**RESEARCH ARTICLE**

Ssc-miR-21-5p regulates endometrial epithelial cell proliferation, apoptosis and migration via the PDCD4/AKT pathway

Renwu Hua¹,*, Xiuling Zhang¹,*, Wenchao Li¹, Weisi Lian¹, Qiaorui Liu¹, Dengying Gao¹, Yueying Wang²,‡ and Minggang Lei¹,3,4,‡

**ABSTRACT**

Endometrial receptivity plays a vital role in successful embryo implantation in pigs. MicroRNAs (miRNAs), known as regulators of gene expression, have been implicated in the regulation of embryo implantation. However, the role of miRNAs in endometrial receptivity during the pre-implantation period remains elusive. In this study, we report that the expression level of Sus scrofa (ssc)-miR-21-5p in porcine endometrium tissues was significantly increased from day 9 to day 12 of pregnancy. Knockdown of ssc-miR-21-5p inhibited proliferation and migration of endometrial epithelial cells (EECs), and induced their apoptosis. We verified that programmed cell death 4 (PDCD4) was a target gene of ssc-miR-21-5p. Inhibition of PDCD4 rescued the effect of ssc-miR-21-5p repression on EECs. Our results also revealed that knockdown of ssc-miR-21-5p impeded the phosphorylation of AKT (herein referring to AKT1) by targeting PDCD4, which further upregulated the expression of Bax, and downregulated the levels of Bcl2 and Mmp9. Furthermore, loss of function of Mus musculus (mmu)-miR-21-5p in vivo resulted in a decreased number of implanted mouse embryos. Taken together, knockdown of ssc-miR-21-5p hampers endometrial receptivity by modulating the PDCD4/AKT pathway.

**KEY WORDS:** Ssc-miR-21-5p, Embryo implantation, AKT, PDCD4, Endometrial epithelial cells

**INTRODUCTION**

The majority of prenatal losses occur during maternal recognition and implantation in pigs (Geisert and Schmitt, 2002; Velych et al., 1997). The synchronous crosstalk between conceptus and uterus has a pronounced effect on successful implantation during the pre-implantation period (Geisert and Velych, 1997; Viganò et al., 2003). On gestation days 5 to 11, the attachment sites of the embryos are determined in the uterus through embryo migration (Dhindsa et al., 1967; Waite and Day, 1967). During days 11 and 12 of pregnancy, porcine conceptuses synthesize and release a large number of estrogens that promote maternal endometrial remodeling (Bazer and Johnson, 2014; Geisert et al., 1990). The maternal endometrium undergoes morphological changes and produces various molecules for embryo implantation (Kyrizakis and Whitemore, 2005). Thus, it is vital to investigate the molecular mechanism underlying porcine endometrial changes that occur before implantation.

MicroRNAs (miRNAs) are non-coding RNAs of ~22 nucleotides in length, and play a vital role in RNA silencing and post-transcriptional regulation of gene expression (Ha and Kim, 2014). Previous studies have indicated that miRNAs exert a significant impact on embryo implantation by regulating endometrial function and embryo development (Liang et al., 2017; Reza et al., 2019). Among those miRNAs, Sus scrofa (ssc)-miR-21-5p is the most highly expressed miRNA in endometrial tissues from both Meishan and Yorkshire pigs during the pre-implantation period (Li et al., 2018b). Ssc-miR-21-5p is upregulated at the implantation site and regulates the expression of matrix metallopeptidase 9 (Mmp9) in endometrial stromal cells via targeting the Reck gene during mouse implantation (Hu et al., 2008). Furthermore, in mouse uterine extracellulare vesicles, Mus musculus (mmu)-miR-21-5p promotes embryo development and the growth of fertilized eggs (Lv et al., 2018). Despite its acknowledged importance, the biological function and regulatory mechanism of ssc-miR-21-5p in the porcine endometrium during the pre-implantation period have not been fully elucidated.

Programmed cell death 4 (PDCD4) is generally considered to be a key regulator of apoptosis (Feng et al., 2018). Protein expression of PDCD4 is not only reduced at the implantation site, but also gradually decreases in mouse endometrium from days 4 to 7 of pregnancy (Zhang et al., 2018). PDCD4 suppresses proliferation and migration of human endometrial cells (HEC-1B), and primary endometrial cells by inhibiting autophagy and the NF-kB/Mmp9 pathway (Li et al., 2018a). Intriguingly, miR-21-5p regulates apoptosis by modulating the PDCD4/AKT (herein AKT refers to AKT1) pathway in SH-SY5Y cells (Feng et al., 2018). However, little is known about the role of PDCD4 in porcine EECs.

In this study, we detected the expression profiles of ssc-miR-21-5p and PDCD4 in endometrium tissues during the pre-implantation period. Further investigation revealed that PDCD4 is a target gene of ssc-miR-21-5p, and ssc-miR-21-5p manipulates proliferation, apoptosis and migration of EECs via the PDCD4/AKT pathway. Additionally, the effect of knockdown of mmu-mir-21-5p on embryo implantation in mice was also explored. Our study provides some important clues for understanding the regulatory mechanism of ssc-miR-21-5p in terms of endometrial receptivity during the pre-implantation period.

**RESULTS**

The expression profile of ssc-miR-21-5p

Real-time quantitative PCR (RT-qPCR) was used to validate the expression levels of ssc-miR-21-5p in various tissues of pigs, and
showed that it was widely expressed in a series of tissues (Fig. 1A). In the endometrial tissues of pigs, the expression level of ssc-miR-21-5p was significantly upregulated from day (D) 9 to D12 of pregnancy (Fig. 1B). Notably, the mature miR-21-5p sequences are highly conserved in mammals, such as pigs, mice and humans (Fig. 1C).

The role of ssc-miR-21-5p in EECs

To investigate the role of ssc-miR-21-5p in EECs, an antagonomir of miR-21-5p (which acts as an inhibitor of miR-21-5p) was transfected into endometrial epithelial cells (EECs). The expression level of ssc-miR-21-5p was decreased after the transfection of antagonomiR-21-5p (Fig. 2A). The Cell Counting Kit-8 (CCK-8) results showed that knockdown of ssc-miR-21-5p significantly inhibited the proliferation of EECs (Fig. 2B). An apoptosis analysis indicated that antagonomiR-21-5p induced the apoptosis of EECs (Fig. 2C,D). A variety of cells undergo apoptosis under the influence of different factors, and are accompanied by a decline in mitochondrial membrane potential (Haebelrein, 2004). The proportion of EECs with stable mitochondrial membrane potential was determined by flow cytometry, and the ratio was decreased after transfection with antagonomiR-21-5p (Fig. 2E,F). Moreover, antagonomiR-21-5p remarkably hindered the migration of EECs (Fig. 2G,H). Altogether, knockdown of ssc-miR-21-5p inhibited the proliferation and migration of EECs, and induced their apoptosis.

Ssc-miR-21-5p directly targets the 3′-UTR of PDCD4

In order to analyze the regulatory mechanism of ssc-miR-21-5p in EECs, a target gene of ssc-miR-21-5p was found. Public websites (such as miRDB, miRBase and TargetScan) were used to identify its targets. After our screening, PDCD4 was selected for further study. The target site sequence in the 3′-UTR of PDCD4 shows high conservation in mammals, including pigs, mice and humans (Fig. 3A). We verified whether ssc-miR-21-5p targets the PDCD4 3′-UTR by constructing luciferase reporter plasmids carrying the PDCD4 3′-UTR with wild-type or base-pair mutant (mut) binding region (Fig. 3B). Luciferase activity was obviously increased when PK-15 cells were co-transfected with antagonomiR-21-5p and a wild-type luciferase reporter (PmirGLO-PDCD4-WT) (Fig. 3B). However, there was no significant difference in the luciferase activity of a mut-type PDCD4 reporter (PmirGLO-PDCD4-Mut) (Fig. 3B). These results suggested that PDCD4 was the target of ssc-miR-21-5p. Furthermore, in order to verify that ssc-miR-21-5p directly targets the 3′-UTR of PDCD4 in EECs, we inhibited ssc-miR-21-5p expression and observed an upregulation of PDCD4 expression at mRNA and protein levels (Fig. 3C,D).

The function of PDCD4 in EECs

RT-qPCR analysis showed that PDCD4 was widely expressed in various tissues of pigs (Fig. 4A). The expression level of PDCD4 in endometrial tissues during the porcine pre-implantation period was detected by immunofluorescence and RT-qPCR. The mRNA expression level of PDCD4 in the endometrium was significantly downregulated from D9 to D12 of pregnancy, which was opposite to the trend of the expression level of ssc-miR-21-5p (Fig. 4B). Immunofluorescence results showed that the protein expression level of PDCD4 was also strikingly downregulated, especially in the luminal epithelium (Fig. 4C).

To clarify the function of PDCD4 in EECs, a small interfering (si) RNA against PDCD4 (si-PDCD4) was transfected into EECs. The mRNA and protein expression levels of PDCD4 were drastically decreased after treatment (Fig. 5A; Fig. 6C,D). CCK-8 analysis indicated that si-PDCD4 significantly promoted the proliferation of EECs (Fig. 5B). The apoptosis analysis showed that suppression of PDCD4 significantly inhibited the apoptosis of EECs (Fig. 5C,D). Intriguingly, the proportion of these cells that have a stable mitochondrial membrane potential was increased after treatment with si-PDCD4 (Fig. 5E,F). Furthermore, the results demonstrated that si-PDCD4 significantly elevated the migration of EECs (Fig. 5G,H). In summary, knockdown of PDCD4 promoted the proliferation and migration of EECs, and inhibited their apoptosis.

Si-PDCD4 rescued the influence of antagonomiR-21-5p in EECs

To verify whether ssc-miR-21-5p affects the proliferation, apoptosis and migration of EECs via targeting PDCD4, si-PDCD4 was transfected to attempt to restore the effect of antagonomiR-21-5p on EECs. Compared with co-transfection with antagonomiR-21-5p and si-NC, co-transfection with antagonomiR-21-5p and si-PDCD4 significantly promoted the proliferation and migration of EECs (Fig. 6A,C). These results indicated that si-PDCD4 could alleviate the inhibitory effects of antagonomiR-21-5p.

---

**Fig. 1. The expression level of ssc-miR-21-5p.** (A) ssc-miR-21-5p was expressed in various tissues of pigs. (B) The expression level of ssc-miR-21-5p in porcine endometrium on D9 and D12 of pregnancy. (C) Protein sequence alignment showing the conservation of miR-21-5p across seven different species. Data are mean±s.d., derived from three independent experiments. *P<0.05 (Student’s t-test).
on the proliferation and migration of EECs (Fig. 6A,C). A cell apoptosis assay showed that the transfection of si-PDCD4 could reduce the proportion of apoptotic EECs caused by antagomiR-21-5p (Fig. 6B). Taken together, the above results proved that knockdown of PDCD4 could reverse the influence of antagomiR-21-5p in EECs.
The regulatory mechanism of ssc-miR-21-5p and PDCD4 in EECs

To study the regulatory mechanism of ssc-miR-21-5p and PDCD4 in EECs, we analyzed the expression of functional regulatory genes by western blotting. Knockdown of ssc-miR-21-5p inhibited the phosphorylation of AKT, which could regulate cell proliferation, cell migration and apoptosis (Fig. 7A, B). Compared with NC, silencing ssc-miR-21-5p significantly induced the level of pro-apoptotic protein Bcl-2-associated X protein (Bax), but decreased the expression of anti-apoptotic gene B-cell leukemia 2 (Bcl2) (Fig. 7A, B). Notably, antagonomiR-21-5p decreased the expression level of migration-related protein Mmp9 (Fig. 7A, B).

Knockdown of PDCD4 promoted the phosphorylation of AKT (Fig. 7C, D). Compared with si-NC, si-PDCD4 significantly decreased the expression of Bax, but increased the expression of Bcl-2 (Fig. 7C, D). Importantly, si-PDCD4 increased the protein expression level of Mmp9 (Fig. 7C, D).

Si-PDCD4 rescued the influence of antagonomiR-21-5p on the protein expression levels of phosphorylated (p)AKT/AKT (ratio of pAKT/AKT), Bax/Bcl2 (ratio of Bax/Bcl2), Mmp9 and PDCD4 (Fig. 7E, F). Altogether, knockdown of ssc-miR-21-5p impeded the phosphorylation of AKT, upregulated the expression of Bax and downregulated the levels of Bcl2 and Mmp9 by targeting PDCD4.

Knockdown of mmu-miR-21-5p results in implantation failure in mice

To further explore the role of miR-21-5p on embryo implantation in vivo, we injected antagoniR-21-5p into the uterine lumen of the mice on day 3 of pregnancy, which is known as the receptive window period (Wang et al., 2019). The number of implantation sites in the uterine horn injected with antagoniR-21-5p was...
significantly lower than in the uterine horn injected with NC on day 7 of pregnancy (Fig. 8A). As shown in Fig. 8B, the protein expression of PDCD4 was significantly higher in the uterine horn injected with antagomiR-21-5p than in the inhibitor NC-injected uterine horn on day 5 of pregnancy (implantation time).

DISCUSSION

Embryo implantation is a vital process, influencing the reproductive traits of litter weight and litter size in pigs (Fu et al., 2016, 2018; Geisert and Schmitt, 2002). EECs undergo functional changes for embryo implantation during the pre-implantation period (Bazer and Johnson, 2014; Burghardt et al., 2009). In this study, we demonstrated that the expression of ssc-miR-21-5p is upregulated in the endometrium before implantation, whereas the expression of PDCD4 is downregulated.

In vitro, knockdown of ssc-miR-21-5p inhibited the phosphorylation of AKT via targeting PDCD4, thus repressing the proliferation and migration of EECs and inducing their apoptosis. Additionally, knockdown of mmu-miR-21-5p resulted in increased expression of PDCD4 in the endometrium, leading to an increased risk of implantation failure.

Endometrial receptivity establishment, which is related to changes in gene expression, is important for the attachment of the conceptus and its firm adhesion to the luminal epithelium (Bauersachs and Wolf, 2015; Geisert and Schmitt, 2002). The main changes of gene expression were localized to the porcine luminal epithelium on D12 of pregnancy, which is the phase of initial maternal recognition of pregnancy (Zeng et al., 2018). A recent study indicated that genes involved in cell proliferation and migration were upregulated in endometrial epithelium collected from D12 pregnant gilts compared with non-pregnant cyclic gilts (Zeng et al., 2018). In this study, we found that ssc-miR-21-5p was upregulated in the endometrium from D9 to D12 of pregnancy. We also observed that the mRNA expression of PDCD4, identified as a target gene of ssc-miR-21-5p, was lower on D12 of pregnancy than on D9 of pregnancy. Therefore, we speculated that ssc-miR-21-5p may play a crucial role in endometrial receptivity by targeting PDCD4 during the pre-implantation period.
Fig. 5. The function of PDCD4 in EECs. (A) The mRNA expression level of PDCD4 after treatment with si-PDCD4. Results are PDCD4 mRNA expression relative to RPS20 expression. (B-F) CCK8 analysis after treatment with si-PDCD4 during EEC proliferation. The effects of si-PDCD4 on apoptosis (C,D) and mitochondrial membrane potential (E,F) were measured by flow cytometry. For C, annexin-V-positive and PI-negative cells were defined as early apoptotic cells, and the late apoptotic cells were defined as annexin-V- and PI-positive cells. The apoptosis rate (D) represents the proportion (%) of early and late apoptotic cells from all detected cells. In E, cells with stable MMP are in the first quadrant. The numbers in the plots for C and E are percentages for each quadrant. (G,H): After treatment with si-PDCD4, cell migration was determined using a transwell assay. In H, relative cell number represents the average cell number found. Data are mean±s.d., derived from three independent experiments. *P<0.05, **P<0.01 (Student’s t-test). Scale bar: 200 μm.
It has been found that miRNAs are associated with diverse functional states of the endometrium (Zheng et al., 2017). A previous study has indicated that the miR-181 family (miR-181a or miR-181c) might regulate embryo implantation in the porcine endometrium by targeting genes with known function in implantation, such as \textit{SPP1}, \textit{ITGB3} and \textit{ESR1} (Su et al., 2014). Highly abundant endometrial miRNAs, such as miR-21-5p, mainly impose strong effects on modulating endometrial remodeling on D12 of pregnancy (Hong et al.,...
Here, we verified that knockdown of ssc-miR-21-5p inhibited proliferation and migration but induced apoptosis of EECs. A previous study indicated that endometrial remodeling, including cell proliferation, cell migration, apoptosis and cytoskeleton organization, plays an important role in successful embryo implantation (Kaczmarek et al., 2020). Moreover, this study demonstrated that loss of function of
miR-21-5p in vivo could result in the upregulation of PDCD4, and a decreased number of implanted embryos. The above data manifest that ssc-miR-21-5p could regulate the function of EECs to affect endometrial receptivity.

MiRNAs in the endometrium influence embryo implantation by targeting genes during the pre-implantation period (Bo et al., 2015; Hu et al., 2008; Jimenez and Mendelson, 2014; Li et al., 2015a,b; Zhang et al., 2017). PDCD4 has been identified as a target gene of mir-21-5p in various cells, such as HEK293 cells (Ajuyah et al., 2019), cardiomyocytes (Xiao et al., 2016), pancreatic β cells (Ruan et al., 2011) and hematopoietic stem cells (Hu et al., 2020). In this study, PDCD4 was first predicted to be a target gene of ssc-miR-21-5p by bioinformatics analyses, and was then verified by RT-qPCR, western blotting and dual-luciferase reporter assays. The results of western blotting and RT-qPCR suggested that ssc-miR-21-5p could not only suppress mRNA translation but also affect mRNA stability. Accordingly, si-PDCD4 rescued the effect on proliferation, apoptosis and migration in EECs treated with antagomiR-21-5p. These findings suggest that ssc-miR-21-5p regulates the proliferation, apoptosis and migration of EECs by targeting PDCD4.

PDCD4 is well known as a protein translation inhibitor and tumor suppressor (Wang and Yang, 2018). PDCD4 can inhibit the helicase activity of EIF4A or directly bind to mRNA to interfere with protein translation (Biyanee et al., 2015; Yang et al., 2004). The translational targets of PDCD4 are associated with cell proliferation, migration and apoptosis (Matsushashi et al., 2019; Wang and Yang, 2018). This study further examined the effect of PDCD4 on the proliferation, migration and apoptosis of EECs. Our results showed si-PDCD4 could promote proliferation and migration, and inhibit apoptosis of EECs. A previous study indicated that uterine homeostasis during early pregnancy and the estrous cycle is principally controlled by apoptosis in the porcine endometrium (Akira et al., 2007). The in vitro findings suggest that the knockdown of PDCD4 influences the uterine receptive function.

The phosphorylation of AKT was reported to play a significant role in cell proliferation, apoptosis and migration (Jiang et al., 2019; Li et al., 2019; Liang et al., 2016; Yang et al., 2018). A previous study indicated that SPP1 can activate the AKT signaling pathway to regulate cell proliferation and the migration of the endometrial luminal epithelium during the peri-implantation period (Johnson et al., 2003). Annexin A8 promotes the proliferation of primary endometrial cells by activating the phosphorylation of AKT in pigs (Jiang et al., 2019). Moreover, the AKT signaling pathway could regulate the protein expression of Bax and Bcl2 in trophoblast cells (Li et al., 2019). It has been previously reported that the AKT signaling pathway is involved in the regulation of Mmp9 expression (Lu and Wahl, 2005). A previous study demonstrated that PDCD4 suppresses mTORC2 activation by inhibiting the translation of Sin1 to attenuate AKT activity (Wang et al., 2017). In this study, we found that knockdown of ssc-miR-21-5p could attenuate the phosphorylation of AKT, and inhibition of PDCD4 could activate the phosphorylation of AKT.

Bcl2 and Bax are cellular apoptosis susceptibility genes, and are apparently involved in the progressive deregulation of apoptosis of EECs (Zhang et al., 2017). Bcl2 family proteins regulate programmed cell death by affecting the permeability of the mitochondrial outer membrane. In this study, we found that ssc-miR-21-5p may regulate the ratio of Bax/Bcl2 and EECs with stable mitochondrial membrane potential to affect apoptosis by targeting PDCD4.

The upregulation of Mmp9, via activation of SPP1 induced by estrogen, may relate to the migration of human primary EECs (Yang et al., 2015). Silencing ssc-miR-21-5p could decrease the protein expressions of Mmp9 to inhibit cell migration (Gui et al., 2016; Hu et al., 2008). PDCD4 could suppress the migration ability of primary endometrial cells and Ishikawa cells through inhibiting the protein expression of Mmp9 (Li et al., 2018a). Consistent with these findings, we found that knockdown of ssc-miR-21-5p suppresses the level of Mmp9, whereas inhibition of PDCD4 has an opposite effect.

In summary, we revealed the expression profiles of ssc-miR-21-5p and PDCD4 in the endometrium from D9 to D12 of pregnancy. Moreover, we provided novel evidence showing that knockdown of ssc-miR-21-5p inhibited the phosphorylation of AKT by targeting PDCD4, which further affected the functions of EECs and the implantation process.

**MATERIALS AND METHODS**

**Ethics approval and consent to participate**

Animal experiments were performed as described previously by Wang et al. (2019). All experiments involving animals were carried out in accordance
with regulations (No. 5 proclamation of the Standing Committee of Hubei People’s Congress) approved by the Standing Committee of the Hubei People’s Congress in China. All animal sample collection procedures were approved by the Ethics Committee of Huazhong Agricultural University. The animals were housed at Jingpin Farm of Huazhong Agricultural University (Wuhan, China), and were slaughtered at the slaughterhouse of Huazhong Agricultural University. Before every gilt was slaughtered they were relaxed with a warm shower, stunned with a low-voltage electric shock to reduce the pain. They were then exsanguinated by puncturing the carotid artery.

**Tissue collection**

Six purebred Meishan gilts with similar weights (120±10 kg), ages (8 months) and genetic backgrounds were selected for this study. Gilts were artificially inseminated using extended semen from one boar (the breeding pig farm of Huazhong Agricultural University) at the onset of estrus (D0), and again 12 h later. Uteri were obtained from gilts slaughtered on D9 (n=3) and D12 (n=3). After slaughter, the uteri were collected and flushed using PBS (pH 7.4) to collect the embryos, and the uteri were then cautiously opened lengthwise at the inner side. The day of pregnancy was confirmed by the size and morphology of conceptuses, as follows: D9, spherical conceptuses with a diameter of 0.5–2 mm; and D12, filamentous forms of conceptuses more than 100 mm long. Samples from the endometrium of the pregnant sows were taken from three locations of each uterine horn proximal, medial, and distal. All tissue samples were frozen in liquid nitrogen and stored at −80°C until RNA was isolated.

**Cell culture**

Six additional non-pregnant gilts (D12 of estrous cycle) at a similar age (8 months) and weight (120±10 kg) were slaughtered for in vitro culture of endometrial epithelial cells. Isolation and culture of porcine primary EECs were carried out as previously described (Wang et al., 2019; Gao et al., 2018). The endometrium was separated and shredded with a sterile scissor. After washing twice with PBS, the tissue pieces were incubated at 37°C for 2.5 h with collagenase I (Gibco) and shaken vigorously every 30 min. Undigest tissue pieces were removed by screen filtration. Then, the filtrate was centrifuged at 500 g for 10 min to remove the supernatant (fraction rich with endometrial stromal cells). The pellet (epithelial-rich fraction) was resuspended twice in PBS and re-centrifuged (500 g, 10 min) twice. The resultant pellets containing EECs were suspended in Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F12; 1:1) medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco), and cultured at 37°C in a 5% CO₂ incubator. Endometrial stromal cells were further removed with 0.25% trypsin without EDTA, after 2 to 4 days. The epithelial cells (purity >95%) characterized as positive for epithelial-specific cytokeratin were then trypsinized with 0.25% trypsin-EDTA, and placed in a cell culture flask (Corning) for subsequent experiments (Fig. S1). PK-15 cells (China Center for Type Culture Collection) were cultured with growth medium (Gibco) containing 10% FBS (Gibco) at 37°C with 5% CO₂.

**RNA extraction and RT-qPCR**

RNA was extracted from cells using TRIZol (Invitrogen) following the manufacturer’s instructions, and the concentration and quality were measured using a NanoDrop 2000 (Thermo Fisher Scientific). Complementary DNA was synthesized using a reverse transcription kit (Takara). Then, Realtime PCR Master Mix (Toyobo) and specific primers for every gene (Table S1) were used to perform RT-qPCR with a real-time system (Roche). The expression levels of ssc-miR-21-5p and the genes were normalized with U6 and RPS20 to obtain the relative expression using the 2^{−ΔΔC_{T}} method, respectively.

**Transfection**

All RNA oligonucleotides were designed and synthesized by GenePharma and are shown in Table S2. EECs were transfected with 100 nM si-RNA or miRNA antagonists in six-well plates with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

**Cell proliferation assay**

A CCK-8 assay (Dojindo) was used to measure cell proliferation 0, 12, 24, 48 and 72 h after transfection, following the manufacturer’s instructions. The optical density (OD) at 450 nm of each well plate was determined using a microplate reader (Bio-Rad).

**Cell apoptosis and mitochondrial membrane potential analysis**

Cell apoptosis analysis was carried out using the Annexin V-FITC/PI apoptosis kit (Dojindo) according to the manufacturer’s protocol. Analyses were performed using a flow cytometer (Beckman). The mitochondrial membrane potential was determined using a flow cytometer, according to the protocol of the KeyGEN JC-1 Apoptosis Mitochondrial Membrane Potential Detection Kit.

**Cell migration assay**

Cell migration was assessed using a transwell assay that had 12 mm polycarbonate membranes of 8.0 μm pore size (Corning). The EECs were resuspended with serum-free medium as a single-cell solution at 4 h post transfection. Approximately 2×10^5 EECs were seeded on the upper chambers, and complete medium with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation at 37°C with 5% CO₂, cells which migrated to the lower chamber were fixed with 4% paraformaldehyde for 5 min, stained with 0.1% Crystal Violet for 5 min, rinsed third in PBS and observed using an Olympus DP80 microscope. The migrating cell numbers were obtained by counting five fields per membrane and represented the average of three independent experiments.

**Western blotting**

Proteins were extracted using RIPA lysis buffer (Servicebio). The sample was denatured by heating, separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Next, the membranes were blocked with 5% bovine serum albumin (BSA) buffer and separately probed with rabbit anti-PDCD4 (Abcam, ab79405), rabbit anti-β-actin (Servicebio, GB11001), rabbit anti-Bax (Servicebio, GB11007), rabbit anti-Bcl2 (Proteintech, 12789-1-AP), rabbit anti-AKT (Proteintech, 10176-2-AP), mouse anti-pAKT (Proteintech, 66444-1-lg) and rabbit anti-Mmp9 (N-terminal) (Proteintech, 10375-2-AP) overnight at 4°C with a final dilution of 1:1000 (v/v). After washing three times, the membranes were incubated with goat anti-rabbit IgG or anti-mouse IgG secondary antibodies (Servicebio) at a 1:2000 dilution (v/v), at 37°C for 1.5 h. The images of membranes treated with enhanced chemiluminescence were captured using a Western Blotting Detection System (Tiangen).

**Plasmid construct and dual-luciferase reporter assay**

The miR-21-5p target genes were predicted using mirBase (www.mirbase.org/), TargetScan (www.targetscan.org/) and miRDB (www.mirdb.org/). To construct reporters for luciferase assays, the fragment containing the binding sites of ssc-miR-21-5p on the 3'-UTR of PDCD4 was cloned into the Pmir-GLO Vector (Promega). The mutant of ssc-miR-21-5p binding sites on the 3'-UTR was generated using mutagenic primers (Table S1) to construct a mutant vector (Pmir-GLO-PDCD4-Mut). Antagomir-21-5p and the dual-luciferase reporter vectors (Pmir-GLO-PDCD4-WT or Pmir-GLO-PDCD4-Mut) were co-transfected into PK-15 cells. Treated cells were collected at 24 h post transfection and the luciferase activity was detected with PerkinElmer 2030 Multilabel Reader (Promega).

**Immunofluorescence assay**

Uterine tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Tissue sections were permeabilized with 0.1% Triton X-100, exposed to blocking solution (PBS/3% BSA) and incubated with anti-PDCD4 (Abcam, ab79405) overnight at 4°C. Next, the sections were incubated with FITC-labeled secondary antibodies for 40 min at room temperature. Nuclei were stained using DAPI. Fluorescent images were taken using an Olympus DP80 digital microscopy camera. All morphometric measurements were performed with three independent individuals in a blinded manner.

**Intrauterine injection of mice**

It is difficult to perform in vivo experiments in pigs, so we used a mouse implantation model based on other studies focusing on pig or human...
implantation (Jiang et al., 2019; Sun et al., 2014; Wang et al., 2019). The date of finding the vaginal suppository after mating was designated as the first day. The intrauterine injection surgery under general anesthesia was performed on the eight mice on D3 of pregnancy in the evening, according to previous studies (Sun et al., 2014; Wang et al., 2019). Inhibitor negative control (NC) and 10 μmol/l antagonimiR-21-5p (5 μl each) were injected into the right and left uterine horns, respectively. The wounds were then sutured and the mice were put under a 37°C warmer until awakening from the anesthesia. To reduce pain, temgesic was injected into the mice at 12, 24, and 48 h after surgery. On D5, the endometria of three mice were isolated to examine the protein expression level of PDCD4. On D7, five mice were killed and their uteri were isolated to record the number of implanted embryos.

**Statistical analysis**

Data were expressed as mean±s.d. derived from at least three independent experiments. A paired two-tailed Student’s t-test was used to perform statistical analysis. P<0.05 was considered as statistically significant (*P<0.05, **P<0.01).

**Acknowledgements**

We would like to thank Dr Ting Ting Kang for her critical reading of the manuscript and valuable input.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


**Funding**

This work was supported by grants from the National Basic Research Program of China (2014CB138504) and the Agriculture Research System of China (CARS-35). The funding bodies did not participate in the design of the study, sample collection, analysis, or in the writing of the manuscript.

**Supplementary information**

Supplementary information available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.248898.supplemental

**Peer review history**

The peer review history is available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.248898.reviewer-comments.pdf


