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Retinoic Acid-Metabolizing Enzyme Cytochrome P450 26a1 (Cyp26a1) Is Essential for Implantation: Functional Study of Its Role in Early Pregnancy

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Vitamin A (VA) is required for normal fetal development and successful pregnancy. Excessive VA intake during pregnancy may lead to adverse maternal and fetal effects. Cytochrome P450 26A1 (cyp26a1), a retinoic acid (RA)-metabolizing enzyme, is involved in VA metabolism. It has been shown that cyp26a1 is expressed in female reproductive tract, especially in uterus. In order to investigate the role of cyp26a1 during pregnancy, we constructed a recombinant plasmid DNA vaccine encoding cyp26a1 protein and immunized mice with the plasmid. Compared to control groups, the pregnancy rate of the cyp26a1 plasmid-immunized mice were significantly decreased ($P < 0.01$). Further results showed that both cyp26a1 mRNA and protein were specifically induced in the uterus during implantation period and localized in the uterine luminal epithelium. Importantly, the number of implantation sites was also significantly reduced ($P < 0.05$) after the uterine injection of cyp26a1-specific antisense oligos or anti-cyp26a1 antibody on day 3 of pregnancy. Accordingly, the expression of RA-related cellular retinoic acid binding protein 1 and tissue transglutaminase was markedly increased ($P < 0.05$) in the uterine luminal epithelium after intrauterine injection treatments. These data demonstrate that uterine cyp26a1 activity is important for the maintenance of pregnancy, especially during the process of blastocyst implantation.

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Vitamin A (VA) and its derivatives play important roles in mammalian reproduction. Severe deficiency of VA results in infertility, or impairs reproduction, mostly due to fetal resorption, stillbirths, and congenital malformations (Bates, 1983; Clagett-Dame and DeLuca, 2002). VA deficiency is also associated with an increased risk of preterm delivery and maternal anemia (Radhika et al., 2002), as well as abnormal placental apoptosis (Antipatis et al., 2002). However, excessive VA intake during pregnancy causes acute maternal hypervitaminosis A and birth defects called retinoic acid (RA) syndrome (Azais-Braesco and Pascal, 2000). RA, as a potent signaling molecule derived from VA, is the functional form of VA in female reproduction (Clagett-Dame and DeLuca, 2002). Its polar metabolites, such as 4-O-RA and 4-OH-RA, have been suggested to be the inactive products of RA. However, some reports have raised the possibility that these polar metabolites have important biological functions (Pijnappel et al., 1993; Sonneveld et al., 1999).

The intracellular level of active RA is dependent on the balance between its synthesis by retinaldehyde dehydrogenases (RALDHs) and its degradation by cytochrome P450 26 (CYP26) enzymes. RALDH1 and RALDH2 are expressed in mouse uterus during ovarian cycle and early pregnancy (Vermot et al., 2000), and their expression is regulated by estrogen in uterus (Deng et al., 2003). The cyp26 family was discovered as a group of RA-metabolizing enzymes responsible for catalyzing the transformation from RA to more polar metabolites for excretion (White et al., 1996, 2000; Taimi et al., 2004; Gu et al., 2006). Cyp26a1, the most important enzyme of the cyp26 family, has been shown to express in female reproductive tract (Vermot et al., 2000). In addition, ovarian steroid hormone progesterone induces the expression of cyp26a1 in mouse uterine luminal epithelium and glandular cells (Jeong et al., 2005; Fritzsche et al., 2007). In endometrial tissue obtained from premenopausal women, the cyp26a1 mRNA level was about 20 times higher in the secretory phase than in the proliferative phase (Deng et al., 2003), but in women with moderate or severe endometriosis, cyp26a1 was significantly down-regulated in both early secretory and midsecretory endometrium (Burney et al., 2007), suggesting its importance in both uterine physiology and pathology.

However, there is still no evidence supporting the roles of cyp26a1 during pregnancy. In this study, we constructed a recombinant plasmid DNA vaccine encoding cyp26a1 protein and immunized mice with the plasmid. The pregnancy rate of the plasmid-immunized mice was significantly decreased. Further results showed that both cyp26a1 mRNA and protein were expressed in the uterine luminal epithelium during blastocyst implantation period. Moreover, the number of implantation sites was markedly reduced after the uterine injection of cyp26a1-specific antisense oligos or anti-cyp26a1

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antibody. All these data suggest an important role of uterine cyp26a1 in the maintenance of pregnancy, especially during the process of blastocyst implantation.

Materials and Methods

Animals

Sexually mature, healthy female Kunming white (KM) and Balb/c strain mice (6–8 weeks old) were purchased from the Institute of Genetics and Development Biology, Chinese Academy of Sciences. Mice were housed in a temperature- and humidity-controlled room with a 12 h light/dark cycle. All animal manipulation procedures were approved by the Institutional Animals Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences. Female mice were caged overnight with male mice of the same strain, and the presence of vaginal plug was considered as D1 of pregnancy. Uteri were excised from D4 to D6 mice and frozen in liquid nitrogen for RNA and protein analysis. Pseudopregnancy was induced by caging adult females with vasectomized males and was confirmed by checking the vaginal plug. The presence of vaginal plug was considered as D1 of pseudopregnancy. To induce superovulation, female mice within their estrous cycle were intraperitoneally (i.p.) injected with 10 IU pregnant mare serum gonadotropin (PMSG) in 0.1 ml saline at 12:00, followed 48 h later by an i.p. injection of 10 IU human chorionic gonadotropin (HCG) in 0.1 ml saline. Following HCG injection, each female was caged with a male overnight.

Plasmid construction

Anti-fertility assay using DNA vaccine was performed as we described previously (Xiang et al., 2003; Shi et al., 2005; Sun et al., 2008). Full-length rat cyp26a1 cDNA was cloned from the uteri of pregnant rats, and specific primers with *Hin*dIII/*Xho*I restriction sites (forward primer: 5′CGAAGCTT (*Hin*dIII) ATGGGGCTCCCGGCGCTGCT3′; reverse primer: 5′CGCTCGAG (*Xho*I) TCAGATATCTCCCTGGAAGTGG3′) were used. The product was purified and cloned into pGEM-T vector (Promega, Madison, WI). Both pGEM-T-cyp26a1 and pCR3.1 vector (Invitrogen, Eugene, OR) were cut by *Hin*dIII/*Xho*I (Promega) at 37°C for 2 h, and then the fragment was ligated into pCR3.1 with T4 ligase (Promega) at 16°C overnight to construct pCR3.1-cyp26a1. The recombinant plasmid pCR3.1-cyp26a1 was digested by *Hin*dIII/*Xho*I at 37°C for 2 h and the insert was sequenced to confirm the correctness of construction. The expression ability of the recombinant plasmid was examined in vitro as described previously (Sun et al., 2008).

Anti-fertility assay

Plasmid pCR3.1-cyp26a1 and pCR3.1 were extracted using Endofree Plasmid Maxi kit (Qiagen, Valencia, CA). Fifty-seven healthy and sexually mature female KM mice and 27 Balb/c mice were used in the experiment. The mice were divided into three groups. One group was immunized with 100 μl saline containing 20 μg pCR3.1-cyp26a1 per mouse as treatment group (KM, n = 18; Balb/c, n = 9), and the other two groups were immunized, respectively, with 100 μl saline (KM, n = 19; Balb/c, n = 9) and 100 μl saline containing 20 μg pCR3.1 (KM, n = 20; Balb/c, n = 9) per mouse as control groups. All the mice were immunized by injecting plasmid into the leg muscle in three different spots. Twenty-four hours before immunization, each mouse was injected with 100 μl 0.25% bupivacaine as adjuvant in the same way. Immunization was performed three times in total with 1-week interval. Within 1 week after the last immunization, the female mice were coupled with male ones by the ratio of 2:1 until vaginal plugs were found. The mice that failed to mate within 1 week were excluded from the final statistics. The total number of the mice which gave birth and the newborns of treatment group were counted and compared with that of control groups.

ELISA

The rat recombinant cyp26a1 protein was obtained as described previously (Xia et al., 2008). Flat-bottomed 96-well microtiter plates (Corning, Corning, NY) were coated overnight at 4°C with 100 μl (1 μg/ml) per well of recombinant cyp26a1 antigen diluted in 0.05 M bicarbonate buffer (pH 9.6), washed three times in PBST (0.1% Tween-20 and 0.01% Triton-X-100 in phosphate-buffered saline (PBS)), and blocked with 1% BSA/PBS at room temperature for 0.5 h. After washing, 50 ml of sera of pCR3.1-cyp26a1 or pCR3.1 immunized mice at serial dilutions from 1:100 to 1:1,000 were added to each well. Normal serum at 1:100 dilution was used as the control. After incubation at 37°C for 2 h, 100 ml of secondary anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Jackson Company, Muskegon, MI) at a dilution of 1:2,000 was added for continual incubation at 37°C for 2 h. The color development kit (R&D Systems Inc., Minneapolis, MN) was used to detect the staining. The reaction was stopped by 1 M H_2SO_4, and the plate was measured with a plate reader (Bio-Rad, Hercules, CA) at 450 nm. Titers were defined as the final dilution giving an optical density of at least 0.1 unit above the optical density of the 1:50 dilution of the pre-immune serum. The pre-immune serum normally had an optical density of <0.1 units.

In situ hybridization

The total RNA of mouse (KM strain) uterus was extracted and reversely transcribed into cDNA. The cyp26a1 cDNA was amplified with forward primer 5′-GAGGAAGTCAGCAGTTGTC-3′ and reverse primer 5′-ATGACCACCAAAGAGGAG-3′ and cloned into pGEM-T vector (Promega) for sequence analysis. The plasmid was amplified with the primers specific to T7 and SP6 promoters to prepare the templates. Digoxigenin (DIG)-labeled sense (SP6) or antisense (T7) cRNA probes were transcribed in vitro using DIG RNA labeling kit (Roche, Mannheim, Germany).

The uteri were cut into 4–7 mm pieces, rapidly frozen in liquid nitrogen and stored at −80°C. Frozen sections (10 μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma–Aldrich, St. Louis, MO)-coated slides. Hybridization was performed as described previously (Ni et al., 2002). Sections were counterstained with 1% methyl green (Sigma–Aldrich). The sense probe was also hybridized and served as a negative control. There was no detectable signal from sense probes.

Real-time PCR

The total RNA of mouse (KM strain) uterus in the different periods of early pregnancy was reversely transcribed into single-stranded cDNA using M-MLV reverse transcriptase (Promega). cDNA was amplified using SYBR Green RealMasterMix (Tiangen, Beijing, China) according to the manufacturer's instructions. Real-time PCR was performed with ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The result was analyzed using ABI Prism 7000 SDS Software. After analysis using the ΔCt method, data were normalized to gapdh expression. Primers used for real-time PCR were as follows: cyp26a1—forward: 5′-CGAGAAGAGATAAA-GAGCAAG-3′, reverse: 5′-TCAGAGCAACCCGAAACC-3′ and gapdh—forward: 5′-TCCCACTCTTCCACCTTCG-3′, reverse: 5′-TCTCTTGCT-CAGTGTCCTTG-3′. To determine the expression of RA-related genes, the uterine luminal epithelium was isolated from the uterus which was treated with anti-cyp26a1 antibody or normal rabbit IgG. The isolation of uterine luminal epithelium was performed as described previously (Zhao et al., 2008). The primer sequences of RA-related genes are listed in Table 5.

Immunohistochemistry

The frozen sections (10 μm) of mouse (KM strain) uterus were mounted on 3-aminopropyltriethoxy-silane-coated slides and fixed in cold acetone for 10 min. After treated by 3% H_2O_2 at room

temperature for 10 min to inhibit endogenous peroxidase activity, the sections were blocked in goat serum at room temperature for 1 h. Then the sections were incubated with rabbit anti-cyp26a1 antibody (Acris, Hiddenhausen, Germany) diluted 1:100 in PBS overnight at 4°C. Normal rabbit IgG was used as negative control. After washing thoroughly in PBS, the sections were incubated with goat anti-rabbit IgG conjugated with HRP diluted 1:200 in PBS at room temperature for 1 h. Color was developed using diaminobenzidine tetrahydrochloride (Sigma–Aldrich). Sections were counterstained with hematoxylin (Sigma–Aldrich).

Western blot

Proteins were extracted from mouse (KM strain) uterus by nondenaturing lysis buffer (Applygen, Beijing, China) and the concentration was determined by Bio-Rad protein assay (Bio-Rad). Uterine proteins were separated on 12% SDS–PAGE and electroblotted onto a nitrocellulose membrane (Pall, New York, NY). After blocking in 5% nonfat milk at 37°C for 3 h, the membranes were incubated with rabbit anti-cyp26a1 antibody (1:100; Acris) or rabbit anti-actin antibody (1:500; Santa Cruz, Santa Cruz, CA) overnight at 4°C. Then the membrane was washed thoroughly in TBST and incubated with goat anti-rabbit IgG conjugated with HRP (Jackson Company) diluted by 1:2,000 in TBST at 37°C for 1 h. Chemiluminescence reaction was performed with enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

Preparation of morpholino oligos

Intrauterine injection of antisense oligonucleotide morpholino oligos (MO) was performed as described previously with minor modification (Luu et al., 2004). The following MOs (Gene Tools, Philomath, OR) were used: cyp26a1 MO, 5′-CATGGCACGC-TTCAGCCTCCCGCGC-3′ (cyp26a1-MO); irrelevant MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (standard control MO, Std-MO); and FITC-labeled standard MO (FITC-MO). The MOs were prepared at a stock concentration of 6 mM. For intrauterine injection, 10 μl of the weak-base delivery reagent, ethoxylated/polyethylenimine (EPEI, Gene Tools) was mixed with 5 μl of MO stock, resulting in 15 μl of solution containing 30 nmol of MO. The MO/EPEI mixture was vortexed for 30 sec and incubated for 20 min at room temperature to allow the MO/EPEI delivery complexes to form before use.

Treatment of early pregnant mice with MOs

Before the intrauterine injection of the MOs, we first analyzed the uterine penetrance of the MOs. We injected 15 μl FITC-MO/EPEI into one uterine horn and Std-MO/EPEI into the contralateral uterine horn of a nonpregnant mouse. Forty-eight hours later, the mice were killed and their uteri were excised and frozen in OCT compound (TissueTek, Sakura, Japan). Ten-micrometer-thick frozen sections were then analyzed under a fluorescence microscope at 488 nm.

For treatment of early pregnant mice with MOs, mice (KM strain) were anesthetized by i.p. injection of ethyl carbamate (20% w/v solution, 1 g/kg; Sigma) at 8:00 am on D3 of pregnancy. The skin on both sides of dorsal midline was incised and each uterine horn was exposed using small forceps. Under a dissecting microscope, MOs were delivered into the uterine lumen through the uterotubal junction using a 26-gauged Hamilton syringe. A volume of 15 μl of cyp26a1 MO/EPEI was injected into one uterine horn, and the same volume of nonspecific Std-MO/EPEI was injected into the contralateral horns, as its own internal control. After administration, the incision was closed and the mice were placed in warmed cages until full recovery from the anesthetic. On the morning of D5, implantation sites were visualized after tail vein injection of trypan blue dye solution (1% in saline, 0.1 ml per mouse) 5 min before the mice were killed and uteri were excised. Numbers of implantation sites were recorded.

Treatment of early pregnant mice with antibody

Intrauterine injection of anti-cyp26a1 antibody was performed as described previously (Ramos et al., 2005). KM strain mice were used in this experiment. The surgical procedure for intrauterine injection of anti-cyp26a1 antibody was similar to that described above. A volume of 10 μl (1 mg/ml) rabbit anti-cyp26a1 antibody (Acris) was intraluminally injected into one uterine horn, and the contralateral horn received the vehicle or similar volume of nonspecific rabbit IgG as its own internal control. To analyze the effect of anti-cyp26a1 antibody on blastocyst implantation after treatment, the mice were euthanized on D5 of pregnancy. The uteri were removed, and the numbers of implantation sites were counted in each uterine horn.

Embryo transfer

On D4 of pregnancy, pregnant donor mice (KM strain) were killed and the uteri were excised, pre-implantation blastocysts were flushed from the uteri with M2 medium (Sigma–Aldrich), washed twice in M2 medium, and transferred to a 20 μl droplet of M16 medium, which contained cyp26a1 MO/EPEI or anti-cyp26a1 antibody at the same concentration as used in intrauterine injection experiment. The droplet was overlaid with paraffin oil (Sigma–Aldrich) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 36 h. Then, the embryos were divided randomly and transferred into the uteri on D4 of pseudopregnancy. The surgical procedure was performed as described above for intrauterine injection experiment. Forty-eight hours later, the mice were killed and the uteri were removed, and the numbers of implantation sites were counted.

Statistical analysis

Values were reported as mean ± SEM. The significance of difference was assessed by one-way ANOVA or paired *t*-test. $P < 0.05$ was considered statistically significant. Statistical analysis was conducted with SPSS 15.0 software (SPSS Software, Chicago, Chicago, IL).

Results

Anti-fertility assay

To determine the role of cyp26a1 during pregnancy, we immunized mice by recombinant plasmid encoding cy26a1 protein. The applicability of DNA vaccine in the studies of reproduction was confirmed elsewhere (Xiang et al., 2003; Shi et al., 2005; Sun et al., 2008). Previously, we showed that heterogenous antigen could not induce host immune tolerance and had no cytotoxic responses that might result in abnormal reproductive function or other damage (Xiang et al., 2003). Here, rat-derived full-length cyp26a1 cDNA (share 94% homology with mouse cyp26a1 cDNA sequence) was used to construct the recombinant plasmid pCR3.1-cyp26a1 DNA vaccine. The expression of cyp26a1 protein by the recombinant plasmid pCR3.1-cyp26a1 DNA vaccine was confirmed in vitro (Fig. S1).

To confirm the production of anti-cyp26a1 antibody by pCR3.1-cyp26a1 in vivo, we performed ELISA assay. The sera samples from the mice immunized with pCR3.1 or pCR3.1-cyp26a1 were analyzed by ELISA. As shown in Figure 1, at 1:100, 1:250, and 1:500 dilutions, the antibody titer of the serum from the mice immunized with pCR3.1-cyp26a1 was higher ($P < 0.05$) than that from the mice immunized with pCR3.1.

Both KM and Balb/c strain mice were immunized for detecting the effect of the pCR3.1-cyp26a1 on the pregnancy rate of mice. As shown in Tables 1 and 2, the pregnancy rate of KM mice immunized with saline or pCR3.1 was 73.7% and 75%, respectively, while it was 77.8% and 88.9%, respectively, in Balb/c mice. In contrast, in the group immunized with pCR3.1-cyp26a1, the pregnancy rate was reduced significantly

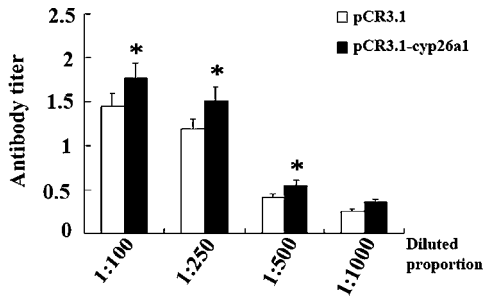

Fig. 1. The antibody titer of the sera samples from the mice immunized with pCR3.1 or pCR3.1-cyp26a1 were analyzed by ELISA. The sera were diluted from 1:100 to 1:1000. *$P < 0.05$.

(38.8% and 55.6% in KM and Balb/c strain mice, respectively) compared to the above two groups, indicating an important role of cyp26a1 in the maintenance of pregnancy. There was no significant difference in the number of newborns among the three groups (saline, pCR3.1 and pCR3.1-Cyp26a1) in KM strain mice (8.9 ± 0.65, 11.5 ± 0.57, and 10.4 ± 1.36, respectively). In Balb/c strain mice, however, the number of newborns in the group immunized with pCR3.1-cyp26a1 significantly decreased (4.6 ± 0.51) compared to that of saline or pCR3.1 group (6.1 ± 0.51 and 6.0 ± 0.38, respectively); this may be due to the strain difference.

Expression pattern of cyp26a1 mRNA and protein in mouse uterus during peri-implantation period and during pseudopregnancy

To further study the role of cyp26a1 in mouse pregnancy, we examined the expression pattern of cyp26a1 mRNA and protein in mouse endometrium during early pregnancy. By in situ hybridization, we did not detect cyp26a1 expression signal on D4 of pregnancy. Interestingly, on D5 and D6, a strong cyp26a1 mRNA expression signal was observed specifically in the luminal epithelium at implantation site (Fig. 2A). To quantify cyp26a1 mRNA expression, real-time PCR was performed. As expected, uterine cyp26a1 expression level was significantly induced on D5 and D6 ($P < 0.01$) (Fig. 2B). We further examined cyp26a1 protein localization in peri-implantation mouse uterus by immunohistochemistry. We showed that cyp26a1 protein staining was also detected strongly and specifically in the luminal epithelium at implantation site on D5 and D6, which was consistent with the result from in situ hybridization (Fig. 2C,D). The expression pattern of cyp26a1 mRNA and protein suggests its potential roles in blastocyst implantation.

To determine whether cyp26a1 expression was dependent on the embryo, cyp26a1 expression during pseudopregnancy was examined. By in situ hybridization and immunohistochemistry, both cyp26a1 mRNA and protein were detected on D5 and D6, but not on D4 during pseudopregnancy (Fig. 3). On D5 and D6, a strong level of cyp26a1 mRNA and protein expression was detected in the uterine luminal epithelium. The similar expression pattern of cyp26a1 in pseudopregnant and normal pregnant mice indicates that the expression of cyp26a1 in endometrium during peri-implantation period is independent on embryo, and its expression is under maternal control.

Intrauterine injection treatments

To further determine the roles of uterine cyp26a1 during peri-implantation period, intrauterine blocking treatments were performed by MOs or antibody injection. First, we examined the uterine penetrance of MOs by fluorescence microscopy assay. As shown in Figure 4A, there was only autofluorescence in the uteri which were treated with unlabeled control standard MOs. However, in the contralateral uteri which were treated with FITC-MOs, fluorescence was observed in luminal epithelium and underlying stroma cells (Fig. 4B). This result indicated that the MOs had penetrated the luminal epithelium and the underlying stromal cells in vivo and there was no significant transfer of MOs between uterine horns. In order to examine the efficacy of the cyp26a1-MOs in inhibiting the production of cyp26a1 protein in vivo, total proteins of the uteri that were treated with cyp26a1-MOs or Std-MOs were extracted and analyzed by Western blot. As shown in Figure 5A, cyp26a1 protein level was remarkably decreased ($P < 0.05$).

We found that the number of implantation sites on D5 was significantly reduced after the intrauterine injection of Cyp26a1-specific MOs on D3 (2.3 ± 0.6) compared to that of Std-MOs group (6.2 ± 0.8) (Fig. 5B and Table 3). To further confirm this implantation-blocking effect of cyp26a1, we performed intrauterine injection of cyp26a1 antibody. By intrauterine injection of anti-cyp26a1 antibody, we again showed that the number of implantation site was decreased in the uteri injected with anti-cyp26a1 (2.7 ± 1.5) antibody compared to the control group (6.7 ± 1.3) (Fig. 5C and Table 3), further confirming the data from the MOs treatments. Taken together, these data demonstrate that the knock-down of cyp26a1 in mouse endometrium during early pregnancy inhibits blastocyst implantation.

To examine whether the MOs or antibody are harmful to pre-implantation embryos, embryo transfer experiment was performed. There was no difference between normal embryos and MOs- or antibody-treated embryos in the number of implantation sites (Table 4), indicating that the MOs or antibody did not affect embryo development, and the embryos surrounded by the intrauterine injected cyp26a1-MO or anti-cyp26a1 antibody were still implantation-competent.

Expression patterns of RA-related genes in mouse uterine luminal epithelium after intrauterine injection

To determine whether cyp26a1 actually reduced the level of RA in uterine luminal epithelium when blastocyst implantation occurred, we examined the expression of RA-related genes

TABLE 1. The pregnant rate and the number of newborns in immunized Kunming white strain mice

Group	Number of mice (with vaginal plug)	Number of mice (gave birth)	Pregnant rate (%)	Number of newborns
Saline (0.9%)	19	14	73.7	8.9 ± 0.65 (n = 124)
pCR3.1	20	15	75	9.1 ± 0.57 (n = 137)
pCR3.1-Cyp26a1	18	7	38.8[a]	10.4 ± 1.36 (n = 73)

Values are mean ± SEM.
[a]The pregnant rate in pCR3.1-Cyp26a1 group was significantly different from control groups ($P < 0.01$); n: total number of newborns in each group. Pregnant rate was the ratio of "number of mice (gave birth)" to "number of mice (with vaginal plug)."

TABLE 2. The pregnant rate and the number of newborns in immunized Balb/c strain mice

Group	Number of mice (with vaginal plug)	Number of mice (gave birth)	Pregnant rate (%)	Number of newborn
Saline (0.9%)	9	7	77.8	6.1 ± 0.51 (n = 43)
pCR3.1	9	8	88.9	6.0 ± 0.38 (n = 48)
pCR3.1-Cyp26a1	9	5	55.6[a]	4.6 ± 0.51 (n = 23)[a]

Values are mean ± SEM.
[a]Both the pregnant rate and the number of newborns in pCR3.1-Cyp26a1 group were significantly different from control groups ($P < 0.01$); n: total number of newborns in each group. Pregnant rate was the ratio of "number of mice (gave birth)" to "Number of mice (with vaginal plug)."

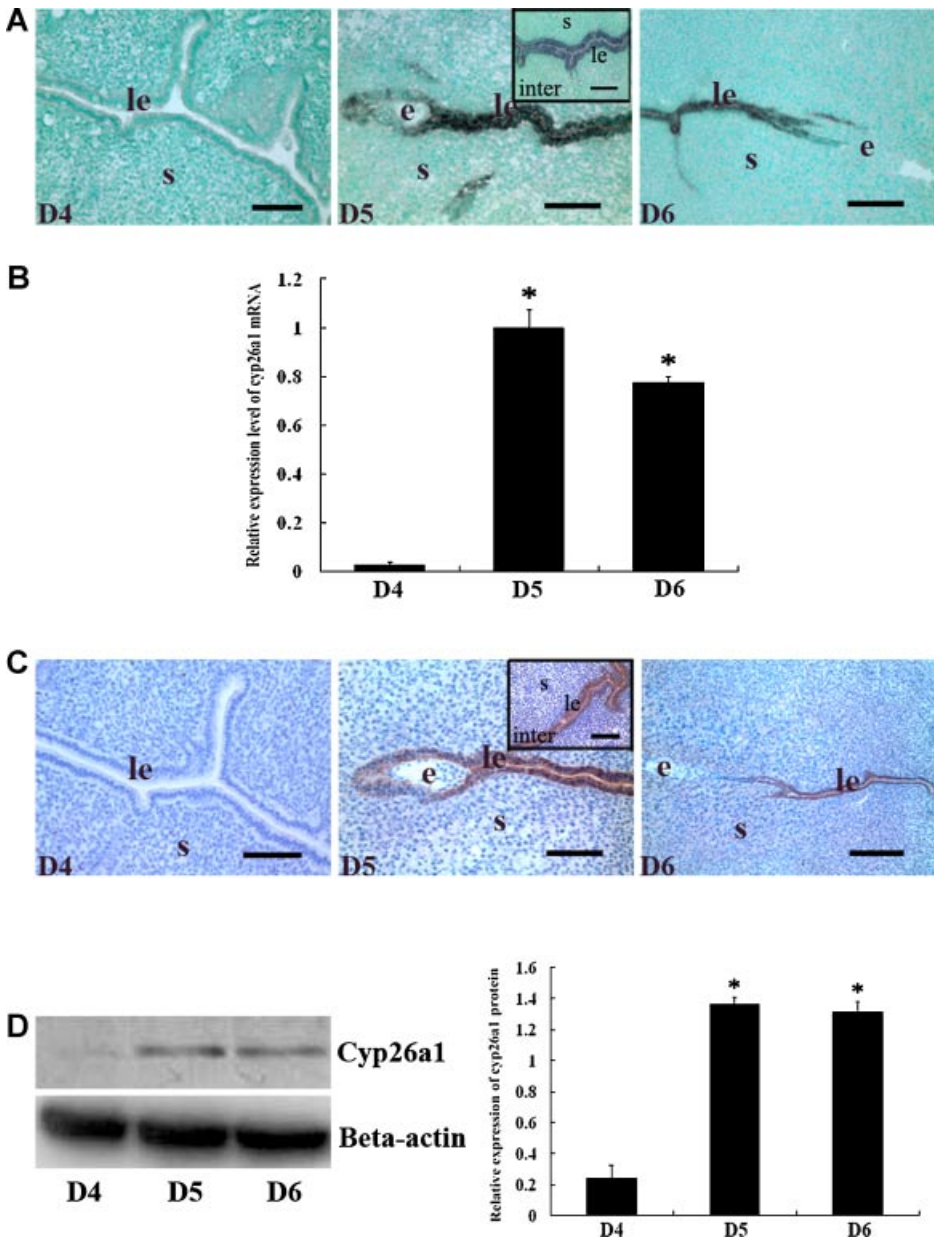

Fig. 2. Cyp26a1 expression during peri-implantation period. A: In situ hybridization of cyp26a1 mRNA expression in mouse endometrium on D4, D5, and D6 of pregnancy. B: Quantification of cyp26a1 mRNA expression in mouse endometrium on D4, D5, and D6 of pregnancy by real-time PCR. The expression level was normalized with Gapdh expression. C: Immunohistochemistry of cyp26a1 protein expression in mouse endometrium on D4, D5, and D6 of pregnancy. D: Western blot analysis of cyp26a1 protein in mouse endometrium on D4, D5, and D6 of pregnancy. The protein level of cyp26a1 was quantitated by measuring OD of each band and normalized with beta-actin protein level. e, embryo; le, luminal epithelium; inter, inter-implantation sites; s, stroma. Bar = 100 μm. *$P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

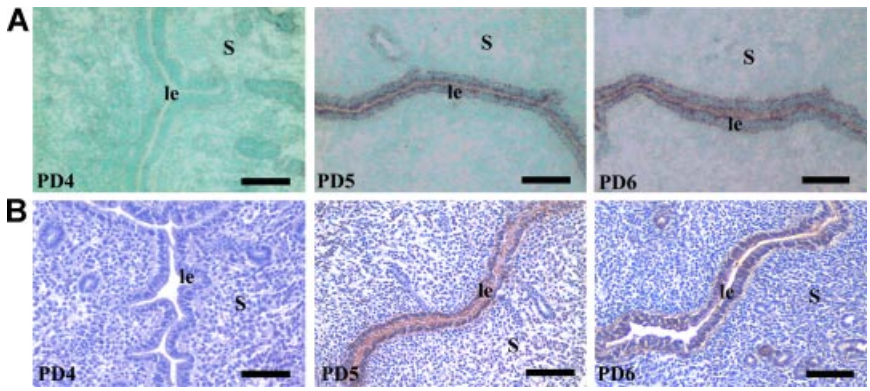

Fig. 3. Cyp26a1 expression during pseudopregnancy. A: In situ hybridization of cyp26a1 mRNA expression in mouse endometrium on D4, D5, and D6 during pseudopregnancy. B: Immunohistochemistry of cyp26a1 protein expression in mouse endometrium on D4, D5, and D6 during pseudopregnancy. le, luminal epithelium; s, stroma. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

after intrauterine injection. These included RA receptors RAR-α, RAR-β, and RAR-γ; RA binding proteins CRABP1 and CRABP2; and RA target gene tissue transglutaminase (tTG). Real-time PCR results showed that CRABP1 and tTG expressions were significantly increased ($P < 0.05$) in the uterine luminal epithelium treated with anti-cyp26a1 antibody compared to that in normal rabbit IgG-treated uterine luminal epithelium (Fig. 6). However, there was no significant difference between the two groups in the level of RAR-α and RAR-γ. CRABP2 and RAR-β were almost not detected in both the groups.

Discussion

In our study, the pregnancy rate of the mice immunized with recombinant plasmid pCR3.1-cyp26a1 was significantly decreased, and more importantly, the number of implantation sites in the mice treated with cyp26a1-specific antisense oligos cyp26a1-MO or anti-cyp26a1 antibody was markedly reduced. These data highlight that cyp26a1 is an important regulator for the maintenance of pregnancy and it is especially essential for blastocyst implantation.

RA, as a potent signaling molecule derived from VA, has been shown to play an important role in female reproduction. RA regulates the differentiation of the uterine epithelium (Ponnamperuma et al., 1999) and the expression of matrix metalloproteases (MMPs) which have been shown to mediate the blastocyst implantation by the reduction of endometrial integrity (Osteen et al., 2003). Moreover, its synthesizing enzymes (RALDH1, RALDH2), receptors (RARs, RXRs), and cellular RA-binding proteins (CRABPI, CRABPII) express coordinately with a temporal- and spatial-specific manner in uterus (Bucco et al., 1996; Zheng and Ong, 1998; Vermot et al., 2000; Fukunaka et al., 2001). Although these data suggest that RA signaling may be important for normal female reproduction, intake of excessive RA during pregnancy has many adverse effects, especially on the development of embryo (Lammer et al., 1985; Ross et al., 2000; Clagett-Dame and DeLuca, 2002). Thus, an RA-metabolizing enzyme appears particularly necessary for protecting tissues from excessive RA. Cyp26a1 was identified as a metabolizing enzyme catalyzing all-trans-RA to more polar metabolites (White et al., 1996). As an RA-metabolizing enzyme, cyp26a1 creates an RA gradient for normal embryonic development (Hernandez et al., 2007; White et al., 2007), and the disruption of the cyp26a1 gene causes many developmental defects (Abu-Abed et al., 2001; Sakai et al., 2001). However, in addition to the process of embryo development, whether the maternal reproductive system also needs such an enzyme to avoid excessive RA for maintaining the pregnancy is unknown. Previous studies show that cyp26a1 has a specific expression and regulation in uterus (Vermot et al., 2000; Deng et al., 2003; Jeong et al., 2005; Burney et al., 2007; Fritzsche et al., 2007). Thus, we speculate that the maternal-derived cyp26a1 may participate in early pregnancy.

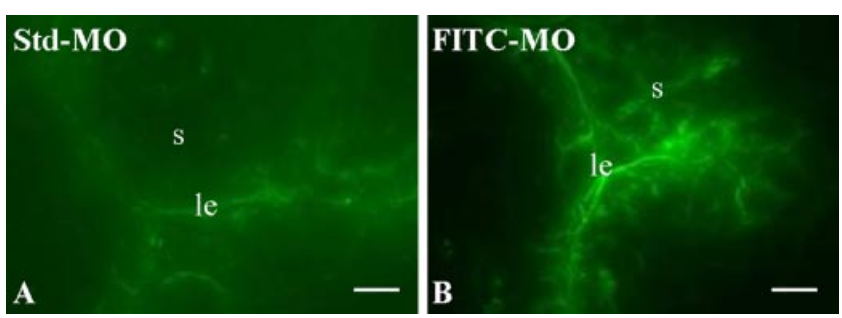

Fig. 4. Assessment of the uterine penetrance of MOs under a fluorescence microscope at 488 nm. A: Uterine horn treated with unlabeled control Std-MOs. B: Uterine horn treated with FITC-labeled control FITC-MOs. le, luminal epithelium; s, stroma. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

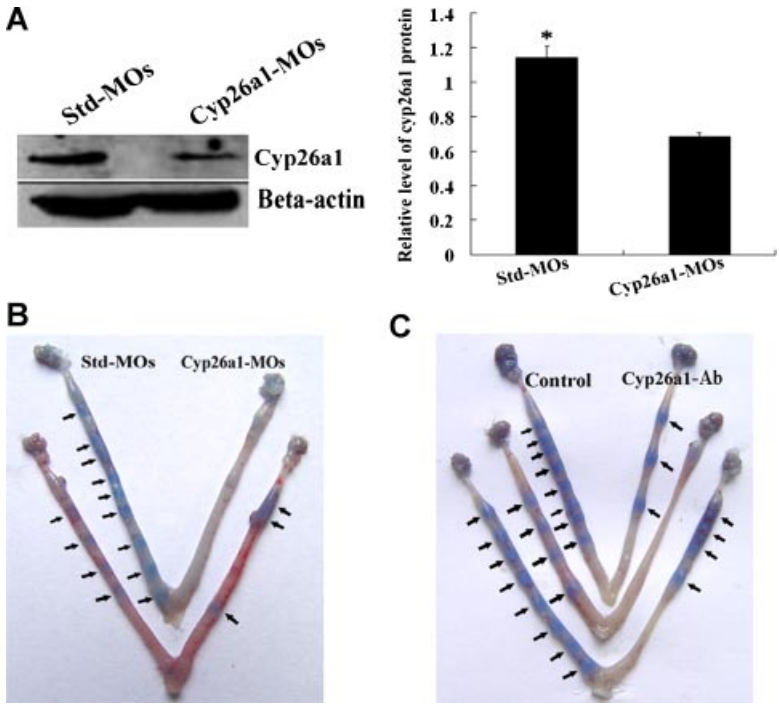

Fig. 5. Intrauterine blocking treatments with Cyp26a1-MOs or anti-cyp26a1 antibody. A: Western blot analysis of cyp26a1 protein in mouse uteri treated with Std-MOs or Cyp26a1-MOs. The protein level of cyp26a1 was quantitated by measuring OD of each band and normalized with beta-actin protein level. B: Uterine horns were injected with Std-MOs or Cyp26a1-MOs. C: Uterine horns were injected with anti-cyp26a1 antibody (Cyp26a1-Ab), and the contralateral horns were treated with vehicle or rabbit IgG as control. Arrows show the blastocyst implantation sites. $^{*}P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In this study, DNA vaccine was adopted to investigate the effect of cyp26a1 on mouse pregnancy. DNA vaccine has many advantages over antigen immunization. It can induce a long duration of antibody response and can generate th1/th2-type immune responses (Davis et al., 1996; Casares et al., 1997; Donnelly et al., 1997). In addition, the antibody produced by DNA vaccine has a greater avidity than the protein-raised antibody, even though the antigen produced by the vaccine was smaller than the antigen administered exogenously (Boyle et al., 1997). Compared to the control groups, the pregnancy rate in both Kunming and Balb/c strain mice immunized with the recombinant plasmid pCR3.1-cyp26a1 was significantly reduced. This result demonstrates that the maternal-derived cyp26a1 is essential for the maintenance of successful pregnancy. In consistent with this result, progesterone, as a steroid hormone that is essential for the establishment and maintenance of pregnancy (Spencer and Bazer, 2002), can induce the expression of cyp26a1 in mice uterus (Jeong et al., 2005; Fritzsche et al., 2007). This suggests that cyp26a1 may act as a downstream effector of progesterone and mediates the establishment or maintenance of pregnancy.

A previous report showed that the expression of cyp26a1 mRNA appeared in the mouse uterine epithelium during pre-implantation period and reached a maximal intensity during implantation period (Vermot et al., 2000). However, in this study, no cyp26a1 mRNA was found in pre-implantation endometrium, while its expression was specifically induced in the uterine luminal epithelium during implantation and post-implantation period. This expression pattern was further confirmed by immunohistochemistry and Western blot. Correspondingly, cyp26a1 protein presented the same expression pattern as its mRNA. Moreover, the expression of cyp26a1 in endometrium during peri-implantation period was independent on embryo, and its expression was under maternal control. These data show that both cyp26a1 mRNA and protein have a specific expression pattern in peri-implantation endometrium. Thus, we hypothesize that cyp26a1 may participate in blastocyst implantation process to support the establishment or maintenance of pregnancy. To confirm this hypothesis, intrauterine blocking treatments were performed. Cyp26a1-specific antisense oligos cyp26a1-MOs or anti-cyp26a1 antibody were injected into mice uterine lumen.

TABLE 3. The number of implantation sites in intrauterine-treated mice

Group	N	Control	Experimental
MOs	6	6.2 ± 0.8	2.3 ± 0.6^{a}
Antibody	3	6.7 ± 1.3	2.7 ± 1.5^{a}

Values are mean $\pm$ SEM.
[a]The number of implantation sites was significantly decreased both in MOs and antibody groups ($P < 0.05$); Std-MOs were used as control in MOs group; Vehicle or rabbit IgG were used as control in antibody group; N: total number of the intrauterine-treated mice.

TABLE 4. The number of implanted embryos in recipient mice

Group	N_1	N_2	N_3
Control	3	10	6.3 ± 0.88
MOs	3	10	5.3 ± 0.33
Antibody	3	10	6.0 ± 0.58

Values are mean $\pm$ SEM. Embryos were flushed from uteri on D4 of pregnancy, and cultured in cyp26a1-MOs or anti-cyp26a1 antibody in vitro for 36 h, then transferred into uteri on D4 of pseudopregnancy. Forty-eight hours later, the number of implantation sites was counted. N_1: total number of recipient mice; N_2: total number of transferred embryos per mouse; N_3: number of implanted embryos per mouse.

TABLE 5. Primer sequences of RA-related genes for real-time PCR assay

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession number
RAR-α	CCTGGACATTGACCTCTG	AGGTCATTGTGTCTTGCTC	NM_009024
RAR-β	ACAAGTCATCGGGCTACC	TTCTTGTTCCTGTCATTCC	NM_011243
RAR-γ	TGGGACAAGTTCAGCGAG	TGTCATAGTGTCCTGCTCTGG	NM_011244
CRABP1	AAACTTACTGGACCCGAGAG	GTGTGTGGAGTAAAGACCC	NM_013496
CRABP2	TGAGGAAGATCGCTGTGG	TTTCCACTCTCCCATTTC	NM_007759
tTG	AAGGCTCTGTCAAGTTCATC	TGGTCATCATTGCAGTTG	NM_009373
GAPDH	TCCCACTCTTCCACCTTCG	TCTCTTGCTCAGTGTCCTTG	BC096042

Both treatments significantly decreased the number of implantation sites, suggesting that cyp26a1 is an important mediator for the blastocyst implantation process. The specific spatiotemporal expression pattern of cyp26a1 in peri-implantation endometrium implies that cyp26a1 participates in implantation, via mediating blastocyst attachment and/or invasion to the endometrium, but not in initial preparations of endometrium.

It has been shown that RA-synthesizing enzymes RALDH1 and RALDH2 are expressed in mouse uterus during early pregnancy (Vermot et al., 2000), and their expression is regulated by estrogen in uterus (Deng et al., 2003). The biological effects of RA are mediated by its binding proteins and receptors. CRABPI and CRABPII are up-regulated in the uterus of pseudopregnant rat after induced by PMSG (Bucco et al., 1996), and the up-regulation of CRABPII in rat uterine epithelial cells correlates with the synthesis of RA (Bucco et al., 1997). This correlation was further confirmed in rat uterine decidual cells (Zheng et al., 2000). Furthermore, CRABPI and CRABPII are also expressed in the rat uterus during peri-implantation period (Zheng and Ong, 1998). RA receptors, RARs, and RXRs have a specific distribution in human endometrium throughout the menstrual cycle (Kumarendran et al., 1996; Fukunaka et al., 2001) and during implantation period (Tarrade et al., 2000). These results indicate that there is an accumulation of RA during early pregnancy, especially during implantation period. In this respect, it is interesting to note that RA strongly inhibits the decidualization of human endometrial stromal cells in vitro (Brar et al., 1996). Furthermore, there is evidence that RA can inhibit the effect of MMPs on reducing the endometrial integrity that is essential for blastocyst implantation (Bruner-Tran et al., 2002). From these data and the present results, it appears that cyp26a1 may be a physiological pathway for balancing the endogenous RA level in the maternal endometrium to support blastocyst implantation. And the expression of cyp26a1 may establish a proper level of RA in endometrium during implantation period to protect the blastocyst from excessive RA. To test this hypothesis, we used HPLC and LC-ESI-MS/MS method to examine the level of RA in the uterine luminal epithelium after intrauterine injection treatments. Unfortunately, because of the limited samples, we did not detect any RA in the uterine luminal epithelium (data not shown). Indeed, previous literature reported that large amounts of tissues are required for the detection of endogenous retinoids (Napoli, 1986). As an alternative, we applied real-time PCR to examine the expression of RA-related genes to indirectly determine the level of RA. Among these genes, CRABP1 and tTG mRNA were significantly increased in the uterine luminal epithelium in which cyp26a1 activity was blocked. A previous study has showed that tTG is a gene whose expression highly correlates with the level of RA in vivo (Verma et al., 1992). Thus, increased tTG may reflect a higher RA level in the uterine luminal epithelium in which the metabolism of RA by cyp26a1 is disturbed. This higher level of RA appears to need more binding protein such as CRABP1 to maintain the physiological level of free RA in cytoplasm. These results indicate that cyp26a1 may actually reduce the level of RA in uterine luminal epithelium to facilitate blastocyst implantation in normal pregnancy. Indeed, excessive RA decreases the viability of mouse blastocysts in vitro, because RA induces cell death and inhibits cell proliferation of blastocysts (Huang et al., 2003; Huang et al., 2005). In addition, blastocysts developed from cow oocytes exposed to retinol were unable to establish pregnancy (Hidalgo et al., 2005).

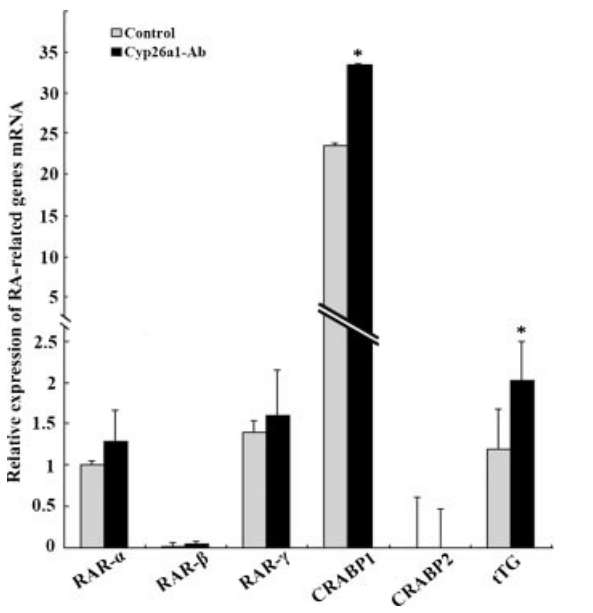

Fig. 6. Quantification of RA-related genes mRNA in mouse uterine luminal epithelium which were treated with anti-cyp26a1 antibody (cyp26a1-Ab) or normal rabbit IgG (control) by real-time PCR. The expression level was normalized with Gapdh expression. *$P < 0.05$.

The metabolism of RA by cyp26a1 produces two effects: the reduction of RA level and the production of RA polar metabolites, such as 4-O-RA and 4-OH-RA. Several reports have indicated that these polar metabolites have important biological functions (Pijnappel et al., 1993; Sonneveld et al., 1999). In contrast, a previous study showed that in the embryo development process, the main function of cyp26 family members is to degrade endogenous RA rather than to synthesize bioactive hydroxylated retinoids (Niederreither et al., 2002). Thus, whether cyp26a1 functions in mouse endometrium to produce biologically active metabolites or rather to inactivate RA to prevent its release in the uterine fluid or down-regulates the RA-sensitive genes, remains to be investigated. Further work is required to clarify the mechanism by which cyp26a1 exerts its function when blastocyst implantation occurred.

In conclusion, we found that immunization with the recombinant plasmid DNA vaccine encoding cyp26a1 protein could reduce the pregnancy rate of the mice. Both cyp26a1 mRNA and protein expression were specifically induced in the

uterine luminal epithelium during implantation. Importantly, the number of implantation sites was also significantly reduced after the uterine injection of cyp26a1-specific antisense oligos or anti-cyp26a1 antibody, and cyp26a1 might actually reduce the level of RA in uterine luminal epithelium when blastocyst implantation occurred. These data demonstrate that uterine cyp26a1 activity is required for the maintenance of pregnancy, especially during the process of blastocyst implantation.

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