Published in final edited form as:

Mol Cell Endocrinol. 2007 March 15; 267(1-2): 26–37.

FSH stimulates ovarian cancer cell growth by action on growth factor variant receptor

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Summary

A number of FSH Receptor (FSH-R) isoforms with distinct structural motifs and signaling paradigms have been described, including a single transmembrane domain variant that functions as a growth factor type receptor (FSH-R3). This study tested the hypothesis that FSH can stimulate ovarian cancer cell proliferation by acting on FSH-R3, using the tumorigenic mouse ovarian surface epithelial cell (MOSEC) line ID8. FSH enhanced ID8 proliferation in a concentration-dependent fashion. Moreover, FSH-treatment of ID8 elicited intracellular events consistent with activation of FSH-R3 and distinct from those associated with activation of the canonical G-protein coupled FSH-R isoform (FSH-R1). Specifically, the FSH-R3 signaling pathway included cAMP-independent activation of ERK downstream of an SNX-482 sensitive component likely to be the Cav2.3 calcium channel. Northern analysis using probes specific for exons 7 and 11 of FSH-R identified consistently only one 1.9 kb transcript. Immunoblot analysis confirmed expression of FSH-R3 but not FSHR-1 in ID8. Together, these data suggest that FSH-R3 signaling promotes proliferation of ovarian cancer cells.

Keywords

Follicle stimulating hormone receptor; ovarian cancer; ovarian surface epithelium

1. Introduction

Ovarian cancer causes more deaths than any other cancer of the female reproductive system in the United States (American Cancer Society, 2005). Although all cell types of the human ovary may undergo neoplastic transformation, the majority of tumors (80–90%) are derived from the ovarian surface epithelium (Scully, 1995;Riman et al., 2004). Epidemiological data show that the risk of epithelial ovarian cancer is decreased by pregnancy and oral contraceptive use, and increased in women over the age of 45 years (Riman et al., 2004). On this basis, it has been proposed that repetitive ovulatory trauma and high circulating concentrations of follicle stimulating hormone FSH and luteinizing hormone LH may contribute to cancer development or progression (Fathalla, 1971;Stadel, 1975;Riman et al., 2004). Experimental models of ovarian cancer support a role for repeated epithelial trauma-repair (Godwin et al., 1992;Testa et al., 1994;Roby et al., 2000), and gonadotropin stimulation in the development and progression of ovarian cancer.

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FSH receptors (FSH-R) are expressed by normal ovarian surface epithelial cells, as well as by epithelial ovarian carcinoma cells and ovarian cancer cell lines (Zheng et al., 2000;Syed et al., 2001;Parrott et al., 2001;Choi et al., 2002;Ji et al., 2004). Moreover, FSH-treatment has been shown to activate pathways associated with cell proliferation and oncogenesis in normal and malignant ovarian surface epithelial cells (Schiffenbauer et al., 1997;Syed et al., 2001;Parrott et al., 2001;Ji et al., 2004;Choi et al., 2004;Choi et al., 2005;Abd-Elaziz et al., 2005). Interestingly FSH levels are reportedly elevated in the peritoneal fluid of cancer patients (Halperin et al., 1999). FSH-R expression was found to be higher in in peritoneal implants and ovarian epithelial tumors than in normal human surface epithelium and fallopian tube (Zheng et al., 1996).

The FSH-R is coded by a single large gene containing 11 exons, and this complex genomic structure favors extensive alternative splicing (Sairam et al., 1996;Sairam et al., 1997;Tena-Sempere et al., 1999). At least three isoforms with distinct structural motifs and signaling paradigms have been cloned (Khan et al., 1993;Sairam et al., 1996;Sairam et al., 1997;Yarney et al., 1997;Babu et al., 1999;Babu et al., 2000;Touyz et al., 2000;Babu et al., 2001). Their exon structures are shown in Figure 1. Other FSH-R splice variants are believed to lack functional significance (Kraaij et al., 1998). Thus, the physiological importance of exon-skipping in the processing of the FSH-R gene remains controversial.

The functionally significant FSH-R isoforms feature a long extracellular domain (>300 amino acids) that is important for ligand binding (Yarney et al., 1993;Sairam et al., 1996;Sairam et al., 1997;Yarney et al., 1997;Babu et al., 1999). The canonical receptor (FSH-R1) is a 7 transmembrane domain protein that is coupled to G_s and activation of adenylate cyclase (Simoni et al., 1997;Babu et al., 2000). One of the splice variants (FSH-R3) has the topology of a growth factor receptor and can promote cell proliferation via a cAMP-independent pathway involving ERK and voltage-dependent calcium channels (Sairam et al., 1997;Babu et al., 1999;Babu et al., 2000;Touyz et al., 2000;Babu et al., 2001). The importance of FSH-R3 in ovarian cancer development and progression has not been investigated previously to our knowledge.

The current study was designed to test the hypothesis that FSH can stimulate cancer cell proliferation by acting on the growth factor type FSH-R. The experimental model was ID8, a tumorigenic cell line derived from mouse ovarian surface epithelial cells (MOSEC) transformed by repeated passage *in vitro* (Roby et al., 2000). MOSEC cells were employed because the expression and functional significance of FSH-R3 have been documented most convincingly in the mouse ovary (Babu et al., 2001). The ID8 cell line was chosen, because functional data and transcriptomic analysis suggest that this murine model is relevant to the human disease and valid as a source of novel and diagnostic targets.(Roby et al., 2000;Urzua et al., 2005;Urzua et al., 2006) Additional consideration was given to the well-characterized ability of ID8 MOSEC to form tumors in immunocompetent mice; this attribute will facilitate translation of our findings to *in vivo* models of ovarian cancer progression.

2. Materials and Methods

2.1 Reagents

Porcine FSH (pFSH) and recombinant human (hFSH) were purchased from the National Hormone & Peptide Program, Harbor-UCLA Medical Center (Torrance, California). Total ERK antibody (sc-94), antibody directed against the N-terminus of FSHR (sc-7798) and all secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Antibody directed against Cav2.3 (anti- α 1E) was purchased from Chemicon International, Inc. (Temecula, California). Antibody against FSH-R3 was based on a previously described epitope

(Babu et al., 1999), and custom ordered from Gallus Immunotech Inc. (Ontario, Canada). SNX82 was purchased from Alomone labs (Jerusalem, Israel). Mouse ovary tissue lysate (INSTA-Blot™) was provided by Imgenex (San Diego, CA). Culture medium was obtained from Mediatech, Inc. (Herndon, CA). Other reagents and chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, Missouri) unless otherwise stated. cDNA constructs encoding FSH-R variants were obtained from R. Sairam at the Clinical Research Institute of Montreal (Montreal, CA).

2.2 Cell culture

A clonal cell line (ID8) of MOSEC transformed by repeated passage was provided by K. Roby at the University of Kansas Medical Center (Kansas City, KS) (Roby et al., 2000). MOSEC were cultured in DMEM supplemented with 4% FBS, 100U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite at 37°C in a humidified atmosphere of 5% CO₂.

PGC-2 cells were obtained from B. Downey (McGill University, Montreal, CA) and maintained at 37°C in a humidified atmosphere of 5% CO₂ in McCoy's modified 5A medium supplemented with 10% FBS, as described previously in detail (Kwan et al., 1996). PGC-2 monolayers were transfected with either FSH-R1 or FSH-R3 using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Stable cell lines were selected and maintained by culture in the presence of G418 (800 µg/ml).

2.3 Assessment of MOSEC proliferation

MOSEC were seeded in 24-well plates at a concentration of 5×10^4 cells/ml in the complete medium in the presence or absence of FSH and SNX-482. At specified time points, cells were washed once with Hank's Balanced Salt Solution (HBSS), lifted with 0.025% trypsin in PBS, and then collected and counted using a hemacytometer.

2.4 cAMP assay

cAMP was measured in cell lysates using an enzyme immunoassay according to the instructions provided by the manufacturer (Assay Designs, Ann Arbor, Miami). IBMX (40 µM) was included in all treatment groups in all experiments.

2.5 Immunoblotting and Determination of ERK Phosphorylation

Whole cell lysates were prepared from ID8 MOSEC cultures in Tris-buffered saline (TBS) containing 1% deoxycholate, 1% Nonidet P-40 and protease inhibitor cocktail (Sigma P-8340, 1:500). Protein concentrations were estimated by using Micro BCA™ protein assay reagent kit (Pierce, Rockford, Illinois). MOSEC lysates were separated by SDS-PAGE (4–12% NuPAGE Novex Bis-Tris with MOPS running buffer, Invitrogen, Carlsbad, California) and transferred to nitrocellulose (0.22 µm pore). Membranes were blocked by incubation at room temperature for 1 hour with TBS containing 0.1% Tween 20, 10% nonfat dry milk and 10% glycerol, and then incubated in primary antibody overnight at 4°C. After washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 hour at room temperature. Bound antibodies were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia). Results were recorded on radiographic film. Equal loading was confirmed either by immunoblotting for actin or by Coomassie-blue staining of gel lanes. In experiments where a single membrane was probed under more than one experimental condition, primary and secondary antibodies were removed using Restore™ Stripping Buffer (Pierce). Primary antibody dilutions were: phosphorylated

ERK1/2 (pERK1/2, 1:1000); total ERK1/2 (tERK 1/2, 1:1000); FSH-R3 (1:1000); Cav2.3 (anti- α_1 E, 1:200); FSH-R (1:250).

For determination of ERK phosphorylation, MOSEC were seeded in six-well plates at a concentration of 5×10^4 cells/ml, and cultured for 4 hours under serum-free conditions before exposure to FSH or SNX-482. After detection of p-ERK1/2, membranes were stripped and re-probed for t-ERK1/2. Densitometry was performed to normalize pERK1/2 to t-ERK1/2 from the same membrane (AlphaEaseFc™ software, Alpha Innotech Corporation).

2.6 DNA isolation and Southern blotting

Genomic DNA was purified from ID8 MOSEC using the SDS proteinase K-phenol-chloroform extraction method (Maniatis et al., 1982). Briefly, the cultures were subjected to centrifugation at $5000 \times g$ for 5 min at 4°C and the resultant cell pellet was homogenized in 2 ml of DNA extraction buffer (0.1M NaCl, 1% SDS, 0.05 M EDTA and 0.05 M Tris-HCl pH 8.0) containing 0.5 mg/ml proteinase K and incubated at 58°C over night. DNA from this mixture was purified by performing phenol/chloroform/isoamyl alcohol extraction and ethanol precipitations (Maniatis et al., 1982). The final DNA pellet was dissolved in 200 μ l of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). The quantity of DNA was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Approximately 40 μ g of genomic DNA was digested with 40 units of EcoRI in a 50 μ l reaction volume at 37°C for 3 h; the reaction was stopped by adding 6X gel loading buffer to 1X final concentration. Ten μ g each of the digested DNA was used per well and resolved on a 0.9% agarose gel for 3 h. The DNA was transferred onto a BrightStar-Plus positively charged nylon membrane (Ambion, Austin, TX) by alkaline downward transfer (1M NaCl and 400 mM NaOH). The membrane was cut to separate the lanes and used for hybridization with 32 P-labeled oligonucleotide probes specific for Exons 7 (7R: 5'ggttccttgatgcacagttgtg) or 11 (11R: 5'cagaaattcactgcctctggcc) or both. Hybridization was performed overnight at 42°C using 10 ml of ULTRAhyb-Oligo hybridization buffer (Ambion) in a roller bottle hybridization incubator (Lab-line Instruments, Melrose Park, IL). End labeling of the oligonucleotides was performed by T4 polynucleotide kinase (Promega, Madison, WI) reaction using γ^{32} P-ATP (Amersham, Piscataway, NJ) according to standard protocols (Maniatis et al., 1982). The blots were then washed twice for 10 min at 42°C with 2X SSC (0.3 M NaCl and 0.03 M sodium citrate) containing 0.5% SDS. Subsequently, the membranes were exposed to radiographic film (BioMax, Eastman Kodak Co., Rochester, NY) at -70°C with an intensifying screen for 4 days, and then the film was developed. The molecular sizes of the hybridized restriction digested DNA fragments were estimated by comparison with molecular standards of λ DNA digested with Hind III enzyme (Promega).

2.7 RNA isolation and Northern blot analysis

Total RNA was isolated from ID8 MOSEC by following the protocols associated with the Tri reagent RNA isolation kit (Sigma-Aldrich). The resultant RNA pellet was washed with 70% ethanol, air dried and resuspended in 80 μ l of TE buffer (pH 8.0). RNA quality was assessed either by visual inspection of 5 μ l of RNA on a 0.8 % formaldehyde agarose gel (Maniatis et al., 1982), or by analysis of the absorption spectrum detected using a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies).

RNA (~30 μ g total RNA per lane) was resolved on a 0.8% formaldehyde agarose gel (105 volts for 2.5 h at room temperature), and then transferred onto the BrightStar-Plus positively charged nylon membrane (Ambion) by alkaline downward transfer method according to the manufacturer's instructions. The membrane was cut to separate each lane containing RNA and

used for hybridization experiment with ^{32}P labeled oligonucleotide probes specific for Exon 7 or 11 or both probes combined (as described above). Conditions for hybridization and washings were optimized such that: hybridization was performed at 42°C overnight using ULTRAhyb-Oligo hybridization buffer (Ambion); blots were washed twice (10 min/wash) with SSC buffer containing 0.5% SDS at 42°C. The membranes were exposed for 4 days to radiographic film (Eastman Kodak) at -70°C with an intensifying screen before the film was developed. The sizes of the hybridization signals were estimated by comparison with the RNA molecular weight markers (Promega).

2.8 Biotinylation

MOSEC were incubated at room temperature with EZ-Link sulfo-NHS-biotin (Pierce, Rockford IL) in PBS, lysed in a buffer containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM Tris-HCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% protease inhibitor cocktail, combined with 200 μl ImmunoPure Immobilized streptavidin (Pierce) and rocked overnight at 4°C. Biotinylated proteins were pelleted by centrifugation and eluted using Laemmli Sample Buffer. The starting material, supernatant, and biotinylated fractions were subsequently subjected to SDS-PAGE and immunoblot analysis as described above.

2.9 Immunocytochemistry

MOSEC were attached to glass slides by cytocentrifugation, and subsequently fixed in acetone for 5 minutes, 2% paraformaldehyde (in PBS) for 15 minutes and cold methanol for 2 minutes. After 3 washes in PBS, cells were permeabilized by 5 minutes incubation in 0.1% Triton-X-100, and blocked in 10% goat serum for 1 hour before overnight incubation at 4°C in primary antibody. Primary antibody dilutions were: FSH-R3 (1:500), Cav2.3 ($\alpha_1\text{E}$, 1:100), or Na^+/K^+ -ATPase (1:200) overnight. Subsequently, slides were washed 3 times in PBS, then incubated in darkness with fluorescein isothiocyanate-conjugated IgG secondary antibody (1:200) for 2 hours at room temperature. After additional washes, Vectashield (Vector Laboratories, Inc., Burlingame, California) was added as an antifade reagent. MOSEC were visualized with a confocal microscope (135-M LSM Microsystem, Carl Zeiss, New York). Slides processed without primary antibody, and incubated with primary antibody in the presence of excess antigen were included to assess staining specificity.

2.10 Statistical Analysis

Data are expressed as mean $\pm$ SEM. Significant differences between groups were identified by ANOVA and multiple comparisons were made using Tukey's procedure (Statistix 8, Analytical Software, Tallahassee, FL). Differences were considered to be significant when $P \leq 0.05$. The numbers of replicates per treatment group are provided in the figures or accompanying legends.

3. Results

3.1 FSH Effects on MOSEC

The ability of FSH to stimulate ID8 MOSEC proliferation was assessed in a series of experiments that employed a range of hormone concentrations and treatment durations. The number of viable MOSEC in culture increased significantly over time under control conditions and in the presence of EGF (10 ng/ml) or FSH (20 ng/ml) (Figure 2A). EGF enhancement of ID8 MOSEC growth is expected (Roby et al., 2000), and was included as a positive control. Interestingly, the effect of FSH on ID8 MOSEC proliferation was significantly greater than that of EGF at 4, 8, 12 and 16 hours of culture. In a separate study using 24 hours of culture as the endpoint, FSH was shown to enhance MOSEC proliferation in a concentration-dependent fashion over a range of 2 to 200 ng/ml (Figure 2B). At concentrations between 2

and 2000 ng/ml, FSH had no stimulatory effects on the accumulation of either estrogen or progesterone in 24 hour ID8 MOSEC cultures (data not shown). There was no significant difference between the effects of the purified and recombinant gonadotropin preparations when recombinant hFSH was tested to rule out any potential effects of low level contaminants in the purified porcine FSH (pFSH) (Figure 2C). Purified porcine FSH was used in all experiments other than Figure 2C.

3.2 Associated Signal Transduction Pathway

The canonical seven-transmembrane, G-protein coupled FSH-R (FSH-R1) is typically associated with a signal transduction pathway that involves stimulation of adenylate cyclase, whereas the growth factor variant FSH-R3 signals via ERK1/2 activation and calcium influx in the absence of increased intracellular concentrations of cAMP (Babu et al., 2000). Specifically, the FSH-R3 receptor has been linked previously to cAMP-independent activation of ERK and to Ca^{2+} influx via voltage-gated channels in two heterologous expression systems, stably transfected immortalized pig granulosa (JC-410) and human embryonic kidney (HEK-293) cells (Babu et al., 2000; Touyz et al., 2000).

The effect of FSH on intracellular cAMP accumulation in ID8 MOSEC was investigated as a first step towards identifying the FSH-R isoform that mediates enhanced cell proliferation. FSH (20 ng/ml) failed to stimulate cAMP accumulation in ID8 cells (Figure 3A) despite the presence of functional adenylate cyclase (Figure 3B). To ensure that our experimental conditions did not interfere with activation of FSH-R1, we examined the effects of FSH on intracellular cAMP in immortalized porcine granulosa cells (PGC-2) stably transfected with either FSH-R1 or FSH-R3. As expected, gonadotropin stimulation of FSH-R1, but not FSH-R3, increased intracellular cAMP (Figure 3D versus 3C). The response of the untransfected ID8 MOSEC to FSH was similar to that of immortalized granulosa cells (PGC-2) stably transfected with FSH-R3 (Figure 3A versus Figure 3C) and unlike that of PGC-2 stably transfected with FSH-R1 (Figure 3A versus Figure 3D).

The effect of FSH on ERK1/2 phosphorylation in ID8 MOSEC was investigated, because ERK activation in the absence of cAMP accumulation has been associated previously with activation of FSH-R3 (Babu et al., 2000). FSH stimulated ERK phosphorylation in these MOSEC in a time-dependent fashion. After hormone addition the phosphorylated forms of ERK1 and ERK2 were evident at 10 minutes, elevated after 30 minutes and declined to basal levels after 60 minutes (Figure 4). These results are similar to those reported previously for immortalized granulosa cells transfected with FSH-R3 (Babu et al., 2000). The importance of ERK activation in FSH-stimulation of ID8 MOSEC growth was confirmed by the effects of the MEK inhibitor PD98059. Pretreatment of cells with PD98059 (100 μM) for one hour not only prevented FSH-activation of ERK (data not shown), but also antagonized FSH-stimulation of cell proliferation. After 16 hours, the number of cells ($10^5/\text{mL}$) was significantly higher in cultures treated with FSH (3.8 ± 0.02 , $n=3$) than in either untreated cultures (2.8 ± 0.03 , $n=3$) or cultures pretreated with PD98059 prior to FSH exposure (2.6 ± 0.16 , $n=3$).

In mammalian cells transfected with FSH-R3, FSH has been shown not only to increase the influx of extracellular calcium, but also to stimulate cell proliferation and ERK phosphorylation in a calcium-dependent fashion (Babu et al., 2000; Touyz et al., 2000). Here, the effects of the calcium channel antagonist SNX-482 were assessed in ID8 MOSEC to determine if the FSH-dependent activation of ERK and stimulation of growth depend on the influx of calcium via voltage-gated channels. SNX-482 blocks the low- to intermediate-voltage-activated calcium currents conducted by $\text{Ca}_v2.3$ channels (Newcomb et al., 1998; Jing et al., 2005). $\text{Ca}_v2.3$ channels or currents have been described in the granulosa and epithelial cells used previously for heterologous expression of FSH-R3 (Berjukow et al., 1996; Ballard H. et al., 2003).

SNX-482 (100 nM) had no effects on either basal cell proliferation or ERK phosphorylation in ID8 MOSEC; however, SNX-482 prevented FSH from stimulating both MOSEC growth and ERK activation (Figure 5). The results obtained using SNX-482 at a 10 nM concentration were similar to those obtained using the 100 nM concentration (data not shown).

3.3 Molecular basis of FSH-R in ID8 MOSEC

The functional data presented above suggest strongly that the FSH effects on MOSEC growth are mediated by the single membrane-spanning domain growth factor receptor FSH-R3 rather than by the seven transmembrane domain, G-protein coupled receptor (GPCR) FSH-R1. The expression of FSH-R3 RNA was examined using Northern blot analysis. The expression of FSH-R1 and FSH-R3 protein was assessed by Western analysis.

The mouse FSH-R3 Exon 11 sequence was identified by bioinformatics. Initially, BLAST analysis at the NCBI database using the previously reported sheep FSH-R3 Exon 11 amino acid sequence (Sairam et al., 1997) identified a human homolog, but not the mouse homolog. Subsequently, nucleotide sequences spanning the human homolog were used in homology searches to identify the mouse FSH-R3 protein coding sequence spanning Exon 11. The analysis aided the identification of several BAC clones spanning the mouse FSH-R gene. One of these (GenBank accession no. **AC166993**) contains the FSH-R locus spanning Exons 2 to 11. Figure 6A is a schematic diagram of the BAC clone showing the EcoRI sites surrounding Exons 7 and 11 along with the predicted restriction digested fragments, and the nucleotide and amino acid sequences spanning Exon 11. Because the 3' end of the untranslated region of the Exon 11 could not be determined, sequence similar in length to that reported for sheep FSH-R3 is shown (Sairam et al., 1997). Two oligonucleotide probes from within Exons 7 and 11 were designed from the complimentary strand and used for mapping the transcription and genomic regions spanning Exon 7 (7R) and Exon 11 (11R); the sequences of the probes are also shown in Figure 6A. Figure 6B is a schematic diagram of the predicted FSH-R3 mRNA.

These Exon 7 and 11 specific oligonucleotide probes were used in both the Southern blot analysis of the EcoRI digested genomic DNA (Figure 6C, left) and Northern blot analysis of the total RNA (Figure 6C, right) isolated from ID8 MOSEC. The Exon 7 probe detected only one 11.6 kb fragment of EcoRI digested DNA. Similarly, the Exon 11 probe hybridized with a 4.3 kb EcoRI restricted fragment in the mouse genomic DNA. The hybridization analysis of the DNA blot with both Exon 7 and 11 probes recognized 11.6 and 4.3 kb fragments. Moreover, these EcoRI cut DNA fragments matched with the predicted fragments identified based on the sequence information reported in the mouse BAC clone (compare Figure 6A and 6C).

Independent of the oligonucleotide probes used, the RNA blot analysis identified only one transcript of about 1.9 kb. This transcript was recognized by both the Exon 7 and 11 probes, hybridized either one at a time or together. The detection of a single 1.9 kb transcript by oligonucleotide primers specific for Exons 7 and 11 is inconsistent with identification of FSH-R1 RNA. To determine if FSH-R1 protein was expressed by ID8 MOSEC, Western blot analysis was performed on mouse ovary tissue and ID8 MOSEC lysates using a commercially available antibody (FSHR (N-20): sc-7798; Santa Cruz) recommended for detection of FSH-R1 (~75 kDa) in mouse, rat and human tissue. This antibody is directed against an N-terminal sequence of the FSH-R and would be expected to detect all functional FSH-R isoforms, including FSH-R1 and FSH-R3.

FSH-R (N-20) detected a prominent band of ~75 kDa consistent with FSH-R1 in the mouse ovary tissue lysate but not the ID8 lysate (Figure 7A). More faint bands of ~50 kDa consistent with FSH-R3 were apparent in both the mouse ovary and ID8 lysates. Neither the 75 kDa nor

the 50 kDa bands were detectable when the primary antibody was pre-incubated with the appropriate blocking peptide.

In contrast to the results obtained with antibody FSH-R (N:20), the antibody directed specifically against FSH-R3 detected prominent protein bands at ~50 kDa in both the mouse ovary tissue and ID8 lysates (Figure 7B). Two bands with slightly lower molecular mass were also detected by the FSH-R3 antibody in the ovary tissue lysate. These may represent partially glycosylated forms of FSH-R3 present in the cell types that comprise the normal mouse ovary (Babu et al., 2001; Menon et al., 2005); however, this hypothesis was not tested experimentally.

3.4 Surface Membrane Localization of FSH-R3 and Cav2.3 proteins in ID8 MOSEC

Surface expression of FSH-R3 protein in MOSEC was confirmed by immunocytochemistry, as well as by surface biotinylation and immunoblot analysis (Figure 8). Surface expression of the SNX-482 sensitive Cav2.3 channel was demonstrated in a similar fashion (Figure 8). The results of confocal microscopy indicate that both the FSH-R3 and Cav2.3 proteins are co-localized with the membrane marker Na^+/K^+ -ATPase. Moreover, after surface biotinylation and immunoblot analysis both FSH-R3 and Cav2.3 are detected in the biotinylated (plasma membrane) fraction. The FSH-R3 antibody detected a single protein band of ~50 kDa, consistent with the results shown in Figure 7.

4. Discussion

The role of gonadotropin stimulation in the development and progression of ovarian cancer is controversial, and the associated signal transduction pathways are defined incompletely. The specific nature of the aberrant transduction pathways can suggest strategic therapeutic targets for treatment of cancer. Thus, it is important to understand the mechanisms by which FSH can affect cancer cell growth and survival.

FSH receptors can be expressed by normal ovarian surface epithelial cells, as well as by epithelial ovarian carcinoma cells and ovarian cancer cell lines (Zheng et al., 2000; Syed et al., 2001; Parrott et al., 2001; Choi et al., 2002; Ji et al., 2004). FSH-treatment has been shown to activate pathways associated with cell proliferation and oncogenesis in normal and malignant ovarian surface epithelial cells (Schiffenbauer et al., 1997; Syed et al., 2001; Parrott et al., 2001; Ji et al., 2004; Choi et al., 2004; Choi et al., 2005; Abd-Elaziz et al., 2005). To date, it has been assumed that FSH actions on ovarian cancer cells were mediated by the canonical 7 transmembrane domain GPCR FSH-R1. However, Northern blot analysis of FSHR transcripts from normal ovarian surface epithelium and ovarian cancer cell lines has demonstrated expression of multiple FSH-R transcripts, with smaller transcripts predominant in surface epithelial and cancer cells compared to granulosa (Parrott et al., 2001; Choi et al., 2002). Furthermore, where expression of FSH-R message has been documented in ovarian tissues and tumors using PCR techniques (Syed et al., 2001; Ji et al., 2004), the oligonucleotide primers have been based on regions of the FSH-R common to FSH-R1 and its functional splice variants (Sairam et al., 1996; Sairam et al., 1997; Babu et al., 1999; Babu et al., 2001).

This investigation was designed to test the hypothesis that FSH can stimulate cancer cell proliferation by acting on FSH-R3, an alternatively spliced FSH-R isoform reported to function as a growth factor type receptor (Sairam et al., 1997; Babu et al., 2000; Babu et al., 2001). The experimental results demonstrate convincingly that FSH promotes proliferation of transformed MOSEC by stimulation of its growth factor variant receptor and subsequent activation of a signal transduction pathway that involves ERK and the Cav2.3 channel.

4.1 FSH-R Isoform Expression

Sequence analysis aided the identification of an FSHR genomic region in a BAC clone that included the Exon 11 sequence absent from FSH-R1 but present in FSHR2/R3. Southern blot analysis confirmed the specificity of these Exon 7- and 11-specific DNA probes as they detected the predicted DNA fragments from the FSHR genomic locus. Northern blot analysis using the probes specific for Exon 7 or 11 alone or together identified only one 1.9 kb transcript. The detection of only one transcript by oligonucleotide probes specific for Exons 7 and 11 suggests that the transcript does not represent FSHR1. Furthermore, the transcript detected is smaller than those of either FSH-R1 or FSH-R2 which exceed 2.4 Kb in size (Sairam et al., 1997; Babu et al., 1999; Parrott et al., 2001). While it is possible that the FSH-R transcript detected in ID8 MOSEC cells could represent either FSH-R2 or FSH-R3 or both as these two transcripts contain Exons 7 and 11 (Figure 1), it is highly unlikely that the 1.9 kb transcript is FSH-R2 based on its size. Additional experiments such as ID8 library construction and cloning could confirm absolutely that this transcript is FSH-R3. Instead, our subsequent experimental efforts were focused on detection of FSH-R1 and FSH-R3 proteins in ID8.

An anti-FSH-R3 antibody directed against an 18 amino acid epitope in sheep Exon 11 (GQREHISEFGLKSKQHPN) recognized only one major protein of ~50 kDa in ID8 MOSEC, as expected from a published study that used a similar antibody to identify FSH-R3 in mouse ovarian membrane fractions (Babu et al., 2001). The Exon 11-specific mouse amino acid sequence homologous to this region (GSEFLNLESKEEAKS) contains 7 amino acids conserved between mouse and sheep FSHR3; it is likely that the immunogenic epitope is within the conserved region. The size of the FSH-R3 protein identified in this study (~50 kDa) is larger than that predicted from the FSHR3 primary structure (~28 kDa), but consistent with that detected previously (Babu et al., 1999; Babu et al., 2000; Babu et al., 2001). The difference between the theoretical and actual molecular mass of FSH-R3 has been attributed to dimerization of the glycosylated receptor protein (Sairam et al., 1997; Babu et al., 2001).

Antibody directed against a region of the FSH-R common to FSH-R1 and FSH-R3 detected a ~75 kDa protein consistent with FSH-R1 in lysates from mouse ovary tissue but not ID8 MOSEC. These data demonstrate convincingly that FSH-R1 is expressed by one or more cell types represented in the ovarian tissue lysate. This finding is unremarkable, as expression of FSH-R1 by granulosa cells of ovarian follicles is well documented (Burns et al., 2001). Interestingly, the FSH-R antibody did detect a prominent band in ID8 MOSEC at ~50 kDa, the apparent molecular mass of FSH-R3. Plasma membrane expression of FSH-R3 in ID8 was confirmed using confocal microscopy and biotinylation. In summary, the analysis of both RNA and protein expression suggest strongly that FSH-R3 is the only FSH-R isoform expressed by ID8 MOSEC, and demonstrate that FSH-R3 protein is expressed on the surface of these tumorigenic cells.

4.2 FSH-R3 Signaling Pathway

Role of MAPK—FSH-treatment of ID8 MOSEC elicited intracellular events similar to those documented in response to activation of the growth factor variant FSH-R3 and distinct from those associated with activation of the G-protein coupled isoform FSH-R1. In ID8 MOSEC treated with FSH, intracellular cAMP was decreased rather than increased; phosphorylation of ERK was evident within minutes of gonadotropin exposure. PD90859 (MEK inhibitor) prevented FSH-stimulation of both ERK activation and ID8 MOSEC proliferation. Thus, the signal transduction pathway involving FSH-R3 activation of MAPK appears to be critical to the FSH stimulated growth of these MOSEC transformed by repeated passage.

FSH has been shown previously to promote the proliferation of a number of immortalized pre-neoplastic and neoplastic human ovarian surface epithelial (IOSE-29, IOSE-29E, HOSE) and ovarian cancer (OVCAR-3, OVCA) cell lines (Syed et al., 2001;Choi et al., 2002). Interestingly, in human ovarian surface epithelial cells transformed by transfection with SV40 T-Antigen in the absence (IOSE) or presence of E-cadherin (IOSE-29E) co-transfection, the proliferative effect of FSH was associated with activation of ERK, but not accumulation of cAMP (Choi et al., 2002). That observation suggests that FSH-R3 signaling could play a role in ovarian carcinogenesis in species other than the mouse. Further investigation of FSH-R expression in malignant human ovarian surface epithelial cells is warranted to determine the functional significance of splice variants other than the canonical GPCR FSH-R1.

Role of Cav2.3 Channel—FSH-stimulation of not only cell proliferation, but also ERK phosphorylation is dependent on the influx of extracellular calcium in JC-410 cells stably transfected with FSH-R3 (Babu et al., 2000). Moreover, in either JC-410 or HEK-293 cells engineered to over-express FSH-R3, the FSH-induced increase in intracellular calcium is rapid, concentration-dependent and absent in Ca^{2+} free buffer or in the presence of chelating agents (Babu et al., 2000;Touyz et al., 2000). Calcium entry has been presumed to occur through L-type voltage-gated channels, because FSH-induced calcium responses were blocked by the calcium channel blocker diltiazem in HEK-293 cells transfected with FSH-R3 (Touyz et al., 2000). While it is obvious from the published data, that calcium influx is a key component of FSH-R3 signal transduction in two heterologous expression systems (Babu et al., 2000;Touyz et al., 2000), the identity of the calcium channel involved is less certain.

Diltiazem is acknowledged to block non-L-type calcium currents at the concentrations employed in previous studies of FSH-R3 signaling (Diochot et al., 1995). The endogenous calcium currents in both HEK-293 cells and pig granulosa cells exhibit voltage-dependence and pharmacological profiles atypical of high voltage-activated L-type currents (Kusaka et al., 1993;Mattioli et al., 1993;Berjukow et al., 1996;Ballard H. et al., 2003). The endogenous calcium current in HEK-293 cells is most similar to the voltage-sensitive current conducted by Cav2.3 (aka $\alpha 1\text{E}$) channels (Berjukow et al., 1996). The low-voltage activated Ca^{+2} current in granulosa cells could also be associated with Cav2.3 channels (Kusaka et al., 1993;Mattioli et al., 1993;Bourinet et al., 1996). Freshly isolated and immortalized porcine granulosa cells have been shown to express the Cav2.3 channel protein (Ballard H. et al., 2003). Furthermore, the Cav2.3 channel has been implicated in IGF-1 regulation of neuroendocrine tumor cells and immature atrial myocytes, suggesting a link between this channel and growth factor signaling (Piedras-Renteria et al., 1997;Larsen et al., 2005;Mergler et al., 2005).

On the basis of the data cited above, it was hypothesized that the FSH-R3 signaling pathway in ID8 MOSEC would include the Cav2.3 calcium channel. Two sets of data were obtained in support of this supposition. First, SNX-482, a relatively specific peptide antagonist of Cav2.3 channels at the 10–100 nM concentrations used in this study, inhibited FSH-stimulation of cell proliferation and ERK phosphorylation in ID8 MOSEC. Additionally, Cav2.3 protein was localized to the surface membrane of these MOSEC. The findings are consistent with previous suggestions that FSH-R3 modulates cell growth via a signaling pathway that includes a voltage-gated Ca^{2+} channel, and they suggest a role for the Cav2.3 channel.

Conclusion—In summary, the data suggest strongly that FSH stimulates the proliferation of malignant ovarian surface epithelial cells by acting on a receptor splice variant, the growth factor type receptor FSH-R3 (Sairam et al., 1997). The associated signaling pathway features cAMP-independent activation of MAPK downstream of an SNX-482 sensitive component likely to be the Cav2.3 channel. In the framework of existing knowledge about the pathogenesis and treatment of epithelial ovarian cancer, the discovery that FSH-R3 can modulate

proliferation of transformed ovarian surface epithelial cells has significant implications, as detailed below.

As a result of the silent nature of early stage ovarian cancer, 70–80% of patients present with disseminated and metastatic ovarian cancer (Hasan et al., 2005). In these cases, treatment consists of tumor debulking followed by cytotoxic chemotherapy. Unfortunately, even in patients who exhibit an initial positive clinical response, development of chemoresistant disease and death are common outcomes (NIH Consensus Conference, 1995; Hasan et al., 2005). Discovery of novel therapeutic targets is needed to advance therapy of ovarian cancer. Moreover, identification of molecular markers that enable prediction of sensitivity to particular drugs and drug combinations will facilitate development of less toxic and more effective treatment protocols. FSHR-3 and Cav2.3 represent previously unexploited therapeutic targets in epithelial ovarian cancer.

Acknowledgements

Helpful comments were provided by Tim Rozell (Kansas State University), Paul Terranova (University of Kansas) and John Davis (University of Nebraska). This work was made possible by NIH R01-HD-36002 (LCF), as well as assistance from the following sources at Kansas State University: COBRE Confocal Microscopy Facility and Molecular Biology Support Cores (NIH P20-RR017686); Terry C Johnson Center for Basic Cancer Research; ADVANCE Career Advancement Program supported by the National Science Foundation under Cooperative Agreement number SBE-0244984. *Note:* Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

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FSH-R1 (GPCR): **FSH-R2 (GPCR Truncation):** **FSH-R3 (Growth Factor Type I Receptor):** **Figure 1.**

Exon Structure of three FSH receptor (FSH-R) splice variants characterized previously by the Sairam Laboratory (Sairam et al., 1996;Sairam et al., 1997;Yarney et al., 1997;Babu et al., 1999;Babu et al., 2000;Touyz et al., 2000;Babu et al., 2001). The full length FSH-R, FSH-R1, is a G-protein coupled receptor (GPCR) with 7 transmembrane domains (TMD) encoded in exon 10. FSH-R2 differs from FSH-R1 only in that the carboxyl terminus is shortened by 25 amino acids; this structural difference results in impaired coupling to adenylate cyclase (Yarney et al., 1997). FSH-R3 contains the first 8 exons of FSH-R1, followed by a single membrane spanning segment (Sairam et al., 1997;Babu et al., 1999). FSH-R3 functions as a growth factor receptor (Babu et al., 2000;Babu et al., 2001). Although the 3' nucleotide sequences of FSH-R2 and FSH-R3 are identical after the splice point, the amino acid sequences differ as the result of a shift in the reading frame. An 18 amino acid epitope unique to FSH-R3 is shown. This sequence was used for generation of antibody against FSH-R3 in previous work (Babu et al., 2001) and in the present study.

Figure 2.

FSH stimulates proliferation of transformed MOSEC. Panel A: ID8 MOSEC were cultured in the absence (Con) and presence of either FSH (20 ng/ml) or EGF (10 ng/ml) for the times indicated on the x-axis. Different superscripts indicate significant difference between treatment groups at same time-point (n=4). Panel B: ID8 MOSEC were cultured for 24 hours in the presence of 0–2000 ng/ml FSH. Asterisks indicate significant difference from control (n=4). Panel C: ID8 MOSEC were cultured for 24 hours in the absence (Con) or presence of either 20 ng/ml purified porcine FSH (pFSH) or 20 ng/ml recombinant human FSH (hFSH). Different superscripts indicate significant difference between treatment groups (n=4).

Figure 3.

Effects of FSH on intracellular levels of cAMP in ID8 MOSEC compared to PGC2 stably transfected with FSH-R splice variants. Panel A: ID8 MOSEC were cultured in the absence (Con) or presence of FSH (20 ng/ml) for 2 hours. Asterisk indicates significant difference from control (n=3). Panel B: ID8 MOSEC were cultured in the absence (Con) or presence of forskolin (10 μM) for 2 hours. Asterisk indicates significant difference from control (n=3). Panel C: PGC-2 cells stably transfected with FSH-R3 were cultured with and without FSH as described above. There was no significant difference in cAMP levels between treated and untreated cultures (n=4). Panel D: PGC-2 cells stably transfected with FSH-R1 were cultured with and without FSH as described above. Asterisk indicates significant difference from control (n=4).

Figure 4. FSH activation of ERK in ID8 MOSEC. Serum-starved cells were stimulated with FSH (20 ng/ml) for 0, 10, 30, or 60 minutes, then lysed. Western analysis of phosphorylated ERK1/2 (top panel) and total ERK1/2 (middle panel) is shown. Summary data (n=4) from densitometric analysis are shown in the lower panel. Different superscripts indicate a significant difference between time-points.

Figure 5.

SNX-482 antagonizes FSH effects on cell proliferation and ERK activation in ID8 MOSEC. Top panel: ID8 MOSEC were cultured for 24 h in the absence (Con) and presence of FSH (20 ng/ml), alone and in combination with the Ca²⁺ channel antagonist SNX-482 (100 nM). Different superscripts indicate significant difference between treatment groups at the same time-point (n=3). Middle Panel: Densitometric analysis of ERK activation in MOSEC treated with FSH (20 ng/ml) for 10 minutes in the absence and presence of SNX-482 (100 nM). Different superscripts indicate significant difference between treatment groups (n=3). Bottom Panel: Exemplar immunoblot showing phosphorylated (pERK) and total (tERK) ERK1/2 in MOSEC ID8 treated as described above.

Figure 6.

Identification of the Exon 11 region and transcriptional analysis of the MOSEC FSHR3. Panel A: Genomic organization of the mouse BAC clone (**AC166993**). Numbers 1 and 199,776 represent the first and last nucleotide of the clone. The positions of Exon 7, the putative Exon 11 and the EcoRI (E) sites surrounding these exons are identified, along with their nucleotide positions and the predicted size of the genomic fragments of EcoRI digested DNA. The sequences of the oligonucleotide probes designed from the complimentary strand and used for mapping the transcription and genomic regions spanning the Exon 7 (7R) and Exon 11 (11R) are shown directly below the schematic. The putative Exon 11 sequence in the BAC clone, predicted amino acid sequence and intron-exon boundary at the 5' end of the Exon 11 (AG/) are shown in the lower-most panel. Panel B: The predicted mouse FSHR3 mRNA. Panel C: For Southern blot analysis (left), ³²P-labeled oligonucleotide probes (7R, 11R or both) were hybridized to individual lanes of EcoRI digested and agarose gel resolved MOSEC genomic DNA transferred to a nylon membrane. Northern blot analysis (right) was performed in a similar fashion using individual membrane strips that contain the MOSEC total RNA resolved

on a formaldehyde agarose gel. Molecular weight markers are shown on the left of the blots. DNA and RNA lanes used for hybridization were identified with the names of the oligonucleotide probes (7R, 11R or 7R/11R).

Figure 7. Immunoblot analysis of FSHR protein expression in MOSEC ID8

Results shown in each panel were replicated three times. Panel A: Left panel shows an immunoblot of FSH-R protein in mouse ovary tissue (mO) and ID8 cell lysates obtained using a primary antibody (FSHR (N-20)) directed against an N-terminal sequence common to FSH-R1 and FSH-R3. Right panel shows the same membrane stripped and subsequently reprobed with primary antibody that was pre-incubated with an excess of epitope-specific peptide. Actin immunoblot (lower panel) is included as a loading control. Panel B: Left panel shows an immunoblot of FSH-R3 protein in mO and ID8 lysates. Right panel shows a similar membrane probed with primary antibody that was pre-incubated with an excess of epitope-specific peptide. Actin immunoblots (lower panel) are provided as loading controls.

Figure 8.

Surface Expression of FSH-R3 and Cav2.3 in ID8 MOSEC. Top Panel: Confocal microscopic images of FSH-R3 (A), Cav2.3 (E) and Na⁺/K⁺-ATPase (B, F) in ID8 MOSEC (bright field images in C, G). Immunofluorescence (IF) of the secondary antibodies to experimental targets is red (A, E), while IF of secondary antibody to the membrane marker (B, F) is green. Yellow color in the merged images (D, H) indicates co-localization of the target and the plasma membrane marker Na⁺/K⁺-ATPase. Middle Panel: Line-scan profile of fluorescence in merged images (D, H of top panel). Red color is used to indicate IF of FSH-R3 (right) and Cav2.3 (left). IF of Na⁺/K⁺-ATPase is represented as green. In images of both FSH-R3 and Cav2.3, the peak IF of target and marker is associated with the surface membrane. Bottom Panel: Immunoblot

analysis of FSH-R3 and Cav2.3 in total cell lysates (Lane 1), supernatant (non-biotinylated) fraction (Lane 2) and streptavidin pulldown (biotinylated) fraction (Lane 4) obtained from ID8 MOSEC subjected to cell surface biotinylation. The negative controls (Lane 3) contain lysis buffer only.