region of *miR-98* are both 8mer and

have different complementarity degrees with *miR-98* in non-responsive region of 3'-UTR (Figs 5B and 6B).

To investigate whether *GDF6* or *FAPP2* is the target gene of *miR-98*, their 3'-UTR fragment containing responsive sites was respectively cloned into pmirGLO control vectors, which have luciferase reporter genes. Recombinant plasmids were co-transfected into HTR8/Svneo cells with *miR-98* mimic, *miR-98* mimic negative control, *miR-98* inhibitor and *miR-98* inhibitor negative control, respectively. Detecting by dual-luciferase assay, the luciferase activity was significantly reduced in *miR-98* mimic group compared to *miR-98* mimic

Figure 6 The prediction and confirmation of the *miR-98* target gene *FAPP2*. (A) *miR-98* conservative binding sites in *FAPP2* 3'-UTR region between different species. (B) Schematic diagram for constructing *miR-98* binding site into pmirGLO vector. (B1 and B2) Confirmation of *miR-98* target gene *FAPP2* through transfecting HTR8/Svneo cells with *miR-98* mimic negative control, *miR-98* mimic, *miR-98* inhibitor negative control and *miR-98* inhibitor, respectively, for dual-luciferase assay. The final concentration of *miR-98* mimic, inhibitor or corresponding controls was 50 nM and the concentration of plasmid DNA was 0.5 ng/μL. (C) Schematic diagram for constructing *miR-98* mutant binding site into pmirGLO vector. (C1) Mutation analysis of the *miR-98* binding site. (D) *FAPP2* protein level in HTR8/Svneo cells treated with *miR-98* mimic and *miR-98* inhibitor. The expression of β-ACTIN served as an internal control. The black histogram represents the optical densities of the signals quantified by ImageJ and normalized with β-ACTIN intensity. (E1 and E2) *FAPP2* expression level in HTR8/Svneo cells treated with *miR-98* mimic and inhibitor. The expression of *FAPP2* in HTR8/Svneo cells were detected by qRT-PCR. *Gapdh* serves as an internal reference. The y-axis displays the relative log2 ratio of *FAPP2* normalized by *Gapdh*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

negative control group (*GDF6*-37%, $P < 0.01$, Fig. 5B1; *FAPP2*-54%, $P < 0.05$, Fig. 6B1). Conversely, the luciferase activity was obviously increased by *miR-98* inhibitor treatment (*GDF6*+19%, $P < 0.05$, Fig. 5B2; *FAPP2*+14%, $P < 0.05$, Fig. 6B2). To further validate the targeting relationship between *GDF6*, *FAPP2* and *miR-98*, mutation of responsive sites in recombinant plasmids was constructed for dual luciferase assay. Unmutated recombinant plasmids were utilized to be control. Under the influence of *miR-98* mimic, the luciferase activity of the control group was significantly lower than that of the mutation group (*GDF6*-51.72%,

$P < 0.05$, Fig. 5C1; *FAPP2*-89.66%, $P < 0.001$, Fig. 6C1). These data suggested that *miR-98* could down-regulate *GDF6* and *FAPP2* expression through binding to their responsive sites at the 3'-UTR. Therefore, it is better understood that both *GDF6* and *FAPP2* are direct target genes of *miR-98*.

To confirm whether *miR-98* could regulate endogenous *GDF6* and *FAPP2* expression, HTR8/SVneo was transfected transiently with *miR-98* mimic and *miR-98* inhibitor, respectively. The Western blot results showed that the *GDF6* protein was significantly decreased after *miR-98* mimic transfected and greatly

Figure 7 Knockdown of *GDF6* attenuates the down-regulation of *miR-98* mediated enhancement of cell growth. *GDF6* siRNA and scramble siRNA were transfected into HTR8/Svneo cells with *miR-98* inhibitor, respectively. (A) Cell proliferation was detected by Edu assay. The photographs are shown at 200 $\times$ and the scale bar is 50 μ m. (B) Cell migration was determined by transwell assay. The photographs are shown at 200 $\times$. The scale bar is 200 μ m. * $P < 0.05$.

increased in the *miR-98* inhibitor transfected group compared to negative control group ($P < 0.05$, Fig. 5D1; $P < 0.05$, Fig. 5D2, Supplementary Fig. 1, see section on supplementary materials given at the end of this article). In addition, as the result of qRT-PCR showed, the level of *GDF6* mRNA was significantly down-regulated by *miR-98* mimic and up-regulated by *miR-98* inhibitor in HTR8/SVneo cells ($P < 0.01$, Fig. 5E1; $P < 0.05$, Fig. 5E2). Similar results were obtained when *FAPP2* expression levels were detected by Western blot and qRT-PCR ($P < 0.05$, $P < 0.01$, Fig. 6D1 and D2; $P < 0.05$, $P < 0.01$, Fig. 6E1 and E2).

miR-98 effect HTR8/SVneo cells proliferation and migration by targeting *GDF6* and *FAPP2*

To further study the possible functions of *miR-98* mediating *GDF6* and *FAPP2*, rescue experiments were conducted on HTR8/SVneo cells. *GDF6* siRNA and *miR-98* inhibitor were co-transfected into HTR8/SVneo cells. As the results show, proliferation capacity of the cells co-transfected by *GDF6* siRNA and *miR-98* inhibitor was significantly decreased compared to that co-transfected by scramble siRNA and *miR-98* inhibitor ($P < 0.05$,

Fig. 7A1). Similar results of proliferation and migration were obtained when the cell was co-transfected with *FAPP2* siRNA and *miR-98* inhibitor ($P < 0.05$, Fig. 8A1 and B1). These findings implied that *miR-98* mediated enhancement on cell proliferation was down-regulated by knockdown of *GDF6* and *FAPP2*. They also suggest that *miR-98* could reduce cell proliferation and migration through suppression of *GDF6* and *FAPP2* expression.

Downregulation of *GDF6* and *FAPP2* expression in the placental villi from MA patients

To further explore the expression profiles of *miR-98* target genes *GDF6* and *FAPP2*, the expression and distribution of *GDF6* and *FAPP2* in human placental tissues with MA were determined by immunohistochemistry. Both *GDF6* and *FAPP2* were widely detected in MA tissues and normal tissues (Fig. 9A). They were mainly localized in villi syncytiotrophoblast, cytotrophoblast and villous stroma. The results reflected that the *GDF6* expression in placental villi of MA patients at each age was slightly less than that of control, but there was no statistical difference (Fig. 9A1). The expression of *FAPP2* in MA patients has great reduce in Y < 25 group and Y30–35

Figure 8 Knockdown of *FAPP2* attenuates the down-regulation of *miR-98* mediated enhancement of cell growth. *FAPP2* siRNA and scramble siRNA were transfected into HTR8/SVneo cells with the *miR-98* inhibitor, respectively. (A) Cell proliferation was detected by Edu assay. The photographs are shown at 200 $\times$. The scale bar is 50 μ m. (B) Cell migration was determined by transwell assay. The photographs are shown at 200 $\times$. The scale bar is 200 μ m. * $P < 0.05$.

group ($P < 0.01$, $P < 0.05$, Fig. 9A2). The expression trend of *GDF6* and *FAPP2* were opposite with *miR-98* in MA placental tissues, indicating that *miR-98* may be involved in the occurrence of MA by negatively regulating *GDF6* and *FAPP2*.

Discussion

In this study, we found that *miR-98* is significantly increased in MA placental villi tissues compared to normal tissues, suggesting that up-regulation of *miR-98* may be associated with the occurrence of MA. As far as we know, our research is the first study to examine the relationship between *miR-98* and the occurrence of MA.

Our findings demonstrate that *miR-98* was up-regulated in the placental villi from MA patients. We further explored the biological functions of *miR-98* in MA and found that overexpression of *miR-98* in HTR8/SVneo trophoblast cells *in vitro* inhibited cells proliferation, activity and migration. Interestingly, it has been found that *miR-98* prevents the proliferation, migration and invasion of cells in many cancers (Ni *et al.* 2015, Fu *et al.* 2018). The role of *miR-98* in

human trophoblastic cells was coincident with that in cancers. Consequently, all the facts show that *miR-98* is a cell growth suppressor. Proliferation, migration and invasion of trophoblastic cells into the maternal endometrium are essential steps in embryo implantation and placentation (Cohen *et al.* 2010). Up-regulation of *miR-98* in MA placental villi tissues may result in a shallow invasion of the trophoblast into the decidua and maternal vessels and affect the fetal nutrition supply and then may cause embryo death and the occurrence of MA.

To clarify the regulatory mechanisms of *miR-98* on cells, we predicted putative targets of *miR-98* by bioinformatic analysis and found that growth and differentiation factor 6 (*GDF6*) and phosphatidylinositol-4-phosphate adaptor protein-2 (*FAPP2*) were highly conserved among different species. In consideration of the abundant expression of *GDF6* and *FAPP2* in placenta, we chose them for further investigation (Fagerberg *et al.* 2014). *GDF6* and *FAPP2* 3'-UTR-mediated luciferase activity is specifically responsive to transfection of *miR-98* mimic and inhibitor. And mutation of seed sequence in the 3'-UTR of *GDF6* and *FAPP2* significantly reduced the binding ability to *miR-98*. Moreover, *miR-98* inversely regulated endogenous *GDF6* and *FAPP2* expression. The

Figure 9 The expression of *GDF6* and *FAPP2* in placental tissues. (A) The expression of *GDF6* and *FAPP2* in MA placental tissues and normal placental tissues was analyzed by immunohistochemistry. The sections were incubated with fetal bovine serum as negative control. The stain was developed with DAB and characterized by yellow-brown positive signal. The scale bar is 100 μ m. CTB: cytotrophoblast; STB: syncytiotrophoblast; VS: villous stroma. The gene expression is reflected by the intensity of positive signals quantitatively analyzed using the ImageJ soft. (A1) The comparison of *GDF6* expression level between MA placental tissues (case group) and normal placental tissues (control group) in different age groups. (A2) Statistics of *FAPP2* expression in different age groups. * $P < 0.05$, ** $P < 0.01$.

down-regulation of *miR-98*-mediated the enhancement of cell growth and invasion and was partially weakened by knockdown of *GDF6* or *FAPP2*. All these facts show that *GDF6* and *FAPP2* are the functional target gene of *miR-98*.

GDF6 governing an embryonic cell gene signature has been shown to play an important role in embryonic skeletogenesis, organogenesis and nervous system development (Settle *et al.* 2003, Nohe *et al.* 2004, Seemann *et al.* 2009, Bragdon *et al.* 2011, Hegarty *et al.* 2013, Yadin *et al.* 2016). *Gdf6a*($-/-$) mutants displayed maximal ocular apoptosis and induced microphthalmia based on the regulation of retinal progenitor cell number and proliferation in the ciliary marginal zone at 28 hours post fertilization (hpf) (French *et al.* 2013, Pant *et al.* 2013). In addition, *GDF6* have been implicated in limb joint formation and chondrogenesis in mammals (Chang *et al.* 1994, Thomas *et al.* 1996). It prevents differentiation of coronal suture mesenchyme in early cranial development and also has an essential role in retinal development (Asai-Coakwell *et al.* 2007, Clendenning & Mortlock 2012). Although the relationship between *GDF6* and abortion has not been reported so far, all the previously mentioned findings suggest that *GDF6* plays an important role in embryo development. High rates of recurrent pregnancy loss/MA were observed in a majority of embryos presenting with recurrent abnormal morphology (Philipp *et al.* 2003, Feichtinger *et al.* 2018). Therefore, we speculated

that the up-regulation of *miR-98* in MA inhibited the expression of *GDF6*, which caused abnormal embryonic development and even death, and then result in MA.

FAPP2 is a phosphoinositide receptor protein and a glycolipid transporter, which plays a key role in the production of glycosphingolipids (GSLs). *FAPP2* overexpression could promote colon cancer cell growth through activation of Wnt/ β -catenin signaling (Chen *et al.* 2019). Wnt/ β -catenin pathway has been confirmed to regulate cell fate determination, differentiation, proliferation and apoptosis during embryonic development, blastocyst activation for implantation and uterine decidualization. While aberrant Wnt signaling can damage embryo implantation and decidualization processes (Katoh & Katoh 2006, Zhang & Yan 2016). The downregulation of *FAPP2* gene could increase tumor cell sensitivity to Fas-induced apoptosis (Tritz *et al.* 2009). These results imply that *FAPP2* can positively regulate tumor cell invasion. Trophoblast invasion during embryo implantation in some aspects resembles tumor cell invasion. Our research demonstrated that *FAPP2* expression had an obvious downward trend in villus tissues of MA patients and that *miR-98* oppositely controlled *FAPP2* expression. On the basis of the previously mentioned results, we speculated that high-expression of *miR-98* in villus tissues of MA patients restrained trophoblast proliferation and invasion by inhibiting *FAPP2* expression, which might lead to abnormal expression of Wnt/ β -catenin signaling and Fas/FasL signaling. These events may result in a shallow of

embryo implantation and abnormal decidualization and finally cause MA.

miRNAs have become an ideal class of biomarker candidates for clinical diagnosis and treatment based on miRNAs stability and tissue specificity. Here, we found that *miR-98* level was significantly increased in Y>35 MA patients. Therefore, we considered to administer *miR-98* inhibitor or compensate *miR-98* target genes, *GDF6* and *FAPP2*, to treat *miR-98*-positive MA patients.

Taken together, our results demonstrate that up-regulation of *miR-98* is associated with the occurrence of MA. *miR-98* prevents proliferation, viability and migration of trophoblast cells by regulating *GDF6* and *FAPP2*. This study may provide new insights into the pathogenesis of MA.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0282>.

Declaration of interest

The authors declared that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H F X and X M conceived and designed the experiments. Q Z, Y C D and L Z performed the experiments. Q Z analyzed the data. Q Z and H F X wrote the paper. HFX and XM performed the final editing of the text.

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