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OOCYTES OF BABOON FETAL PRIMORDIAL OVARIAN FOLLICLES EXPRESS ESTROGEN RECEPTOR β mRNA

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Abstract

In fetal ovaries of estrogen-suppressed baboons, we have previously shown that follicle numbers were 50% lower than in estrogen-replete animals and contained oocytes with a reduced number of microvilli. In the baboon fetal ovary, although estrogen receptor (ER) α and β have been detected by immunocytochemistry in granulosa cells, it is not known whether oocytes express ER. Because the actions of estrogen are mediated by interaction with cell-specific receptors, the current study determined whether ER α/β mRNA were expressed in oocytes of baboon fetal ovaries obtained on day 165 (term = day 184) of gestation. Oocyte nuclei and cytoplasm from primordial follicles were isolated by laser capture microdissection and ER α , ER β , GATA-4 (granulosa cell specific marker) mRNAs and 18S rRNA determined by RT-PCR and products verified by sequencing. ER β mRNA was expressed in oocytes of 5 of 5 fetuses. In contrast, fetal oocytes did not express ER α mRNA. Although 18S rRNA was expressed in all oocytes, GATA-4 mRNA was not detected in oocytes and only detected in granulosa cells confirming purity of oocytes sampled. We conclude that oocytes of the fetal baboon ovary express ER β mRNA, thereby providing a mechanism by which estrogen regulates oocyte function, e.g. microvillus development.

Keywords

Ovary; Estrogen Receptor; Fetus; Primate

INTRODUCTION

Ovarian function in the adult is dependent upon developmental events that occur during fetal life, most notably the formation of a pool of primordial follicles comprised of healthy oocytes surrounded by, and in contact with, healthy granulosa cells (1). By midgestation in the baboon (2) as in the human (3,4), oocytes begin to become enveloped by granulosa cells to initiate formation of primordial follicles. Follicle formation is important since it has been proposed that oocytes that enter and then arrest in the diplotene stage of meiosis will regress unless enveloped by granulosa cells (5).

We previously showed that compared to estrogen-replete baboons, in animals in which estrogen levels were suppressed by administration of an aromatase inhibitor throughout the

second half of gestation, fetal ovarian follicle numbers were decreased by 50% whereas the number of interfollicular germ cell nests comprised of oocytes and pregranulosa cells was increased (6). Moreover, in the majority of follicles that were formed in ovaries of estrogen-suppressed baboon fetuses, the oocytes were highly vacuolated and appeared unhealthy, whereas the surrounding granulosa cells appeared intact. Most importantly, oocytes in follicles of estrogen-suppressed animals exhibited a marked reduction in the size and number of microvilli (7), structures essential for uptake of substrates from surrounding granulosa cells (8–10). Based on these results and our studies showing that the effects of estrogen depletion were prevented in fetuses treated concomitantly with aromatase inhibitor and estradiol, we have proposed that estrogen promotes fetal ovarian folliculogenesis and formation of healthy oocytes by controlling development of oocyte microvilli (11, for review).

Although we previously demonstrated that proteins for estrogen receptor (ER) α and ER β (2) were expressed in nuclei of surface epithelium and pregranulosa cells at midgestation and in surface epithelium and interfollicular germ cells nests as well as several but not all granulosa cells in late baboon gestation, it remains to be determined whether ER is expressed in fetal oocytes. Therefore, to determine whether estrogen can directly act on the oocyte to regulate cell-specific expression of molecules involved in oocyte function, e.g., microvilli development, in the current study oocytes free of surrounding granulosa cells were isolated from primordial follicles by laser capture microdissection (LCM) and real time reverse-transcription (RT) polymerase chain reaction (PCR) was utilized to ascertain whether mRNA for ER α/β is expressed in fetal oocytes from ovaries of near-term fetal baboons. To confirm purity of LCM captured oocytes, mRNA for GATA-4, which in the ovary is expressed only by granulosa cells (12,13) was also determined.

MATERIALS AND METHODS

Animals

Ovaries from five baboon (*Papio anubis*) fetuses were obtained on days 160–175 of gestation (term = day 184) as described previously (6). Briefly, baboons were sedated with ketamine (10 mg/g BW), anesthetized with isoflurane, and after obtaining maternal and umbilical blood samples, the placenta and the fetus were delivered by cesarean section and the fetus sacrificed with an overdose of sodium pentobarbital. Fetal ovaries were excised, trimmed off fat, and weighed and fixed in 10% buffered formalin and embedded in paraffin. Archived samples of adult baboon ovary (n = 2) and endometrium (n = 3) frozen in liquid nitrogen and stored at -80°C as well as formalin-fixed-paraffin-embedded (n = 2) or frozen (n = 1) ovaries from adult baboons obtained at random times during apparently normal menstrual cycles were also analyzed. All baboons were cared for and used strictly in accordance with U.S. Department of Agriculture regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication 85-23, 1985). The Institutional Animal Care and Use Committee of the Eastern Virginia Medical School approved the experimental protocol employed in this study.

Slide Preparation for RNA Extraction

Randomly selected sections (4 μm) of paraffin-embedded fetal and adult baboon ovaries were preheated at 55°C for 30 min to increase adhesion to Superfrost microscope slides (Fisher Scientific Co., Arlington, VA) and then deparaffinized by sequential 5 min incubation (twice) in xylenes (Fisher) and subsequent treatment (30 sec) with 100%, 95% and 70% ethanol. After washing in distilled water (30 sec), slides were stained (20–30 sec) with Arcturus blue stain (Arcturus Engineering Co., Mountain View, CA), treated with 95% ethanol (1 min; twice), 100% ethanol (1 min) and xylenes (5 min) and then air dried (60 min).

Laser Capture Microscopy

An Arcturus PixCell II LCM system equipped with Olympus microscope (Arcturus Engineering, Inc.) was employed to capture the nuclei and immediately surrounding cytoplasm of oocytes from primordial follicles of fetal ovaries (80–200 oocytes isolated and pooled from 2–3 sections/animal) and primordial/primary (86–200 oocytes/animal), secondary (24–55 oocytes/animal) and antral (5–36 oocytes/animal) follicles of adult ovaries. In selected instances, a second capture was performed on the same slides to isolate after retrieval of oocytes the remaining single layer of granulosa cells from approximately 120 primordial follicles. A single LCM cap (Arcturus CapSure™ HS LCM Caps, Arcturus Engineering, Inc.) stored at room temperature was used per tissue section. Optimal conditions for LCM capture of oocytes included a laser power of 40–80 mW, a pulse duration of 1.5–2.5 msec, and a laser spot size of 7.5 μm . The LCM cap with captured cells (83–200 oocytes or granulosa cells from 120 primordial/primary follicles) was then tightly fitted to an Eppendorf tube containing lysis buffer (Rneasy, Qiagen, Valencia, CA) and inverted several times and vortexed for 1 minute at room temperature, then incubated at 42°C for 30 min. Lysates were immediately processed for RNA extraction.

RNA extraction

Total RNA was extracted from LCM captured oocytes and granulosa cells by Nonidet P-40-guanidine isothiocyanate extraction/silica gel spin column centrifugation (Rneasy[®] Mini Kit, Qiagen) following the manufacturer's protocol. RNA was eluted from columns in 30 μl ribonuclease-free water. Total RNA was extracted from adult whole ovary and endometrium according to the modified method of Chirgwin *et al.* (14) and as described previously (15, 16). Briefly, tissues were homogenized in 4 M guanidine isothiocyanate/0.83% 2 β -mercaptoethanol, extracted with chloroform:isoamyl alcohol, and RNA isolated by 5.7 M cesium chloride gradient centrifugation. Although the amount of total RNA obtained from LCM captured cells was too low to reliably quantify by spectrophotometry, our laboratories previously showed that integrity of RNA extracted from baboon glandular epithelial cells isolated by LCM was preserved (17).

Reverse Transcription (RT), Real Time Polymerase Chain Reaction (PCR)

Extracted total RNA was reverse transcribed in a 20 μl reaction mixture containing: 1 mM each deoxy-nucleotides, 5 mM MgCl₂, 1 $\times$ PCR buffer, 1U/ μl RNase inhibitor, 2.5 U/ μl Moloney murine leukemia virus (MMLV) reverse transcriptase and 2.5 μM random hexamers (GeneAmp[®] RNA PCR Kit, Applied Biosystems by Roche, Branchburg, NJ) in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Cambridge, MA). Two μl (or in repeat analyses, 3 μl) of the RT mixture was added to separate PCR reaction mixtures (20 μl final volume) containing 4 mM MgCl₂ and 20 pmol of each primer (LightCycler Fast Start DNA Master SYBR Green I; Roche Diagnostics, Mannheim, Germany). Negative controls in which either the RT enzyme or RNA was not added to the RT mixture or to which water solvent was added were performed to test for genomic DNA contamination and specificity of primers, respectively and PCR performed for up to 45 cycles in a programmable Roche LightCycler (Roche Diagnostics). Oligonucleotide primers for ER α , ER β , 18S rRNA and GATA-4 were synthesized by Invitrogen Life Technologies (Rockville, MD) and selected from human cDNA sequences described by Green *et al.* (18), Ogawa *et al.* (19), Torczynski *et al.* (20), and Huang *et al.* (21), respectively. Primers were designed with LC Primer probe with human gene sequences from NCBI (National Center for Biotechnology Information, Bethesda, MD). BLASTn (NCBI) searches against dbEST and the nonredundant set of GenBank, EMBL, and DDBJ database sequences confirmed the total gene specificity of the nucleotide sequences chosen for the primers.

ER α forward primer: 5' - AAATTCAGATAATCGACGCC - 3' (position 816–835) and ER α reverse primer: 5' - AGCCAGAAAAGCATAGGG - 3' (position 1137–1120).

ER β forward primer: 5' - ATCTGTATGCGGAACCT - 3' (position 708–724) and ER β reverse primer: 5' - TCATTATGTCCTTGAATGCT - 3' (position 963–944).

18S rRNA forward primer: 5' - TCAAGAACGAAAGTCGGAGG - 3' (position 1126–1145) and 18S rRNA reverse primer: 5' - GGACATCTAAGGGCATCACA - 3' (position 1614–1595).

GATA-4 forward primer: 5' - GTTTCTAGCACCGAGGA - 3' (position 2175–2191) and GATA-4 reverse primer: 5' - GCCGTTTGGCTAAACT - 3' (position 2626–2611).

PCR products were separated on 2% agarose gel, photographed using Polaroid 667 film (Polaroid Corp., Cambridge, MA) and purified using a Mini Elute Gel Extraction Kit (Qiagen, Inc., Valencia, CA) for sequencing. All real-time RT-PCR analyses were performed in duplicate and repeated at least twice.

Analysis (Microchemical Core Facility, San Diego State University, CA) of PCR products revealed approximately 98–99% sequence identity of the baboon ER α and ER β and 95% sequence identity of the baboon GATA-4 to respective human sequences.

Immunocytochemistry

The immunocytochemical detection of ER β protein was determined using immunofluorescence essentially as described previously (22). Briefly, randomly selected paraffin-embedded sections (4 μ m) of fetal ovaries were mounted onto Superfrost microscope slides (Fisher Scientific), heat fixed and endogenous peroxidase blocked. Sections were incubated for 48 h (4°C) with rabbit polyclonal antibody to ER β (Abcam, Inc., Cambridge, MA) diluted (5 μ g/ml) in 5% normal goat serum (NGS). Sections were then washed and incubated with biotinylated anti rabbit second antibody (Dako Corp., Carpinteria, CA) and protein localized by immunofluorescence using streptavidin conjugated with Alexa Fluor 488 Green (Molecular Probes, Inc., Eugene, OR) diluted 1:500 with 5% NGS and treated with Sudan Black (Sigma Chemical Corp., St. Louis, MO) to quench autofluorescence. Slides were rinsed, treated with propidium iodide (1.0 μ g/ml PBS) to stain nuclei red and following application of mounting media, were sealed with nail polish and examined using an Olympus BX41 microscope (Optical Elements Corp., Melville, NY) equipped with an Olympus DP70 digital camera.

RESULTS

Figure 1 illustrates LCM isolation of oocytes from near-term baboon fetal ovaries fixed in formalin, paraffin-embedded and sections processed as described above. The figure shows a section of fetal ovary before (A) and after (B) oocyte capture from a primordial follicle. Importantly, a single oocyte nucleus and surrounding cytoplasm were captured onto the LCM cap (C) without disrupting the granulosa cells of the follicle.

Adult baboon whole ovary expressed the expected 322 and 259-bp mRNAs for ER α (crossing point 23–25 cycles) and ER β (crossing point 26–28 cycles), respectively, an anticipated 489-bp 18S rRNA (crossing point 8–10 cycles) and mRNA for GATA-4 (crossing point 32–34 cycles) detected as a 452-bp product (Fig 2A). In contrast, although adult baboon endometrium also expressed mRNA for ER α and ER β as well as 18S rRNA, the 452-bp mRNA product for the granulosa cell-specific marker GATA-4 was not detected (Fig 2B). All PCR products expressed in the ovary and endometrium exhibited 95%–99% sequence identity to respective human sequences. Moreover, melting curves for all PCR products detected in endometrium

and adult whole ovary (not shown) were homogenous and exhibited distinct single melting peaks.

GATA-4 mRNA was not detected in oocytes isolated by LCM from fetal ovaries (Fig 3A) or adult oocytes from primary, secondary or antral follicles (not shown) confirming the purity of the oocyte populations. In contrast, 18S rRNA was detected (crossing point 28–33 cycles) in all oocytes isolated by LCM (Fig 3B). However, as anticipated, GATA-4 mRNA was expressed as a 452-bp product in granulosa cells isolated by LCM from primordial follicles of fetal (Fig 3A) and adult primordial follicles (not shown).

The 259-bp mRNA for ER β was detected in fetal oocytes (Fig 3B and Table 1) captured from ovaries of 5 of 5 baboons. In contrast, ER α mRNA was not detected in oocytes from fetal ovaries. All fetal oocytes isolated expressed 18S rRNA (Fig 3B and Table 1) for which the melting curves also exhibited a single peak. ER β mRNA and 18S rRNA were also detected in oocytes isolated from primary, secondary and antral follicles of ovaries of adult baboons (Table 2). However, mRNA for ER α although not detected in oocytes from secondary or antral follicles was detected in oocytes from primary follicles in 2 of 3 adult ovaries. In contrast, mRNA for GATA-4 was not detected in any oocyte isolated from adult follicles.

Immunocytochemistry confirmed that ER β protein was expressed by and localized primarily to nuclei of oocytes and granulosa cells of primordial follicles in the near term baboon fetal ovary (Fig 4A). Specificity was confirmed by absence of signal in sections incubated without primary antibody (Fig 4B).

DISCUSSION

The results of the current study are the first to unequivocally demonstrate expression of ER β , but not ER α , mRNA in a homogeneous population of fetal oocytes isolated by LCM from ovaries obtained from baboons in late gestation. The current results support and extend our previous findings in which we demonstrated using Northern blot and immunocytochemistry that the near-term baboon fetal ovary (2) expressed mRNA and protein for both ER α and ER β and that protein for ER α was not detected in oocytes. Rather, ER α protein was detected primarily in nuclei of pregranulosa cells of germ cell nests and granulosa cells of several but not all primordial follicles, whereas ER β was extensively expressed in nuclei of granulosa cells of all primordial follicles. In our previous study, however, we could not confirm presence or absence of ER β protein in fetal oocytes which most likely reflected limitation and sensitivity of procedures employed. However, using immunofluorescence, the current study showed that nuclei of oocytes as well as granulosa cells of primordial follicles expressed ER β protein. In a study of human fetal ovaries, although ER α and ER β proteins were detected and localized to granulosa cells of primordial follicles, proteins also appeared to be detected in oocytes of primordial follicles (23). However, as pointed out by the authors (23) “immunostaining was clearly localized to the cytoplasm and only occasional nuclear staining was detected”. These discrepancies most likely reflect differences in methodology e.g. time required to obtain and fix tissues; procedures for immunocytochemical analysis. The results of the current study also indicate that expression of ER β mRNA in the oocyte of the fetus is maintained into adulthood and thus manifest in oocytes of primordial/primary, secondary and antral follicles in the adult baboon ovary. In previous studies (2,15), we showed that in adult baboon ovary ER α protein was expressed in, but limited to, granulosa cells surrounding the oocyte of antral follicles, whereas ER β protein was abundantly expressed in granulosa cells of antral as well as preantral and less developed (e.g. primary) follicles. ER α has also been localized to granulosa cells of antral follicles in the human (24–26). Although transcripts for ER α / β mRNA have been detected in human cumulus-oocyte complexes and oocytes, mRNA was not detected in granulosa/cumulus cells from *in vitro* fertilization and benign gynecological specimens (27).

Collectively, the current study and our previous studies further document that the fetal oocyte, as well as fetal granulosa cells of primordial follicles of the baboon fetal ovary, are estrogen-target cells.

We previously demonstrated (6,7) that in estrogen-suppressed baboons, fetal ovarian follicle numbers were reduced by approximately 50%, and that more than 70% of the follicles formed appeared unhealthy and contained oocytes with a marked reduction in size and number of microvilli, structures critical for nutrient uptake from surrounding granulosa cells (8–10). Because follicle numbers and microvilli were normal in fetuses treated with estradiol, we proposed that estrogen regulates fetal ovarian folliculogenesis and development of follicles with oocytes comprised of microvilli and thus presumably long-term survival (11). The present study shows that ER β mRNA is expressed in oocytes of fetal baboons, thereby providing a mechanism for estrogen to exert a direct action on the oocyte to control expression of the components, e.g. ezrin, (28–30), that are required to form the microvilli. However, it remains to be determined whether oocyte ER β mRNA expression is altered in estrogen-suppressed baboons in which oocyte microvilli development is markedly reduced.

In the current study, we also showed that mRNA for GATA-4 was not expressed in baboon fetal oocytes, but was detected in granulosa cells of primordial follicles from which oocytes were isolated by LCM. These findings are consistent with results reported in human fetal oocytes in which GATA-4 mRNA and protein were also specifically localized by *in situ* hybridization and immunocytochemistry, respectively, to granulosa cells and absent from oocytes (13). Thus, the results of the current study not only confirm that GATA-4 mRNA is a specific marker of granulosa cells in the baboon fetal and adult ovary, but also document that oocytes isolated by LCM from follicles of fetal as well as adult ovaries are not contaminated with cytoplasm from surrounding granulosa cells.

In summary, the results of the current study show that the oocytes of fetal primate ovary express ER β mRNA, thereby providing a mechanism by which estrogen regulates folliculogenesis and development of microvilli in fetal ovarian oocytes. In addition, the combination of LCM and real time RT-PCR provide a significant technological advance to study the cell-specific effects of estrogen on oocyte-granulosa cell development and expression of mRNA and proteins that regulate follicle development and function.

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Figure 1.

Photomicrograph of hematoxylin/eosin histology illustrating isolation by laser capture microdissection (LCM) of oocytes from primordial follicles of fetal ovaries obtained from baboons in late gestation. (A) Primordial follicle showing an oocyte surrounded by granulosa cells before LCM. (B) Remaining granulosa cells after removal of the oocyte nucleus and immediately associated cytoplasm. (C) Oocyte nucleus/cytoplasm collected on LCM cap. Original magnification = 200×

Figure 2.

A, B: Representative real time RT-PCR of GATA-4, ER α and ER β mRNA and 18S rRNA in whole ovary (A) and endometrium (B) from an adult baboon. Total RNA was reverse transcribed and added to separate PCR reaction containing oligonucleotide primers and PCR performed for 45 cycles. The 452-bp GATA-4, 322-bp ER α , 259-bp ER β , and 489-bp 18S, products were separated on agarose gels (M, marker ladder).

Figure 3.

Representative real time RT-PCR of GATA-4 (452-bp), ERα (322-bp) ERβ (259-bp) mRNA and 18S rRNA (459-bp) expression in oocytes and granulosa cells isolated by LCM from primordial follicles of paraffin-embedded fetal ovaries obtained from 3 (panel A) to 4 (panel B) baboons in late gestation. Total RNA was extracted, reverse transcribed and PCR products were separated on agarose gels.

Figure 4.
Representative photomicrograph of ER β protein in oocytes and granulosa cells of primordial follicles of the baboon fetal ovary in late gestation. Paraffin-embedded sections were incubated with primary antibody and stained green with streptavidin conjugated with AlexaFluor 488. Original magnification = $\times 400$; $\times 1000$ insert

Table 1Expression of ER α , ER β , GATA-4 mRNA and 18S rRNA in fetal baboon oocytes^a.

	# fetuses expressing ER α mRNA	# fetuses expressing ER β mRNA	# fetuses expressing 18S rRNA	# fetuses expressing GATA-4 mRNA
Primordial oocytes from baboon fetuses (n = 5)	0	5	5	0

^a Oocytes isolated by LCM from primordial follicles of paraffin-embedded baboon fetal ovaries obtained on day 165 of gestation (term = day 184).

Table 2

Expression of ER α , ER β and GATA-4 mRNA, and 18S rRNA in oocytes of follicles isolated from ovaries of adult baboons.^a

	# animals expressing ER α mRNA	# animals expressing ER β mRNA	# animals expressing 18S rRNA	# animals expressing GATA- 4 mRNA
Primary oocytes	2	3	3	0
Secondary oocytes	0	2	3	0
Antral oocytes	0	3	3	0

^aOocytes isolated by LCM from follicles of 3 paraffin-embedded adult baboon ovaries.