Luteinizing Hormone–Induced Up-Regulation of ErbB-2 Is Insufficient Stimulant of Growth and Invasion in Ovarian Cancer Cells

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Abstract

The effects of luteinizing hormone (LH), a gonadotropic hormone implicated in the development of ovarian cancer, are mediated by specific binding to its G protein–coupled receptor, the LH receptor (LHR). Activated LHR initiates second messenger responses, including cyclic AMP (cAMP) and inositol phosphate. Because cAMP increases expression of ErbB-2, a receptor tyrosine kinase whose overexpression in cancers correlates with poor survival, we hypothesized that LH may regulate ErbB-2 expression. Cell surface LHR expression in stable transformants of the ErbB-2–overexpressing ovarian cancer cell line SKOV3 was confirmed by PCR and whole-cell ligand binding studies. Second messenger accumulation in the LHR-expressing cells confirmed signaling through Gs and Gq. Western blots of total protein revealed that LHR introduction up-regulated ErbB-2 protein expression 2-fold and this was further up-regulated in a time- and dose-dependent manner in response to LH. Forskolin and 8Br-cAMP also up-regulated ErbB-2 in both LHR-expressing and mock-transfected cells, indicating that regulation of ErbB-2 is a cAMP-mediated event. Kinase inhibitor studies indicated the involvement of protein kinase A–mediated, protein kinase C–mediated, epidermal growth factor receptor–mediated, and ErbB-2–mediated mechanisms. The LH-induced up-regulation of ErbB-2 was insufficient to overcome the negative effects of LH on proliferation, invasion, and migration. A molecular signature for this nonaggressive phenotype was determined by Taqman array to include increased and decreased expression of genes encoding adhesion proteins and metalloproteinases, respectively. These data establish a role for LH and LHR in the regulation of ErbB-2 expression and suggest that, in some systems, ErbB-2 up-regulation alone is insufficient in producing a more aggressive phenotype.
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Introduction

Luteinizing hormone (LH) is the pituitary hormone responsible for inducing ovulation in premenopausal women. The ovulatory process involves extensive tissue remodeling, proteolytic activity, and changes in proliferation, which parallel many cancer-associated processes (1-3). Epidemiologic evidence implicates the pituitary-derived gonadotropic hormones, follicle-stimulating hormone (FSH) and LH, in the development of ovarian cancer by correlating an increased risk of ovarian cancer with physiologic conditions resulting in increased exposure to LH and FSH, such as incessant ovulation, infertility treatments, and menopause (4, 5). *In vivo*, LH influences cellular processes, including adhesion (6), apoptosis (7, 8), anchorage-independent growth (9), angiogenesis (10), and proliferation in ovarian cancer cells or in ovarian epithelial cells, the putative precursor cell to epithelial ovarian cancer (11-13).

The LH receptor (LHR) is a G protein–coupled receptor that is activated on binding of either LH or chorionic gonadotropin (CG), the LH homologue that maintains progesterone production during pregnancy (14). Ligand-activated LHR is capable of signaling through Gs, Gq/11, and Gi/o, although the actions mediated by LHR through Gs are the most studied (14-16). The LHR-initiated second messenger responses, cyclic AMP (cAMP), inositol 1,4,5-trisphosphate (IP3), 1,2-diacylglycerol, and intracellular calcium, stimulate protein kinase cascades that are associated with mitogenic signaling (17). Recently, the actions of LH during ovulation were shown to involve the release of the epidermal growth factor (EGF)-like factors epiregulin and amphiregulin that act as mediators of the ovulatory response (18, 19).

ErbB-2 is a member of the EGF receptor (EGFR) family of receptor tyrosine kinases. Heterodimerization of ErbB-2 with other EGFR family members results in phosphorylation and activation of the dimer partner (20, 21). In cases of ErbB-2 overexpression, homodimerization and autophosphorylation of ErbB-2 can also occur (22). The activated receptor dimer subsequently activates intracellular signaling pathways that regulate biological variables of proliferation, differentiation, and cell motility and survival (23-25). ErbB-2 is often overexpressed in cancers of the breast, ovary, prostate, and colon, and overexpression of ErbB-2 in breast and ovarian cancers is associated with poor survival and resistance to chemotherapy (26-28). When overexpressed in breast cancer,

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ErbB-2 is a valuable therapeutic target and the antibody treatment Herceptin is clinically effective in such cases (21). ErbB-2 expression is induced by cAMP in Schwann cells (29) and mammary cells (30). Because cAMP is the second messenger resulting from LH signaling through Gs and Gq, we hypothesized that LH signaling may induce expression of ErbB-2.

The goal of this study was to determine the role of LHR signaling and its relationship to ErbB-2 in ovarian cancer cells. We introduced LHR to the ErbB-2–overexpressing ovarian cancer cell line SKOV3 and used this model system to study LHR-associated changes in ErbB-2 expression as well as ErbB-2–regulated biological variables. We have shown that LHR expression and activation up-regulate ErbB-2 expression through a protein kinase A (PKA)–dependent and protein kinase C (PKC)–dependent mechanism and that reduced invasion and migration are associated with LHR expression. These data suggest that up-regulation of ErbB-2 in this system is an insufficient stimulant of an aggressive phenotype.

Results

Stable Expression of LHR in SKOV3 Cells

SKOV3 cells were transfected with either full-length hLHR-pcDNA3 or empty vector and subsequently selected for G-418 resistance. Two surviving clonal cell lines, SLHR1 and SMOCK2, were chosen for characterization of the transcript expression, ligand binding, cAMP, and inositol phosphate signaling properties. Nested PCR was used to determine the presence or absence of hLHR transcripts in each cell line (Fig. 1). The expected 711-nucleotide band was visible in the PCR product of the SLHR1 cell line (Fig. 1, *lane 2*) but absent from the SKOV3 and SMOCK2 cell lines (Fig. 1, *lanes 2* and *4*, respectively). The SLHR1 PCR product was determined to represent the hLHR transcript sequence by cloning the PCR product into the TOPO-TA cloning vector and sequencing the insert from the resulting vector.

SLHR1 Cells Express Functional Cell Surface LHR That Signals through Gs and Gq

To confirm receptor expression on the cell surface, we did whole-cell competitive and saturation binding assays with ^{125}I-CG. For competitive binding assays, confluent cultures of SLHR1 and SMOCK2 cells were incubated with 250 pmol/L ^{125}I-CG in the presence or absence of competing CG. The IC_{50} for CG binding in SLHR1 cells was determined to be 0.8 nmol/L (Fig. 2A, *solid line*). SMOCK2 cells showed no specific binding of ^{125}I-CG (Fig. 2A, *dotted line*), consistent with the lack of LHR transcript in this cell line. Saturation binding experiments were done only on SLHR1 cells, and K_d and B_{max} were determined to be 0.3 nmol/L and ~12,000 receptors per cell, respectively (Fig. 2B).

The coupling of cell surface LHR to G protein–associated second messengers was confirmed by measuring cAMP production in response to CG and LH (Fig. 2C) and inositol phosphate accumulation in response to LH (Fig. 2D). SLHR1 cells exhibited a dose-dependent increase in cAMP production in response to both CG and LH stimulation. Similarly, IP increased 2-fold in SLHR1 cells in response to incubation with 153 nmol/L LH. In contrast, there was no increase over basal concentrations of cAMP or IP in response to either LH or CG in SMOCK2 cells. The basal cAMP level of SLHR1 cells (5.2 ± 1.1 pmol/mL) was determined to be 4.5-fold greater than that of SMOCK2 cells (1.2 ± 0.1 pmol/mL).

LH Regulates ErbB-2 Expression in SLHR1 in a Time- and Dose-Dependent Manner

Because the basal cAMP levels of SLHR1 were 4.5-fold greater than SMOCK2, we determined the basal protein expression level of ErbB-2 and its phosphorylated form, phospho-ErbB-2, in these cell lines. Western blots of total protein probed for ErbB-2 or phospho-ErbB-2 revealed 2.9-fold higher basal expression of ErbB-2 in SLHR1 cells compared with SMOCK2 cells (Fig. 3A and B) with a concomitant increase in phospho-ErbB-2. To test for the ability of LH to regulate ErbB-2 expression, SLHR1 and SMOCK2 cells were incubated with 16.7 nmol/L LH over the course of 20.5 hours. Significant increases in ErbB-2 and phospho-ErbB-2 expression were observed in incubations of SLHR1 cells with LH for 8 and 20.5 hours (Fig. 3C and D). Shorter incubations of SLHR1 cells or incubations of SMOCK2 (data not shown) cells with LH revealed no changes in ErbB-2 or phospho-ErbB-2 expression. The dose-dependent nature of ErbB-2 regulation by LH in SLHR1 cells was determined by incubating SLHR1 cells with increasing concentrations of LH for 18 hours. Significant increases in ErbB-2 and phospho-ErbB-2 expression were observed at 16.7 pmol/L LH (Fig. 3E and F). The apparent EC_{50} of LH regulation on ErbB-2 expression was determined to be ~32 pmol/L.

LH Regulation of ErbB-2 Expression Is cAMP Dependent

To test the hypothesis that LH regulates ErbB-2 expression in ovarian cancer cells through its cAMP second messenger, SLHR1 and SMOCK2 cells were incubated with the LHR ligand, LH, with the stable synthetic cAMP analogue, 8Br-cAMP, or with forskolin, which produces cAMP through the direct activation of adenylyl cyclase. Western blots of total protein probed for ErbB-2 revealed that only the LHR-expressing SLHR1 cell line showed an increase in ErbB-2 expression when treated with LH. ErbB-2 protein expression

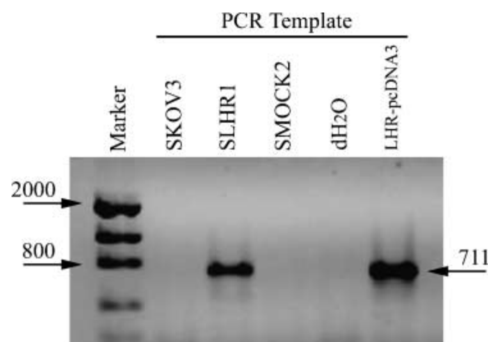

FIGURE 1. LHR transcript in cell lines: agarose gel electrophoresis of nested PCR products from reactions with cDNA from SKOV3, SLHR1, and SMOCK2 cells. Control PCRs were run with distilled water (*dH2O*) and plasmid template.

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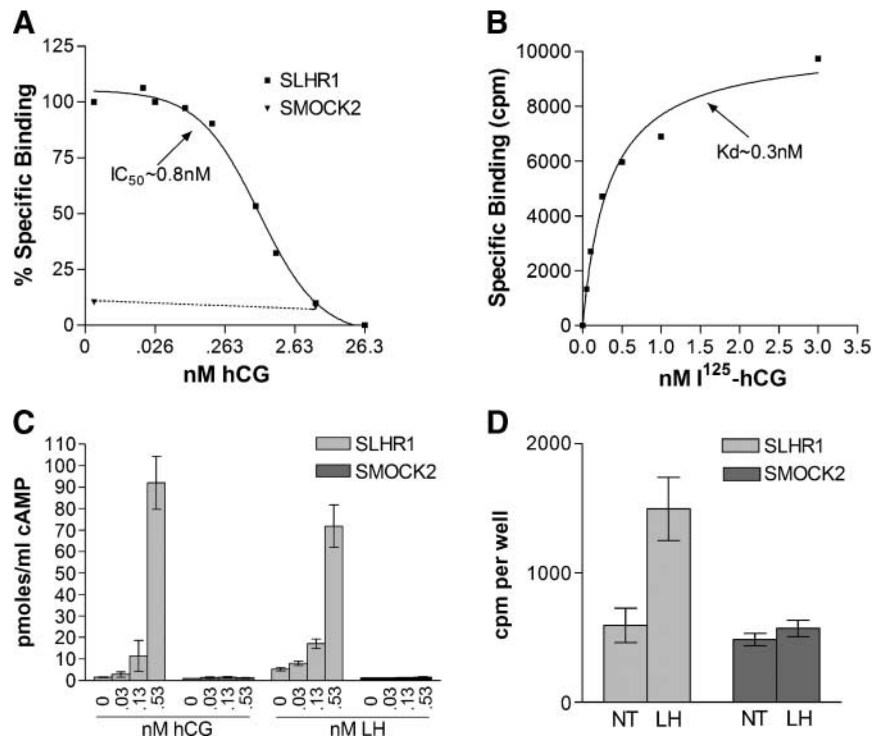

FIGURE 2. SLHR1 cells express functional receptor at the cell surface. **A.** Competitive binding curve: whole cells were incubated with 250 pmol/L ^{125}I-CG in the presence of increasing concentrations of competing CG. SLHR1 cells (*solid line*) bound CG, whereas SMOCK2 cells did not (*dotted line*). **B.** Saturation binding curve: whole SLHR1 cells were incubated with increasing concentrations of ^{125}I-CG without competitor. **C.** cAMP production in response to LHR ligand: increased cAMP accumulation was detected only in whole-cell lysates of SLHR1 cells incubated with LHR ligand. **D.** IP production in response to LH: increased IP production was detected in SLHR1 cells incubated with 13 nmol/L LH but not in nontreated (*NT*) controls.

increased ~1.6-fold in SLHR1 cells treated with 1.67 nmol/L LH for 24 hours (Fig. 4A). Both cell lines, however, up-regulated ErbB-2 protein expression in response to 8Br-cAMP (5 mmol/L) and forskolin (1 μmol/L) over the same time period. Furthermore, Western blots of total protein probed for phospho-ErbB-2 revealed a concomitant increase in the phospho-ErbB-2 in treatments that up-regulated ErbB-2 protein expression (data not shown).

Inhibitors of PKA, PKC, EGFR Kinase, and ErbB-2 Kinase Inhibit the Up-Regulation of ErbB-2 by LH

To determine the signaling pathways involved in the regulation of ErbB-2 expression by LH, we examined the ability of several pharmacologic inhibitors to block the up-regulation of ErbB-2 by LH (Table 1). Western blots of total protein probed for ErbB-2 and phospho-ErbB-2 indicated that inhibitors of PKC (Fig. 4C and D), PKA (Fig. 4E and F), EGFR kinase (Fig. 4G and H), and ErbB-2 kinase (Fig. 4I and J) block the ability of LH to up-regulate ErbB-2 expression. Inhibitors of extracellular signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein/ERK kinase, phosphatidylinositol 3-kinase, and a broad-spectrum metalloproteinase inhibitor were ineffective inhibitors of ErbB-2 up-regulation by LH (data not shown).

LHR Associated with Reduced Invasion, Migration, and Proliferation of Ovarian Cancer Cells

The effects of LH or the LHR receptor agonist CG on ovarian cancer cell proliferation are controversial and have been reported to be stimulatory (12, 31) as well as suppressive (11, 32). We obtained 7-day growth curves for SLHR1 and SMOCK2 cells in the presence or absence of 16.7 nmol/L LH (Fig. 5A and B) and compared the slopes of the growth curves. SLHR1 cells grown in the presence of 16.7 nmol/L LH proliferated significantly slower ($P \leq 0.001$) than vehicle-treated SLHR1 cells (Fig. 5A). LH incubation did not affect the growth rate of SMOCK2 cells (Fig. 5B) and there was no difference between the basal growth rates of SLHR1 and SMOCK2 cells.

The ability of the SLHR1 and SMOCK2 cells to invade through the synthetic basement membrane Matrigel was measured (Fig. 5C). The basal invasive index of SLHR1 cells was 3.4 ± 0.3–fold lower ($P = 0.003$) than SMOCK2 cells, indicating that SLHR1 cells have a significantly reduced invasive potential. Further stimulation of SLHR1 cells or SMOCK2 cells with LH had no effect on invasion (data not shown).

The migratory properties of SLHR1 and SMOCK2 as well as the influence of LH on the migratory properties of these cells were investigated. Serum-starved, confluent cultures were scratched with a pipette tip to mimic a wound and treated for 48 hours with either vehicle, 0.5% fetal bovine serum (FBS), 16.7 nmol/L LH, 1 μmol/L forskolin, or 8BR-cAMP. Although SLHR1 and SMOCK2 basal wound closure were not different, wound closure induced by 0.5% FBS was 1.5-fold greater ($P \leq 0.004$) in SLHR1 cells than in SMOCK2 cells. LH reduced wound closure in only SLHR1 cells ($P \leq 0.0001$), but wound closure was inhibited in both SLHR1 and SMOCK2 cells by forskolin ($P \leq 0.0002$) and the synthetic cAMP analogue 8Br-cAMP ($P \leq 0.0001$).

LHR Expression Results in Changes in Gene Expression

The basal RNA expression levels of 95 genes associated with cell adhesion, invasion, and LHR signaling (Supplementary Table S1) were measured using a real-time expression array

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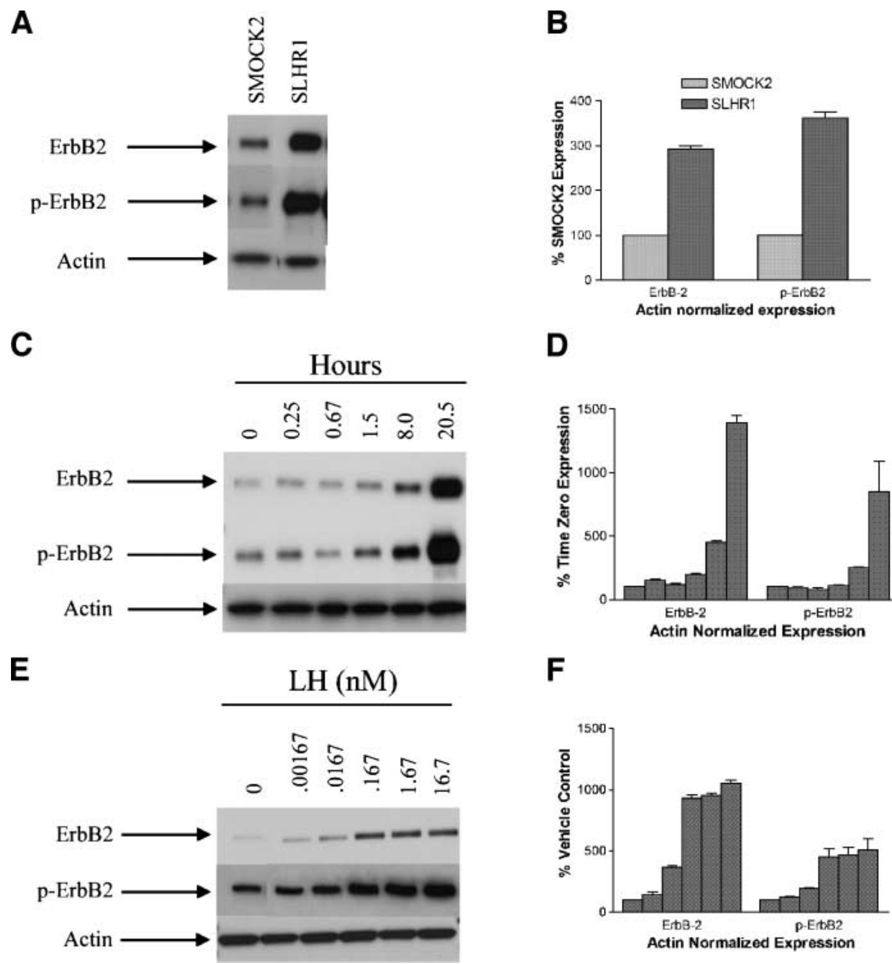

FIGURE 3. LHR/LH regulation of ErbB-2. **A.** Western blot representing basal protein expression levels of ErbB-2 and phospho-ErbB-2 in SLHR1 and SMOCK2. **B.** Graphic representation of ErbB-2 and phospho-ErbB-2 Western blot densitometry readings normalized to actin. **C.** Western blot representing ErbB-2 and phospho-ErbB-2 up-regulation in SLHR1 cells during time course incubation with 16.7 nmol/L LH. **D.** Actin-normalized ErbB-2 and phospho-ErbB-2 Western blot densitometry readings for the time course (**C**). **E.** Western blot representing the dose-dependent up-regulation of ErbB-2 and phospho-ErbB-2 in response to incubating SLHR1 cells with various LH concentrations for 18 h. **F.** Actin-normalized ErbB-2 and phospho-ErbB-2 Western blot densitometry readings for the dose-response curve.

format. Filtering expression data for expression levels lower than the recommended assay detection limit ($C_t \leq 34$) eliminated 42 genes. After calculating the $\Delta\Delta C_t$ values, 31 additional genes were removed by filtering expression data for genes showing less than a 2-fold difference between SLHR1 and SMOCK2. The remaining 22 genes (Fig. 6A) represent genes whose expression at the RNA level is significantly affected by the introduction of functional LHR to the cell line. Overexpression of LHR increased RNA expression of *BRCA1, COL4A3, COL4A3BP, EGFR, LAMC3, matrix metalloproteinase (MMP) 24, SERPINE2,* and the stably transfected *LHCGR*. Down-regulated genes include *IGFBP5, IGFBP6, ITGB3, ITGB6, LAMA3, LAMC2, MMP10, MMP13, MMP1, MMP2, SPARC, SPP1*, and *TIMP3*.

LHR Stimulation in SLHR1 Cells Regulates Gene Expression

The effect of adding LH to LHR-expressing cells on gene expression of 95 genes associated with cell adhesion, invasion, and LHR signaling was determined in a time course stimulation experiment. SLHR1 cells were incubated with or without 13.2 nmol/L LH for 0, 0.5, 5, 24, and 48 hours. The expression array C_t value of untreated cells was subtracted from that of treated cells for each time point and the data were normalized to the zero time point. Genes whose expression was below the recommended lower limit were removed (35 genes), as were genes whose expression did not change more than 2-fold over the stimulation time course (34 genes).

The 26 genes whose expression changed at least 2-fold relative to basal expression for at least one time point are presented in Fig. 6B. Addition of LH to SLHR1 cells resulted in an increase in LHR expression and a decrease in expression of the endogenous hormone-specific subunits CGB (CG β) and LHB (human LH β). LHR-mediated up-regulation during the stimulation time course was observed for *ERBB2, IGF1R, COL4A3, COL4A4, NID2, ITGB8, LAMA3,* and *PLAUR,* whereas down-regulation was observed for *LAMC2, MMP1, ITGB3, PLAU,* and *SERPINE1*.

Discussion

Ovarian cancer is the leading cause of death from gynecologic malignancy in U.S. women (33). The gonadotropin stimulation theory postulates a role for gonadotropic hormones, including LH, in the development of ovarian cancer (34, 35). Epidemiologic evidence correlates physiologic conditions that lead to high gonadotropin levels with an increased risk of ovarian cancer (5). LHR is expressed in the putative precursor cell to ovarian cancer, the ovarian surface epithelium (OSE),

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and in 40% of ovarian cancers (8, 12), although one study correlated a decreased expression of LHR with increasing grade of ovarian cancer (36). LHR is also expressed in other nongonadal tissues, such as brain, skin, and mammary gland (37-39). Whereas the proliferative effects of LH in human OSE are reported to be stimulatory (12, 40), proliferation of ovarian cancer cells is reported to be stimulated (12, 31), inhibited (11), or unaffected by LH or its homologue CG (7). Thus, the experimental systems used to study the biological influence of LHR in ovarian cancer have had a profound effect on the conclusions.

The receptor tyrosine kinase ErbB-2, an EGFR family member, is believed to play a pivotal role in the aggressive clinical course of breast cancer patients whose tumors overexpress the protein (23). In fact, the ErbB-2 antibody treatment Herceptin is clinically effective in treating a subset of breast

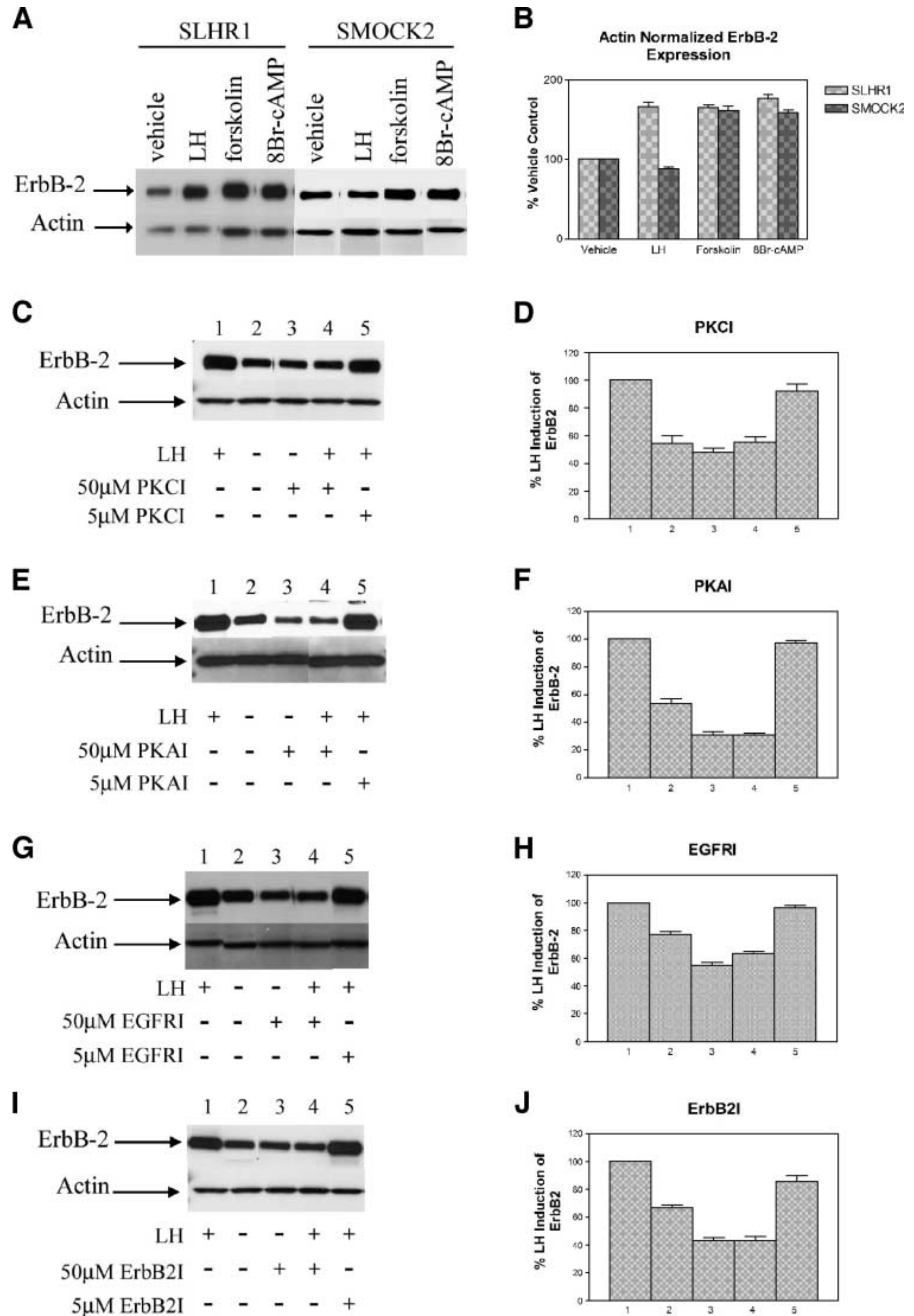

FIGURE 4. LH-induced ErbB-2 expression is a cAMP-mediated event involving PKA, PKC, EGFR, and ErbB-2. **A.** ErbB-2 and actin probed Western blots of total protein from overnight incubations of SLHR1 or SMOCK2 cells in LH, forskolin, or 8Br-cAMP. **B.** Graphic representation of ErbB-2 Western blot densitometry readings normalized to actin. **C, E, G,** and **I.** Western blots of total protein showing inhibition of LH-induced ErbB-2 up-regulation in SLHR1 cells. Cells were preincubated for 1 h with inhibitors of PKC (**C**), PKA (**E**), EGFR kinase (**G**), or ErbB-2 kinase (**I**) and incubated for an additional 18 h with or without LH. **D, F, H,** and **J.** Graphic representation of ErbB-2 Western blot densitometry readings normalized to actin. Cells were preincubated for 1 h with PKC (**D**), PKA (**F**), EGFR kinase (**H**), or ErbB-2 kinase (**J**) and incubated for an additional 18 h with or without LH.

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Table 1. Effects of Inhibitors on LH-Mediated Up-Regulation of ErbB-2 and Phospho-ErbB-2

Target	Inhibitor	Inhibits LH-Induced Up-Regulation of ErbB-2*
PKC	GF109203X	Yes
PKC	Ro-31-8220	Yes
PKA	H-89	Yes
Metalloproteinase	GM 6001	No
PI3K	LY 294002	No
ERK1/2	PD 98059	No
MEK	U0126	No
EGFR kinase	AG-1478	Yes
ErbB-2 kinase	AG-825	Yes

Abbreviations: MEK, mitogen-activated protein/ERK kinase; PI3K, phosphatidylinositol 3-kinase.
*These results are based on Western blots, some of which are shown in Fig. 4.

cancer patients (21). Overexpression of ErbB-2 also correlates with poor outcome in ovarian cancer (41, 42), although antibody therapy for ErbB-2 is not used clinically for the treatment of ovarian cancer. ErbB-2 is expressed in OSE cells (43) in approximately 25% to 30% of ovarian cancer (41, 44, 45) and in cultured ovarian cancer cells, including SKOV3 cells.

SKOV3 cells overexpress ErbB-2 as a result of gene amplification and have been used for several years as a model system for the influence of ErbB-2 overexpression on proliferation and malignancy in ovarian cancer (46, 47). Yu et al. (47) injected SKOV3 cells into immunodeficient mice, isolated a secondary cell line that expressed 2-fold greater levels of ErbB-2 than the parental SKOV3, and correlated this increased ErbB-2 expression with increased malignancy. Furthermore, Hsieh et al. (48) created SKOV3 analogues with a range of reduced ErbB-2 expression and correlated increased ErbB-2 expression with an increase in EGF-mediated proliferation. The model system described here also defines an SKOV3-derived cell line with increased ErbB-2 expression that correlates with the introduction and stimulation of LHR.

In the studies presented here, we introduced the LHR to the LHR-negative, ErbB-2–overexpressing SKOV3 cell line (49) and used this model system to determine the effects of LHR on ErbB-2 expression. The ligand binding and second messenger responses of the model system were carefully defined and confirmed the presence of ~12,000 active cell surface LHRs per SLHR1 cell. Typical LH target cells, granulosa and Leydig cells for example, are reported to express between 2,000 and 60,000 receptors per cell (50-54), so the number of receptors per cell for this model system falls well within the range of reported normal receptor expression. Furthermore, the binding constants (K_d, ~0.3 nmol/L; IC_{50}, ~0.8 nmol/L) determined for SLHR1 cells were similar to those published for LHR in transiently transfected COS7 or HEK293 cells (55-57). The LH-induced cAMP and IP3 responses in SLHR1 cells again confirm that active cell surface LHR is present in SLHR1 cells and that LHR in these cells is capable of signaling through Gs and Gq.

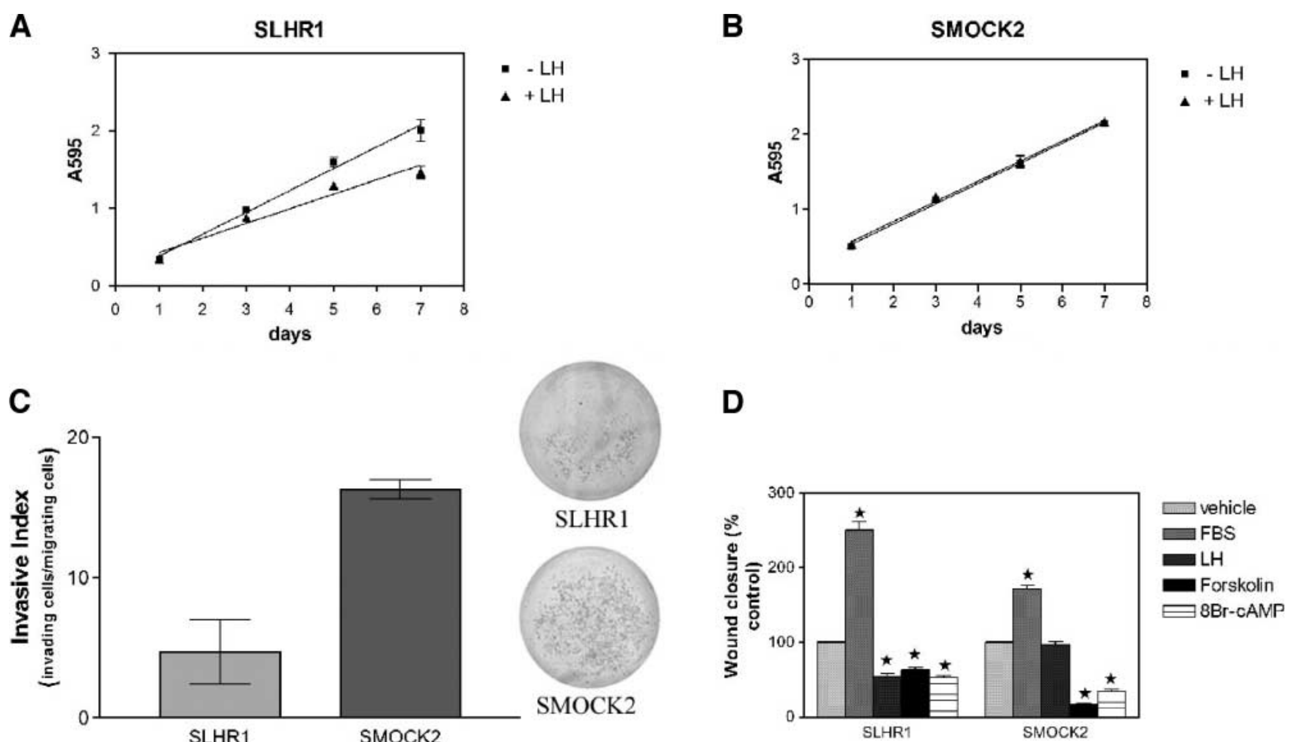

FIGURE 5. Biological consequences of LHR introduction and LH stimulation. **A.** Seven-day growth curve of SLHR1 cells grown in the presence (▲) or absence (■) of 16.7 nmol/L LH revealing an antiproliferative effect of LH ($P \leq 0.001$). **B.** Seven-day growth curve of SMOCK2 cells grown in the presence (▲) or absence (■) of 16.7 nmol/L LH revealing no effect of LH on proliferation in SMOCK2 cells. **C.** Lowered basal invasive index of SLHR1 cells ($P \leq 0.003$). Insets are representative membranes showing invasive cells after incubation for 24 h on Matrigel-covered 8-μm pore membranes. **D.** Reduced wound closure associated with cAMP-mediated events. Bars labeled with a star represent statistically different wound closure compared with control for that cell line.

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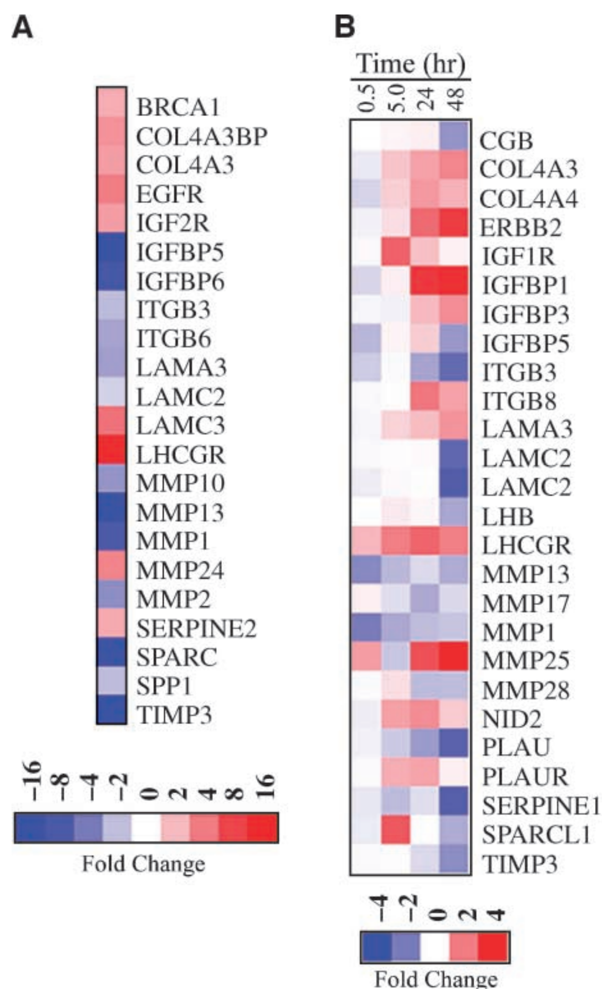

FIGURE 6. Gene expression changes associated with LHR expression and stimulation. **A.** Basal expression changes: RNA from SLHR1 or SMOCK2 cells was converted to cDNA and the expression of 95 genes was assayed by quantitative reverse transcription-PCR using ABI Taqman Expression Arrays. Expression was normalized to SMOCK2. **B.** Expression changes associated with LHR stimulation: SLHR1 cells were incubated in 13 nmol/L LH for 0, 0.5, 5, 24, and 48 h and assayed as in **A**. Expression changes were normalized to basal expression levels (zero time point).

A role for LHR signaling in the regulation of ErbB-2 expression is documented here by the increased SLHR1 basal levels of ErbB-2 protein and the time- and dose-dependent increase in ErbB-2 expression in response to LH. LH incubations longer than 8 hours were required to up-regulate ErbB-2 in SLHR1 cells, suggesting that the induction of transcription factors may be critical to the process. The apparent EC_{50} (32 pmol/L) of LH-induced ErbB-2 up-regulation is consistent with a specific interaction with LHR, characterized by an approximate 0.3 nmol/L K_d of CG binding to SLHR1 cells. The LH-induced up-regulation of ErbB-2 was determined to be a cAMP-mediated event by documenting increased ErbB-2 expression in both SLHR1 and SMOCK2 cells in response to forskolin and 8Br-cAMP. These data indicate that in this model system, introduction of LHR up-regulates ErbB-2, that LH incubation in LHR-expressing cells further up-regulates ErbB-2, and that this up-regulation correlates with cAMP and IP3 accumulation, variables associated with LHR activation. Although some studies have linked FSH receptor overexpression to increased ErbB-2 expression (43) and others have shown an up-regulation of EGFR by both FSH and LH (13), the data presented here represent, to the best of our knowledge, the first demonstration that LHR expression and activation up-regulate ErbB-2 protein expression.

Intracellular phosphorylation cascades activated by G protein–coupled receptors constitute one cellular mechanism for responding to external stimuli and regulating physiologic events (58). To define some of the intracellular phosphorylation pathways involved in LH-induced ErbB-2 up-regulation, the ability of specific kinase inhibitors to block the LH-induced ErbB-2 up-regulation was investigated. Our second messenger accumulation data suggested that LHR in SLHR1 cells signals through Gs and Gq, which lead to the activation of PKA and PKC, respectively (15). Accordingly, the PKA and PKC inhibitors blocked LH-induced ErbB-2 up-regulation in SLHR1 cells. Whereas Gq coupling leads to PKC activation, EGFR signaling also can activate PKC and LH has been shown to cause the release of EGFR ligands in some reproductive cells (19, 59). Based on this reasoning, an EGFR kinase and an ErbB-2 kinase inhibitor were tested and both inhibitors blocked the LH-induced up-regulation of ErbB-2. LH is also known to cause the release of heparin-bound EGF by activating MMPs at the cell surface (3, 58). In our system, however, the broad-spectrum MMP inhibitor was ineffective at blocking LH-induced ErbB-2 up-regulation, suggesting that the activation of protease activities is not involved in the LH-induced up-regulation of ErbB-2.

The kinase inhibitor studies suggest the possibility of three different phosphorylation pathways involved in the LH-induced up-regulation of ErbB-2 protein: the canonical Gs/cAMP/PKA pathway, the Gq-coupled LH activation of PKC, and a third pathway involving the activation of PKC through the release of EGFR ligands. Recently, the viability of the EGFR activation pathway in the ability of LH to activate ERK1/2 has been shown. Using a coculture model, Shiraishi and Ascoli (59) show that the LHR ligand CG stimulates the release of soluble factors, which activate EGFR and subsequently ERK1/2, in an autocrine/paracrine fashion. Although many elements of this pathway, including secretion/release of EGFR ligands and EGFR activation, remain undefined in SLHR1 cells by the data presented here, it certainly remains a viable possible mechanism for the LH-induced ErbB-2 up-regulation based on the EGFR kinase inhibition experiments and the fact that PKC inhibitors also abrogate LH-induced up-regulation of ErbB-2.

Up-regulation of ErbB-2 has been shown to increase proliferation, invasion, and migration in several human cancers (26, 60), and studies in SKOV3 cells document a correlation between increased ErbB-2 protein expression and increased proliferation (47, 48). However, in our SKOV3-based model system coexpressing LHR and ErbB-2, the LH-induced up-regulation of ErbB-2 was insufficient in overcoming the negative effects of LHR on invasion, proliferation, or wound-induced migration and did not correlate with a more aggressive phenotype. The negative effects of LHR on proliferation and invasion documented here are consistent with previous studies (11, 32, 61). Furthermore, in MCF7 human breast cancer cells,

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expression of neuregulin, an ErbB ligand, increased proliferation more than did ErbB-2 overexpression, suggesting that mere overexpression of ErbB-2 does not create a dominant proliferative phenotype (62).

Molecular evidence supporting a decreased invasive phenotype of SLHR1 cells is provided by our gene expression studies. With the exception of *MMP24,* decreased basal expression was shown for all MMPs on the array. In addition, stimulation of the receptor decreased expression of *PLAU,* the enzyme that converts plasminogen to the active protease plasmin. These data suggest a lowered proteolytic potential for the LHR-expressing cells and thus a lower invasive potential. Confirmation of these expression changes at the protein level, especially for *MMP25,* is needed to establish the lower proteolysis associated with these cells.

Increased gene expression of basement membrane components and cell adhesion molecules (e.g., *COL4A4, NID2, ITGB8,* and *LAMA3*) was also detected in our LHR-expressing cells and supports a decreased migratory phenotype of SLHR1 cells. One role of normal OSE is to contribute to the repair of the wound created by ovulation through synthesis of basement membrane components (4). Although the increased basement membrane and adhesion molecule expression that we noted may represent an invocation of normal OSE function, it may also contribute to a less invasive phenotype by favoring cell-cell interactions over cell migration in these ovarian cancer cells.

In summary, the studies presented here elucidate a role for LHR in the regulation of ErbB-2 protein expression. We show that LHR introduction and stimulation with LH up-regulates ErbB-2 expression in an ovarian cancer cell model coexpressing LHR and ErbB-2. ErbB-2 up-regulation in this model was shown to be mediated by cAMP, and involve PKA-mediated, PKC-mediated, EGFR-mediated, and ErbB-2–mediated mechanisms. The LH-induced up-regulation of ErbB-2 was insufficient to overcome the negative effects of LH on proliferation, invasion, and migration in this model system. Evidence for this phenotype is offered in the lowered MMP expression and increased expression of adhesion molecules exhibited by SLHR1 cells. Further studies should determine the predominant intracellular signaling mechanism of LH-induced up-regulation and may reveal additional cell types or systems in which LH regulates ErbB-2. Further investigation is also needed to determine what additional factors or conditions, if any, will stimulate a more aggressive phenotype in this LHR/ErbB-2 coexpression system.

Materials and Methods

Materials

Human LH and CG were obtained from Dr. A.F. Parlow (National Hormone and Peptide Program, Torrance, CA). 8Br-cAMP was obtained from Sigma Chemical Co. The inhibitors and selection agents, including AG825, GF 109203X, G-418 Sulfate, H-89, PD 98059, RO-31-8220, AG-1478, GM6001, LY 294002, and U0126, were obtained from EMD Biosciences.

Cell Culture and Stable Lines

SKOV3 ovarian cancer cells (American Type Culture Collection) were maintained in DMEM (Life Technologies) containing 5% FBS (Hyclone), 10 mmol/L HEPES (Sigma), and antibiotics/antimycotics (1,000 units/mL penicillin, 1 mg/mL streptomycin, and 2.5 μg/mL amphotericin B; Sigma). Cells were subcultured weekly, at a 1:6 ratio, by harvesting with trypsin (0.05%) containing 0.02% EDTA (Sigma). Stable hLHR-pcDNA3 and empty vector transformants of SKOV3 cells were generated by incubating transiently transformed cells in selection medium for 3 wk. SKOV3 cells (70% confluent, six-well plates) were transiently transfected in the presence of serum with 0.5 μg plasmid DNA using Lipofectamine (Invitrogen) according to the manufacturer's protocols. Two days after transfection, the cultures were split 1:100 into selection medium [DMEM, 5% FBS, 10 mmol/L HEPES, antibiotics, and 1 mg/mL geneticin (Life Technologies)] and allowed to grow for 3 wk, replacing medium every 5 d. Surviving colonies were isolated via cloning cylinders and the cultures were expanded.

RNA Isolation, cDNA Synthesis, and Taqman Arrays

Confluent cultures were harvested in Trizol (Molecular Research Corp.), and crude total RNA was isolated according to the manufacturer's protocols. The crude RNA was DNase treated (RQ1-DNase, Promega) for 30 min at 37°C and further purified over RNeasy columns (Qiagen). Purified RNA was quantified by UV spectrometry and the quality was assessed by gel electrophoresis. Purified RNA (1 μg) was converted to cDNA using random primers and reverse transcriptase (High-Capacity cDNA Archive kit, Applied Biosystems, Inc.) in a 40 μL total reaction volume. cDNA from this reaction was used directly for first-round PCRs or Taqman Low Density Arrays (Applied Biosystems). Control cDNA synthesis reactions without reverse transcriptase enzyme were used to confirm the removal of DNA from the purified RNA. The internal control for Taqman Low Density Arrays was 18S RNA. Each assay appeared in duplicate on the array and arrays were done twice on each sample. Data were analyzed according to published methods (63). The C_t values of the internal control were subtracted from the C_t value of the gene of interest to obtain the ΔC_t value for each sample. When comparing the basal expression of SLHR1 and SMOCK2 cells, the $\Delta\Delta C_t$ value represents the difference between the SLHR1 and SMOCK2 gene of interest ΔC_t values. When analyzing the LH stimulation time course in SLHR1 cells, the $\Delta\Delta C_t$ value represents the difference between the ΔC_t value at time zero and the ΔC_t value following LH incubation. $\Delta\Delta C_t$ values were visualized using TreeView.[1]

PCR

Nested PCR (iTAQ DNA Polymerase, Bio-Rad, Inc.) was used to show the presence of hLHR transcripts in the cell lines. A standard 25 μL PCR contained 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each deoxynucleotide triphosphate, 0.5 unit enzyme, 200 nmol/L each primer, and 4 μL template DNA. The first round of amplification contained cDNA, representing 25 ng total RNA, as template and amplified the 2,000-nucleotide

[1] http://rana.lbl.gov/EisenSoftware.htm

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product between primers hLHR-97F (CCCTGAGCCCTG-CAACTG, exon 1) and hLHR-2097R (CTCTCACTTGACT-CACCGAC, exon 11). The reaction was run for 40 cycles with a 65°C annealing temperature and a 2-min, 10-s product extension time. The second round of amplification contained 4 μL of round one PCR product as template DNA and amplified the 700-nucleotide product between primers hLHR-297F (ATGCCTTTGACAACCTCCTC, exon 3) and hLHR-1008R (GAGAGTGAACTGAGTGGCTG, exon 11). The second round was also run for 40 cycles with a 65°C annealing temperature and a 55-s extension time. The product size was determined by gel electrophoresis. The hLHR fragment amplified from SLHR1 cell line was cloned into the TOPO-TA (Invitrogen) vector and sequenced to verify that the PCR product represents that of the hLHR transcript. Amplification of the housekeeping gene *GAPDH* [GAPDH-287F: GAAATCCCATCACCATCTTCCAG (exon 5); GAPDH-599R: CTTTGGTATCGTGGAAGGACTCAT (exon 7)] was done on the cDNA to confirm equal loading of RNA into the cDNA synthesis reaction and to confirm the absence of DNA from the purified RNA. The hLHR-pcDNA3 plasmid and distilled water served as positive and negative controls for the PCRs, respectively.

Cell Culture Growth Curves

Confluent cultures were harvested and the collected cells were plated in 96-well plates at a density of 5,000 per well. The following day, medium was replaced in all wells and either PBS (Life Technologies) or LH (16.7 nmol/L) was added to the cells. Cell numbers were estimated in triplicate wells every other day for 7 d using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Medium was replaced every other day to ensure continuous hormone availability. Statistical analysis was done on triplicate experiments. A linear regression and statistical analysis of the growth curves was done in Prism (version 3.02 for Windows; GraphPad Software) and the resulting slope represented the growth rate of the cell culture. A two-way ANOVA analysis was done using the variables of time and treatment.

Invasion Assay

Cell cultures were maintained for 2 d in low growth medium [phenol red–free DMEM (Sigma), 5% heat-inactivated, dextran-coated charcoal-stripped FBS (Hyclone), 10 mmol/L HEPES, and antibiotics as described above] to withdraw hormones and growth factors. The withdrawn cells (10,000) were plated in low growth medium on rehydrated 24-well BD Biocoat Matrigel Invasion Chambers (BD Biosciences). The bottom wells were supplemented with 5% FBS to establish chemotaxis across the invasion chamber. Migration assays were done using control membranes without Matrigel (Fisher) plated with serial dilutions of the hormone-withdrawn cells. After incubation at 37°C (48 h for invasion, 18 h for control membranes), cells remaining on the top of membrane were gently scraped off with a cotton swab. Cells were fixed to the membrane in ice-cold methanol (2 min) and stained for 2 min (0.25% crystal violet in 25% methanol). Whole membranes were scanned on an Epson Perfection 3170 Scanner and cells were counted using ImageJ 1.34s software.[2] Invasion results are presented as the invasive index, the number of invading cells divided by the number of migrating cells. Statistical analysis was done on triplicate experiments. A t test was done with Prism (version 3.02 for Windows).

Hormone Binding Assays

CG was radioiodinated using the Iodo-Gen iodination kit from Pierce Biotechnology, Inc. with $Na^{125}I$ from Amersham Pharmacia Biotechnology, Inc. For competitive binding studies, confluent six-well plates were washed twice with binding buffer (278 mmol/L sucrose, 0.1% glucose, 5 mmol/L HEPES, 5 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1 mmol/L $NaHCO_3$, 1 mmol/L $CaCl_2.2H_2O$, and 1.2 mmol/L KH_2PO_4, containing 0.1% bovine serum albumin) and incubated with 250 pmol/L ^{125}I-CG for 5 h at 37°C containing varying concentrations of unlabeled competitor CG (Sigma). Medium was aspirated and the cells were washed twice with PBS and harvested in 1 N NaOH. Wells were washed with PBS and pooled with the cell harvest. Raw counts were measured on a Wallac 1470 Wizard Gamma Counter. For saturation binding studies, confluent six-well plates were washed twice with binding buffer and incubated for 5 h at 37°C with varying concentrations of ^{125}I-CG in the presence (nonspecific binding) or absence (total binding) of 1 μg/mL unlabeled CG. Medium was aspirated and the cells were washed twice with PBS and harvested in 1 N NaOH. Wells were washed with PBS and pooled with the cell harvest. Raw counts were measured on a Wallac 1470 Wizard Gamma Counter. Data from triplicate experiments were analyzed and binding variables were determined in Prism (version 3.02 for Windows).

cAMP Signaling Assay

Confluent six-well cultures were washed twice in binding buffer (see Hormone Binding Assay methods above) containing 0.1% bovine serum albumin and incubated for 30 min in binding buffer containing 0.8 mmol/L isobutylmethylxanthine (Sigma). The medium was replaced with the binding buffer/bovine serum albumin/isobutylmethylxanthine medium containing various concentrations of LH and cultures were incubated for 30 min at 37°C. Medium was aspirated and accumulated cAMP was recovered by lysing cells in ice-cold ethanol for 30 min. The ethanol-soluble fraction was transferred to 1.5 mL centrifuge tubes and dried in a SpeedVac, and the cAMP levels were determined by RIA (Perkin-Elmer Life Sciences). Data from triplicate experiments were analyzed in Prism (version 3.02 for Windows).

Inositol Phosphate Signaling Assay

Confluent cultures (six-well plates) were incubated overnight in inositol-free, serum-free DMEM (Specialty Medium, Inc) containing 2.5 μCi/mL *myo*-[1,2-3H(N)]inositol (American Radiochemicals, Inc). The following day, the inositol-labeled cells were washed with inositol-free, serum-free DMEM and incubated for 30 min at 37°C in the above

[2] http://rsb.info.nih.gov/ij/

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medium containing 10 mmol/L LiCl. Cells were stimulated with hormone for an additional 30 min at 37°C in the LiCl medium. To assay for inositol phosphate accumulation, cells were disrupted and harvested by incubating in ice-cold 50 mmol/L formic acid for 15 min at 4°C. Samples were neutralized with 150 mmol/L ammonium hydroxide and the inositol phosphate derivatives were isolated by ion exchange chromatography. Data from triplicate experiments were analyzed in Prism (version 3.02 for Windows).

Protein Isolation and Western Blot

Cultures were scraped into ice-cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (Santa Cruz Biotechnology). Collected cells were incubated on ice for 60 min and DNA was sheared by drawing the lysate through a 21-gauge needle. The lysate was cleared by centrifugation at 14,000 × g, 4°C for 10 min. The pellet was discarded and the protein concentration of the supernatant was determined with the bicinchoninic acid protein assay (Pierce Biotechnology). Total cellular protein (10-20 μg) was resolved by gel electrophoresis on a 4% to 20% polyacrylamide gel, transferred to polyvinylidene difluoride, and immunoblotted using standard methods. Membranes were blocked (2% bovine serum albumin in PBS) for 2 h at room temperature and incubated overnight with dilutions of primary antibody in blocking solution. The antibodies for actin, ErbB-2, or phospho-ErbB-2 (Santa Cruz Biotechnology) were diluted 1:500 and 1:1,000, respectively. Membranes were incubated in secondary antibody and specific antibody-protein interactions were visualized using chemiluminescence detection (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer Life Sciences). For inhibitor studies, SLHR1 cells were preincubated with inhibitor at two concentrations, 5 and 50 μmol/L, for 1 h and for an additional 24 h with inhibitor plus LH (1.67 nmol/L) before protein isolation. Densitometry information on specific protein bands was obtained by applying the Gel Analysis function of the ImageJ 1.34s software to scanned Western images. The data presented in figures are representative Westerns of duplicate experiments.

Wound Healing Migration Assay

Cells were plated into 24-well dishes at a density of 10,000 per well. After reaching confluency, cells were withdrawn from hormones by incubating overnight in serum-free medium. The following morning, each well was scratched with a pipette tip to remove cells and mimic a wound area. The medium was replaced with serum-free medium with or without added FBS, hormone, forskolin, or 8Br-cAMP, and a digital picture of each well was recorded. At 24 h after the initial dosing, medium and treatments were refreshed, and at 24 and 48 h after initial dosing, pictures were recorded. Each migration experiments contained duplicate wells for each treatment and each experiment was repeated thrice. The area of the wound was determined using ImageJ 1.34s software and the wound closure was calculated. Results were normalized to wound closure in serum-free medium and the results presented were analyzed in and graphed in Prism (version 3.02 for Windows).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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