miR-3074-5p/CLN8 pathway regulates decidualization in recurrent miscarriage

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Abstract

Decidualization is essential for the successful establishment of pregnancy, and the dysregulated decidualization may lead to early pregnancy loss. It was previously reported by us that miR-3074-5p could promote apoptosis but inhibit invasion of human extravillus trophoblast (EVT) cells in vitro, and the expression level of miR-3074-5p in villus tissues of recurrent miscarriage (RM) patients was significantly increased. The aim of this study was to preliminarily explore the role of miR-3074-5p played in the decidualization of human endometrial stromal cells (ESCs). It was found that the decidual expression level of miR-3074-5p in RM patients was remarkably higher than that in the control group. The overexpression of miR-3074-5p in the immortalized human ESC line, T-HESCs, showed suppressive effects not only on the cell proliferation, as well as the intracellular expression levels of cyclin B1 (CCNB1), CCND1 and CCNE1 but also on the in vitro-induced decidualization. CLN8 mRNA, encoding an endoplasmic reticulum (ER)-associated membrane protein, was validated to be directly targeted by miR-3074-5p. And, the expression level of CLN8 was continuously increased along with the decidualization process, whereas down-regulated CLN8 expression could inhibit the decidualization of T-HESCs in vitro. Furthermore, contrary to the increased expression level of miR-3074-5p, a significantly decreased CLN8 expression was observed in decidual tissues of RM patients. Collectively, these data suggested that an increased miR-3074-5p expression in ESCs might cause early pregnancy failure by disturbing decidualization of ESCs via the miR-3074-5p/CLN8 pathway, providing a potential diagnostic and therapeutic target for RM.

Introduction

Recurrent miscarriage (RM), defined as two or more consecutive pregnancy loss prior to the 20th week of gestation in humans, is a common pregnancy-related complication that affects 2–5% of couples trying to conceive. Despite considerable advances that have been achieved in reproductive biology and medicine, the cellular and molecular mechanisms of RM pathology remain to be largely unknown (Larsen et al. 2013). Successful embryo implantation is essential for the establishment of pregnancy, and decidualization is integral to the implantation process. Dysfunctional decidualization can lead to recurrent implantation failure, recurrent miscarriage, and other pregnancy disorders (Tamura et al. 2014, Liao et al. 2015). Decidualization, characterized by endometrial stromal cells (EnSCs) proliferation and differentiation, coincides with the establishment of uterine receptivity. And, EnSCs derived from RM patients showed an abnormal response to in vitro decidualization stimuli (Salker et al. 2010). Thus, a better understanding of the regulation of EnSCs decidualization would undoubtedly contribute to deepening our insight into the pathogenesis of RM.

miRNAs, the single-stranded ncRNA molecules, participate in the regulation of cell proliferation, apoptosis, and invasiveness (Khawar et al. 2019). Accumulating evidence has suggested that the dysregulation of miRNAs expression is involved in various human reproductive diseases, and a number of differentially expressed miRNAs have been screened out in RM patients (Wang et al. 2016). It has been demonstrated that, during the implantation process, miRNAs play fundamental roles in early embryo development (Bernstein et al. 2003) and the formation of endometrial receptivity through regulating uterine gene expression in mice (Hu et al. 2008). In humans, a number of miRNAs were also identified to be...
associated with the endometrial receptivity, indicating that miRNAs have important roles in decidualization and embryo implantation (Sha et al. 2011, Altmae et al. 2013). However, the exact bio functions of miRNAs in decidualization, as well as their dysfunctions in early pregnancy failure, still need to be further investigated.

In our previous studies, a number of differentially expressed miRNAs in placental villus tissues between RM patients and gestational age-matched normal pregnant women were screened out by using deep sequencing analysis, and the villus miR-3074-5p expression was subsequently validated to be significantly increased in RM patients (Gu et al. 2016); furthermore, the overexpression miR-3074-5p was accompanied by the enhanced apoptosis and reduced invasiveness of human extravillous trophoblast cells (EVTs), suggesting that miR-3074-5p might be involved in the pathogenesis of RM by interfering with the normal activities of EVTs (Gu et al. 2018). Given the success of embryo implantation and placentation depends on the reciprocal communication between blastocyst and endometrium, we supposed that miR-3074-5p might also participate in the pathogenesis of RM by disturbing decidualization. Thus, in this study, the difference in decidual miR-3074-5p expression level between RM patients and the control group was detected, the effect of the up-regulated miR-3074-5p expression on decidualization was examined by using the immortalized human EnSC line T-HESC as the in vitro model, and the downstream target of miR-3074-5p was identified to preliminarily explore the role of miR-3074-5p played in decidualization. Meanwhile, the differentially expressed gene profile in miR-3074-5p overexpressed T-HESC was also established to screen the secreted proteins that presented changed peripheral blood plasma levels in RM patients, with a view to identify the potential biomarkers for RM.

Materials and methods
Sample collection

Human decidual tissues and peripheral blood samples of RM patients and normal pregnant women (control group) at early pregnancy were collected at the Department of Gynecology and Obstetrics, The Second Hospital of Tianjin Medical University, Tianjin, China. The current pregnancy losses of RM patients were objectively confirmed by transvaginal ultrasound examination. Classical risk factors including abnormal parental karyotypes, uterine anatomical abnormalities, infectious diseases, luteal phase defects, diabetes mellitus, thyroid dysfunction, and hyperprolactinemia were excluded. In parallel, control group women, who had no history of miscarriage and were undergoing legal elective abortions, were enrolled and also checked for classical risk factors for early pregnancy losses. After separation of decidual tissues from chorionic villi, the decidua tissues were immediately put in phosphate-buffered saline and transported to the laboratory, tissues were washed twice in phosphate-buffered saline at room temperature to remove excess blood, and then cut into small pieces. The collected decidual tissues were frozen and stored at −80°C. Whole venous blood samples used for plasma separation were collected in EDTA-containing tubes and then immediately centrifuged at 1600 g for 10 min to separate the plasma samples. The plasma samples were carefully transferred into new tubes and stored at −80°C until use. Written informed consent was obtained from all patients who provided tissue samples. All experiments involving human were approved by the Medical Ethics Committees of The Second Hospital of Tianjin Medical University (KY2017K002), and the Shanghai Institute of Planned Parenthood Research (Ref # 2013-7, 2013-12, and PJ2018-06).

RNA isolation

Total RNA was extracted from decidual samples and cells using TRIzol reagent according to the manufacturer’s protocol (Invitrogen). The concentration of total RNA products was measured by NanoDrop (Thermo Scientific), and RNA integrity was checked with a Bioanalyzer2100 (Agilent).

Real-time PCR for miR-3074-5p

Total RNA was reversely transcribed using miRNA-specific reverse primers (Ribobio, Guangzhou, China) to obtain cDNA. Real-time PCR was carried out using the FastStart Universal SYBR Green Master kit (Roche Diagnostics) and analyzed using Roche LightCycler480 (Roche). The primers used for real-time PCR of miR-3074-5p were from a Bulge-Loop TM miRNA PCR Primer Set (RiboBio). Primer efficiencies were determined by the standard curve. Briefly, PCR reactions were amplified with different amounts of the same cDNA sample by sequential dilution (five concentrations with a dilution factor 1:10), and after the PCR was run, the results were evaluated by using the software. The acceptable primer efficiency should be higher than 90%. The relative expression level of miR-3074-5p was calculated by the efficiency-corrected ΔCt method and normalized to the endogenous control snRNA U6.

In situ hybridization (ISH)

In our previous study, two pairs of decidual samples (RM1 and RM2 in the RM group and CON1 and CON2 in the control group) were chosen randomly for the ISH analysis. In this study, the same decidual tissues were used for miR-3074-5p ISH analysis as previously described (Yang et al. 2018). Briefly, frozen 10 μm serial sections were fixed in 4% paraformaldehyde and followed by hybridization with LNA microRNA probes (Exiqon, Copenhagen, Denmark) specifically against miRNA-3074-5p. After blocking, sections incubated with alkaline phosphatase-conjugated anti-digoxin antibody (1:200, Roche and BCIP/NBT (Promega) were used as a substrate to visualize the stained signals. The scramble miRNA (nonspecific control) probe was used as the negative control. After that, the human decidua slides were incubated with HLA-G antibody (1:800, Abcam) or IgG antibody to identify which cell type expresses miRNA-3074-5p in decidua tissues. Further incubated with horseradish peroxidase-conjugated secondary antibody.
(Vector, 1:500) and visualized with DAB (Dako Cytomation) solution. They were then counterstained with Nuclear Fast Red (Beijing Dingguo Changsheng Biotechnology, Beijing, China) and mounted with neutral balsam. The detailed information about probe sequences was presented in Supplementary Table 1 (see the section on supplementary materials given at the end of this article).

**Construction of lentiviral vector expressing miR-3074-5p**

The mature fragment of miR-3074-5p or its NC fragment was respectively linked upstream of the GFP in the pSicoR vector (a gift from Prof Haibin Wang, School of Medicine, Xiamen University, China) to generate the recombinant vectors pSicoR-3074-5p and pSicoR-NC. The sequence of the miR-3074-5p was verified by direct sequencing (Xiamen BoRui biotechnology co., Ltd, Xiamen, China). Then, the 293T cells were used for lentiviruses packaging by transfecting with 10 μg of lentiviral packaging mix (6 μg pSPAX and 4 μg pMD2G) and 8 μg pSicoR-3074-5p (or pSicoR-NC) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s manual. The lentiviral supernatants were collected and filtered through a 0.45 μm low protein binding filter to remove cellular debris. Then, lentiviruses were concentrated by ultracentrifugation (2 h at 50,000 g) at 4°C and stored at −80°C as aliquots.

**Culture and in vitro decidualization of T-HESC**

The immortalized human endometrial stromal cell line T-HESC was purchased from the American Type Culture Collection (ATCC® CRL-4003™) and cultured as described (Liao et al. 2015). Briefly, the medium used for proliferation was phenol red-free DMEM/F-12 (Gibco), supplemented with 10% charcoal-stripped fetal bovine serum (CS-FBS) (Biological Industries, Cromwell, CT), 3.1 g/L glucose (Sigma), 1 mM sodium pyruvate (Sigma), 1.5 g/L sodium bicarbonate (Sigma), 1% insulin-transferrin-selenium (ITS, Gibco), 500 ng/mL puromycin (Sigma), and 50 mg/mL penicillin-streptomycin (Gibco). T-HESC cells were cultured in six-well plates until they reached 70% confluence for the subsequent assays. For the in vitro decidualization, T-HESC cells were cultured in minimal medium (phenol red-free DMEM/F-12 containing 2% CS-FBS) in the presence of stimuli (0.5–10 nM bucladesine (cAMP, MCE, Monmouth Junction, NJ), 10 nM β-estradiol (E2, Sigma), and 1 mM medroxyprogesterone 17-acetate (MPA, Sigma)) for 6 days, renewing the stimuli every 48 h. The expression levels of decidualization markers, decidual prolactin (PRL), forkhead box O1 (FOXO1) and insulin-like growth factor-binding protein 1 (IGFBP1) in T-HESC cells were evaluated at different time points after the decidualization stimuli treatment.

**Lentiviral vector transfection of T-HESC**

For experiments involving lentiviral vector transfection, T-HESC were plated in a 3.5 cm culture dish at a density of 1 × 10^6 cells per dish, cultured to 70–80% confluency. Then, T-HESC were infected with the lentiviruses containing pSicoR-3074-5p or pSicoR-NC (as negative control). The culture media were refreshed 24 h after infection. The infected efficiency of the lentiviruses was observed by fluorescence microscope at 48 h and total RNA was extracted at 72 h after infection. MiR-3074-5p overexpression efficiency was detected by qPCR analysis.

**MTS assay**

T-HESC were seeded into a 96-well plate at a density of 1500 cells per well and incubated at 37°C in a humidified incubator in an atmosphere of 5% CO₂. After 12-h incubation with a serum-free culture medium, the cells were infected with LV-3074-5p or LV-NC. The culture media were refreshed after 24 h, and cell viability was measured using MTS Cell Proliferation Assay Kit (Cat. No. G5430, Promega). Briefly, MTS solution was added to the culture medium at different time points and incubated for 2 h. The absorbance of samples was then measured at 490 nm using a plate reader (Promega GloMax). This experiment was independently repeated at least three times.

**Cell cycle assay**

To gain insights into the mechanism involved in overexpression miR-3074-5p-induced proliferation inhibition in T-HESC, the cell cycle distribution was detected by flow cytometry (MoFloTM XDP, Beckman, Germany). The T-HESCs transfected with lentiviruses containing pSicoR-3074-5p or pSicoR-NC were harvested and centrifuged at 800 g/min for 5 min. The supernatant was discarded, the cells were washed twice with cold phosphate-buffered saline and fixed in 75% cold ethanol for 30 min, and then resuspended the cells in 500 μL PBS containing 30 ng/mL propidium iodide (PI) for 30 min at room temperature in light-free condition. The stained cells were analyzed by flow cytometry to obtain cell cycle profiles (MoFloTM XDP). This experiment was independently repeated at least three times.

**EdU incorporation and staining**

The effect of miR-3074-5p overexpression on T-HESCs proliferation was determined by EdU incorporation assay using the Cell-Light TM EdU Imaging Detecting Kit according to the manufacturer’s instructions (RiboBio). Briefly, T-HESCs were cultured in 24-well plates at a density of 8 × 10⁴ cells/well and transfected with lentiviruses containing pSicoR-3074-5p or pSicoR-NC for 48 h. The cells were subsequently incubated with 50 μM EdU for 2 h at 37°C, fixed with 4% formaldehyde for 30 min at room temperature, and permeabilized with 0.5% Triton X-100 for 10 min. Then, the cells were incubated with 1× Apollo reaction mix for 30 min. DNA was stained with 1× Hoechst 33342 for 30 min, and then the samples were directly observed by fluorescence microscopy (DFC420C; Leica). This experiment was independently repeated at least three times.

**Immunofluorescence staining**

For phospho-histone H3 (pH3) detection, cultured T-HESCs were washed in PBS and fixed with PBS containing 4% paraformaldehyde for 20 min. After being washed in PBS, the
cells were incubated in a blocking buffer (0.2% Triton-X100 and 10% BSA in PBS) for 1 h. Next, the cells were incubated with a blocking buffer containing pH3 antibody (1:200 dilution, CST, Danvers, MA) at 4°C overnight. Then the cells were incubated with fluorescence (cyanine 3)-conjugated goat anti-rabbit IgG (1:200, Invitrogen) secondary antibody. The cell nuclei were stained with DAPI (Sigma). The results were recorded using Leica laser confocal microscope (Leica). This experiment was independently repeated at least three times.

RNA-seq analysis

Two groups of cells were used for RNA-seq: (Group 1) T-HESCs collected at proliferation stage after lentiviruses containing pSicoR-3074-5p or pSicoR-NC transfection, and (Group 2) T-HESCs cells transfected with lentiviruses containing pSicoR-3074-5p or pSicoR-NC and collected after 24 h of induced decidualization. Total RNA was extracted by TRIzol (Invitrogen) and used for RNA-seq analysis. The construction of the cDNA library and sequencing were performed by the BGISEQ-500 platform. Briefly, purified mRNA was fragmented into small pieces with fragment buffer. Then first-strand cDNA was generated using random hexamer primed RT, followed by second-strand cDNA synthesis. The cDNA fragments obtained from the previous step were amplified by PCR, and the products were validated on the Agilent Technologies 2100 bioanalyzer for quality control. The double-stranded PCR products from the previous step were heated denatured and circularized by the splint oligo sequence to get the final library. Raw sequencing reads were cleaned by removing adaptor sequences, that is, reads in which unrecognized bases with a frequency > 10% and low-quality reads. Clean reads were aligned to the human hg19 genome (GRCh37) using HISAT/Bowtie2. The expression levels for each of the genes were calculated and normalized to fragments per kb of exon model per million mapped reads (FPKM) by Expectation Maximization (RSEM). Then, we identified differentially expressed genes (DEGs) between the LV-NC transfected T-HESCs and the LV-3074 transfected T-HESCs based on the following criteria: false discovery rate ≤ 0.01 and an absolute value of log2 ratio ≥ 2. DEGs were used for the subsequent analyses.

Construction of lentiviral vector expressing Ceroid-Lipofuscinosis, Neuronal 8 (CLN8)

The plasmid pLVX-IRE5-Tomato (a gift from Prof Haibin Wang, School of Medicine, Xiamen University, China), which recombined from pLVX-IRE5-zsGreen by replacing the fluorescence reporter gene zsGreen with Tomato, was used for the reconstruction of a lentiviral vector containing CLN8. The human CLN8 gene was amplified from the T-HESC used for the reconstruction of a lentiviral vector containing the fluorescence reporter gene zsGreen with Tomato, was recombined from pLVX-IRES-zsGreen by replacing the LV-NC transfected T-HESC and the LV-3074 transfected T-HESC with the fluorescence reporter gene zsGreen with Tomato, was recombined from pLVX-IRES-zsGreen by replacing the LV-NC transfected T-HESC and the LV-3074 transfected T-HESC with the fluorescence reporter gene zsGreen with Tomato.

E. coli competent cells stbl3 (GeneCopoeia, Rockville, MD). The recombinant plasmid was verified by sequencing. The vector was named pLVX-CLN8. For lentivirus packaging, the 293T cells were transfected with 10 μg of lentiviral packaging mix (6 μg pSPAX and 4 μg pMD2G) and 8 μg pLVX-CLN8 (or pLVX-IRES-Tomato) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's manual. The lentiviral supernatants were collected and filtered through a 0.45 μm low protein binding filter to remove cellular debris. Then lentiviruses were concentrated by ultracentrifugation (2 h at 50,000 g) at 4°C and stored at −80°C as aliquots.

Rescue assay

T-HESCs were plated in a 3.5 cm culture dish at a density of 3.5 × 10^4 cells per dish and incubated 12–24 h at 37°C in a humidified incubator in an atmosphere of 5% CO2 before infection. Then T-HESCs were infected with the lentiviruses containing pSicoR-3074-5p or pSicoR-NC (as negative control). After 24 h, the culture media was refreshed and T-HESCs were infected with the lentiviruses containing pLVX-CLN8 (LV-CLN8) or pLVX-IRES-Tomato (LV-Tomato, as negative control). The infected efficiency of the lentiviruses was observed by fluorescence microscope at 48 h and total protein was extracted at 72 h after LV-CLN8 or LV-Tomato infection. The total protein was analyzed by Western blotting to verify the rescue efficiency.

Western blotting

Cultured T-HESCs were collected in lysis buffer (Beyotime, China), and the lysate was centrifuged to collect the supernatant. The human decidual tissues were quickly frozen in liquid nitrogen, granulated into a fine powder, and homogenized in lysis buffer (Beyotime, China). Protein concentrations were measured by Bradford assay (Bio-Rad), and 50 μg of total protein was separated on a 12% acrylamide gel and then transferred electrophoretically onto nitrocellulose membranes (Millipore). The membranes were blocked with 5% defatted milk in shaking table at room temperature for 1 h and incubated overnight at 4°C with specific primary antibodies Cyclin B1 (Abcam, 1:500 dilution), Cyclin E1 (Abcam, 1:1000 dilution), Cyclin D1 (Invitrogen, 1:1000 dilution), FOXO1 (1:1000 dilution, CST, Boston, Massachusetts), IGFBP1 (Abcam, 1:1000 dilution), PRL (R&D, 1:100 dilution), CLN8 (1:1000 dilution, Novus, Littleton, Colorado), GAPDH (Abmart, 1:1000 dilution) and Tublin (1:3000 dilution, Abclonal, Wuhan, China), followed by incubation with the appropriate secondary antibodies. The blot was developed using a PhosphaGLO AP Substrate Kit (KPL, Gaithersburg, MD) according to the manufacturer’s protocol. The band intensities were quantified by densitometry using the ImageJ software (U.S. National Institutes of Health, MD).

Real-time PCR analysis for decidual markers, GDF15, ITH13, and CLN8

Total RNA (1 μg) was reverse transcribed by First Strand cDNA Synthesis Kit (Toyobo, FSK-100) to obtain cDNA. Real-time PCR was carried out using Real-time PCR Master Mix (SYBR Green) (QPK-201, Toyobo, Saitama, Japan) and analyzed using
LightCycler480 (Roche). Melt curve analysis was subsequently conducted to monitor the amplification specificity. All primers for the real-time PCR are listed in Supplementary Table 2. The relative miRNAs expression was calculated by the efficiency-corrected ΔCt method and normalized to the endogenous control GAPDH. Each sample in each group was measured in triplicate, and the experiment was repeated at least three times.

**Recombinant plasmid construction and luciferase activity assay**

To validate whether the CLN8 gene is a target of miR-3074-5p, the 3'-UTR of the CLN8 gene was amplified from human genomic DNA using primers (5'-GGCGATCGCTCGAGACAGGTACCGGTCAGATT-3'; 5'-GGCCAGCCGCGCTTTCTGATTTACTCTGAGG-3'). Mutant CLN8 3'-UTR was generated on the miR-3074-5p target recognition sites (seed sequences) through site mutations (5'-TCAGGGAACTTACTTCCGCTGCCACCTC-3'; 5'- GAGGTGGCAGCGGAAGTAAGTTCCCTGA-3'). Both the WT and mutated 3'-UTRs of the CLN8 were cloned into the psiCHECK-2 dual-luciferase reporter vector (Promega Corp) between Not I and Xho I restriction sites. The constructed recombinant plasmids were confirmed by sequencing.

293T cells were maintained in DMEM medium supplemented with 10% FBS, 1 mM sodium pyruvate, and 1 mM penicillin/streptomycin and transfected with miR-3074-5p mimic and control (50 nM; Ruibo) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, the day before transfection, the cells were plated with a normal growth medium to achieve 70 to 80% confluence at the time of transfection. miRNAs and/or DNA plasmids were diluted in Opti-MEM (Life Technologies). For reporter assays, 293T cells were transiently transfected with reporter constructs together with miRNA mimics/inhibitors and mimics/inhibitor control, and the ratio of Renilla to firefly luciferase was measured using the Dual-Luciferase Reporter Assay System (Promega Corp).

**siRNA transfection in T-HESCs**

Human CLN8 targeting siRNA (CLN8-siRNA, CGCTAATCATTAATCCATA) and its negative control small RNA (CLN8-NC) were purchased from GenePharma, Shanghai, China. Before the in vitro decidualization T-HESCs, CLN8-siRNA or CLN8-NC was respectively transfected into cultured T-HESCs according to the Lipofectamine® RNAiMAX protocol (Thermo). Briefly, 7.5 μL of Lipofectamine® RNAiMAX transfection reagent was mixed with 25 pmol of CLN8-siRNA (or CLN8-NC) to form complexes, and this mixture was subsequently dispersed into each well of a six-well culture plate. The experiments in each group were repeated three times under the same conditions.

**ELISA**

The commercial sandwich ELISA kit (CUSABIO, Houston, TX) was used to detect peripheral plasma levels of growth differentiation factor 15 (GDF15) and inter-alpha-trypsin inhibitor heavy chain 3 (ITIH3) in RM patients and the control group according to the manufacturer's protocol. Absorbance was read using Infinite 200 Pro M Plex (TECAN). Absorbance readings were taken at 450 nm. A standard curve using recombinant GDF15 or ITIH3 was run concurrently in every plate using dilution buffer provided by the manufacturer and the sample concentration was calculated based on the standard curve and dilution factor.

**Statistical analysis**

All values were presented as the mean ± S.E.M., as determined from at least three independent experiments. Statistical significance was assessed by one-way ANOVA with Tukey multiple comparison test or evaluated by two-way ANOVA test with Sidak's multiple comparison using GraphPad Prism 6. A P value < 0.05 was considered statistically significant. Pearson correlation analysis was used for determining the association between miR-3074-5p and CLN8. The receiver operating characteristics (ROC) curve was also generated to estimate the protein sensitivity and specificity using Stata 10.0 software package (StataCorp).

**Results**

**The decidual expression level of miR-3074-5p was significantly increased in RM patients**

In our previous study, a remarkably up-regulated villus expression of miR-3074-5p was observed in RM patients (Gu et al. 2016). Here, the decidual miR-3074-5p expression level of RM patients was compared to that of the control group. In total, 11 RM patients who had experienced at least two consecutive spontaneous early miscarriages before the 12th gestational week were recruited. Meanwhile, 11 well-matched controls were concurrently enrolled. No significant differences in the average age and gestational week at sampling were observed between RM patients and the control group (Supplementary Table 3). The expression level of miR-3074-5p was detected by using qPCR analysis, and the results showed that the decidua miR-3074-5p expression level of the RM group was significantly higher than that of the control group (Fig. 1A). To localize the miR-3074-5p expression in human decidua tissues at early pregnancy, the ISH/IHC experiment was performed by using HLA-G protein as the marker of EVTs. The IHC results showed that HLA-G positive cells were detected in decidua tissues, indicating that these decidua tissues originated from the embryo implantation site. As expected, the ISH results showed that miR-3074-5p signals were located both in maternal decidua stromal cells (DSCs) and embryonic EVTs (Fig. 1B).

**Overexpression of miR-3074-5p attenuated the cell proliferation activity of T-HESCs**

During the embryo implantation process, uteri EnSCs undergo dynamic proliferation activity prior to the decidualization. Thus, we detected the effect of the
up-regulated miR-3074-5p expression on the cell-proliferation activity of T-HESCs. The results of the MTS assay (Fig. 2B) and the EdU incorporation assay (Fig. 2D) showed that the overexpression of miR-3074-5p in T-HESCs led to a significantly reduced cell-proliferative capability. Meanwhile, the miR-3074-5p overexpression resulted in a remarkable decrease in the proportion of T-HESCs in the S-phase, while the proportion of the G0/G1 phase exerting a significant rise (Fig. 2C). Furthermore, the mitotic activity of T-HESCs was visualized by staining of p-Histone H3 (pH3, ser10), a unique mitotic phase marker, and the results showed that the pH3 expression was drastically reduced in miR-3074-5p-overexpressed T-HESCs (Fig. 2E). These results suggested that overexpression of miR-3074-5p could attenuate T-HESCs proliferation by inhibiting the cell-cycle switch from S-phase into the G2/M phase. Given cyclins/CDKs play critical roles in the regulation of the cell cycle, we, therefore, observed the effects of miR-3074-5p overexpression on expression levels of CCNB1, CCND1 and CCNE1 in T-HESCs by Western blot analysis. The results showed that the expression levels of CCNB1, CCND1 and CCNE1 were significantly down-regulated in the miR-3074-5p overexpressed T-HESCs (Fig. 2F and G), indicating the increased miR-3074-5p expression might inhibit T-HESCs proliferation ability through its effect on cell cycle via cyclins/CDKs pathway.

**Overexpression of miR-3074-5p impaired the in vitro decidualization of T-HESCs**

Uterine stromal cell decidualization is characterized by stromal cell proliferation and differentiation. On the basis that overexpression of miR-3074-5p inhibited the cell proliferation activity of T-HESCs and the decidual miR-3074-5p expression level was increased in RM patients, the following research focuses on the influence of miR-3074-5p overexpression on the decidualization of T-HESCs in vitro. The results showed that the expression levels of three decidual markers, FOXO1, IGFBP1, and PRL, were significantly inhibited by the overexpression of miR-3074-5p in T-HESCs (Fig. 3A and B), suggesting that the increased expression of miR-3074-5p inhibited the decidualization process in vitro.

To further explore the downstream molecular pathway underlying the effects of miR-3074-5p on decidualization of EnSCs, we screened out the potential targets involved in the miR-3074-5p functional pathway by following three candidate target pipelines: (1) to compare the transcriptomes between miR-3074-5p-overexpressed T-HESCs and NC-treated T-HESCs, and 56 down-regulated genes in miR-3074-5p-overexpressed cells were identified; (2) to compare the transcriptomes between the decidualized miR-3074-5p-overexpressed T-HESCs and decidualized NC-treated T-HESCs, and 52 down-regulated genes in decidualized miR-3074-5p-overexpressed cells were identified (Supplementary Table 4); (3) to search the TargetScan database, and 5443 predicted miRNA targets of miR-3074-5p was screened out (data are not shown). To narrow down the downstream molecular gene list underlying the effects of miR-3074-5p on decidualization, we overlapped the above two RNA-seq data with TargetScan. Then, by overlapping these three groups of genes, CLN8, ACO1, TGFβ2 and KLHL23 were identified to be candidate targets of miR-3074-5p in T-HESCs related to the decidualization process (Fig. 3C).

**CLN8 expression level was increased during the in vitro decidualization process of T-HESCs**

As CLN8 is involved in the endoplasmic reticulum (ER) function regulation, and it was reported that impaired ER homeostasis contributed to RM pathogenesis (Persaud-Sawin et al. 2007, Guzel et al. 2017), we, therefore, focused our attention on this gene, and subsequently,
observed the alteration of CLN8 expression level in T-HESCs during the process of decidualization. The results of Western blot and real-time PCR analyses showed that the expression level of CLN8 was significantly increased during in vitro decidualization of T-HESCs (Fig. 3D and E).

**Down-regulated CLN8 expression impeded the in vitro decidualization of T-HESCs**

To further explore the role of CLN8 in the decidualization of HESCs, the CLN8 expression was knocked down in cultured T-HESCs through the transfection of CLN8-siRNAs. As a result, the CLN8 expression level was significantly reduced in CLN8-siRNA-transfected T-HESCs (siCLN8) compared to that in the negative control (NC)-transfected T-HESCs (siNC), and this reduction was correlated with a significant decrease in FOXO1, IGFBP1, and PRL expression, indicating that the down-regulated CLN8 expression inhibited the in vitro decidualization of T-HESCs (Fig. 3F, G and H). Rescue experiments showed that the CLN8 overexpression could effectively reverse the decidualization defect caused by miR-3074-5p overexpression (Fig. 3I).

**CLN8 was validated to be a direct target gene of miR-3074-5p**

To confirm that CLN8 was a direct target gene of miR-3074-5p, luciferase reporter constructs containing WT and mutant 3'-UTR of the human CLN8 gene were constructed (Fig. 4A). The pSiCHECK-2 luciferase reporter plasmids with the WT or mutated 3′UTR of CLN8 were transiently transfected into 293T cells along with miR-3074-5p mimic or NC. We found that cells co-transfected with miR-3074-5p mimic and WT CLN8 3′-UTR reporter plasmid showed a significant decrease in reporter activity, whereas no differences were observed when the cells were transfected with the CLN8-mut reporter (Fig. 4B), indicating that the 3'-UTR of CLN8 was the directed target of miR-3074-5p.
The decidual CLN8 expression level was significantly reduced in RM patients

Although CLN8 was identified as a target gene for miR-3074-5p, it is unknown whether there was a correlation between decidual expressions of miR-3074-5p and CLN8 in RM patients. Thus, we examined the expression of miR-3074-5p and CLN8 simultaneously in another seven-pair decidual tissues of RM patients and the control group (Supplementary Table 5). By Western Blot, we found that the CLN8 protein expression level in decidual tissues of RM patients was significantly decreased compared to that in the control group (Fig. 4C and D). The results of real-time PCR showed that, in consistent with the anterior data of this study (Fig. 1A), the decidual miR-3074-5p expression level was significantly increased in RM samples compared to that in control samples (Fig. 4E), which showed an inverse correlation in decidual expression levels between miR-3074-5p and CLN8. In order to find possible connection between changes in expression of CLN8 and miR-3074-5p, we performed Pearson correlation analysis, and positive correlation ($r=0.7871$, $P=0.0357$) between CLN8 and miR-3074-5p was observed in RM patients but not in control samples ($r=0.3822$, $P=0.3975$) (Fig. 4F and G).

GDF15 and ITIH3 were identified as potential biomarkers of RM

As shown in Fig. 3, we screened out the potential targets involved in the miR-3074-5p functional pathway by RNA-seq. We noticed that there are some secretory proteins in the downstream molecular gene list, and the secretory proteins could serve as potential biomarkers of RM. We picked some secretory proteins according to published research on reproductive biology, including GDF15, ITIH3, ACO1, and GSTM1 (Supplementary Table 4). Subsequently, we detected and compared the peripheral blood plasma levels of GDF15, ITIH3, ACO1, and GSTM1 between RM patients and controls to determine whether they might be a viable biomarker for RM.
candidate for RM detection. In total, 34 RM patients and 31 matched controls were concurrently enrolled. No significant differences in the average age and gestational week at sampling were observed between RM patients and controls (Supplementary Table 6).

The peripheral blood samples of RM patients and the control group were collected. We found that the plasma level of GDF15 in RM patients was significantly lower compared to the controls (Fig. 5A), whereas the ITIH3 level was significantly higher than that in the control group (Fig. 5B). ACO1 and GSTM1 expression were low to undetectable in controls and RM plasma. The ROC curve was generated based on these results to estimate GDF15 and ITIH3 sensitivity and specificity in RM detection. We found that the combination of plasma GDF15 and ITIH3 (Model 1) discriminated the RM with a sensitivity of 67.65% and a specificity of 96.67%. While GDF15 (Model 2) or ITIH3 (Model 3) alone respectively showed a sensitivity of 79.41% and specificity of 90.32% and a sensitivity of 38.34% and specificity of 90.00% (Fig. 5C and Table 1). To further verify the potential association between miR-3074-5p and GDF15/ITIH3, we performed miR-3074-5p overexpression in H-TESC. The results showed that the mRNA expression of GDF15 and ITIH3 significantly decreased after miR-3074-5p overexpression (Fig. 5D).

**Discussion**

This study demonstrated at the first time that the decidual expression of miR-3074-5p was significantly increased, whereas that of its target, CLN8, was remarkably decreased in RM patients. The up-regulated miR-3074-5p expression showed the obvious inhibitory effects both on proliferation and decidualization of the
human endometrial stromal cell line, T-HESC, in vitro. The down-regulated CLN8 expression in T-HESC was also accompanied by a significantly reduced decidualization activity, and the increased CLN8 expression could effectively reverse the inhibitory effects of miR-3074-5p overexpression on the decidualization of T-HESC. GDF15 and ITIH3 were screened out to be differentially expressed in miR-3074-5p overexpressed T-HESC, and their circulating concentrations in RM patients were obviously altered.

Being stable in human peripheral blood, miRNAs are promising novel biomarkers of human diseases and a number of miRNAs have been recently identified to be involved in the pathogenesis of RM (Hosseini et al. 2018, Yang et al. 2018). It was found in our previous study that the miR-3074-5p expression at the implantation sites was decreased compared to that at the non-implantation sites during early pregnancy in mice (the nucleotide sequences of miR-3074-5p are conserved between humans and mice) (Gu et al. 2016), and the miR-3074-5p expression in placental villus of RM patients was significantly increased, and such an abnormally increased miR-3074-5p expression in EVTs could disturb the embryo implantation by attenuating the invasion of EVTs (Gu et al. 2018), indicating that up-regulated miR-3074-5p expression at the mater–fetal interface in the early pregnancy might be involved in the pathogenesis of RM. Given that successful embryo implantation depends both on the appropriate invasive ability of embryonic EVTs and the synchronous decidualization of maternal EnSCs (DeMayo & Lydon 2020, Deryabin et al. 2020), we supposed that aberrant decidual miR-3074-5p expression might be also associated with the RM pathogenesis. Thus, we detected the decidual miR-3074-5p expression at early pregnancy, and as expected, its expression in RM patients was significantly increased compared to controls.

Decidualization of EnSCs, characterized by cell proliferation and differentiation, is an important event during embryo implantation and pregnancy establishment (Das 2009), and it has been recognized that the deficiencies in the decidualization process were involved in RM pathology (Ni & Li 2017). With a view to determine whether the up-regulated decidual miR-3074-5p expression is the cause or consequence of the early pregnancy loss, we examined the effects of up-regulated miR-3074-5p expression on proliferation and decidualization of human EnSCs. Interestingly, it was observed that the miR-3074-5p overexpression in T-HESC not only caused the reduction of cell-proliferation ability by inducing the cell-cycle arrest in the G0/G1 phase but also showed an inhibitory effect on the induced decidualization in vitro, indicating that the abnormally increased miR-3074-5p expression in EnSCs might damage the embryo implantation process by interfering with the decidualization of EnSCs. Taking into consideration that the up-regulated miR-3074-5p expression in EVTs could lead to the reduction of cell invasiveness and the enhancement of cell apoptosis (Gu et al. 2018), we speculated that the increased miR-3074-5p expression at the maternal–fetal interface might cause early pregnancy loss by disturbing synergistically the EVTs invasion and EnSCs decidualization.

However, to our knowledge, although there are lots of predicted targets of miR-3074-5p, the rare target has been validated so far. Thus, in order to identify the candidate targets of miR-3074-5p in EnSCs to further reveal the molecular pathway of roles miR-3074-5p played in EnSCs, we herewith searched out more than 5400 predicted targeted genes of miR-3074-5p from the public database and respectively screened out 56 and 52 down-regulated genes from non-decidualized or decidualized miR-3074-5p overexpressed T-HESC. And then, candidates were narrowed down to four genes (CLN8, ACO1, TGFB2, and KLHL23) by overlapping the above-mentioned three candidate gene pools. Among these four genes, only CLN8 was authenticated herewith to be a direct target of miR-3074-5p. Subsequently, it was found that the CLN8 expression level in T-HESC was increased during the decidualization process, and the down-regulated CLN8 expression in T-HESC led to the reduction of decidualization. More interestingly, the increased CLN8 expression could significantly reverse the inhibitory effect of the up-regulated miR-3074-5p

<table>
<thead>
<tr>
<th>Roc analysis</th>
<th>Prediction for clinical pregnancy outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuROC (95% CI)</td>
<td>Model 1</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>0.871 (0.786, 0.958)**</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>67.65</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>96.67</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>72.50</td>
</tr>
<tr>
<td>Correctly classified (%)</td>
<td>72.50</td>
</tr>
<tr>
<td>Cut-off value</td>
<td>90.00</td>
</tr>
</tbody>
</table>

*No significant difference in ROC area between Model 1 and Model 2. **Significant difference in ROC area between Model 1 and Model 3 ($\chi^2 = 10.32, P = 0.0013$).
expression on decidualization of T-HESC, suggesting that the abnormally increased miR-3074-5p expression might inhibit the decidualization of T-HESC by targeting the CLN8 protein expression.

CLN8, as a 33 kDa endoplasmic reticulum (ER)-associated membrane protein, localizes to the ER/ER-Golgi intermediate compartment (ERGIC) (Passantino et al. 2013, di Ronza et al. 2018) and participates in the ER-to-Golgi transfer of lysosomal enzymes, and the deficiency in CLN8 function could cause the lysosomal storage disorder, neuronal ceroid lipofuscinosi (NCL) (Luzio 2018). The ER acts as the specific cellular site of synthesis, folding, and structural maturation and trafficking of secretory and cell-surface proteins, and recent evidence showed that impaired ER homeostasis contributed to reproductive physiology and pathology including implantation and placentation, pregnancy complications and preterm birth (Guzel et al. 2017). It was reported that CLN8 interacts with protein phosphatase 2A (PP2A) in human fibroblasts (Adhikari et al. 2019), and as a major intracellular serine/threonine phosphatase, PP2A regulates a number of signaling pathways, including the cyclins/CDKs pathways (Lin et al. 2018, Pippa et al. 2020). CCNB1, CCND1 and CCNE1 have been reported to be involved in human EnSCs proliferation (Das 2009), and in the present study, decreased expression levels of these three cyclins were observed in miR-3074-5p-overexpressed T-HESC. Thus, we supposed that in human EnSCs, miR-3074-5p might inhibit the cell proliferation through the CLN8-PP2A-cyclins/CDKs pathway, and this speculation should be authenticated in our next investigation.

As the increased decidual miR-3074-5p expression was detected in RM patients, the decidual CLN8 expression level should be reduced in RM patients if miR-3074-5p is really involved in the pathogenesis of RM by targeting CLN8. As expected, it was found that the miR-3074-5p expression was significantly increased, whereas the CLN8 expression was remarkably reduced in decidual tissues of RM patients compared to that of controls, supporting the premise that the increased miR-3074-5p expression in EnSCs might inhibit the CLN8 expression, and then dysregulate the proliferation and decidualization of EnSCs, leading to early pregnancy loss. However, it should be noted that the confidence of the results was weakened by such a small sample size, thus, this phenomenon should be confirmed in large size of decidual samples.

In recent years, the identification of non-invasive predictive biomarkers for RM diagnosis has been paid close attention (Rull et al. 2013). Unfortunately, being consistent with the results of bio-information analysis, we have failed in the detection of the peripheral blood level of miR-3074-5p by qPCR analysis in this study (data not shown). Thus, we have tried to screen out the potential circulating biomarkers of RM from the above-mentioned differentially expressed genes in miR-3074-5p overexpressed HSCs. Five secretory proteins, GDF15, ITIH3, Aconitase 1 (ACO1), glutathione S-transferase M1 (GSTM1) and C4B, were selected to be evaluated in this study. ELISA results showed that in RM patients, the circulating concentration of GDF15 was significantly increased, whereas that of ITIH3 was obviously decreased. But, due to the lack of appropriate commercial ELISA kits, the data of circulating concentrations of ACO1, GSTM1 and C4B were not qualified for evaluation (data not shown).

GDF15, as a member of the transforming growth factor (TGF)-β superfamily (Mullican et al. 2017), is expressed at the undetectable level under normal physiological in most somatic tissues of human, but, in some pathological conditions, such as inflammation and cancer, its expression level is highly increased, and especially, it is abundantly expressed by the syncytiotrophoblast (STB) in placenta (Wischhusen et al. 2020). Although the association of circulating level of GDF15 in the second and third trimester with the PE (Wertaschnigg et al. 2020) or pregnancy-associated vomiting (Petry et al. 2018) has been evaluated, however, the association of its circulating level in the first trimester with RM has not yet been reported. ITIH3 belongs to the ITIH gene family, and ITIHs play important roles in inflammation and carcinogenesis in humans (Hamm et al. 2008), as well as in the maintenance of the uterine surface glyocalyx during placenta development in pig (Geisert et al. 2003). ITIH3 is most recognized to interact with hyaluronan.
to stabilize the matrix (Zhuo and Kimata 2008), and is involved in TGFβ-regulated ECM function in human trabecular meshwork (HTM) cells (Raghunathan et al. 2015). Both GDF15 and ITIH3 are involved in the TGFβ pathway, which is associated with the activation of the inflammatory response during the pathological process of RM (Ogasawara et al. 2000, Darmochwal-Kolarz et al. 2017). Interestingly, TGFβ was identified to be a candidate target of miR-3074-5p by RNA-seq analysis in this study. Furthermore, secretory proteins expressed by various cells under certain pathological conditions are potential biomarkers (Stastna & Van Eyk 2012), thus, we preliminarily evaluated the potentiality of circulating levels of GDF15 and ITIH3 as the candidate biomarkers for the prediction of RM by ROC analysis. The results showed that the circulating concentration of GDF15 only and that of the combination of GDF15 and ITIH3 presented promising predictive factors for RM. Then, we further investigated whether miR-3074-5p was capable of regulating the mRNA expression of GDF15 and ITIH3. We found that the miR-3074-5p was involved in the GDF15 and ITIH3 mRNA expression regulation, and the detailed mechanism needs further investigation. These data will be useful for the identification of potential markers in RM clinics.

In summary, the decidual expressions of miR-3074-5p and its target gene CLN8 were respectively increased and decreased in RM patients. The overexpression of miR-3074-5p could inhibit the in vitro decidualization of human EnSCs, and such an inhibitory effect could be specifically reversed by up-regulated CLN8 expression. GDF15 and ITIH3 were screened out to be differentially expressed from the miR-3074-5p overexpressed EnSCs, and their circulating levels in RM patients were significantly changed compared to the control group (Fig. 6). These data suggested that miR-3074-5p/CLN8 pathway might participate in the pathogenesis of RM, and GDF15 and ITIH3 might be novel circulating biomarkers of RM.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/REP-21-0032.

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
NM and XW contributed equally to this work. XZ and YG conceived of and designed the study. NM, XW, YS, and QY contributed to experiment and data analysis. BJ and TZ contributed to sample collection and clinical diagnosis. YM and QZ involved in data analysis. XW and XZ wrote the manuscript.

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