Retinoic acid metabolizing enzyme CYP26A1 is implicated in rat embryo implantation

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BACKGROUND: The retinoic acid metabolizing enzyme Cyp26a1 plays a pivotal role in vertebrate embryo development. Cyp26a1 was characterized previously as a differentially expressed gene in peri-implantation rat uteri via suppressive subtracted hybridization analysis. However, the role of Cyp26a1 in rat embryo implantation remained elusive.

METHODS: The expression of Cyp26a1 in the uteri of early pregnancy, pseudopregnancy and artificial decidualization was detected by northern blotting, real time-PCR, in situ hybridization, western blotting and immunofluorescent staining. The effect of Cyp26a1 on apoptosis of endometrial stromal cells (ESCs) isolated from rat uteri was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and Hoechst staining. Apoptosis-related proteins in ESCs were detected by western blotting.

RESULTS: Cyp26a1 showed distinctive expression patterns in embryos and uteri during the peri-implantation period, with a remarkable increase (P < 0.01 versus Days 4–5) in mRNA and protein in the implantation phase (Days 5.5–6.5 of pregnancy). CYP26A1 was specifically localized in glandular epithelium, luminal epithelium and decidua basalis. The level of CYP26A1 protein was significantly increased in uteri of artificial decidualization (P < 0.01 versus control). Forced Cyp26a1 overexpression significantly reduced the sensitivity of ESCs to etoposide-induced apoptosis, with reductions in p53 (P < 0.01) and Fas (P < 0.05) proteins versus control, while in contrast, FasL (P < 0.01) and proliferating cell nuclear antigen (P < 0.05) proteins increased.

CONCLUSIONS: Cyp26a1 is spatiotemporally expressed in the uterus during embryo implantation and decidualization. Overexpression of Cyp26a1 attenuates the process of uterine stromal cell apoptosis, probably via down-regulating the expression of p53 and FasL.

Key words: Cyp26a1 / embryo implantation / rat / uterus apoptosis

Introduction

Retinoic acid (RA), the active metabolite of vitamin A, plays critical roles in normal embryonic development (Kopinke et al., 2006; Chambers et al., 2007) and normal pregnancy maintenance (Zheng and Ong, 1998; Ruhl et al., 2006). RA is the ligand for nuclear RA receptors (RARs, β and γ) that act in heterodimeric combinations with retinoid-X receptors to regulate the transcription of target genes containing RA-response elements (Chambon, 1996). RA deficiency causes embryonic malformations, such as abnormal eye, brain, heart, somite and limb development (Morriss-Kay and Sokolova, 1996; Niederreither et al., 1999, 2000; White et al., 2000). Excess of RA also causes severe malformations and inhibits the decidualization of stromal cells in vitro (Brar et al., 1996; Abu-Abed et al., 2001).

The RA-metabolizing enzyme (CYP26A1) is a key enzyme of all trans RA inactivation, which can metabolize RA into more polar hydroxylated and oxidized derivatives (White et al., 1996; Fujii et al., 1997). Cyp26a1 gene was originally cloned from wound epithelium of zebrafish regenerating the caudal fin (White et al., 1996). To date, mouse (Fuji et al., 1997; Ray et al., 1997; Abu-Abed et al., 1998), human (White et al., 1997), Xenopus laevis (Hollemann et al., 1998), chick (Swindell et al., 1999) and rat (Wang et al., 2002) homologues have also been cloned.

Cyp26a1 plays an important role during embryogenesis. For example, Cyp26a1 is required in the anterior region of the gastrulating mouse embryo to prevent teratological effects that may result from RA signaling (Ribes et al., 2007). Mutant mice that lack Cyp26 exhibited anomalies, including caudal agenesis, similar to those induced by administration of excess RA (Abu-Abed et al., 2003). The lack of
Cyp26 also resulted in homeotic transformation of vertebrae as well as in misspecification of the rostral hindbrain associated with anterior expansion of RA-positive domains (Sakai et al., 2001). However, limited data are available about the pathophysiological significance of Cyp26a1 during rat embryo implantation.

In the present study, by applying multiple approaches, we have demonstrated that Cyp26a1 is spatiotemporally expressed in the rat uterus during embryo implantation and decidualization. An excess of Cyp26a1 attenuates the sensitivity of endometrial stromal cells (ESC)s to etoposide-induced apoptosis via down-regulating the expression of p53 and FasL.

Materials and Methods

Experimental animals and protocols

Sexually mature, healthy female Sprague-Dawley rats (n = 100, 220–260 g body weight) were purchased from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China). Rats were housed in a temperature- and humidity-controlled room with a 12 h light/dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Institute of Zoology, Chinese Academy of Sciences. Individual estrous female rats were caged overnight with a male Sprague-Dawley rat, and the presence of a vaginal plug or sperm was considered as Day 1 of pregnancy (g.d.1). Uteri were excised from g.d.3 to g.d.8 rats and fixed with 4% paraformaldehyde solution (Sigma, St Louis, MO, USA) for in situ hybridization, or frozen in liquid nitrogen for RNA and protein analysis.

Pseudopregnancy was induced by caging adult female rats with vasectomized males, and the presence of a vaginal plug was considered as Day 1 of pseudopregnancy. The uteri were collected from Day 4 to 5.5 of pseudopregnancy. The uteri were embedded in 10% formalin for 24 h and then cross-sectioned (8 μm) from a g.d.5.5 rat and cloned into a pGEM-T plasmid (Promega, Madison, WI, USA). Fluorescein-labeled antisense or sense cRNA probes were transcribed in vitro using a RNA Colour Kit (T7 for sense, SP6 for antisense; Amersham Biosciences), then the probes were incubated with 0.4 mmol/l NaHCO3 and 0.6 mmol/l Na2CO3 at 60°C for 40 min to allow hydrolysis into small fragments (200–230 bp). Frozen sections (8 μm) of pregnant or pseudo-pregnant uteri were hybridized with fluorescein-labeled antisense probe in 50% formamide buffer at 55°C for 16 h. The fluorescein-labeled cRNA probe was detected using an anti-fluorescein antibody at a dilution of 1:500. Finally the color reaction was developed with alkaline phosphatase substrate solution (BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) overnight in the dark at 4°C. The sections were hybridized with a sense probe as a negative control. Samples were viewed using a Eclipse 80i microscope (NIKON, Japan). The mean optical densities (MOD) of positive signals were determined by NIS-Elements BR Image processing and analysis system (NIKON).

Real time RT–PCR

Total RNAs from adult rat uteri were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA (2 μg) from each tissue was used as a template for reverse transcription using Superscript III (Invitrogen).

Real-time PCR assay was performed on an ABI PRISM 7000 SDS (Applied Biosystems, Foster city, CA, USA) in a final volume of 25 μl solution containing 1 × SYBR Green PCR Master Mix (Applied Biosystems) and primers at a final concentration of 300 nM. The primers were as follows: forward primer 5'T-TCCGGTGGCTCTGAAACT-3' and reverse primer 5'GTTTCGGAGAAGCGGGTGAAT-3'. Triplicate experiments were performed for each sample. All quantifications were normalized to an endogenous control Gapdh. Gene expression was measured by the quantification of unknown cDNA relative to a calibrator sample serving as a physiological reference. The data were processed using SDS 2.2 software (Applied Biosystems).

In situ hybridization

The whole coding sequence of Cyp26a1 cDNA was amplified from uterine tissue of a g.d.5.5 rat and cloned into a pgEM-T plasmid (Promega, Madison, WI, USA). Fluorescein-labeled antisense or sense cRNA probes were transcribed in vitro using a RNA Colour Kit (T7 for sense, SP6 for antisense; Amersham Biosciences), then the probes were incubated with 0.4 mmol/l NaHCO3 and 0.6 mmol/l Na2CO3 at 60°C for 40 min to allow hydrolysis into small fragments (200–230 bp). Frozen sections (8 μm) of pregnant or pseudo-pregnant uteri were hybridized with fluorescein-labeled antisense probe in 50% formamide buffer at 55°C for 16 h. The fluorescein-labeled cRNA probe was detected using an anti-fluorescein antibody at a dilution of 1:500. Finally the color reaction was developed with alkaline phosphatase substrate solution (BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) overnight in the dark at 4°C. The sections were hybridized with a sense probe as a negative control. Samples were viewed using a Eclipse 80i microscope (NIKON, Japan). The mean optical densities (MOD) of positive signals were determined by NIS-Elements BR Image processing and analysis system (NIKON).

Western blotting analysis

Whole uterus was lysed with protein extraction reagent [50 mmol/l Tris–HCL (pH 7.4), 150 mmol/l NaCl, 0.25% sodium deoxycholate, 1% Triton X-100, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF) and 1 μg/ml aprotinin]. PMSF and aprotinin were added just prior to use. Total proteins (50 mg) were separated by electrophoresis and blotted onto nitrocellulose (NC) membrane (Amersham Biosciences) by electrophoretic transfer in 25 mmol/l of Tris and 192 mmol/l of glycine buffer (pH 8.3). The membranes were incubated with anti-CYP26A1, anti-p53 (sc-6243, SantCruz Biotechnology Inc., CA, USA), anti-Fas (sc-716, SantCruz), anti-FasL (sc-834, SantCruz) or anti-β-actin (sc-1616-R, SantCruz) polyclonal antibodies and anti-proliferating cell nuclear antigen (PCNA) (ZM-0213, Sant-Cruz) monoclonal antibody. The specific protein-antibody complex was detected using horse-radish peroxidase (HRP)-conjugated immunoglobulin (lgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Detection of the chemiluminescence reaction was carried out using the enhanced chemiluminescence (ECL) kit (Fierce, Appleton, WI, USA). All experiments were repeated at least three times. The bands were analyzed using the Quantity One analyzing system (Bio-Rad).

Immunofluorescence

After fixation in 4% paraformaldehyde, embryos and sections (8 μm) from uteri of pregnant and pseudo-pregnant rats were permeabilized in 0.1%
Triton X-100 (Sigma) at room temperature, incubated with rabbit anti-CYP26A1 antibody at 4°C, and then incubated in fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories). Nuclei were stained with 0.01 mg/ml propidium iodide (Sigma). Sections or embryos were viewed under a laser-scanning confocal microscope (ZEISS LSM 510 META, Germany). To evaluate the specificity of the antibodies, negative control staining was performed by substituting normal rabbit serum for the primary antibody. The MOD of positive staining were determined by NIS-Elements BR Image processing and analysis system.

**Preparation and treatment of ESCs**

ESCs were enzymatically isolated from the uteri of g.d.5 rats according to our previously described method (Xia et al., 2007).

The whole coding sequence of CYP26A1 cDNA was cloned into the eukaryotic expression vector pCR3.1. PCR3.1-Cyp26a1 was transfected into ESCs using lipofectamine 2000 (Invitrogen) essentially according to the manufacturer’s manual. The transfected cells were cultured in the medium with 500 μg/ml Geneticin G418 (determined by the killcurve assay; Sigma) to select the cells that exhibit resistance to this marker 2 days after transfection. Total protein from cultured cells was extracted to detect the expression of CYP26A1 after Geneticin G418 screening for 3 weeks.

The antitumour agent etoposide (BioVision, Mountain View, CA, USA) was prepared as a stock solution in dimethylsulphoxide (DMSO; Sigma). The final concentration of DMSO in cultures was 0.1%, which did not induce apoptosis. Before treatment, cells were seeded in culture medium, allowed to attach overnight and made quiescent by 24 h incubation in 2% serum medium. Then cells were treated with 5 mM etoposide dissolved in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Hyclone, Logan, UT, USA) supplemented with 2% serum for 48 h. The control group was treated with DMEM/F12 supplemented with 2% serum alone.

**Detection of apoptosis by TUNEL and Hoechst staining**

*In situ* detection of apoptotic cells was performed on adherent cells cultured on chamber slides by using *in situ* Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was conducted according to the manufacturer’s instructions. For the correlation of TUNEL with nuclear morphology, cells were counterstained with phosphotidylinositol (5 μg/ml). Apoptotic cells were counted in different optical fields (magnification ×400) selected in a random manner, and at least 500 cells were counted for each sample. To confirm the specificity of TUNEL, cells were treated with 1 μg/ml of DNase I (Sigma) at room temperature for 10 min to create the positive control. Terminal deoxynucleotidyl transferase (TdT) was omitted from the labeling reaction mixture in the negative control. Samples were viewed by fluorescence microscopy (ZEISS LSM 510 META).

The fragmented nuclei were stained with Hoechst 33342 (Sigma), diluted with phosphate-buffered saline and added to the medium to a final concentration of 10 μg/ml. Cells were incubated for 20 min at room temperature and visualized with a fluorescence microscope (ZEISS LSM 510 META).

**Statistical analysis**

All values are reported as the mean ± SEM. Statistical analysis was conducted by one-way analysis of variance. When significant effects of treatment were indicated, the Student–Newman–Keuls multi-range test was employed among the groups using the Statistical Package for the Social Sciences version 14.0. A value of P < 0.05 was considered statistically significant.

**Results**

**Spatiotemporal expression of Cyp26a1 in the rat uterus during the peri-implantation period**

Cyp26a1 mRNA and protein showed distinctive expression patterns in the uterus during the peri-implantation period. Northern blotting...
analysis showed that the expression of Cyp26a1 mRNA was barely detected in the preimplantation period (g.d.3–5), but mRNA levels markedly increased in the implantation period (g.d.5.5–g.d.6), followed by a decrease in the post-implantation period (g.d.7–g.d.8) (Fig. 1A). Real-Time PCR analysis revealed that the Cyp26a1 transcript level was very low on Days 4, 4.5 and 5 of pregnancy, but Cyp26a1 was strongly expressed on Days 5.5, 6 and 6.5 of pregnancy, followed by sharply decreased expression on Day 7 of pregnancy. (Fig. 1B).

The expression pattern of CYP26A1 protein during the peri-implantation period was determined by western blotting analysis. Anti-CYP26A1 polyclonal antibody was obtained from the immunized rabbit using the purified CYP26A1 recombinant protein. CYP26A1 protein in the uterus of early pregnant rats (A), pseudo-pregnant rats (B) and artificial decidualization (C) was detected by western blotting. Total protein was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), then transferred to NC membrane. The membrane was probed with anti-CYP26A1 antibody. The specific protein-antibody complex was detected using HRP-conjugated goat anti-rabbit Ig and an ECL detection system. Chemiluminescence was analyzed using Bio-Rad quantity one software (Bio-Rad). The curve and histogram represent the optical densities of the signals quantified by densitometric analysis and represented as CYP26A1 intensity/β-ACTIN intensity. *P < 0.05 versus d4, **P < 0.01 versus non-infused. All values are mean ± SEM.

**Figure 2.** Determination of CYP26A1 protein in rat uterus in early pregnancy, pseudopregnancy and artificial decidualization. Anti-CYP26A1 polyclonal antibody was obtained from the immunized rabbit using the purified CYP26A1 recombinant protein. CYP26A1 protein in the uterus of early pregnant rats (A), pseudo-pregnant rats (B) and artificial decidualization (C) was detected by western blotting. Total protein was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), then transferred to NC membrane. The membrane was probed with anti-CYP26A1 antibody. The specific protein-antibody complex was detected using HRP-conjugated goat anti-rabbit Ig and an ECL detection system. Chemiluminescence was analyzed using Bio-Rad quantity one software (Bio-Rad). The curve and histogram represent the optical densities of the signals quantified by densitometric analysis and represented as CYP26A1 intensity/β-ACTIN intensity. *P < 0.05 versus d4, **P < 0.01 versus non-infused. All values are mean ± SEM.

**Localization of Cyp26a1 in rat embryos and uteri during the peri-implantation period**
To further study the role of CYP26A1 in embryo implantation, we examined its localization in both embryos and uteri during the peri-implantation period.
CYP26A1 protein was mainly localized in the cell membrane of 2-cell, 4-cell and 8-cell embryos (Fig. 3Aa–c). In 16-cell embryos and morulae, CYP26A1 protein was mainly localized in the cytoplasm (Fig. 3Ad and e). At the blastocyst stage, strong signals were found in the trophectoderm and no signals were observed in the inner cell mass (Fig. 3Af).

The expression of CYP26A1 protein was more intense in 2-cell, 4-cell and 8-cell embryos than that in 16-cell embryos, morulae and blastocysts (Fig. 3B).

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The cell-specific expression profile of Cyp26a1 mRNA in the rat uterus during early pregnancy was determined by in situ hybridization. The signals for Cyp26a1 were very low in the uterus on g.d.4 (Fig. 4Aa). On g.d.5 prior to the initiation of implantation, a modest signal level was primarily detected in the glandular and luminal epithelium (Fig. 4Ab). Strong signals of Cyp26a1 mRNA in the glandular/luminal epithelium and modest signals in the stroma/decidua basalis (Stroma between the myometrium and the embryo is called the ‘decidua basalis’ after implantation) were found on g.d.5.5–7 (Fig. 4Ac–e). The specificity of hybridization was confirmed by the lack of signal in tissue sections hybridized with a Cyp26a1 sense probe (Fig. 4Af). Compared with that on g.d.4, the mRNA level of Cyp26a1 was significantly increased (P < 0.01) in the glandular and luminal epithelium on g.d.5.5 and g.d.6 (Fig. 4B).

The signals of Cyp26a1 mRNA in stroma/decidua basalis were more intense on g.d.4 and g.d.5 than that on g.d.5.5, g.d.6 and g.d.7 (P < 0.05; Fig. 4B).

The distribution of CYP26A1 protein in the rat uterus during early pregnancy was determined by Immunofluorescence.
CYP26A1 protein was hardly detected in the uteri on g.d.4 (Fig. 5Aa). Strong signals of CYP26A1 were found in glandular/luminal epithelium and stroma on g.d.5.5 and g.d.6 (Fig. 5Ab and c). On g.d.7 and g.d.8, CYP26A1 protein was mainly localized in glandular/luminal epithelium and decidua basalis (Fig. 5Ad–f). A modest enhancement was observed in myometrium after g.d.5. There was no immunoreaction in the control sections incubated with normal rabbit serum (Fig. 5Af). The positive staining of CYP26A1 protein in stroma/decidua basalis and luminal and glandular epithelium was more intense on g.d.5.5 than that on g.d.4 ($P < 0.01$). The signals of CYP26A1 in glandular epithelium, stroma/decidua basalis and myometrium were stronger on g.d.6, g.d.7 and g.d.8 than that on g.d.4 ($P < 0.05$), except for luminal epithelium on g.d.6 ($P < 0.01$; Fig. 5B).

**Figure 4** In situ localization of Cyp26a1 mRNA in the uterus of early pregnant and pseudo-pregnant rats. Uteri sections from Day 4 (Aa), Day 5 (b), Day 5.5 (c), Day 6 (d) and Day 7 (e) of pregnancy and Days 4 (g), Day 5 (h) and Day 5.5 (i) of pseudopregnancy were subjected to in situ hybridization using fluorescein-labeled cRNA antisense probe specific to Cyp26a1. The stain was developed with alkaline phosphatase substrate solution (BCIP/NBT). (f) represents uterus section from Day 5 of pregnancy hybridized with a sense probe for Cyp26a1 as a negative control. Red arrows indicate hybridization signals. The photographs are at ×200 original magnification. m, myometrium; v, uterine blood vessels; db, decidual basalis; mg, maternal gland; s, stroma; le, luminal epithelium; gd, gestational day; pgd, pseudo gestational day. The histogram represents the MOD of positive signals of CYP26A1 in pregnancy (B) and pseudopregnancy (C) that were determined by NIS-Elements BR Image processing and analysis system. *$P < 0.05$, **$P < 0.01$. All values are mean ± SEM.
Temporal and spatial expression of Cyp26a1 in rat uteri of pseudopregnancy and artificial decidualization model

To further explore whether Cyp26a1 expression was associated with the initiation and progression of the embryo implantation process, Cyp26a1 expression in pseudo-pregnant and artificial decidualizing uteri was examined by western blotting and in situ hybridization. A low level of CYP26A1 was found in uteri on Day 4 of pseudopregnancy. The CYP26A1 protein level was markedly increased on Day 5.5 to Day 6 of pseudopregnancy (P < 0.05; Fig. 2B). Consistent with western blotting results, in situ hybridization analysis revealed that stronger signals...
Expression of CYP26A1 protein in cultured ESCs

ESCs were isolated from the g.d.5 rat uteri. Cells were incubated with rabbit anti-CYP26A1 antibody prepared by our laboratory. A weak CYP26A1 signal in the cytoplasm was detected in ESCs (Fig. 6Aa). Expression of transfected Cyp26a1 was identified with the specific antibody by immunofluorescence and western blotting. A strong signal was found in the cytoplasm. There was no immunoreaction in ESCs incubated with normal rabbit serum as the negative control (Fig. 6Ac). The protein level of CYP26A1 was higher in ESCs transfected with pCR3.1-Cyp26a1 than in cells transfected with pCR3.1 or normal ESCs (Fig. 6B). These observations show that the ESCs transfected with recombinant vector express CYP26A1 proteins in culture.

Diminution of susceptibility to etoposide-induced apoptosis by Cyp26a1

TUNEL was performed to investigate the effect of Cyp26a1 on apoptosis of ESCs (Fig. 7A and B). Apoptotic cells were counted in different optical fields (magnification ×400) selected in a random manner, and at least 500 cells were counted for each sample. In situ TUNEL labeling showed that TUNEL-positive cells constituted ~10–12% of the normal ESCs and pCR3.1 expression ESCs. Although TUNEL-positive cells (18% of total cell number) were slightly increased in ESCs expressing pCR3.1-Cyp26a1, overexpression of Cyp26a1 apparently had no significant effect on the apoptotic status. To further assess the potential role of Cyp26a1 in stromal cell apoptosis, ESCs were treated with etoposide (5 μM, 48 h). TUNEL assay demonstrated that TUNEL-positive cells constituted ~78% of the normal ESCs and pCR3.1 expression ESCs, whereas TUNEL-positive cells (43% of total cell number) were remarkably decreased in ESCs expressing pCR3.1-Cyp26a1. Cells stained after treatment with DNase I were used as a positive control, and those stained without TdT were used as a negative control.

To confirm the above results, we examined nuclear morphological changes of ESCs by Hoechst 33342 staining (Fig. 8). The cells undergoing apoptosis demonstrated apoptotic chromatin changes: chromatin condensation, nuclear segmentation and crescent-shaped chromatin etc. Hoechst 33342 staining showed that forced expression of Cyp26a1 in ESCs had no detrimental effects on nuclear condensation and fragmentation of ESCs. After etoposide treatment, large portions of the normal and pCR3.1 expression ESCs displayed nuclear fragmentation and condensation. ESCs expressing pCR3.1-Cyp26a1 had reduced the number of cells exhibiting nuclear condensation and crescent-shaped chromatin compared with that in control groups (P < 0.05).

Effect of Cyp26a1 on apoptosis-related gene expression

To reveal the underlying mechanism and account for the anti-apoptosis function of Cyp26a1 in ESCs, we analyzed the expression of some proliferation and apoptosis-related genes by western blotting. Overexpression Cyp26a1 had no significant effect on the expression of p53 and PCNA but increased the expression of Fas (P < 0.01) and FasL (P < 0.05). After the etoposide treatment, both p53 (P < 0.01) and Fas (P < 0.05) protein levels were reduced, whereas the FasL (P < 0.01) and PCNA (P < 0.05) protein levels were enhanced in ESCs expressing pCR3.1-Cyp26a1 (Fig. 9).
**Discussion**

Cyp26a1 is a highly conserved component of the RA signaling pathway, which belongs to the cytochrome P450 (CYP) superfamily and metabolizes RA into various derivatives (Abu-Abed et al., 1998). Previous studies focused on the effect of Cyp26a1 on vertebrate embryonic development (Abu-Abed et al., 2001; Ribes et al., 2007). In this study, we examined the expression and role of Cyp26a1 in rat uteri during early pregnancy in order to provide insight into the physiological functions of Cyp26a1 during embryo implantation.

Wang et al. (2002) first cloned rat Cyp26a1 cDNA from the liver. The nucleotide sequence predicts a 497-amino-acid protein whose sequence is 94% identical to that of mouse and 91% homologous to human Cyp26a1. In our previous study, suppression subtractive hybridization was utilized to analyze differentially expressed genes between the preimplantation and implantation periods, and Cyp26a1 was identified as a differentially expressed gene. We had cloned rat full-length Cyp26a1 cDNA from the uterus of Day 5.5 of pregnancy by the method of rapid amplification of 5’ and 3’-cDNA ends (GenBank accession number DQ317305).
To study the role of Cyp26a1 during embryo implantation, we first examined its temporal and spatial distribution in both the pre-implantation embryo and the uterus during the peri-implantation period. CYP26A1 protein was mainly localized in the cell membrane at 2-cell, 4-cell and 8-cell embryos. At the morula stage, CYP26A1 protein was mainly localized in the cytoplasm. At the blastocyst stage, strong signals were found in the trophectoderm with no signals in the inner cell mass. Gene expression in the developing preimplantation embryos is essential to the establishment of the dorso-ventral axis at the 2-cell stage, the initiation of compaction at the 8-cell stage and formation of trophectoderm and inner cell mass. During human embryo implantation, trophectoderm mediates adhesion of the blastocyst to the uterine epithelium (Sugihara et al., 2007). Our observation of high expression of Cyp26a1 at various stages of preimplantation embryos with cell-specific expression in the trophectoderm suggests that Cyp26a1 may participate in embryo cleavage and attachment processes.

We also provided evidence that Cyp26a1 was differentially expressed in rat uteri during the peri-implantation period, showing a sharp increase in the implantation period. CYP26A1 immunoreactivity was hardly detected in uteri during the preimplantation period. In implantation and post-implantation periods, luminal epithelium/glandular epithelium was the major site of CYP26A1 expression. This result was consistent with previous reports by Vermot et al.

Figure 8  Hoechst 33342 staining of ESCs. (A) The cells undergoing apoptosis demonstrated apoptotic chromatin changes: nuclear segmentation, and chromatin condensation under a fluorescence microscope. Arrows indicate typical morphological features of apoptotic cells (nuclear shrinkage and DNA fragmentation) observed in treated ESCs. The cells with nuclear shrinkage and DNA fragmentation were counted in different optical fields (magnification × 400) selected in a random manner, and at least 500 cells were counted for each sample. (a) Normal ESCs; (b) ESCs expressing pCR3.1; (c) ESCs expressing pCR3.1-Cyp26a1; (d) Normal ESCs after 5 μM etoposide treatment; (e) ESCs expressing pCR3.1 after treatment with 5 μM etoposide; (f) ESCs expressing pCR3.1-Cyp26a1 after treatment with 5 μM etoposide. The scale bar represents 50 μM. (B) Histogram of ratio of apoptotic cells. The results were expressed as ratio of apoptotic cells vs total ESCs. *P < 0.05. All values are mean ± SEM.
(2000), Fritzsche et al. (2007) and Han et al. (2010) who showed that Cyp26a1 expression was high in mouse luminal epithelium by the time that blastocysts reached the uterus and started to implant. In addition, we observed that Cyp26a1 expression was also detected in the decidua basalis and the myometrium during the implantation period, which may be related to species differences and the application of different hybridization probes. In the Han et al. (2010) study, mice were used to investigate Cyp26a1 expression and a partial sequence of Cyp26a1 mRNA was used as the hybridization probe. In our study, rats were used to study Cyp26a1 expression and the whole coding sequence of Cyp26a1 mRNA, hydrolysed into small fragments (200–230 bp), was used as the hybridization probe so that the signal was more intense than that of partial sequence.

The increase of uterine cyp26a1 expression in the implantation period indicated its possible involvement in embryo implantation and decidualization. In preparation for implantation, the endometrium undergoes differential cellular changes (Psychoyos, 1986). The

**Figure 9** The effect of CYP26A1 on apoptosis- and proliferation-related proteins. Western blotting was utilized to analyze the expression of apoptosis- and proliferation-related proteins in ESCs. The membrane was incubated with antibodies against p53, Fas, FasL and PCNA and detected by the ECL system. The expression of β-actin served as an internal control. *P < 0.05, **P < 0.01. All values are mean ± SEM.
endometrium is the inner membrane of the mammalian uterus. Prior to the initiation of implantation, the morphological and biochemical changes occur in endometrial luminal epithelium and the luminal epithelium acquires the ability to adhere (receptivity) to the developing blastocyst (Demir et al., 2002; Dominguez et al., 2003; Spencer et al., 2007). Successful implantation results from initial apposition and adhesion of the blastocyst to the apical surface of the luminal epithelia (Abrahamsohn and Zorn, 1993). So the increased expression of CYP26A1 in luminal epithelia may play an important role in endometrial receptivity. After implantation, stromal cells in endometrium, under the influence of the invading trophoblast, differentiate into large, rounded, glycogen-filled decidual cells (Akcai et al., 1996; Sheikh et al., 2007). So an increase of CYP26A1 expression in the stroma during the implantation phase may contribute to stromal-decidual transformation. With the progression of pregnancy, the uterus has to respond to the unique physical challenge of changing mechanical strain owing to uterine occupancy by the invasion of trophoblast cells, formation of the placenta and growth of the fetus. The myometrium must differentially respond to these mechanical strains to allow for appropriate growth and in utero survival of the fetus (Caspo et al., 1965; Wu et al., 2008). In early pregnancy, the myometrium remodeling and myometrial cell hyperplasia were beneficial to increase uterus volume and invasion of trophoblast (Shynlova et al., 2006, 2009; Wu et al., 2008). So the increased expression of CYP26A1 in myometrium during the implantation phase may contribute to pregnancy adaption with myometrial cell proliferation. All these findings suggest that Cyp26a1 may play an important role in the process of embryo implantation and decidualization.

In this study, we also explored the involvement of Cyp26a1 in uterine functions during pseudopregnancy and experimentally induced decidualization. Our results showed that the expression level of Cyp26a1 was significantly increased in uteri from Day 5.5 of pseudopregnancy and in artificially induced decidua in rats. These results indicate that decidual expression of Cyp26a1 may not be embryo-dependent, confirming its implications during uterine decidualization.

Embryo implantation is regulated not only by cell differentiation, but also by apoptosis. During blastocyst implantation and uterine decidualization, apoptosis occurs in specific uterine cells (Schlacke et al., 1985; Parr et al., 1987; Welsh and Enders, 1991). Uterine homeostasis is also mainly controlled by apoptosis during early pregnancy (Okano et al., 2007). However, over apoptosis can result in pregnancy loss (Savion et al., 2002; Tayade et al., 2006).

The effect of Cyp26a1 on apoptosis in ESCs isolated from rat uteri on g.d.5 was determined in order to investigate whether CYP26A1 was involved in apoptosis during embryo implantation. In situ TUNEL labeling showed that TUNEL-positive cells constituted ~10–12% of the total ESCs in both normal and pCR3.1 expression groups. TUNEL-positive cells were slightly increased in ESCs expressing pCR3.1-CYP26A1 (~18% of total cell number). After etoposide treatment, the number of TUNEL-positive cells was much lower in ESCs overexpressing Cyp26a1 than in the control group, indicating that overexpression of Cyp26a1 can protect the ESCs viability and significantly reduce the sensitivity of ESCs to etoposide-induced apoptosis. Further analysis revealed that the expression of apoptosis-related proteins, p53 and Fas, was reduced, whereas that of FasL and PCNA was increased upon etoposide treatment in ESCs overexpressing Cyp26a1. p53, as a tumor-suppressor gene plays a critical role in triggering apoptosis (Levine, 1997). Down-regulation of p53 expression implies that the apoptotic signal is inhibited in the ESCs with overexpression of Cyp26a1. During normal pregnancy, the decidua is populated by a variety of leukocytes (Mincheva-Nilsson et al., 1994). Maternal decidua cell-derived FasL may be involved in preventing the recruitment of Fas-bearing leukocytes at the maternal–fetal interface through apoptosis induction by Fas/FasL interaction (Qiu et al., 2005). Fasl might restrain the local immune response by induction of apoptosis of CD4+ T lymphocytes in epithelial and mesenchymal cells of mouse uterus (Imara et al., 2005). Fas can mediate receptor-specific apoptotic signals (Rothstein et al., 2000). Up-regulation of Fasl and down-regulation of Fas in the ESCs overexpressing Cyp26a1 suggest that forced expression of Cyp26a1 may inhibit ESCs over-apoptosis and assist with maternal immune tolerance by Fas/FasL-dependent mechanisms. Together, these results support the hypothesis that Cyp26a1 might regulate apoptosis homeostasis, and overexpression of Cyp26a1 may be beneficial to embryo implantation under abnormal gestation. Therefore, we speculate that inhibition of Cyp26a1 expression might be deleterious to early pregnancy.

In conclusion, Cyp26a1 was differentially expressed in rat uteri during the peri-implantation period. Results obtained during pseudopregnancy and artificial decidualization imply that decidualization induces the expression of Cyp26a1. In addition, overexpression of Cyp26a1 significantly reduces the sensitivity of ESCs to etoposide-induced apoptosis, via regulating the expression of p53 and Fas/FasL. This study has provided new insights into the understanding of the Cyp26a1 function during embryo implantation.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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