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Short Communication

Follicle-Stimulating Hormone Receptor Is Expressed in Human Ovarian Surface Epithelium and Fallopian Tube

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The cellular expression of pituitary gonadotropin receptors in gonadal tissues is poorly defined because of the lack of suitable reagents. In this study, we developed* in situ *hybridization and reverse transcription polymerase chain reaction techniques for the evaluation of follicle-stimulating hormone receptor (FSHR) expression in the ovary and fallopian tube. Using a single-strand RNA probe, we demonstrated that FSHR mRNA expression is strongest in Graafian follicles. Within these developing follicles, granulosa cells showed the greatest expression, although both theca interna and theca externa were also positive, interna greater than externa. Granulosa cells in both primary and primodial follicles were positive, with primordial follicles showing only weak focal positivity. Ovarian surface epithelium and fallopian tube epithelium, not previously recognized to express FSHR, were both strongly positive. The FSHR expression in the ovary and fallopian tube was confirmed by reverse transcription polymerase chain reaction. Our results indicated that the FSHR is expressed in a cell-specific fashion at different stages of follicular development and is also expressed in ovarian surface and fallopian tube epithelia. The presence of FSHR in ovarian surface epithelium and of gonadotropin-binding sites in ovarian neoplasms provide additional evidence supporting the derivation of epithelial ovarian tumors from the surface epithelium and should promote heightened interest in the gonadotropin theory of ovarian tumorigenesis. More importantly, this study shows the feasibility of evaluating FSHR expression by both* in situ *hybridization and reverse transcription polymerase chain reaction. Application of these techniques to tumor specimens will help to elucidate the role of gonadotropins and their receptors in the carcinogenesis of gynecological tumors.(Am J Pathol 1996, 148:47–53)

Follicle-stimulating hormone (FSH) is a glycoprotein hormone synthesized in the pituitary and secreted in both males and females. The follicle-stimulating hormone receptor (FSHR) and the luteinizing hormone receptor (LHR) act synergistically and play pivotal roles in reproductive physiology. FSHR is a cell surface receptor present mainly on gonadal cells and acts through interactions with G proteins.[1,2] The predicted membrane topography of these receptors is that they traverse the membrane with seven helical domains, with an extracellular amino terminus and an intracellular carboxy terminus.[2,3] The transmembrane domains of FSHR share approximately 70% sequence homology with other members of the G-protein-coupled family, such as LHR and thyrotropin-stimulating hormone receptor.

By ligand-based assays, FSHR expression has been localized to all ovarian granulosa cells and testicular Sertoli cells, whereas LHR was found mainly on ovarian theca, interstitial, and luteal cells and granulosa cells of the Graafian follicles and on

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testicular Leydig cells.[4] The fallopian tube, a structure that undergoes cyclic changes in response to ovarian steroid hormones,[5–7] was not considered traditionally to be a direct target organ for gonadotropins. A recent study, however, identified functional expression of LHR as well as the gene of its ligand (hCG/LH) in human fallopian tube,[8] suggesting the likely involvement of gonadotropins and their receptors in the physiological functions of the fallopian tube.

The possible roles of these receptors in gynecological tumors are also unclear. It is known that the age-specific incidence of ovarian cancer rises slowly in the early forties, then increases rapidly, coinciding with the onset of menopause and the rise of pituitary gonadotropin levels.[9] This observation raises the possibility that gonadotropins may be linked to the development of ovarian cancer; the expression status of FSHR and LHR in these tumors is thus of considerable interest. A major obstacle to the study of tissue expression of FSHR and LHR, however, has been the lack of suitable reagents. Most studies to date relied on ligand assays,[10–13] which are technically cumbersome and do not show clear cellular localization. This is in sharp contrast to the analysis of other hormonal receptors, eg, estrogen, progesterone, and androgen receptors, for which antibodies are commercially available and widely used in diagnostic pathology. In this study, we developed *in situ* hybridization (ISH) and reverse transcription polymerase chain reaction (RT-PCR) assays to circumvent this technical difficulty and to evaluate the expression of FSHR in human ovary and fallopian tube.

Materials and Methods

Tissues

Fresh human adult ovarian and fallopian tube tissues were obtained from the Department of Pathology, The New York Hospital/Cornell Medical Center. The tissues were collected from salpingo-oophorectomy specimens removed for benign diseases from women of childbearing age, were embedded in OCT compound, and snap-frozen in liquid nitrogen.

Oligonucleotide Primers

Oligonucleotide primers were designed based on published FSHR sequences[14] and commercially synthesized (Operon Technologies, Alameda, CA). The 5′ primer was derived from exon 1 (5′-GAGAGCAAGGTGACAGAGATTCC-3′, nucleotides 97 to 120), and the 3′ primer was from exon 5 (5′-CCTTTTGGAGAGAATGAATCTT-3′, nucleotides 439 to 417). This primer pair covered a region involved in FSH binding and signal transduction, as suggested by Sprengel et al.[2] and Tilly et al.[15] This region of human FSHR was chosen because it shares less than 50% homology with other members of the pituitary-derived tropic hormone receptor family, such as LHR and thyrotropin-stimulating hormone receptor, excluding the possibility of cross-hybridization. In addition, these primers, located at different exons, effectively rule out the possibility of false positive PCR results caused by DNA contamination.

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated by the guanidine thiocyanate-CsCl gradient method.[16] Three micrograms of RNA were reverse transcribed into cDNA in a 20-μl reaction, following the protocol of GeneAmp RNA PCR Kit (Perkin Elmer-Cetus, Emeryville, CA). The PCR reaction mixture contained 10 μl of the RT product, 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 1 μmol/L of oligonucleotide primers, and 1.25 U of *Taq* DNA polymerase (Perkin Elmer-Cetus). The samples were subjected to 35 cycles of amplification in a thermal cycles (Perkin Elmer-Cetus) with 1 minute of denaturation at 94°C, 1 minute of primer annealing at 60°C, and 1 minute of primer extention at 72°C. Primers amplifying the p53 gene (exon 5 and exon 6) sequences[17] were included as the positive control. Two negative controls were included for all experiments, using distilled H_2O as the substrate for RT and for PCR, respectively. After PCR, 10-μl aliquots of PCR products were analyzed by 1.5% agarose gel or 8% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

Plasmid Cloning and Preparation of RNA Probes

For preparation of single-strand RNA probes, the PCR-amplified FSHR cDNA fragment was isolated from the agarose gel, electroeluted, and cloned into PCR direct-cloning vector pT7Blue (Novagen). The plasmid clone containing the appropriate DNA insert was selected and digested with *Bam*HI and *Pst*I, and the DNA insert was subcloned into pBluescript KS^+ vector (Strategene, La Jolla, CA). Single-strand antisense RNA probe incorporating digoxigenin-labeled UTP (Boehringer Mannheim Biochemicals, In-

dianapolis, IN) was then synthesized with the RNA transcription kit (Strategene) and T3 polymerase (GIBCO-BRL, Gaithersburg, MD), following the manufacturer's protocol. The sense RNA probe was also synthesized as a negative control, with T7 polymerase (GIBCO-BRL). With 1 μg of purified DNA template, the typical riboprobe yield was 10 μg, as assessed by Genius System Boehringer Mannheim. The RNA probes were aliquoted and stored at −20°C.

DNA Sequence Analysis

The FSHR cDNA in pBluescript KS^+ was DNA sequenced to confirm the cloned sequence. Plasmid DNA was prepared with Wizard Minipreps (Promega, Madison, WI), and double-strand dideoxy DNA sequencing was carried out with Sequenase DNA sequencing kit (USB, Cleveland, OH), following manufacturers' protocols.

In Situ *Hybridization*

Tissue sections on Probe-On slides (Fisher Scientific, Pittsburgh, PA) were deparaffinized, dehydrated, and treated with pepsin (2.5 mg/ml in 0.12 N HCl, pH 2) at 45°C for 5 to 10 minutes. The pepsin concentration used was determined through titration for optimal signal. Prehybridization was then performed for 1 hour at 45°C in hybridization buffer (50% v/v formamide, 5X standard saline citrate (SSC), 2% w/v blocking reagent (Boehringer Mannheim), 0.1% w/v *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate) in a moist chamber. Probes were preheated to 68°C for 5 minutes, immediately chilled, and used for hybridization. Titration of the probes between 0.05 and 8 μg/ml indicated that 4 μg/ml was the optimal probe concentration for maximal signal with minimal background. Hybridization was conducted in a moist chamber for 12 hours at 45°C. The slides were washed for 10 minutes with 2X SSC, followed by two 15-minute washes with 0.1X SSC at 45°C. The signal was detected with an alkaline phosphatase color reaction using antidigoxigenin antibody coupled to alkaline phosphatatse (Boehringer Mannheim), following the manufacturer's protocol. 4-Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate were used as the substrate, generating purple end products. The incubation was performed in the dark at room temperature for 3 to 15 hours depending on the signal development. The reaction was stopped by washing for 5 minutes at room temperature with 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0. Slides were counterstained with 1% fast red for 2 minutes and mounted with Permount (Fisher).

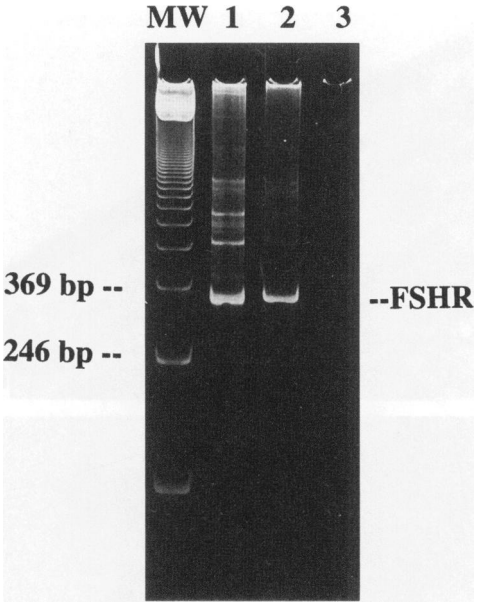

Figure 1. *Analysis of FSHR mRNA expression by RT-PCR. Total RNA from ovary* (lane 1) *and fallopian tube* (lane 2) *show the predicted 343-bp amplification product, indicating FSHR mRNA expression. Liver RNA* (lane 3) *is negative, and testicular RNA is positive (data not shown).*

Results

Detection of FSHR mRNA Expression with RT-PCR

RT-PCRs were performed on total RNA from ovary, fallopian tube, testis, and liver. The RNA integrity was confirmed in all preparations by successful amplification of p53 sequences (from exon 5 to exon 6, data not shown). FSHR amplification results showed FSHR mRNA expression in both human ovarian and fallopian tube, as indicated by the presence of 343-bp PCR products (Figure 1). Human testis, used as a positive control, showed PCR product of identical size (data not shown). Human liver, in contrast, was negative for FSHR expression (Figure 1).

To confirm that the amplified sequence is the FSHR gene product, the RT-PCR product was cloned and sequenced. The DNA sequence obtained was identical to the published human FSHR sequence.[14]

In Situ *Hybridization*

The FSHR expression in the ovary was localized by ISH with digoxigenin-labeled antisense riboprobe.

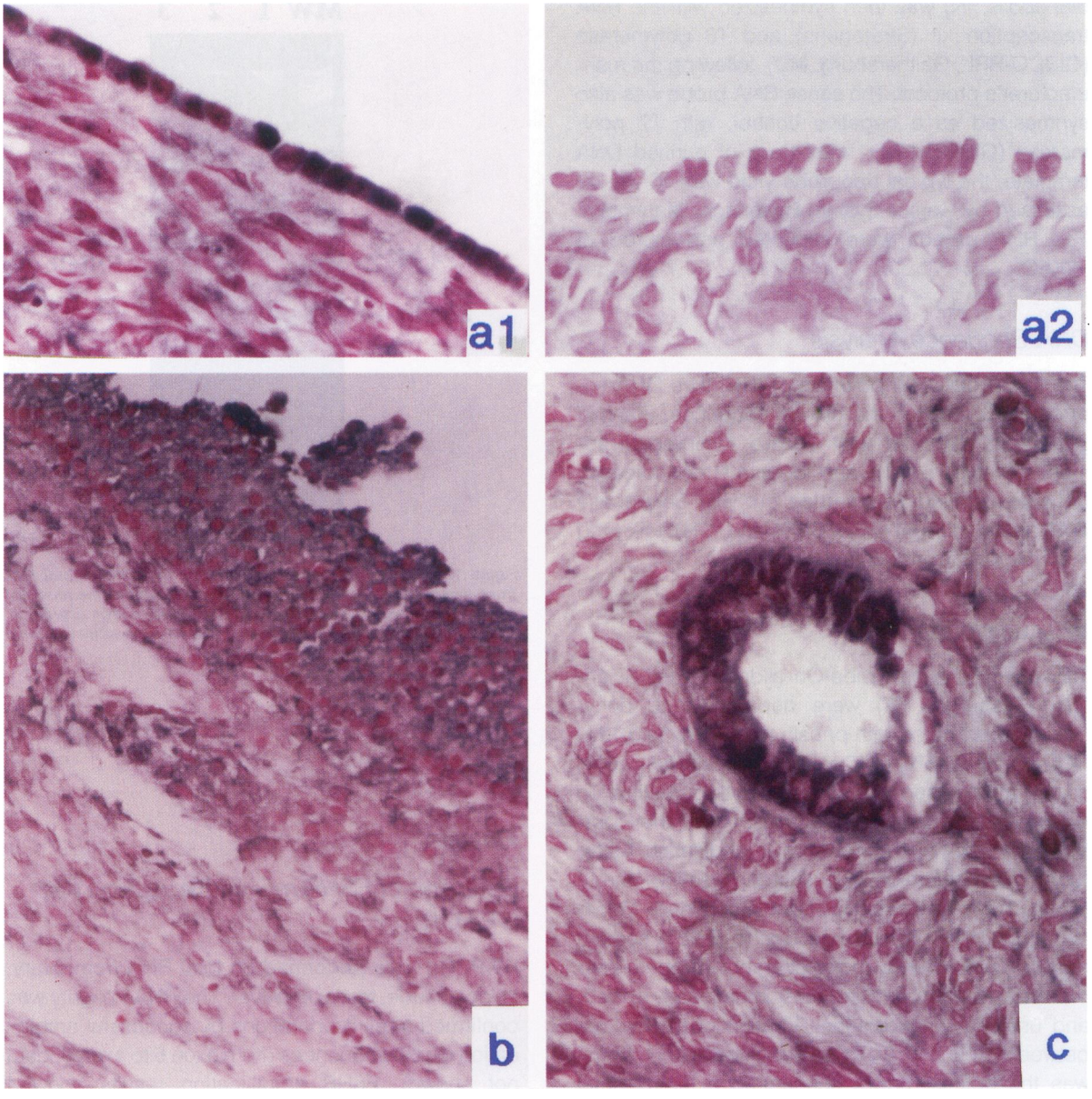

Figure 2. *Analysis of FSHR mRNA expression in ovary by* in situ *hybridization. Positive expression is indicated by dark purple cytoplasmic staining, as is seen in ovarian surface epithelium* (a1), *a Graafian follicle* (b), *and a primary follicle* (c). *Negative control with sense probe* (a2) *was negative on ovarian surface epithelium. In Graafian follicles, mRNA expression is seen in both granulosa cells and theca layers. Ovarian stroma* (a *and* c) *show variable background staining with no discrete cytoplasmic localization. Magnification,* ×*400* (a *and* c) *and* ×*200* (b).

Results showed the presence of FSHR transcripts in surface epithelium (celomic epithelium), Graafian follicles, and primary follicles. The expression in ovarian surface epithelium was uniform and of moderate intensity (Figure 2a1), in contrast to the negative result with the control sense probe (Figure 2a2). In the Graafian follicles, strong staining was seen in all granulosa cells (Figure 2b). Theca cells exhibited slightly weaker signals, interna greater than externa. Granulosa cells in primary follicles showed moderate positivity (Figure 2c), and primordial follicle showed only focal weak positivity, suggesting a low level of expression (data not shown). Corpus luteum and corpus albicans were FSHR mRNA negative.

ISH analysis of the fallopian tube showed abundant diffuse mRNA expression in the mucosal epithelium but not in the smooth muscle and interstitial tissue (Figure 3). No obvious difference in FSHR mRNA expression was seen between ampulla and isthmus or between ciliated and nonciliated cells. Evaluation of nine specimens showed no apparent cyclic variation of FSHR mRNA expression.

To demonstrate the specificity of the hybridization signal, the following controls were performed: (1)

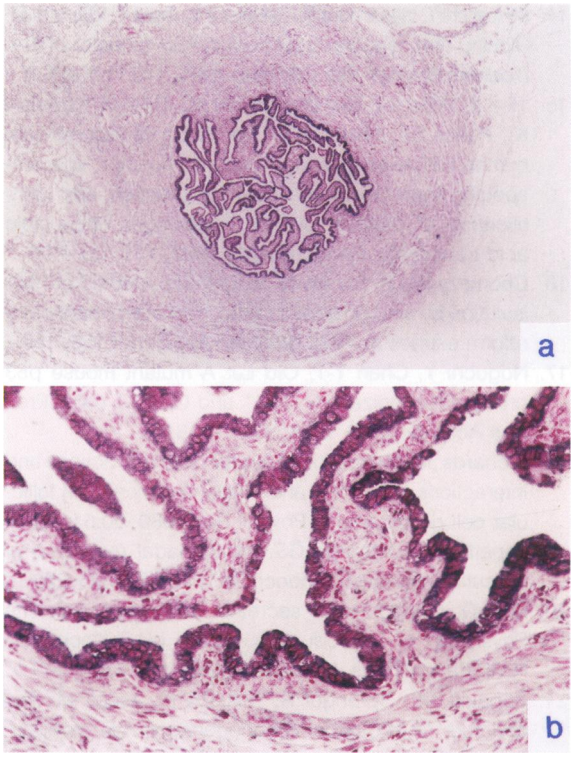

Figure 3. *Analysis of FSHR mRNA expression in fallopian tube, showing strong expression in tube epithelium but not in the muscular layer or interstitium. Magnification,* ×*63* (a) *and* ×*400* (b).

substitution of the antisense probe with a digoxigenin-labeled sense probe (Figure 2a2), (2) RNAse (20 μg/ml) pretreatment of the tissue sections at 37°C for 15 minutes, and (3) excess complementary sense RNA probe. No signal was obtained after any of these three treatments. The hybridization signal was also abolished when the digoxigenin-labeled probe was omitted, ruling out the possibility of endogenous alkaline phosphatase activity.

Discussion

Ovarian follicular development is dependent on an appropriate milieu of steroid and gonadotropins for its maintenance and eventual maturation during the reproductive cycle.[18,19] Folliculogenesis depends not only on the circulating levels of the gonadotropins but also on the expression of gonadotropin receptors by follicle cells in the ovary.[20] Despite recent advances in understanding the hormonal regulation of the ovarian gonadotropin receptor mRNAs in animal studies,[4,21,22] little information is available regarding expression of FSHR mRNA in human gonadal tissue. Recently, Hecket and Griswold[23] demonstrated that two FSHR transcripts of 4.5 and 2.6 kb are expressed in rat testicular Sertoli cells and that these mRNAs are regulated in the stages of the seminiferous epithelium. Camp et al[4] showed that the rat FSHR mRNA levels are regulated in a complex fashion during the recruitment, maturation, and ovulation of the ovarian follicles. To examine FSHR mRNA expression in the human ovarian and fallopian tissues, we have developed a highly sensitive and specific PCR-based assay to detect FSHR mRNA in total RNA samples. As a verification of this assay, we examined FSHR expression in these tissues with both RT-PCR and nonradioactive ISH procedures. Using both methods, we observed that both human ovary and fallopian tissues express FSHR mRNA. With ISH, we demonstrated that FSHR mRNA was expressed in follicles of all maturation stages, strongest in the Graafian follicles and weakest in primordial follicles. This is in agreement with previous *in vitro* and radiolabeled ligand-binding studies,[4,24,25] in which it was suggested that the granulosa cells of nearly all follicles have receptors for FSH. Our observation that both theca interna and theca externa also express FSHR mRNA, however, has not been previously reported. The functional significance of this expression remains to be elucidated.

The epithelial neoplasms of the ovary are generally accepted as originating from the surface epithelium (modified mesothelium or surface celomic epithelium).[26,27] Several lines of evidence suggest that these ovarian epithelial tumors may be target tissues of gonadotropins: (1) a significant number of human ovarian tumors have been shown to contain binding sites for gonadotropins by biochemical binding studies,[11,12,28–30] (2) the growth of cell lines derived from ovarian epithelial tumors can be stimulated by gonadotropins,[31,32] and (3) ovarian tumors develop in animal models after prolonged treatment with exogenous gonadotropins or elevated levels of endogenous gonadotropins.[33,34] In this study, we found that the ovarian surface epithelium does indeed express an abundant level of FSHR mRNA, providing another piece of supporting evidence for the gonadotropin theory of ovarian tumorigenesis. Analysis of FSHR expression in ovarian neoplasms will help shed light on this question, and experiments are currently underway.

Another intriguing, previously unrecognized observation we noted was the extragonadal expression of FSHR mRNA in the mucosal epithelium of the fallopian tube. The presence of FSHR, considered in conjunction with the recent finding that the fallopian tube also expresses LHR,[8] suggests that gonadotropins may directly regulate the physiological functions of the tube, eg, the transport, maturation, and fertilization of oocytes, and initial embryonic development. Tumors of

the fallopian tube are similar pathologically to ovarian tumors of surface epithelial origin.[27] The establishment of ISH and RT-PCR techniques now allows us to evaluate FSHR expression in these tumors and study the possible role of FSH and FSHR in the tumorigenesis of the ovary and fallopian tube.

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