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Expression and localization of the 20α-hydroxysteroid dehydrogenase (HSD) enzyme in the reproductive tissues of the cynomolgus monkey *Macaca fascicularis*

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ABSTRACT

This study was conducted to characterize and functionally analyze the monkey 20α-hydroxysteroid dehydrogenase (20α-HSD) in the ovary, placenta, and oviduct. We focused on 20α-HSD mRNA expression and protein localization in monkey reproductive tissues and the molecular characterization of the promoter region. Reverse transcription-polymerase chain reaction (RT-PCR) monkey 20α-HSD mRNA was more strongly detected in the ovary at pre-ovulation than in the placenta and oviduct at pre-parturition. The mRNA was approximately 1.2 kb in size and the expression was high in the ovary, which was the same as the RT-PCR result. We also produced His tagged 20α-HSD proteins by using an *Escherichia coli* expression system. In a western blot for the 20α-HSD protein, only 1 band of approximately 37-kDa was detected in the ovary, oviduct tissue, and recombinant protein produced in the Chinese hamster ovary (CHO) cell line. However, in the placenta, additional 2 bands (35 and 39 kDa) were detected.

Immunohistochemical analyses suggested that the monkey 20α-HSD protein was localized mainly in the syncytiotrophoblast of the placenta and the isthmus cells of the oviduct. According to promoter analyses with the enhanced green fluorescent protein (EGFP) gene, the monkey 20α-HSD promoter was efficiently expressed in the CHO-K1 cell line; however, the promoter was not expressed in bovine fetal fibroblast (bFF) cell. Taken together, our study showed that the 20α-HSD mRNA and protein are coordinately expressed in the ovary at pre-ovulation and in the placenta and oviduct at pre-parturition. Therefore, monkey 20α-HSD in the placenta, ovary and oviduct plays an important role in the estrous cycle, pregnancy, and parturition.

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1. Introduction

In all mammalian species, progesterone is essential for preparing the body for pregnancy and also for maintaining pregnancy, if it occurs, by preparing the endometrium for possible implantation and inhibiting uterine contraction until birth. Aldo-keto reductases (AKRs) belong to a superfamily of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductases that act on a wide range of substrates, including simple carbohydrates, steroid hormones, and endogenous prostaglandins [1]. These enzymes, which require nicotinamide cofactors for catalysis, have similar cofactor-binding sites, even among proteins with less than 30% amino acid sequence identity [2].

Dihydrodiol dehydrogenase 1 (DD1), which is also referred to as AKR1C1, is a member of the AKR superfamily [1]. AKR1C1 is also called 20α-hydroxysteroid dehydrogenase (20α-HSD) because of its high 20α-HSD activity [3,4]. The DD1 and DD4 (also referred to as AKR1C4) enzymes have been purified from livers of the cynomolgus monkey and the Japanese monkey. The sequence identity between DD1 and DD4 is 83%. In the Japanese monkey, DD1 mRNA was detected in the liver, kidney, intestine and adrenal gland, while DD4 was expressed only in the liver and kidney. A difference in the isoenzyme-mediated metabolism of steroids and xenobiotics has been suggested between monkeys and humans [5]. These distribution patterns in Japanese monkey tissues differ from those in humans. In humans, AKR1C1 is ubiquitously expressed, while AKR1C4 is expressed only in the liver. In humans, DD1 purified from the liver has been shown to possess 20α-HSD enzyme activity [6,7]. Monkey 20α-HSD (also referred to as DD1 and AKR1C1) mRNA is highly expressed in the kidney, stomach, and liver. Comparison of the deduced amino acid sequence of the monkey enzymes with the corresponding human and mouse enzymes shows that monkey 20α-HSD shares approximately 94% and 76% sequence identity with the human and mouse counterparts, respectively [8].

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The 20α-HSD protein predominantly converts progesterone into its biologically inactive form, 20α-hydroxyprogesterone (20α-OHP) and plays a crucial role in the termination of pregnancy and initiation of parturition [9]. Accordingly, 20α-HSD in the placenta may be involved in reducing the cytotoxic effects of progesterone in the developing fetus. In addition, progesterone has been shown to be synthesized in the brain, where it stimulates myelin formation. The presence of 20α-HSD in the brain suggests that it could play a role in the regulation of myelin formation. The role of the 20α-HSD enzyme in human skin has not been well-defined [3]. It has been shown that treatment with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) increased 20α-HSD activity and resulted in a concomitant decrease in progesterone concentration [10]. The activity of 20α-HSD is suppressed by prolactin during luteal regression and is increased at the end of pseudopregnancy and pregnancy [11]. Aspirin, a well-known salicylic acid-based drug, and its metabolite salicylic acid were shown to inhibit AKR1C1 activity [12].

Several 20α-HSD isoforms have been identified. Seong et al. [9,13] purified 20α-HSD, and found 2 distinct 20α-HSD molecules (HSD-1 and HSD-2). Jayasekara et al. [14] observed that 20α-HSD mRNA was localized mainly in the endometrial epithelium on the caruncle side of the goat placenta during mid-to-late pregnancy. Considerable 20α-HSD enzyme activity was also detected in the cytosolic fraction of the placenta and in the intercaruncular region of the uterus. During early pregnancy in humans, the level of 20α-HSD mRNA expression in the secretory phase endometrium was significantly higher than that in the proliferative phase endometrium and chorionic tissues. These results suggested that progesterone itself contributes to the regulation of local progesterone concentration through 20α-HSD in endometrial stromal cells during the peri-implantation period [3]. To gain more knowledge about 20α-HSD, in the present study, we characterized the tissue distribution of mRNA and cellular localization of 20α-HSD in the pre-ovulation and pre-parturition reproductive tissues of the cynomolgus monkey.

2. Materials and methods

2.1. Animal tissues and cell lines

Tissue samples were obtained from the Korea National Primate Research Center (Ohchang, Korea). Ovaries from *Macaca fascicularis* were collected at pre-ovulation and were minced. After obtaining the ovum, the ovarian cells were collected and centrifuged. The supernatant was discarded and cell debris was collected. Placenta and oviducts from an 8-year-old female rhesus monkey of Chinese origin were collected by cesarean section in the pre-parturition period. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. All animal housing and experiments were performed in accordance with the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Institutional Animal Care and Use Committee Guidelines (Accepted No. KRIBB-AEC-09017). Chinese hamster ovary (CHO-K1) cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Bovine fetal fibroblast (bFF) cells were established in our lab as previously described [15].

2.2. Total RNA isolation

Total RNA was extracted using TRIzol reagent (Invitrogen, NY, USA). Monkey tissues (100 mg) were added to 1 mL of TRIzol reagent and homogenized. Then, 0.2 mL chloroform was added per 1 mL of TRIzol reagent, and the samples were vigorously vortexed for 15 s. Samples were centrifuged at 12,000 × *g* for 15 min at 4 °C. After centrifugation, the upper aqueous phase was carefully transferred into a fresh tube without disturbing the inter face and 0.5 mL isopropyl alcohol was added. Then, the tube was centrifuged for 10 min by 12,000 × *g* at 4 °C. The supernatant was completely removed. The RNA pellet was washed twice with 75% ethanol-diethylpyrocarbonate-treated water (DEPC water), and then the tube was air-dried for 15 min. Finally, 50 μL RNase-free water was added to the tube, and the tube was incubated for 10 min at 56 °C. The concentration and purity of RNA was determined by spectrophotometry at 260 nm and 280 nm.

2.3. cDNA synthesis and reverse transcription-polymerase chain reaction

cDNA synthesis was performed using the SuperScript™ First-Strand Synthesis System according to the manufacturer's instructions. The RNA-primer mixture was prepared as follows: 5 μg RNA, 1 μL of 10 mM dNTP mix, 1 μL of Oligo-dT, and up to 10 μL DEPC water were incubated at 65 °C for 5 min and then placed on ice for at least 1 min. Then the reaction mixture (2 μL of 10× RT buffer, 4 μL of 25 mM $MgCl_2$ and 2 μL of 0.1 M dithiothreitol (DTT)] was prepared. RNaseOut recombinant RNase inhibitor (1 μL) and 9 μL reaction mixture were added, mixed gently, and incubated at 4 °C for 2 min. SuperScript II RT (1 μL) was added to each tube and incubated at 42 °C for 50 min. Finally, the reaction was terminated by incubation at 70 °C for 15 min; then, the reaction mixtures were chilled on ice and centrifuged. RNase H (1 μL) was added to each tube and incubated for 20 min at 37 °C. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the AccuPower RT-PCR Kit (RT/PCR PreMix). Total RNA (1.0 μg) extracted from monkey placenta, ovary, or oviduct was mixed with the reverse primer (5′-TAA TTG AAG ATT TTT CAG TGA CCT-3′), incubated at 70 °C for 5 min, and then placed on ice. The forward primer (5′-GCC TTG GCA AAA AAG CAC AAG-3′) was then added, and the reaction volume was made to 20 μL with DEPC water. PCR was performed under the following conditions: 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, which was followed by a final extension at 72 °C for 8 min. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for the normalization of monkey 20α-HSD expression and the primer sequences of the forward and reverse primers were 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′, respectively. The expected length of the PCR fragment was 452 bp. The PCR conditions were 26 cycles for 10 s at 98 °C, 20 s at 55 °C, and 20 s at 72 °C. Gel electrophoresis was used to analyze 10 μL of the PCR products.

2.4. Northern blot analysis

For northern blot analysis, RNA electrophoresis was performed on an agarose gel containing 10× MOPS and 37% formaldehyde. The total RNA concentration from monkey ovary and placenta was adjusted to 10 μg/μL. After electrophoresis, the RNA was transferred to a membrane, which was incubated overnight in 20× SSC. The probe was prepared by purifying the sample after PCR amplification. Probe labeling was performed with the DIG DNA Labeling Kit. The membrane was pre-hybridized for 1 h at 68 °C and hybridized with DIG-labeled monkey 20α-HSD cDNA with gentle rocking at 68 °C overnight. The membrane was subsequently washed 2 times with buffer (2× SSC/0.1% SDS) at 68 °C for 5 min with gentle rocking, and 2 times with 0.5× SSC/0.1% SDS at 68 °C for 15 min with gentle rocking. Anti-DIG antibody (5 μL) was mixed with blocking reagent and added to the membrane and then incubated at room temperature (RT) for 1 h. The membrane was washed 2 times with washing buffer at RT for 15 min, and equilibrated with detection buffer at RT for 5 min. The band was detected by the addition of CDP-star.

2.5. Production of monkey 20α-HSD protein in a bacterial system

Monkey AKR1C1 protein was expressed in the *E. coli* using a pET15b expression plasmid vector. Three colonies were selected to produce the recombinant protein. To prepare the His-tagged recombinant protein, monkey 20α-HSD cDNA was cloned into the pET15b vector. This vector includes 6-histidine amino acid tag at the N-terminus. The positive clones were cultured for 3 h at 37 °C and then expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and the clones were incubated at 25 °C overnight. The expressed protein was purified by nickel nitrilotriacetic acid (Ni-NTA) chromatography. Monkey 20α-HSD protein was detected by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining.

2.6. Construction of expression transfer vector and transient expression into CHO cell lines

The full-length monkey 20α-HSD cDNA was amplified with specific primers containing an *Apa*I site at the 5′-end and a *Kpn*I site at the 3′-end. The PCR fragments were ligated into the PCR2.1 vector and sequenced. The monkey 20α-HSD gene was digested with *Apa*I and *Kpn*I restriction enzymes, and the digested fragments were then ligated into the eukaryotic expression vector pcDNA3 that had been digested with the same enzymes. Each vector was completely sequenced to confirm the presence of the Kozak site and to rule out the possibility of PCR errors. The expression vectors were transfected into CHO-K1 cells by using the Lipofectamine 2000 transfection method according to the manufacturer's instructions. After 48 h, the cells were collected in a tube and were centrifuged at 15,000 rpm for 10 min, and the cell debris was recovered.

2.7. Western blot analysis

Ovarian, oviduct, and placental tissues (10–20 mg) were minced, and total protein was extracted using the PRO-PREP protein extraction solution. The samples, which included the transfected CHO cell debris, were then homogenized in 600 μL PRO-PREP solutions. Cell lysis was induced by incubating the cells for 30 min on ice. After blotting, the membrane was blocked with 1% blocking reagent for 1 h and incubated with a 1:1500 dilution of a polyclonal rabbit antiserum against bovine 20α-HSD (produced in our lab) for 1 h. The membrane was then incubated with a 1:2000 dilution of a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody for 30 min. Finally, the membrane was incubated with 3 mL Lumi-Light substrate solution for 5 min and exposed to X-ray film for 1–10 min.

2.8. Immunohistochemical analyses

Samples of pre-parturition oviduct and placenta were first fixed in 10% neutral buffered formalin at room temperature for 24 h and washed with phosphate-buffered saline (PBS). Next, the fixed samples were rehydrated in graded ethanol (EtOH; 100%, 2×; 95%, 1×; 70%, 1×; and 50%, 1×for 3 min each) and embedded in paraffin. For immunostaining, paraffin-embedded monkey tissues were sectioned (5 μm) and mounted onto poly L-lysine-coated slides. Then, the mounted tissues were deparaffinized in xylene and graded alcohol and then boiled in 10 mM sodium citrate buffer (pH 6.0) for 3 min in a microwave oven. Slides were cooled for 20 min on ice and by rinsing in distilled water and then incubated with 3% hydrogen peroxide solution for 10 min. Slides were blocked with 1% horse serum and 5% goat serum for 1 h at room temperature and then incubated overnight with a polyclonal rabbit antiserum against bovine 20α-HSD at 4 °C. The negative control slides were incubated with blocking solution. After rinsing 3 times with PBS, the slides were incubated with biotinylated anti-rabbit immunoglobulin secondary antibody for 2 h. The slides were immunostained using the ABC detection kit and stained with diaminobenzidine (DAB). Finally, the slides were counterstained with hematoxylin, dehydrated in graded alcohol and xylene, and protected using coverslips.

2.9. Promoter cloning by long-amplification PCR and construction of a green fluorescent protein vector

Genomic DNA was extracted using the QIAamp DNA Mini Kit, according to the manufacturer's specifications. Placental genomic DNA (5 μg) was completely digested with an appropriate restriction enzyme (*Sau3*AI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, or *Xba*I). DNA was purified using EtOH precipitation, and then finally, it was dissolved in 10 μL TE buffer. The digested DNA and the cassette fragments were mixed, and the reaction mixture was incubated at 16 °C overnight. Next, we subjected the ligation samples to long-amplification PCR (LA-PCR). The first reaction mixture contained the cassette C1 primer (5′-GTA CAT ATT GTC GTT AGA ACG CGT AAT ACG ACT CA-3′), the gene-specific monkey RT1 primer (5′-CAT GAA GTG ACC ATC ATT CAG CTT-3′), 1 μL DNA, 0.4 mM dNTPs, 10× LA buffer, and 1 unit of TaKaRa LA Taq DNA polymerase. The reaction mixture was subjected to pre-denaturation at 95 °C for 5 min, then 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 4 min, and then a final extension at 72 °C for 10 min. The reaction mixture was then cooled to 4 °C. The PCR products were analyzed by electrophoresis. The second and third PCR reaction mixtures contained the cassette C2 primer (5′-CGT TAG AAC GCG TAA TAC GAC TCA CTA TAG GGA GA-3′) and either the gene specific monkey RT2 primer (5′-CAT GAA GTG ACC ATC ATT CAG CTT-3′) or the monkey RT3 primer (5′-CAC ACA CTG ATG TTT CGA ATC CAT-3′) and 1 μL of the first PCR product. This reaction was performed under the same conditions as the first PCR. The gel-purified PCR products were ligated into the cloning vector pCR2.1. Plasmids were purified and confirmed by sequencing (2005 bp). Finally, we identified the positive clone in the *Sal*I-digested sample.

Next, we constructed an expression vector with green fluorescent protein (GFP) under the control of the monkey 20α-HSD promoter. The promoter gene was inserted into pCR2.1 that was cut with *Eco*RV and *Xho*I. We amplified a 984-bp fragment containing the GFP gene (749 bp) and the bGHpA region (235 bp) by using the following primers (sense primer: 5′-GAT ATC CGA TGG TGA CGA AGG GCG AGG A-3′; antisense primer: 5′-CTC GAG TCC CCA GCA TGC CTG CTA TT-3′) with *Eco*RV and *Xho*I enzyme sites from the pEGFP-C2 vector. The amplified gene was ligated to the 20α-HSD promoter. Restriction mapping was used to confirm the orientation.

2.10. Detection of GFP expression in mammalian cell lines

CHO-K1 and bFF cell lines were cultured in either Ham's F12 medium or Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 U/mL), streptomycin (50 mg/mL), glutamine (2 mM), and 10% fetal bovine serum (FBS). The constructed vector (monkey 20α-HSD promoter + enhanced green fluorescent protein [EGFP]) was transfected into the cell lines by using the liposome formulation (Lipofectamine 2000) transfection method, according to the manufacturer's instructions. The EGFP-C2 vector was used as a positive control. One day before transfection, the cells were plated at 0.5×10^5 to 1×10^5 cells per well in 500 μL growth medium without antibiotics. Two samples of vector and Lipofectamine were mixed gently and incubated for 20 min at RT. DNA–lipofectamine complexes (250 μL) were added to each well. After 4–6 h, 250 μL of 20% FBS was added to the well, and the cells were incubated for 24 h. On the next day, the cells were washed 2 times, and then, 300 μL

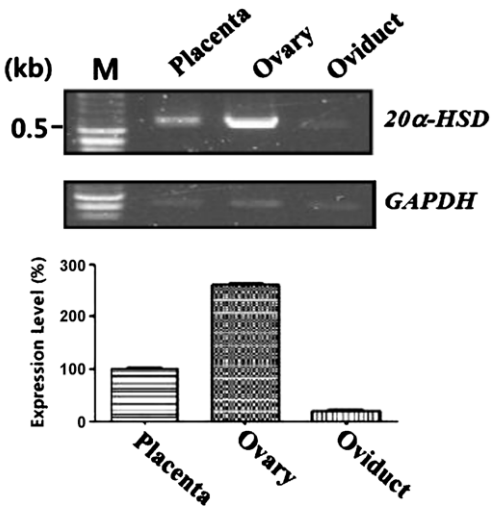

Fig. 1. 20α-HSD mRNA expression in the placenta, ovary, and oviduct by RT-PCR. Ovary was collected from pre-ovulation. Placenta and oviduct tissues were obtained by laparotomy under general anesthesia pre-parturition. Total RNA was extracted and then subjected to RT-PCR. The amplified products of *20α-HSD* gene and *GAPDH* were separated on agarose gel and stained with ethidium bromide. Representative results are shown; graphs show the average ± SEM of 3 independent experiments.

CHO-SFM-II was added. Finally, GFP expression was observed using a Nikon Eclipse TE-2000-E confocal microscope.

3. Results

3.1. Expression of monkey 20α-HSD mRNA

Using primers specific for monkey 20α-HSD, mRNA expression levels were analyzed by RT-PCR. We determined the expression of 20α-HSD mRNA in the ovary during pre-ovulation and in the oviduct and placenta at pre-parturition. The expression of 20α-HSD mRNA was seen in all tissues (Fig. 1). Expression was high in the ovary during pre-ovulation. However, 20α-HSD mRNA expression was lower in the oviduct than in the placenta at pre-parturition. To our knowledge, this was the first report of 20α-HSD mRNA expression in the oviduct. For northern blot analysis, total RNA was extracted from the ovary and placenta. A monkey 20α-HSD mRNA band with a size of approximately 1.2 kb was observed. Similar to the RT-PCR result, the mRNA level was higher in the ovary than in the placenta, and it was strongly detected in the ovary tissue (Fig. 2). This was because the progesterone level was the lowest at the time of ovulation.

3.2. Western blotting analysis in the placenta, ovary, and oviduct

As shown in Fig. 3A, monkey 20α-HSD protein was expressed in the ovary during pre-ovulation and in the oviduct and placenta at pre-parturition. Recombinant protein was also expressed in CHO cells (Fig. 3B). The recombinant protein was detected as a single band about 37-kDa in size, as was the protein in ovary and oviduct tissues. However, we detected 2 other bands (at approximately 35- and 39-kDa) in the placenta tissues (Fig. 3A). A comparison of the expression level of 20α-HSD protein with that of mRNA in the ovaries at pre-ovulation showed a similar pattern. The 20α-HSD protein was more highly expressed in the ovary than in the other tissues analyzed in this study. Interestingly, 3 protein bands were detected in the placenta. This observation was not consistent with the northern blot result in which only one band was found. We did not analyze the other 2 bands (35- and 39-kDa).

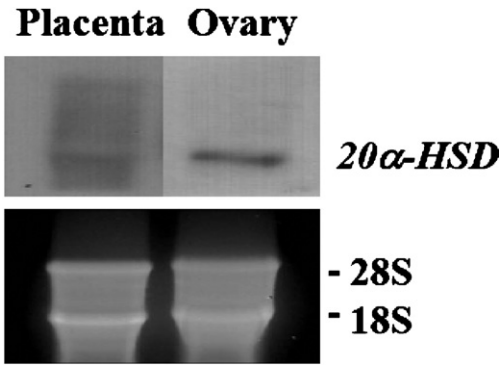

Fig. 2. Northern blot analysis of monkey 20α-HSD mRNA expression in the placenta at pre-parturition and ovary at pre-ovulation. Blots shown are the results of a representative experiment.

3.3. Production of monkey 20α-HSD protein in the CHO cell line and E. coli

Monkey 20α-HSD protein was expressed in *E. coli* by using the pREST expression plasmid vector. However, the level of protein expression was very low (data now shown). Next, we expressed monkey 20α-HSD protein by using the pET15b expression vector, which includes a 6-His-tag coding sequence at the N-terminus. Protein was detected at the predicted molecular size of 39.1 kDa by SDS-PAGE with Coomassie blue staining (Fig. 3B). Finally, the recombinant His-tagged protein was purified by using Ni-NTA chromatography. Expression vector pcDNA3 was transiently transfected into CHO-K1 cells and the lysates from these cells were collected and subjected to SDS-PAGE.

3.4. Immunolocalization of monkey 20α-HSD protein in the oviduct and placenta at pre-parturition

To determine the cell types responsible for 20α-HSD protein expression in the oviduct and placenta, immunohistochemical

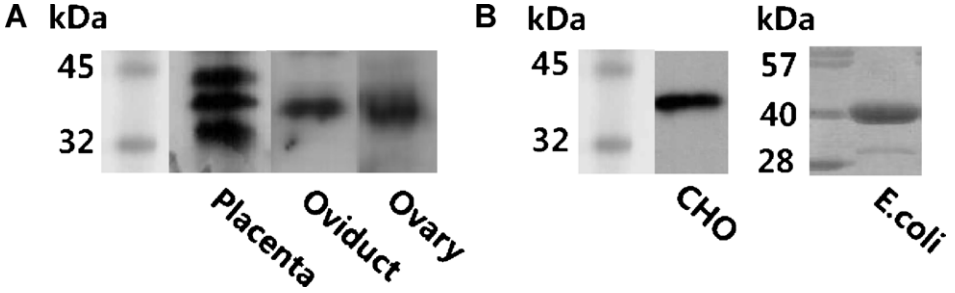

Fig. 3. Western blot analysis of monkey placenta, ovary, and oviduct (A) and expression of recombinant monkey 20α-HSD in mammalian cells and *E. coli* system (B). The proteins of extracted from tissues and recombinant 20α-HSD expressed into the CHO and *E. coli* were transferred onto a PVDF membrane. Protein on the blot was detected with rabbit anti-bovine 20α-HD (1:1000), followed by staining with anti-rabbit IgG-POD as secondary antibody (1:400).

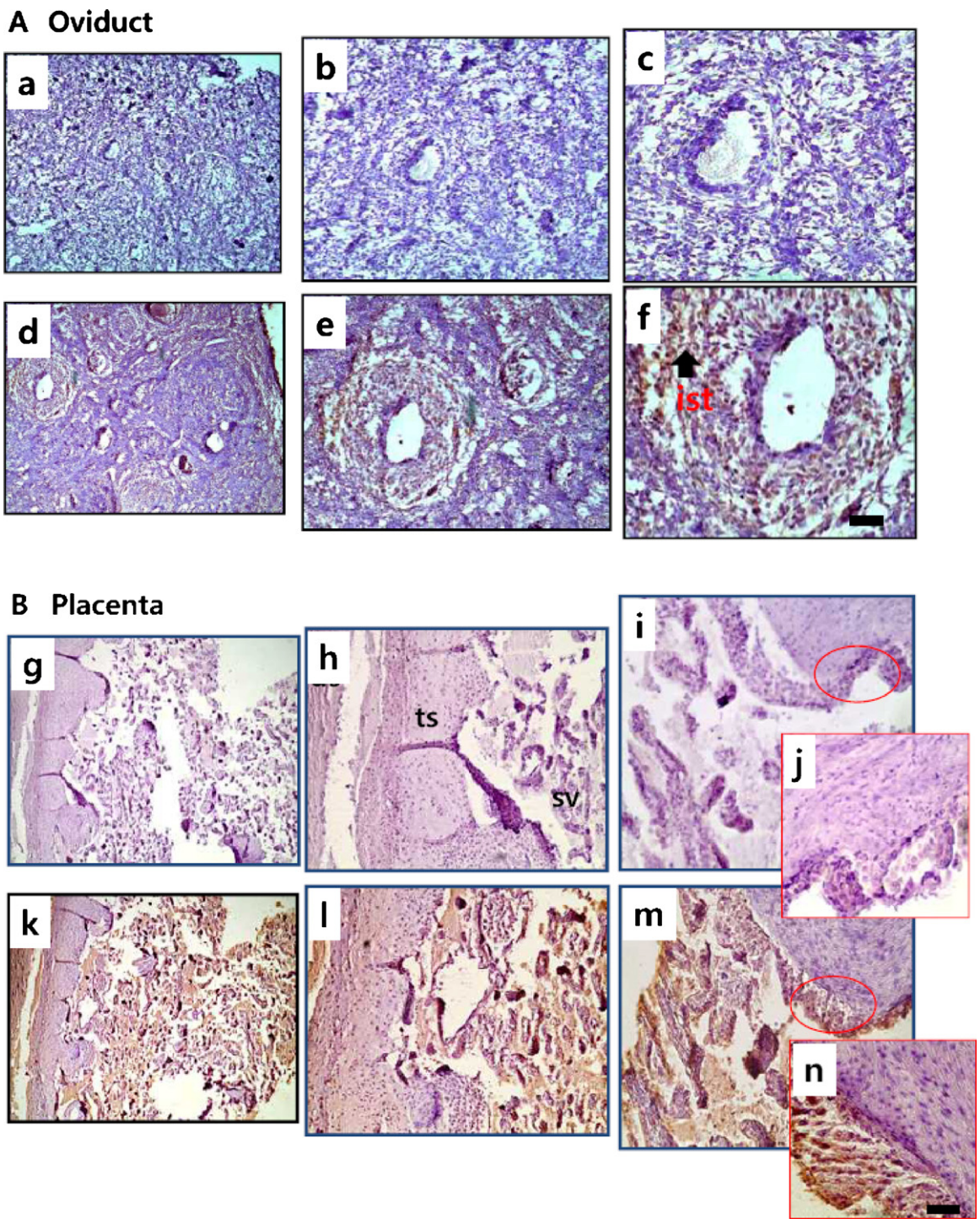

Fig. 4. Localization of monkey 20α-HSD protein expression in the oviduct and placenta at pre-parturition. (A) Oviduct at pre-parturition. Control was stained with pre-immune serum (upper panel). Specific signal was localized in the isthmus cells of muscularis layer as shown in arrow. (B) Placenta at pre-parturition. 20α-HSD expression was localized in the syncytial villi of syncytial trophoblast (under panel). Immunohistochemical method was performed using a Vectastain ABC kit. Control (oviduct: a–c; placenta: g–i) and 40× (g and k); 100× (a, d, h and l); 200× (b, e, i and m); 400× (c, f, j and m). TS, trophoblast; SV, syncytial villi. Black bars = 50 um.

analysis was performed on these 2 tissues. A polyclonal rabbit antiserum against bovine 20α-HSD produced in our lab was used as the primary antibody. In the oviduct, the 20α-HSD protein was localized in the isthmus cells of the muscularis layer, as shown by the arrow in Fig. 4A. The protein was also localized in the syncytial villi of syncytio trophoblasts (Fig. 4B). We should have analyzed protein localization in the ovary tissue by using immunohistochemical analysis. However, we did not analyze the ovarian tissues because after obtaining the ovary, we immediately minced and recovered the cells.

3.5. Characterization of the monkey 20α-HSD gene promoter

The monkey 20α-HSD gene promoter was cloned from monkey placenta at pre-parturition by using LA-PCR. A 2000-bp fragment containing the 20α-HSD 5′-flanking region was amplified from the

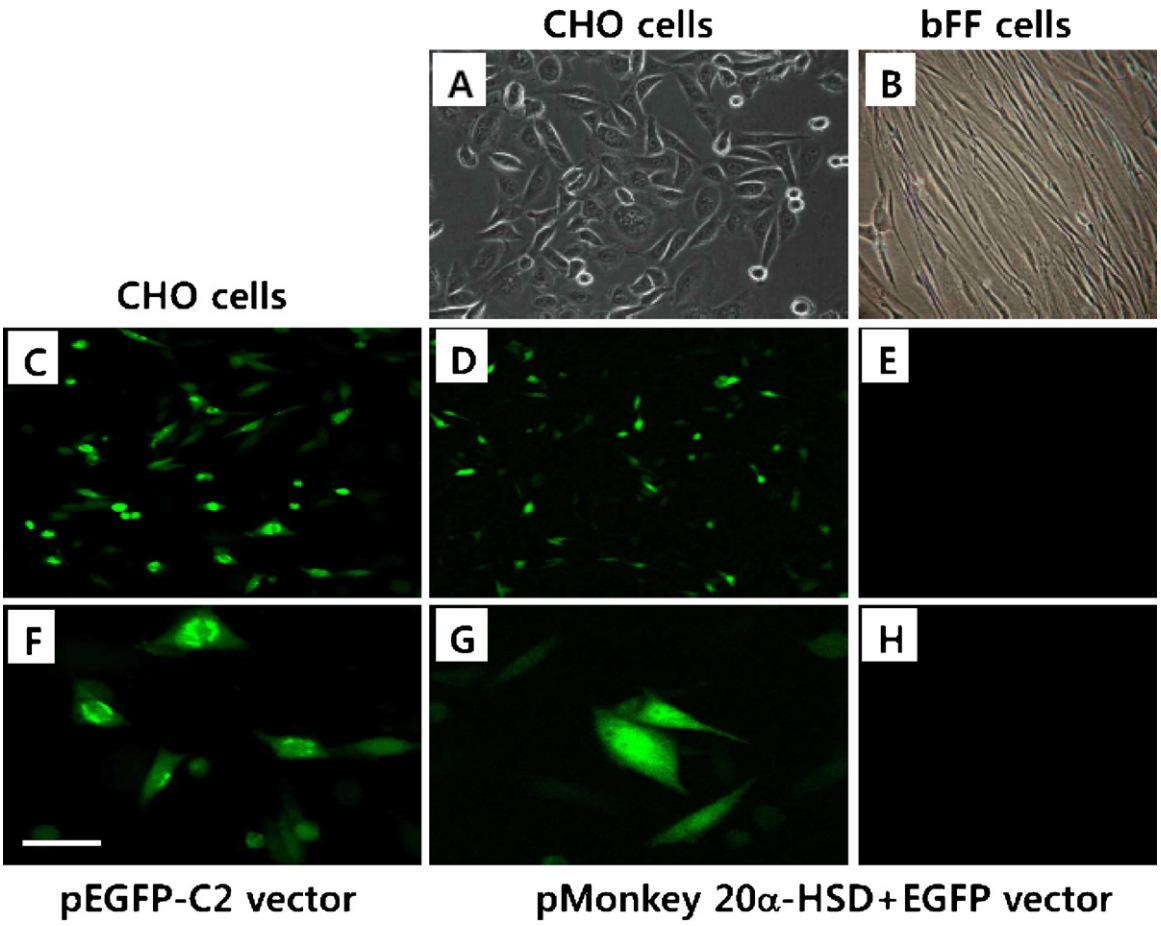

Fig. 5. Detection of GFP expression under monkey 20α-HSD promoter in mammalian cell lines. The constructed vectors (pEGFP-C2 and pMonkey 20α-HSD+EGFP) were transfected into mammalian cells (CHO: A, D and G; bFFs: B, E and H) using monkey promoter vector (pEGFP-C2: C and F; monkey 20α-HSD promoter + EGFP gene: A, B, D, E, G and H). After transfection at 4–6 h, the well was added 250 μL of 20% FBS. And the cells were incubated for 24 h. Next day, the cells were washed 2 times and added 300 μL of CHO-SFM-II. Finally, GFP expression was checked observed using a Nikon Eclipse TE-2000-E confocal microscope. C, D, and E: 100×; E, F, and H: 400×. White bars = 50 um.

*Sal*I enzyme-digested template. The PCR product was cloned and sequenced. The nucleotide sequence of the promoter was determined from −2002 bp to the ATG codon (data not shown).

This promoter region included 2 consensus sequences, a TATA box (−52 → −55 bp) and CCAAT boxes (−59 → −63 bp and −103 → −107 bp). The promoter region also included 22 CpG sites. There were 5 sites from −2005 to −1475 bp, 11 sites from −1139 to −695 bp, and 6 sites from −695 to −47 bp (data not shown). In order to determine the 20α-HSD promoter function in mammalian cells, we made a construct containing the EGFP gene under the control of the monkey 20α-HSD promoter. The vector constructs were transfected into CHO-K1 cells (Fig. 5D) and bFFs (Fig. 5E), and the pEGFP-C2 vector was used as a positive control (Fig. 5C and F). As shown in Fig. 5C and D, the pEGFP and 20α-HSD vectors were efficiently expressed in the CHO cell line. However, we did not detect any GFP expression in the bFF cell line (Fig. 5E); therefore, the vector was not functional in this bovine somatic cell line. We suggest that while the monkey 20α-HSD promoter is functional in cell lines established from ovary, it is not functional in the bFF cell line.

4. Discussions

In this study, we determined the expression and molecular characterization of monkey the 20α-HSD gene in the ovary at pre-ovulation, placenta and in the oviduct at pre-parturition by RT-PCR, northern blotting, and immunohistochemical analysis. In addition, the promoter region was characterized using mammalian cell lines. Higaki et al. [3] reported the expression of monkey 20α-HSD mRNA in the liver, intestine, adrenal gland, and kidney. Liu et al. [8] showed high expression levels in the stomach, liver, kidney, and mammary gland and moderate levels of 20α-HSD mRNA in the ovary, adrenals and colon. In this study, we also detected the expression of the monkey 20α-HSD gene in the reproductive tissues. Expression of 20α-HSD mRNA was high in the ovary at pre-ovulation, while it was moderate in the placenta and low in the oviduct at pre-parturition. By northern blotting, a 1.2 kb mRNA fragment was clearly detected in ovary.

DD1 was reported as the major form of DD in cynomolgus monkey liver, as determined by the purification of the DD enzyme in this form [16] and indanol dehydrogenase [17] from Japanese monkey liver. The high expression level of DD1 in the monkey liver is an additional difference from the human liver, in which at least AKR1C1, AKR1C2, and AKR1C4 mRNA are expressed nearly equally [18] and activity [6,19]. Higaki et al. [3] reported that the distribution pattern of the DD1 transcript in Japanese monkey tissues differs from the ubiquitous expression of the AKR1C1 transcript in human tissues. Monkey 20α-HSD is expressed abundantly in the stomach and in the liver, while it is moderately expressed in the kidney, mammary gland, ovary, and adrenal gland. Low expression levels of 20α-HSD were found in the prostate, endometrium, myometrium, and colon [8]. However, mRNA expression in the placenta and oviduct at pre-parturition was not analyzed. Our result was nearly identical in that mRNA expression was low in the ovary at pre-ovulation. We found that monkey 20α-HSD mRNA was also

expressed in the placenta and oviduct at pre-parturition. This result provides the basis for future investigations on the regulation and function of this enzyme in monkeys.

Goat 20α-HSD mRNA was expressed in the placenta and the intercaruncular part of the uterus during mid-to-late pregnancy but was not expressed in the adrenal gland, liver, or spleen during pregnancy [20]. Goat 20α-HSD mRNA expression was low or at a minimum level in the placenta on day 40 of pregnancy, but it increased by day 90 and remained high until parturition [14]. Human 20α-HSD is highly expressed in the liver, mammary gland, and brain, whereas the expression is lower in the prostate, testis, and uterus and is remarkably low in the adrenal gland [4]. PCR with human 20α HSD-specific primers detected expression not only in the ovary, uterus, and placenta but also in many other tissues such as the heart, liver, adrenal gland, kidney, muscle, peripheral blood lymphocytes, and testis [7]. However, in our study, we only determined 20α-HSD expression in the placenta, ovary, and oviduct. The monkey 20α-HSD shows a distribution profile that is similar to its human homolog, and it is expressed in a large variety of tissues [8]. The function of monkey 20α-HSD expressed in the reproductive tissues remains to be clarified. The expression of 20α-HSD in the ovary and placenta suggest that this enzyme could play a major role in the catabolism of steroids.

In contrast to those in rats, high 20α-OHP levels are present in the blood throughout the estrous cycle in goats, and plasma progesterone and 20α-OHP concentrations are roughly parallel to each other during the estrous cycle [21]. In macaque monkeys, the progesterone level is low (<0.5 ng/mL) during the follicular phase, and the level begins to increase about 1 or 2 days before ovulation [22]. Recombinant monkey 20α-HSD protein, with a molecular weight of 37 kDa, was detected in CHO-K1 cells, and the protein was also detected as a single band in the ovary and oviduct. Its molecular weight was similar to the protein from other species. However, we detected 2 additional bands (about 35- and 39-kDa) in the placenta. These results suggest that the 20α-HSD protein has a key role during the estrous cycle and pregnancy in the ovary, oviduct, and placenta. Interestingly, 3 protein bands were detected in the placenta and this finding was inconsistent with the results of northern blot, in which only 1 band was obtained. These 2 bands need to determine whether these proteins are 20α-HSD isotypes or not. Furthermore, we have to determine the amino acid sequence and molecular mechanism for the production of the other 2 bands detected in placenta. The expression of monkey 20α-HSD in the placenta and in the oviduct during pre-parturition may contribute significantly to the progression of pregnancy by controlling peripheral progesterone concentrations. This is consistent with the view that functional luteolysis is associated with an increase in 20α-HSD activity.

To our knowledge, this is the first study to determine the localization of the 20α-HSD protein in the oviduct and placenta at pre-parturition. Monkey 20α-HSD protein was localized primarily in the isthmus cells of the oviduct and in the syncytiotrophoblasts cells of the placenta at pre-parturition. Histologically, the oviduct consists of the mucosa and muscularis. The muscularis layers of the oviduct tissue are not sufficient in the monkey tissue. The isthmus is the narrowest portion (2–3 mm in diameter) of the parts located in the peritoneal cavity. Syncytiotrophoblasts are multinucleated cells that form the outermost fetal component of the placenta. Increasing their surface area enables nutrient exchange between the mother and the fetus. Immunohistochemical analysis showed that in the rat, the 20α-HSD protein was expressed in decidual cells and trophoblastic giant cells on day 10 as well as spongiotrophoblasts and the visceral yolk sac cells on day 21 of pregnancy [23]. In the mouse, *in situ* hybridization analysis indicated that 20α-HSD mRNA was localized in endometrial epithelial cells, maternal placental endothelial cells, and fetal epidermal cells during pregnancy [24]. We determined the localization of the bovine 20α-HSD protein during the estrous cycle. Bovine 20α-HSD protein was localized in the large luteal cells of the late estrous cycle. In addition, the deer 20α-HSD protein was localized specifically in the basal part of primary chorionic villi and the chorionic stem villi of placenta and was strongly detected in the cell of the corpus luteum on day 30 of pregnancy (unpublished data).

In the present study, the monkey 20α-HSD promoter was cloned and the transcription regulation was analyzed in mammalian cells. The promoter included several putative binding sites for different transcription factors like Ap-1, Sp-1, Oct-1, GATA-1, GATA-2, GATA-3, HSF-2, XFD, CRE-BP, IRF-1, 2, Sox-5, GR, and others. We determined the expression of GFP under the control of the monkey 20α-HSD promoter in mammalian cells, and the results suggested that the promoter is active in cell lines established from the ovary but not in the bFF cell line. Nishizawa et al. [5] suggested that up to 3 kb upstream of the promoters of the human 20α-HSD, bile acid-binding protein (BABP) [AKR1C2], prostaglandin F synthase (PGFS) [AKR1C3], and DD4 (AKR1C4) genes were highly conserved. The transcription factor-binding motifs were found to be almost identical between 20α-HSD and BABP. They used granulose cells from porcine ovarian follicles and reported that the luciferase activity of constructs harboring 20α-HSD and BABP promoters was high in the absence of $PGF_{2\alpha}$ and oxytocin. $PGF_{2\alpha}$ and oxytocin are involved in pregnancy and parturition [25,26]. Treatment with $PGF_{2\alpha}$ and oxytocin increased the luciferase activity of the 20α-HSD promoter 1.7-fold and that of the BABP construct, 1.2-fold. $PGF_{2\alpha}$ stimulation of 20α-HSD gene expression requires the Nur77 transcription factor, which is induced by $PGF_{2\alpha}$ in the corpus luteum of pregnant rats before the induction of 20α-HSD [27,28]. Whether the Nur77 elements found in the 20α-HSD promoter play a role in regulating the transcription of this gene remains an intriguing possibility. Two other hormones that have been shown to down-regulate the levels of 20α-HSD mRNA during pregnancy are prolactin (PRL) and progesterone [29]. Sp-1 family members play a significant role in the expression of the 20α-HSD gene in mouse luteal cells [30]. The monkey 20α-HSD promoter contains specific binding sites for Ap-1 (ATGTCTCAT) and Sp-1 (GGGGCGTTGC) between 276 and 72 bp.

In summary, to the best of our knowledge, this is the first study to determine the localization of the 20α-HSD protein in the placenta and oviduct at pre-parturition. Our study also showed that monkey 20α-HSD mRNA and protein are coordinately expressed in the placenta, ovary, and oviduct. We identified the expression pattern of the 37-kDa 20α-HSD protein and found that monkey 20α-HSD protein was localized specifically in the syncytiotrophoblast of the placenta and in the isthmus cells of the oviduct. The presence of 20α-HSD in the placenta and oviduct indicates that it plays a pivotal role in monkey reproductive physiology. Further studies need to be conducted to determine the functional significance of 20α-HSD during pregnancy and parturition in monkey.

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