RESEARCH

miR-183-5p regulates uterine receptivity and enhances embryo implantation

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Abstract

Receptive endometrium is a prerequisite for successful embryo implantation, and it follows that poor endometrial receptivity is a leading cause of implantation failure. miRNAs play important roles as epigenetic regulators of endometrial receptivity and embryo implantation through post-transcriptional modifications. However, the mechanisms of action of many miRNAs are poorly understood. In this study, we investigated the role of the miR-183 family, comprising three miRNAs (miR-183-5p, miR-182-5p, and miR-96-5p) in endometrial receptivity and embryo implantation. The miR-183 family shows estrogen-dependent upregulation in endometrial Ishikawa (IK) cells. The miR-183 family also has a positive role in migration and proliferation of IK cells. Furthermore, JAr spheroid attachment experiments show that attachment rates were significantly decreased after treatment of IK cells with inhibitors for miR-183-5p and miR-182-5p and increased after treatment with miR-183-5p-mimic and miR-96-5p-mimic, respectively. The downstream analysis shows that catenin alpha 2 (*CTNNA2*) is a potential target gene for miR-183-5p, and this was confirmed in luciferase reporter assays. An *in vivo* mouse pregnancy model shows that inhibition of miR-183-5p significantly decreases embryo implantation rates and increases *CTNNA2* expression. Downregulation of *CTNNA2* in endometrial cells by miR-183-5p may be significant in mediating estrogenic effects on endometrial receptivity. In conclusion, miR-183-5p and the *CTNNA2* gene may be potential biomarkers for endometrial receptivity and may be useful diagnostic and therapeutic targets for successful embryo implantation.

Key Words

- ▶ miR-183-5p
- ▶ uterine receptivity
- ▶ embryo implantation
- ▶ *CTNNA2*

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Introduction

Endometrial receptivity is critical for successful embryo implantation. It refers to the restricted period when the uterus acquires a suitable morphological and functional state, compatible with blastocyst adhesion and implantation. This period spanning between 20 and

24 days of the human menstrual cycle is often described as the ‘window of implantation’ (Strowitzki *et al.* 2006). During the process of implantation, an intimate connection is established between the blastocyst and maternal endometrial surface, forming the placenta and

providing a connection between the growing fetus and maternal circulation. For successful embryo implantation, a healthy, functional embryo at the blastocyst stage and receptive maternal endometrium are necessary (Achache & Revel 2006). Recent studies show that embryo implantation and endometrial receptivity are regulated by classic transcriptional mechanisms and several other epigenetic phenomena, including DNA methylation, histone modification and small non-coding RNA (miRNA) modifications (Bartel 2004, Ha & Kim 2014).

The miRNA molecules of approximately 20–22 nucleotides have important roles in RNA silencing and post-transcriptional gene regulation. Several studies have reported their roles in embryo implantation (Bartel 2004, Ha & Kim 2014). The discovery of extracellular miRNAs opened up a new avenue for implantation studies (Liang *et al.* 2017). Recent studies show that miRNAs play protagonist roles in cellular processes including cellular differentiation, proliferation, migration, and apoptosis (Liang *et al.* 2017). Several studies have reported different roles of miRNAs in uterine receptivity. Zheng *et al.* (2017) found that miR-200c may impair uterine receptivity and maybe useful as a biomarker for clinical application. Yang *et al.* (2018) showed that miR-543 was associated with endometrial receptivity, and its downregulation might affect embryo implantation. The miR-183 family, comprising three miRNAs (miR-183-5p, miR-182-5p, and miR-96-5p) with sequence homology, has been reported in the human uterus (Estella *et al.* 2012, Vilella *et al.* 2015); however, its target role in endometrial receptivity and the ultimate effect on embryo implantation remains unclear.

In this study, we explored the role of the miR-183 family in endometrial receptivity and implantation. We found that miR-183-5p could regulate alpha catenin 2 gene (*CTNNA2*) that might mediate endometrial receptivity. *CTNNA2* is a cytoskeleton protein responsible for cell–cell adhesion, and it negatively regulates the Wnt signaling pathway (Gates & Peifer 2005, Sun *et al.* 2014). Exploring the function of the miR-183 family and their potential target genes in endometrial receptivity and embryo implantation may improve our understanding of the respective mechanisms.

Materials and methods

Cell culture

This research study was approved by the research and ethical committee of the Women's Hospital, Zhejiang University, Hangzhou, China. Ishikawa (IK) cells,

human endometrial cell line (American Type Culture Collection) were cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; vol/vol), 100 U/mL streptomycin and 100 U/mL penicillin at 37°C plus 5% CO₂.

Transfection of IK cells with miRNA inhibitors and mimics

The inhibitors for miRs are chemically synthesized, complementary, antisense, single-stranded oligonucleotides to their target: endogenous, mature miRNA (Bai *et al.* 2017). Inhibitors for miR-183-5p (I-miR-183-5p), miR-182-5p (I-miR-182-5p) and miR-96-5p (I-miR-96-5p), and negative controls were manufactured by Applied Biological Materials Inc. (abm), Richmond, BC, Canada. Mimics for miR-183-5p (miR-183-5p-mimic), miR-182-5p (miR-182-5p-mimic) and miR-96-5p (miR-96-5p-mimic), and negative controls were obtained from Genepharma (Shanghai, China). The sequences of inhibitors and mimics are shown in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article). Negative controls (NCs) were validated by random sequences, tested on mammalian cells and tissues, and shown to produce no identifiable effects on known miR function.

Before transfection, IK cells were cultured in normal medium containing estradiol (E₂) (Sigma) for 48 h. In the inhibition experiments, IK cells in each group were treated with negative control and 10^{−7} M E₂ (NC+10^{−7} M E₂ group), I-miR-183-5p and 10^{−7} M E₂ (I-miR-183-5p+10^{−7} M E₂ group), I-miR-182-5p and 10^{−7} M E₂ (I-miR-182-5p+10^{−7} M E₂ group), and, I-miR-96-5p and 10^{−7} M E₂ (I-miR-96-5p+10^{−7} M E₂ group), respectively. In the mimic experiments, IK cells in each group were treated with negative control and 10^{−9} M E₂ (NC+10^{−9} M E₂ group), miR-183-5p-mimics and 10^{−9} M E₂ (miR-183-5p-mimic+10^{−9} M E₂ group), miR-182-5p-mimic and 10^{−9} M E₂ (miR-182-5p-mimic+10^{−9} M E₂ group), and miR-96-5p-mimic and 10^{−9} M E₂ (miR-96-5p mimic+10^{−9} M E₂ group), respectively. IK cells were also treated with 10^{−9} M E₂ alone (10^{−9} group) and 10^{−7} M E₂ alone (10^{−7} M E₂ group) respectively in order to verify the estrogenic effects.

When cells reached 70–90% confluence, cells were transfected with 5 nM inhibitors, 5 nM mimics and their negative controls respectively using RNAiFectin™ (Cat. No. G073) according to the manufacturer's guidelines (Applied Biological Materials Inc.). After transfection, the transfection medium was substituted with normal medium and cultured for different intervals based on

the purposes of different experiments. Protein and RNA were collected for Western blot and real-time PCR (Q-PCR) analyses.

Quantitative real-time PCR

After transfection and subsequent incubation, IK cells were scraped using cell scraper and TRIzol was added. Cell suspension from each well was collected in Eppendorf tubes, and total miRNA extraction was undertaken using phenol chloroform extraction method. RT to cDNA was performed using Mir-X™ miRNA First-Strand Synthesis kit, according to manufacturer's guidelines (Clontech Laboratories, Inc.). cDNA synthesis reaction contained mRQ buffer (5 µL), mRQ enzyme (1.25 µL) and RNA sample (3.75 µL, 0.25–8 µg), making the total volume 10 µL. Incubation was performed in thermocycler for 1 h at 37°C followed by termination at 85°C for 5 min to inactivate the enzyme. Then 90 µL ddH₂O was added to make the final volume 100 µL. Q-PCR was performed using Mir-X miRNA qRT-PCR SYBR Kit (Cat. No. 638314, Clontech Laboratories). Q-PCR reactions were carried out in 10 µL reaction mixture containing 1 µL of cDNA sample and master mix containing ddH₂O (3.6 µL), SYBR premix (5 µL), miR-specific primer (0.2 µL) and mRQ 3' primer (0.2 µL). The quantitative Q-PCR analysis was carried out in an Applied Biosystems 7500 Fast (ABI) according to SYBR® qRT-PCR manufacture's guidelines. The expression levels were quantified by measuring cycle threshold (Ct) values and normalized to U6-snRNA (endogenous control for miRNA detection) or *GAPDH* (endogenous control for mRNA) using the $2^{-\Delta\Delta Ct}$ method. The sequence of primers is given in Supplementary Table 2.

JAr spheroid attachment to IK cells monolayer

Multicellular spheroids of human choriocarcinoma cells (HTB 144, American Type Culture Collection) (Kolibianakis *et al.* 2003) were applied to IK cells monolayer. The IK cells were pretreated with 10^{-9} M or 10^{-7} M E₂, cultured for 48 h followed by transfection using miRNA inhibitors or mimics for miR-183-5p, miR-182-5p, and miR-183-5p. JAr spheroids were prepared according to a standard procedure (Hohn *et al.* 2000) and transferred onto the surface of confluent IK cells monolayer for 1 h (100 spheroids/well in 24-well plates). Non-adherent spheroids were detached by centrifugation of the plate at 10 *g* for 10 min, keeping cell surface facing down. JAr spheroids were counted under a light microscope and the attachment rate was calculated for each well as follows: Attachment rate = ratio

of the number of spheroids attached to the number of spheroids seeded. The experiment was repeated at least three times.

Cell migration assay

IK cells were cultured in six-well plates. In the inhibition experiments, after transfection, cells were wounded by removing a 500 to 700 µm wide strip with a standard 200 µL pipette tip and photographed, designated as time 0 h. The cells were then incubated for another 24 h and were photographed. The distance of migration was calculated using ImageJ software (National Institutes of Health).

Cell proliferation assay

The confluent cultured IK cells were trypsinized and suspended in RPMI-1640 medium with 10% FBS. The cells ($1 \times 10^4/\text{cm}^2$) were plated in 96-well plates. After each treatment, cell counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) (Gao *et al.* 2018) was used to examine viable cells and proliferation rates. The solution of CCK-8 (10 µL) was added to each well. After 1–4 h the absorbance (OD value) was measured at 450 nm using micro-plate reader. The OD value at 450 nm is proportional to the number of viable cells and proliferation. Each group was in triplicate, so the average OD value for each group was taken.

Target gene prediction

Target gene prediction was performed using online Target predicting software including Target scan (www.targetscan.org) and miRDB (www.mirbase.org).

Luciferase reporter assay

The human 3'-UTR of *CTNNA2* gene was cloned downstream of *hRluc* (humanized Renilla luciferase) gene into REPORT™ Dual-Luciferase miRNA Target Expression Vector (RIBBIO, Guangzhou, China), and a mutant plasmid was constructed with site-directed mutagenesis in the seed region for miR-183-5p. Firefly luciferase gene was used as a normalization reporter within the same plasmid backbone. 293T cells were seeded into a 96-well plate at 1.5×10^4 cells/well and allowed to adhere overnight. Cells were co-transfected with pmiR-RB-REPORT™ luciferase reporter plasmids (50 ng/well) (RIBBIO) and hsa-miR-183-5p mimic (final concentration at 50 nM) using Lipofectamine 3000 (0.225 µL/well) (RIBBIO) following

Lipofectamine 3000 Reagent Protocol. The medium was removed at 48 h post transfection. PBS (35 μ L) and an equal volume of Dual-Glo[®] Luciferase Reagent were added to each well, mixed and shaken at 25°C for 10 min. The mixture was transferred into 96-well LUMITRAC[™] 200 white microplates, and the firefly luminescence was measured. Stop &Glo[®] Reagent (35 μ L, RIBBIO) was replenished in each well, mixed and shaken at 25°C for 10 min. The *Renilla* luminescence was measured.

Western blot analysis

Tissue lysis was done using RIPA lysis buffer containing protease inhibitor cocktail. Protein concentrations were determined using the BCA kit (Thermo Scientific). Samples at 20 μ g/lane were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membrane (Schleicher & Schuel Bioscience GmbH, Dassel, Germany). After blocking in 5% non-fat milk for 2 h, the membranes were incubated with primary antibodies including rabbit monoclonal anti-CTNNA2 antibody (1:2000, Cat# ab76015, Abcam Inc.), rabbit monoclonal anti-GSK3 β antibody (1:5000; Cat# ab32391, Abcam), and, rabbit monoclonal β -Actin antibody (1:5000; Santa Cruz Biotechnology) at 4°C overnight. After several washes with Tris-buffered saline and 0.2% Tween 20, the membranes were incubated with the appropriate secondary antibody for 1 h at room temperature. Protein intensities were determined and analyzed using Odyssey[®] Imager (LI-COR, Lincoln, NE, USA). The relative protein levels were quantified using Image J software and were normalized to GAPDH or β -actin.

Animal experiments

All animal protocols were approved by the Animal Care and Use Committee, School of Medicine, Zhejiang University. Female ICR mice aged 6–8 weeks in estrus were bred with male ICR mice aged 8–10 weeks, and the day when vaginal plugging was observed was considered

as day 1 of pregnancy. At day 3, pregnant mice were anesthetized at 09:00 h, and, 10 μ L solution containing 1 μ g miR-183-5p inhibitor (GenePharma) was injected into the right horn of uterus of each mouse, while the left horn was injected with normal saline. Mice were dissected at day 7 of pregnancy to observe the embryo implantation rate, and, uterine endometrium samples were collected for protein and miRNA analyses.

Statistical analysis

GraphPad Prism 6 (GraphPad Software) was used for statistical analysis. Student *t*-test was used to determine the significant difference between two groups. All experiments were performed in triplicate. Differences at $P < 0.05$ were considered significant.

Results

miR-183 family is upregulated in IK cells by estrogen

We examined the expression levels of the miR-183 family including miR-183-5p, miR-182-5p and miR-96-5p in IK cells treated with different concentrations of E_2 for 48 h. 10^{-9} M E_2 is a basic and physiological concentration, whereas 10^{-7} M E_2 , a high concentration, could be observed in the period of embryo implantation and pregnancy (Abbassi-Ghanavati *et al.* 2009, Berkane *et al.* 2017). Our results showed that, compared to 10^{-9} M E_2 , treatment of IK cells with 10^{-7} M E_2 significantly upregulated expression levels of miR-183-5p, miR-182-5p and miR-96-5p (Fig. 1).

miR-183 family mediates estrogenic effects on migration and proliferation of IK cells

Compared to 10^{-9} M E_2 , treatment of IK cells with 10^{-7} M E_2 significantly increased migration (Fig. 2A and D) and proliferation (Fig. 2E) of IK cells. Treatment of IK cells with the miR-183 family inhibitors including I-miR-183-5p, I-miR-182-5p and I-miR-96-5p significantly attenuated the effects of 10^{-7} E_2 on migration (Fig. 2A and B) and

Figure 1

IK cells were treated with different concentrations of estradiol (E_2) (mol/L). E_2 dose dependently increased the expression levels of miR-183-5p (A), miR-182-5p (B), and miR-96-5p (C) in IK cells. ** $P < 0.01$, compared with control (10^{-9} E_2). Number of repeated experiments (n) = 3.

proliferation (Fig. 2C). Contrary to inhibition, treatment of IK cells with the miR-183 family mimics including miR-183-5p-mimic, and miR-96-5p-mimic but not miR-182-5p-mimic, significantly promoted migration (Fig. 3A and B). Cell proliferation was also promoted by the three mimics in the presence of 10^{-9} M E_2 (Fig. 3C). These results suggest that the miR-183 family may play an important role in regulating the migration and proliferative capacity of IK cells by estrogen.

miR-183 family improves JAr spheroid attachment process

To verify that the endometrial miR-183 family mediates embryo implantation, a JAr-endometrial cell model was deployed in further experiments. Multiple JAr spheroids were added to the IK cell monolayer, and, the attachment rates were calculated (Fig. 4A, B and C). We found that, compared to 10^{-9} M E_2 , treatment of IK cells with 10^{-7} M E_2 , significantly increased JAr attachment rates (Fig. 4F), as we have previously reported (Zou *et al.* 2011, Ullah *et al.* 2017). Inhibition of miR-183-5p and miR-182-5p, but not miR-96-5p, decreased the JAr attachment rates in the presence of 10^{-7} M E_2 (Fig. 4D). However, treatment of IK cells with miR-183-5p-mimic and miR-96-5p-mimic, but not miR-182-mimic, significantly increased the JAr attachment rates in the presence of 10^{-9} M E_2 (Fig. 4E).

These findings suggest that the miR-183 family may play an important role in embryo implantation.

CTNNA2 is a potential target gene of miR-183-5p

As the miR-183 family may play a significant role in both endometrial receptivity and embryo implantation, we tried to predict the target genes of this miRNA family by using bioinformatics software as shown in 'Materials and methods'. *CTNNA2* (Catenin Alpha 2) was predicted as one of the target genes regulated by miR-183 family, and previous study showed that *CTNNA2* expression levels were significantly different between the receptive and non-receptive phase of the female reproductive cycle (Carson *et al.* 2002, Horcujadas *et al.* 2006, Díaz-Gimeno *et al.* 2011).

We found that, compared to 10^{-9} M E_2 , 10^{-7} M E_2 significantly downregulated the expression of *CTNNA2* (Fig. 5A). To further verify the binding of miR-183 to the 3'-UTR of *CTNNA2* mRNA, we cloned the wild-type (WT) 3'-UTR and mutant-type (MUT) 3'-UTR target sequences into the luciferase reporter vector (pmiR-RB-REPORT™ vector). The sequences of wild-type and mutant 3'-UTR of *CTNNA2* constructs are shown in Supplementary Table 3. Binding of miR-183-5p to its seed sequence within the miRNA recognition element (MRE) at the 3'-UTR of *CTNNA2* mRNA is indicated in Fig. 5B. The luciferase

Figure 2

Effects of miR-183-5p, miR-182-5p and miR-96-5p inhibitors on cell migration and proliferation of IK cells. E_2 at 10^{-7} M significantly increased cell migration (A and D) and proliferation (E). Treatment of IK cells with inhibitors (I-miR183-5p, I-miR182-5p and I-miR96-5p) significantly reduced 10^{-7} M E_2 -induced cell migration (A and B) and cell proliferation (C). * P < 0.05 and ** P < 0.01, compared with corresponding controls. Number of repeated experiments (n) = 3.

Figure 3

Effect of miR-183-5p, miR-182-5p and miR-96-5p mimics in cell migration and proliferation. Treatment of IK cells with mimics for miR-183-5p and miR-96-5p significantly increased cell migration in the presence of 10⁻⁹ M E₂ (A and B). Cell proliferation also increased significantly by mimics for miR-183-5p, miR-182-5p and miR-96-5p, in the presence of 10⁻⁹ M E₂ (C). *P < 0.05 and ***P < 0.001, compared with corresponding controls. Number of repeated experiments (n) = 3.

activity of *Renilla luciferase* significantly decreased after co-transfection of the WT luciferase reporter construct and miR-183-5p-mimic, whereas no significant change was observed in enzymatic activity when co-transfection of a mutant luciferase reporter construct and miR-183-5p-mimic was performed (Fig. 5C), suggesting that miR-183-5p could regulate *CTNNA2* expression.

To verify the regulatory role of miR-183-5p in *CTNNA2* levels, we performed Western blot in IK cells transfected with I-miR-183-5p in the presence of 10⁻⁷ M E₂, or miR-183-5p-mimic in the presence of 10⁻⁹ M E₂. We found that *CTNNA2* was significantly upregulated by I-miR-183-5p, and, downregulated by miR-183-5p-mimic (Fig. 5D and E). Q-PCR analysis also showed that the expression levels

of *CTNNA2* mRNA were increased in IK cells transfected with I-miR-183-5p, and, decreased in IK cells transfected with miR-183-5p-mimic (Fig. 5G).

It has been shown that the Wnt pathway play an important role in endometrial receptivity (Tepekoy *et al.* 2015). GSK3β (Glycogen Synthase Kinase 3 Beta) is a negative regulator of Wnt/β-catenin pathway (Ma *et al.* 2014, Ahmed *et al.* 2016, Zhang & Yan 2016). We found that I-miR-183-5p not only upregulated *CTNNA2* levels in IK cells, but also increased GSK3β protein and GSK3β mRNA levels (Fig. 5D, F and H). On the other hand, miR-183-5p-mimic attenuated levels of both *CTNNA2* and GSK3β protein and mRNA (Fig. 5D, F and H). These results suggest that the Wnt pathway might be regulated by miR-183-5p.

Figure 4

Effects of miR-183-5p, miR-182-5p and miR-96-5p on JAR spheroid attachment to IK cell monolayer. JAR spheroids attached to IK cells at 10x (A), 20x (B) and 40x (C) magnification. E₂ at 10⁻⁷ M significantly increased JAR spheroid attachment rate (F). Attachment rates were significantly reduced after IK cells were treated with I-miR-183-5p and I-miR-182-5p, respectively (D), and, significantly increased after IK cells were treated with miR-183-5p-mimic and miR-96-5p-mimic, respectively (E). *P < 0.05 and **P < 0.01, compared with corresponding controls. Number of repeated experiments (n) = 3.

Figure 5

miR-183-5p targeting *CTNNA2* was validated by luciferase reporter assay. Binding of miR-183-5p to its seed sequence within 3'UTR of *CTNNA2* mRNA (B). Renilla luciferase (Rluc) activity was significantly decreased after co-transfection of wild type *CTNNA2* 3'UTR and miR-183-5p mimic, while there is no significant decrease by using mutant *CTNNA2* 3'UTR (C). Q-PCR analysis shows that *CTNNA2* mRNA expression levels in IK cells were significantly decreased by E_2 at 10^{-7} M (A) and miR-183-5p-mimic (G), and, increased by I-miR-183-5p (G). Western blot analyses show increased levels of *CTNNA2* (D and E) and *GSK3β* (D and F) proteins in IK cells transfected with I-miR-183-5p, and, decreased levels of *CTNNA2* (D and E) and *GSK3β* (D and F) proteins in IK cells transfected with miR-183-5p-mimic. *GSK3β* mRNA levels are also increased by I-miR-183-5p and decreased by miR-183-5p-mimic (H). * $P < 0.05$ and ** $P < 0.01$, compared with corresponding controls. Number of repeated experiments (n) = 3.

Inhibition of miR-183-5p attenuated uterine receptivity and embryo implantation in mouse model

We further verified roles of miR-183-5p in uterine receptivity and embryo implantation by an *in vivo* mouse model. Inhibitor for miR-183-5p was injected into one horn of mouse uterus while normal saline was injected into the other horn as control at day 3 of pregnancy (Fig. 6A). Inhibition of miR-183-5p significantly decreased embryo implantation rate in mouse dissected at day 7 of pregnancy (Fig. 6B). Meanwhile, we also found that I-miR-183-5p not only inhibited the expression level of miR-183-5p (Fig. 6D), but also upregulated expression levels of both *CTNNA2* (Fig. 6C and E) and *GSK3β* (Fig. 6C and F) in the horn of uterus treated with miR-183-5p inhibitor as compared to the control horn. The results suggest that *CTNNA2* and *GSK3β* might be involved in the regulatory

role of miR-183-5p in endometrial receptivity and embryo implantation.

Discussion

The establishment of receptive endometrium is essential for successful embryo implantation. Poor endometrial receptivity is one of the major causes of implantation failure and infertility (Fatemi & Popovic-Todorovic 2013). Currently, there is great interest in the identification of miRNAs as markers for the detection of impaired endometrial receptivity, altered embryo development and implantation failure (Nothnick 2012). Several studies examined whether miRNAs were involved in the process of uterine receptivity and embryo implantation (Chakrabarty *et al.* 2007, Chu *et al.* 2015, Zheng *et al.* 2017).

Figure 6

Mouse uterus was injected with normal saline (NS) and I-miR-183-5p at day 3 of pregnancy, and, observed on day 7 (A). Number of embryo implantation was significantly decreased in the uterine horn injected with I-miR-183-5p (B). Western blot analysis shows that levels of CTNNA2 and GSK3 β were significantly increased in the uterine horn injected with I-miR-183-5p (C, E and F). Q-PCR analysis shows that miR-183-5p levels were also decreased in uterine horn injected with I-miR-183-5p (D). * $P < 0.05$ and ** $P < 0.01$, compared with corresponding controls. Number of mouse (n) = 5.

In the present study, we showed for the first time, the roles of the miR-183 family comprising three miRs (miR-183-5p, miR-182-5p, and miR-96-5p) in endometrial receptivity and embryo implantation.

Previous studies demonstrated that miR-183 was upregulated in the endometrial fluid in the late secretory phase (Vilella *et al.* 2015), and, the miR-183 family was significantly downregulated during human endometrial stromal decidualization (Estella *et al.* 2012). Using the IK cell line, we identified that E_2 significantly upregulated expression levels of the miR-183 family, and this family has positive roles in migration and proliferation of IK cells. By using *in vitro* JA r spheroid assay, we found that miR-183-5p played a significant role in embryo attachment as inhibiting miR-183-5p considerably reduced attachment rates, and, miR-183-5p-mimic increased attachment rates.

Our *in vivo* mouse model also showed that inhibition of miR-183 levels results in low embryo implantation rates.

We evaluated potential target genes of the miRs mentioned above by using mi-RNA target predicting software. The identified genes (CTNNA2, LRP6, KCNJ14, HOXA9, and FGF-9) have been reported to be downregulated in the secretory phase of the female reproductive cycle in different studies (Carson *et al.* 2002, Horcajadas *et al.* 2006, Díaz-Gimeno *et al.* 2011). Luciferase reporter assay in the present study validated that CTNNA2 gene is a potential target gene of miR-183-5p.

The complexity of tight junctions significantly reduce in receptive-phase endometrium, and, less complex tight junctions may assist penetration of blastocyst and endometrium (Murphy *et al.* 1992). Another study demonstrated that components of gap junctions and adhesion decreased during the pre-implantation period to facilitate trophoblast invasion through uterine epithelium (Grund & Grummer 2018). Alpha-catenin also regulates actin dynamics, negatively correlating cell adhesion with cell proliferation (Kobiela & Fuchs 2004). Activation of Wnt/ β -catenin pathway increases uterine receptivity and adhesion of embryo and endometrial cells (Zheng *et al.* 2017). CTNNA2 is responsible for cell-cell adhesion by maintaining the integrity of the cadherin-catenin complex and linking adherent junctions to the actin cytoskeleton (Gates & Peifer 2005). The present study showed that miR-183-5p may increase cell migration and proliferation of endometrial cells and downregulate expression levels of CTNNA2 and GSK3 β that is a negative regulator of Wnt/ β -catenin pathway (Ma *et al.* 2014, Ahmed *et al.* 2016, Zhang & Yan 2016). Our data indicate that miR-183-5p might be involved in promoting the effects of estrogen on endometrial receptivity and embryo implantation by decreasing endometrial cell-cell adhesion, and, the junctional complexity of uterine epithelial cells.

Our *in vivo* mouse model showed that inhibition of miR-183 levels resulted in low embryo implantation rate, and, higher CTNNA2 and GSK3 β levels in uterus. Recently, it was reported that the inactivation of Wnt/ β -catenin signaling impaired receptive uterine endometrium formation (Zheng *et al.* 2017). We proposed a hypothetical model showing how miR-183-5p regulates the CTNNA2 gene and results in downstream changes, which can ultimately affect the receptivity of endometrium (Fig. 7).

Embryonic implantation in humans depends on the interaction of the embryo with the receptive endometrium. The roles of E_2 in embryo implantation are contradictory in different studies. High levels of estrogen can be observed in women during *in vitro* fertilization

Figure 7

Hypothetical model shows the role of miR-183-5p in regulating endometrial receptivity via targeting *CTNNA2* gene.

and embryo transfer (IVF-ET), and, high doses of estrogen are also used in endometrium preparation in patients underlying frozen embryo implantation (Berkane *et al.* 2017). Several studies demonstrated that supraphysiological E_2 levels were not detrimental to IVF-ET outcomes (Jing *et al.* 2019, Sekhon *et al.* 2019), although previous studies suggested that exposure to high levels of E_2 during IVF treatment in the early follicular phase might be related to a lower chance of pregnancy and IVF outcome (Kolibianakis *et al.* 2003). Our previous study and the present studies also showed that 10^{-7} M E_2 promoted JAr spheroid attachment on IK cells (Ullah *et al.* 2017).

In the present study, we demonstrated a part of the roles and underlying mechanisms of miR-183 family in uterine receptivity and embryo implantation. The roles of other potential target genes of miR-183 family involved in the process of endometrial receptivity and embryo implantation may be important for the clinical application of the miR-183 family as a biomarker for implantation failures, and, new strategy for infertility treatment.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0184>.

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

RA, K U and J-Z S designed the experiments. RA performed the experiments, T U R helped to analyze the data. L-Y J, Y C, H-Y P and Q-J W helped in performing experiments. RA, H-F H and J-Z S wrote and edited the paper.

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