Mmu-microRNA-200a Overexpression Leads to Implantation Defect by Targeting Phosphatase and Tensin Homolog in Mouse Uterus

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Abstract

Successful mouse embryo implantation requires a receptive uterus and an activated blastocyst. A large number of genes, cytokines, and other factors are involved in the process. MicroRNAs (miRNAs) regulate the expression of many genes, and previous studies have investigated the relationship between miRNA expression and embryo implantation. In this study, we show that mmu-microRNA-200a (mmu-miR-200a) is expressed in a spatiotemporal manner during implantation in mouse uterus and found that phosphatase and tensin homolog (*PTEN*), *SON*, and programmed cell death 4 (*Pdcd4*) are the target genes of mmu-miR-200a by bioinformatics analysis. In vitro gain and loss of function experiments confirm that *PTEN*, a critical gene for cell proliferation and apoptosis, is the target gene of mmu-miR-200a. Our experiments also show that injection of the uterine horn with mmu-miR-200a lentivirus leads to a decreased implantation rate. Collectively, our results suggest that mmu-miR-200a affects embryo implantation by regulating *PTEN* protein expression. Thus, clarifying the physiological functions of uterine miRNAs will help to elucidate the embryo implantation process and may even contribute to curing infertility and inventing new contraceptives.

Keywords

implantation, mouse, microRNA

Introduction

MicroRNAs (miRNAs) are noncoding RNAs of 18 to 25 nucleotides in length and can be found in diverse species.¹ The miRNAs mediate posttranscriptional gene regulation by affecting mRNA translation and/or stability.²⁻⁴ The miRNAs have spatiotemporal expression specificity, such that miRNAs are expressed in specific tissues during certain organisms' developmental stages.⁵ The expression of most mammalian miRNAs is spatiotemporally regulated and influences a variety of biological processes, including development, tissue morphogenesis, tissue identity maintenance, cell growth, differentiation, apoptosis, and metabolism.⁴ Recent reports of severe phenotypic abnormalities in miRNA-knockout mouse models further document the crucial roles of miRNAs as regulators of gene expression.^{6,7}

Successful embryo implantation requires the synchronous development of the receptive uterus and competent blastocyst.⁸ Although in vitro fertilization and embryo transfer techniques have been partially successful in overcoming human infertility, the embryo implantation rates remain disappointingly low, which may be partially due to the presence of a nonreceptive uterus at the time of embryo implantation. Endocrine, paracrine, autocrine, and/or juxtacrine factors,^{8,9} including *Ihh*,¹⁰ *Bmp2*,¹¹

Lif,¹² *Msx1*,¹³ and *Muc1*,¹⁴ are also involved in the process of implantation. The process of murine implantation is divided into prereceptive (D1-D3), receptive (D4, the window of implantation), and refractory phases (D5),¹⁵ the transition of which are regulated by estrogen (E2) and progesterone (P4), hormones that are secreted from the ovary.¹⁶ A short delay of on-time implantation leads to poor pregnancy outcomes and spontaneous pregnancy loss.¹⁷⁻¹⁹ Thus, the presence of a receptive uterus is critical for a successful pregnancy, and understanding the molecular circumstance of the uterus is beneficial for improving infertility. Recent reports have shown that miRNAs are indeed involved in implantation by regulating their target genes.^{20,21} Chakrabarty found that cyclooxygenase 2 (COX-2), the target gene of microRNA-101 (miR-101) and miR-199a, is an essential

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gene for implantation and decidualization.²² Hu performed locked nucleic acid (LNA)-modified microarrays to distinguish miRNA expression patterns at mouse implantation sites and investigated the expression and regulation of differentially expressed miRNAs and their target genes.²³ According to our previous studies, mmu-microRNA-200a (mmu-miR-200a) expression in the mouse endometrium decreased after implantation. Other studies have shown that miR-200a expression varies in endometrial diseases (endometrial carcinoma and endometriosis)^{24–26} as well as in various cancers and other diseases.^{27–36} Cell proliferation and invasion are 2 biological processes characteristic of both tumor and embryo implantation. In our studies, we demonstrate that mmu-miR-200a has an important role in embryo implantation and that phosphatase and tensin homolog (*PTEN*) is the target gene of mmu-miR-200a. The *PTEN* regulates cell proliferation and apoptosis in many types of cancers, and its expression is widely reported to be regulated by miRNAs.^{37–39}

Materials and Methods

Animals

NIH mice 6- to 8-week-old and weighing 25 to 30 g were used. The Chongqing Medical University Animal Care and Use Committee approved all our experiments. Animals were provided by the Chongqing Medical University Experimental Animal Centre in accordance with the institute's animal welfare policy (certificate no. SCXK [YU] 2007-0001, Chongqing, CHN). Females were mated with fertile males at a 2:1 ratio to induce pregnancy. The appearance of the vaginal plug was taken as day 1. Pregnant mice were sacrificed at 1000 hours, and the endometria were then removed and stored for analysis.

Total RNA Extraction

Total RNA was extracted from mouse endometrial tissues using Trizol reagent (TAKARA Biotechnology Co, Ltd, Dalian, China), according to the manufacturer's instructions. The extracted RNA was then stored at -20°C . RNA quantification and purity were assessed by measuring the samples' optical density at 260 and 280 nm. Total RNA integrity was examined using agarose gel electrophoresis.

Real-Time Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (PCR) was performed as described previously.⁴⁰ The PCR primers that were used are listed in Table 1. U6 was used for normalization.

Bioinformatics Analysis

Target gene prediction was performed with online tools including miRGen,⁴¹ Targetscan,⁴² and Pictar.⁴³

Table 1. Primers Used to Perform Real-Time miRNA RT-PCR.

Primers	Sequence (5' → 3')
miR-200a	
RT primer	Forward: GTCGTATCCAGTGCAGGGTCCGA Reverse: GGTATTGCGACTGGATACGACacatcg
PCR primer	Forward: GGCTAACACTGTCTGGTAACGATG Reverse: GTGCAGGGTCCGAGGT
U6	Forward: GCTTCGGCAGCACATATACTAAAAT Reverse: CGCTTCACGAATTTGCGTGTTCAT
PTEN	
PCR primer	Forward: CCGTTACCTGTGTGTGGTGATATC Reverse: GAATGTATTTACCCAAAAGTGAacatt

Abbreviations: miRNA, microRNA; miR-200a, microRNA-200a; PTEN, phosphatase and tensin homolog; RT-PCR, reverse transcriptase polymerase chain reaction.

In Situ Hybridization

The Mmu-miR-200a-specific probes and a negative control (Scrambled) were purchased from Exiqon (Denmark). In situ hybridization was performed using a kit from the Peking Dinguo Biotechnology Company (China), according to the manufacturer's instructions. Briefly, frozen uterine tissue were sectioned and fixed with 4% paraformaldehyde at room temperature for 10 minutes. Fixed samples were incubated with acetylate for 10 minutes and then treated with protease K at 37°C for 7 minutes. The samples were incubated in prehybridization solution for 3 hours at 55°C in a humidified chamber. After addition of denatured probes (40 $\mu\text{mol/L}$) to each of the samples, the samples were incubated overnight at 55°C . The samples were then washed with standard sodium citrate and incubated with rabbit anti-bovine serum albumin (1:100 dilution) at 37°C for 1 hour. The samples were incubated with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (1:100 dilution) for 1 hour; the endometria were then stained with nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate at room temperature for 33 minutes.

Injection

The Mmu-miR-200a lentivirus was synthesized by Shanghai Shengbo Biotechnology Company (China). Day 2 pregnant mice were randomly divided into control and experimental groups; each group included 7 mice. In the control group, nothing was injected into the left cornu uteri, but distilled water was injected into the right cornu uteri. In the experimental group, mmu-miR-200a lentivirus was injected into the left cornu uteri, and distilled water was injected into the right cornu uteri as described previously.⁴⁴ Uteri from all the mice were removed and examined on day 7 of pregnancy.

Cell Culture and Transfection

Primary cells were isolated from the uteri of mice that were sacrificed on pregnancy day 4. After the uteri were removed, they were washed with phosphate-buffered saline, cut into

small pieces, and treated with 1 mL of 10% trypsin at 4°C for 1 hour. The uteri were then trypsinized at 20°C for 1 hour and at 37°C for 10 minutes. Trypsin digestion was terminated by adding Dulbecco-modified Eagle medium (DMEM)/F12 containing 10% fetal bovine serum (Hangzhou Evergreen Biological Engineering Materials Co, Ltd, China). Uterine stromal cells were collected by centrifugation at 500 rpm for 10 minutes. The cells were seeded in 50-mL culture flasks at a density of 1×10^6 cells/mL and cultured in DMEM/F12 medium (HyClone Laboratories, Inc, South Logan, Utah) and supplemented with 10% fetal bovine serum. The cells were incubated at 37°C in a 5% CO₂ atmosphere. We identified the stromal cells using vimentin antibodies and chose cultures that were more than 95% pure for transfection.

Lipofectamine 2000 (Lipo2000) was purchased from Invitrogen (Grand Island, New York). The mmu-miR-200a mimic and inhibitor were purchased from Guangzhou Ruibobio Co, Ltd (Guangzhou, China). The cells were plated at a density of 5000 cells/well in 96-well plates. The miR-200a mimic, miR-200a inhibitor, negative controls, and lipofectamine 2000 (lipo2000) were diluted with DMEM/F12 medium, which was not supplemented with fetal bovine serum, and incubated for 5 minutes at room temperature after being mixed slightly. The miR-200a mimic, which was combined with lipo2000 (final concentration of 20 nmol/L), the miR-200a inhibitor, which was also mixed with lipo2000 (final concentration of 100 nmol/L), and the negative controls were prepared 1 day after cell plating and added to the cells after 20 minutes incubation at room temperature. The medium was replaced with fresh DMEM/F12 medium (supplemented with 10% fetal bovine serum), 4 to 6 hours after transfection. The cells were harvested after culturing for 48 hours.

Dual-Luciferase Activity Assay

Primary stromal cells were cultured in 24-well plates and transfected with pcDNA3/enhanced green fluorescent protein (EGFP)/PTEN, pcDNA3.1(+), and pcDNA3.1/pri-miR-200a. pcDNA3.1(+) is an empty plasmid, and pri-200a is a mmu-miR-200a precursor that is synthesized by chemical processes. pcDNA3/EGFP/PTEN included a sequence of PTEN that was predicted to interact with mmu-miR-200a. The MU-pcDNA3/EGFP/PTEN plasmid included a mutated sequence of PTEN that was predicted to interact with mmu-miR-200a. Both the plasmids contained EGFP sequence coding green fluorescence protein. Transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instruction. Firefly and renilla luciferase activity were measured 48 hours after transfection and were assessed using the dual-luciferase reporter assay.

Western Blotting

Uterine samples from the control and experimental groups were extracted into ice-cold radioimmunoprecipitation assay buffer (1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl,

1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, and 50 mmol/L Tris-HCl, pH 7.4) and supplemented with a complete protease inhibitor cocktail. Protein concentration was determined using a bicinchoninic acid protein assay kit, according to the manufacturer's protocol (Sigma Chemical Co, St. Louis, Missouri). Western blotting was conducted as described previously.⁴⁵

Statistical Analysis

Statistical analyses were performed with SPSS 13.0 (SPSS Inc, Chicago, Illinois). Data from the 2 groups were compared with the Student *t* test. Differences found between groups with *P* values < .05 were considered to be statistically significant. Prism GraphPad 5.0 was used for statistical analysis.

Results

The mRNA Expression of mmu-miR-200a in Peri-Implantation Mouse Endometrium

To examine the differential expression of mmu-miR-200a in the prei-implantation mouse endometrium, we collected endometria from murine uteri on pregnancy days 4 to 6. The data from real-time quantitative PCR showed that the expression level of mmu-miR-200a was highest on day 4 and decreased gradually on days 5 and 6 (Figure 1A). Moreover, mmu-miR-200a expression was much lower at day 5 implantation sites than that at the interimplantation sites (Figure 1B). We also found that mmu-miR-200a was mainly expressed in stromal cells, and its expression was very low in epithelial and glandular cells (Figure 1C).

Prediction and Confirmation of miR-200a Target Genes

We screened the target genes of mmu-miR-200a by investigating the Pictar, MirGen, and Targetscan databases,²⁵⁻²⁷ we chose the genes that were shared by the 3 databases, and then selected the genes that were specifically associated with cell proliferation and apoptosis. We ultimately obtained 3 potential target genes: *Son*, programmed cell death 4 (*Pdcd4*), and *PTEN* (Table 2).

To confirm the results obtained from database search, we transfected primary stromal cells with mmu-miR-200a mimic, mmu-miR-200a inhibitor, and their corresponding mmu-miR-200a controls. The SON, PDCD4, and PTEN expressions were assessed by Western blotting (Figure 2A). Our results indicated that the expression of *Son* and *Pdcd4* in stromal cells did not change after transfection. However, the PTEN expression was downregulated when mmu-miR-200a was overexpressed in stromal cells but was upregulated when stromal cells were transfected with mmu-miR-200a inhibitor. In addition, we utilized real-time quantitative PCR to determine the mRNA expression of *Pten* in stromal cells, and our results showed that the expression level of *Pten* was not changed before and after transfection (Figure 2B). These results suggest that *Pten* is the target gene of mmu-miR-200a, and the level of PTEN protein is

Figure 1. Expression pattern of mmu-miR-200a in mouse endometria. A, Expression levels of mmu-miR-200a mRNA from pregnancy phases days 4 to 6, as measured by real-time PCR. * $P < .05$ when comparing day 5 and 6 with day 4. B, Real-time PCR assessment of mmu-miR-200a mRNA expression levels on pregnancy day 5; expression levels were measured in implantation and interimplantation sites. U6 was used as a normalization control. The Mmu-miR-200a expression values from endometria of pregnant mice were compared with those from phase day 4; these values represent the average $\pm$ SD. Microarray analysis was performed by Shanghai Kangcheng Bioengineering Co, Ltd. D4, D5, and D6 represent pregnancy days 4, 5, and 6 respectively. IM and inter-IM represent implantation site and interimplantation site, respectively. * $P < .05$ for day 5 expression levels at interimplantation versus implantation sites. C, Mmu-miR-200a in situ hybridization in the mouse uterus. The Mmu-miR-200a expression levels were determined with probes from Exiqon (Denmark). Positively stained tissue (in blue) represents an area of miRNA expression. Scrambled represents the staining seen in our negative control. Days 4 to 6 are shown at $\times 400$ magnification. Mmu-miR-200a indicates mmu-microRNA-200a; mRNA, messenger RNA; miRNA, microRNA; PCR, polymerase chain reaction; SD, standard deviation.

Figure 3. The Mmu-miR-200a target gene confirmation. Mouse primary stromal cells were cotransfected with pcDNA3.1(+) vector and 1 of the following plasmids: mmu-miR-200a precursor (pri-200a), MU-pcDNA3/EGFP/PTEN, or pcDNA3/EGFP/PTEN. Luciferase activity was examined after 48 hours. pcDNA3.1(+) was an empty plasmid; pri-200a was a chemically synthesized mmu-miR-200a precursor. pcDNA3/EGFP/PTEN included a sequence of PTEN predicted to interact with mmu-miR-200a. The MU-pcDNA3/EGFP/PTEN plasmid contained a mutated sequence of PTEN and was predicted to interact with mmu-miR-200a. Both plasmids contained an EGFP sequence that coded for green fluorescence. Luciferase activity was not affected when the cells were transfected with MU-pcDNA3/EGFP/PTEN. However, luciferase activity decreased by about 35% when the cells were transfected with the miR-200a precursor. * $P < .05$ when compared to levels from transfection with pcDNA3/EGFP/PTEN. EGFP indicates enhanced green fluorescent protein; Mmu-miR-200a, mmu-microRNA-200a; PTEN, phosphatase and tensin homolog.

Table 3. Primers Used to Perform miRNA and PTEN RT-PCR.

Primers	Sequence (5' → 3')
miR-200a	Forward: CGCGGATCCACAGCCATCTTCCCTCCTG
PCR primer	Reverse: CCGGAATTCGGAGCTGACACAGGCCCTC
PTEN	Forward: ACGGGATCCGTCTGTAAACAGCCTTACTC
PCR primer	Reverse: CAGGAATTCAATCAGTTTAAAGTGGAGTTT
	Mutation: CAGGAATTCAATCAGTTTAAAGTGG
	Reverse: AGTTTATTTGTGAAAGAGATAAAGGGTTGTTAAC

Abbreviations: miR-200a, microRNA-200a; miRNA, microRNA; PTEN, phosphatase and tensin homolog; RT-PCR, reverse transcriptase polymerase chain reaction.

lentivirus, which was designed to overexpress mmu-miR-200a, was injected the left uterine lumen, and distilled water was injected into the right uterine lumen in the experimental group. The uterus were collected 5 days (day 7) after injection, the number of implantation sites in the left and right uterine horns of control group was 6.71 ± 1.78 and 6.57 ± 1.72 , respectively. However, the average number of implantation sites in the left uterine horn was much lower than that in the right in the experimental group. Moreover, the average number of embryos in the left and right uterine in the experimental group was 2.71 ± 1.38 and 7.14 ± 1.77 , respectively. In addition, the average number of implantation sites in the left experimental uterine horn was also lower than that in the control mice (Figure 5A-D). There

were no differences in the number of implantation sites in the control mice (Figure 5A). Figure 5C and D showed the efficiency of mmu-miR-200a overexpression after the injection of lentivirus and distilled water, respectively. The green fluorescence represents the efficiency of viral infection, indicating the effectiveness of the lentivirus. Lentivirus inducing the overexpression of mmu-miR-200a was injected on day 2, and uterine protein was extracted 72 hours later for PTEN expression analysis (Figure 5E). We observed that uterine PTEN expression was downregulated after induction of mmu-miR-200a overexpression, when compared to that in the controls. These results suggest that upregulated mmu-miR-200a lead to reduced pregnancy rate.

Figure 4. Immunohistochemical staining of phosphatase and tensin homolog (PTEN) in pregnant mice uteri. Control represents staining seen in the negative control. Representative uterine tissue sections from days 4 to 6, and the control are shown at $\times 400$ magnification.

Discussion

Most mammalian miRNAs participate in the regulation of gene expression and are involved in many cellular processes including cell growth, differentiation, apoptosis, metabolism, and spatio-temporal tissue structure regulation.^{4,46} In the human genome, approximately 1000 miRNAs regulate about 30% protein-encoding genes.^{42,47-49} Many of these miRNA-regulated genes are crucial for physiological processes and can be targeted for transcriptional regulation using gene knockout technology. For example, miR-7 is a tumor suppressor that targets Yin Yang 1 (YY1) in patients with colon cancer. The YY1 facilitates cancer cell growth by inhibiting p53 and promoting Wnt signaling pathways and also serves as an independent prognostic biomarker for patients with colorectal cancer.⁵⁰ Moreover, miRNAs also contribute to essential physiological processes in the body. For instance, miR-208 is required for cardiomyocyte hypertrophy, fibrosis, and expression of β -major histocompatibility complex in response to stress and hypothyroidism.⁵¹ These findings demonstrate that miRNAs play an integral role in physiological and pathological process.

Embryo implantation is a complicated physiological process that involves interactions between the uterus and the blastocysts, and differential gene expression is also crucial for the implantation process. In our study, mmu-miR-200a expression decreased during days 4 to 6. Epithelial cells also stop proliferating and begin to differentiate on day 4. Stromal cells start to proliferate following an upregulation of P4 levels,⁵² and epithelial cells begin to undergo apoptosis after embryo implantation.⁵³ *PTEN*, the target gene of mmu-miR-200a, plays an important role in cell proliferation and apoptosis. The *PTEN* expression was upregulated during days 4 to 6, and this pattern was in contrast to that of mmu-miR-200a but was consistent with that of cell proliferation and apoptosis. Therefore, we could get the conclusion that mmu-miR-200a may have a function during the embryo implantation process.

Moreover, previous studies have shown that miRNAs are involved in implantation by regulating their target genes.^{20,21} Chakrabarty found that COX-2, the target gene of miR-101 and miR-199a, is essential for implantation and decidualization.²² Before Chakrabarty investigations, scholars found that E2 and

Figure 5. The function of Mmu-miR-200a in embryo implantation. A, The number of implanted embryos after injecting nothing into left cornu uteri and injecting distilled water into right cornu uteri between 0800 and 0900 hours on pregnancy day 2 was used as a control. B, The number of implanted embryos in the left uterine horns after injecting mmu-miR-200a overexpression lentivirus between 0800 and 0900 hours on pregnancy day 2 was markedly decreased compared with the right uterine horns after injecting distilled water as a control. The number of implanted embryos after injecting nothing into the left cornu uteri was same as that in the control. C, The efficiency of mmu-miR-200a overexpression after injecting lentivirus. The green fluorescence represents the efficiency of viral infection. D, The efficiency of mmu-miR-200a overexpression after injecting distilled water. E, A quantitative representation of the results is shown in (A) and (B). The data shown are an average $\pm$ SD. L and R represent left and right, respectively. * $P < .05$ versus control. F, Western blot of PTEN protein expression after injecting mmu-miR-200a overexpression lentivirus. The Mmu-miR-200a lentivirus injection between 0800 and 0900 hours on pregnancy day 2, and extracted protein 72 hours later. The PTEN protein expression levels in the uterus were downregulated after the mmu-miR-200a lentivirus injection, when compared to levels found in the control. Untreated and 200a-Lv represent control and mmu-miR-200a lentivirus, respectively. Mmu-miR-200a indicates mmu-microRNA-200a; PTEN, phosphatase and tensin homolog; SD, standard deviation.

P4 could not regulate COX-2 expression.⁵⁴ The mechanism of COX-2 regulation was unclear. However, Chakrabarty discovered that miRNA regulated COX-2 expression for the first time. He confirmed that miRNA participated in embryo implantation through its target gene. In the present study, we used different strategies to find that the miRNA-mmu-miR-200a and its target gene-Pten are essential for embryo implantation. Hu performed LNA-modified microarrays to distinguish miRNA expression patterns at implantation sites in the mouse uterus. He also investigated the expression and regulation of target genes of differentially expressed miRNAs.²³ We narrowed our focus down to a specific miRNA for these investigations, despite having performed a vast microarray analysis. In summary, we launched an in-depth analysis of how the uterus enters into the receptive phase and the factors involved in the embryo implantation process.

We used functional analyses to verify that mmu-miR-200a was involved in embryo implantation. After injecting 1 side of the uterine lumen with lentivirus designed to overexpress mmu-miR-200a, we found that implantation rates were decreased significantly compared with that of the control. Thus, we confirmed that mmu-miR-200a participates in the implantation process. Although the mechanism regarding regulation of embryo is still unclear, we can postulate that mmu-miR-200a target gene, *PTEN*, is involved. Previous studies have reported that loss of *PTEN* resulted in cancer susceptibility and tumor progression, suggesting that *PTEN* overexpression may be an attractive target for cancer prevention and therapy. *PTEN* has tumor suppressive effects, regulating PI3K-dependent and -independent pathways. Elevated *PTEN* levels also negatively regulate 2 of the most pronounced metabolic features of tumor cells: glutaminolysis and the Warburg effect.⁵⁵ Additionally, Tashiro et al ascertained that *PTEN* is a tumor suppressor gene that plays a significant role in the pathogenesis of the endometrioid type of endometrial carcinoma.⁵⁶ More importantly, Chen et al observed that *PTEN* has a role in the mouse embryo implantation.⁵⁷ Consistent with previous studies, we observed that *PTEN* expression was upregulated during pregnancy days 4 to 6, with changes that were observed mainly in stromal cells and epithelial cells. The Mmu-miR-200a was expressed at higher levels at interimplantation sites than that at implantation sites on day 5, indicating that *PTEN* also had higher levels of expression at implantation sites than at interimplantation sites. Moreover, luminal epithelial cells start to differentiate on day 4, and *PTEN* also regulates the apoptosis of these cells during this stage. The *PTEN* may inhibit excessive proliferation of stromal cells, keeping the uterus in a good condition for implantation. The proliferation and apoptosis of epithelial and stromal cells were not influenced significantly when there is no embryo at interimplantation sites; therefore, the *PTEN* expression level was lower at the interimplantation sites when compared to that at the implantation sites.

In summary, we applied the microarray analysis in investigating mmu-miR-200a and confirmed the mmu-miR-200a expression pattern during the embryo implantation. We also

demonstrated that *PTEN* is the target gene of mmu-miR-200a. Moreover, we observed that mmu-miR-200a regulates embryo implantation, and that *PTEN* is an important effector in this process. We will continue to investigate the roles of *PTEN* in detail during both stromal cell and embryo implantation in our future study.

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Declaration of Conflicting Interests

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