Activation of Extracellular Regulated Kinase Pathways in Ovarian Granulosa Cells by the Novel Growth Factor Type 1 Follicle Stimulating Hormone Receptor: Role in Hormone Signaling and Cell Proliferation.

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SUMMARY

Follicle stimulating hormone (FSH) regulated growth and function of the ovarian follicle was previously thought to be mediated solely through activation of Gs-coupled receptors. In this study, we show for the first time that this function is predominantly mediated through the alternatively spliced and novel growth factor type 1 receptor (oFSH-R3) that is also present in the ovary. Immortalized granulosa cells lacking endogenous FSH receptors, when transfected with either oFSH-R3 cDNA (JC-R3) or the Gs-coupled oFSH-R1 (JC-R1), expressed the corresponding glycosylated receptor. In JC-R3 or JC-R1 cells labeled with bromodeoxyuridine (BrdU) or $[^3]H$ thymidine, FSH stimulated the cells to progress through S-phase and divide. The growth promoting effect of recombinant FSH in JC-R3 cells was preceded by the rapid activation of ERK1 and ERK2. This effect was hormone specific and transient. In JC-R3 cells inhibitors like calphostin C, PD98059, Ag 18, or calcium chelators EGTA or BAPTA/AM inhibited both MAP kinase activation and BrdU incorporation. FSH induced phosphorylation of the FSH-R3 receptor was blocked by pretreating cells with Calphostin C. There was no cAMP induction by FSH in JC-R3 cells. The cAMP independent growth promoting effect of FSH is mediated by activation of Ca$^{2+}$ and MAP kinase dependent pathways. Thus, alternative splicing of a G-protein coupled receptor creates the expression of a novel receptor motif that can mediate a widely recognized function of the glycoprotein hormone.
INTRODUCTION

The ovarian follicle is among the most prolific of normal tissues undergoing rapid cellular proliferation and differentiation to accommodate the development and maturation of the ovum that is vital for propagation of the species. Two dimeric pituitary gonadotropins, follicle stimulating hormone or follitropin (FSH) and luteinizing hormone or lutropin (LH) that belong to the complex glycoprotein hormone family are critically involved in the mechanisms regulating follicle status and development. The actions of gonadotropic hormones are mediated by binding to high affinity receptors present on plasma membranes of target gonadal cells. FSH regulates a large number of genes encoding nuclear, cytoplasmic, membrane associated (1), and secreted proteins (2). The full length FSH receptor (hereafter called FSH-R1) belongs to a super family of G-protein coupled receptors, which interact with intracellular effector system through seven transmembrane domains (3-5). This FSH-R1 receptor like LH and TSH (thyroid stimulating hormone-thyrotropin) receptors has a large extracellular (EC) amino terminal domain comprised of more than 300 amino acid residues. The EC domains of these receptors are encoded by multiple exons and contain leucine rich repeat sequences that are thought to be important for ligand binding. According to present evidence, the FSH receptor is coded by a single large gene (80-100 kb), in various species (3, 6). Alternative pre m-RNA splicing is a wide spread theme for gene regulation and for generating isoform variants as this mechanism will ensure molecular diversity for cellular regulation in instances where only one gene exists (7). There are many reports on the identification of various alternatively spliced transcripts for most glycoprotein hormone receptors including that of FSH in the ovary and testis, the two exclusive targets of the hormone action (4, 5, 8, 9). In our previous investigations, we have reported the cloning of
several alternatively spliced FSH receptors displaying different structural motifs including a dominant negative receptor (designated FSH-R2) (5, 11), a growth factor type 1 receptor (called FSH-R3) (10, 12), and a potential soluble form (called FSH-R4) (13, 14). The biochemical characteristics of the ovine FSH-R3 receptor motif (10) including the identification of protein expression in the ovary and testes have been described (12).

Follicles in the ovary are either quiescent or committed to one of the two developmental pathways: growth or atresia/apoptosis. Although most of the growth in the follicles is attributed to the proliferation of granulosa cells under the tropic influence of FSH, the molecular mechanisms and the exact receptor motifs that participate in the process are poorly understood. Recent evidence derived from the generation of knockout mutants for the FSH-ß subunit (lacking the hormone dimer) (15) and all forms of the FSH receptor (16) in the mouse substantiate the importance of the hormone-receptor interaction in follicular development. The presence of underdeveloped ovarian follicles in our FSH-R knockout mouse suggests that FSH signaling is critical in the final phases of follicular growth and maturation (16).

Recent studies of numerous cells undergoing proliferation in response to various extracellular signals have demonstrated the activation of mitogen activated protein kinases (MAP kinases) or extracellular regulated kinases (ERK). The ERK cascade consists of 3-kinase modules that include a MAPK which is activated by a MAPK/ERK kinase (MEK), which is in turn activated by another MEK kinase (MEKK) (17). Multiple mammalian MAPK pathways have been identified, of which the ERK cascade is the best characterized. It consists of Raf isoforms, MEK1/2 and ERK1/ ERK2 and is regulated by Ras. In addition, it is also known that protooncogenes, the normal cell progenitors of oncogenes may regulate the growth and differentiation of normal cells. Accordingly, a number of observations like the transient increase in c-fos, c-myc expression,
MAP kinase activation were reported in primary granulosa cell cultures in response to FSH (18-20) and some of the actions are apparently mediated by cAMP independent pathways (19, 20). This raises the critical question whether all FSH actions on target cells are mediated by one type of receptor (R1), the heptahelical transmembrane form that is coupled to the activation of adenylyl cyclase producing cyclic AMP as the second messenger? As the FSH receptor gene undergoes extensive alternative splicing (3, 6) and most of the earlier observations in literature are made with primary cultures of target cells that may express heterogeneous populations of FSH receptors, it is conceivable that other FSH receptor motifs may be implicated in signaling events that contribute to cell proliferation. In view of its novel structural features, the alternatively spliced receptor 39 kDa FSH-R3 identified in the developing ovary (12) that is distinctly different from R1 becomes a good candidate. Like other growth factor type I receptors, the FSH-R3 has a single transmembrane domain and undergoes dimerization in response to the action of FSH (10). We have also shown that this R3 type receptor but not the Gs coupled R1 receptor mobilizes Ca\textsuperscript{2+} influx into the cell through L-Voltage Ca\textsuperscript{2+} channels (21). The experiments presented herein were designed to test for the potential role of this growth factor type I receptor in FSH mediated MAP kinase signaling in target granulosa cell proliferation. The availability of a granulosa cell line (JC-410) (22, 23) that lost innate expression of gonadotropin receptors during spontaneous immortalization but retained its steroidogeneic capability has provided us an ideal system to understand the role of the alternatively spliced FSH receptor in hormone signaling. The results of the current study provide the first direct evidence strongly implicating a role for the novel growth factor type I receptor in activating extracellular regulated kinase pathways by mechanisms that are independent of the cAMP pathway.
MATERIALS AND METHODS

Reagents - Highly purified recombinant hormones rhFSH (AFP 8468A), rhCG (AFP8456A) and rhLH (1295 RL) were obtained from the National Institutes of Health, Bethesda, MD. For iodination, highly purified hFSH prepared in our laboratory was used. Anti rFSHR (W970) a polyclonal antibody against rFSHR (sequence 150-183) was kindly provided by J. Dias (State of New York, Dept. of Health, NY). Anti oFSHR ab (J25) is a rabbit polyclonal antibody obtained by immunizing E. coli expressed R4 type oFSHR (24). Other antibodies used in this study are P44/42 MAP kinase, phospho specific P44/42 MAP kinase, Akt and phospho specific Akt (New England Biolabs), anti-BrdU antibody (DaKo Diagnostic, Ontario), anti Glucose-6-Phosphate dehydrogenase antibody (Sigma, St. Louis, Mo). Ag 18 was kindly furnished by Dr.A.Levitzki of Jerusalem, Israel. All other inhibitors used in this study were purchased from Calbiochem.

Cell Culture, Transfections and Signaling - The immortalized pig granulosa cells (JC-410) were handled as described earlier (22), except that DMEM-F12 medium was used for cell culture instead of M-199. pcDNA1 Neo vector (Invitrogen) containing the complete coding sequence for oFSH-R1 of 678 residues (11) and oFSH-R3 of 242 residues (10) of the respective mature proteins was transfected into JC-410 cells. For making stable cell lines the calcium precipitate protocol was used. Densely growing foci of transformed cells were visualized, selected for 8 weeks using G418 (GIBCO, Mississauga, ON 400µg/ml) and expanded into cell lines. Initial screening for cells expressing the oFSH-R3 protein was performed by $^{125}$I-hFSH binding and responsive cells were further used for signaling studies in serum deprived medium in the presence of stimulators or inhibitors as described in the individual experiments outlined below.
Ligand Binding Assay - This was performed according to published protocols (10, 11). Highly purified hFSH was labeled with $^{125}$I by lactoperoxidase method (specific activity 100,000cpm / ng) and incubated with granulosa cells expressing FSH-R3 or R1 or the vector control. The membranes were incubated for 12 hours at 25°C with 100,000 cpm/ml of $^{125}$I FSH in presence or absence of excess unlabeled oFSH. Specific binding was determined in presence of 1 µg of unlabeled oFSH. The number of receptor molecules present in either JC-R3 or JC-R1 cells was determined as described earlier (10).

Genomic DNA and Reverse Transcriptase Polymerase Chain Reaction - Cells were washed with PBS and lysed in lysis buffer (0.2 mM Tris pH 8.0; 0.1 M EDTA, 1% SDS and 100 µg/ml proteinase K) and digested for 3 hrs at 50°C with gentle shaking. Salt concentration was adjusted to 0.2 M NaCl and DNA was extracted using Phenol-Chloroform. PCR using genomic DNA was performed with neomycin specific primers Fwd AAGGGACTGGCTGCTATTG (RS 003) and Rev AGAAAGCGGCACCATTTC (RS 004) to amplify a 348 bp fragment. RNA from cells expressing FSH-R3 or FSH-R1 and cells transfected with pcDNA1 vector alone were extracted using midi RNA isolation kit (Ambion, USA) and reverse transcribed using first strand synthesis kit (Ambion, USA). The cDNAs were used to amplify both oFSH-R3 and oFSH-R1 using specific primers SFR1 (ATGTGTTCTCCAACCTGCCCA) and TB21 (CTGACTCGAGCTAATTTGGATGGCTTGCT) for an expected size of 517 bp for R3 and SFR1 and SFR11( CATCATCTTCTGCAAAGAGA) for R1 with an expected size of 690bp. The products were separated on 1.5 % agarose gel and stained with ethidium bromide.

Cell Surface Expression of oFSH-R3 and oFSH-R1 in Granulosa Cells by Immunocytochemistry - The cells (1 x 10$^6$/ml) transfected with/without FSH-R3 or FSH-R1 were harvested and fixed in 1% buffered paraformaldehyde (pH 7.4) for 30 min at 4°C. The cells were washed thrice with 3 ml of
staining buffer (PBS containing 1% bovine serum albumin) and resuspended in 100 µl of staining buffer containing the FSH-R antisera (1:100) raised in rabbits and incubated at RT for 1 h. They were again washed and incubated with 100 µl of staining buffer containing fluoresceinated goat anti-rabbit IgG (1:50 dilution) in dark for 30 min at RT. After one more final wash they were resuspended in 1 ml staining buffer. The green fluorescence intensity of cells was measured in a Coulter flow cytometer at 530 nm. Control cells were processed in the same manner excluding the primary antibody. The antibodies used were characterized previously and shown to react with FSH receptor in cells or gonadal tissues (12, 24, 25).

Deglycosylation of oFSH-R3 in JC-R3 Cells - The cells were scraped in cold PBS and collected after centrifugation at 800Xg for 10 min at 4°C. They were suspended in lysis buffer and left on ice for 30 min to solubilize membrane proteins. The solubilized receptor was deglycosylated using N-Glycosidase F (Boehringer-Mannheim), which cleaves all N-linked glyco moieties of the FSH receptor (26). Approximately 200 µg of membrane protein was suspended in 0.1M Tris-HCl buffer pH 7.4 with 0.1 mM PMSF and incubated with 50U/ml of N-Glycosidase F. After incubation for 4 hours at 30°C, 100µg equivalent protein was reduced in 1X SDS sample buffer and subjected to SDS-PAGE followed by immunoblotting using the FSH-R3 specific peptide antibody (12).

Western Blot Analysis - Granulosa cells expressing the cloned oFSH-R3 or oFSH-R1 were stimulated in serum free medium as described in figures. In preliminary studies, we used natural and highly purified hFSH and hLH or hCG prepared in our laboratory. In subsequent experiments all data reported herein were derived from the use of recombinant human glycoprotein hormones (see methods). Cells collected by scraping in cold PBS, were washed and homogenized in lysis buffer (20mM Tris HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5
mM sodium pyrophosphate, 1mM Na$_3$VO$_4$ and 0.1mM PMSF). Protein content in the samples was estimated by the Bio-Rad protein assay. For verifying the expression of FSH-R3 ~100 µg protein was used whereas for MAP kinases 5 µg of total solubilized protein was adequate. Proteins were separated on 10% SDS–PAGE minigel and transferred on to PVDF (Dupont) membrane. After blocking non-specific sites, membranes were incubated with anti-MAP kinase (1:1000), phospho MAP-Kinase (1:1000), oFSH-R3 IgG (1:2000) or FSHR ab (1:2000) in TBS containing 1% (w/v) skim milk powder and 0.05% Tween-20 for 12 hours at 4°C. The washed blots were incubated with goat anti rabbit IgG conjugated to HRP (Sigma) at 1:3000 dilution. Bound antibody was visualized using the Luminol (Boehringer-Mannheim) chemiluminescent detection system.

Quantification of Granulosa Cell Proliferation by BrdU Incorporation and Dual Parameter Flow Cytometry - The quantification of BrdU-positive cells by flow cytometry can be considered as a measure of actively dividing S-phase cells (27). Quantification of BrdU-labeled cells was done according to the procedure described earlier (28). Granulosa cells transfected with/without FSH-R3 or FSH-R1 receptor were growth arrested by serum-starvation for 16-24 hrs and treated with various concentrations of human recombinant FSH and 50 µM BrdU. After one hour of incubation at 37°C, the cells were washed thrice with Dulbecco's phosphate buffered saline (Ca$^{2+}$ and Mg$^{2+}$-free) and fixed in 70% chilled ethanol at a concentration of 1 X 10$^6$ per ml. An aliquot of ethanol-fixed cells (2 X 10$^6$) was washed, pepsinized, acid denatured and incubated with 200 µl of a monoclonal antibody against BrdU at 1:40 dilution for 1 hr in dark. The cells were washed and incubated with 200 µl of FITC-conjugated goat anti-mouse IgG (Sigma, St Louis) at 1:10 dilution for 30 min in dark and cells were counter-stained with propidium iodide staining solution containing 100 µg per ml DNAse-free RNAase. Control cells were processed similarly without
incubating with BrdU. The intensity of green fluorescence of FITC staining and red fluorescence of PI stained cells were measured at 530 and 620 nm respectively in a Coulter Flow Cytometer.

*Measurement of $^3$H]Thymidine Incorporation* - Granulosa cells (JC-R3, JC-R1 or JC-Vector) were plated at a density of 2.5x10$^4$ cm$^2$ in 24 well tissue culture plates and grown for 2 days without a medium change. They were rinsed once with serum-free DMEM and serum starved for 16 hrs by incubating with DMEM. The medium was changed to DMEM containing varying concentrations of rFSH plus 1 µCi/well of $[^3]$H thymidine (specific activity 79 Ci/mmol). The cells were incubated at 37°C for 24 hrs and the amount of $[^3]$H thymidine incorporated in to DNA was determined as described previously (10).

*Phosphorylation of Intact JC-R3 Cells and Immunoprecipitation.* JC-R3 cells plated in 100 mm dishes were biosynthetically labeled in phosphate free DMEM medium with 100µci/ml of $^{32}$P orthophosphate for 3 hours at 37 °C under 5%CO$_2$ as described by others (29) and stimulated for another 30 min with 10 ng/ml of rFSH. The plates were placed on ice and cells were scraped into cold buffer (0.15M NaCl, 20 mM HEPES pH 7.4 containing mixture of protease inhibitors and 0.5% NP-40). Cells were solubilized on ice for 30 min and centrifuged at 100,000 Xg for 30 min. The soluble receptor in the supernatant was immunoprecipitated using anti-FSH R3 IgG antibody and resolved on SDS-PAGE in presence of thiol reducing agents. Autoradiographs of dried gels were obtained using intensifying screen.

*Steroid Hormone (Estradiol), cAMP Production and Protein Assays* - Stable granulosa cells expressing oFSH-R3 (JC-R3) or oFSH-R1 (JC-R1) were evaluated for their capacity to stimulate estradiol production. Estradiol-17β was estimated by RIA in 200µl of culture medium, as previously described in the presence or absence of 0.1µM androstendione (22). For cAMP estimation, 2’-O monosuccinyl adenosine 3’:5’ cyclic monophosphate methyl ester was labeled
with Na$^{125}$ I using chloramine T. Cells were cultured in DMEM/F12 medium containing 0.1mM 3-isobutyl-1-methyl Xanthine (IBMX, Sigma). The cAMP produced was measured using the double antibody precipitation method (5) and expressed as picomole/µg of protein. Cells were washed twice with PBS and solubilized in cell lysis buffer (20mM Tris–HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100 and 0.1 mM PMSF) and cellular protein content was determined with Bio-Rad protein assay kit.

Statistical Analysis - All data are expressed as mean ± SEM and analyzed by one way ANOVA. A value of P< 0.05 was considered to be statistically significant.
RESULTS

Establishment of Stable Granulosa Cells Expressing Alternatively Spliced Growth Factor Type 1 Receptor (JC-R3) - The role of alternatively spliced FSH-R3 signaling was evaluated in granulosa cell line (JC-410) which had lost the responsiveness to gonadotropins during spontaneous immortalization (22), but retained expression of several genes required for steroidogenesis. The retention of these phenotypic features is critical in examining all phases of hormone action. Accordingly this ovarian cell line provides a convenient in vitro model for creating responsive target cells with discrete receptor motifs. Following transfection of granulosa cells with the oFSH-R3 or oFSH-R1 construct in the pcDNA1 Neo vector, successful transfectants were identified and selected by their resistance to G418 (400 µg/ml) for 8 weeks. The stable cell lines are, hereafter named JC-R3 and JC-R1 respectively. Further screening of these stable cell lines was done by PCR to confirm the expression of neomycin. Neomycin (348bp) was amplified only in the cell lines transfected with FSH-R3 or FSH-R1(Fig. 1A). The expression of oFSH-R3 and oFSH-R1 was also confirmed in these cells by RT-PCR using primers specific for FSH-R3 and FSH-R1. A 517 bp product specific to FSH-R3 was amplified only in FSH-R3 transfected cell lines (Fig. 1A) but not with vector alone. Similarly a 690bp product, which spans between exon 3 to 10 for FSH-R1, was expressed only in JC-R1 cells. These results not only showed the faithful expression of R3 type receptor in the JC-R3 cell lines and R1 in JC-R1 cells but also confirmed the absence of any endogenous FSH receptors in the vector transfected cells.

Radio Ligand Binding Assay and Cell Surface Expression - The cell surface expression and $^{125}$I hFSH binding to JC-R3 or JC-R1 membranes are shown in Fig. 1B. The binding of $^{125}$I hFSH was effectively competed by unlabelled FSH but not the structural homologous hormone LH. In
the same experiment granulosa cells transfected with vector alone showed no specific binding of labeled FSH. Previously, we have reported that HEK 293 cells expressing the same FSH-R3 or FSH-R1 cDNA show high affinity binding (Kd = 0.17- 0.27nM) to labeled FSH (10) similar to receptors in the target tissue (Kd =0.66nM). From the binding data in this study the number of receptor molecules present in the JC-R3 and JC-R1 were estimated to be approximately equal (∼2000 R/ cell). Taken together these data confirm that the first 8 exons, which account for about 61% of the extracellular domain of the R1 receptor, is sufficient for efficient hormone binding when it is combined with a single transmembrane protein segment that allows cell surface expression (10) (see below).

Analysis of intact JC-R3 or JC-R1 cells with selected and previously characterized antibodies using flowcytometry also showed that both these receptors were present on the cell surface. The two antibodies used in this study are rabbit polyclonal antibodies prepared against segments of the extracellular domain of the FSH receptor. The polyclonal J25 reacts with determinants within the exon 1-4 of oFSH-R and W970 is a peptide antibody to rat FSH-R (150-183). Both antibodies have been used earlier to detect FSHR’s in the target and transfected cells (25, 30). Fig. 1C shows the histograms depicting right shift of antibody bound to cells expressing FSH-R3 (upper panel) and FSH-R1 (lower panel), whereas these antibodies did not show any specific binding with JC-410 cells transfected with vector alone (data not shown). Thus, we were able to demonstrate the specificity of the system by detecting the capability of JC-R3 cells for expression of R3 protein on the cell surface and R1 protein in JC-R1 cells.

Western blotting of extracts of membranes solubilized from JC-R3, using R3 specific peptide antibody, detected a band of approximately 39 kDa (Fig. 2A). In previous studies we had used this antibody to show the expression of FSH-R3 both in the sheep ovarian and testicular
membranes (12). The apparent increase in the molecular weight that is different from the value predicted from the cDNA sequence (12) is suggestive of the covalent modification of one or both of the two potential N-glycosylation sites in FSH-R3. Previously published data suggest that in both FSH-R and LH/hCG receptors, glycosylation patterns influence the migration in SDS-PAGE and these receptors are sensitive to N-glycosidase F treatment (26, 31, 32). Accordingly, we tested for the presence of glycosylation sites (at Asn 174 and 182) of the receptor in transfected cells as described earlier with slight modifications (33). Digestion of soluble cell extracts expressing FSH-R3 with N-Glycosidase F led to a decrease in the Mr by ~ 8-10 kDa, which was clearly evident by the downward shift in protein mobility. Thus, it appears that the N-linked oligosacharides of expressed R3 receptor are accessible to the action of N-Glycosidase F. The results suggest that recombinantly expressed FSH-R3 in the granulosa cells is a glycoprotein that is N-glycosylated at both sites as predicted by its cDNA structure.

As a further confirmation of specificity, we have verified by Western immunoblotting the soluble extracts of adult mouse ovarian tissues using antibody directed against C-terminal end of R3. The immunoblot revealed a distinct band with the normal mouse ovary of a size similar (39 kDa) to that of cells expressing this receptor (Fig. 2A). It may be noted that in the same blot the ovarian extract of the FSH-R knockout (-/-) mouse does not show any immunoreactivity indicating the specificity of the R3 peptide antibody (13). The bottom panel of Fig. 2A shows the blot probed with GAPDH antibody revealing approximately equal loading for all lanes. The immunoblot with both mouse ovary extracts and cells expressing R1 and R3 treated with an FSHR antibody (J25) which recognizes both the forms of receptors is shown in Fig. 2B. The expression of corresponding proteins in respective transfected cells identifies bands of the correct
size only in extracts of +/- mouse ovary. These data confirm for the first time that a receptor equivalent to FSH-R3 is also present in the mouse ovary.

*Cyclic AMP Production in JC-R3 and JC-R1 Cells* - As shown in Fig. 3, when JC-R3 or JC-R1 and vector control cells were stimulated with different concentrations of rFSH, there was no accumulation of cyclic AMP in JC-R3 cells despite the presence of the phosphodiesterase inhibitor. These results, verified several times, suggest that the R3 receptor expressed in the granulosa cells is not coupled to the activation of cAMP unlike FSH-R1 type of receptor. In JC-R1 cells expressing the Gs coupled receptor, cAMP increase was evident in presence of 1ng/ml FSH. (Fig. 3). The latter is consistent with numerous reports showing that FSH-R1 in HEK 293 cells is efficiently coupled to this signaling pathway (3, 5, 11). Forskolin, a non-hormonal adenylyl cyclase activator, promptly increased cAMP production in JC-R3 cells in a concentration dependent manner from 1-10 µM indicating that following FSH-R3 cDNA transfection the G-protein coupled signaling apparatus is fully intact.

*FSH-Induced Phosphorylation* - The results presented in Fig. 4 show that cells expressing oFSH-R3 respond to rFSH (10ng/ml) or PMA (200 nM) with a 4 fold increase in receptor phosphorylation compared to unstimulated cells. The FSH induced phosphorylation was partially blocked by pretreating the FSH-R3 expressing cells with PKC inhibitor Calphostin C, suggesting that FSH-R3 receptor phosphorylation is mediated by either PKC or one or more other kinases.

*Regulation of MAP Kinase Pathway in JC-R3 Cells* - The regulation of MAP kinase pathway in JC-R3 cells was monitored by measuring the total phosphorylation pattern and activation of two MAP kinase isoforms, ERK1 and ERK2. Cells were maintained in serum free medium for 12 hours to arrest growth and then stimulated with the hormone. The cells were harvested at different time periods and subjected to Western blot analysis using antibody that specifically
recognizes the phosphorylated form of ERK1 and ERK2 (Fig. 5A). Remarkably, these phosphorylated forms of ERK1 and ERK2 became quickly detectable within 5 min of hormone addition and reached maximum at 10 min and then declined to almost basal level by 60 min. However, repeated experiments confirmed that FSH is unable to activate other kinases including both P38 and SAPK/JNK in JC-R3 cells (data not shown). This is probably because these two pathways are primarily associated with cellular stress responses, a signaling mechanism that may not be mediated by the FSH-R3 in the granulosa cell. Fig. 5B shows the concentration (FSH) dependent increase in ERK1 and ERK2 activation in JC-R3 cells. It may be noted that a very low concentration of the hormone FSH (0.1ng/ml ≈ 3 X 10^{-12} M) is sufficient to double ERK1 and ERK2 activation, and this effect is enhanced with increasing FSH up to 100 ng/ml.

As it is known that PKC’s are involved with activation of ERK MAP kinase pathway, we studied the effect of both PKC inhibitor (Calphostin C) and MEK inhibitor (PD98059) on FSH action in JC-R3 cells (Fig. 5A). Pretreatment of cells either with PD98059 or Calphostin-C at a concentration of 10^{-5} mole/L for 30 min prior to FSH stimulation blocked the activation of ERK1 and ERK2 indicating the potential role of PKC pathways in the activation of MAP kinases. However, as the compound H89, which is a PKA inhibitor did not produce a comparable effect significant involvement of cAMP pathways in the activation of MAP kinases is unlikely in JC-R3 cells. This result is in agreement with the lack of cAMP production by FSH in JC-R3 cells (Fig. 3). The specificity of FSH induced activation of ERK1 and ERK2 in JC-R3 cells is supported by data shown in Fig. 5C. The JC-R3 cells stimulated with 10% serum for 10 min served as positive control, whereas the JC-410 or vector transfected cells stimulated under identical conditions with FSH did not show any activation of ERK1 and ERK2. Similarly the homologous hormone, rhCG, did not have any effect on activation of MAP kinases in JC-R3 cells (Fig. 5C). Compared to the
two-fold activation with 0.1 ng/ml FSH (Fig. 5B) rhCG at 100 ng/ml was without effect. Results were similar using highly purified natural hCG (data not shown). In the light of these novel observations, we compared the effects of the hormone in JC-R1 cells designed to express the \( G_s \)-coupled receptor. It was already shown (Fig. 3) that FSH activates adenylyl cyclase promptly in these cells. However, under conditions identical to robust activation of the ERK1 and ERK2 in JC-R3 cells, FSH stimulation of this parameter was very low or marginal in JC-R1 cells (compare Figs. 6 and 5B). Likewise, forskolin that enhanced cAMP in JC-R3 cells had no influence on ERK1 and ERK2 activation (Fig. 6 bottom panel). These data clearly suggest that in the granulosa cell system as studied here, intracellular cAMP is unable to induce the activation of ERK1/2.

**Role of Calcium** - Downward changes in cellular \( \text{Ca}^{2+} \) are detrimental in the regulation of Ras/MAP kinase pathway through mechanisms that are not completely understood. Pretreating the JC-R3 cells with either intracellular calcium chelator BAPTA/AM \((10^{-2} \text{ M})\) or exposing the cells to an extracellular calcium chelator like EGTA \((10^{-3} \text{ M})\), completely blocked MAP kinase activation (Fig. 7). This suggests that calcium influx plays a major role in the FSH controlled events that may induce cell proliferation. We also tested whether activation of PKC will have any effect on MAP kinases in these cells and as seen in Fig. 7, PMA at a concentration of 200 nM, induced a transient increase in ERK1/2 phosphorylation suggesting positive regulation of PKC in MAP kinase activation. Pretreating JC-R3 cells with Ag 18 \((10^{-5} \text{ M})\), a tyrosine kinase inhibitor also reduced the FSH-induced ERK1 and ERK2 activation. The lower panel shows the expression of MAP kinase verified as internal control.

**Role of PI-3 Kinase Pathway in ERK1/ERK2 Activation in JC-R3 Cells** - Since the role of PI-3 pathway as an upstream regulator of MAP kinase activation was reported in some cell lines
(34, 35), we examined the activation of Akt (protein kinase), a downstream regulatory molecule in PI-3 pathway in JC-R3 cells. FSH did not activate Akt phosphorylation (Fig. 8A). However, in cells treated with serum Akt phosphorylation was clearly evident. When we pretreated JC-R3 cells with the specific PI-3 kinase inhibitor LY294002 there was no effect on ERK1/2 activation (Fig. 8B). Together these data suggest that FSH has no influence on PI-3 activation and this pathway is not involved in the activation of ERK1/2 in JC-R3 cells.

**Role of MAP Kinases in the Growth Promoting Effects of FSH in JC-R3 Cells.** - To examine the contribution of MAP kinase signaling to the stimulatory effect of FSH on cell cycle progression, the cells were treated with hormone and BrdU incorporation in S-phase cells was quantitated by flow cytometry. Fig. 9A compares the response of FSH in JC-R3 or JC-R1 and JC-Vector cells. Biphasic effects were evident in both JC-R3 and JC-R1 cells. In JC-R3 cells maximal stimulation was seen with 1ng/ml FSH. There was a 2.5 fold increase in the BrdU positive cells that gradually decreased with increasing FSH concentration. At high hormone concentration there was no effect (Fig. 9A). Under identical conditions, JC-R1 cells also showed a moderate increase in BrdU positive S-phase cells. Fig. 9B shows the time course of BrdU incorporation in JC-R3 cells with increase seen up to 3 hours. Long period growth promoting action of FSH was measured using $[^3]$H thymidine incorporation in both JC-R3 and JC-R1 cells at two hormone concentrations that was stimulatory in the above study (Fig. 9A). At 24 hrs DNA synthesis (Fig. 9C) is significantly higher in JC-R3 than in JC-R1 under identical conditions. Fig. 10A shows that inhibition of MAP kinase activation with MEK inhibitor PD 98059 prior to BrdU treatment effectively blocked the BrdU incorporation in JC-R3 cells indicating the potential role of MAP kinases in granulosa cell proliferation. However, pretreating cells either with KT 5720 or H89 (10$^{-5}$ M) caused no change in BrdU incorporation confirming that MAP kinase activation in these
cells is mediated by cAMP independent pathways. Parallel experiments with JC-R1 cells (Fig. 10B) show that blocking MEK activity by PD98509 or inhibiting intra cellular calcium has no effect on BrdU labeling but a partial reduction is seen with PKA inhibitor H89. In sum these data suggest the presence of potential alternative pathways in granulosa cell proliferation.

**Steroidogenic Response in Granulosa Cells** - JC-R3 cells were stimulated in minimal medium with rhFSH in presence or absence of 0.1µM androstenedione, which serves as the substrate for the enzyme aromatase. This enzyme converts the androgen to the phenolic steroid estradiol-17β. Fig. 11 (top panel) reveals a concentration dependent increase in estradiol-17β production. Significant stimulation evident at 10 ng/ml of FSH increased in a linear manner up to 200 ng/ml, the highest concentration that was tested in our study. However, it should be noted that these effects, which normally become apparent only after prolonged (24-48 h) incubation of granulosa cells, are weak when compared to the rapid and robust stimulation of other parameters shown in previous figures (Figs. 5, 8, 9, 10) for JC-R3 cells. Since the steroidogenic action of FSH mediated by the Gs coupled human FSH-R1 is well established for progesterone production in transfected mouse adrenal Y1 cells (36), we compared the actions of the hormone in granulosa cells transfected individually with the two receptors. Fig. 11 (bottom) depicts the estradiol production in transfected cells lines expressing either R1 or R3 receptors. Cells with R3 produced a three-fold increase in estradiol production compared to untransfected cells in presence of 0.1µM androstendione. Cells expressing R1 caused a seven-fold increase in estradiol. As the cells bearing equivalent number of receptors were treated under identical conditions and the effect was reproducible, the differences in the estradiol production induced by these two receptors may be attributable to differences in their mode of action during steroidogenesis. Irrespective of the type
of FSH-R present in the transfected granulosa cell, addition of the non-hormonal agent forskolin resulted in the same level of activation of steroidogenesis (Fig. 11B).
DISCUSSION

During the growth and maturation of ovarian follicles, the granulosa cells, which are the major cell type in this structure that also contains the developing ovum, proliferate rapidly and undergo differentiation in a precisely ordered sequence. This process includes the acquisition of receptors for the glycoprotein hormone FSH in the early stages of growth. It is known that FSH, a major regulator of receptor(s) induction, increases FSH receptor mRNA and protein levels both in vivo and in vitro (37, 38). The rapid increase in FSH induced DNA synthesis in the immature ovary well before cAMP production (39), increased uptake of (3H)-thymidine in preantral follicles (40), imply that growth has been initiated by allowing the cells to enter the cell cycle. Subsequently, check points of the cell cycle like cyclin D2 (41) and other control mechanisms determine the path of the granulosa cells towards further development and differentiation (42). However, the precise molecular nature of FSH receptors or the signaling mechanisms that mediate hormonal induction of such mitotic activities during the early stages of follicular growth have remained a mystery. According to the present dogma in the glycoprotein hormone receptor field, the diverse action(s) of the glycoprotein hormones is assumed to be mediated solely by the G-protein coupled heptahelical transmembrane receptor entity (43). Having cloned novel structural motifs of the functional FSH receptor that differ from this topography and documented expression of the corresponding protein in the ovary (12), we launched a systematic attempt to define the role of the growth factor type I receptor in ovarian cells to identify its signaling pathways. While this report focuses on revealing the properties of the novel receptor FSH-R3, we have included some studies comparing the Gs coupled receptor providing data on relative differences in signaling.
Although variants of G-protein coupled receptors suggestive of differential coupling to diverse signaling pathways are documented (44), there had been no report of the creation of a new receptor motif by alternative splicing mechanisms (10, 12). The functional data presented here introduce a new paradigm in the study of cell regulation by complex hormones and reveal that novel receptor motifs created by splicing and expressed in the gonads are linked to discrete signaling pathways. The recent availability of ovarian granulosa cells that can be maintained in culture without differentiation and functional endogenous FSH receptors (22, 23) (Fig. 1 this study) has afforded us the opportunity to recreate target cells with unique receptor forms. In this manner, signal transduction including steroidogenesis by individual receptor motifs can be investigated without interference of cross talk that complicate analysis in cells derived from primary cultures of animal tissues that may express more than one type of receptor.

Most previous studies have been conducted in non-target cells (4, 10, 21, 25, 36, 45, 46) that are incapable [except the Y1 cells used by Kelton et al. (36)] of performing a steroidogenic function. The demonstration of the R3 receptor on the surface of granulosa cells extends previous findings of the expression of the novel receptor that had been initially observed in HEK 293 cells (10, 12). The immunoblot data (Fig. 2) using two different antibodies further suggests that this receptor was expressed as a glycoprotein of Mr 39 kDa. Evidence derived from enzymatic deglycosylation of R3 transfected cells and the apparent higher Mr in SDS-PAGE suggest that both the consensus sites at Asn\textsubscript{174} and Asn\textsubscript{182} of the mature R3 receptor protein are glycosylated.

The growth factor receptor type I motif for FSH was initially cloned from the sheep testis (14) and ovary (12). Expression of the corresponding protein has also been verified (12). The universal participation of such alternatively spliced receptors in signaling processes requires confirmation in other species. Thus, our first demonstration that the R3 receptor also exists in the mouse ovary
(Fig. 2) is significant. The specificity of our detection system for R3 protein was evident in the Western blots because only the wild type +/+ mouse ovary extracts showed the expression of this receptor protein with a migration very similar to that of the receptor in JC-R3 transfected cells. The complete absence of a protein band corresponding to this as well as R1 in the ovaries of FSH receptor knockout (-/-) mouse also proves that the entire repertoire of FSH receptor motifs were eliminated in our knockout strategy causing sterility in females (16).

Based on recent x-ray crystallographic evidence, the glycoprotein hormone FSH and other members of this family have been modeled (47) as having structural features that include the cystine knot motif that are also present in many ligands that act as growth factors. The receptors for these ligands and their signaling properties are different from the G-protein coupled receptors. The discovery of a growth factor type receptor R3 for FSH is consistent with the structural predictions for the hormone and is strongly supported by the data described in this study which show that signaling mechanisms other than the G-proteins are also utilized by the hormone. Thus, its linkage to the activation of MAP kinase pathways as demonstrated here now provides a new perspective in understanding glycoprotein hormone action. The MAP kinase signaling cascade, an important regulator of cell cycle progression, has been used as a biochemical marker to evaluate the status of hormones and growth factors as mitogens (48).

The FSH-R3 receptor itself consists near its carboxyl terminus the sequence PVILSP (10, 12, 14), which represents a potential consensus motif (PXnS/TP where X is basic or neutral residue) for phosphorylation by MAP kinases (49). Because this motif appears only after alternative splicing at the 8th exon of the FSH receptor gene (10, 14) we can argue that the change must have structural and functional significance. Structurally, the change led to the appearance of a single transmembrane domain for FSH-R3 as well as the other accompanying motifs that couple
to alternative signaling pathways. This consensus site is present in the analogous hTSH receptor variant in the thyroid where this transcript is also generated by alternative splicing at exon 8 (50).

As both FSH and TSH are mitogenic hormones and stimulate cell proliferation in their respective target tissues, presence of the R3 type of motif may have great significance in receptor function and dynamics. FSH induced receptor phosphorylation (Fig. 4) in JC-R3 cells suggest that some of the serine residues in C-terminal end may be involved in the modification. However, the mechanisms by which the receptor is phosphorylated remain to be clarified. Receptor autophosphorylation is unlikely because the C-terminus lacks intrinsic kinase domains. The effects of gonadotropin receptor phosphorylation in general have recently been reviewed (51) and other investigators studying the R1 type FSH receptor in transfected HEK 293 cells (52) have shown that phosphorylation modulates receptor uncoupling and internalization.

Gonadotropins are known to increase the MAP kinases in ovarian granulosa cells collected from intact animals (18, 19). The differences in the activation of ERK1/ERK2 by cAMP and gonadotropins (LH and FSH) may suggest that gonadotropins activate these enzymes via pathways that operate, at least in part independent of cAMP (19). However, the precise receptor mechanisms identifying how FSH may regulate MAP kinase activation and its subsequent role in granulosa cell proliferation was unknown. It has long been appreciated that a significant elevation of intracellular cAMP levels may potently inhibit cell proliferation and division (53) but at lower concentration may have opposite effect (54). However, FSH induced cell proliferation in ovarian follicles is a major effect of the hormone, that may be incompatible with elevation of cAMP, a second messenger that is clearly elevated by the R1 receptor (3, 36, 45, 46). Based on the data presented in Figs. 3, 5, 6, 8 and 9, we may conclude that FSH signaling through the R3 receptor is a major mechanism of MAP kinase activation and cell proliferation independent of the cAMP
pathway. The absence of ERK1/2 activation by forskolin (Fig. 6B) an agent that promptly elevated cAMP in JC-R3 cells, and the complete abrogation of hormone-induced cell proliferation by PD98059, a specific inhibitor of MAP kinase activity, clearly justifies such a conclusion. Thus, our data provides a mechanistic explanation for inferences drawn from studies on FSH action in primary cultures (19). FSH action in JC-R3 cells increased BrdU labeling of granulosa cell nuclei, in a hormone specific manner. The biphasic nature of this response and maximal stimulation obtained at low concentration (Fig. 9) although intriguing, is typical of the behavior exhibited by growth promoting hormones. This is further confirmed by long term growth promoting effects of FSH both in R3 and R1 cells by $[^3]$H thymidine incorporation. Our data with JC-R3 cells bearing a cloned FSH-R are consistent with the earlier observation of Bley et al (55), who also found that FSH had biphasic response on ovarian cellular growth in primary cultures. Inhibition of phosphorylation of MEK1 by the specific MEK kinase inhibitor PD 98059 or chelating intracellular calcium by BAPTA/AM completely blocks the proliferative effects of FSH. These results signify that activation of MEK1 is important for proliferative actions of FSH in granulosa cells that express the R3 receptor. Under identical conditions blocking MEK1 activity or chelating intracellular calcium had no effect on proliferation in JC-R1 cells (Fig. 10).

It has been well established that growth factors activate MAP kinases in a variety of cell types (48) and that inhibitors of the activation of the MAPK cascade block the mitogenic action of the growth factors (56). These observations have been confirmed and extended in the present study with the novel glycoprotein hormone receptor motif FSH-R3. As both H89 and K57032, that are PKA inhibitors did not significantly influence the FSH induced proliferation and activation of MAP kinase in FSH-R3 transfected cells, our conclusion that the FSH-R3 receptor utilizes other pathways to activate MAP kinase instead of cAMP/PKA appears justified. This is further
supported by the absence of cAMP production by FSH in FSH-R3 transfected cells (Fig. 3). Our investigations, designed solely to understand the signaling properties of the novel FSH-R3 in an individual setting in cells that express only one type of receptor does not preclude a role for theGs coupled FSH-R1 and the PKA pathway in cell proliferation. Our results indeed support that FSH-R1 may also coupled to cell proliferation through other mechanisms which need to be clarified in more detail. In addition to the differences noted above, the phenotypic characteristics of granulosa cells bearing one or the other FSH receptor appears to be different. While JC-R1 cells that produce cAMP in response to FSH promptly show the cell rounding phenomenon as reported in other studies (36), the JC-R3 cells that activate ERK1/2 do not display this behavior (data not shown). Therefore, it is possible that the preponderance of one or the other form of FSH receptors varying as the ovarian granulosa cells mature might dictate the selection of different signaling pathways and cross talk that is likely to occur during rapid developmental changes.

The relation between hormone action, Ca$^{2+}$ influx, MAP kinase activation, and cell proliferation and steroidogenesis in JC-R3 cells can be integrated by our investigations. In a previous study, we reported that, FSH induces Ca$^{2+}$ influx in FSH-R3 transfected HEK 293 cells. through L-voltage dependent pathways (21). As the hormone induces Ca$^{2+}$ in primary granulosa or Sertoli cells but not in R1 transfected cells (see 21, 57), participation of a different receptor motif is likely. Taken together with the results of the present study, the implication is clear that FSH induces calcium influx in target granulosa cells via the FSH-R3 and the increased Ca$^{2+}$ in the cytosol in turn leads to MAP kinase activation.

Ca$^{2+}$ influx is well known to activate MAP kinase in cultured cells by several mechanisms (58, 59) including PKC activation of Ras (60). Since FSH-induced ERK activation in JC-R3 cells is inhibited by a PKC inhibitor, the involvement of PKC pathways upstream of ERK signaling is
most likely. This conclusion is further supported by data showing inhibition of ERK activation by intracellular or extracellular Ca\textsuperscript{2+} chelators. Recent reports that PI-3 kinase can be involved in the regulation of ERK kinase pathway and PI-3 kinase can also be activated by Ca\textsuperscript{2+} influx (59) have led to the implication of PI-3 kinase in downstream control of Ras in some cellular systems (34, 35, 61-64). More importantly the PI-3 kinase activity is also able to modulate the ERK/MAP kinase pathway (34, 35). Since we did not observe any effect of the hormone on phosphorylation of Akt and furthermore a PI-3 kinase inhibitor did not influence the MAP kinase activation, we suggest that mediation of FSH effects by PI-3 kinase is unlikely in the JC-R3 cells.

Another important new observation emerging from the current study is that the growth factor type I receptor of FSH is also coupled to steroidogenic machinery albeit weakly. Therefore, a second pathway in addition to the classical Gs-coupled signaling mechanism may also be operative in granulosa cells. A hallmark of FSH action in the ovarian granulosa cell is the synthesis of estrogen, which subsequently activates transcription of numerous genes via its nuclear receptors. Based on the data shown in Fig. 11, the conclusion is inescapable that cells expressing FSH-R3 indeed secreted estradiol-17\textbeta into the medium in response to the hormone. However, under identical conditions the full length FSH-R1, which activates the Gs-signaling systems is more efficient in inducing steroidogenesis. The identification of two different pathways for hormone signaling including steroidogenesis may represent a back up mechanism utilized by a dynamic system such as the granulosa cell that has to perform diverse functions.

In conclusion, these results for the first time demonstrate that a novel type of FSH receptor (R3) exhibiting features of a growth factor type I receptor is primarily responsible activation of the ERK pathway in ovarian granulosa cells. This was essentially independent of cAMP/PKA mediated events but dependent upon Ca\textsuperscript{2+} influx and PKC pathway. The identification of putative
transducers that directly induces Ca$^{2+}$ influx and activation of MAP kinases remain to be determined. Future challenges include the recognition and eventual identification of interacting protein(s) partners for each specific FSH receptor type within the target cell. Differences in modulation of these events may dictate the final commitment of the cell to undergo proliferation and/or produce steroids.
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REFERENCES

1. Loganzo, F. Jr. and Fletcher, P. W. (1992) Mol. Endocrinol. 6, 1259-1267
2. Griswold, M.D. (1993) in The Sertoli Cell. (Russell, L.D. and Griswold, M.D., eds) pp. 493-508, Cache River Press, Clearwater, FL.
3. Simoni, M., Gromoll, J., and Nieschlag, E. (1997) Endocr. Rev. 18, 739-773
4. Braun, T., Schofield, P. R., and Sprengel, R. (1991) EMBO J. 10, 1885-1890
5. Sairam, M. R., Jiang, L. G., Khan, H., and Yarney, T. A. (1996) Biochem. Biophys. Res. Commun. 226, 717-722
6. Sairam, M. R. and Subbarayan, V. S. R. (1997) Mol. Reprod. Dev. 48, 480-487
7. Sharp, P. A. (1994) Cell 77, 805-815
8. Loosfelt, H., Misrahi, M., Atger, M., S alleles, R., Vu Hai-Luu Thi, M. T., Jolivet, A., Guiochon-Mantel, A., Sar, S., Jallal, B., Garnier, J., and et al (1989) Science 245, 525-528
9. Themmen, A. P. N., Kraaij, R., and Grootegoed, J. A. (1994) Mol. Cell Endocrinol. 100, 15-19
10. Sairam, M. R., Jiang, L. G., Yarney, T. A., and Khan, H. (1997) Mol. Reprod. Dev. 48, 471-479
11. Yarney, T. A., Jiang, L. G., Khan, H., MacDonald, E. A., Laird, D. W., and Sairam, M. R. (1997) Mol. Reprod. Develop. 48, 458-470
12. Babu, P. S., Jiang, J., Sairam, A. M., Touyz, R. M., and Sairam, M. R. (1999) Mol. Cell. Biol. Res. Commun. 2, 21-27
13. Yarney, T. A., Fahmy, M. A., Sairam, M. R., Khan, H., and MacDonald, E. A. (1997) *J. Mol. Endocrinol.* **18**, 113-125

14. Khan, H., Yarney, T. A., and Sairam, M. R. (1993) *Biochem. Biophys. Res. Commun.* **190**, 888-894

15. Kumar, T. R., Wang, Y., Lu, N., and Matzuk, M. M. (1997) *Nat. Genet.* **15**, 201-204

16. Dierich, A., Sairam, M. R., Monaco, L., Fimia, G. M., Gansmuller, A., LeMeur, M., and Sassone-Corsi, P. (1998) *Proc. Natl. Acad. Sci. (USA)* **95**, 13612-13617

17. Elion, E. A. (1998) *Science* **281**, 1625-1626

18. Das, S., Maizels, E. T., DeManno, D., St.Clair, E., Adam, S. A., and Hunzicker-Dunn, M. (1996) *Endocrinology* **137**, 967-974

19. Cameron, M. R., Foster, J. S., Bukovsky, A., and Wimalasena, J. (1996) *Biol. Reprod.* **55**, 111-119

20. Pennybacker, M. and Herman, B. (1991) *Mol. Cell. Endocrinol.* **80**, 11-20

21. Touyz, R.M., Jiang, L., and Sairam M.R. (2000) *Biol. Reprod.* **62**, 1067-1074

22. Chedrese, P. J., Rodway, M. R., Swan, C. L., and Gillio-Meina, C. (1998) *J. Mol. Endocrinol.* **20**, 287-292

23. Rodway, M. R., Swan, C. L., Gillio-Meina, C., Crellin, N. K., Flood, P. F., and Chedrese, P. J. (1999) *Mol. Cell Endocrinol.* **148**, 87-94

24. Khan, H., Jiang, L. G., Jayashree, G. N., Yarney, T. A., and Sairam, M. R. (1997) *J. Mol. Endocrinol.* **19**, 183-190

25. Liu, X., DePasquale, J., Griswold, M. D., and Dias, J. A. (1994) *Endocrinology* **135**, 682-691

26. Davis, D., Liu, X., and Segaloff, D.L. (1995) *Mol. Endocrinol.* **9**, 159-170.
27. Gasinska, A. and Wilson, G. D. (1988) *Br. J. Radiol.* **61**, 133-139
28. Terry, N. H., White, R. A., Meistrich, M. L., and Calkins, D. P. (1991) *Cytometry* **12**, 234-241
29. Nakamura, K., Krupnick, J. G., Benovic, J. L., and Ascoli, M. (1998) *J. Biol. Chem.* **273**, 24346-24354
30. Moudgal, N. R., Sairam, M. R., Krishnamurthy, H. N., Sridhar, S., Krishnamurthy, H., and Khan, H. (1997) *Endocrinology* **138**, 3065-3068
31. Dattatreyamurty, B. and Reichert, L. E., Jr. (1992) *Endocrinology* **131**, 2437-2445
32. Minegishi, T., Delgado, C., and Dufau, M. L. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 1470-1474
33. Touraine, P., Beau, I., Gougeon, A., Medwi, G., Desroches, A., Richard, C., Detoeuf, M., Daniel, B., Pieur, M., Zorn, J. R., Milgrom, E., Kutenn, F., and Misrahi, M. (1999) *Mol. Endocrinol.* **23**, 1844-1854
34. Grammer, T. C. and Blenis, J. (1997) *Oncogene* **14**, 1635-1642
35. King, W. G., Mattaliano, M. D., Chan, T. O., Tsichlis, P. N., and Brugge, J. S. (1997) *Mol. Cell. Biol.* **17**, 4406-4418
36. Kelton, C. A., Cheng, S. V. Y., Nugent, N. P., Schweickhardt, R. L., Rosenthal, J. L., Overton, S. A., Wands, G. D., Kuzeja, J. B., Luchette, C. A., and Chappel, S. C. (1992) *Mol. Cell. Endocrinol.* **89**, 141-151
37. Camp, T. A., Rahal, J. O., and Mayo, K. E. (1991) *Mol. Endocrinol.* **5**, 1405-1417
38. LaPolt, P. S., Tilly, J. L., Aihara, T., Nishimori, K., and Hsueh, A. J. W. (1992) *Endocrinology* **130**, 1289-1295
39. Delidow, B. C., White, B. A., and Peluso, J. J. (1990) *Endocrinology* **126**, 2302-2306
40. Roy, S. K. and Treacy, B. J. (1993) *Fertil. Steril.* **59**, 783-790

41. Sicinski, P., Donaher, J. L., Geng, Y., Parker, S. B., Gardner, H., Park, M. Y., Robker, R. L., Richards, J. S., McGinnis, L. K., Biggers, J. D., Eppig, J. J., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1996) *Nature* **384**, 470-474

42. Robker, R. L. and Richards, J. S. (1998) *Biol. Reprod.* **59**, 476-482

43. Ji, T. H., Grossman, M., and Ji, I. (1998) *J. Biol. Chem.* **273**, 17299-17302

44. Chew, S.L. (1997) *Trends Endocrinol. Metab.* **8**, 405-413

45. Sprengel, R., Braun, T., Nikolics, K., Segaloff, D. L., and Seeburg, P. H. (1990) *Mol. Endocrinol.* **4**, 525-530

46. Yarney, T. A., Sai, M., Khan, H., Ravindranath, N., Payne, S., and Seidah, N. G. (1993) *Mol. Cell Endocrinol.* **93**, 219-226

47. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature* **369**, 455-461

48. Campbell, J. S., Seger, R., Graves, J. D., Graves, L. M., Jensen, A. M., and Krebs, E. G. (1995) *Recent Prog. Horm. Res.* **50**, 131-159

49. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) *J. Biol. Chem.* **266**, 22159-22163

50. Graves, P. N., Tomer, Y., and Davies, T. F. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1135-1143

51. Ascoli, M. (1996) *Biochem. Pharmacol.* **52**, 1647-1655

52. Quintana, J., Hipkin, R. W., Sanchez-Yague, J., and Ascoli, M. (1994) *J. Biol. Chem.* **269**, 8772-8779

53. Cook, S. J. and McCormick, F. (1993) *Science* **262**, 1069-1072

54. Frodin, F., Peraldi, and Obberghen, E.V. (1994) *J. Biol. Chem.* **269**, 6207-6214
55. Bley, M. A., Saragueta, P. E., and Baranao, J. L. (1997) *J. Steroid Biochem. Mol. Biol.* **62**, 11-19

56. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489-27494

57. Shibata, E. F., Matsuda, J. J., Volk, K. A., Collison, K. A., and Segaloff, D. L. (1992) *Endocrinology* **131**, 979-981

58. Thomas, S. M., DeMarco, M., D'Arcangelo, G., Haledoua, S., and Brugge, J. S. (1992) *Cell* **68**, 1031-1040

59. Miller, T. M., Tansey, M. G., Johnson, E. M. Jr., and Creedon, D. J. (1997) *J. Biol. Chem.* **272**, 9847-9853

60. Hallberg, B., Ashcroft, M., Loeb, D. M., Kaplan, D. R., and Downward, J. (1998) *Oncogene* **17**, 691-697

61. Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) *Nature* **370**, 508-509

62. Kodaki, T., Woscholski, R., Hallberg, B., Rodriguez-Viciana, P., Downward, J., and Parker, P. J. (1994) *Current Biology* **4**, 798-806

63. Rodriguez-Viciana, P., Warne, P. H., Vanhaesebroeck, B., Waterfield, M. D., and Downward, J. (1996) *EMBO Journal* **15**, 2442-2451

64. Klinghoffer, R. A., Duckworth, B., Valius, M., Cantley, L., and Kazlauskas, A. (1996) *Mol. Cell Biol.* **16**, 5905-5914
rhFSH, recombinant human Follicle-Stimulating Hormone (follitropin); rhCG, recombinant-Human Chorionic Gonadotropin; LH, Luteinizing Hormone (lutropin); ERK, extra cellular regulated kinase; ERK1/2-P, Activated ERK; MAPK, mitogen activated protein kinase; cAMP, 3’,5’- cyclic AMP; DMEM, Dulbecco's modified Eagle's medium.
FIGURE LEGENDS

Fig. 1. Expression of the oFSH-R3, oFSH-R1 Receptors in Stably Transfected Granulosa Cell. Panel A: Verification by PCR of Neomycin and receptors in transfected cells. Genomic DNA was extracted from granulosa cells by phenol–chloroform method and subjected to PCR (95°C for 4 min denaturation, 94°C 45 sec, 54°C 45 sec and 72°C 1.5 min for 30 cycles with final extension at 72°C of 10 min) using primers specific for neomycin SC 004 and SC 003 (fragment size 348bp). To verify R3 and R1 receptor expression, RT PCR was done on total RNA isolated from vector, oFSH-R3 and oFSH-R1 cDNA transfected granulosa cells as described in the methods and subjected to reverse transcription using MMLV RT( Ambion) for 1 hour at 42°C. The cDNA was further subjected to PCR (95°C for 4 min denaturation, 94°C 1 min, 55°C 1.5 min, 72°C 1.5 min for 30 cycles and final extension at 72°C for 10 min) using R3 and R1 specific primers. The PCR products separated on 1.5% agarose gel are shown along with the positions of known DNA markers on the left. Only JC-R3 and JC-R1 not Vector transfected cells show specific expression of FSH-R3 (517 bp) and FSH-R1(690 bp) respectively. Panel B shows hormone binding in granulosa cells: Membranes were prepared from cells as described in the methods for measuring radioligand (125IhFSH) binding during 12 hours at 25°C. Equivalent amounts of JC- vector, JC-R3 and JC-R1 were incubated in presence or absence of 1 µg of unlabeled purified hormone (oFSH) along with the labeled hormone. To determine hormonal specificity of binding, purified oLH was used as a competitor instead of oFSH with JC-R3 membranes. Note that specific binding is observed only with JC-R3 and JC-R1 cells. Panel C: Analysis of cell-surface expression of oFSH-R3 by cytofluorimetry: Cells were prepared and analyzed with the control and experimental antibodies as described in the methods.
Histograms are presented showing the binding of two polyclonal antibodies against FSH-R (W970 and J25) to granulosa cells expressing receptors (Upper panel - JC-R3 cells). The panel shows the reaction of S.AbFITC (solid line), normal rabbit serum (bold solid line) as controls. Specific antibody binding indicated by the right shift are shown by J25 (dashed line) and W970 (fainter dotted line). J25 is an antibody to the recombinant oFSH receptor protein and W970 is a rat FSH-R peptide antibody (see methods). The lower panel represents the cell surface expression of oFSH-R1 in JC-R1 cells.

Fig. 2. Characterization of oFSH-R3 Expression by Immunoblotting and Enzymatic Deglycosylation. Granulosa cells expressing FSH-R3 (JC-R3) or FSH-R1 (JC-R1) and without (JC-Vector) were solubilized using NP-40 for immunoblotting. Extracts of JC-R3 cells were incubated in the absence or presence of N-glycosidase F before analysis. For examination of receptor in the mouse, extracts prepared both from FSH receptor knockout mouse FORKO (-/-) and wild-type mouse (+/+) ovarian membranes were utilized. An equal amount of protein (100 µg) was loaded to each well and subjected to SDS-PAGE, followed by transfer to PVDF membrane. The blot was incubated with anti oFSH-R3 peptide IgG (1:2000) in Fig.2A, or FSH-R antibody (J25) in Fig.2B, and bound antibody detected using peroxidase conjugated anti-rabbit IgG antibody and a chemiluminescence detection system. The respective lanes are identified with the estimated Mr shown on the right side. Note that the R3 protein band is not detected in the ovary of FSH receptor knockout mouse. The blot (Fig.2A) was stripped and reprobed to check for normalization and internal control (G6PDH) for each sample. In Fig.2B the extracellular domain antibody detects both R1 and R3 in the +/+ but not -/- mouse ovary. Respective receptor bands are observed in JC-R3 and JC-R1 cells.
Fig. 3. **cAMP Production in JC-R3 and JC-R1 Cells:** Granulosa cells transfected with oFSH-R3 (JC-R3) or oFSH-R1 (JC-R1) and without receptor (JC-Vector) were cultured in serum free DMEM-F12 medium. Cells were stimulated either with rFSH or different concentration of forskolin for 1 hour at 37°C in presence of 0.1mM IBMX (isobutyl methyl xanthine). After 1 hour the cAMP produced in the cells was measured by RIA using double antibody binding method. Protein content in the lysates was determined by Bio-Rad protein assay. Compared to the statistically significant increase in cAMP at 1ng/ml for JC-R1 there is no such effect for JC-R3 cells even at 1000 ng FSH / ml. Stimulation by forskolin is shown only for JC-R3 cells.

Fig. 4. **Phosphorylation of the oFSH-R3 in Stably Transfected Granulosa Cells.**

The JC-R3 granulosa cells stably expressing oFSH-R3 were biosynthetically prelabeled with $^{32}$P for 4 hr and further incubated with or without 10 ng/ml rhFSH for 45 min as indicated. In some experiments, cells are treated with either PMA for 20 min or pretreated with PKC inhibitor Calphostin C for 30 min prior to FSH stimulation. Immunoprecipitates of FSH-R3 were prepared using R3 specific antibody as described under material and methods. The results presented at the bottom are densitometric scan of representative autoradiographs obtained using equal amount of protein used for immunoprecipitation. Lane 1-JC-vector, 2-5 JC-R3 treated as shown.

Fig. 5. **Activation of MAP Kinases (ERK1/ERK2) by FSH in JC-R3 Granulosa Cells:** Panel A: Serum starved cells were stimulated with FSH as indicated. In some experiments, cells were pretreated for 30 min to PD98059, Calphostin C and H89 at a concentration of $10^{-5}$ mole/L before agonist (FSH) stimulation. Cells were harvested in lysis buffer as described in materials and methods and equal protein concentrations (5 μg) were analyzed by Western blot using anti-phospho specific ERK (p44/42) antibody. The intensity of the band in each lane demonstrates the degree of increase in ERK (ERK1+ERK2) phosphorylation in cells (ERK 1/2-P). The middle
section of this panel represents unphosphorylated MAP kinase (ERK1/2) activities in the same blot. The lower section summarizes the densitometric scanning of phosphorylated MAP kinases. To get a better idea of the relative activation status, respective lanes in each treatment were scanned by densitometry and the data expressed as a ratio by dividing the intensity of ERK1/2-P in each lane by the corresponding value for ERK 1/2.

Panel B: For examining hormone dependent phospho specific MAP kinase (ERK1/ERK2) activation, the cells were stimulated for 10 min with increasing concentration of rhFSH (0.1, 1, 10, 100 ng/ml respectively). Blots were probed as above in panel A. The middle section represents MAP kinase (ERK1/2) expression in the same blot. Bands were scanned by densitometry and relative activation status was calculated as in Fig 5A. Note that activity is doubled at 0.1ng FSH / ml (~3x10^{-12} M).

Panel C: Hormone (FSH) specific activation of MAP kinase in JC-R3 cell lines: Serum starved cells either JC-410, JC-vector and JC-R3 cells were stimulated with either rFSH or rhCG (with different concentrations) as shown in the figure for 10 min. In Ag18 treated cells, cells were preexposed at a concentration of \(10^{-5}\) mole/L for 30 min. The cells are lysed in lysis buffer as described in methods and equal amount of protein loaded on 10% SDS-PAGE and immunoblotted using phospho specific anti ERK1/ERK2 MAP kinase antibody. Serum treated JC-R3 cells serve as a positive control for comparison. Note the lack of activation by rhCG at any concentration. The lower section shows the expression of MAP kinase in the same blot after reprobing.

Fig.6. Influence of cAMP Activating Mechanisms on ERK1/ERK2 Phosphorylation in JC-R1 and JC-R3 Cells.
A. Serum starved JC-R1 cells were stimulated with 100ng/ml of rFSH for different time intervals and probed as indicated in previous figures. The intensity of the band in each lane represents the degree of ERK1/ERK2 phosphorylation. Cells treated with 10% serum for 10 min served as a positive control. Equal loading is shown by ERK1/2 intensity in each lane. B. Serum starved JC-R3 cells were stimulated with different concentrations of forskolin (µM) for 10 min as shown in the Fig. The intensity of the band in each lane represents the degree of ERK1/ERK2 phosphorylation. There is no activation by forskolin at any concentration. 10% serum stimulated cells for 10 min served as a positive control. Relative ERK 1/2 activation status in both panels was calculated as in Fig 5A and is shown on the right.

Fig. 7. **Effect of Intra or Extracellular Calcium Chelator on FSH Induced ERK1/ERK2 Activation in JC-R3 Cells.** Serum starved JC-R3 granulosa cells were stimulated with either rhFSH (100 ng/ml) or PMA (200 nM) for 10 min. In some experiments, cells were pretreated for 30 min with intra or extracellular Ca²⁺ chelator BAPTA/AM (10⁻²mole/L) or EGTA (10⁻³ mole/L). The cells were lysed in lysis buffer as described in methods, 5 µg of soluble cell protein was separated on 10% SDS-PAGE and transferred to PVDF membrane. The blot was probed with phospho specific ERK1/2 antibody (1:1000) and same blot was stripped and reprobed with ERK1/2 antibody to evaluate equal loading of protein (see bottom).

Fig. 8. **The Effect of FSH on the PI-3 Kinase Pathway in JC-R3 Granulosa Cells:** A. Granulosa cells expressing R3 were stimulated with rhFSH (100 ng/ml) for different time periods or with 10 % serum for 30 min. Cell protein extract was prepared as described in methods. An equal amount (100 µg) protein was separated on 10% SDS-PAGE and immunoblotted with phospho-Akt (1:1000) and Akt (1:1000) antibodies as shown in figure. B. JC-R3 cells stably expressing R3 receptor were pretreated for 30 min with different
concentrations of PI-3 kinase inhibitor LY294002 as shown. Subsequently the cells were stimulated with FSH 100ng/ml for 10 min as described in the methods. The upper panel represents the phospho specific ERK1/ERK2 activation and lower panel represents ERK1/ERK2 expression.

Fig. 9. Mitogenic Actions of FSH on Transfected Granulosa Cells: The top section (A) shows responses of granulosa cell proliferation induced by increasing concentration of FSH: Granulosa cells transfected with either oFSH-R1 or oFSH-R3 and vector control were growth arrested in serum-free medium and treated with varying concentrations of rhFSH and BrdU for 1 hour. BrdU labeling indices of S-phase cells are represented in the fig as % of BrdU positive cells. Data are the mean ± SEM of triplicate determinations. In the middle section (B) are shown time course of mitogenic action of rhFSH on JC-R3 cells: Both JC-Vector and JC-R3 granulosa cells in serum-free medium were treated with rFSH and BrdU as indicated. Cells were harvested at different times after FSH treatment for analysis. The bottom section (C) shows DNA synthesis as measured by thymidine incorporation in cultured granulosa cells expressing either FSH-R3 or FSH-R1 and with out receptor (JC-Vector). Cells were incubated with or without FSH (1ng/ml and 10ng/ml) for 24 hrs in the presence of 1 μCi [3H] thymidine. The data are mean ± SEM (n = 3 per experiment) with * indicating statistically significant differences from control.

Fig.10. Effect of Specific Signaling Pathway Inhibitors on Proliferation in JC-R3 and JC-R1 Cells. A. shows the inhibition of FSH induced proliferation in JC-R3 cells by MEK inhibitor PD98059 and intracellular calcium inhibitor BAPTA/AM. JC-R3 cells were treated with PD98059 (10⁻⁵ M), BAPTA/AM (10⁻² M), KT5720 (10⁻⁵ M), H89 (10⁻⁵ M) or LY294002 (10⁻⁶ M) for 30 min prior to agonist stimulation (1 ng/ml for 1 hr) and BrdU labeling. BrdU labeled S-phase cells are expressed as relative % of BrdU positive cells. Significant differences are represented by the asterisk.
B. represents the effect of different inhibitors on FSH induced proliferation in JC-R1 cells. JC-R1 cells were treated with PD98059 (10⁻⁵ M), BAPTA/AM (10⁻² M), H89 (10⁻⁵ M) and LY294002 (10⁻⁶ M) for 30 min prior to FSH (1 ng/ml, 1hr) stimulation and BrdU labeling. BrdU labeled S-phase cells are expressed relative % of BrdU positive cells. Significant differences are represented by the asterisk.

Fig. 11. Activation of Steroidogenesis in Granulosa Cells Transfected with FSH-R3: Top: Granulosa cells were cultured in DMEM containing 5% FBS for 24 hours, washed with PBS and incubated for additional 12 hours in serum free medium. Cells were then stimulated with rhFSH in presence or absence of 0.1µM androstenedione as substrate for the enzyme aromatase for 48 hours. Bottom: For the experiment with JC-R3 and JC-R1 cells the respective cDNA’s are transfected and cultured for 12 hours in DMEM with 5% FBS. Subsequently, cells in serum free medium were stimulated with rFSH (100 ng/ml) in presence of 0.1 µM androstenedione for an additional 48 hours. In both sets of experiments the culture media were then collected and analyzed for estradiol production by RIA. Each point represents the mean ± SEM of three experiments, repeated twice. Significant differences are represented by the asterisk. Note that both JC-R3 and JC-R1 cells treated with forskolin produce the same amount of estrogen.
Fig. 1B

|       | JC- Vector | JC-R3 | JC-R1 |
|-------|------------|-------|-------|
| hFSH $[^{125}I]$ | + +        | + ++  | + ++  |
| oFSH (1μg/ml)    | -          | + -   | + -   |
| oLH (1μg/ml)     | -          | - +   | - +   |
Fig. 3

![Graph showing cAMP production over FSH and Forskolin concentrations.]

- **Y-axis**: cAMP production (pmol/mg of protein)
- **X-axis**: FSH (ng/mL) and Forskolin (μM)

Lines represent:
- **JC-Vector**
- **JC-R3**
- **JC-R3 + Forskolin**
- **JC-R1**
10 min stimulation

| Condition           | ERK1/2-P | ERK1/2 |
|---------------------|----------|--------|
| rhFSH (100ng/ml)    | -        | +      |
| BAPTA/AM            | -        | -      |
| EGTA                | -        | -      |
| PMA                 | -        | -      |

Fig. 7
**Fig. 8**

**A**

| Min  | 0 | 5 | 10 | 30 | 60 | +ve (serum) |
|------|---|---|----|----|----|-------------|
| Phospho-Akt |   |   |    |    |    |             |
| Akt |   |   |    |    |    |             |

**B**

| LY 294002 μM | No FSH | 0 | 0.5 | 1 | 5 |
|-------------|--------|---|-----|---|---|
| ERK1/2-P    |        |   |     |   |   |
| ERK1/2      |        |   |     |   |   |
Fig. 9

A

% of BrdU positive cells

0 0.1 1 10 100 200

ng/ml

B

% of BrdU positive cell

0 1 2 3 4

Time (hrs)

C

[3H] Thymidine incorporated (% Basal)

0 100 150 350

FSH (ng/ml) 24 hrs

JC-V  JC-R3  JC-R1
Fig. 11

JC-R3

Estradiol pg/μg of protein

|       | JC-410 | JC-R3 | JC-R3 | JC-R1 | JC-R1 |
|-------|--------|-------|-------|-------|-------|
| FSH (100 ng/ml) | +      | +     | -     | +     | -     |
| Forskolin (2 μM) | -      | -     | +     | -     | +     |
Activation of Extracellular Regulated Kinase Pathways in Ovarian Granulosa Cells by the Novel Growth Factor Type I Follicle Stimulating Hormone Receptor: Role in Hormone Signaling and Cell Proliferation
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