TSH/miR-17-5p/ZNF367 axis is related to spontaneous abortion in patients with TSH above 2.5 mIU/L

Yang Yang1, Jiashu Li1, Yingying Zhou1,2, Wen Dai1,3, Weiping Teng1 and Zhongyan Shan1

1Department of Endocrinology and Metabolism, Institute of Endocrinology, NHC Key Laboratory of Diagnosis and Treatment of Thyroid Diseases, The First Affiliated Hospital of China Medical University, China Medical University, Shenyang, Liaoning, People’s Republic of China
2Department of Endocrinology and Metabolism, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, People’s Republic of China
3Department of Gynecology, Affiliated Hospital of Guizhou Medical University, Guiyang, Guizhou, People’s Republic of China

Correspondence should be addressed to Z Shan: shanzhongyan@medmail.com.cn

Abstract

Elevated thyroid-stimulating hormone (TSH) is associated with an increased risk of spontaneous abortion (SA); however, the associated mechanism remains unclear. This study aimed to investigate the expression of miRNAs and pathogenesis in the chorionic villi of TSH > 2.5 mIU/L-related SA patients. The chorionic villi were collected from pregnant women in the first trimester with TSH > 2.5 mIU/L with or without SA, as well as TSH < 2.5 mIU/L with or without SA to determine the level of miRNA expression. Differentially expressed miRNAs were confirmed by qRT-PCR in a total of 92 subjects. Cell Counting Kit-8 (CCK8), wound healing, transwell assays, and Western blotting were used to measure cellular biological functions and related proteins in HTR-8/SVneo cells. The potential mechanisms were determined using a Luciferase reporter assay and rescue experiment. Compared with normal pregnant women, miR-17-5p was decreased and zinc finger protein 367 (ZNF367) was upregulated in the chorionic villi of TSH > 2.5 mIU/L-related SA patients. Using HTR-8/SVneo cells, we demonstrated that elevated TSH inhibited miR-17-5p expression, as well as trophoblast migration and invasion. The overexpression of miR-17-5p targeted and inhibited ZNF367 expression promoting the biological function of trophoblasts. Further studies confirmed that ZNF367 interference partially reversed the biological function of the miR-17-5p inhibitor on HTR-8/SVneo cells. Taken together, our results showed that miR-17-5p promoted the biological function of trophoblasts by suppressing ZNF367.

Key Words

- TSH > 2.5 mIU/L
- spontaneous abortion
- trophoblast
- miR-17-5p
- ZNF367

Introduction

Serum thyroid-stimulating hormone (TSH) is a first-line indicator for the diagnosis of hypothyroidism. According to the latest American Thyroid Association (ATA) pregnancy guidelines, it is recommended that if pregnancy-specific TSH cannot be obtained, 4.0 mIU/L can be used as the cut-off point for the diagnosis of hypothyroidism (Alexander et al. 2017). However, several epidemiological studies have shown that TSH > 2.5 mIU/L during early pregnancy is associated with adverse pregnancy outcomes (Karakosta et al. 2012, Hernández et al. 2018), including spontaneous abortion (SA) (Negro et al. 2010, Kianpour et al. 2019, Li et al. 2019). Moreover, a meta-analysis has
shown that once subclinical hypothyroidism (SCH) is diagnosed using TSH > 2.5 mIU/L or pregnancy-specific diagnostic criteria, the risk of abortion will increase (Zhang et al. 2017a). In addition, it is more beneficial to pregnancy if the levels of TSH are controlled to levels less than 2.5 mIU/L in early pregnancy (Zhang et al. 2017a).

Early SA, defined as a spontaneous loss of pregnancy before 12 weeks, is one of the most common pregnancy complications. Embryo implantation and human placenta development are critical for a successful pregnancy. Accompanying implantation, trophoblast cells give rise to extravillous trophoblasts (EVTs) which migrate from the attached embryo and invade the uterine endometrium to anchor placenta and remodel spiral artery (Staun-Ram & Shalev 2005, Zhang et al. 2017b). A few studies have shown the non-thyroidal action of TSH on liver and vascular endothelial cells (Yan et al. 2016, Wang et al. 2020). Thyroid-stimulating hormone receptor (TSHR) mRNA and protein expression are decreased in the uterus of overt and subclinical hypothyroid rats before and at 5 days of pregnancy (Shan et al. 2019), which indicates that TSH might be involved in SA pathogenesis. However, the mechanism by which TSH > 2.5 mIU/L leads to SA, and the effect of TSH on trophoblasts remain largely unclear.

miRNA (miRNA) is a type of single-stranded RNA molecule with approximately 23 nucleotides in length (Bartel 2009, Tétreault & De Guire 2013). Previous reports have shown that miRNAs are associated with pregnancy-related diseases, including gestational diabetes (Zhao et al. 2011), pregnancy loss (Hong et al. 2018), and preeclampsia (Murphy et al. 2015). miRNAs can regulate the process of pregnancy by affecting uterine receptivity and trophoblast invasion (Zheng et al. 2017, Ding et al. 2019). miR-17-5p is a member of the miRNA17-92 cluster located on human chromosome 13q31.3. Recent studies have indicated that miR-17-5p is involved in different cancers as well as metabolism and inflammatory diseases (Liu et al. 2018a, Kuo et al. 2019). miR-17-5p was significantly decreased in the serum of patients with endometriosis (Wang et al. 2018). Compared with healthy conceptus, miR-17-5p was downregulated in the endometrium of the porcine arresting conceptus (Bidarimath et al. 2015). Some members of the miRNA17-92 cluster have been confirmed to promote the invasion and migration of trophoblasts (Xu et al. 2014, Tian et al. 2020). However, no studies have presented evidence of the miR-17-5p function in SA or abnormal TSH to date. In our previously published work, we found that miR-940 and miR-486-5p expression were elevated in the SCH combined with SA group compared with the control group (Zhou et al. 2018).

ZNF367 belongs to the ZNF transcription factor family, which has diverse functions including DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding (Laity et al. 2001). Recent studies have shown that several zinc finger proteins are involved in trophoblast invasion and differentiation (Chiu & Chen 2016, DaSilva-Arnold et al. 2019). While the function of ZNF367 in trophoblasts remains unknown.

In the present study, we sought to determine the differential expression of miRNAs in the chorionic villi of TSH > 2.5 mIU/L-related SA patients, as well as the potential roles of TSH and differentially expressed miRNA in regulating the process of SA.

Materials and methods

Patients and samples

Pregnant women undergoing a natural or induced abortion within the first 12 weeks of pregnancy at the Gynecology Clinic of the First Affiliated Hospital of the China Medical University were enrolled. Blood samples were measured for the levels of TSH, free thyroxine (FT4), free triiodothyronine (FT3), thyroid peroxidase antibody (TPOAb), and thyroglobulin antibody (TgAb) using an electrochemiluminescence immunoassay (Roche Diagnostics). Fresh chorionic villi were collected from the participants and stored at −80°C until further use. General information was recorded for all subjects. The following inclusion criteria were used for participant selection: (i) ultrasound diagnosis of pregnancy revealed stillbirth or normal pregnancy within 12 weeks; (ii) FT4 and FT3 within the normal reference range with negative TPOAb and TgAb; (iii) no history of other abortion-related diseases; (iv) no medication history affecting abortion; (v) no previous history of SA; vi) no other autoimmune diseases. The participants were divided into four groups based on the level of TSH and embryo survival: (i) group 1 (G1): TSH > 2.5 mIU/L combined with SA; (ii) group 2 (G2): TSH > 2.5 mIU/L without SA; (iii) group 3 (G3): TSH < 2.5 mIU/L with SA; (iv) normal pregnant women (CON): TSH < 2.5 mIU/L without SA. The experimental procedures were approved by the China Medical University Ethics Committee and were consistent with the Declaration of Helsinki. All participants signed written informed consent forms.

miRNA-seq analysis

The total RNA in the villous tissues was isolated using TRIzol reagent (Invitrogen). RNA integrity number (RIN)
or RNA quality number (RQN) is measured by fragment analyzer. RNA with RIN/RQN ≥ 7.0 was considered as high-quality RNA which was selected for miRNA-seq. RNA samples from G1 (n = 3), G2 (n = 3), G3 (n = 3), and CON participants (n = 3) were sent to The Beijing Genomics Institute (Beijing, China) for the analysis of miRNA expression. Large-scale miRNA expression profiling was achieved using miRNA-seq (BGISEQ-500). The 18–30nt RNA was separated from total RNA by PAGE electrophoresis and the 5-adenylated, 3-blocked ssDNA linker was connected to it. Added primers for RT and extension to synthesize a strand of cDNA, the used highly sensitive polymerase to amplify cDNA, enrich and amplify library yield. During the analysis, the data were filtered first to obtain clean tags, and the clean reads were compared to the reference base set and other small RNA databases using the comparison software AASRA. We used TPM to standardize the expression level of small RNAs so that the processing can avoid the influence of different sequencing quantities on the quantitative accuracy. We then performed multiple pairwise comparisons, in order to obtain more miRNAs for PCR verification under the premise of ensuring quality; we selected differentially expressed miRNAs according to the criteria of fold change ≥ 1.5 and P value < 0.05.

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

The total RNA of the villous tissues was extracted using TRIzol reagent (Invitrogen). The quality and concentrations of total RNA were measured using Nanodrop (Thermo Scientific), and RNA was considered as good quality when ratios of A260/A280 were 1.9–2.0. cDNA for miRNA and mRNA for qRT-PCR was synthesized using Mir-XTM (TaKaRa), and RNA was considered as good quality when the ratio of A260/A280 was 1.9–2.0. cDNA for miRNA and mRNA for qRT-PCR was synthesized using Mir-XTM (TaKaRa), and Prime Script RT Master Mix (TaKaRa), respectively. qRT-PCR using SYBR Green detection chemistry (TaKaRa) was performed with a LightCycler® 480 Instrument (Roche). PCR primer sequences for miRNA and mRNA were presented in Table 1. The relative level of miRNA and mRNA expression was normalized to that of the internal control U6 and GAPDH, respectively.

Western blotting

The total villous tissue and cellular protein were extracted by Total Protein Extraction Kit (KeyGEN, China). The BCA method was used to detect the concentration of protein. The protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes. After blocking for 2 h in 5% skim milk at room temperature, the membranes were incubated with the desired primary antibodies overnight (4°C): TSHR (1:1000 dilution; ZSGB-Bio, China), matrix metalloproteinase 2 (MMP2), MMP9, proliferating cell nuclear antigen (PCNA), and ZNF367 (1:1000 dilution; Abcam, USA), and GAPDH (1:2000 dilution; ZSGB-Bio, China). The membranes were subsequently incubated with a secondary antibody (1:5000 dilution; ZSGB-Bio, China) for 2 h at room temperature. The immunoreactive bands were detected by chemiluminescence (Thermo, USA). ImageJ software was used to quantify the signal intensities of the bands.

Cell culture and transfection

The human extravillous trophoblast cell line (HTR-8/SVneo) was purchased from the American Type Culture Collection (ATCC) via a reagent trader. HTR-8/SVneo was cultured in Dulbecco’s modified Eagle’s medium (DMEM, HyClone) supplemented with 10% PAN fetal bovine serum (FBS) (PAN-Biotech, Germany), 100 U/mL penicillin, and streptomycin, and incubated at 37°C with 5% CO2. miR-17-5p mimic and inhibitor, short interfering RNA (siRNA) targeting ZNF367, and their corresponding negative controls were obtained from Gene Pharma (Suzhou, China). When the cells reached 60–70% confluency in six-well plate, a mixture of Lipofectamine 3000 (Invitrogen) and miR-17-5p mimic/ inhibitor or si-ZNF367 was added following the manufacturer’s instructions. The siRNA and control sequences were si-ZNF367 (sense): 5’-GCCUGAGCGAGAUCACCCATT-3’, si-ZNF367 (antisense): 5’-UGGGUGAACUGUCAGGGT-3’, control was 5’-UUCUCCGAACGUGUCAGUTT-3’.

Immunofluorescence

HTR-8/SVneo cells on coverslips were fixed in 4% paraformaldehyde for 15 min and washed three times with PBS. The cells were permeabilized using Triton X-100 for

*Table 1*  Primsers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17-5p</td>
<td>Sense</td>
<td>CAAAGTGGCTTAGTGCAGTGA</td>
</tr>
<tr>
<td>U6</td>
<td>Sense</td>
<td>CTGCCAGTGGACGCACT</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TCGAGTAATCCACTGGTGTGA</td>
</tr>
<tr>
<td>ZNF367</td>
<td>Sense</td>
<td>TGCATGGGGTGAATCTGCAAG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GAAAGGTCAGGTGCGAATG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>GAAGATGGTATGGGATTTTC</td>
</tr>
</tbody>
</table>

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20 min and blocked in 5% BSA for 1 h. An anti-TSHR antibody (1:100) was used as a primary antibody, and an anti-rabbit fluorescein conjugate as the secondary antibody was used to incubate the cells.

**Cell counting kit-8 (CCK-8) assay**

A density of 2500 cells per well were seeded into 96-well plates (five replicate wells for each condition) and subsequently transfected with miR-17-5p mimic/inhibitor, si-ZNF367, or control. At 24, 48, 72, and 96 h post-transfection, 10 μL of the CCK-8 reaction solution (MCE, USA) was added to each well. After a 3 h incubation at 37°C, the absorbance was measured at a wavelength of 450 nm.

**Wound healing assay**

A total of 2 × 10^4 cells per well were seeded into six-well plates and transfected or stimulated. After 36 h of transfection or stimulation and upon reaching confluence, a thin scratch was made on the monolayer using a 200 μL pipette tip. The cells were washed three times with PBS to remove any cellular debris. Images in five non-overlapping observation fields were randomly captured for each group. The six-well plate was placed in the incubator. After 24 h, the scratches were observed under an inverted microscope, and pictures were obtained to record the changes in the scratches from each group.

**In vitro migration/invasion assays**

At 36 h post-transfection, HTR-8/SVneo cells were digested and seeded into the corresponding upper Matrigel-coated inserts at a density of 2.5 × 10^4 cells/well in 200 μL FBS-free DMEM media. A total of 600 μL DMEM media containing 15% FBS was used as a chemoattractant in the lower chamber. After a 24-h incubation, the inserts were removed, fixed with 4% paraformaldehyde for 30 min, and finally stained with 0.5% crystal violet for 10 min. The five randomly selected non-overlapping fields of invasive cells in each well were visualized and photographed at 100x magnification. A migration assay was performed similar to that described in the invasion assay, except that the inserts were not conducted with Matrigel and the cell density was 2 × 10^4 cells/well.

**Dual-luciferase assay**

HTR-8/SVneo cells were seeded into six-well plates at a density of 2 × 10^4 cells/well. After a 24-h incubation, the cells were co-transfected with ZNF367 3'-UTR WT/ Mutant vector (Gene Pharma, China) and an internal control Renilla luciferase-expressing vector (Promega), together with miR-17-5p mimic or the corresponding control. At 48 h post-transfection, the cells from each of the different groups were digested and seeded into 96-well plates at a density of 2 × 10^4 cells/well (three replicate wells for each condition). The 96-well plates were incubated for 6 h, and the luciferase activity was assessed using a Dual-Luciferase Reporter Assay Kit (Promega) in accordance with the manufacturer's instructions.

**Statistical analysis**

Normally and non-normally distributed data for the clinical characteristics of the included participants were expressed as the means ± s.d. and the median (interquartile range), respectively. For normally distributed data, a one-way ANOVA and least significant differences (LSD) post-hoc multiple comparisons test were conducted. A Kruskal–Wallis test was used to analyze the non-normally distributed data. For in vitro experiments, the significant difference between the two groups was determined using the Student’s t-test. A Spearman correlation test was used to analyze the correlation between miR-17-5p expression and the TSH level. All data were analyzed with SPSS 25.0 software (SPSS Inc). When the P-value was <0.05, the result was considered to be statistically significant.

**Results**

**miRNA-Seq of chorionic villi in TSH > 2.5 mIU/L-related SA patients and in controls**

Table 2 shows the general characteristics and laboratory test results of the pregnant women included in the present study. The serum TSH levels were higher in G1 and G2 than in G3 and CON. Except for TSH levels, there were no significant differences in clinical factors, including maternal age, height, weight, FT4, FT3, TPOAb, and TgAb levels. We selected three subjects out of four groups to perform miRNA-Seq using the RNA extracted from random sites of chorionic villi. Compared with CON group, there are a total of 218 known miRNAs, and 80 novel miRNAs were found to be up- or downregulated by at least 1.5-fold in chorionic villi of G1 (P < 0.05). Further, 642 differentially expressed miRNAs were selected when compared SCH+SA with SCH or SA by at least 1.5-fold change. Then we intersected the results of the two screenings and finally selected 145 relative
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Table 2 Thyroid function test and demographic data of the study participants.

<table>
<thead>
<tr>
<th></th>
<th>TSH &gt; 2.5 mIU/L with SA</th>
<th>TSH &gt; 2.5 mIU/L without SA</th>
<th>TSH &lt; 2.5 mIU/L with SA</th>
<th>Normal pregnant women</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>22</td>
<td>24</td>
<td>24</td>
<td>25</td>
<td>0.533</td>
</tr>
<tr>
<td>Age (years) a</td>
<td>30.36 ± 3.09</td>
<td>30.24 ± 3.91</td>
<td>31.21 ± 6.26</td>
<td>29.28 ± 4.16</td>
<td>0.642</td>
</tr>
<tr>
<td>Height (cm) b</td>
<td>163 (160–165.5)</td>
<td>162 (160–165)</td>
<td>162 (160–166.5)</td>
<td>164 (161–169)</td>
<td>0.363</td>
</tr>
<tr>
<td>Weight (kg) b</td>
<td>58.5 (53.75–62)</td>
<td>55 (52.13–60)</td>
<td>60 (53.88–65.75)</td>
<td>54 (48.5–65)</td>
<td>0.073</td>
</tr>
<tr>
<td>Smoking</td>
<td>1/22</td>
<td>0/24</td>
<td>0/22</td>
<td>1/22</td>
<td>NA</td>
</tr>
<tr>
<td>Drinking</td>
<td>2/22</td>
<td>1/24</td>
<td>3/24</td>
<td>1/22</td>
<td>NA</td>
</tr>
<tr>
<td>FT4 (pmol/L) a</td>
<td>13.53 ± 1.23</td>
<td>13.85 ± 1.16</td>
<td>13.52 ± 1.46</td>
<td>13.91 ± 1.82</td>
<td>0.706</td>
</tr>
<tr>
<td>FT3 (pmol/L) b</td>
<td>4.62 ± 0.54</td>
<td>4.45 ± 0.46</td>
<td>4.51 ± 0.43</td>
<td>4.43 ± 0.39</td>
<td>0.525</td>
</tr>
<tr>
<td>TSH (mIU/L) a</td>
<td>2.74 (2.60–3.28)</td>
<td>2.89 (2.66–3.32)</td>
<td>0.96 (0.62–1.43)</td>
<td>1.10 (0.81–1.51)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TPOAb (IU/mL) b</td>
<td>0.32 (0.06–0.86)</td>
<td>0.26 (0.11–0.93)</td>
<td>0.27 (0.12–0.46)</td>
<td>0.16 (0.10–0.34)</td>
<td>0.336</td>
</tr>
<tr>
<td>TgAb (IU/mL) b</td>
<td>1.84 (0.91–4.2)</td>
<td>1.77 (1.11–2.71)</td>
<td>1.06 (0.80–2.12)</td>
<td>1.28 (0.97–2.5)</td>
<td>0.073</td>
</tr>
</tbody>
</table>

aVariables are presented as mean ± s.d. analyzed using a one-way ANOVA; bVariables are presented as the median (interquartile range) and analyzed using a Kruskal–Wallis test.

FT3, free triiodothyronine; FT4, free thyroxine; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; TSH, thyroid-stimulating hormone.

Figure 1
The relative miR-17-5p expression in the chorionic villi of TSH > 2.5 mIU/L with spontaneous abortion (SA) group and other groups. (A) Heat-map of the differential miRNAs in the chorionic villi of four groups and three samples per group. (B) Comparison of miR-17-5p in the chorionic villi of 22 TSH > 2.5 mIU/L with SA, 21 TSH > 2.5 mIU/L without SA, 24 TSH < 2.5 mIU/L with SA, and 25 control (CON) groups, as determined by qRT-PCR. Data are presented as the median with a range. A Kruskal–Wallis test was used to analyze the differences between groups; *P < 0.05 and **P < 0.01. (C) The ability of miR-17-5p to differentiate TSH > 2.5 mIU/L with SA patients from other groups was shown by receiver-operating characteristic (ROC) curves. (D) A Spearman correlation test showed miR-17-5p was negatively associated with serum thyroid-stimulating hormone (TSH) levels. (E) The level of thyroid-stimulating hormone receptor (TSHR) was measured in each of four groups by Western blotting. Data are presented as the mean ± s.d., as analyzed by a one-way ANOVA; **P < 0.01. A full color version of this figure is available at https://doi.org/10.1530/JME-20-0335.
miR-17-5p was down-regulated in the chorionic villi of TSH > 2.5 mIU/L-related SA patients and associated with TSH levels

We subsequently selected several miRNAs to detect expression in the villous specimens derived from 22 TSH > 2.5 mIU/L-related SA patients (G1), 21 TSH > 2.5 mIU/L pregnant women without SA (G2), 24 SA patients with TSH < 2.5 mIU/L (G3), and 25 normal pregnant women (CON) (Supplementary Fig. 1). According to the results of PCR and the relative published article, we chose miR-17-5p for further analysis. Compared with the G3 and CON groups, miR-17-5p was downregulated in TSH > 2.5 mIU/L combined with SA patients (Fig. 1B). Except U6, we also chose RNU44 as an internal control to evaluate the relative expression of miR-17-5p in villi. When RNU44 was selected as the internal control, the PCR result was consistent with U6 as the internal control (Supplementary Fig. 2). To evaluate the sensitivity and specificity of miR-17-5p in the diagnosis of TSH > 2.5 mIU/L combined with SA,
we performed receiver operating characteristic curves with an area under the curve of 0.76 (Fig. 1C). As shown in Fig. 1D, miR-17-5p expression was negatively correlated with serum TSH levels ($r = -0.371; P < 0.01$). The villous specimens from G1 exhibited significantly higher levels of TSHR protein expression compared with that of the CON group (Fig. 1E). Based on these findings, we speculated that miR-17-5p might play an important role in TSH > 2.5 mIU/L-related SA.

High concentration of TSH inhibited miR-17-5p expression, as well as the migration and invasion of HTR8/SVneo cells in vitro

Furthermore, we evaluated the level of miR-17-5p in HTR8/SVneo cells exposed to TSH. TSHR mRNA (present within 30 cycles of amplification) and protein expression were detectable in HTR-8/SVneo cells (Supplementary Fig. 3A and B). Compared with 0.1 mIU/mL or 1 mIU/mL TSH, 10 mIU/mL TSH significantly inhibited the expression of miR-17-5 (Fig. 2A). Wound-healing and transwell assays were applied to investigate the effect of TSH on HTR8/SVneo cell migration and invasion. The results indicated that 10 mIU/mL TSH could significantly inhibit HTR8/SVneo cell migration and invasion compared with 0.1 or 1 mIU/mL TSH stimulation (Fig. 2B and C). The level of MMP2 and MMP9 protein expression was decreased when trophoblasts were exposed to 10 mIU/mL TSH compared with 0.1 or 1 mIU/mL TSH exposure (Fig. 2D). Therefore, we demonstrated that a high concentration of TSH inhibited the migration and invasion ability of HTR8/SVneo cells accompanied by decreased miR-17-5p expression.

miR-17-5p enhanced HTR8/SVneo cell proliferation, migration, and invasion

To assess the role of miR-17-5p in regulating trophoblast function, we cultured HTR8/SVneo cells in vitro for further investigation. A CCK-8 assay was used to detect cellular proliferation, and the results showed that the proliferation of miR-17-5p-overexpressing cells was increased compared with the control ($P < 0.05$) (Fig. 3A). We subsequently found that the proliferation of HTR8/SVneo cells transfected with miR-17-5p inhibitor was significantly decreased ($P < 0.05$) (Fig. 3B). Wound-healing and transwell assays were conducted to explore trophoblast migration and invasion. Compared to the corresponding control cells, the fusion area and number of cells migrating/invading through transwell pores were significantly increased following transfection with miR-17-5p mimic (Fig. 3C and D).

As expected, miR-17-5p-knockdown significantly reduced HTR-8/SVneo cell invasion and migration (Fig. 3C and D). The level of PCNA, MMP2, and MMP9 protein expression was consistent with the biological function results (Fig. 3E and F). In addition, the ectopic expression and inhibition of miR-17-5p were confirmed by qRT-PCR after transfection (Supplementary Fig. 3C and D). These results suggested that miR-17-5p enhanced trophoblast proliferation, migration, and invasion.

ZNF367 was a target of miR-17-5p

To explore the mechanism of miR-17-5p-mediated biological function, TargetScan 7.2, miRDB, miRTarBase, and miRanda were utilized to predict the direct target genes of miR-17-5p. ZNF367 was selected as a putative miR-17-5p target for further research. Three predicted positions for miR-17-5p binding to ZNF367 mRNA were shown in the 3'-UTR: two were located at 163 bp–169 bp and 311–317 bp, which are highly conserved across species, and the other was located at 1178–1184 bp, which is poorly conserved. To investigate whether ZNF367 inhibition by miR-17-5p occurred via these predicted binding sites, the above three sites were mutated and referred to as miR-17-5p mutant (Fig. 4A). A dual-luciferase reporter assay was performed to demonstrate that ZNF367 could be inhibited by miR-17-5p. miR-17-5p markedly decreased the luciferase activity of WT ZNF367 3'-UTR in HTR-8/SVneo cells, whereas the suppressive effect was abrogated after mutating the ZNF367 3'-UTR binding site (Fig. 4B). The level of ZNF367 mRNA increased in miR-17-5p-knockdown cells, whereas its expression was declined in miR-17-5p-overexpressing cells (Fig. 4C). The level of ZNF367 protein expression coincided with changes in the level of mRNA in miR-17-5p-knockdown and miR-17-5p-overexpressing cells (Fig. 4D). To investigate the expression of ZNF367 in the chorionic villi, we examined ZNF367 mRNA and protein expression in the chorionic villi of the TSH > 2.5 mIU/L with SA, TSH > 2.5 mIU/L without SA, TSH < 2.5 mIU/L with SA, and CON groups. As expected, the TSH > 2.5 mIU/L with SA group exhibited significantly higher levels of ZNF367 mRNA and protein expression (Fig. 4E and F). In addition, the level of miR-17-5p expression in the villous tissue was negatively correlated with the level of ZNF367 mRNA expression (Fig. 4G). Moreover, the level of ZNF367 protein expression was increased when trophoblasts were exposed to 10 mIU/mL TSH compared with 0.1 or 1 mIU/mL TSH exposure (Fig. 4H).
The inhibition of ZNF367 promoted HTR8/SVneo cell proliferation, migration, and invasion

Given that miR-17-5p targeted ZNF367 and inhibited its expression, we explored the biological function of ZNF367 in HTR8/SVneo cells. Supplementary Figure 3E and F show the transfection efficiency of ZNF367 inhibition in HTR-8/SVneo cells. The results of the CCK-8 assay showed that the inhibition of ZNF367 increased trophoblast cell proliferation compared with control (P < 0.05) (Fig. 5A). Wound-healing and transwell assays were conducted to explore trophoblast migration and invasion. Compared to the corresponding control, the fusion area and the number of cells...
invading and migrating through transwell pores were significantly increased after ZNF367 inhibition (Fig. 5B and C). The level of PCNA, MMP2, and MMP9 protein expression was increased (Fig. 5D). Taken together, these results indicated that ZNF367 inhibited trophoblast proliferation, migration, and invasion.

**Overexpression of miR-17-5p could partly restore high TSH-induced inhibition of migration and invasion in HTR-8/SVneo cell**

To confirm whether the biological suppressive effects of high TSH were mediated by miR-17-5p in HTR-8/SVneo...
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Discussion

miRNAs are involved in a variety of regulatory processes related to growth and development. Abnormal miRNA expression is related to disease occurrence, which can be used as potential molecular biomarkers for the diagnosis of various diseases (Thai et al. 2010). In our previously published work, a miRNA array was conducted to characterize the miRNA expression patterns in the serum of SCH patients with SA. miR-940 and miR-486-5p were upregulated in the serum of SCH combined with SA patients (Zhou et al. 2018). This is the first study to explore the pathogenesis of TSH > 2.5 mIU/L-associated SA and the effects of TSH on trophoblasts. In the present study, we demonstrated that the expression of miR-17-5p in the chorionic villi was downregulated in TSH > 2.5 mIU/L-related SA patients compared with TSH < 2.5 mIU/L with SA and CON groups. Furthermore, we showed that a high concentration of TSH inhibited trophoblast invasion

Figure 5

ZNF367 inhibition promoted the proliferation, migration, and invasion ability of HTR-8/SVneo cells. (A) A CCK-8 assay was used to evaluate cellular proliferation following ZNF367 inhibition in HTR-8/SVneo cells. Data are presented as the mean ± s.d., as analyzed by an independent sample t-test; *P < 0.05. (B) A wound-healing assay was used to analyze the migratory ability of HTR-8/SVneo cells transfected with si-ZNF367 and corresponding control. Data are presented as the mean ± s.d., as analyzed by an independent sample t-test; ***P < 0.01. (C) Transwell assays with or without Matrigel were used to determine the invasion and migration of HTR-8/SVneo cells after transfection with si-ZNF367 and the corresponding control. Data are presented as the mean ± s.d. as analyzed by an independent sample t-test; *P < 0.05 and ***P < 0.01. (D) The expression of the biological function-related protein (PCNA, MMP2, and MMP9) was assessed by Western blotting in ZNF367-inhibited HTR-8/SVneo cells. Data are presented as the mean ± s.d. as analyzed by an independent sample t-test; *P < 0.05 and **P < 0.01. A full color version of this figure is available at https://doi.org/10.1530/JME-20-0335.
and migration by regulating the expression of miR-17-5p and ZNF367.

Several studies have indicated that the endothelial dysfunction caused by abnormal TSH is related to increased osteopontin, integrin αvβ3, vascular cell adhesion molecule-1 (VCAM-1), and endothelial nitric oxide synthase (eNOS) induced by TSH (Yan et al. 2016, Jiang et al. 2018). Previous reports have shown that the expression of TSHR increased when pinopodes appear and endometrial receptivity is established, suggesting that TSH may have local effects on the endometrium and embryo (Aghajanova et al. 2011). Moreover, TSH increased the expression of leukemia inhibitory factor (LIF) and its receptor in endometrial mesenchymal cells, which may further participate in endometrial glucose transport (Aghajanova et al. 2011). However, whether TSH plays a role in the biological function of trophoblasts and placental development remains unknown. In the current study, we found that a high concentration of TSH inhibited cell migration and invasion as well as the expression of miR-17-5p in HTR-8/SVneo cells. We also identified miR-17-5p was negatively correlated with TSH. Therefore, TSH has a potential role in trophoblast biological function.

miR-17-5p is a member of the miRNA17-92 cluster located on human chromosome 13q31.3. Different members of the same cluster may have similar biological functions. Four out of six members in the miR-17-92 cluster (miR-17, miR-18a, miR-19b, and miR-92a) were decreased in severe preeclampsia (sPE) placentas, while the opposite results were observed in other studies (Chen & Wang 2013, Xu et al. 2014). It has been shown that miR-18 belonging to miR-17-92 clusters can promote the invasion of trophoblasts, and miR-19b expression is reduced in the chorionic villi of patients with recurrent SA (Xu et al. 2014, Tian et al. 2020). In the porcine endometrium, downregulation of miR-17-5p is found in arresting conceptus compared with healthy conceptus (Bidarimath et al. 2015). Previous studies have observed the differential expression of miR-17-5p in pregnancy-related diseases; however, no studies have presented evidence of the miR-17-5p function in SA or abnormal TSH to date. In this study, we used an extravillous trophoblast line (HTR-8/SVneo...
To research the role of miR-17-5p on trophoblasts, miR-17-5p was overexpressed or knockdown in HTR-8/SVneo cells. Furthermore, functional assays were conducted to prove that miR-17-5p promoted proliferation, migration, and invasion in HTR-8/SVneo cells. Proliferation-related protein PCNA and metastasis-associated proteins MMP2, MMP9 were detected via Western blotting. PCNA, MMP2, MMP9 were increased by miR-17-5p in HTR-8/SVneo cells. Based on the above results, we found that miR-17-5p is involved in the pathogenesis of abortion by affecting the proliferation, migration, and invasion of trophoblast cells.

Since four bioinformatics prediction websites predict ZNF367 to be a target of miR-17-5p, we used a dual-luciferase reporter assay and PCR to verify this prediction. The ZNF367 gene (also known as ZFF29 or ZFP367) was originally isolated from human fetal liver erythroid cells (Asano et al. 2004). ZNF367 is central in gene co-regulation networks during aging (Baumgart et al. 2014), and a recent study showed that ZNF367 is a new modulator of neuroblast proliferation during embryonic neurogenesis (Naef et al. 2018). In addition, ZNF367 has been found to play a critical role in spermatogonial stem cell regulation and cancer development (Jain et al. 2014, Liu et al. 2018b).

We first explored the biological function of ZNF367 on trophoblasts. We found that the level of ZNF367 mRNA and protein was upregulated in the chorionic villi of TSH > 2.5 mIU/L with SA patients and negatively correlated with miR-17-5p. Combined with the luciferase results, we found that ZNF367 was the target gene of miR-17-5p. Further functional assays confirmed that ZNF367 knockdown...
promoted the invasion and migration of HTR-8/SVneo cells, indicating ZNF367 is involved in the process of miR-17-5p regulating the biological functions of the trophoblast.

Further, we conducted rescue experiments to investigate the functional relationship of TSH, miR-17-5p, and ZNF367. The results showed that suppression of ZNF367 could rescue the inhibitory effect of miR-17-5p inhibitor on trophoblast biological function. However, this mitigation effect is only a partial alleviation, suggesting that except ZNF367, there may be other target genes involved in this process. In addition, we also indicated that the overexpression of miR-17-5p also could rescue the effect of a high concentration of TSH on trophoblast biological functions.

However, we did not record human chorionic gonadotropin (hCG) when the participants were enrolled. In addition, the study work only utilized one cell line, HTR-8/SVneo cells. Further research is required to identify the function of miR-17-5p in other trophoblast cell lines or primary cells.

In summary, this is the first study to explore the pathogenesis of TSH > 2.5 mIU/L-associated SA and the effects of TSH on trophoblasts. miR-17-5p was decreased in the chorionic villi of TSH > 2.5 mIU/L-related SA patients, whereas the expression of TSHR and ZNF367 protein were increased. A high concentration of TSH inhibited the invasion and migration of trophoblasts, as well as miR-17-5p expression. Furthermore, the inhibition of miR-17-5p suppressed trophoblast proliferation, invasion, and migration by targeting ZNF367. There is a need for additional studies on other differentially expressed miRNAs and the mechanism of miRNA-associated pathogenesis of TSH > 2.5 mIU/L-related SA.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JME-20-0335.

Declaration of interest
The authors declare 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
Y Y designed and performed the study, collected samples, analyzed data and drafted manuscript; J L helped to collect the samples, improved the experimental design; Y Y Z and W D helped to collect samples and detected thyroid function; W P T: critically revised the report; Z Y S conceived the study, interpreted the data, and critically revised the report. All authors have approved the final version.

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