Genes &
Diseases

FULL LENGTH ARTICLE

miR-21a inhibits decidual cell apoptosis by targeting Pdcd4

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Abstract   Decidualization of endometrial stromal cells (ESCs) accompanied with embryo implantation is a key process in mammalian reproduction. Evidence suggests that maintenance of decidual cells function is essential. As a critical part in post-transcriptional gene regulation, microRNAs (miRNAs/miR) have been confirmed to be involved in decidualization. However, whether microRNAs regulate decidual cells function has not been reported. Aiming to clarify the role and potential mechanism of miRNAs in decidual cells, artificial induced decidualization model in mice was established. There are 94 differentially expressed miRNAs (≥two-fold change) between decidualized and non-decidualized tissues, including 60 upregulated and 34 downregulated miRNAs. Of the differentially expressed miRNAs, mmu-miR-21a is up-regulated. RT-qPCR also confirmed the up-regulation of mmu-miR-21a following decidualization *in vivo* and *in vitro*, and bioinformatic analysis and luciferase activity assay revealed Pdcd4 to be the target gene of mmu-miR-21a. Inhibition of mmu-miR-21a restrained secretory function of decidual cells induced by mESCs, accompanied with increase of Pdcd4 expression and resulted in the increase of cell apoptosis. In addition, we also determined the expression of hsa-miR-21 and Pdcd4 in human proliferative endometrial tissues and decidua tissues. hsa-miR-21 showed higher expression in human decidua tissues compared with proliferative endometrial tissues, while expression of Pdcd4 was contrary to that of hsa-miR-21. Similarly, cell apoptosis increased significantly in human endometrial stromal cell line in response to inhibition of hsa-miR-21. Collectively, we conclude that mmu-miR-21a/hsa-miR-21 may play a key

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Introduction

It has been well delineated that mammal pregnancy involves a series of physiological process including implantation, decidualization and placentation before parturition.[1,2] As a transient tissue, decidua begins to develop in mice when the blastocyst is attached to endometrium on day 4.5 of gestation. In the following 3 days, the decidual cells around the attachment site of the embryo proliferate and differentiate vigorously, and become larger in size, usually in a bi-nucleated or polyploid status.[3] During this period, the endometrium undergoes considerable changes, including angiogenesis and tissue remodeling in preparation for pregnancy.[4] Tissue remodeling and angiogenesis in decidua area can provide nutrients to the implanted embryo for further development. Decidual tissue not only can limit excessive trophoblast invasion, thus preventing pathologies such as placenta accrete, but also can protect embryos from maternal immune rejection.[5] Dysregulated decidualization may result in compromised embryo-maternal communication as well as recurrent abortion.[6] Therefore, maintenance of the function of decidual cells is crucial to a successful pregnancy and parturition.

To date, several transcription factors, autocrine/paracrine factors and post-transcriptional factors have been confirmed to collectively regulate the process of decidualization Being 18- to 24-nucleotide non-coding RNAs, miRNAs can regulate gene expression in diverse organisms at the post-transcriptional level, specifically, through the cleavage or transcriptional inhibition of mRNAs.[7,8] Nevertheless, only a few miRNAs that are directly responsible for the repressed expression of hundreds of proteins have so far been functionally characterized, despite of a large quantity of miRNAs having been cloned or predicted.[9–12] According to the previous studies, miRNAs are highly involved in the process of female reproduction from oogenesis to fertilization, implantation, and even placentation, and miRNA dysregulation may lead to a variety of reproductive disorders, including polycystic ovarian syndrome,[13] and endometriosis.[14] Several studies published recently focused on the role of miRNAs in embryo implantation. Li et al reported in 2014 that mmu-miR-193 could regulate the expression of growth factor receptor-bound protein 7 in mouse uterus[15]; Yang et al found that mmu-miR-96 were associated with the apoptotic process of stromal cells as well as decidual cells by regulating Bcl 2[16]; and Sirohi et al revealed that ormeloxifene, an estrogen receptor modulator, could induce miR-140 and act on insulin-like growth factor 1 receptor, and consequently hinder embryo implantation in rat uterus.[17] However, little is known about the exact regulatory effect of miRNAs on endometrial decidual cells.

In order to have a deeper understanding of the role of miRNAs on endometrial decidual cells and the underlying molecular mechanisms, we constructed mouse models of artificially induced decidualization, by which we spotted 94 differentially expressed miRNAs (≥two-fold). It was noticed that in decidualized endometrium, the mmu-miR-21a expression was significantly up-regulated. Shi-Jun Hu et al revealed that mmu-miR-21a was highly localized in subluminal stromal cells. Reck was confirmed to be the target gene of miR-21a, which was closely related to embryo implantation primarily by inhibiting Mmp-9 activity. These findings imply that mmu-miR-21a may work as a regulator for the reconstruction and maintenance of the decidual areas.[9] MicroRNAs may play a regulatory role through multiple target genes, and as an important regulator in the establishment of decidua,[18] Pdcd4 attracted our attention due to the binding site of mmu-miR-21a in its 3′UTR. Pdcd4 is a pivotal tumor-suppressor against the occurrence and development of a variety of cancers such as breast cancer[19] and hepatocellular carcinoma.[20] It is believed that Pdcd4 inhibits tumorigenesis by regulating apoptosis. Wang et al once reported that Pdcd4 mediated the apoptosis of gastric carcinoma cells by suppressing FLICE-inhibiting protein (FLIP), a negative apoptosis regulator.[21] Moreover, there are studies revealing an inverse correlation between the level of miR-21 and Pdcd4 expression in breast cancer cells[22] and stem cells,[23] as well as in contrast-induced apoptotic renal cells.[24] Therefore, we hypothesized that mmu-miR-21a may work as a key factor in decidual cells by targeting Pdcd4.

In this study, we clarified the expression, the role and potential mechanism of mmu-miR-21a in decidual cells in mice. Additionally, human endometrial tissues and cell line were employed to validate the role and potential mechanism of hsa-miR-21. These findings would complement and improve the molecular mechanism of maintenance of decidual function.

Materials and methods

Animals and human tissues collection

8 weeks old of virgin female Kunming mice (*Mus musculus*) weighed 20–25 g were purchased from the Laboratory Animal Center of Chongqing Medical University Chongqing, China (Certificate: SICXK (YU) 2007–0001). The adult mice were settled in a sterilized animal room at 22 °C ± 2 °C, with a controlled 12-h light/dark cycle and a relative humidity of 55% ± 10%. Water and food were ad libitum. All

animal procedures were approved by the Ethics Committee of Chongqing Medical University (20170016) on 1 March 2017, and informed consents were given by all the human subjects.

The male mice, after vasectomy, were mated with female mice, and the first day of pseudopregnancy (pd 1) was marked by the vaginal plug in the next morning. We infused 10 μl of corn oil into one uterine horn of the mice on pd4 to obtain the artificially induce decidualization (ID), with the other horn without any infusion used as the induced decidualization control (IDC). 30 mice were euthanized on pd 8 by cervical dislocation to collect the endometrium, which was then preserved in liquid nitrogen for miRNA sequencing, DGE profiling, real-time qPCR, and western blotting. 30 mice were euthanized on pd4 by cervical dislocation to isolate primary endometrial stromal cells.

A total of eight human subjects were recruited as the control group from the pregnant women who visited the Outpatient Department of Gynecology, the First Affiliated Hospital of Chongqing Medical University from August to October, 2017. 5 out of the 8 patients received induced abortion and were absent from any signs of pregnancy disorders, and the other three were under surgical management for missed-abortion (at 6−9 wk of gestation). Analysis of baseline data in age and weight of the women as well as their gestational weeks showed no significant differences among these subjects. The inclusion criteria included: (1) positive results in pregnancy test; (2) normal menstrual cycle; (3) negative findings in genetic disease history; (4) absence of sexually transmitted diseases; (5) absence of immunodeficient diseases; (6) absence of endocrine diseases; (7) absence of chromosomal abnormalities; (8) absence of abnormality in uterine anatomy by ultrasound; and (9) normal embryo sizes corresponding to the gestational ages.

miRNAs and mRNA sequencing

miRNAs Sequencing and bioinformatics analysis were performed by Beijing Genomics Institute (BGI) technology. The small RNA digitalization analysis based on Hiseq high-throughput sequencing took the SBS-sequencing by synthesis, which can decrease the loss of nucleotides caused by the secondary structure. The 49 nt sequence tags from Hiseq sequencing went through the data cleaning analysis first, which includes getting rid of the low-quality tags, 5′ adaptor contaminants from the 50 nt tags, to get credible clean tags. Then the length distribution of the clean tags and common and specific sequences between samples were summarized. Then the standard analysis annotated the clean tags into different categories and took those which cannot be annotated to any category to predict the novel miRNA and seed edit of potential known miRNA.

Isolation of mESCs, *in vitro* decidualization and cell transfection

The primary stromal cells were isolated according to the procedure published in previous study.[25] On pd 4, after removed from the mice, the uterus were minced and then put in 5 mL of Hank's balanced salt solution (HBSS) with pancreatin (Sigma) and dispase (Roche, Indianapolis, IN) at 4 °C for 2 h, followed by another 30 min of standing at 37 °C. 500 g of the digested tissues were measured for 5-min centrifugation, with the supernatant being discarded. Following three times of washing procedure, the tissues were transferred into collagenase I purchased from Invitrogen (Carlsbad, CA) and stayed at 37 °C for 30 min, accompanied by vigorous shaking every 10 min to fully disperse the stromal cells in the supernatant. 70-μm nylon mesh was employed for filtration to collect 1000 g of filtered tissue debris mixture for another 5-min centrifugation. The supernatant was discarded to obtain the stromal cells, which were then resuspended in Dulbecco Modified Eagle Medium F-12 (DMEM-F12; Sigma) containing 10% charcoal-stripped fetal bovine serum purchased from Biological Industries (Kibbutz Beit Haemek, Israel). The cells, after transferred to culture plates or flasks, were incubated for 1 h to remove the medium with unattached cells. After the cell purity was identified by vimentin and Cytokeratin 7 (Sangon, China), the cells with a purity of greater than 95% were artificially induced decidualization by adding 10 nmol/L estradiol-17β (E2) and 1 μmol/L progesterone (P4) (Sigma) into the wells. After 96 h, cells were transfected with mmu-miR-21a inhibitor (RiboBio, Guangzhou, China) by Lipofectamine™ 2000 (lipo 2000; Invitrogen) under the manufacturer's instructions. The cells were harvested after 48 h of transfection.

Culture and human ESC transfection

ATCC®CRL-4003™, the human endometrial stromal cell line (hESC), was purchased from American Type Culture Collection (ATCC), and cultured as previously described (Salamonsen, Hannan, & Dimitriadis, 2007). 1% antibiotics and puromycin were mixed with 10% charcoal stripped fetal calf serum and transferred to DMEM/F12, which was used to culture the human stromal cells, followed by an 8-day *in vitro* decidualization using 10 nM estradiol-17 β, 1 μM medroxy-progesterone acetate as well as 0.5 mM 8-Br-Camp (Sigma). Replenishment of the media was performed at a 48-h interval. Then cells were transfected with hsa-miR-21 inhibitor (RiboBio, Guangzhou, China), and harvested after 48 h for Flow Cytometric Analysis.

Real-time qPCR

Endometrial tissues and cells were used to extract total RNAs with RNAiso plus reagent under the instruction of the manufacturer. The total RNAs, after treated with DNase I, were collected for cDNA synthesis in a reaction system (10 μl) using a RT-qPCR kit purchased from TaKaRa Biotechnology (Dalian, China). The specific primers for mmu-miR-21a, U6, Pdcd4, β-actin genes, as well as decidual/trophoblast prolactin-related protein (Dtprp), a marker for decidualization (Das et al, 2009), are listed in Table 1. Transcripts quantification was carried out with SYBR Green, and Bio-Rad CFX Manager 3.1 Detection System (USA) was employed for PCRs. Experiments were performed in triplicate for each sample, and $2^{-\triangle\triangle Ct}$ method was used to calculate the relative gene expression in different

Table 1   Primer Sequences for qPCR.

Genes		Sequence of primers 5′ to 3′
mus-Pdcd4	Forward	ACTGACCCTGACAATTTAAGCG
	Reverse	TTTTCCGCAGTCGTCTTTTGG
homo-Pdcd4	Forward	AGGGAAGGTTGCTGGATAGGC
	Reverse	TACCCCAGACACCTTTGCCTC
mmu-miR-21a	Forward	GCTAGCTTATCAGACTGATG
	Reverse	GTGCAGGGTCCGAGGT
hsa-miR-21-5p	Forward	GGCTAGCTTATCAGACTGATGTTG
	Reverse	CATCAGTCTGATAAGCTAGCC
Dtprp	Forward	AGCCAGAAATCACTGCCACT
	Reverse	TGATCCATGCACCCATAAAA
β-actin	Forward	TGGAATCCTGTGGCATCCATGAAAC
	Reverse	TAAAACGCAGCTCAGTAACAGTCCG
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT

tissue samples, with β-actin as the internal control of mRNA and U6 as the internal control of miRNA.

Western blotting

The cells were processed in lysis buffer to obtain the samples, which were then boiled for 10 min in $5 \times$ sodium dodecyl sulfate (SDS) loading buffer, followed by SDS-polyacrylamide gel electrophoresis (PAGE) with 10% gel. The separated bands were then transferred to poly-vinylidene difluoride membranes for an overnight 4 °C incubation with the following antibodies: anti-Pdcd4 (D29C6), anti-Caspase-3 (#9662), anti-cleaved Caspase-3 (#9664), anti-β-actin (#3700) were purchased from Cell Signaling Technology (Danvers, MA, USA), anti-BAX(ab32500; Abcam Cambridge, MA, USA), and anti-BCL2 (cat. no. sc-509; Santa Cruz, Biotechnology, Inc., Dallas, TX, USA). The membrane was washed three times with PBST, 15 min for each time, and then incubated with goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (BA1054, BA1050, BOSTER, China). The washing process was repeated after 1 h of incubation. The expression of protein was detected by chemiluminescent reaction (Millipore, USA) under the manufacturer's instruction and analyzed with Quantity One 4.6 software. Protein expression was normalized to β-actin.

Flow cytometric analysis of apoptosis

After the transfection with mmu-miR-21a and hsa-mmu-21a inhibitor and the negative controls, the mESCs and hESCs were harvested for a 15-min incubation with AnnexinV and PI at room temperature, followed by quantitative apoptosis analysis using a FACS Vantage SE flow cytometer (BD Biosciences, USA). All the experiments were conducted in triplicate.

Dual-luciferase reporter assay

Sequence alignment using the public bioinformation resources from National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/gene/27250) confirmed the

identities of the base sequences of the Pdcd4 3′-untranslated region (UTR) fragments. TargetScan databases (http://www.targetscan.org/vert_72/) was employed to predict the target, with a result showing two potential target sites of mmu-miR-21a in the 3′-UTR of the Pdcd4 mRNA sequence. A plasmid construct was collected from a random 3′-UTR site, and employed as the negative control. PCR amplification was conducted for the two predicted target sites as well as for the negative control using human genomic DNA as the template. pmiR-RB-REPORT vector purchased from Promega (Fitchburg, WI, USA) was used to clone the PCR products. The reporter plasmid was constructed and designated as hPDCD4 3′-UTR. The reporter plasmid and 50 nM miR-21a mimic or miRNA negative control from Guangzhou RiboBio (Guangzhou, China) were co-transfected into the 293 T cells, followed by incubation for 48 h. Dual-luciferase reporter system purchased from Promega was used to determine the cell extracts under the instruction of the manufacturer.

Statistical analysis

Each of the experiments was performed at triplicate or more times for each group. SPSS 18.0 (SPSS, Chicago, IL, USA) was employed for data analysis, with Student's t-tests used for data comparison for pairing groups, and one-way analysis of variance (ANOVA; Student-Newman-Keuls) for multiple groups. $P < 0.05$ was considered as significant.

Results

Sequencing analysis of miRNA expression in the ID and IDC

miRNAs sequencing assays were conducted to differentiate the expression of miRNAs in endometrium between ID and IDC groups. Compared with IDC group, a total of 94 miRNAs demonstrated a significant difference ($\geq$two-fold change) in their expression levels in DC group, including 60 upregulated and 34 downregulated miRNAs. As for mmu-miR-21a, a 2.07-fold increase was observed in the ID group, which is a remarkable inter-group difference. Fig. 1 shows miRNAs with large differences in expression between the two groups.

Expression of mmu-miR-21a in mouse endometrium following artificially induced decidualization *in vivo* and *in vitro*

Aiming to confirm the different expression of mmu-miR-21a in endometrium before and after decidualization, we constructed an artificially induced decidualization model *in vivo* and *in vitro*. Fig. 2A shows the uterus after artificially induced decidualization, robust deciduoma appeared on the induced side. The horn mass of induced side was significantly higher than the control side (Fig. 2B), and the expression levels of Dtprp increased significantly in the deciduoma (Fig. 2C). These data confirmed successful induction of decidualization *in vivo*. The results of Real-time
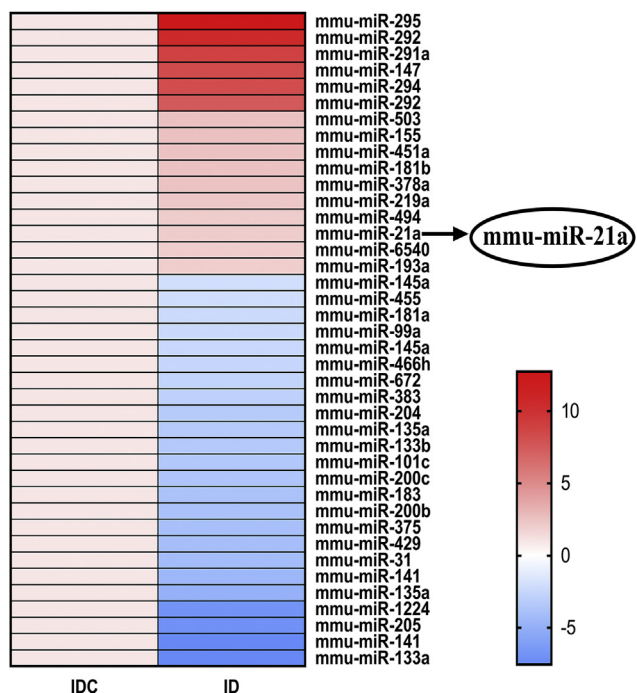
Figure 1 Sequencing of miRNAs and mRNAs for the models of ID and IDC. Profiling of miRNAs in the uterus of mice showing a two-fold or greater change in the ID compared with the IDC group. The red color represents a relatively higher expression, and the blue color represents a relatively lower expression in the ID group as compared to the control group. ID, induced decidualization; IDC, induced decidualization control.

PCR showed that mmu-miR-21a was evidently upregulated (Fig. 2D). Mouse primary endometrial stromal cells were isolated, and the results of Vimentin and Cytokeratin fluorescence staining showed that the purity of cells exceeded 95% (Fig. 2E). The expression levels of Dtprp and mmu-miR-21a were also higher after induced decidualization by E2 and P4 *in vitro* (Figs. 2F and G). These results confirmed that expression of mmu-miR-21a increased significantly accompanied with decidualization.

The function of mmu-miR-21a on decidualized cells

To further investigate the role of mmu-miR-21a in decidualized cells, mmu-miR-21a inhibitor was employed to downregulate expression of mmu-miR-21a in decidual cells. The expression of mmu-miR-21a was downregulated after transfection with mmu-miR-21a inhibitor. Compared with the non-induced cells, the expression levels of Dtprp in the cells treated with hormones alone and treated with hormones and inhibitors were both significantly higher. However, the Dtprp mRNA levels in the cells treated with hormones and inhibitors decreased compared with that in the cells induced by hormones only (Figs. 3A and B). The results of flow cytometry revealed decreased cell survival and increased cell apoptosis after transfected with mmu-miR-21a inhibitor in decidualized cells (Figs. 3C—E). The protein levels of cleaved Caspase-3, Caspase-3, and BAX in the decidualized cells increased after transfected with

mmu-miR-21a inhibitor, while expression of BCL2 decreased compared with those in the control group as well as in the negative control group (Figs. 3F and G).

Identification of Pdcd4 as a target gene of mmu-miR-21a

To explore the regulatory mechanism of mmu-miR-21a on decidual cells function, we conducted an online search to explore the target gene of mmu-miR-21a using the TargetScan databases (http://www.targetscan.org/vert_72/), with a result suggesting that Pdcd4 may be a putative mRNA target of mmu-miR-21a (Table 2). Moreover, mRNAs sequencing assays display that Pdcd4 exhibited a negative correlation with the expression of mmu-miR-21a, and a 2.43-fold decrease was observed in its expression in the ID group. The results of Western blot showed that the expression of Pdcd4 significant increase in the decidualized cells transfected with mmu-miR-21a inhibitor (Fig. 4A).

Luciferase reporter assay was performed to validate the targeting relationship between mmu-miR-21a and Pdcd4. The 3′-UTR fragment of Pdcd4-WT containing mmu-miR-21a binding sites was cloned into the downstream of the firefly luciferase reporter gene in pmiR-RB-REPORTTM Vector (designated as Pdcd4-WT + mmu-miR-21a) in dual luciferase assay (Fig. 4B), and Pdcd4-WT was co-transfected with miR-21a mimics. Compared with N-control, the relative luciferase activity in 293T cells after co-transfection with mmu-miR-21a mimics and Pdcd4 was decreased approximately 33.1% ($P < 0.01$). Furthermore, the luciferase activity had no effect in cells co-transfected with miR-21a mimics and Pdcd4-Mut (Fig. 4C).

Expression and regulation of hsa-miR-21 in human decidual cells

To explore the role of hsa-miR-21 in human decidual cells, we also detected the expression of hsa-miR-21 and Pdcd4 in proliferative endometrial tissues ($n = 4$) and decidua tissues ($n = 4$) in human. The results showed that expression of hsa-miR-21 in the decidua tissues was significantly increased compared with the proliferative endometrial tissues, while the Pdcd4 mRNA levels in the decidua tissues were lower than that in the proliferative endometrial tissues (Fig. 5A). Consistent with the findings from animal models, flow cytometry also revealed accelerated apoptosis in the human cell lines treated with hormones and hsa-miR-21 inhibitor *in vitro*, as shown in Figs. 5B—D.

Discussion

Decidualization is a pivotal process for embryo implantation as well as the subsequent placental formation, during which the endometrial stromal fibroblasts are differentiated into decidual cells, accompanied by morphological and biochemical transformation.[26] The extracellular matrix produced by decidualized cells can work as a physical barrier against trophoblast invasion, whereas the microenvironment established by decidua and the resident leukocytes was chemoattractive for trophoblast invasion.[27]
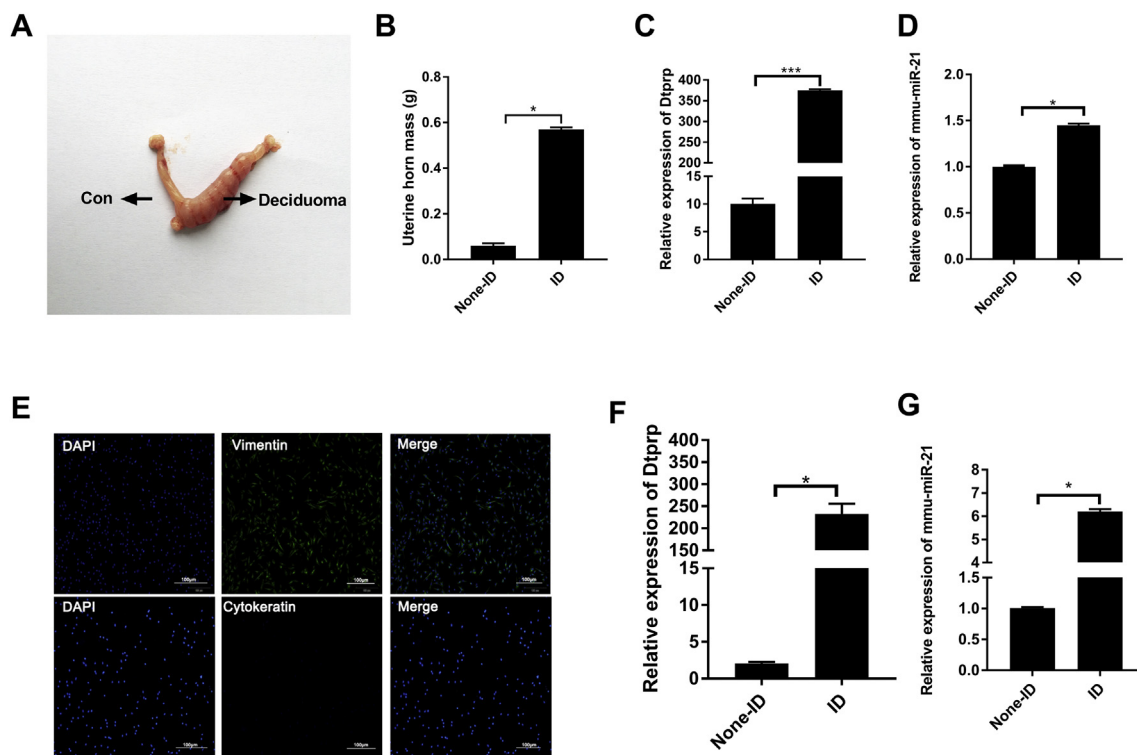
Figure 2 Expression of mmu-miR-21a in mouse endometrium after artificially induced decidualization *in vivo* and *in vitro*. **(A)** Uterus collected from a representative mouse, with oil injection into the right horn as the physical stimulus to induce decidualization and the left horn without oil injection as the internal control. **(B)** The uterine horn mass. **(C)** The relative expression level of Dtprp *in vivo*. **(D)** The relative expression levels of mmu-miR-21a *in vivo*. **(E)** Immunofluorescence analysis of Vimentin and Cytokeratin in primary endometrial stromal cells. **(F)** The relative expression level of Dtprp *in vitro*. **(G)** The relative expression level of mmu-miR-21a *in vitro*. Data represent the mean $\pm$ SD. *represents $P < 0.05$, ***represents $P < 0.01$.

Endometrium decidualization is believed to be a critical process for establishment of pregnancy. However, there are few reports on the maintenance of decidual cells function, especially the regulation of miRNA on decidual cells. In this study, we use sequencing methods to screen for miRNAs that may play an important role in decidual cells.

Among the selected miRNAs, we have found that miR-21 has been widely reported to be involved in the regulation of the endometrial function. The involvement of miR-21 in diseases such as endometriosis, leiomyoma, cervical and endometrial cancer has been identified.[28] Buscaglia and Li reported a directly regulatory effect of miR-21 on PTEN, TPM1, Bcl-2, and other oncogenes,[22] and thereafter many studies focused on the target genes of miR-21, particularly PTEN and Bcl-2.[29–31] Although it is known that these miR-NAs can target various mRNAs involved in a variety of cellular activities, including proliferation and development, differentiation, apoptosis, migration and invasion, as well as the key processes for successful pregnancy,[32] their roles and expression patterns in regulating decidual cells apoptosis remain ambiguous. Therefore, based on previous research and the results of the sequencing assays, we established *in vivo* and *in vitro* artificial decidualization models to further investigate the correlation between mmu-miR-21a and decidual cells, by which a remarkable increase in the level of mmu-miR-21a was observed in decidualization group as compared to the control group

(Fig. 2). In addition, as a response to embryonic stimuli *in vitro*, decidualization is associated with ESCs apoptosis via downregulated activation of Akt signal pathway, suggesting that ESCs apoptosis may also play a crucial role in implantation *in vivo*.[33] A recent study revealed that miR-378a-3p might be responsible for early pregnancy loss by targeting Caspase-3 and as a consequence, inducing decidual cell apoptosis.[34] According to recent publications, apoptosis of decidual cells are important events in the establishment and maintenance of decidual. To explore the regulatory mechanism of miR-21a on Pdcd4 in decidual cells, we investigated the downstream signaling molecules and mitochondrial-pathway in this study. In our study, inhibited the expression of mmu-miR-21a upregulated Pdcd4 expression resulted in an elevation of cleaved Caspase-3, Caspase-3 and BAX/BCL2 in decidual cells with inhibited miR-21 (Fig. 3D), implying that mmu-miR-21a may function by inhibition of Pdcd4. These results also indicate a possible mechanism that miR-21-mediated down-regulation of Pdcd4 could cause abnormal apoptosis and disturbing the establishment and maintenance of decidualization.

Direct evidence was also found in this study supportive of our assumption that mmu-miR-21a functions in decidual cells by targeting Pdcd4. Using target prediction software, we identified Pdcd4 as the 3′-UTR target of mmu-miR-21a. Considering the high conservation of the seed sequence of mmu-miR-21a for 3′-UTR of Pdcd4 across different
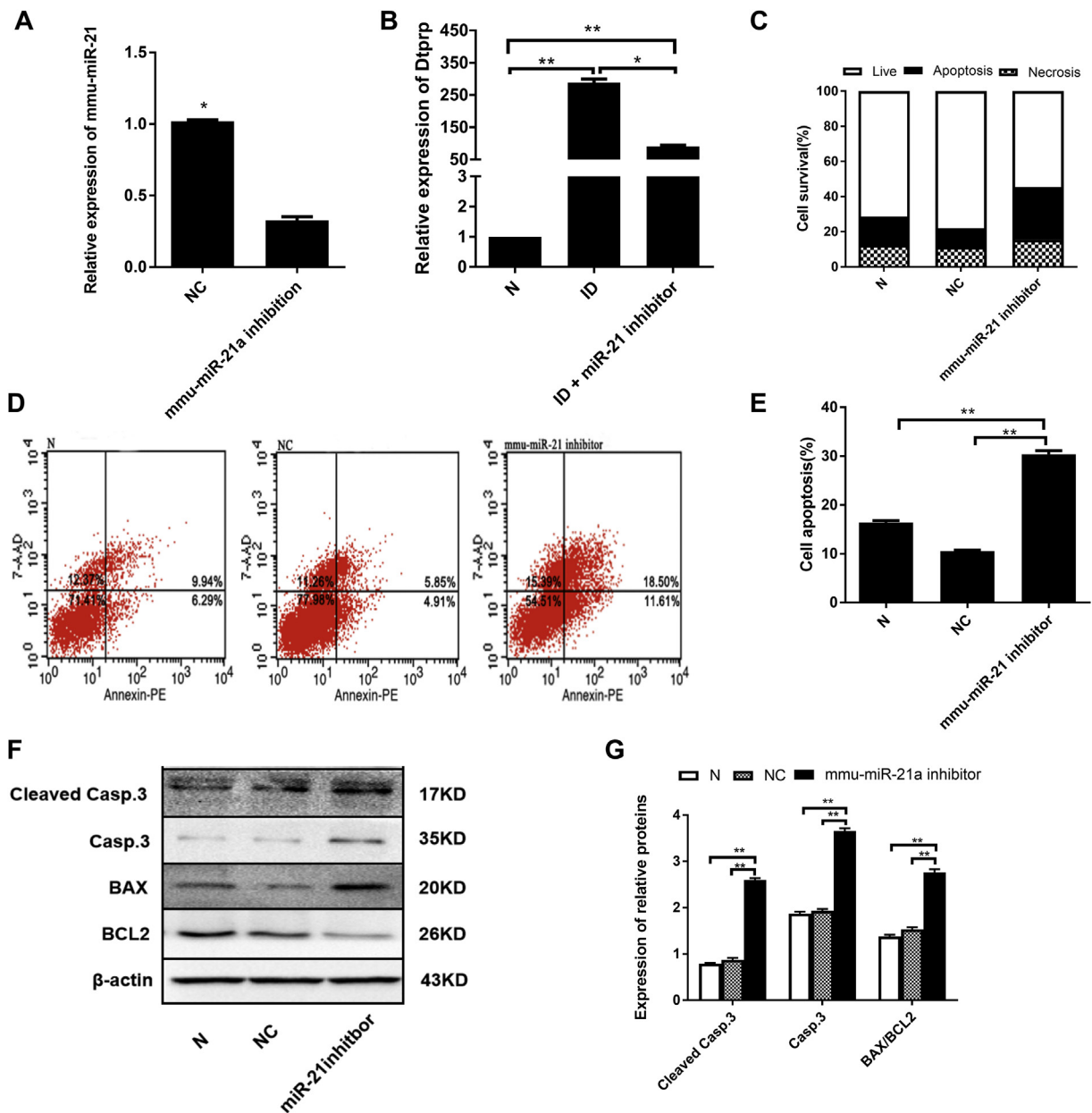
Figure 3 The effects of mmu-miR-21a on ESC decidualization. **(A)** The relative expression of mmu-miR-21a in decidualized cells after transfection with the mmu-miR-21a inhibitor. **(B)** The relative expression of Dtprp mRNA in decidualized cells after transfection with the mmu-miR-21a inhibitor. **(C)** Data on cell survival. **(D)** Flow cytometry analysis for ESC apoptosis for N, NC, and mmu-miR-21a inhibitor groups. **(E)** Statistical analysis of the cell apoptosis rate. **(F)** Western blotting analysis of cleaved Caspase-3, Caspase-3, and BAX/BCL2 proteins in ESCs after the cells were transfected with the mmu-miR-21a inhibitor. **(G)** Quantitative analysis of relative protein expression using western blotting. Data represent the mean ± SD. * represents $P < 0.05$, ** represents $P < 0.01$.

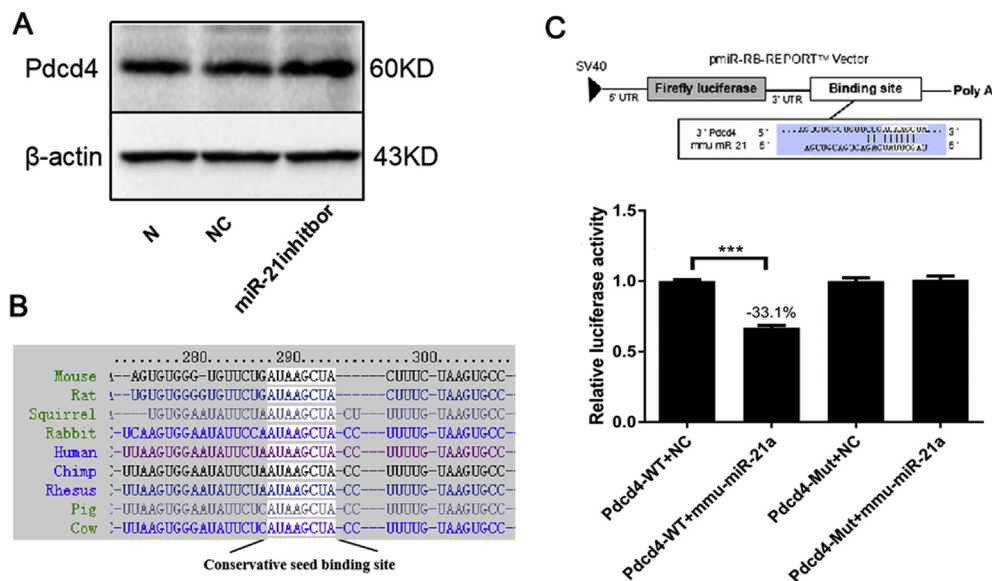| Table 2 Target Genes of mmu-miR-21a.	
Target gene name	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 289−296 of Pdcd4 3′ UTR	5′ ...AGUGUGGGUGUUCUGAUAAGCUA...
	|||||||||||
mmu-miR-21a	3′ AGUUGUAGUCAGACUAUUCGAU

Figure 4 Prediction and identification of Pdcd4 as a target gene of mmu-miR-21a. **(A)** Western blotting analysis of Pdcd4. **(B)** The 3′-UTR fragment of pdcd4-WT of mouse containing miR 21 binding sites was cloned into the downstream of firefly luciferase reporter gene in pmiR-RB-REPORTTM Vector in dual luciferase assay. **(C)** 293T cells were co-transfected with mmu-miR21 mimic and Pdcd4-WT or Pdcd4-Mut for dual luciferase assay. *** represents $P < 0.01$.

mammalian species (Fig. 4A), we conclude that Pdcd4 carrying the 3′-UTR is the target gene of mmu-miR-21a (Fig. 4A). Pdcd4 has been identified as a tumor suppressor gene and a target gene of miR-21 in a variety of malignant tumor cells.[35–38] In the present study, an evidently increased Pdcd4 expression was observed in artificially induced decidualized cells with mmu-miR-21a inhibitor, indicating the regulatory effect of miR-21 on Pdcd4
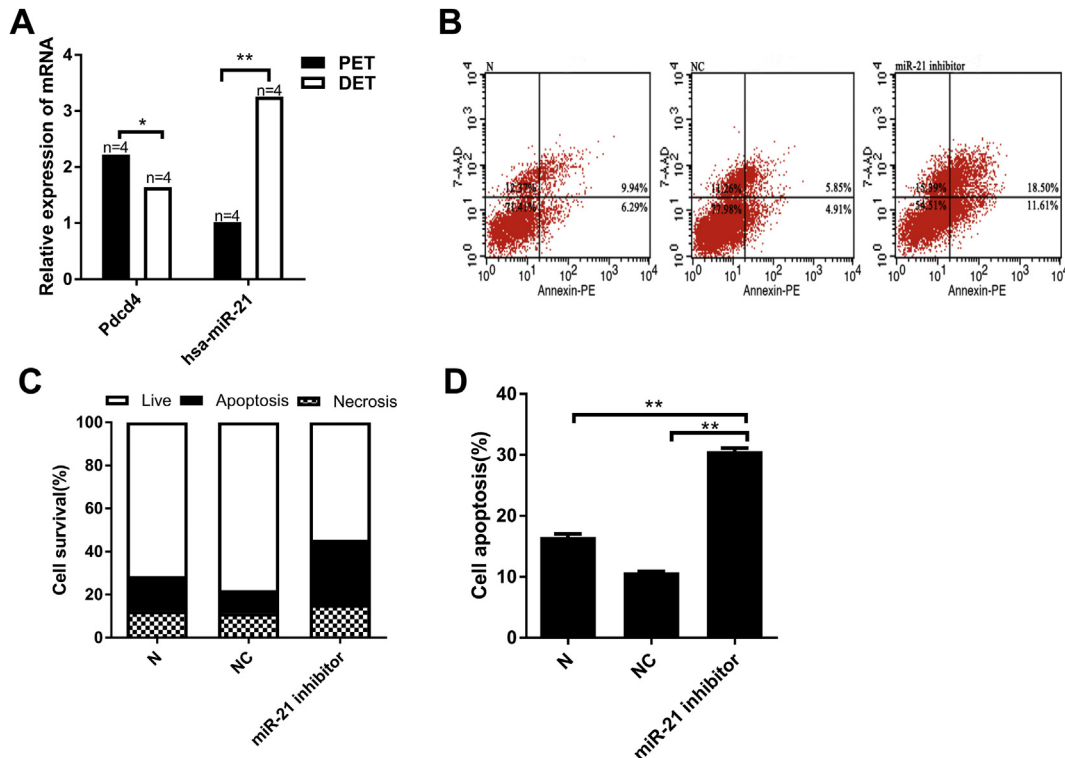

Figure 5 The expression of miRNA21 and Pdcd4 in human endometrium. **(A)** The relative expression levels of Pdcd4 and miR-21 in human endometrium. $n = 4$. **(B)** Flow cytometry analysis of apoptosis in human endometrium in N, NC, and miR-21 inhibitor groups. **(C)** Data on cell survival. **(D)** Statistical analysis of the cell apoptosis rate. PET, proliferative endometrial tissue; DET, decidualized endometrial tissue. *represents $P < 0.05$, **represents $P < 0.01$.

expression in decidual cells. Moreover, we also found that the expression of hsa-miR-21 and Pdcd4 in the proliferative endometrial tissues and decidual tissues was negatively correlated (Fig. 5A), and hsa-miR-21 is similarly involved in the apoptosis regulation of human decidual cells.

In summary, this study first revealed that mmu-miR-21a play an important role in decidual cells by inhibiting cell apoptosis via targeting Pdcd4, which is critical for mouse and human pregnancy establishment and maintenance. The data suggests a new pathogenic mechanism of deficiencies in maintenance of decidual cells function, and the specific molecular mechanism still needs to be further studied.

Conflict of Interests

The authors have no conflict of interest.

Acknowledgements

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