Expression and function of *Pdcd4* in mouse endometrium during early pregnancy

Yue Zhang^{1,*}, Mingyun Ni^{1,*}, Na Liu¹, Yongjiang Zhou², Xuemei Chen¹, Yubin Ding¹, Junlin He¹, Yingxiong Wang¹, Xueqing Liu¹, Yanqing Geng¹ and Liling Xie³

¹Laboratory of Reproductive Biology, School of Public Health and Management, Chongqing Medical University, Chongqing, People's Republic of China, ²Maternal, Child and Adolescent Health, School of Public Health, Hainan Medical College, Hainan, People's Republic of China and ³The First Affiliated Hospital of Chongqing Medical University, Chongqing, People's Republic of China

Correspondence should be addressed to Y Geng or L Xie; Email: aqing000@163.com or 582570124@qq.com

*(Y Zhang and M Ni contributed equally to this work)

Abstract

Embryo implantation is a complex process involving synchronised crosstalk between a receptive endometrium and functional blastocysts. Apoptosis plays an important role in this process as well as in the maintenance of pregnancy. In this study, we analysed the expression pattern of programmed cell death 4 (*Pdcd4*), a gene associated with apoptosis in the mouse endometrium, during early pregnancy and pseudopregnancy by real-time quantitative polymerase chain reaction, *in situ* hybridisation, Western blotting and immunohistochemistry. The results showed that *Pdcd4* was increased along with days of pregnancy and significantly reduced at implantation sites (IS) from day 5 of pregnancy (D5). The level of *Pdcd4* at IS was substantially lower than that at interimplantation sites (IIS) on D6 and D7. In addition, *Pdcd4* expression in the endometrium was reduced in response to artificially induced decidualisation *in vivo* and *in vitro*. Downregulation of *Pdcd4* gene expression in cultured primary stromal cells promoted decidualisation, while upregulation inhibited the decidualisation process by increasing apoptosis. These results demonstrate that *Pdcd4* is involved in stromal cell decidualisation by mediating apoptosis and therefore plays a role in embryo implantation in mice.

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Introduction

Embryo implantation is a multifactorial and complex event that results in the establishment of a successful pregnancy. During this process, a well-designed molecular dialogue between a potential embryo and the maternal endometrium is necessary to complete implantation through positioning, adhesion and invasion. These independent but inseparable events involve several specific regulatory factors, such as hormones, cytokines and the expression of corresponding receptors and co-receptors (Paria *et al.* 1993, Bowen & Burghardt 2000, Dey *et al.* 2004). When the blastocyst adheres to the endometrial epithelium, stromal cells near the blastocyst begin to proliferate and differentiate into decidual cells like 'fertile soil' to aid successful embryo implantation. The establishment and maintenance of normal decidua play vital roles in embryo implantation and development. Any errors in these processes can result in a failed pregnancy (Huet-Hudson *et al.* 1990, Bigsby & Li 1994, Das *et al.* 1997, Everett *et al.* 1997, Carson 2000).

According to a WHO comparative report, at least one in four couples suffers from primary or secondary infertility in developing countries; in developed countries, the incidence of infertility is approximately 15% and has remained stable since the turn of the twentieth century (Messinis *et al.* 2016). In failed human pregnancies, an estimated 30% of conceptions are lost following implantation in the third or fourth week of gestation (Larsen *et al.* 2013). Although a variety of assisted reproductive technologies have been developed, and medical technology has been improved, the rate of successful pregnancy has not obviously increased. Therefore, exploring the factors and mechanisms contributing to failed pregnancies is important for treating infertility and other related diseases.

Many studies have explored the roles of proliferation and differentiation during the processes of decidualisation and implantation; however, the role of apoptosis and related molecular events remains unclear. In recent years, apoptosis has attracted more and more attention by scholars. For example, the cytokine receptor

syndecan1 (SDC1) and its ligands, which are known to play a role in the apoptosis of tumour cells, are also involved in the implantation process (Boeddeker & Hess 2015). However, there are no reports on whether the programmed cell death 4 (*Pdcd4*) is related to embryo implantation.

PDCD4 is a highly conserved protein with two basic domains at the N/C-terminus and is known to inhibit carcinogenesis, tumour progression and invasion by preventing gene transcription and translation (Zhang *et al.* 2016). PDCD4 can also regulate several biological function via several factors and pathways, such as S6K1, microRNA-21 (miR-21), mTOR and pAkt (Lankat-Buttgereit & Goke 2009). In addition, PDCD4 was shown to be downregulated in several cancers, such as thyroid adenoma, colon carcinoma, oesophageal cancer and ovarian cancer (Avi Ashkenazi 1998, Green & Reed 1998, Mudduluru *et al.* 2007, Ding *et al.* 2016). In hydatidiform mole tissues and choriocarcinoma cells, PDCD4 was negatively regulated by overexpression of miR-21 and promoted proliferation, migration and invasion (Wang *et al.* 2017).

This study aimed to explore the expression pattern and function of the *Pdcd4* gene in mouse endometrium during early pregnancy and pseudopregnancy. Our findings also clarified the role of *Pdcd4* in decidualisation and helped elucidate the molecular mechanisms involved in implantation.

Materials and methods

Ethical approval and reagents

Mice (*Mus musculus*) were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China (Certificate: SICXK (YU) 2007-0001)). All animals/human procedures were approved by the Ethical Committee of Chongqing Medical University, and informed consent was obtained from all human subjects.

Major reagents: All primers were synthesised by Songon Biotech Co., Ltd. (Shanghai, China). RNAiso Plus Reagent (D9108B), SYBR Green and a reverse transcription kit (DRR047A) were purchased from TaKaRa. The oligonucleotide probe for the *Pdcd4* gene labelled with digoxigenin was designed and synthesised by Dingguo BioTechnology Co., Ltd. (Beijing, China), and the oligonucleotide sequence was as follows: 5'-GACACTGAATGTGAACCCCACTGACCCT-3'. The *in situ* hybridisation kit was purchased from the same company. The anti-PDCD4 (D29C6), anti- β -actin (#3700), anti-cleaved caspase3 (#9664) and anti-caspase3 (#9662) antibodies used in Western blotting (WB) were purchased from Cell Signaling Technology. The anti-BAX (ab32503) and anti-BCL2 (ab32124) antibodies were purchased from Abcam. The anti-PDCD4 (ab79405) antibody used in immunohistochemistry (IHC) was also purchased from Abcam. A diaminobenzidine kit (DAB ZL-9018) was used.

Animals and tissue collection

Adult virgin female and male Kunming mice (20–25 g, 8 weeks old) were housed in a specific pathogen-free animal room under a controlled photoperiod (12 h light/12 h darkness) at $22 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ relative humidity. All mice had access to water and food *ad libitum*. Female mice were mated with the fertile males or vasectomised males at a ratio of 3:1 to induce pregnancy and pseudopregnancy, and the day of appearance of a vaginal plug was considered day 1 of pregnancy (D1) or day 1 of pseudopregnancy (PD1). The pregnant and pseudopregnant mice were randomly assigned to several groups (D1, D4–D7 and PD1–PD7), with 8 mice per group. The implantation sites (IS) on D5 tissues were identified by intravenous injection of trypan blue through the tail vein. The mice were killed between 08:00 and 09:00 h, and part of the endometrium was collected and stored in liquid nitrogen for real-time quantitative polymerase chain reaction (RT-qPCR), WB and *in situ* hybridisation (ISH); the remaining sample was fixed in 4% paraformaldehyde for immunohistochemistry (IHC) (Ding *et al.* 2012).

An established procedure for artificially inducing decidualisation (ID) *in vivo* was adopted from a previous publication (Long *et al.* 2015). Briefly, one uterine horn from each PD4 mouse was injected with 10 μL corn oil, while the other uterine horn did not receive any treatment and served as a negative control (Non-ID). The mice were killed by cervical dislocation four days later. After the wet weight of the uterine tissues was measured, part of the uterine tissue was stored in liquid nitrogen for RT-qPCR and WB, and the rest was fixed in 4% paraformaldehyde for IHC.

All human samples were obtained from pregnant women visiting the clinic at the Department of Gynecology in the First Affiliated Hospital of Chongqing Medical University from August to October 2014 ($n=16$). The samples were obtained from the control group (the patients underwent induced abortion surgery without any signs of pregnancy disorders, $n=8$), threatened-abortion patients ($n=5$) and missed-abortion patients ($n=3$) by surgical operation (at 6–9 weeks of pregnancy). There were no significant differences in age, weight and the gestational weeks among the participants. (The inclusion criteria used to select human participants for this study were as follows: (1) Positive pregnancy test; (2) Regular menstrual cycle; (3) No history of genetic disease; (4) No sexually transmitted disease; (5) Non-immunodeficient; (6) No endocrine disease; (7) No chromosomal abnormalities; (8) Normal uterine anatomy (when examined by ultrasound); (9) Embryo sizes consistent with gestational age.)

Real-time quantitative polymerase chain reaction

Total RNA was extracted from endometrial tissues and cells using RNAiso Plus Reagent according to the manufacturer's protocol. The cDNA was synthesised from an appropriate quantity of total RNA treated with DNase I in a 10 μL reaction system using an RT-qPCR kit. β -actin, a housekeeping gene, was used to normalise the data (Kang *et al.* 2002, Ozpolat *et al.* 2007). The specific primers used for the *Pdcd4*, *Dtprp* (decidual/trophoblast prolactin-related protein, a marker for

Table 1 Primer sequences for qPCR.

Genes		Sequence of primers 5'–3'
<i>Pdcd4</i>	Forward	ACTGACCCTGACAATTTAAGCG
	Reverse	TTTTCGCGAGTCGTCTTTTGG
<i>Dtprp</i>	Forward	AGCCAGAAATCACTGCCACT
	Reverse	TGATCCATGCACCCATAAAA
β -actin	Forward	TGGAATCCTGTGGCATCCATGAAAC
	Reverse	TAAAACGCAGCTCAGTAACAGTCCG

in vitro decidualisation) (Liang *et al.* 2010) and β -actin genes are shown in Table 1. Transcripts were quantified using SYBR Green, and the PCR analyses were performed on a Bio-Rad CFX Manager 3.1 Detection System (USA). The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels of *Pdcd4* in the endometrium on different days of pregnancy for different model systems.

Western blotting

Proteins were extracted from the endometrial tissues and cells by using lysis buffer. Protein concentrations were determined with a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocol. Samples were boiled in 5× sodium dodecyl sulphate (SDS) sample loading buffer for 10 min and loaded onto a 10% SDS-PAGE gel. Samples were electrophoresed for 100 min and were then transferred onto PVDF membranes. Membranes were blocked for 1 h at room temperature in PBST containing 5% skim milk or BSA powder. Membranes were incubated with the following antibodies overnight at 4°C: anti-PDCD4 (1:1000 dilution), anti-Cleaved Caspase3 (1:1000 dilution), anti-Caspase3 (1:1000 dilution), anti-BAX (1:1000 dilution), anti-BCL2 (1:500 dilution) and anti- β -actin (1:2000 dilution) and washed three times (for 15 min each) with PBST. The washing processes were repeated after incubation for 1 h with the goat anti-rabbit IgG or goat anti-mouse IgG. The protein expression was detected by ECL Plus reagent according to the manufacturer's protocol and analysed using Quantity One 4.6 software.

In situ hybridisation

According to a previous report (Ni *et al.* 2002), uteri were cut into pieces and flash frozen in liquid nitrogen. Frozen sections (10 μ m) were mounted continuously and fixed in 4% paraformaldehyde solution in PBS for 1 h. After three washes with PBS, the tissues were treated with Triton (1%) for 20 min. After three washes with PBS, the sections were prehybridised in a hybridisation buffer at 37°C for 2–3 h and then hybridised in hybridisation buffer with DIG-labelled antisense or sense RNA probe (1–5 mg/mL) at 55°C for 16 h. After hybridisation, the sections were washed in 5× SSC, 2× SSC, 0.5× SSC and 0.2× SSC twice for 10 min each. Nonspecific binding in the sections was blocked with 0.5% block mix, and then, the sections were incubated with goat anti-DIG antibody. The final signal was visualised with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The sections were counterstained with methyl green. The sections treated with the DIG-labelled sense probe were negative. The images were captured by using an Olympus microscope (BX40, Olympus).

Immunohistochemistry

Tissue samples were embedded in paraffin according to standard histological procedures and sectioned to obtain continuous sections of 4 μ m thickness. The sections were deparaffinised in xylene and rehydrated in descending concentrations of ethanol after antigen retrieval in sodium citrate buffer and cooled down to room temperature. The tissue sections were blocked in 10% normal goat serum for 30 min and incubated with anti-PDCD4 antibody (1:300 dilution) at 4°C overnight. Then, the tissue sections were incubated with a secondary antibody (goat anti-rabbit IgG) at 37°C for 30 min. Next, the sections were incubated with streptavidin-conjugated horseradish peroxidase at 37°C for 30 min. The staining was performed by using DAB substrate for 5 min at room temperature and terminated by rinsing with water. The sections were subsequently stained with haematoxylin. The images were captured by using an Olympus microscope (BX40, Olympus).

Isolation of endometrial stromal cells (ESCs) and *in vitro* decidualisation

Mouse uterine stromal cells were isolated as previously described (Osteen *et al.* 1989). The uteri obtained from PD4 mice were washed thoroughly with D-Hanks solution. In addition, they were cut into small fragments with sterile scissors. The uterine tissues were placed in an appropriate amount of HBSS containing 1% wt/vol trypsin and 6 mg/mL dispase at 4°C for 2 h, and then, they were incubated at 37°C for 30 min. The digested uteri were gently agitated by pipetting to dislodge sheets of luminal epithelial cells. After centrifugation, the supernatant containing sheets of epithelial cells was discarded. The remaining tissues were rinsed twice with HBSS and digested in an appropriate amount of HBSS containing 0.15 mg/mL collagenase I (Invitrogen) at 37°C for 30 min. The mixture was vigorously shaken every 10 min until the supernatant became turbid with dispersed stromal cells. The stromal cells were filtered through a 70 μ m nylon filter. Then, they were centrifuged, and the supernatant was removed by using HBSS to wash stromal cells twice. The cells were resuspended in a complete medium consisting of Dulbecco's modified Eagle's medium-nutrient mixture F-12 Ham (DMEM-F12, Sigma) with 20% charcoal-stripped foetal bovine serum.

Cells were plated into 6-well cell culture plates at a density of 2×10^5 cells per plate. Fresh culture medium containing 10 nmol/L oestradiol-17 β (E_2) and 1 μ mol/L progesterone (P_4) were added in each well to artificially induce decidualisation.

Digital gene expression profiling

Ovariectomised mice were treated with subcutaneous injections of corn oil (0.1 mL/mouse, control), E_2 (100 ng/mouse) and P_4 (2 mg/mouse) to establish the steroid hormone processing model. The mice were killed, and the uteri were collected at 24 h after each treatment. The tissues were sent to the Beijing Genomics Institute (BGI) and Digital gene expression profiling was performed by BGI Technology.

The inhibition and overexpression of *Pdcd4* in ESCs (cell transfection)

Seed cells were 70–90% confluent under normal growth conditions (typically 37°C and 5% CO₂) before transfection. The siRNA specifically targeting mouse *Pdcd4*, (siRNA-1496/843/1096, 50 nmol/L, GenePharma, China), an overexpression vector of *Pdcd4*, pCMV6-*Pdcd4* and pCMV6-vector (515 ng/μL and 603 ng/μL, Origene, Rockville, MD, USA) were mixed with Lipofectamine 3000 Reagent in appropriate proportions. The mixtures were incubated at room temperature for 5–10 min to allow complex formation. The DNA/siRNA–lipid complexes were added dropwise onto cells. The plates were then gently swirled to uniformly distribute the transfection complexes among all cells. The cells were incubated at 37°C and 5% CO₂ and kept under observation. If cytotoxicity was observed after 6–8 h, the complexes were removed, and fresh culture medium was added to the cells.

To investigate whether the down/upregulation of PDCD4 affects the establishment or maintenance of decidua function, we established several groups as follows: the ESCs were treated with siRNA-1096/pCMV6-*Pdcd4* and then treated with E₂ and P₄ to analyse the effects of down/upregulation of PDCD4 on the establishment of decidualisation by measuring *Dtprp* mRNA (siRNA-1096/pCMV6-*Pdcd4*+ID, ID followed by transfection). Similarly, decidualisation was induced in ESCs, and the cells were treated with siRNA-1096/pCMV6-*Pdcd4* to explore the effects on the maintenance of decidua (ID+siRNA-1096/pCMV6-*Pdcd4*, transfection followed by ID). Cells without any treatment were depicted as Con.

Flow cytometry for apoptosis analysis

ESCs transfected with pCMV6-*Pdcd4* and the negative control were harvested and incubated with Annexin V and PI at room temperature for 15 min in the dark and then quantitatively analysed using a FACS Vantage SE flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (Liu *et al.* 2013).

Statistical analysis

Each experiment was repeated at least 3 times in each group. The collected data were analysed using SPSS, ver. 16.0 software (SPSS). Student's *t*-tests were performed to compare data in experiments having only two groups, while the data from experiments with multiple groups were analysed by one-way ANOVA (Students–Newman–Keuls). *P* < 0.05 was considered significant.

Results

Expression of *Pdcd4* in mouse endometrium during early pregnancy

To investigate the role of *Pdcd4* in mouse endometrium, we examined the *Pdcd4* mRNA and protein levels in the endometrium by qPCR and WB. The results indicated that the *Pdcd4* mRNA and protein levels were increased as the days of pregnancy increased and were reduced significantly at IS from D5. Furthermore, the *Pdcd4*

mRNA and protein levels at IS were substantially lower than those at interimplantation sites (IIS) on D6 and D7 (Fig. 1A, B and C). ISH was performed to detect the localisation of *Pdcd4* mRNA in the endometrium, and the results indicated that *Pdcd4* was primarily located in the cytoplasm of luminal epithelium (LE) and glandular epithelium (GE) on D1, while positive signals were observed in the cytoplasm of ESCs from D4 (Fig. 1D). The expression pattern of PDCD4 protein detected by IHC was similar to that of the mRNA, but the protein was located in both the nuclei and cytoplasm (Fig. 1E).

Expression of *Pdcd4* in mouse endometrium during pseudopregnancy

To explore whether the changes of *Pdcd4* expression in mouse endometrium were induced by a blastocyst signal, we examined *Pdcd4* levels in the endometrium of pseudopregnant mice. There were no significant differences in *Pdcd4* gene expression found among PD1–PD7 (Fig. 2A, B and C). *Pdcd4* was predominantly observed in the LE and GE (Fig. 2D). These results indicated that the differential expression of *Pdcd4* in mouse endometrium was triggered by a blastocyst signal.

Expression of *Pdcd4* in mouse endometrium after artificially induced decidualisation *in vivo* and *in vitro*

To further explore the association between *Pdcd4* and decidualisation of the endometrium, we established artificially induced decidualisation models *in vivo* and *in vitro*. As shown in Fig. 3A and B, uterine swelling occurred after injection of corn oil, and the mass increased approximately 20 times. Both *in vivo* and *in vitro*, the expression levels of *Pdcd4* were significantly decreased in response to artificially induced decidualisation (Fig. 3C, D, E and F). In addition, we also investigated the effects of oestrogen and progesterone on PDCD4 using digital gene expression profiling since they are key regulators during pregnancy. The results showed that oestrogen had an effect on PDCD4 (Table 2).

Downregulation of *Pdcd4* promotes decidualisation of mouse ESCs

The ESCs of PD4 mice were used to confirm the effect of *Pdcd4* on endometrial decidualisation. Figure 4A shows the identification of ESCs by immunofluorescence assays. The ESCs were transfected with siRNA and harvested 48 h later to detect the *Pdcd4* mRNA and protein levels. The siRNA segment that had the maximum silencing effect (siRNA-1096) was chosen from the three segments (siRNA-1496, 843, 1096) for the next experiment (Fig. 4B, C and E). The Cleaved Caspase3, Caspase3 and BAX/BCL2 proteins were detected by WB. Cell apoptosis was inhibited after downregulation of *Pdcd4* (Fig. 4D and E). Figure 4F shows that the *Dtprp* levels

Figure 1 Expression of *Pdcd4* in mouse endometrium during early pregnancy. (A) The relative mRNA expression of *Pdcd4* in mouse endometrium. (B) Western blotting analysis of PDCD4 protein expression by Western blotting. (C) Quantitation of PDCD4 protein expression by Western blotting. (D) The localisation of *Pdcd4* mRNA was determined using ISH; the yellowish-brown stain indicates a positive signal. (E) The localisation of PDCD4 protein was determined by IHC; the yellowish-brown stain indicates a positive signal. DC, decidualised cells; EM, embryo; ESC, endometrial stromal cells; GE, glandular epithelium; IS, implantation sites; IIS, interimplantation sites; LE, luminal epithelium; # indicates compared with D5IS; * and ** indicate $P < 0.05$; ** indicates $P < 0.01$.

of the ID group were significantly higher than those in the control group, which indicated that the artificially induced decidualisation model *in vitro* was established successfully. Simultaneous comparison showed that the *Dtprp* mRNA level of the ID+siRNA-1096 (transfection followed by ID) group was significantly higher than

that of the ID group. However, there was no significant difference between the siRNA-1096+ID (ID followed by transfection) group and the ID group. These data indicated that downregulation of *Pdcd4* expression promotes the maintenance of the decidualisation process by inhibiting apoptosis.

Figure 2 Expression of *Pdcd4* in mouse endometrium during early pseudopregnancy. (A) The relative mRNA expression of *Pdcd4* in mouse endometrium. (B) Western blotting analysis of PDCD4 protein in mouse endometrium. (C) Quantitation of PDCD4 protein expression by Western blotting. (D) The localisation of PDCD4 protein was determined by IHC; the yellowish-brown stain indicates a positive signal. ESC, endometrial stromal cells; GE, glandular epithelium; LE, luminal epithelium.

Figure 3 Expression of *Pcdcd4* in mouse endometrium during artificially induced decidualisation *in vivo* and *in vitro*. (A) Uterus from one representative mouse. The image shows the horn that received a physical stimulus (oil injection) to induce deciduoma formation on the left and the right as the internal control, which did not receive an oil injection. (B) The masses of the deciduoma and control uterine horns. (C) The level of *Pcdcd4* mRNA in mouse endometrium. (D) Western blotting analysis of PDCD4 protein in mouse endometrium. (E) Quantitation of PDCD4 protein expression by Western blotting. (F) The localisation of PDCD4 protein was determined by IHC; the yellowish-brown stain indicates a positive signal. DC, decidualised cells; ESC, endometrial stromal cells; GE, glandular epithelium; LE, luminal epithelium. *Indicates $P < 0.05$, **indicates $P < 0.01$.

PDCD4 expression in human endometrium

The PDCD4 protein levels in different types of human endometrial samples during spontaneous abortion (control group, threatened-abortion group, missed-abortion group) were detected. We found that the PDCD4 protein levels in the endometrium of the threatened-abortion and missed-abortion groups were substantially higher than those in the control group (Fig. 5A and B).

Overexpression of *Pcdcd4* inhibits decidualisation of ESCs from mouse endometrium

Since PDCD4 protein levels in human endometrial samples from the threatened-abortion and missed-abortion groups were significantly higher than those in endometrial samples from the control group, we investigated the effects of *Pcdcd4* overexpression in mouse ESCs. Figure 6A, B and C reveal that the ESCs transfected with pCMV6-*Pcdcd4* had a significant increase in *Pcdcd4* mRNA and PDCD4 protein levels compared to those in the control and negative control (pCMV6-vector) groups. The results of our flow cytometric experiments coupled with the upregulated levels of Cleaved Caspase3, Caspase3 and BAX/BCL2 proteins indicated that *Pcdcd4* overexpression increases apoptosis in ESCs (Fig. 6B, C, D, E and F). Furthermore, the results of our experiments involving artificially induced decidualisation indicated

that overexpression of the *Pcdcd4* gene could inhibit the establishment of decidualisation (pCMV6-*Pcdcd4*+ID, ID followed by transfection). For the maintenance of the decidua (ID+pCMV6-*Pcdcd4*, transfection followed by ID), a decrease in the expression levels of *Dtprp* was observed, but this trend was not statistically significant (Fig. 6G and H).

Discussion

Successful embryo implantation is a fundamental step in the overall process of pregnancy. For implantation, not only does the conceived embryo need to become an active blastocyst, but this step must also synchronise with endometrial receptivity. The decidual cells are special tissues around the implantation site, which is composed of a variety of cellular components, and the establishment of decidualisation is regulated by hormones as well as the expression of various genes. This process plays a critical role in the maintenance of the early pregnancy (Pijnenborg 2002, Wang & Dey 2006, Ramathal et al. 2010, Zhang et al. 2013, 2015).

Table 2 The results of digital gene expression profiling.

Genes	Control	Oestrogen	Progesterone
<i>Pcdcd4</i>	331	91**	301

**Indicates compared with control and $P < 0.01$.

Figure 4 Effect of downregulated *Pdcd4* on decidualisation of stromal cells. (A) Immunofluorescence analysis of VIMENTIN protein in ESCs. Green fluorescence indicates a positive signal. Scale bar=200 μ m. (B) The relative mRNA expression of *Pdcd4* in ESCs after transfection with siRNA; siRNA-1096 showed the maximum silencing effect and was chosen for the next experiment. (C) Western blotting analysis of PDCD4 protein in ESCs after transfection with siRNA; siRNA-1096 showed the maximum silencing effect and was chosen for the next experiment. (D) Western blotting analysis of Cleaved Caspase3, Caspase3, BAX and BCL2 proteins in ESCs after transfection with siRNA-1096. (E) Quantitation of relative protein expression by Western blotting. (F) The relative mRNA expression of *Dtprip* in different treatment groups and Con. *Indicates $P < 0.05$, **indicates $P < 0.01$.

During the early pregnancy of mice, D4-D7 of pregnancy was the key period of the embryo implantation (Wang & Dey 2006, Li *et al.* 2007, Ramathal *et al.* 2010). The attachment reaction occurs on D4, when the uterus becomes receptive to implantation. Then, epithelial cells around the blastocyst undergo apoptosis, which causes the epithelium around that area to disintegrate to allow blastocyst invasion and implantation. While this occurs, the stromal cells near the blastocyst begin to proliferate and differentiate into decidual cells (Carson 2000, Boeddeker & Hess 2015). We found that the *Pdcd4* levels increased up to D4 to allow epithelial disintegration, which is necessary for embryo implantation. In addition, we found that the *Pdcd4* mRNA and protein levels in the endometrium of IS on D5 were significantly higher than those at IS on D6 and D7 and that *Pdcd4* gene expression was primarily observed in the decidual area. These results indicate that after embryo implantation, as the establishment and development of the decidua progresses, *Pdcd4* expression is reduced to allow continued maintenance of

decidua. These findings are consistent with the results of our *in vivo* and *in vitro* experiments involving artificially induced decidualisation before or after transfection with siRNA-1096. Furthermore, downregulation of *Pdcd4* expression had little effect on the proliferation and differentiation of stromal cells but was important

Figure 5 The expression of PDCD4 protein in human endometrium during spontaneous abortion (Con, control group; T, threatened-abortion; M, missed-abortion). (A) Western blotting analysis of PDCD4 protein in the Con, T, and M groups. (B) Quantitation of PDCD4 protein expression by Western blotting.

Figure 6 The effect of *Pdc4* overexpression on decidualisation of stromal cells. (A) The relative level of *Pdc4* mRNA in ESCs after transfection with the overexpression vector of *Pdc4*. (B) Western blotting analysis of PDCD4, Cleaved Caspase3, Caspase3, BAX and BCL2 proteins in ESCs after transfection with the overexpression vector of *Pdc4*. (C) Quantitation of relative protein expression by Western blotting. (D) The results of flow cytometry for apoptosis analysis of ESCs among the Con, pCMV6-vector and pCMV6-*Pdc4* groups. (E) The data for cell survival presented in D. (F) The statistical analysis of the cell apoptosis rate presented in D. (G) The relative mRNA expression of *Dtpm* after treatment with pCMV6-vector/*Pdc4*+ID. (H) The relative mRNA expression of *Dtpm* after treatment with ID+pCMV6-vector/*Pdc4*. *Indicates $P < 0.05$, **indicates $P < 0.01$.

for the apoptosis of decidua. To investigate whether changes in *Pdc4* gene expression were induced by embryo signals rather than by hormonal regulation, we conducted experiments on pseudopregnant mice. The results clearly verified our hypothesis.

The normal low expression of *Pdc4* has favourable effects on decidualisation; however, what about abnormal expression? We observed that the protein levels of PDCD4 in the threatened-abortion and missed-abortion groups were notably increased. Hence, we hypothesised that the high levels of PDCD4 could be a contributing factor to the occurrence of abortion. As PDCD4 protein increased in the overexpression model, apoptosis was enhanced accordingly. Meanwhile, *Pdc4* overexpression had adverse effects on the establishment of decidualisation. However, for the maintenance of decidua function, we observed a downward trend of *Dtpm* mRNA level, but this result was not significant. We attributed this to the survival time of stromal cells, which is only 6–7 days *in vitro*. After artificially induced decidualisation, the activity and function of stromal cells were weakened, and it was difficult to achieve an effective transfection. These present results further illustrated the detrimental effects of high levels of PDCD4 on decidualisation and hence on pregnancy.

Data from recent publications, as well as our results from this study, indicate that apoptosis plays a major role in the female reproductive tract. Apoptosis of

ESCs can be initiated via an intrinsic or mitochondria-associated pathway. The aggregation of cytochrome C activates Caspase9 and consequently Caspase3 to induce apoptosis (Joswig *et al.* 2003). Our results demonstrated that overexpression of the *Pdc4* gene increases apoptosis in ESCs via the mitochondria-associated pathway. Several studies reported that non-differentiated cells are resistant to apoptosis – even after exposure to death receptors – because these cells have the potential to proliferate and differentiate. Accordingly, after artificially induced decidualisation, differentiated ESCs showed high sensitivity to Caspase3 activation (Boeddeker & Hess 2015). This phenomenon probably occurs because the decidual cells surrounding the embryo have higher expression of pro-apoptotic proteins than the adjacent stromal cells, which express anti-apoptotic BCL2 proteins instead. Collectively, neither a premature nor delayed end of decidualisation can result in a normal pregnancy. Although proliferation and differentiation of stromal cells are very important for the formation of the decidua, timely apoptosis is equally important for this process (Mikhailov 2003).

In summary, appropriate expression of the *Pdc4* gene in mouse endometrium plays an important role in the establishment and maintenance of decidua function. Thus, we hypothesised that *Pdc4* gene expression affects embryo implantation. Abnormal expression of the *Pdc4* gene in the endometrium is

likely to be detrimental to pregnancy. These results could help develop novel strategies or new therapeutic targets for treating disorders of the reproductive system, including infertility.

Declaration of interest

The authors report that no conflict of interest exists to prejudice the impartiality of the research reported.

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