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# Endometrial pyruvate kinase M2 is essential for decidualization during early pregnancy

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## Abstract

Embryo implantation is essential for normal pregnancy. Decidualization is known to facilitate embryo implantation and maintain pregnancy. Uterine stromal cells undergo transformation into decidual cells after embryo attachment to the endometrium. Pyruvate kinase M2 (PKM2) is a rate limiting enzyme in the glycolysis process which catalyzes phosphoenolpyruvic acid into pyruvate. However, little is known regarding the role of PKM2 during endometrial decidualization. In this study, PKM2 was found to be mainly located in the uterine glandular epithelium and luminal epithelium on day 1 and day 4 of pregnancy and strongly expressed in the decidual zone after embryo implantation. PKM2 was dramatically increased with the onset of decidualization. Upon further exploration, PKM2 was found to be more highly expressed at the implantation sites than at the inter-implantation sites on days 5 to 7 of pregnancy. PKM2 expression was also significantly increased after artificial decidualization both *in vivo* and *in vitro*. After PKM2 expression was knocked down by siRNA, the number of embryo implantation sites in mice on day 7 of pregnancy was significantly reduced, and the decidualization markers BMP2 and Hoxa10 were also obviously downregulated *in vivo* and *in vitro*. Downregulated PKM2 could also compromise cell proliferation in primary endometrial stromal cells and in Ishikawa cells. The migration rate of Ishikawa cells was also obviously suppressed by si-PKM2 according to the wound healing assay. In conclusion, PKM2 might play an important role in decidualization during early pregnancy, and cell proliferation might be one pathway for PKM2 regulated decidualization.

**Key Words**

- pyruvate kinase M2
- decidualization
- embryo implantation
- proliferation

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## Introduction

In mammals, the reproductive process represents a complex and highly coordinated biological phenomenon, which involves embryo implantation, decidualization and placental development (Cakmak & Taylor 2011, Hemberger *et al.* 2019). It is a synchronized molecular dialogue between embryo and maternal endometrium, which is conducted via cytokines as well as by the expression of corresponding receptors (Hess *et al.* 2007, Boeddeker *et al.* 2015). During the early stage of pregnancy, embryo implantation and endometrial decidualization are considered as two of the most crucial steps which have a determinant impact on successful

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pregnancy (Patterson *et al.* 2017). After embryo implantation, stromal cells around the implantation site experience proliferation and differentiation to form decidual cells, known as decidualization (Gao *et al.* 2015). The process of endometrial decidualization is accompanied by a source of physiological and morphological changes including secretory transformation of uterine glands, influx of specialized uterine natural killer cells and vascular remodeling (Gellersen *et al.* 2007). Any aberration in decidualization is considered to be responsible for placental distortion and adverse pregnancy outcomes (Garrido-Gomez *et al.* 2017). The blastocyst enters the uterine cavity and invades the uterus by positioning and adhering, which needs the cooperation of hormones, cytokines, metabolism, adhesion molecules, immune system amongst others (Carson *et al.* 2000). However, the underlying mechanisms of embryo implantation remain unclear.

It is well known that cells are mainly metabolized by the mitochondrial uptake of pyruvate and oxygen into the tricarboxylic acid cycle, producing ATP to support cell growth and development (Nath & Villadsen 2015). However, in certain pathophysiological states, cells utilize glycolysis for the production of ATP, known as the Warburg effect (Sancho *et al.* 2016). As early as the 1920s, German biologist Otto Warburg found that, in a normal oxygen environment, tumor cells ingest large amounts of glucose in order to cope with their rapid proliferation and differentiation. In the Warburg effect, pyruvate is converted into lactic acid in the cytoplasm and produces a small amount of ATP, rather than entering the Krebs cycle in the mitochondria to produce ATP (Chen *et al.* 2018). Previous studies have suggested that the Warburg effect is a major pathway in providing energy production in tumor cells, and a potential role in the reproductive process of mammals has also been reported recently (Metallo & Vander Heiden 2013, Ferramosca & Zara 2014, Mordhorst *et al.* 2016).

Glycolysis contains three irreversible steps, regulated by hexokinase (HK) or glucokinase (GK), phosphofructokinase (PFK), and pyruvate kinase (PK) (Wilson 2017). Pyruvate kinase is considered to regulate whether phosphoenolpyruvate is used to make glucose or pyruvate (Iqbal *et al.* 2014). Pyruvate kinase contains four isoforms (PKM1, PKM2, PKR and PKL), and each of them has multiple expression patterns in different tissues (Iqbal *et al.* 2014). Studies have shown that PKM2 is mainly expressed in embryonic tissues and proliferating cells, especially tumor cells (Smith & Sturmey 2013). However, little is known regarding the role of PKM2 during endometrial decidualization. At the beginning of decidualization, the stromal cells immediately surrounding the implanting blastocyst proliferate and form the primary decidual zone late on day 5. This is followed by the cessation of proliferation of stromal cells in the primary decidual zone and proliferation of stromal cells outside the primary decidual zone by day 6, forming the secondary decidual zone (Tan *et al.* 1999). It is reported that the expression of PKM2 is closely related to the proliferation and differentiation of tumor cells (Liang *et al.* 2016). So we speculated that PKM2 may also be involved in endometrial decidualization during early pregnancy.

Therefore, we aimed to investigate the expression and biological functions of PKM2 in endometrial stromal cells during decidualization *in vivo* and *in vitro*. The present study is the first to provide evidence that PKM2 was essential for endometrial decidualization.

## Methods and materials

### Animal

All the Kunming mice were purchased from the experimental animal center of Chongqing Medical University and all animal procedures were approved by the Ethics Committee of Chongqing Medical University, China. Kunming mice (6–8 weeks) were fed at an independent ventilated germ-free cage at a constant temperature under a 12 h light:12 h darkness cycle. Female mice in estrus were mated with male mice or vasectomized male mice overnight in a 3:1 ratio. The vagina of female mice was examined next morning at 08:00 h, and the morning following the appearance of a vaginal plug was defined as day 1 of pregnancy (D1) or day 1 of pseudopregnancy (PD1). After the mice were killed by cervical vertebra dislocation, the uterus of the mice was separated and stored at −80°C for real time PCR and Western blot. The remaining uterine section was fixed in 4% paraformaldehyde for immunohistochemistry. The implantation sites (IS) on day 5 of pregnancy were displayed by tail i.v. injection of 0.1 mL of 1% Chicago blue (Sigma) in 0.85% sodium chloride. The IS and inter-implantation sites (IIS) were separately collected (Long *et al.* 2019).

The model of artificial induced decidualization in mice was established as follows: PD4 female mice were anesthetized with 5% lidocaine, and 25 μL of corn oil was injected into the uterine horn on one side, while the

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other side was not treated as the control. Mice artificially induced decidualization were sacrificed in PD8.

### Cell culture and cell transfection

Ishikawa was obtained from ATCC and cultured in RPMI-1640 with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were placed in a 37°C, 5% $CO_2$ concentration incubator. Transfections of si-PKM2 were performed using lipofectamine 2000 (Lip 2000; Invitrogen). Four μL of si-PKM2 and 2 μL of lipofectamine 2000 were separately diluted with 50 μL DMEM-F12/RPMI-1640 for 5 min and mixed at room temperature away from light. The compounds were added to orifice plates at equal levels. Penicillin/streptomycin and Amphotericin B-free DMEM-F12/RPMI-1640 medium were added to make the total volume was 500 μL. The si-PKM2 and negative control were purchased from GenePharma (Shanghai, China). The si-PKM2 sequences are 5′-tcctgtggctggactacaa-3′ for mice endometrial stromal cells and is 5′-gctgtggctctagacacta-3′ for Ishikawa cells.

### Human decidual tissue

Human decidual tissues were collected in the First Affiliated Hospital of Chongqing Medical University. Normal decidua tissues were obtained from healthy volunteer women undergoing legal elective termination at 7–10 weeks of gestation. The other decidual tissues were obtained from the patients with missed abortion. There was no significant difference of age and BMI between the patients who underwent termination and those who had missed abortions. All patients included in the current study provided written consent before surgery, and the Ethics Committee of Chongqing Medical University approved the study protocol.

### Real time PCR

Real time PCR experiments were performed as described previously (Long *et al*. 2019); detailed experimental methods are as follows: total RNA was extracted from the mouse endometrial tissues using TRIzol reagent (TaKaRa). RNA purity was assessed by measuring OD at 260 and 280 nm and A260/A280 ≥1.8 was the standard. Its integrity was assessed by agarose gel electrophoresis. cDNA synthesis was completed with 1 μg total RNA using the First Strand Synthesis for RT-PCR kit (Takara). cDNA was stored at −20°C until real-time RT-PCR analysis. The sequences of all oligonucleotide primers are listed as follows: PKM2: forward: 5′-cagagaaggtcttcctggctca-3′, reverse: 5′-gccacatcactgccttcagcac-3′; Dtprp: forward: 5′-agccagaaatcactgccact-3′, reverse: 5′-tgatccatgcaccca taaaa-3′; β-actin: forward: 5′-tcctatgtgggtgacgaggc-3′, reverse: 5′-tacatgcctggggtgttgaagtct-3′. Real-time RT-PCR was performed with SYBR Premix Ex Taq kits (Takara) and the Bio-Rad CFX96 Real-Time System (Bio-Rad). The real-time RT-PCR master mixture (15 μL) consisted of 7.5 μL of 2× SYBR Premix Ex Taq, 0.6 μL of 10 pmol/mL primers, 1.2 μL of cDNA, and 5.1 μL of double-distilled $H_2O$. The PCR conditions were as follows: initial denaturation at 95°C for 30 s, 40 cycles of 10 s at 95°C, 30 s at 65°C, and 30 s at the corresponding primer melting temperature (Tm). Experiments were performed in triplicate for each sample. cDNA template was replaced with nuclease-free water for negative control. Melting curves of the products were obtained after cycling using a stepwise increase in temperature from 55°C to 95°C. Relative gene expression were calculated with the $2^{-\Delta\Delta Ct}$ method, with β-actin used as the internal control.

### Western blot

Detailed methods were described according to the established method (Long *et al*. 2019) . The proteins from the uterine tissues and cell samples were extracted by commercial kits from Beyotime Biotechnology (Beijing, China). The protein concentration was determined by bicinchoninic acid assay method according to the manufacturer's instruction (Beyotime Biotechnology). Proteins were resolved by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% skim milk or BSA at 37°C for 80 min and incubated with the primary antibodies (the primary antibodies information is shown in Table 1) overnight at 4°C. Blots were washed and then probed with HRP-conjugated secondary antibodies (Boster, Pleasanton, CA, USA). The positive bands were detected by chemiluminescent reaction (Millipore). Image collection and densitometry analysis were performed by Quantity One version 4.5.0 analysis software (Bio-Rad).

### Immunohistochemistry

The uterus was extracted and fixed in 4% paraformaldehyde solution for 4–6 h. It was dehydrated in an alcohol series (75%, 85%, 95%, and 100%) and then embedded in paraffin. Five micrometer sections were cut. Antigen retrieval was performed in ethylenediaminetetraacetic acid antigen retrieval solution (Beyotime) for 15 min at

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**Table 1** Primary antibody list.

| Antibody name | Company | Cat No. | The concentration for WB | The concentration for IHC | The concentration for IF |
|---|---|---|---|---|---|
| PKM2 | CST | 4053s | 1:1000 | 1:200 | |
| BMP2 | abcam | ab14933 | 1:500 | | |
| HOXA10 | SANTA | sc-271428 | 1:500 | | |
| PCNA | CST | 2586s | 1:1000 | 1:300 | |
| CyclinD3 | CST | 2936s | 1:1000 | 1:200 | |
| P-ERK | CST | 9910 | 1:1000 | | |
| ERK | CST | 9910 | 1:1000 | | |
| Ki67 | Bioss | bs-23103R | | 1:100 | 1:50 |
| β-Actin | sigma | A5441 | 1:1000 | | |
| GAPDH | abcam | Ab181602 | 1:2000 | | |

95°C followed by cooling naturally to room temperature. Endogenous peroxidase activity was inhibited by incubation with 3% $H_2O_2$ for 10 min. Nonspecific binding in tissue sections was blocked with 10% goat serum for 30 min. Sections were incubated with primary antibody (the primary antibodies information is shown in Table 1) overnight at 4°C and with the corresponding biotinylated secondary antibodies for 1 h at room temperature. It was followed by incubation with an avidin-biotinylated peroxidase complex system (Zhongshan Biosciences, Beijing, China) for 30 min. The chromogenic reaction was developed by the incubation with diaminobenzidine (Zhongshan Biosciences) for 3 to 5 min and terminated with water. Then, the sections were lightly counter stained with hematoxylin. Finally, the image was captured using a microscope and quantified by Image J software.

## Isolation and artificial decidualization of mice endometrial stromal cells *in vivo*

The induction of decidualization *in vitro* has been described previously (Long *et al.* 2019). Artificial decidualization of endometrial stromal cells was as follows: The concentration of 10 nmol/L $E_2$ (Sigma) and 1 μmol/L $P_4$ (Sigma) was added in the medium at a ratio of 1:1. The cell was cultured for 48–72 h.

## Immunofluorescence

The isolated endometrial stromal cells were cultured into a 24-well plate containing coverslips. When the density of adherent cells was about 80%, the cell slides were fixed with ice methanol for 15 min, and PBS was washed three times for 3 min each time. The cells were perforated by incubating with 0.5% Triton X-100 solution for 20 min at room temperature. After washing three times, the cell slides were incubated with the rabbit polyclonal anti-Ki67 (Bioss, Woburn, MA, USA) overnight at 4°C. Slides were washed and incubated with FITC-labeled fluorescent rabbit antibody for 1 h at 37°C. The plate was incubated with DAPI for 10 min and sealed with anti-fluorescence quenching tablets. Finally, the image was taken using a microscope and quantified by Image J software.

## Uterine horn injection

It is reported that uterine horn injection of siRNA had an inhibited effect on implantation rate in mice (Chen *et al.* 2017). To determine the effect of PKM2 on embryo implantation *in vivo*, si-PKM2 was injected into the uteri of mice. Briefly, siRNA was injected into one horn of uteri on the morning of day 3 of pregnancy and the other horn received scrambled control siRNA or water injection which served as control (Li *et al.* 2011). Then the mice uterine on day 7 of pregnancy was collected for observation.

## Wound healing assay

A migration assay was then performed in the presence or absence of PKM2 at 0 and 24 h. After transfected with Si-PKM2, wounds were made by perpendicular linear scratch using a pipette tip. The area of cell migration was measured using Image J software. Cell migration was measured as the ratio of the migrated area relative to the cell-free area of the initial scratch.

## Statistics

Statistical analyses were performed using SPSS 18.0 (SPSS). Graphing was performed using GraphPad Prism 7.0 software. Data are expressed as mean ± S.E. Comparisons were calculated using *t*-test and adjusted by Bonferroni and Holm for multiplicity. More details for statistics is shown in each figure legend. $P<0.05$ was considered statistically significant.

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## Results

### Expression of PKM2 in mice uterine during early pregnancy and pseudopregnancy

To explore the role of endometrial PKM2 during early pregnancy, immunohistochemistry was first performed to localize the expression of PKM2 in mouse uteri from day 1 to 7 of pregnancy (D1 to D7). As shown in Fig. 1A, PKM2 was mainly located in the uterine glandular and luminal epithelium on D1 and D4. With the onset of implantation on D5, PKM2 was highly expressed in the stromal cells surrounding the blastocyst (Fig. 1A). Accompanied with embryo adhesion and invasion into the endometrium, stromal cells transform into decidual cells, which is termed as decidualization (Zhang *et al.* 2013). At the endometrial implantation site on D6 and D7, PKM2 was strongly expressed in decidual cells (Fig. 1A and B). Real-Time PCR was then used to quantify the transcription level of PKM2 in mice uterus from D5 to D7. It showed that the level of endometrial PKM2 mRNA was much higher at the implantation sites than that at the inter-implantation sites (Fig. 1C). Western blot analysis also revealed a significantly high expression level of PKM2

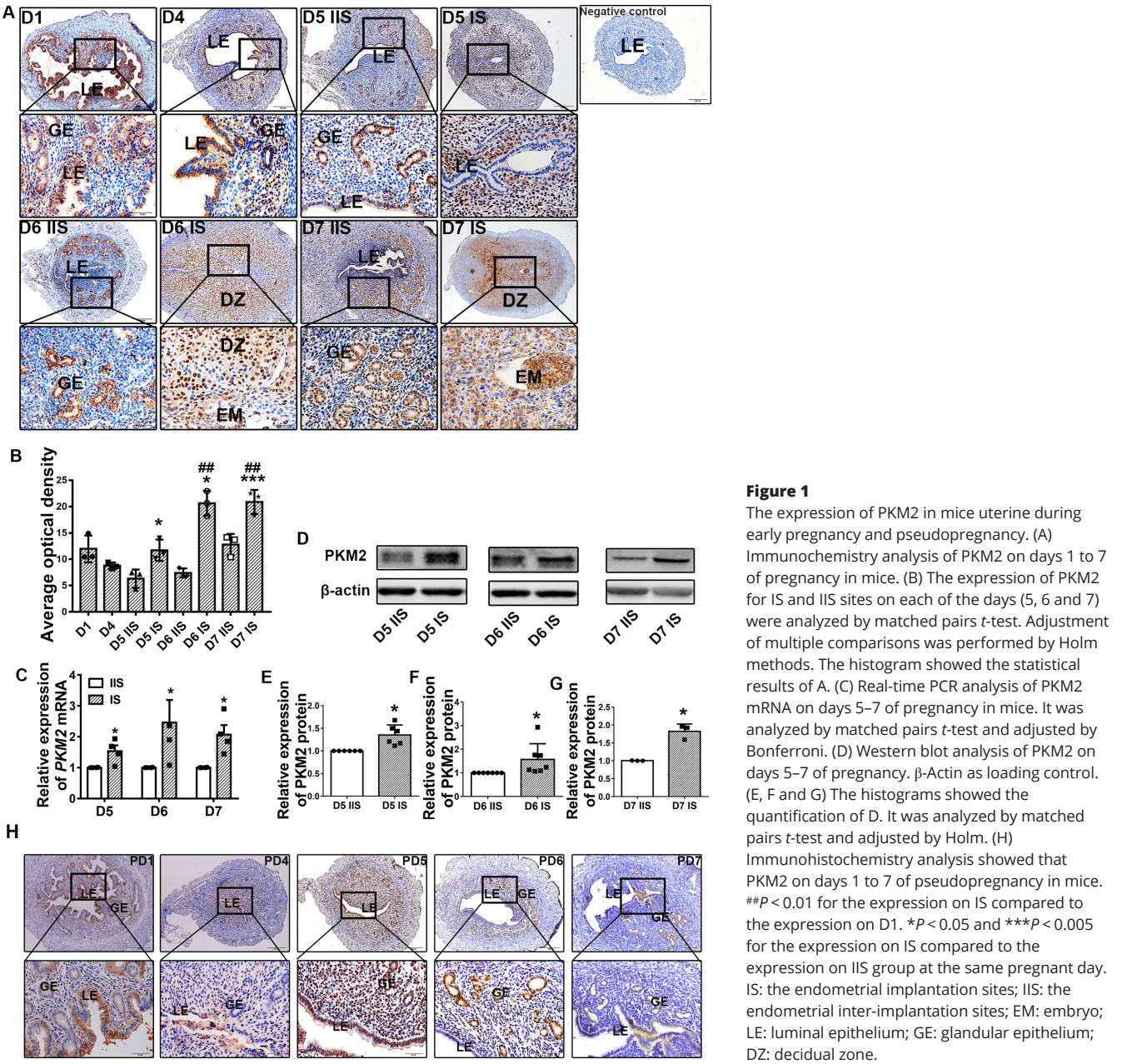


**Figure 1**
The expression of PKM2 in mice uterine during early pregnancy and pseudopregnancy. (A) Immunochemistry analysis of PKM2 on days 1 to 7 of pregnancy in mice. (B) The expression of PKM2 for IS and IIS sites on each of the days (5, 6 and 7) were analyzed by matched pairs *t*-test. Adjustment of multiple comparisons was performed by Holm methods. The histogram showed the statistical results of A. (C) Real-time PCR analysis of PKM2 mRNA on days 5–7 of pregnancy in mice. It was analyzed by matched pairs *t*-test and adjusted by Bonferroni. (D) Western blot analysis of PKM2 on days 5–7 of pregnancy. β-Actin as loading control. (E, F and G) The histograms showed the quantification of D. It was analyzed by matched pairs *t*-test and adjusted by Holm. (H) Immunohistochemistry analysis showed that PKM2 on days 1 to 7 of pseudopregnancy in mice. $^{\#\#}P < 0.01$ for the expression on IS compared to the expression on D1. $^{*}P < 0.05$ and $^{***}P < 0.005$ for the expression on IS compared to the expression on IIS group at the same pregnant day. IS: the endometrial implantation sites; IIS: the endometrial inter-implantation sites; EM: embryo; LE: luminal epithelium; GE: glandular epithelium; DZ: decidual zone.

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at the implantation site on D5, D6, and D7 (Fig. 1D, E, F and G). Pseudopregnancy mice model was represented to identify whether PKM2 expression was triggered by embryonic signaling. Immunohistochemistry analysis showed that PKM2 was also mainly located in the uterine glandular and luminal epithelium in day 5 and 6 of pseudopregnancy (Fig. 1H). It showed that PKM2 expression pattern is not dependent on the presence of blastocyst.

## Artificial decidualization could enhance PKM2 expression and down regulation of PKM2 could result in abnormal decidualization both *in vivo* and *in vitro*

Accompanied with embryo adhesion and invasion into the endometrium, the endometrium experienced decidualization, which is essential for the successful pregnancy of mice (Long *et al.* 2019). Decidualization can be induced naturally by blastocysts. In addition, it can also be induced by intraluminal means of oil infusion in pseudopregnant mice. Here, we performed an *in vivo* decidualization model for further exploration of PKM2 expression. Briefly, 25 μL corn oil was injected into one horn of uteri on day 4 of pseudopregnancy in the morning (stimulated), whereas the other horn without treatment served as the control (unstimulated). On the morning of day 8 of pseudopregnancy, the uterus was collected for analysis. The significantly increased wet weight and Dtprp mRNA expression in stimulated horn of uterine suggested that artificial decidualization model *in vivo* was successfully established (Fig. 2A, B and C). Immunohistochemistry demonstrated PKM2 was primarily highly expressed in decidual cells in the oil-injected side, compared with control side (Fig. 2D and E). Besides, in the control side, PKM2 was mainly located in the uterine glandular and luminal epithelium, which was similar with that in the pseudopregnant mice uterus (Fig. 2D and E). Real-time PCR and Western blot showed that PKM2 was significantly higher in the oil-injected side than that in the control side (Fig. 2F, G and H).

To further investigate whether PKM2 was involved in decidualization, primary stromal cells isolated from the uteri of nonpregnant mice were treated with $E_2$ and $P_4$ for artificial decidualization *in vitro*. After stromal cells were induced to decidual cells, the cells were much larger, polygonal, rounded, and giant mono- or bi-nuclear and there was a significant increase of Dtprp mRNA which is a reliable marker for mouse decidualization (Fig. 3A and B).

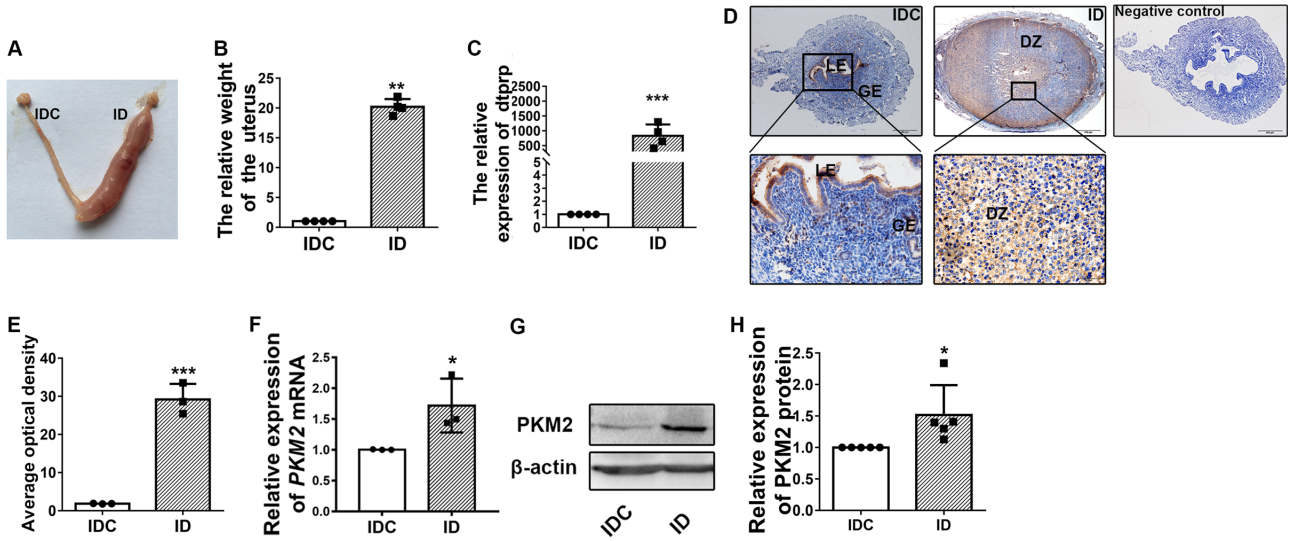


**Figure 2**

PKM2 was significantly increased after experimental decidualization in pseudopregnant mice *in vivo*. Artificial decidualization was experimentally induced by injection with corn oil into one horn of uterus (stimulated, ID), whereas the other horn received no infusion and served as the control (unstimulated, IDC). (A) The picture showed the uterine gross morphology. The stimulated uterine horns formed robust deciduoma. (B) The stimulated and unstimulated uterine horn gross weight are shown as the histogram. (C) The mRNA expression of Dtprp, a marker gene of decidualized cells, was significantly increased on the stimulated horn of uterus. (D) Immunochemistry analysis of PKM2 on the control side and the oil-injected side. (E) The histogram shows the statistical results of D. (F) Real-time PCR analysis of PKM2 mRNA on the control side and the oil-injected side. (G) Western blot analysis of PKM2. β-Actin as loading control. (H) The histograms showed the quantification of G. ID: oil side, artificially induced decidualization; IDC: control side, nothing injected; LE, luminal epithelium; GE, glandular epithelium; DZ, decidual zone. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$ for the ID group compared to the IDC group. It was analyzed by 1-sample *t*-test. A full color version of this figure is available at https://doi.org/10.1530/JOE-19-0553.

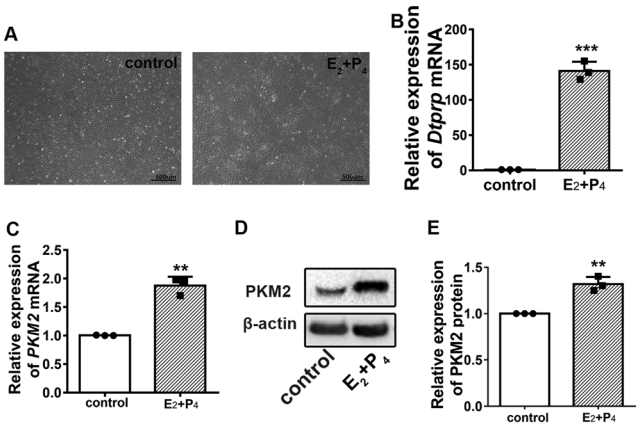


**Figure 3**
PKM2 was obviously increased after experimental decidualization in primary stromal cells mice *in vitro*. Uterine primary stromal cells isolated from the uteri of nonpregnant mice were treated with estrogen and progesterone for artificial decidualization *in vitro*. (A) After experimental decidualization by E2 and P4 *in vitro*, white light photograph showed the cells were much larger, polygonal, rounded, and giant mono- or bi-nuclear. The significantly increased mRNA expression of Dtprp (B), a marker gene of mice decidualized cells, and the obviously enhanced PKM2 mRNA (C) and protein (D and E) expression were observed after primary stromal cells were artificially decidualized. $^{**}P < 0.01$ and $^{***}P < 0.005$ for the $E_2$ and $P_4$ artificially decidualized group compared to the control group. It was analyzed by 1-sample *t*-test.

At the same time, a significant induction of PKM2 mRNA and protein in decidual cells was exhibited after stromal cells induced to decidual cells (Fig. 3C, D and E). Combined with the data shown in *in vivo* decidualization, it suggested the potential role of PKM2 in the decidualization during early pregnancy.

To further identify the regulation of PKM2 in decidualization during early pregnancy of mice, we then used siRNA specifically targeting PKM2 to knock down the expression of PKM2 in primary mice endometrial stromal cells. It showed that si-PKM2 significantly downregulated PKM2 expression compared with scrambled control siRNA (Fig. 4A, B, C and D). The decidualization markers BMP2 and Hoxa10 were also obviously downregulated after PKM2 expression was knocked down (Fig. 4C, D, E and F). Next, the function of PKM2 in decidualization during early pregnancy was also explored by injecting si-PKM2 into the uterine of mice. Briefly, siRNA was injected into one horn of uteri on the morning of day 3 of pregnancy and the other horn received scrambled control siRNA or water injection which served as control (Li *et al.* 2011). As shown in the results, the number of embryo implantation sites on day 7 of pregnancy was significantly reduced in the PKM2-siRNA treated horn (Fig. 4G and H).

## Silencing PKM2 could compromise cell proliferation during decidualization

The regulation of PKM2 on cell proliferation was widely studied in tumors (Lunt *et al.* 2015, Liu *et al.* 2016). During mice decidualization, uterine stromal cells first undergo widespread proliferation and then differentiation into decidual cells (He *et al.* 2015). We next explored whether PKM2 was involved in cell proliferation during decidualization. After PKM2 was down-regulated by si-PKM2, Western blot showed a dramatically reduced expression of cell proliferation markers PCNA, cyclinD3, and p-ERK in stromal cells decidualized artificially (Fig. 5A and B). Ishikawa cell line was later employed to confirm the role of PKM2 in regulating proliferation. Knock down of PKM2 in Ishikawa cells by siRNA could result in a significantly decreased protein expression of PCNA and cyclin D3 (Fig. 5C and D). The migration rate of Ishikawa cells was also obviously suppressed by si-PKM2 according to the wound healing assay (Fig. 5E and F). Immunofluorescence was performed to examine the expression of Ki67, which is another proliferation marker. It showed a significant reduction of Ki67 expression (Fig. 5G). These results implied that down-regulation of PKM2 could compromise cell proliferation during decidualization.

## Endometrial PKM2 was down regulated in the patients with missed abortion during early pregnancy

To explore the role of PKM2 in the endometrium during missed abortion, the decidua tissues of humans were collected for our study. As exhibited in Fig. 6, both Western blot and immunohistochemistry analysis revealed down regulation of PKM2 in decidua from patients who suffered missed abortion. Furthermore, significant reduction of proliferation markers PCNA, cyclin D3 and Ki67 were also observed in patients who suffered missed abortion (Fig. 6).

## Discussion

Decidualization is known to facilitate implantation and maintain pregnancy (Cho *et al.* 2019). Uterine stromal cells undergo transformation into decidual cells after embryo attachment to the endometrium, which occur at 24:00 h on day 4 of pregnancy. On day 5 of pregnancy, the stromal cells around the implantation chamber experience rapid proliferation and differentiation forming the primary decidual zone (PDZ). From day 6, stromal cells next to the PDZ transform into decidual

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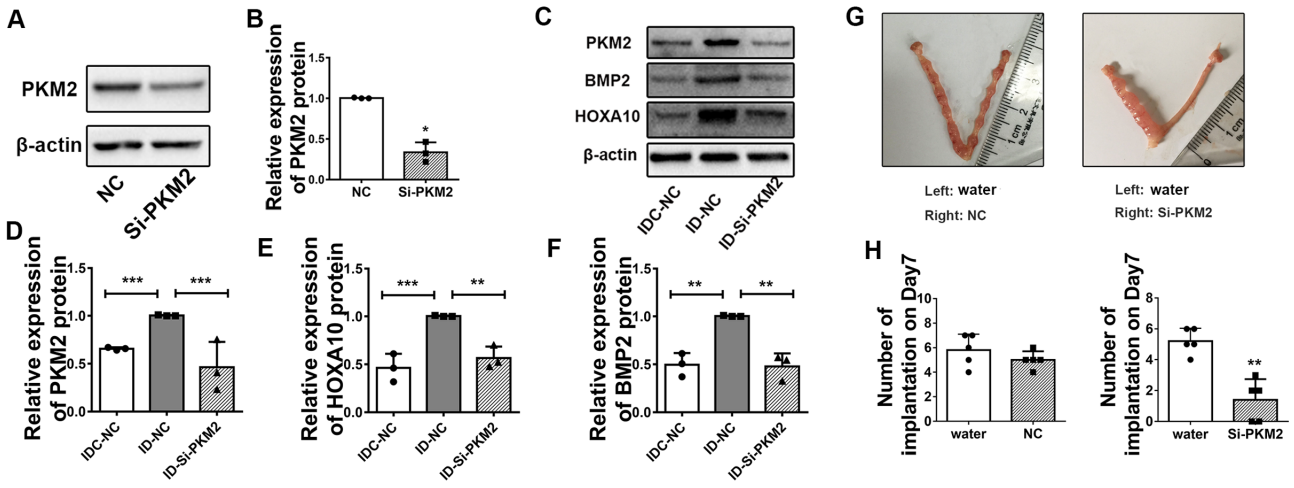


**Figure 4**

Down regulation of PKM2 could result in abnormal decidualization both *in vivo* and *in vitro*. (A) siRNA specifically targeting PKM2 was used to knock down the expression of PKM2 in primary mice endometrial stromal cells (mESCs). (B) It was analyzed by 1-sample *t*-test and the histogram showed the statistical results of A. (C) The decidualization markers BMP2 and Hoxa10 were also obviously downregulated after PKM2 expression was knocked down in mESCs. (D, E and F) The histograms showed the statistical results of C. Differences between IDC-NC vs ID-NC and ID-NC vs ID-Si-PKM2 were analyzed by matched 1-sample *t*-test and adjustment of multiple comparisons was performed by Bonferroni methods. (G) siRNA was injected into one horn of uteri on the morning of day 3 of pregnancy and the other horn received scrambled control siRNA or water injection which served as control. (H) The number of embryo implantation sites on day 7 of pregnancy was significantly reduced in the PKM2-siRNA treated horn. Differences between water vs NC and water vs Si-PKM2 were analyzed by matched pairs *t*-test and adjusted by Bonferroni. ID: cells were artificially decidualized by E2 and P4; IDC: cells were not decidualized by E2 and P4; NC: negative control for Si-PKM2. **P* < 0.05, ***P*<0.01, ****P* < 0.005.

cells forming the secondary decidual zone (SDZ) (Gao *et al*. 2015). Decidual tissue performs a multi-functional role in fetal-maternal immuno-tolerance and placental development, as well as nourishing the embryo during early pregnancy. It is responsible for fulfilling the metabolic requirement of the embryo in the early stage of pregnancy (Wang *et al*. 2016, 2018). Previous evidence showed that glycolysis from glucose to lactate was increased rapidly during decidualization (Kommagani *et al*. 2015, Zuo *et al*. 2015). Additionally, it is reported that failure of decidualization may be due to improper glucose uptake and/or metabolism in endometrial stromal cells (Frolova *et al*. 2011). PKM2 is a rate-limiting enzyme in glycolysis which catalyzes phosphoenolpyruvic acid into pyruvate (Liu *et al*. 2016). PKM2 is essential during cellular metabolism for the aerobic glycolysis which promotes tumorigenesis (Israelsen *et al*. 2013). The role of PKM2 in the glucose metabolism of pregnancy was examined and analyzed in the current study. We observed that expression of PKM2 was mainly located in the uterine glandular epithelium and luminal epithelium on day 1 and day 4 of pregnancy and strongly expressed in the decidual zone after embryo implantation. PKM2 mRNA and protein were dramatically increased with the onset of decidualization. In the pseudopregnancy model, PKM2 was expressed in uterine glandular epithelium and luminal epithelium. Stromal cells did not transform into decidual cells without the attachment of the embryo to the endometrium. No significant increase in PKM2 was found from day 5 of pseudopregnancy. Taken together, this data in pregnancy and pseudopregnancy models indicated PKM2 has a potential function during post-implantation uterine decidualization. Consistent with these results, the role of PKM2 during decidualization was further investigated *in vivo* and *in vitro*. Immunocytochemistry data demonstrated that PKM2 was extensively expressed in decidual cells. Moreover, both PKM2 mRNA and protein level was increased dramatically in decidual tissue.

To better understand the functional role of PKM2, PKM2-siRNA was employed. PKM2-siRNA injection significantly decreased the number of embryo implantation sites, mainly due to compromised decidualization. This was confirmed in artificially decidualized endometrial stromal cells *in vitro*. Decidualization related factors BMP2 and HOXA10 were expressed at a low level in artificially decidualized cells transfected with si-PKM2. Considering that PKM2 expression at the implantation site was not dependent on the presence of active blastocysts, we speculate that PKM2 might be important for decidualization in mice.

As we know, decidualization is accompanied by cell proliferation and cell differentiation (Israelsen *et al*. 2013). During this process, stromal cells experience proliferation and differentiation into decidual cells

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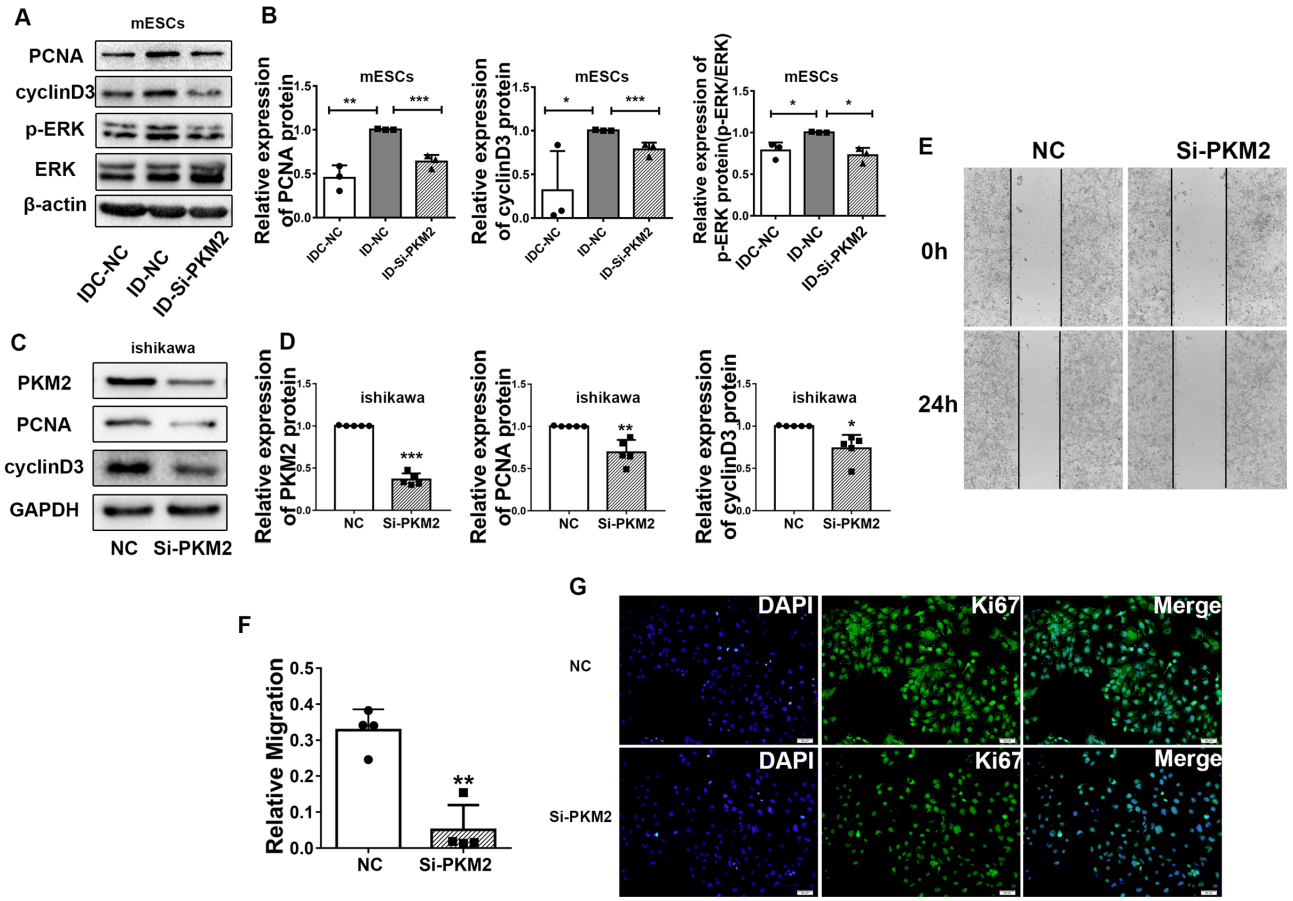


**Figure 5**

Silencing PKM2 could compromise cell proliferation. (A) After PKM2 was downregulated by si-PKM2, Western blot showed a dramatical reduction of cell proliferation markers PCNA, cyclinD3 and p-ERK expression in decidual induced uterine primary stromal cells. (B) The histograms showed the quantification of the Western blot analysis in A. Differences between IDC-NC vs ID-NC and ID-NC vs ID-Si-PKM2 were analyzed by 1-sample *t*-test and adjusted by Bonferroni for multiplicity. (C) Knock down of PKM2 in Ishikawa cells by siRNA also could result in a significant decreased expression of PCNA and cyclin D3 protein. (D) It was analyzed by 1-sample *t*-test and the histograms showed the quantification of the Western blot analysis in C. β-Actin as loading control. (E) The migration rate of Ishikawa cells was obviously suppressed by si-PKM2 according to the wound healing assay. (F) It was analyzed by 1-sample *t*-test and the histograms showed the statistical results of E. (G) Immunofluorescence analysis of Ki67 protein in Ishikawa cells after transfected with PKM2-siRNAs. ID: cells were artificially decidualized by E2 and P4; IDC: cells were not decidualized by E2 and P4; NC: negative control for Si-PKM2. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

(Lim & Wang 2010, Zhang *et al*. 2013). The proliferation related markers, PCNA, cyclinD3 and ERK regulate decidualization during pregnancy (Correia-da-Silva *et al*. 2004, Mestre-Citrinovitz *et al*. 2015, Li *et al*. 2016). It is reported that irregular endometrial stromal proliferation and differentiation often lead to some serious outcomes, such as recurrent miscarriage, preeclampsia, intrauterine growth restriction, and so on (Achache & Revel 2006, Zhou *et al*. 2017). Another proliferation marker, Ki67, is expressed at a significantly lower level in the villi of patients with recurrent miscarriage (Wu *et al*. 2017). Moreover, a study demonstrated that lack of uterine stromal cell proliferation could impair uterine decidualization in mice (Winuthayanon *et al*. 2017). PKM2, a key enzyme in the glycolytic pathway, is reported to regulate cancer metabolism by regulating cell proliferation and survival (Ao *et al*. 2017). It is reported that PKM2 induces cancer proliferation by activating the EGFR signaling pathway (Hsu *et al*. 2016). A study in ovarian cancer demonstrated that si-PKM2 markedly inhibits cell proliferation and results in cell cycle arrest at the G0/G1 phase (Miao *et al*. 2016). Another study revealed that placental tissues from preeclampsia patients had elevated levels of p-PKM2, p-ERK, and ERK in the cytoplasm (Bahr *et al*. 2014). In this study, we examined cell proliferation in artificially decidualized endometrial stromal cells after PKM2-siRNA treatment. Down regulated PKM2 could result in a significantly decreased expression of PNCA, cyclinD3, and p-ERK. These results suggest that PKM2 might regulate endometrial stromal cells decidualization

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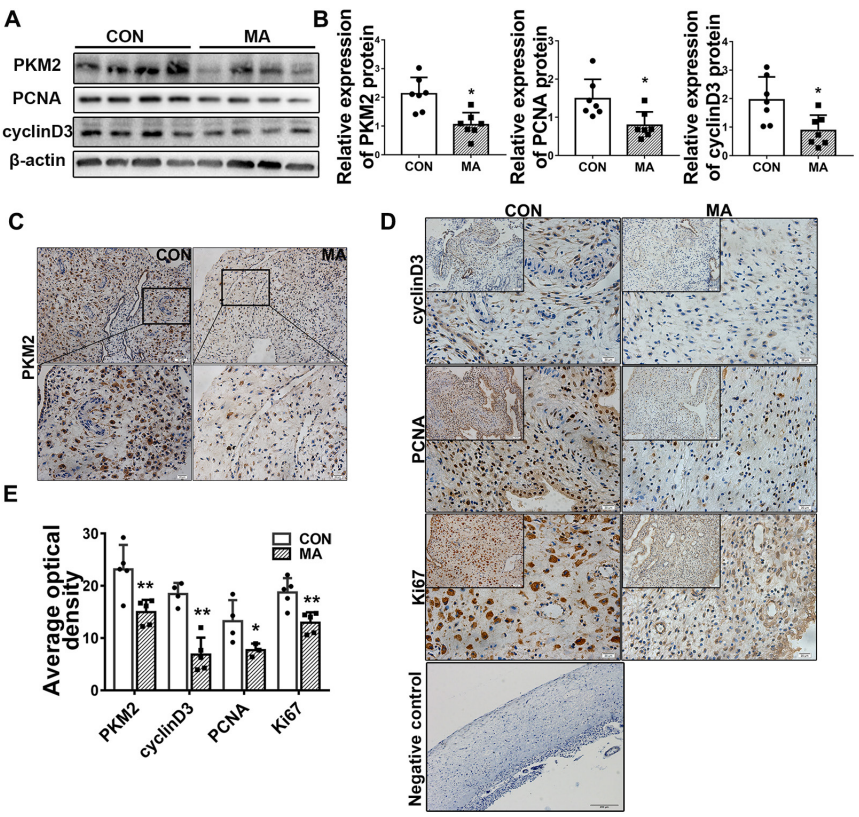


**Figure 6**
PKM2 is down regulated in the endometrium of women suffering miss abortion during early pregnancy. (A) Western blot analysis showed protein level of PKM2 and proliferation markers in human decidua. (B) It was analyzed by 2-sample *t*-tests for two independent samples and the histogram showed the quantification of the Western blot analysis in A. β-Actin was used as loading control. (C and D) Immunochemistry analysis of PKM2 and proliferation markers in human decidua. (E) It was analyzed by 2-sample *t*-tests for two independent samples and the histogram showing the quantification of the results for C and D. $*P < 0.05$, $**P < 0.01$. Con: normal decidua tissues were obtained from healthy volunteer women undergoing legal elective termination at 7–10 weeks of gestation; MA: decidual tissues were obtained from the patients with missed abortion. A full color version of this figure is available at https://doi.org/10.1530/JOE-19-0553.

via cell proliferation. In addition, the regulation of PKM2 on proliferation was confirmed in Inshikawa cells. The proliferative capacity of Ishikawa cells was obviously decreased after PKM2 was down regulated. Lastly, further exploration in women suffering 'missed abortion' miscarriage also revealed the correlation between down regulated PKM2 and cell proliferation.

In our study, we found the expression and biological function of PKM2 in endometrial stromal cells during decidualization *in vivo* and *in vitro*. PKM2 is highly expressed in mouse decidua and it could regulate decidualization by cell proliferation. Moreover, we also found down-regulation of PKM2 and cell proliferation in women suffering missed abortion. However, further investigations will be needed to uncover the details of the involved mechanisms.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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