Differential expression and regulation of Tdo2 during mouse decidualization

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Abstract
Tryptophan 2,3-dioxygenase (Tdo2) is a rate-limiting enzyme which directs the conversion of tryptophan to kynurenine. The aim of this study was to examine the expression and regulation of Tdo2 in mouse uterus during decidualization. Tdo2 mRNA was mainly expressed in the decidua on days 6–8 of pregnancy. By real-time PCR, a high level of Tdo2 expression was observed in the uteri from days 6 to 8 of pregnancy, although Tdo2 expression was observed on days 1–8. Simultaneously, Tdo2 mRNA was also detected under in vivo and in vitro artificial decidualization. Estrogen, progesterone, and 8-bromoadenosine-cAMP could induce the expression of Tdo2 in the ovariectomized mouse uterus and uterine stromal cells. Tdo2 could regulate cell proliferation and stimulate the expression of decidual marker Dtprr in the uterine stromal cells and decidual cells. Overexpression of Tdo2 could upregulate the expression of Ahr, Cox2, and Vegf genes in uterine stromal cells, while Tdo2 inhibitor 680C91 could downregulate the expression of Cox2 and Vegf genes in uterine decidual cells. These data indicate that Tdo2 may play an important role during mouse decidualization and be regulated by estrogen, progesterone, and cAMP.

Key Words
► uterus
► female reproduction
► pregnancy
► uterine–embryo interaction
► mouse

Introduction
Tryptophan is an essential amino acid that is required for the biosynthesis of proteins and also a precursor of neurotransmitter 5-hydroxytryptamine (serotonin) which has been identified within the CNS, vasculature, gastrointestinal tract, and female reproductive tract (Stone & Darlington 2002, Doherty et al. 2011). In mammals, most of tryptophan was metabolized along the kynurenine pathway and tryptophan metabolism was closely correlated with pregnancy (Stone & Darlington 2002, Schröcksnadel et al. 2006). During pregnancy, decreased tryptophan concentration and increased concentrations of kynurenines were observed in human plasma and in the uteri of mice and cows (Minatogawa et al. 2003, Schröcksnadel et al. 2006, Groebner et al. 2011). Previous studies have found that tryptophan metabolism was mainly dependent on tryptophan 2,3-dioxygenase (Tdo2) and indoleamine 2,3-dioxygenase (Ido) (Stone & Darlington 2002). Ido was expressed in dendritic cells, macrophages, giant trophoblasts in mice, and in extravillous trophoblast and villous trophoblast in humans, and could prevent rejection of the allogeneic fetus by suppressing the activation of maternal decidual T lymphocytes (Munn et al. 1998, Saito et al. 2007). During the gestation period, Ido was strongly expressed in the mouse concepti and placenta from days 8.5 to 12.5 of post-coitus (Suzuki et al. 2001, Minatogawa et al. 2003). Unexpectedly,
tryptophan-degrading activity was also observed on days 5.5–9.5 of post-coitus, and was not inhibited by the Ido inhibitor 1-methyltryptophan (Suzuki et al. 2001, Minatogawa et al. 2003). These results indicate that tryptophan metabolism in early gestation is due to Tdo2 activity.

Tdo2 is an initial and rate-limiting enzyme in the metabolism of tryptophan and might catalyze the oxidative cleavage of the indole ring of 1-tryptophan to N-formylkynurenine in the kynurenine pathway (Thackray et al. 2008). Tdo2-deficient mice displayed an elevation of plasma tryptophan, making more of this amino acid available for uptake into the brain, where it is converted to serotonin (Kanai et al. 2009). Tdo2 has also been identified in skin, brain, epididymis, testis, and early concepti, although it was mainly expressed in the liver (Suzuki et al. 2001, Minatogawa et al. 2003, Britan et al. 2006). Accumulating data have shown that Tdo2 was expressed in endometrial stromal cells and could be regulated by Hoxa10 gene, which was essential for decidualization (Benson et al. 1996, Germer et al. 2009, Doherty et al. 2011, Zhang et al. 2013). According to our (B Guo and X-C Tian unpublished observations) micro-array data, Tdo2 was strongly expressed by day 8 decidua and decidualoma under artificial decidualization compared with the untreated uterine horn. However, the expression and regulation of Tdo2 in mouse uterus during decidualization have not been defined so far. Thus, this study was undertaken to examine the expression and regulation of Tdo2 in the mouse uterus during early pregnancy, by in situ hybridization and real-time PCR.

The implantation sites on day 5 were identified by i.v. injection of 0.1 ml of 1% Chicago blue (Sigma) in 0.85% sodium chloride.

Artificial decidualization

Artificial decidualization was induced by intraluminally infusing 25 μl of sesame oil into one uterine horn on day 4 of pseudopregnancy, while the contralateral uninjected horn served as a control. The uteri were collected on day 8 of pseudopregnancy. Decidualization was confirmed by weighing the uterine horn and by histological examination of the uterine sections.

Steroid hormonal treatments

Mature female mice were ovariectomized and, after 2 weeks, given a single s.c. injection of estrogen (100 ng/mouse) or progesterone (2 mg/mouse) (Guo et al. 2012). The uteri were then collected 1, 3, 6, 12, and 24 h after steroid treatment. To examine whether nuclear receptors for estrogen or progesterone are involved in steroid hormonal regulation, ovariectomized mice received injections of ICI 182 780 (an estrogen receptor antagonist) or RU486 (a progesterone receptor antagonist) 1 h before estrogen or progesterone injection respectively. Each mouse received an injection of ICI 182 780 and RU486 at a dose of 500 μg and 1 mg respectively (Guo et al. 2012). All steroids and antagonists were dissolved in sesame oil and injected subcutaneously. Controls received the vehicle only (0.1 ml/mouse).

In situ hybridization

Total RNA from the mouse uteri was reverse-transcribed and amplified with Tdo2 primers. Tdo2 forward primer 5’-GGCATGGCTGGAAAGAACAC-3’ and reverse primer 5’-CTCCCTGGAGTGACGTTAT-3’ were designed based on the sequence of the Mus musculus Tdo2 gene (GenBank accession number: NM_019911). The amplified fragment (257 bp) of Tdo2 was cloned into pGEM-T plasmid (pGEM-T Vector System 1, Promega) and verified by sequencing. Tdo2-containing plasmid was amplified with the primers of T7 and SP6 to prepare templates for labeling (T7, 5’-TAATACGACTCACTATAGGG-3’; SP6, 5’-CATACGATTTAGGTGACACTATAG-3’). Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed in vitro using a DIG RNA labeling kit (Roche Diagnostics GmbH).

The frozen sections (10 μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides

Materials and methods

Animals

Matured Kunming white strain mice (6–8 weeks old) were caged in a controlled environment with a cycle of 14 h light:10 h darkness. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. To confirm reproducibility of results, at least three mice per group were used in each stage or treatment in this study.

Pregnancy and pseudopregnancy

Adult female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy by coagining respectively (day 1 = day of vaginal plug). On days 1–4, pregnancy was confirmed by recovering embryos from the oviducts or uterus.

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and fixed in 4% paraformaldehyde solution in PBS. Hybridization was performed as described previously (Tian et al. 2013). The sections were counterstained with 1% methyl green in 0.12 M glacial acetic acid. The sense probe was also hybridized and served as a negative control. There were no detectable signals from sense probes.

**Real-time PCR**

Total RNAs from mouse uteri or cultured cells were isolated using TRIPURE Reagent according to the manufacturer’s instructions (Roche) and reverse-transcribed into cDNA using M-MLV reverse-transcriptase (Promega). The reverse transcriptase reaction was performed at 42 °C for 60 min with 2 μg total RNA in 25 μl volume. For real-time PCR, cDNA was amplified using FS Universal SYBR Green Real Master (Roche) with a Bio-Rad CFX96 Real-Time Detection System. The conditions used for real-time PCR were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate. The results were analyzed using CFX Manager Software (Foster City, California, USA). After analysis using the 2^(-ΔΔCt) method, data were normalized to Gapdh expression. Primer sequences for real-time PCR are listed in Table 1.

**Isolation of uterine stromal cells and in vitro decidualization**

Uterine stromal cells from day 4 of pregnancy were isolated and cultured, as described previously (Tian et al. 2013). Uterine stromal cells were induced for in vitro decidualization with a fresh medium supplemented with progesterone (1 μM) and estrogen (10 nM) in DMEM–F12 with 2% charcoal-treated FBS (Biological Industries Ltd., Kibbutz Beit Hemeek, Israel).

**Steroid hormonal treatments in vitro**

The cultured stromal cells were treated with 100 nM of progesterone or 0.1 nM of estrogen respectively. For further studies, cells were pretreated with RU486 (1 μM) or ICI 182 780 (100 nM) antagonist for 2 h before the addition of progesterone or estrogen respectively. Then cells were collected at 24 h for further quantitative analysis using real-time PCR. All steroids and antagonists were dissolved in ethanol. Controls received the vehicle only.

**Isolation of uterine decidual cells**

Uteri were collected aseptically on day 7 of pregnancy and were trimmed of mesentery and fat, slit longitudinally, and the conceptuses were removed. The remaining tissues were washed three times with HBSS and further digested with 1 mg/ml collagenase I for 30 min at 37 °C with shaking. The digested tissues were filtered and centrifuged at 200 g. The cell pellets were washed twice with HBSS and resuspended in a complete medium consisting of DMEM–F12 with 10% heat-inactivated FBS. The viable cells were counted by trypan blue staining using a hemocytometer. The cells were plated onto 35-mm culture dishes at the concentration of 1 × 10^6 cells/ml. After an initial culture for 30 min, the medium was changed to remove free-floating cells. The isolated decidual cells were further cultured in fresh complete medium at 37 °C with 5% CO₂ before treatments.

**Plasmid construction and transfection**

A 1221-bp Tdo2 cDNA fragment isolated from the mouse uterus was amplified by PCR, using the following primers with EcoRv/xhoI restriction sites: 5'-GATATC (EcoRv) ATGAGTGTTGCCCCTTTGC-3’; 5'-CTCGAG (XhoI)

### Table 1: Primers for real-time PCR

<table>
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<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Accession numbers</th>
<th>Size (bp)</th>
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<tr>
<td>Tryptophan 2,3-dioxygenase (Tdo2)</td>
<td>CTGGGGGATCCTCAGGCTAT</td>
<td>NM_019911</td>
<td>165</td>
</tr>
<tr>
<td>Decidual/trophoblast PRL-related protein (Dttpp)</td>
<td>TGTCACTGTACCTGCGTGTAGCAGAAATACGCACCT</td>
<td>NM_010088</td>
<td>119</td>
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<td>Aryl hydrocarbon receptor (Ahr)</td>
<td>CCGAAGGACACGGAAATCCAA</td>
<td>NM_013464</td>
<td>244</td>
</tr>
<tr>
<td>Cyclooxygenase 2 (Cox2)</td>
<td>CACCTTCCAGGAAGTCACCCCATC</td>
<td>NM_011198</td>
<td>178</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (Vegf)</td>
<td>CTGGTCGTGAAGAAACTGACATCTCACCT</td>
<td>NM_001025257</td>
<td>90</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)</td>
<td>GCCTTCCGTGTTCCTACCC</td>
<td>NM_008084</td>
<td>102</td>
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TCAATCCGATTCATCGCTGC-3'. The amplified product was purified and cloned into pGEM-T vector. Both pGEM-T-Tdo2 and pcDNA3.1 vectors were cut by EcoRv/XhoI (TaKaRa, Dalian, China) at 37 °C for 1 h, and then the fragment was ligated into pcDNA3.1 with T4 ligase (Promega) at 4 °C overnight to construct pcDNA-Tdo2 (pc-Tdo2). An empty pcDNA3.1 expression vector was used as a control.

Transfection of uterine stromal cells was performed according to the manufacturer’s protocol for Lipofectamine 2000 (Invitrogen). After transfection with control plasmid (empty pcDNA3.1 vector) or pc-Tdo2 plasmid, stromal cells from day 4 pregnant mice were collected or induced for in vitro decidualization for 48 h.

**Figure 1**

*In situ* hybridization of Tdo2 expression in mouse uteri during early pregnancy on days 3 (B), 5 (C), 6 (D), 7 (E), and 8 (F). No hybridization signals were seen in mouse uterus on day 7 of pregnancy when DIG-labeled Tdo2 sense probe was used to replace the antisense probe as a negative control (A). Asterisks indicate embryo. Bars = 60 μm.

**Cell proliferation**

Proliferation assays were performed using MTS reagent (Promega) according to the manufacturer’s directions. Uterine stromal cells were seeded at a density of 1×10^5 per well in 96-well plates and cultured in the DMEM–F12 medium containing 2% heat-inactivated FBS. The cells were treated for 24 h with a medium containing 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 μM Tdo2 inhibitor 680C91 after being induced for in vitro decidualization for 24 h. Finally 20 μl of MTS reagent was added to each well and incubated for 4 h. The absorbance was measured at 490 nm using a 96-well plate reader. Simultaneously, uterine decidual cells treated with 10 μM Tdo2 inhibitor 680C91 and stromal cells after transfection with pc-Tdo2 plasmid...
were also analyzed post addition of MTS. Every experiment was performed in triplicate.

Statistical analysis
All the experiments were independently repeated at least three times. The significance of differences was analyzed by one-way ANOVA using the SPSS Software Program (SPSS, Inc.). The differences were considered significant at $P<0.05$.

Results

Td02 mRNA expression during early pregnancy

*In situ* hybridization was used to examine the spatial distribution of *Td02* mRNA in mouse uterus. No *Td02* mRNA signal was detected in the uterus from days 1 to 5 of pregnancy (Fig. 1B and C). On day 6 of pregnancy, a high level of *Td02* mRNA signal was observed in decidualized cells surrounding the implanting embryo (Fig. 1D). On day 7, *Td02* expression was similar to that observed on day 6 but at much higher levels and scopes (Fig. 1E). When *Td02* antisense probe was replaced by *Td02* sense probe, there was no corresponding signal in the uterus on day 7 of pregnancy (Fig. 1A). On day 8, *Td02* mRNA was primarily localized in decidualized cells of the mesometrial region (Fig. 1F). In addition, *Td02* mRNA was not detected in the embryos from days 5 to 8 of pregnancy by *in situ* hybridization (Fig. 1C, D, E and F).

To quantify *Td02* mRNA expression, real-time PCR was performed. *Td02* was highly expressed in the uterus from days 6 to 8 of pregnancy, although *Td02* expression was detected throughout days 1–8 (Fig. 2).

Td02 mRNA expression during pseudopregnancy

There was no detectable *Td02* mRNA signal in the uterus from days 1 to 5 of pseudopregnancy by *in situ* hybridization (Fig. 3A). However, a high level of *Td02* expression was detected on day 5 of pseudopregnancy by real-time PCR, although *Td02* expression was seen from days 1 to 5 (Fig. 3B).

Td02 expression under artificial decidualization

*Td02* mRNA signal was strongly detected in the decidualized cell under artificial decidualization, but not found in uninjected control uterus (Fig. 3A). By real-time PCR analysis, a significantly higher level of *Td02* expression was detected in the decidualized uterus compared with the control uterus (Fig. 3C).

Td02 expression under *in vitro* decidualization

Primary stromal cells isolated from mouse uterus on day 4 of pregnancy were treated with a combination of estrogen and progesterone to induce *in vitro* decidualization. The success of decidualization was confirmed by a reliable marker for decidualization, decidual/trophoblast PRL-related protein (*Dtprp*) which was detected in the decidualized stromal cells after 24 h with significant time-dependent increases at 48 h and a maximum at 96 h (Fig. 4A). Likewise, elevated expression levels of *Td02* were also observed in the decidualized stromal cells with a time-dependent increase (Fig. 4B).

Regulatory effects of cAMP and H89 on *Td02* expression

Initiation of the decidual process requires elevated intracellular cAMP levels and sustained activation of the protein kinase A (PKA) pathway (Gellersen & Brosens 2003). To examine the effects of cAMP and H89 on *Td02* expression, we treated the uterine stromal cells with cAMP analog 8-bromoadenosine-cAMP (8-Br-cAMP, 500 μM) and PKA inhibitor H89 (10 μM). The results showed that *Td02* expression was gradually increased after uterine stromal cells were treated with 8-Br-cAMP and reached the highest level at 24 h (Fig. 5A). The 8-Br-cAMP stimulation of *Td02* expression was also dramatically altered by the addition of the PKA inhibitor H89 (Fig. 5B).
Regulatory effects of steroid hormones on Tdo2 expression

To determine whether steroid hormones could regulate Tdo2 expression, ovariectomized mice were given a single injection of oil (control), estrogen, or progesterone, and then Tdo2 mRNA was detected in the uteri by in situ hybridization and real-time PCR. The in situ hybridization results showed that neither estrogen nor progesterone had any obvious effects on the expression of Tdo2 (data not shown). By real-time PCR analysis, Tdo2 mRNA expression was gradually enhanced in the uteri of ovariectomized mice and reached a peak at 24 h after injection of progesterone (Fig. 6B). However, the upregulation of progesterone was significantly blocked by pretreatment with RU486 antagonist (Fig. 6D). Likewise, injection of estrogen also resulted in an increase in uterine Tdo2 mRNA level at 24 h (Fig. 6A). After pretreatment with ICI 182 780, Tdo2 mRNA expression significantly declined compared with that of estrogen treatment only (Fig. 6C).

In the in vitro cultured stromal cells, both estrogen and progesterone could induce Tdo2 mRNA expression at 24 h (Fig. 6E and F). However, the estrogen-induced stimulation of Tdo2 expression was blocked by estrogen receptor antagonist ICI 182 780 (Fig. 6E). A similar result was also observed for RU486 treatment (Fig. 6F).

Effect of Tdo2 on decidualization

Stromal cell proliferation is the first step of decidualization. To examine the effect of Tdo2 on stromal cell proliferation, we treated stromal cells with Tdo2 inhibitor 680C91 after...
induction for in vitro decidualization for 24 h. The results showed that proliferation activity of stromal cells was significantly decreased by 5 and 10 μM Tdo2 inhibitor 680C91 (Fig. 7A). In contrast, proliferation of uterine stromal cells displayed a significant increase at 48 h after transfection with pc-Tdo2 plasmid (Fig. 7C). Additionally, the Tdo2 inhibitor 680C91 could also inhibit the proliferation activity of uterine decidual cells at 24 h (Fig. 7B).

To further verify the effect of Tdo2 on decidualization, we transfected uterine stromal cells with the pc-Tdo2 expression plasmid and examined the expression of Tdo2 and Dtpp by real-time PCR. The results showed that Tdo2 mRNA expression was significantly upregulated in the pc-Tdo2-transfected cells compared with the pcDNA3.1-transfected cells (Fig. 8A). Meanwhile, overexpression of Tdo2 could induce the expression of Dtpp (Fig. 8B). Under in vitro decidualization, Tdo2 and Dtpp mRNA levels were also significantly elevated by Tdo2 overexpression compared with those of controls (Fig. 8C and D). In contrast, the Tdo2 inhibitor 680C91 could inhibit the expression of Dtpp gene under in vitro decidualization and in the uterine decidual cells (Fig. 8E and F).

**Regulatory effects of Tdo2 on Ahr, Cox2, and Vegf expression**

In the in vitro cultured stromal cells, overexpression of Tdo2 could result in an increase in uterine aryl hydrocarbon receptor (Ahr), cyclooxygenase 2 (Cox2), and vascular endothelial growth factor (Vegf) mRNA levels at 48 h (Fig. 9A, C and E). In contrast, the Tdo2 inhibitor 680C91 could inhibit the expression of Cox2 and Vegf genes in the uterine decidual cells (Fig. 9D and F), whereas it had hardly any effect on Ahr expression (Fig. 9B).

**Discussion**

In this study, we have investigated the expression and regulation of Tdo2 in mouse uterus during early pregnancy in order to provide insight into the physiological function of Tdo2 during decidualization. The results showed that the Tdo2 mRNA signal was strongly detected in the decidua on days 6–8 of pregnancy using real-time PCR and in situ hybridization. This was consistent with previous results (Tatsumi et al. 2000). Likewise, increased
expression of Tdo2 was confirmed by using artificial decidualization and an in vitro decidualization system. These results indicate that Tdo2 may be involved in mouse decidualization. Decidualization is a process which is characterized by uterine stromal cells proliferating and differentiating into decidual cells with polyploidy (Das 2009). This study showed that Tdo2 could regulate the proliferation of uterine stromal cells and decidual cells. Moreover, overexpression of Tdo2 could stimulate the expression of the decidual marker Dtprp in uterine stromal cells, while inhibition of Tdo2 could cause a decrease in Dtprp mRNA level in uterine stromal cells and decidual cells. These results confirm the effect of Tdo2 on decidualization. Because both inhibition and targeted disruption of Tdo2 could lead to an increase in available tryptophan and circulating serotonin levels (Salter et al. 1995, Kanai et al. 2009), Tdo2 expression in decidualized cells demonstrated that Tdo2 might increase tryptophan catabolism in the decidual zone and decrease available tryptophan for production of serotonin that could inhibit the process of decidualization (Mitchell et al. 1983), which further supports a role for Tdo2 in decidualization.

Figure 6
Hormonal regulation of Tdo2 expression. (A) Real-time PCR analysis of Tdo2 expression in ovariectomized mouse uterus after estrogen (E) treatments for 0, 1, 3, 6, 12, and 24 h. (B) Tdo2 expression in ovariectomized mouse uterus after injection of progesterone (P). (C) Tdo2 expression in ovariectomized mouse uterus after injection of estrogen and ICI 182 780. (D) Tdo2 expression in ovariectomized mouse uterus after injection of progesterone and RU486. (E) Tdo2 expression after stromal cells were treated with estrogen, or both estrogen and ICI 182 780. (F) Tdo2 expression after stromal cells were treated with progesterone, or both progesterone and RU486. Data are presented as mean±S.E.M. Bars with different letters at the top differ significantly.

Figure 7
Effects of Tdo2 on cell proliferation. (A) Uterine stromal cells were treated with various concentration of Tdo2 inhibitor 680C91 after induction for in vitro decidualization for 24 h. Cell proliferation was determined by MTS assay. (B) Uterine decidual cells after 10 μM Tdo2 inhibitor 680C91 treatment were examined by MTS assay. (C) Uterine stromal cells after transfection with pc-Tdo2 plasmid were analyzed by MTS assay. Data are presented as mean±S.E.M. Bars with different letters at the top differ significantly.
**Figure 8**
Effects of Tdo2 on Dtprp expression. (A) Tdo2 mRNA expression following Tdo2 overexpression. (B) Effects of Tdo2 overexpression on Dtprp mRNA expression. (C and D) Effects of Tdo2 overexpression on Tdo2 and Dtprp mRNA expression. After transfection with control plasmid (empty pcDNA3.1 vector) or Tdo2 overexpression plasmid (pc-Tdo2) for 6 h, the stromal cells were induced for in vitro decidualization for 48 h. (E) Effects of Tdo2 inhibitor 680C91 on Dtprp expression in the uterine stromal cells. Stromal cells were treated for 48 h with Tdo2 inhibitor 680C91 after induction for in vitro decidualization for 24 h. (F) Effects of Tdo2 inhibitor 680C91 on Dtprp expression in the uterine decidual cells. After uterine decidual cells were treated with Tdo2 inhibitor 680C91 for 24 h, Dtprp mRNA expression was determined by real-time PCR. Data are presented as mean ± S.E.M. Bars with different letters at the top differ significantly.

*Tdo2* is a rate-limiting enzyme of the tryptophan degradation pathway, producing kynurenine (Thackray et al. 2008). In the glioma cell lines, kynurenine might activate the *Ahr* which is a ligand-activated transcription factor and might play a pivotal role in the physiology of reproduction, including regulation of ovarian function, establishment of an optimum environment for fertilization, and maintenance of pregnancy (Hernández-Ochoa et al. 2009, Opitz et al. 2011). This result showed that *Tdo2* could modulate the expression of *Ahr* in the uterine stromal cells. Further study found that *Ahr* could induce transcription of *Cox2* in breast cancer MCF7 cells (Degner et al. 2007). *Cox2* is an inducible rate-limiting enzyme in the biosynthesis of prostaglandins and implicated in mouse decidualization (Lim et al. 1997, Diao et al. 2007, Zhang et al. 2013). Inhibition or deficiency of *Cox2* could result in defective decidualization (Lim et al. 1997, Diao et al. 2007).

In this study, *Cox2* expression was stimulated by *Tdo2* overexpression in the uterine stromal cells and down-regulated by a *Tdo2* inhibitor in the uterine decidual cells, indicating that *Tdo2* might regulate mouse decidualization through influencing the expression of *Cox2*. Because *Cox2* could promote uterine angiogenesis primarily via the *Vegf* system (Matsumoto et al. 2002), we examined the effect of *Tdo2* on the expression of *Vegf*. Indeed, *Tdo2* might also modulate the expression of *Vegf* in uterine stromal cells and decidual cells. These results indicate that *Tdo2* might direct uterine angiogenesis by affecting the expression of *Cox2* and *Vegf* during decidualization.

Decidualization of endometrial stromal cells is a prerequisite for maintenance of pregnancy, and regulated by ovarian estrogen and progesterone (Zhang et al. 2013). Ovariectomy could lead to a reduction in the *Tdo2* activity in the homogenates of liver from mature rats.
Indeed, progesterone could upregulate the expression of Tdo2 mRNA in ovariectomized mouse uterus and uterine stromal cells. Moreover, the upregulation could be antagonized by the progesterone receptor antagonist RU486, indicating its requirement for this induction. Likewise, estrogen could also induce Tdo2 expression via estrogen receptors in ovariectomized mouse uterus and uterine stromal cells. However, another study found that neither estrogen nor progesterone had any obvious effect on the induction of Tdo2 mRNA in ovariectomized mouse uterus (Tatsumi et al. 2000). The discrepancy might be attributable to differences in sensitivity between real-time PCR and northern blot. Simultaneously, cAMP might also induce the decidualization of endometrial stromal cells and stimulate the expression of decidual marker genes. Moreover, cAMP might also mediate progesterone-dependent decidualization (Brar et al. 1997). Further study found that cAMP triggered decidualization with a sustained activation of the PKA pathway that could sensitize the stromal cells to the activity of progesterone (Gellersen & Brosens 2003, Logan et al. 2013). This study showed that cAMP could stimulate the expression of Tdo2 in the uterine stromal cells and that the stimulation was blocked by the PKA inhibitor H89, indicating that the action of cAMP on Tdo2 expression was mediated by the PKA signaling pathway. In addition, the pattern of Tdo2 expression on days 1–5 of pseudopregnancy was similar to that on days 1–5 of pregnancy, showing that Tdo2 expression was independent of the embryo. In conclusion, Tdo2 may play an important role during mouse decidualization and be regulated by estrogen, progesterone, and cAMP.

**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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**Figure 9**
Regulatory effects of Tdo2 on Ahr, Cox2, and Vegf expression. (A) Effects of Tdo2 overexpression on Ahr mRNA expression. After transfection with control plasmid (empty pcDNA3.1 vector) or Tdo2 overexpression plasmid (pc-Tdo2) in the stromal cells, Ahr mRNA expression was determined by real-time PCR. (B) Effects of Tdo2 inhibitor 680C91 on Ahr expression in the uterine decidual cells. (C) Effects of Tdo2 overexpression on Cox2 mRNA expression. (D) Effects of Tdo2 inhibitor 680C91 on Cox2 expression in the uterine decidual cells. (E) Effects of Tdo2 overexpression on Vegf mRNA expression. (F) Effects of Tdo2 inhibitor 680C91 on Vegf expression in the uterine decidual cells. Data are presented as mean ± S.E.M. Bars with different letters at the top differ significantly.
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