RESEARCH

miR-23a-3p increases endometrial receptivity via CUL3 during embryo implantation

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Abstract

A receptive endometrium is required in a successful embryo implantation. The ubiquitination-induced β -catenin degradation is related to the implantation failure. This study aimed to elucidate whether miR-23a-3p regulates endometrial receptivity via the modulation of β -catenin ubiquitination. The expressions of miR-23a-3p and CUL3 were detected in endometrial epithelial cells (EECs) isolated from pregnant mice and in hormone-induced EEC-like Ishikawa cells. The ubiquitination experiment was performed to explore the effect of CUL3 and miR-23a-3p on β -catenin ubiquitination level. The trophoblast attachment was detected by co-culturing JAR (choriocarcinoma cell line) spheroids with Ishikawa cell monolayers. miR-23a-3p was upregulated while CUL3 was downregulated in EECs at day 4 after pregnancy compared with day 1, as well as in hormone-induced Ishikawa cells. miR-23a-3p positively regulated the protein level of β -catenin without affecting the mRNA level. The ubiquitination and degradation of β -catenin was suppressed by miR-23a-3p, while it was promoted by CUL3. Immunoprecipitation confirmed the binding between CUL3 and β -catenin. Luciferase reporter assay confirmed the target relationship between miR-23a-3p and CUL3. The ubiquitination of β -catenin was modulated by the miR-23a-3p/CUL3 pathway. The overexpression of miR-23a-3p promoted JAR spheroid attachments in Ishikawa cells. miR-23a-3p is beneficial for the endometrial receptivity and embryo implantation, whose mechanism is partly through the modulation of CUL3/ β -catenin.

Key Words

- ▶ embryo implantation
- ▶ endometrial receptivity
- ▶ ubiquitination
- ▶ CUL3
- ▶ miR-23a-3p

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Introduction

Embryo implantation is the first step to establish a successful pregnancy, which requires a normal endometrial receptivity and a functional embryo (Garrido-Gomez *et al.* 2012). With the attachment between trophoblasts in embryo and endometrial epithelial cells (EECs) in the uterus, a synchronized dialogue is started between embryonic and maternal tissues (Cagil *et al.* 2011). During this process, estrogen (E) and progesterone (P) secreted

by the ovaries play an important role, which promote the transformation of endometrium toward secretory and receptive condition in order to receive the embryo (Lim & Wang 2010). At the same time, many molecules, including leukemia inhibitory factor (LIF), integrin $\alpha\beta 3$, and osteopontin (OPN), were released by EECs to enhance the endometrial receptivity (Schmitz *et al.* 2014, Zheng *et al.* 2016, Berneau *et al.* 2019). Therefore, the

EEC-mediated endometrial receptivity plays a critical role in embryo implantation, whose mechanisms have been largely unknown.

The Wnt- β -catenin signaling pathway is well identified in regulating embryo implantation process (Sonderegger *et al.* 2010). As reported, the Wnt- β -catenin signaling pathway is critically involved in trophoblast implantation and adhesion to maternal uterine tissues (Hess *et al.* 2007, Liu *et al.* 2010). Both Wnt and β -catenin proteins are key components of the Wnt- β -catenin signaling pathway. When the Wnt ligand associates with the receptors on the cell surface, the Wnt- β -catenin signaling pathway is turned on and large amounts of β -catenin protein transport into nucleus, thus exerting the transcriptional functions. When the Wnt ligand is blocked, the Wnt- β -catenin signaling pathway is turned off and β -catenin proteins are ubiquitinated, thus gradually being degraded by the proteasomes (Liu *et al.* 2002). Therefore, the inhibition of β -catenin ubiquitination is beneficial for the maintenance of endometrial receptivity and the promotion of trophoblast implantation. CUL3, also known as Cullin 3, modulates the process of ubiquitination and is reported to negatively regulate β -catenin protein expression via increasing its ubiquitination (Angers *et al.* 2006). However, whether CUL3 is involved in regulating EEC-mediated endometrial receptivity by interacting with β -catenin remains unclear.

miRNAs are a group of endogenous ncRNAs encoded by the genome of almost all species (Wang *et al.* 2018). The mature miRNA is about 19–25 nucleotides in length and is derived from the initial transcript pri-miRNA in the nucleus. By binding with the 3'UTR of targeted mRNA, miRNAs exert the regulatory function via degrading the target mRNAs (Beermann *et al.* 2016). Several miRNAs have been identified to modulate the embryo implantation process, such as miR-199a, miR-101, and miR-21 (Chakrabarty *et al.* 2007, Hu *et al.* 2008). miR-23a-3p is reported to regulate cell proliferation and differentiation in various types of cells (Godfrey *et al.* 2018, Zhao *et al.* 2019). Although it is rarely reported to regulate the embryo implantation process, a study conducted by Yang *et al.* (2018) suggested that miR-23a-3p was lowly expressed in recurrent miscarriage patients and that lower serum level of miR-23a-3p was related to the failure of embryo implantation. Notably, we found that there are predicted binding sites between miR-23a-3p and CUL3 mRNA, suggesting the potential effect of miR-23a-3p on CUL3 expression.

In the current study, the expressions of miR-23a-3p and CUL3 were detected in EECs isolated from pregnant

mice and in hormone-induced EEC-like Ishikawa cells. Meanwhile, the effect of miR-23a-3p/CUL3 on ubiquitination of β -catenin was also elucidated, hoping to provide the evidence for the mechanisms underlying EEC-mediated endometrial receptivity during embryo implantation.

Materials and methods

Isolation of EECs from pregnant mice

The female Kunming mice (6-week old) were bought from the Shanghai Lab Animal Research Center (Shanghai, China). They were divided into two groups according to the pregnant date, the 1-day group (1 day after the pregnancy) and the 4-day group (4 days after the pregnancy). There were six mice in each group. EECs were isolated from uterine tissues from the two groups as previously described (Pan *et al.* 2017). Briefly, the uterine horns were cut into small pieces on ice, followed by washing twice using Hanks' balanced salt solution (HBSS) which was deprived with Mg^{2+}/Ca^{2+} . To digest the tissues, 7 g/L trypsin and 18 g/L dispase (Sigma) were added for 30 min at 4 °C before being placed for another 30 min at room temperature. After collecting the epithelial plaques, we washed the collected cells twice using HBSS. Then they were cultured in 12-well plates with complete media (10% charcoal-stripped fetal bovine serum (FBS), 1% penicillin/streptomycin, and phenol-red-free DMEM/F12).

Culture and treatment of Ishikawa cells

The immortalized human uterine endometrial adenocarcinoma cell line (Ishikawa) was bought from the ATCC. The culture medium consisted of RPMI 1640 supplemented with 5% FBS. Cells were stored in a standard tissue culture incubator at 37 °C, with 5% CO₂ and 95% humidity. The medium was changed to phenol-red-free RPMI 1640 containing 5% charcoal-stripped FBS 24 h prior to cell treatment. Ishikawa cells were treated with different concentrations of 17 β -estradiol (E; 10⁻⁹, 10⁻⁸, and 10⁻⁷ mol/L), different concentrations of progesterone (P; 10⁻⁶, 10⁻⁵, and 10⁻⁴ mol/L), or combinedly treated with 10⁻⁸ mol/L E+10⁻⁶ mol/L P (Zhu *et al.* 2013). Ishikawa cells were treated with these hormones for 96 h.

Quantitative real-time PCR (qRT-PCR)

To extract RNAs from cells and tissues, the TRIzol reagent (Thermo Fisher Scientific) was used. To obtain cDNAs

reversely transcribed from RNA, the RT Kit (Thermo Fisher Scientific) was used. The SYBR-Green MicroRNA Kit and SYBR Green PCR Kit (Takara) were used to perform the qRT-PCR assay, which was conducted on ABI 7500 FAST Real-Time PCR System (Thermo Fisher Scientific). The expression levels were determined using $2^{-\Delta\Delta Ct}$ method. U6 was used as the control for miR-23a-3p, and GAPDH was used as the control for CUL3, Wnt1, and CTNNB1 mRNA.

Western blot analysis

Proteins were extracted by the AllPrep Protein/RNA/DNA Mini Kit (Qiagen). The primary antibodies used in this study included anti-CUL3 (ab75851, 1/20,000, Abcam), anti-Wnt1 (ab15251, 1/5000, Abcam), anti- β -catenin (ab6302, 1/4000, Abcam), anti-LIF (ab113262, 1 μ g/mL, Abcam), anti-Integrin α v (ab179475, 1/5000, Abcam), anti-integrin β 3 (ab119992, 1/1000, Abcam), anti-OPN (ab75285, 1/500, Abcam), and anti- β -actin (ab8226, 1/500, Abcam). The secondary antibodies used included horseradish peroxidase-conjugated anti-rabbit IgG (ab205718, 1/2000, Abcam). Expression was visualized with the ChemiDoc XRS system (Bio-Rad) and the ECL enhanced reagent (Thermo Fisher Scientific).

Ishikawa cell transfection

The miR-23a-3p mimic/inhibitor and their negative controls (pre-NC and NC) were purchased from Ribobio Technology (Guangzhou, Guangdong, China). The CUL3 overexpression plasmid vector (pcDNA-CUL3) and its negative control (pcDNA), as well as the siRNA against CUL3 (siRNA-CUL3) and its negative control (siRNA) were constructed by Genechem Corporation (Shanghai, China). Lipofectamine 2000 (Thermo Fisher Scientific) was used in cell transfection.

Ubiquitination assay and cycloheximide (CHX) experiment

The effect of miR-23a-3p/CUL3 on the ubiquitination of β -catenin was analyzed by co-transfecting Ishikawa cells with HA-Ub and miR-23a-3p mimic/pcDNA-CUL3, with MG132 (10 μ g/mL) treatment for 2 h before detection. The cell lysates were immunoprecipitated with anti- β -catenin, and the ubiquitination levels of β -catenin were detected by immunoblotting with anti-HA antibody.

For the CHX experiment, Ishikawa cells were transfected with miR-23a-3p mimic/pcDNA-CUL3,

followed by the addition of 50 μ g/mL CHX. The relative expression of β -catenin at 0, 6, 9, 12, and 24 h after CHX treatment was detected by using Western blot analysis.

Immunoprecipitation assay

The immunoprecipitation assay is conducted by microMACS protein A/G microbeads magnetic separation (MedChemExpress, Monmouth Junction, NJ, USA). In brief, total protein (100 μ g) was incubated on ice for 30 min with protein A/G microbeads (20 μ L) and 2 μ g of the IgG or anti- β -catenin. During a magnetic field, the magnetically labeled immune complex was passed over a microcolumn, followed by washing with the lysis buffer. Then the sodium dodecyl sulfate gel loading buffer was used to elute the immunoprecipitated proteins from the column, followed by immunoblotting with β -catenin antibody or CUL3 antibody.

Luciferase reporter assay

CUL3 3'UTR which contains the predicted binding sites of miR-23a-3p was amplified by using PCR. Mutant CUL3 3'UTR was synthesized by Invitrogen. The products were cloned into the psi-CHECK2 vector (Promega). 293T cells were plated in 96-well plates, followed by the transfection with 100 ng mutant (Mut) or WT CUL3 3'UTR vectors, together with 30 pmol miR-23a mimic/inhibitor. Cells were harvested for the luciferase assay after 48 h. Then, we used Dual-Luciferase Reporter Assay System (Promega) to evaluate the Renilla and firefly luciferases activities, and the value was normalized to the firefly luciferase activity.

JAR spheroid attachment to Ishikawa cell monolayers

The human choriocarcinoma cell line (JAR) was purchased from ATCC and was cultured in RPMI 1640 medium (5% FBS) in a standard tissue culture incubator. The co-culture of JAR spheroids and Ishikawa cells was performed as previously described (Li *et al.* 2017). The JAR cells were then trypsinized, washed, and seeded in a 6-well plate at a concentration of 2×10^5 cells per well. Then they were shaken overnight. Ishikawa cells were plated in 12-well plates (2×10^5 cells per well). Ishikawa cells were transfected with miR-23a-3p mimic followed by the treatment of 10^{-8} mol/L 17 β -estradiol (E) for 24 h. The JAR spheroids (45 spheroids/dish) were transferred onto the surface of Ishikawa cell monolayers for 7 h at 37 °C with 5% CO₂. Then the medium was removed

and the plates were centrifuged for 10 min at 10 *g*. The attached spheroids were counted. The attachment rate was calculated (attached spheroid number/seeded spheroid number).

Immunofluorescence analysis

The JAR cells were probed with the antibody against PCNA (1:100, 2 μ g/mL, ab92552, Abcam). Then they were incubated with IgG Alexa 488 (ab150077, Abcam) for 1 h at a dilution of 1:200. After being washed by 0.1% BSA in PBS, the cells were overlaid with DAPI. The images were taken at 7 and 24 h after the co-culture by using a confocal microscope (Carl Zeiss). The spheroid expansion was calculated as the ratio of the spheroid diameter at 24 h to the spheroid diameter at 7 h.

Statistical analysis

Data are shown as mean $\pm$ S.E.M. To compare the differences between the two groups, the Student's *t*-test was used. To compare the differences among multiple groups, the one-way ANOVA and Tukey's post hoc test was used, and $P < 0.05$ was considered significant.

Results

miR-23a-3p is upregulated while CUL3 is downregulated in EECs during pregnancy and in E/P-induced Ishikawa cells

EECs were isolated from uterine tissues of pregnant mice at day 1 and day 4, then the expressions of miR-23a-3p and CUL3 were detected using qRT-PCR and Western blot analysis. As indicated in the 4-day group, the relative expression of miR-23a-3p was significantly upregulated compared with the non-pregnant group and the 1-day group (Fig. 1A, $P < 0.01$), and the mRNA expression and the protein expression of CUL3 were downregulated (Fig. 1B, $P < 0.05$). On the other hand, the expressions of miR-23a-3p and CUL3 were also detected in Ishikawa cells which were treated with different concentrations of estradiol (E) and/or progesterone (P). With the increasing concentration of E (from 10^{-9} to 10^{-7} mol/L), miR-23a-3p expression did not show a concentration-dependent manner, while with the increasing concentration of P (from 10^{-6} to 10^{-4} mol/L), miR-23a-3p showed a concentration-dependent upregulation in Ishikawa cells (Fig. 1C). In addition, the combined treatment of E (10^{-8} mol/L)+P (10^{-6} mol/L) caused a marked

Figure 1

Expressions of miR-23a-3p and CUL3 in EECs during pregnancy and E/P-induced Ishikawa cells. EECs were isolated from uterine tissues of non-pregnant mice ($n = 6$) and pregnant mice at day 1 ($n = 6$) and day 4 ($n = 6$), then the expressions of miR-23a-3p (A) and the mRNA and protein expressions of CUL3 (B) were detected using qRT-PCR and Western blot analysis. Ishikawa cells were treated with different concentrations of estradiol (E) and/or progesterone (P) for 96 h. The expressions of miR-23a-3p (C) and the mRNA and protein expressions of CUL3 (D) were detected using qRT-PCR and Western blot analysis. * $P < 0.05$.

enhancement of miR-23a-3p in comparison with E (10^{-8} mol/L) or P (10^{-6} mol/L) treated alone (Fig. 1C). Similarly, with the increasing concentration of P (from 10^{-6} to 10^{-4} mol/L), CUL3 showed a concentration-dependent downregulation in Ishikawa cells, and the combined treatment of E (10^{-8} mol/L)+P (10^{-6} mol/L) caused a significant reduction of CUL3 in comparison with E (10^{-8} mol/L) or P (10^{-6} mol/L) treated alone (Fig. 1D).

miR-23a-3p negatively regulates the expression of CUL3

The online database Targetscan (www.targetscan.org) and RAID (<http://www.rna-society.org/raid2/>) predicted the binding between miR-23a-3p and CUL3 3'UTR in several species (Fig. 2A). After transfecting miR-23a-3p mimic/inhibitor into Ishikawa cells, we confirmed the transfection efficacy as shown in Fig. 2B. We mutated the potential binding sites and cloned them into a plasmid reporter vector. The results of the luciferase reporter assay indicated that overexpressing miR-23a-3p reduced the relative luciferase activity in the unmutated group (the WT CUL3 group), while knocking down miR-23a-3p increased the relative luciferase activity in the unmutated group (the WT CUL3 group). Both overexpressing or

knocking down miR-23a-3p did not change the luciferase activity in the mutated group (the Mut CUL3 group) (Fig. 2C). Meanwhile, the simultaneous knockdown of miR-23a-3p and CUL3 reduced the enhancement of CUL3 mRNA and protein levels which were raised by miR-23a-3p knockdown (Fig. 2D). Ishikawa cells were transfected with miR-23a-3p mimic or co-transfected with CUL3 overexpressing plasmid (pcDNA-CUL3). The co-transfection restored the defect of both mRNA and protein expressions of CUL3 (Fig. 2E). These data indicated that miR-23a-3p negatively regulated the expression of CUL3 in Ishikawa cells.

The role of miR-23a-3p on the Wnt- β -catenin signaling pathway

The expressions of the key components of the Wnt- β -catenin signaling pathway, including Wnt1 and β -catenin, were detected after transfection. The result showed that both mRNA and protein levels of Wnt1 was not significantly changed after miR-23a-3p overexpression or knockdown (Fig. 3A). The mRNA level of CTNNB1 (the encoding gene of β -catenin) was not much changed, whereas the protein level of β -catenin was upregulated by miR-23a-3p overexpression and was downregulated

Figure 2

miR-23a-3p negatively regulates the expression of CUL3. (A) The predicted binding sites between miR-23a-3p and CUL3 3'UTR via online database Targetscan (www.targetscan.org) and RAID (<http://www.rna-society.org/raid2/>). (B) Ishikawa cells were transfected with miR-23a-3p mimic/inhibitor for 48 h to overexpress/knockdown miR-23a-3p. The transfection efficacy was confirmed using qRT-PCR. (C) The 293T cells were transfected with miR-23a-3p mimic/inhibitor together with WT or mutant (Mut) CUL3 3'UTR reporter vector. The relative luciferase activity was detected using luciferase reporter assay. (D) Ishikawa cells were transfected with miR-23a-3p inhibitor or co-transfected with small interfering RNA (siRNA) against CUL3 (siRNA-CUL3). The mRNA and the protein levels of CUL3 were detected using qRT-PCR and Western blot analysis, respectively. (E) Ishikawa cells were transfected with miR-23a-3p mimic or co-transfected with CUL3 overexpressing plasmid (pcDNA-CUL3). The mRNA and the protein levels of CUL3 were detected using qRT-PCR and Western blot analysis, respectively. * $P < 0.05$.

Figure 3

The role of miR-23a-3p on the Wnt- β -catenin signaling pathway. Ishikawa cells were transfected with miR-23a-3p mimic/inhibitor for 48 h to overexpress/knockdown miR-23a-3p. (A) The mRNA and protein expressions of Wnt1 were detected using qRT-PCR and Western blot analysis. (B) The mRNA level of CTNNB1 (the encoding gene of β -catenin) and the protein level of β -catenin were detected using qRT-PCR and Western blot analysis. * $P < 0.05$.

by miR-23a-3p knockdown (Fig. 3B). These data indicated that miR-23a-3p affect the Wnt- β -catenin signaling pathway via positively regulating the protein expression of β -catenin in Ishikawa cells.

miR-23a-3p suppresses ubiquitination of β -catenin via inhibiting CUL3 expression

Given that the protein level of β -catenin was regulated by miR-23a-3p, we further investigated the effect of miR-23a-3p on the ubiquitination of β -catenin. The IP assay indicated the binding between β -catenin and CUL3 (Fig. 4A). After overexpressing miR-23a-3p in Ishikawa cells, the ubiquitin-modified β -catenin was reduced (Fig. 4B). Meanwhile, after inhibiting protein synthesis via CHX, the degradation of β -catenin was suppressed by miR-23a-3p overexpression (Fig. 4C), suggesting miR-23a-3p inhibited the degradation of β -catenin via suppressing its ubiquitination. After overexpressing CUL3 in Ishikawa cells, the mRNA expression of CTNNB1 (the encoding gene of β -catenin) was not significantly changed, whereas the protein level of β -catenin was downregulated (Fig. 4D), indicating that CUL3 modulated the β -catenin protein expression. In addition, CUL3 overexpression promoted the β -catenin ubiquitination (Fig. 4E) and the β -catenin degradation (Fig. 4F). In addition, the simultaneous knockdown of miR-23a-3p and CUL3 negated the promotion of β -catenin ubiquitination caused by miR-23a-3p knockdown (Fig. 4G). These findings demonstrated that miR-23a-3p suppressed ubiquitination of β -catenin via inhibiting CUL3 expression.

miR-23a-3p promotes JAR spheroid attachments in Ishikawa cells via suppressing CUL3 expression and increasing β -catenin expression

Ishikawa cells were transfected with miR-23a-3p mimic or co-transfected with CUL3 overexpressing plasmid (pcDNA-CUL3). The overexpression of miR-23a-3p increased the protein levels of β -catenin, LIF, integrin α v, integrin β 3, and OPN, while such response was negated by the co-transfection with pcDNA-CUL3 (Fig. 5A), suggesting endometrial receptivity was modulated by miR-23a-3p/CUL3 pathway. Furthermore, overexpressing miR-23a-3p markedly increased JAR spheroid attachments in Ishikawa cells (Fig. 5B) and promoted JAR spheroid expansion at 24 h in comparison with 7 h (Fig. 5C). Taken together, our findings indicated that miR-23a-3p promoted JAR spheroid attachments in Ishikawa cells via targeting CUL3 and suppressing the ubiquitination of β -catenin.

Discussion

A complex series of events are involved in the embryo implantation, causing the establishment of an initial connection between embryonic and maternal tissues (Diedrich *et al.* 2007). The failure of embryo implantation is often related to the poor quality of embryo and poor endometrial receptivity. As the endometrial receptivity is relatively easy to manually intervene than embryo quality, researchers have been focused on the mechanisms of endometrial receptivity in recent years (Haouzi *et al.* 2009). In the current study, we found a novel regulatory molecule, miR-23a-3p that modulates endometrial

Figure 4

miR-23a-3p suppresses the ubiquitination of β -catenin via inhibiting CUL3 expression. (A) The IP assay was used to identify the binding between β -catenin and CUL3. (B) Ishikawa cells were transfected with miR-23a-3p mimic and HA-Ub (Ubiquitin) with MG132 (10 μ g/mL) treatment for 2 h before detection. The cell lysates were immunoprecipitated with anti- β -catenin, and the ubiquitination levels of β -catenin were detected by immunoblotting with anti-HA antibody. (C) Ishikawa cells were transfected with miR-23a-3p mimic and treated with CHX which is a protein synthesis inhibitor. The protein level of β -catenin was detected using Western blot analysis at 0, 6, 9, 12, and 24 h after CHX treatment. (D) Ishikawa cells were transfected with pcDNA-CUL3 to overexpress CUL3. The mRNA expression of CTNNB1 (the encoding gene of β -catenin) and the protein level of β -catenin were detected using qRT-PCR and Western blot analysis, respectively. (E) Ishikawa cells were transfected with pcDNA-CUL3 and HA-Ub with MG132 (10 μ g/mL) treatment for 2 h before detection. The cell lysates were immunoprecipitated with anti- β -catenin, and the ubiquitination levels of β -catenin were detected by immunoblotting with anti-HA antibody. (F) Ishikawa cells were transfected with pcDNA-CUL3 and treated with CHX. The protein level of β -catenin was detected using Western blot analysis at 0, 6, 9, 12, and 24 h after CHX treatment. (G) Ishikawa cells were transfected with HA-Ub and miR-23a-3p inhibitor or co-transfected with siRNA-CUL3, followed by MG132 (10 μ g/mL) treatment for 2 h before detection. The cell lysates were immunoprecipitated with anti- β -catenin, and the ubiquitination levels of β -catenin were detected by immunoblotting with anti-HA antibody. $*P < 0.05$.

receptivity via CUL3/ β -catenin pathway, providing evidence for the mechanisms underlying endometrial receptivity and embryo implantation.

miRNAs are a subset of ncRNAs with a length of less than 20 nucleotides. The regulatory functions of miRNAs are mainly exerted by two ways (Beermann *et al.* 2016). On the one hand, by completely binding with the 3'UTR of targeted mRNA, miRNAs promote the degradation of the mRNA, thus inhibiting gene expressions. On the other hand, by partly binding with the 3'UTR of targeted mRNA, miRNAs inhibit translation of the mRNA, thus suppressing gene expressions. In our study, the regulatory function of miR-23a-3p on CUL3 was elucidated by using luciferase reporter assay, as the mutation in the

predicted binding sites did not change the luciferase activities either overexpressing miR-23a-3p or knocking down miR-23a-3p, while the WT groups did change the luciferase activities. Additionally, studies have revealed that miRNAs can be sponged by long lncRNAs, which is known as the competing endogenous RNA (ceRNA) mechanism. miR-23a-3p was also reported to be sponged by some lncRNAs. For example, Zhao *et al.* (2019) demonstrated that lncRNA NEAT1 could compete with the mRNA to bind with miR-23a-3p, therefore negating the inhibition of miR-23a-3p to gene expressions. Since several lncRNAs, such as lncRNA ENST00000433673 (Li *et al.* 2019) and lncRNA PTENP1 (Takamura *et al.* 2019), have been reported to be involved in the regulation of

Figure 5

miR-23a-3p promotes JAR spheroid attachments in Ishikawa cells via suppressing CUL3 expression and increasing β -catenin expression. Ishikawa cells were transfected with miR-23a-3p mimic or co-transfected with CUL3 overexpressing plasmid (pcDNA-CUL3). (A) The levels of endometrial receptivity-proteins, including LIF, integrin αv , integrin $\beta 3$, and OPN, and the protein levels of Wnt1 and β -catenin were detected using Western blot analysis. (B) The spheroid attachments were performed by co-culturing human choriocarcinoma cell line (JAR, 45 spheroids/dish) with the Ishikawa cell monolayer after transfecting Ishikawa cells with miR-23a-3p mimic and the treatment of 10^{-8} mol/L 17β -estradiol for 24 h. The attachment rate was calculated as the ratio of the attached spheroid number to the seeded spheroid number. (C) Immunofluorescence analysis of JAR spheroids after the co-culture. The spheroid expansion was calculated as the ratio of the spheroid diameter at 24 h to the spheroid diameter at 7 h. * $P < 0.05$.

embryo implantation. Whether the ceRNA mechanisms exist in the miR-23a-3p/CUL3 pathway deserves further investigations.

Ubiquitination involves a cascade of reactions which are catalyzed by ubiquitin enzymes, including E1, E2, and E3 (Zheng & Shabek 2017). The ubiquitin-activating enzyme E1 transfers ubiquitin to the active site cysteine of the ubiquitin-conjugating enzyme E2. Then the ubiquitin ligase E3 transfers ubiquitin to the substrate from E2. Among them, Cullin-RING ligases (CRLs) are the most common family of E3 (Petroski & Deshaies 2005). CUL3 is a subunit of CRLs that helps form a central stalk-like scaffold to constrain the substrate binding (Petroski & Deshaies 2005). A previous study has revealed that CUL3 was lowly expressed in the placental villi from pre-eclamptic patients and that, in the maternal decidua, lack of CUL3 resulted in repressed invasion of trophoblast cells (Zhang *et al.* 2015). Their study suggested that CUL3 may play a critical role in the embryo implantation process. Since the ubiquitination of β -catenin is causally related to the poor endometrial receptivity and implantation failure (Hess *et al.* 2007, Liu *et al.* 2010), we supposed that the ubiquitin-associated protein CUL3 might affect embryo implantation process via modulating β -catenin. The overexpression

of CUL3 increased the ubiquitin-bound β -catenin and the degradation speed, while the overexpression of miR-23a-3p reduced the ubiquitin-bound β -catenin and the degradation speed. Meanwhile, the simultaneous knockdown of miR-23a-3p and CUL3 negated the promotion of β -catenin ubiquitination caused by miR-23a-3p knockdown. Our findings demonstrated that the β -catenin ubiquitination was modulated by the miR-23a-3p/CUL3 pathway. Notably, the expression of Wnt1 was not affected by the miR-23a-3p/CUL3 pathway in the current study. However, Wnt proteins have been reported to participate in blastocyst development and implantation both in mice and human (Taylor 2000, Mohamed *et al.* 2004). More evidence is still needed on the effects of Wnt protein in implantation regulation.

Inevitably, several limitations exist in our study. The co-culture model is just a surrogate which may not fully represent embryo implantation *in vivo*, and a lack of *in vivo* animal studies is a main limitation of this study. In the current study, the experiments with human EECs and blastocysts have shown that overexpression of miR-23a-3p promotes JAR spheroid attachments in Ishikawa cells, strongly proving the benefit of miR-23a-3p for the endometrial receptivity and embryo implantation. In consideration of the high conservation of miR-23a-3p

and its CUL3-binding site in human and murine, we did not repeat the experiments in murine. Experiments with murine EECs and blastocysts maybe a good supplementary, before an available animal model developed, which requires further efforts.

In conclusion, our study indicated that miR-23a-3p is beneficial for improving endometrial receptivity and trophoblast attachments during embryo implantation. Meanwhile, miR-23a-3p suppressed ubiquitination of β -catenin via inhibiting CUL3 expression. Our findings provide a novel insight into the mechanisms underlying embryo implantation.

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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Ethics approval and consent to participate

This study was approved by the Institute Research Medical Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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