Endocrine, *vol. 22, no. 2, 135–141, November 2003* 0969–711X/03/22:135–141/$25.00

Differential Expression of GRK Isoforms in Nonmalignant and Malignant Human Granulosa Cells

Denise Walker King, Rosemary Steinmetz, Heather A. Wagoner, Tamara S. Hannon, Lin Yuan Chen, Erica A. Eugster, and Ora Hirsch Pescovitz

Section of Pediatric Endocrinology/Diabetology, Wells Center for Pediatric Research, Department of Pediatrics, Physiology and Biophysics, Indiana University School of Medicine, and the James Whitcomb Riley Hospital for Children, Indianapolis, IN

Granulosa cell tumors are serious ovarian neoplasms that can occur in women of all ages. While there have been numerous attempts to understand the cause of these malignancies, the pathogenesis of granulosa cell tumors (GCTs) still remains largely unknown. G-protein coupled receptor kinases (GRKs) are important regulators of signal transduction through the process of receptor desensitization and internalization. Receptors that are regulated by GRKs are members of the large family of seven-transmembrane receptors and include the follicle stimulating hormone receptor (FSHR). In granulosa cells, the FSH signaling system is responsible for cell proliferation, differentiation, and steroidogenesis. In the studies presented, we examined GRK mRNA and protein expression in nonmalignant human granulosa cells, in KGN cells, a human GCT cell line, and in a panel of human GCT samples. The KGN tumor cells express significantly less GRK4 α/β protein and higher levels of GRK2 and GRK4 γ/δ protein as compared to nonmalignant human granulosa cells. In human GCT samples, GRK4 α/β protein was detected in 3 of the 13 tumor samples, whereas γ/δ proteins expression was detected in all samples. These findings suggest that GRK protein expression is altered in GCTs and may be involved in the pathogenesis of these tumors.

Key Words: Ovary; granulosa cell tumors; GRKs; cell signaling.

Introduction

Granulosa cell tumors (GCTs) account for 6–10% of all ovarian cancers *(1,2)*, and, although they are one of the most prevalent forms of ovarian cancer in pediatric patients, the pathogenesis of these tumors is poorly understood. The follicle-stimulating hormone (FSH) receptor is critical to normal granulosa cell function, and it has been suggested that a mutation in the FSH receptor resulting in its constitutive activation may promote GCT formation *(3–6)*. Several laboratories have investigated this theory; however, no gain of function mutations have been identified within the receptor coding sequence. A second hypothesis focuses on mutations in the stimulatory Gsα subunit of the heterotrimeric G-protein as possible causes of these tumors. Again, numerous investigators have been unable to identify Gsα mutations, similar to those found in McCune–Albright syndrome, which are linked to GCT formation *(3,7)*. However, it has been reported that GCTs have developed in patients receiving clomiphene citrate and/or supraphysiologic doses of gonadotropins *(8)*, suggesting that overstimulation of the FSH pathway may be correlated with tumor initiation or progression in some individuals. Thus, whereas the receptor itself and the Gsα subunit do not appear to be involved in most cases of GCT formation, abnormalities in components of the FSH-receptor pathway distal to the receptor may play a role.

The duration of G-protein coupled receptor signaling is regulated, in part, by the members of the family of G-protein coupled receptor kinases (GRKs). Activated receptors are phosphorylated by GRKs resulting in the dissociation of the G-protein from the receptor and the concomitant association of the receptor with an arrestin molecule. This association targets the receptor for desensitization and internalization. We hypothesize that a perturbation in the process of receptor uncoupling and desensitization, as a result of altered GRK expression or activity, could result in uncontrolled receptor signaling. In the case of the FSHR, this would likely lead to increased ovarian stimulation and cell proliferation. Furthermore, in neoplastic granulosa cells such as those found in GCTs, a disruption in receptor signal termination may function as a tumor promoter.

Six different GRK isoforms have been identified to date *(9–17)*. Of these six, GRKs 2, 3, 5, and 6 are ubiquitously expressed *(18)*. GRK1 (rhodopsin kinase) has been detected only in the visual system *(19)*, while GRK4 expression has been found in the testis *(13,20,21)*, brain *(22,23)*, and human myometrium *(24)*. Additionally, GRKs 4 and 6 are expressed as multiple splice variants. The variants for GRK4 are designated as α, β, γ, and δ. GRK4 α is encoded by all 16 exons

Received June 9, 2003; Revised August 5, 2003; Accepted August 5, 2003.
Author to whom all correspondence and reprint requests should be addressed: Rosemary Steinmetz, PhD, Riley Hospital, Room A5984, 702 Barnhill Dr., Indianapolis, IN, 46202. E-mail: rsteinme@iupui.edu

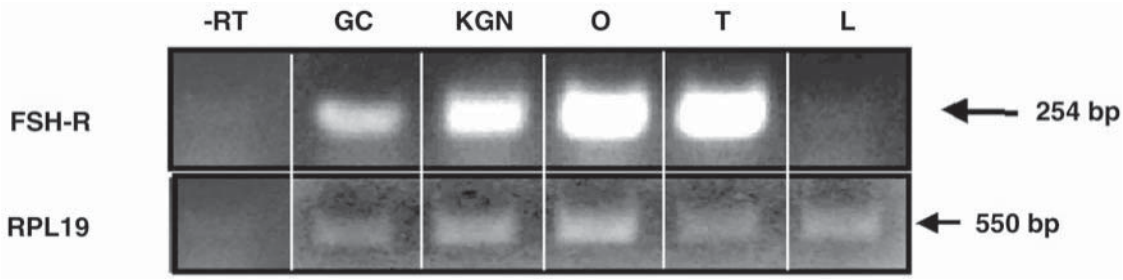

Fig. 1. FSH receptor mRNA expression in human granulosa cells. FSH receptor mRNA was detected using 5 μg of total RNA extracted from human IVF-granulosa cells (GC), KGN tumor cells, and ovary (O), testis (T), and lung (L) tissue. Primers specific to the FSH receptor spanning exons 9 and 10 were used to amplify a 254 bp portion of the receptor. PCR was conducted for 30 cycles and products were run on a 1% agarose gel. RT-PCR experiments were repeated twice to confirm the results.

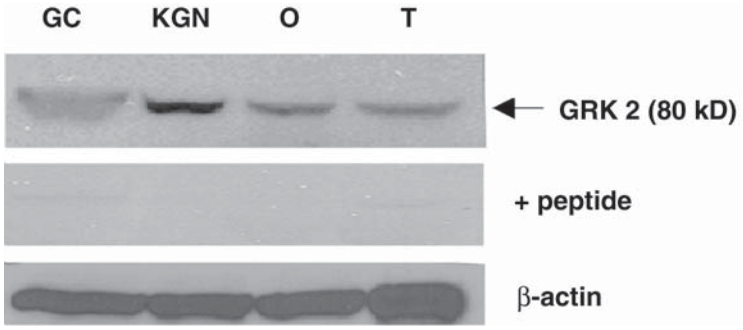

Fig. 2. Western blot analysis of GRK2 expression in human granulosa cells. GRK2 expression was determined using 13 μg of total protein extracted from human granulosa cells (GC), KGN tumor cells, and whole ovary (O) and testis (T). Proteins were separated on a 10% polyacrylamide gel and membranes were probed with a GRK2-specific antibody. A band detected at approx 80 kDa (predicted size for GRK2) was differentially expressed in all samples. Specificity of the antibody was confirmed using a blocking peptide (+ peptide). β-actin antibody was used to demonstrate equivalent protein loading. The blot shown is a representative of three Western blots experiments, each using different protein lysates prepared from KGN and IVF-GC cells.

of the GRK4 gene; while the β, γ, and δ splice variants are truncated, lacking amino acid sequences that are encoded by exons 2 and 15. Three GRK6 splice variants designated A, B, and C, have also been described. These variants differ only in their C-terminal region and result from differential splicing of expressed sequence encoded by exon 16.

The goals of this study were to compare GRKs 2, 3, 4, 5, and 6 expression in nonmalignant and malignant human granulosa cells in order to determine whether patterns of expression vary between these cell types.

Results

FSH Receptor Expression

Because it was hypothesized that abnormal FSH receptor signaling may be involved in the pathogenesis of GCTs, we first confirmed that the KGN cells used for these studies express FSHR mRNA using qualitative RT-PCR analysis. Total RNA extracted from in vitro fertilization (IVF)-derived granulosa cells (GC), whole ovary and testes served as positive controls. FSH receptor mRNA was detected in the GCs and KGN cells, as well as in the whole ovary and testis RNAs (Fig. 1). FSHR mRNA expression in the GCs appeared weaker compared to the other RNA samples, which may be the result of partial FSH receptor down-regulation following supraphysiologic levels of gonadotropins achieved during in vitro fertilization procedures. RNA prepared from lung tissue did not express FSH receptor mRNA, as expected, and was used as a negative control. Because it has previously been reported that KGN cells express functional FSHRs *(25)*, further verification of receptor expression was not performed.

GRK Protein Expression in Granulosa Cells and KGN Granulosa Cell Tumor Cells

Western blot analysis for GRKs 2, 3, 4, 5, and 6 was performed in order to better determine the endogenous protein expression of each of these GRKs in the cells and tissues examined. A protein product of approx 80 kDa, which is similar in size to what has previously been reported for GRK2 *(26,27)*, was detected in all of the samples (Fig. 2, arrowhead). Expression of this band was most pronounced in the KGN cells as compared to the nonmalignant GCs, ovary, and testis samples. When blots were probed with an antibody for GRK3 and GRK5, we were unable to detect either of these proteins in the granulosa cells, KGN cells, ovary, or testis samples (data not shown).

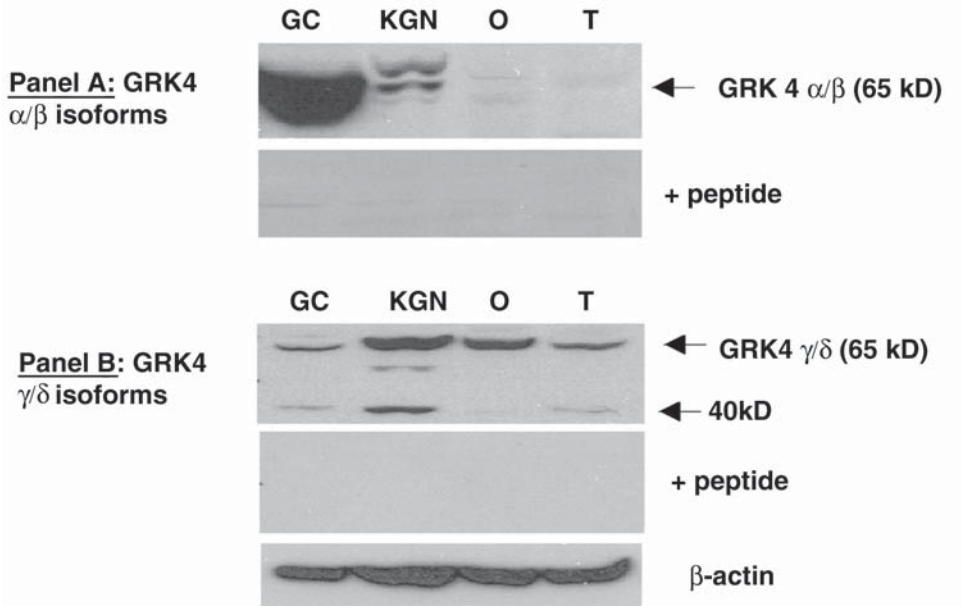

Fig. 3. Western blot analysis of GRK4 expression in human granulosa cells. GRK4 expression was determined using 13 μg of total protein extracted from human granulosa cells (GC), KGN tumor cells, and whole ovary (O) and testis (T). **Panel A**: GRK α/β protein expression was detected using the K-20 antibody. In the KGN and granulosa cell lysates a 65 kDa (expected size for the α or β variant) was detected. **Panel B**: GRK4 γ/δ expression was detected using the I-20 antibody. Specificity of the antibody was confirmed using a blocking peptide (+ peptide). β-actin antibody was used to demonstrate equivalent protein loading. Results shown are a representative of three separate Western blot experiments, each using different protein lysates.

GRK4 protein expression was analyzed using two different antibody preparations, the K-20 antibody, which cross-reacts with both the α and β variants (α/β), and the I-20 antibody, which cross-reacts with both the γ and δ variants (γ/δ). In the enriched GC extracts, a prominent band at approx 65 kDa (Fig. 3, Panel A) was observed, which is the expected molecular weight for the α/β variant. KGN cells expressed less of this product than do the nonmalignant GCs. In the whole ovary and testis protein lysates, this 65 kDa product was weakly detected. Immunoblotting for the γ /δ variants resulted in the detection of a 65 kDa protein in all four samples (Fig. 3, Panel B). A protein band of approx 40 kDa was also detected using the I-20 antibody that may represent another GRK4 splice variant or possibly a degradation product, as previously suggested *(24)*. Both GRK antibodies were tested for specificity using neutralizing peptides specific for each and shown in the panel labeled + peptide.

The results of the Western analyses for GRK2 and GRK4 were then quantified by densitometry. The 65 kDa protein bands for the GRK4 α/β and γ /δ variants in KGN cells were compared to those detected in the nonmalignant granulosa cells. For GRK2, the level of expression of the 80 kDa band was compared in both cell types. Results of these analyses are shown in Fig. 4. The most striking difference was seen for GRK4 α/β expression, which was approximately sixfold higher in the nonmalignant granulosa cells as compared to KGN cells. GRK2 and GRK4 γ /δ were found to be expressed approximately twofold higher in the KGN cells as compared with the nonmalignant GCs.

Western blot analysis using an antibody that cross-reacts with all three GRK6 variants was also performed on granulosa cells, KGN cells, and ovary and testis protein lysates. Similar protein bands were seen in all of the samples tested with no apparent differences in protein expression (data not shown), suggesting that GRK6 is likely not to be involved in the pathogenesis of GCTs.

GRK Expression in Human Granulosa Cell Tumors

The pattern of GRK expression in human GCT samples was next examined. Western analyses for GRK2, GRK4 α/β, and GRK4 γ/δ were performed using total proteins prepared from 13 human GCTs obtained from the Indiana University School of Medicine Tumor Bank. Tumors were designated as either primary (P, $n = 8$) or metastatic (M, $n = 4$) based on the pathology report. One sample was identified only as a sex-cord tumor of the ovary (S) without further classification. All samples were snap frozen in liquid nitrogen and stored at –80°C until protein lysates were prepared.

GRK2 expression in the tumor samples appeared similar to that seen in the KGN cells. The 80 kDa product was detected in both primary and metastatic tumor samples. Although there was some variation in the level of expression, all of the tumor samples expressed this product (Fig. 5). When the GRK2 antibody was preincubated with the specific blocking peptide, this band was almost completely blocked.

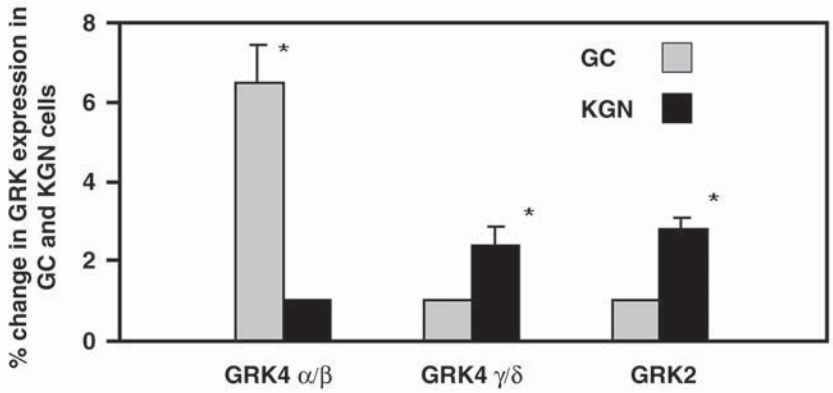

Fig. 4. Quantification of Western blot analysis showing the difference in expression of GRK2and GRK4 variants in KGN granulosa tumor cells as compared to nonmalignant human granulosa cells. The 65 kDa bands for GRK4 α/β and γ/δ and the 80 kDa band for GRK2 were quantified by densitometry and normalized to β-actin expression. Results shown are the mean ± SEM GRK expression calculated from three separate Western blot experiments. *$p \leq 0.05$.

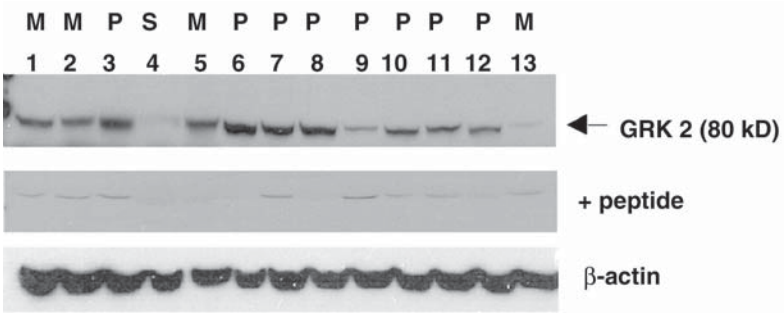

Fig. 5. Western blot analysis of GRK2 expression in human granulosa cell tumors. Protein expression of GRK2 using 13 μg of total protein lysates from 13 granulosa cell tumor specimens that were classified as either primary (P) or metastatic (M). Sample 4 is a sex cord tumor (S) of the ovary with no further description given. The arrowhead on the right indicates the 80 kDa band specific for GRK2. The "+ peptide" blot was incubated with primary antibody that was pretreated with excess blocking peptide specific for GRK2. β-actin antibody was used to demonstrate equivalent protein loading. Results shown are representative of three separate experiments.

GRK4 α/β variants were only weakly detected in tumor samples 6, 8, and 10, all of which were primary in origin (Fig. 6, Panel A, arrowheads). In sample 4, a second band was also detected (*); however, the molecular weight of the product was less that that of the GRK4 α/β variants detected in samples 6, 8, and 10 and may represent a degradation product or splice variant. A prominent protein band at approx 60 kDa was seen in all of the samples. When Western blots were preincubated with a blocking peptide for the α/β variants this band was partially blocked. Immunoblotting for GRK4 γ/δ variants, detected a protein band of the predicted size (65 kDa) in all of the tumor samples (Fig. 6, Panel B).

Discussion

In females, granulosa cells are the only cell type that expresses FSH receptors. Owing to this restricted pattern of expression, the FSH receptor pathway has been considered a provocative candidate to be involved in the initiation and/or progression of GCTs. However, to date, no mutations of the FSH receptor or the associated G-protein have been found to be associated with granulosa cell tumors. In this study, we have extended the search for a FSH pathway-associated abnormality that might be involved in granulosa cell tumorigenesis and focused on the family of intracellular kinases that are key modulators of G-protein coupled receptor activity through their regulation of receptor desensitization and internalization following ligand binding *(28, 29)*. The results of our studies determined that there are differences in the expression of several GRK variants in malignant as compared with nonmalignant granulosa cells.

This is the first report characterizing endogenous expression patterns for GRK variants in malignant and nonmalignant human granulosa cells. Although the GRK 4 α/β variants (the 65 kDa protein product) are expressed at high levels in the nonmalignant cells, their expression in the KGN tumor

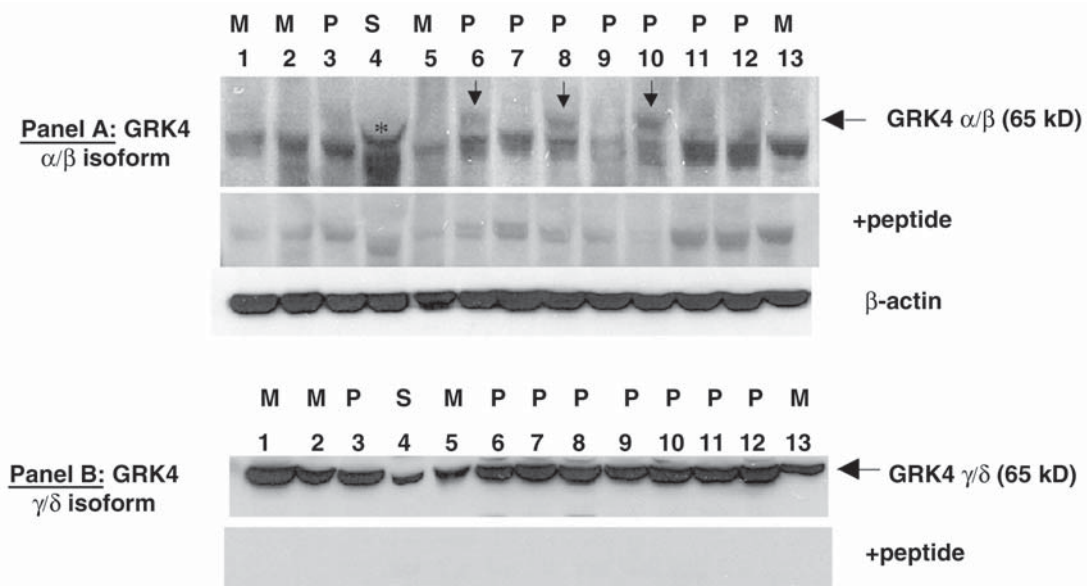

Fig. 6. Western blot analysis of GRK4 expression in a panel of human granulosa cell tumors. **Panel A:** GRK4 α/β variants were detected in tumor samples 6, 8, and 10 (arrows). In sample 4 a second protein band was detected (asterisk), which was not of the expected size for the GRK4 α/β and may represent a degradation product or splice variant. **Panel B:** protein expression of GRK4 γ/δ in the same tumors. The "+ peptide" blot in each was incubated with primary antibody pretreated with excess blocking peptide specific for either the K-20 or I-20 antibody. β-actin antibody was used to demonstrate equivalent protein loading. Results shown are a representative of three experiments.

cells and in the human tumor samples is greatly diminished. In the human GCT samples, only 3 of the 13 samples expressed the α/β variants. In contrast, GRK4 γ/δ variants (65 kDa protein) appear to be overexpressed in the KGN cells as compared to the nonmalignant cells and they were detected in all of the tumor samples. GRK2 protein expression was readily detected in the KGN tumor cells as compared to the IVF-GC. A similar pattern of GRK2 expression was also detected in the human tumor samples, where it was expressed in the majority of samples.

In heterologous systems using MSC-1 and 293 mouse Sertoli cell lines, overexpression of GRK2, 4, and 6 induces FSH receptor phosphorylation following treatment of these cells with FSH *(30)*. Our finding that both GRK2 and GRK4 are differentially expressed in the nonmalignant as compared to malignant granulosa cells suggests that these differences could play a role in granulosa cell tumorigenesis, possibly through regulation of FSH receptor signaling. It is possible that a decrease in GRK4 α/β variant expression could lead to decreased kinase activity and impairment of receptor desensitization. However, further study to determine whether kinase activity is impaired in these tumors and if either of these GRKs is coupled to the FSH receptor in human granulosa cells is needed to confirm this possibility.

It has also been shown that although GRK6 can phosphorylate the FSH receptor, this phosphorylation does not induce receptor desensitization and internalization *(30)*. Thus, our finding that GRK 6 expression was not different between nonmalignant and malignant cells implies that GRK6 does not play a key role in FSHR activity in either normal or malignant granulosa cells. However, it is clear that GRK6 is expressed in the ovary as well as in the testis.

These studies are also the first to identify the presence of GRK4 variants in human female gonadal tissue. Earlier studies in males found GRK4 expression restricted to the testis, whereas, in females, GRK4 was detected by Western analysis in uterine myometrium *(24)*. Additionally, although our studies were not designed to localize GRK4 to a specific ovarian cell type, the results of our studies strongly suggest that GRK4 protein expression is predominantly localized to the granulosa cell compartment based on the Western blot results seen for the enriched IVF granulosa cell samples and KGN cells as compared with the whole ovary samples.

We do not yet know what physiologic significance these differences in GRK expression represent; however, they suggest that the differential expression of either GRK2 or GRK4 isoforms may result in impaired FSH receptor uncoupling and desensitization. Failure of the receptor to be properly desensitized could lead to prolonged activation of the receptor and may play a role during the transformation of granulosa cells from a nonmalignant to a malignant phenotype or in the progression of the tumor. A recent report demonstrating that a decrease in GRK expression in differentiated thyroid carcinoma is associated with decreased homologous desensitization and increased cAMP levels supports this hypothesis *(31)* and suggests that abnormalities in GRK

expression and activity may constitute a new family of intracellular regulators that participate in the pathogenesis of specific endocrine cancers.

Materials and Methods

Human Tissue Samples

All nonmalignant granulosa cells, whole ovary tissue, and GCTs samples used in these studies were obtained following patient signed consent. All protocols for cell and tissue collection were approved by the Indiana University Institutional Review Board.

Human GCT tumor samples ($n = 13$) were chosen based on pathologic evaluation and were classified as either primary (P) or metastatic (M). One tumor was classified as a sex-cord tumor without further identification. All tumor samples were identified by coded numbers only.

Nonmalignant granulosa cells were harvested from discarded follicular fluid collected during oocyte retrieval for in vitro fertilization at the Methodist Hospital Reproductive Biology Laboratory. Follicular fluid was collected and granulosa cells were isolated as previously described *(32)*. For this, follicular fluid was centrifuged at 750*g* over a ficoll gradient. Red blood cells were pelleted and the granulosa cells were collected from the interface, washed in DMEM/HamF12 media, before use in Western blot analysis.

Normal ovary samples ($n = 3$) were obtained from the National Disease Research Interchange (Philadelphia, PA) and were accompanied by pathology reports. The ages of the ovary donors were 40, 41, and 51 yr, and all were undergoing total hysterectomies. Whole normal testes ($n = 2$) were received from the Indiana University Tissue Bank. These samples were accompanied by pathology reports identifying that the samples were normal.

Human Granulosa Cell Tumor Cell Line

KGN cells, a human GCT cell line, were kindly provided by Dr. Yoshihiro Nishi and the RIKEN Cell Bank, Tsukuba Science City, Ibaraki, Japan *(25)*. KGN tumor cells were maintained in culture with DMEM/HamF12 (1:1 vol/vol), 10% FBS, and 1% penicillin/streptomycin. Medium was changed every 3 d and cells were passaged by trypsinization every 2 wk. For Western blotting and RNA extraction, cells were allowed to grow to confluence before harvesting.

Reagents

Tissue culture reagents and Superscript First Strand Synthesis System for RT-PCR were purchased from Invitrogen/Gibco (Carlsbad, CA). All anti-GRK antibodies, blocking peptides, and control cell lysates (Ramos and BJAB cell protein lysates) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody, goat anti-rabbit horseradish peroxidase, was purchased from Cell Signaling Technology (Beverly, MA). Tissue Protein Extraction Reagent (TPER) and the BCA Protein Assay kit were purchased from Pierce (Rockford, IL). Complete Mini protease inhibitor tablets were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Tri-reagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH). Human testis total RNA was purchased from Clontech (Palo Alto, CA), and human whole lung and ovary RNA from Stratagene (La Jolla, CA). Primers for PCR were synthesized by Invitrogen/GIBCO (Carlsbad, CA).

Western Blotting

Both KGN and human granulosa cells were washed twice in 1X PBS. Human GCT samples, normal whole ovary, and normal whole testis (approx 50 mg of each tissue) were pulverized in liquid nitrogen. Both cells and tissues were then lysed with TPER buffer containing Complete Mini protease inhibitor tablets. Lysates were collected, and total protein concentration was determined using the BCA Protein Assay kit. Ramos and BJAB cell lysates were utilized as positive controls for GRK 2, 3, 4, and 6. Protein lysates (13 μg total protein/lane) from all of the cells and tumor samples were electrophoresed in a 10% polyacrylamide gel. Polyclonal antibodies used to detect human GRKs were GRK2 (C-15), GRK4 (K-20) recognizing α and β splice variants only, GRK4 (I-20) recognizing γ and δ splice variants only. The specific GRK isoform blocking peptides were GRK2 (C-15)P, GRK4 (I-20)P for GRK4 α/β, GRK4 (K-20)P for the GRK4 γ/δ variants, GRK5 (C-20)P, and GRK6 (C-20)P. Dilutions varied from 1:1000 to 1:3000 for these primary antibodies, and the secondary antibody, goat anti-rabbit horseradish peroxidase was used at a dilution of 1:5000. Individual blocking peptide concentrations were calculated based on antibody concentration and were used at molar excess. A monoclonal antibody against β-actin (Sigma) was used to detect any differences in sample loading. The Western blots for GRK analysis of granulosa cells, KGN cells, and whole ovary and testis lysates were performed three times using different cell and tissue lysates. Western blots of the human tumor samples were repeated twice using fresh protein lysates prepared from these samples.

Qualitative RT-PCR Analysis

RNA was isolated from KGN cells, human granulosa cells, whole ovary, testis, and lung using Tri-reagent. Complementary DNA (cDNA) was produced from these RNAs using the Superscript First Strand Synthesis System for RT-PCR. FSH receptor mRNA expression was determined using the following primer pair: 5'-AGTAGTCTTAGGCT ACAGAAG, 3'-CCCATGTGAAGATATCATGGG. PCR using these primers results in 254 bp product. Riboprotein L-19 (RPL-19) expression served as a control for RNA loading and degradation and was determined using the following primer pair, 5'-ATGGAAGCCAGCCTCACCTAT, 3'-TTCCT TGGTCTTAGACCTGCG. All PCR experiments

were repeated at least two times. RT-PCR reactions that did not contain RNA or reverse transcriptase were performed as controls for DNA contamination. Both reactions failed to amplify products.

Acknowledgments

This work was supported in part by funding from Genentech 993-C01 and Clarian Health VFR25 (EAE); Genentech 300-FO5 (TSH); Showalter Trust and the Center for Excellence in Women's Health Grant (RS); National Institutes of Health Grant FO3 HD041977-01 (OHP) and by the Riley Memorial Association.

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