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 *Dtprp* 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

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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,

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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 L-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, Germeyer *et al*. 2009, Doherty *et al*. 2011, Zhang *et al*. 2013). According to our (B Guo and X-C Tian unpublished observations) microarray data, *Tdo2* was strongly expressed by day 8 decidua and deciduoma 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.

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 cocaging respectively (day 1 = day of vaginal plug). On days 1–4, pregnancy was confirmed by recovering embryos from the oviducts or uterus. 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′-CTCCCTGGAGTGCACGGTAT-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

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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 $2^{-\Delta\Delta Ct}$ 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% CO_2 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) ATGAGTGGGTGCCCGTTTGC-3′; 5′-CTCGAG (XhoI)

Table 1 Primers for real-time PCR

Genes	Primer sequences	Accession numbers	Size (bp)
Tryptophan 2,3-dioxygenase (*Tdo2*)	CTGGGGGATCCTCAGGCTAT TGTCACTGTACTCGGCTGTG	NM_019911	165
Decidual/trophoblast PRL-related protein (*Dtprp*)	AGCCAGAAATCACTGCCACT TGATCCATGCACCCATAAAA	NM_010088	119
Aryl hydrocarbon receptor (*Ahr*)	CCGAAGCACACGCAAATCAA CCCTTCCAGGGAAGTCCAAC	NM_013464	244
Cyclooxygenase 2 (*Cox2*)	CATCCCCTTCCTGCGAAGTT CATGGGAGTTGGGCAGTCAT	NM_011198	178
Vascular endothelial growth factor (*Vegf*)	ACGTCAGAGAGCAACATCACC CTGTGCTGTAGGAAGCTCATCTC	NM_001025257	90
Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)	GCCTTCCGTGTTCCTACCC TGCCTGCTTCACCACCTTC	NM_008084	102

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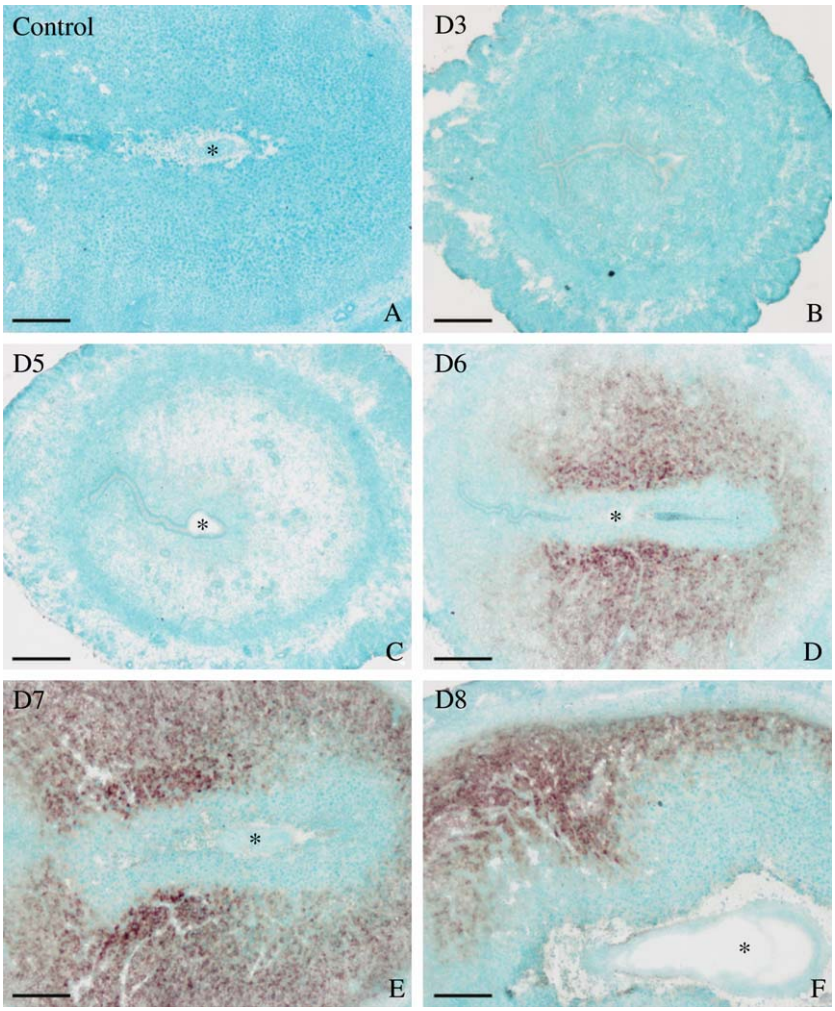

Figure 1
In situ hybridization of *Tdo2* expression in mouse uteri during early pregnancy on days 3 (B), 5 (C), 6 (D), 7 (E), and 8 (F). No hybridization signals were seen in mouse uterus on day 7 of pregnancy when DIG-labeled *Tdo2* sense probe was used to replace the antisense probe as a negative control (A). Asterisks indicate embryo. Bars = 60 μm.

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.

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

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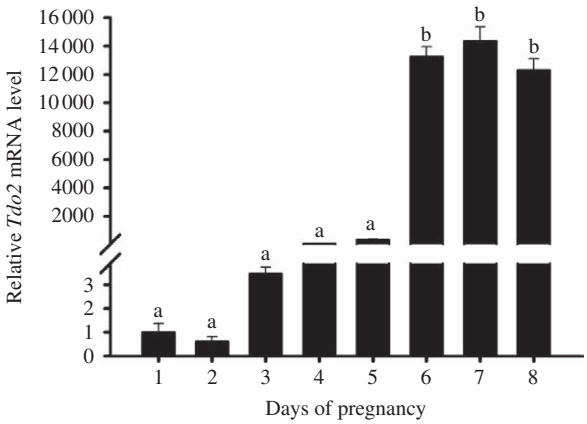

Figure 2
Real-time PCR analysis of *Tdo2* expression in mouse uterus on days 1–8 during pregnancy. Data are presented as mean±S.E.M. Bars with different letters at the top differ significantly.

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

Tdo2 mRNA expression during early pregnancy

In situ hybridization was used to examine the spatial distribution of *Tdo2* mRNA in mouse uterus. No *Tdo2* mRNA signal was detected in the uteri from days 1 to 5 of pregnancy (Fig. 1B and C). On day 6 of pregnancy, a high level of *Tdo2* mRNA signal was observed in decidualized cells surrounding the implanting embryo (Fig. 1D). On day 7, *Tdo2* expression was similar to that observed on day 6 but at much higher levels and scopes (Fig. 1E). When *Tdo2* antisense probe was replaced by *Tdo2* sense probe, there was no corresponding signal in the uterus on day 7 of pregnancy (Fig. 1A). On day 8, *Tdo2* mRNA was primarily localized in decidualized cells of the mesometrial region (Fig. 1F). In addition, *Tdo2* 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 *Tdo2* mRNA expression, real-time PCR was performed. *Tdo2* was highly expressed in the uteri from days 6 to 8 of pregnancy, although *Tdo2* expression was detected throughout days 1–8 (Fig. 2).

Tdo2 mRNA expression during pseudopregnancy

There was no detectable *Tdo2* mRNA signal in the uteri from days 1 to 5 of pseudopregnancy by *in situ* hybridization (Fig. 3A). However, a high level of *Tdo2* expression was detected on day 5 of pseudopregnancy by real-time PCR, although *Tdo2* expression was seen from days 1 to 5 (Fig. 3B).

Tdo2 expression under artificial decidualization

Tdo2 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 *Tdo2* expression was detected in the decidualized uterus compared with the control uterus (Fig. 3C).

Tdo2 expression under *in vitro* decidualization

Primary stromal cells isolated from mouse uteri 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 *Tdo2* were also observed in the decidualized stromal cells with a time-dependent increase (Fig. 4B).

Regulatory effects of cAMP and H89 on *Tdo2* 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 *Tdo2* 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 *Tdo2* 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 *Tdo2* expression was also dramatically altered by the addition of the PKA inhibitor H89 (Fig. 5B).

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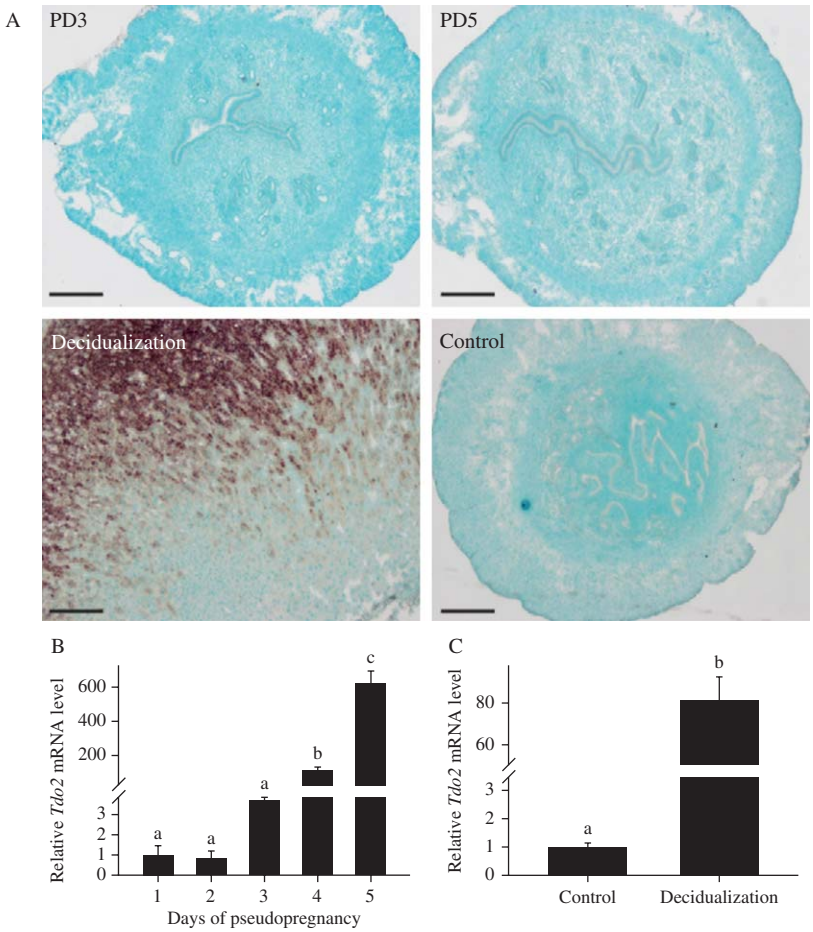

Figure 3

Tdo2 expression during pseudopregnancy and artificial decidualization. (A) *In situ* hybridization of *Tdo2* expression in mouse uteri during pseudopregnancy on days 3 (PD3) and PD5, and after artificial decidualization. Bar = 60 μm. (B) Real-time PCR analysis of *Tdo2* expression in mouse uterus on days 1–5 during pseudopregnancy. (C) Real-time PCR analysis of *Tdo2* expression under artificial decidualization. Data are presented as mean ± S.E.M. Bars with different letters at the top differ significantly.

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

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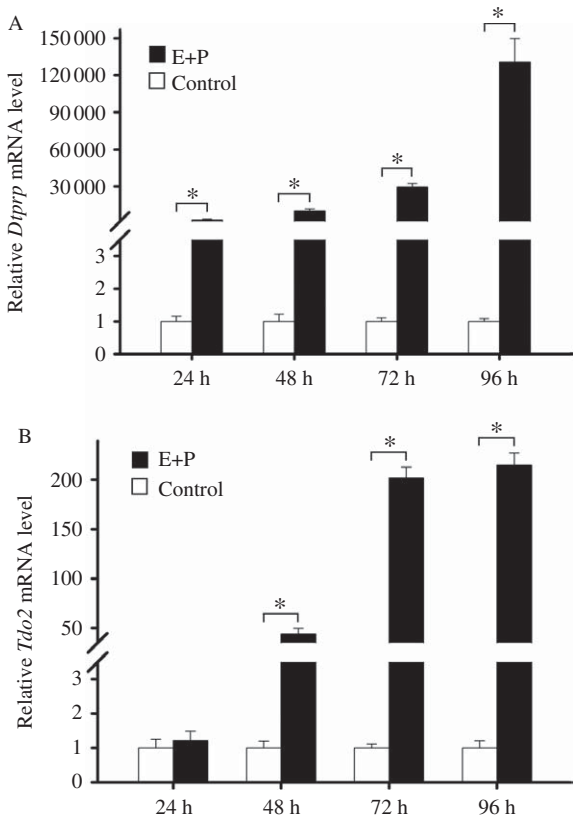

Figure 4
Real-time PCR analysis of *Dtprp* (A) and *Tdo2* (B) expression in *in vitro* decidualization of uterine stromal cells. Data are presented as mean±S.E.M. *Statistical significance ($P<0.05$); E, estrogen; P, progesterone.

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 *Dtprp* 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 *Dtprp* (Fig. 8B). Under *in vitro* decidualization, *Tdo2* and *Dtprp* 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 *Dtprp* 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

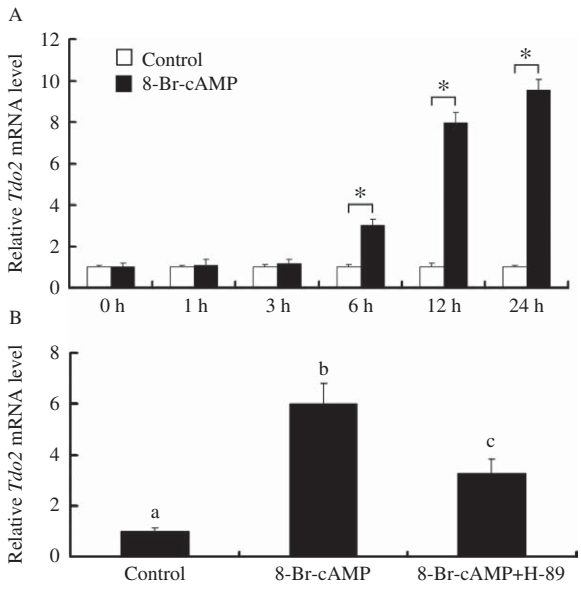

Figure 5
Effects of 8-Br-cAMP and H89 on *Tdo2* expression in the uterine stromal cells. (A) *Tdo2* expression in the uterine stromal cells after 8-Br-cAMP treatment. Data are presented as mean±S.E.M. *Statistical significance ($P<0.05$). (B) *Tdo2* expression after stromal cells were treated with 8-Br-cAMP, or both 8-Br-cAMP and H-89. Bars with different letters at the top differ significantly.

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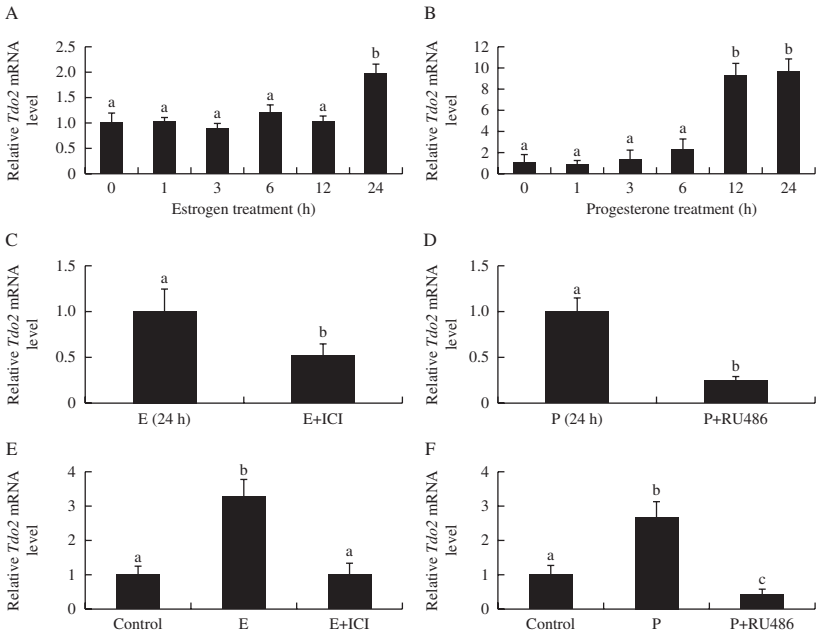

Figure 6
Hormonal regulation of *Tdo2* expression. (A) Real-time PCR analysis of *Tdo2* expression in ovariectomized mouse uterus after estrogen (E) treatments for 0, 1, 3, 6, 12, and 24 h. (B) *Tdo2* expression in ovariectomized mouse uterus after injection of progesterone (P). (C) *Tdo2* expression 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.

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.

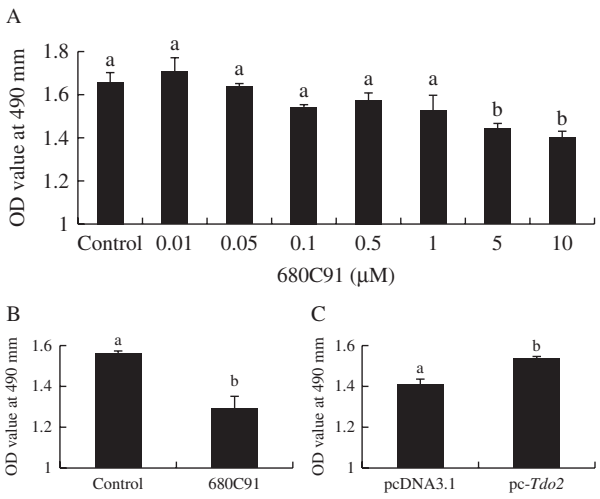

Figure 7
Effects of *Tdo2* on cell proliferation. (A) Uterine stromal cells were treated with various concentration of *Tdo2* inhibitor 680C91 after induction for *in vitro* decidualization for 24 h. Cell proliferation was determined by MTS assay. (B) Uterine decidual cells after 10 μM *Tdo2* inhibitor 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.

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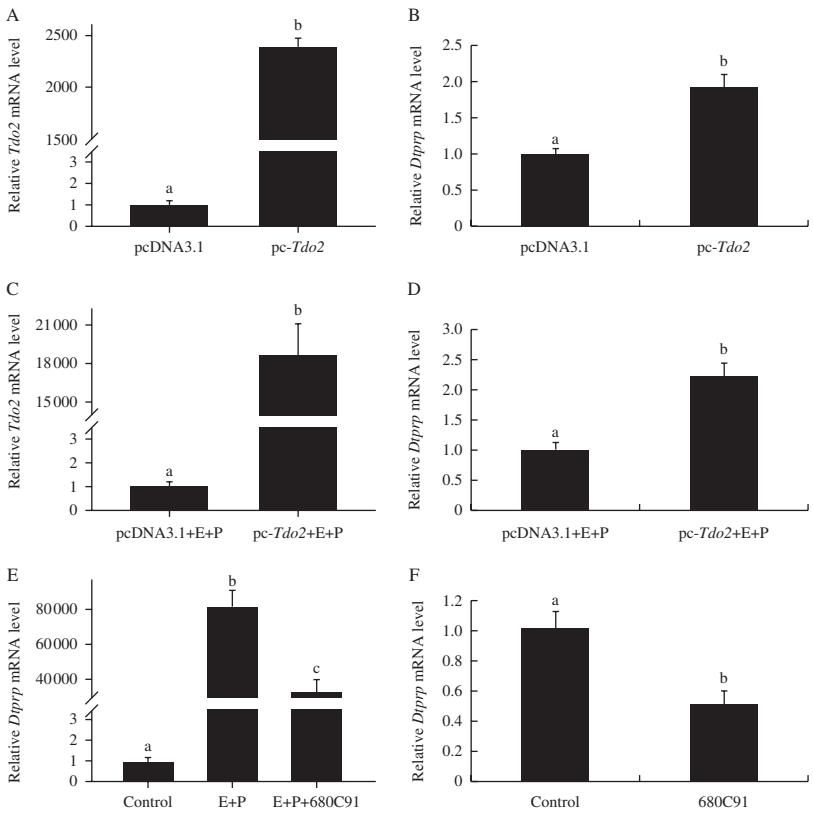

Figure 8

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

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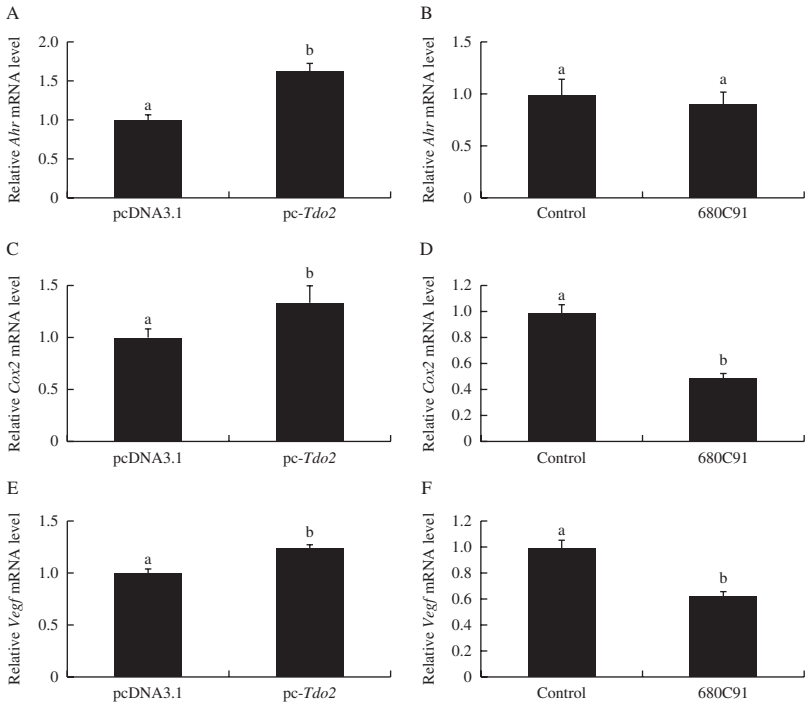

Figure 9

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.

(Bender *et al.* 1983, Oxenkrug 2010), indicating that ovarian hormones could regulate the expression of *Tdo2*. 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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