Published in final edited form as:

Exp Cell Res. 2008 January 1; 314(1): 25–37.

A co-culture system reveals the involvement of intercellular pathways as mediators of the lutropin receptor (LHR)-stimulated ERK1/2 phosphorylation in Leydig cells

Koji Shiraishi and Mario Ascoli

Department of Pharmacology, Carver College of Medicine, The University of Iowa, Iowa City, IA, 52242

Abstract

Co-cultures of lutropin receptor (LHR) positive and negative Leydig cells were used to test the hypothesis that the LHR provokes phosphorylation of the extracellular regulated kinases (ERK1/2) using intracellular and intercellular pathways. Addition of hCG to MA-10 cells (LHR positive) stimulates phosphorylation of the EGF receptor (EGFR) and ERK1/2 whereas addition of hCG to I-10 cells (LHR negative) does not. Addition of hCG to co-cultures of MA-10 and I-10 cells rapidly stimulates the phosphorylation of the EGFR and ERK1/2 in I-10 cells, however. Transfection of interfering constructs show that the LHR-mediated activation of Fyn in MA-10 cells is necessary for the phosphorylation of the EGFR and ERK1/2 in I-10 cells. This pathway can also be demonstrated in MA-10 cells but the phosphorylation of ERK1/2 in MA-10 cells also involves a second pathway mediated by protein kinase A (PKA). We propose that the LHR-mediated stimulation of the ERK1/2 cascade in Leydig cells depends on two independent pathways. One is intracellular and is mediated by PKA. The second is mediated by Fyn and it involves the release of soluble factors that act to phosphorylate the EGFR in an autocrine/paracrine fashion.

Keywords

Leydig cells; Lutropin receptor; G protein coupled receptors; Epidermal growth factor; Extracellular regulated kinases; Src family kinases; Cell culture; Co-culture; Autocrine signaling; Paracrine signaling

Introduction

Lutropin (LH) and choriogonadotropin (CG) are homologous pituitary and placental hormones, respectively, that modulate the proliferative and differentiated functions of testicular Leydig cells by engaging the LHR [reviewed in refs. 1-3]. The LHR is a G protein-coupled receptor (GPCR) that activates the G_s , $G_{i/o}$ and $G_{q/11}$ families of G proteins [4] but it is generally agreed that its ability to modulate the differentiated functions of Leydig cells is mediated by the activation of the G_s /cAMP/protein kinase A (PKA) pathway [1-3].

Address correspondence to: Mario Ascoli, Ph.D., Department of Pharmacology, 2-319B BSB, 51 Newton Road, The University of Iowa, Iowa City, IA 52242, Email mario-ascoli@uiowa.edu, Voice 319-335-9907, Fax 319-335-8930.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Recent studies from this and other laboratories have also shown that activation of the LHR in primary cultures of rat Leydig cells or MA-10 Leydig tumor cells leads to an increase in the phosphorylation of ERK1/2 [5-8]. Measurements of Ras activation, expression of dominant-negative mutants of Ras and addition of pharmacological inhibitors of MEK have shown that the LHR-induced activation of the ERK1/2 cascade occurs through the classical Ras/Raf/MEK pathway [5,7]. The LHR-provoked activation of Ras seems to involve the Gs/cAMP/PKA pathway because dominant-negative constructs and pharmacological inhibitors of this pathway interfere with Ras activation and ERK1/2 phosphorylation [5,6,8]. We have also shown, however that the LHR activates Fyn [9], induces the tyrosine phosphorylation of the EGFR, the tyrosine phosphorylation of Shc and the formation of complexes containing Shc and Sos [7]. Although the activation of these tyrosine kinase cascades seems to be independent of the Gs/cAMP/PKA pathway, they also contribute to the LHR-provoked activation of Ras and the phosphorylation of ERK1/2 because these can be inhibited with dominant-negative constructs or pharmacological inhibitors of Fyn or the EGFR [7,8].

It has become recently clear that agonist binding to some GPCRs (notably those coupled to $G_{i/o}$ and $G_{q/11}$) results in the stimulation of certain metalloproteases that cleave the precursor forms of EGF-like growth factors thus releasing the mature, soluble forms that subsequently bind to and activate the EGFR in the same cell or in neighboring cells [10-15]. Since in some cases this pathway is mediated by a GPCR-induced activation of the Src family of kinases [10-15] it is possible that the LHR-induced activation of Fyn can provoke the release of mature, soluble forms of EGF-like factors that subsequently bind to and activate the EGFR leading to the activation of Ras and the ERK1/2 cascade. On the other hand Src family kinases can also directly phosphorylate the EGFR and Shc [14,16-19] and the LHR-activated Fyn could more directly lead to the activation of Ras and the ERK1/2 cascade in this fashion. Lastly, it is not known if these tyrosine kinase cascades and PKA activate Ras and the ERK1/2 cascade in a coordinate or independent fashion.

Here we use a Leydig cell co-culture system that allows for the detection of intracellular and intercellular signaling pathways leading to the phosphorylation of the EGFR and ERK1/2. Using this system we show that the LHR mediated, Fyn-dependent activation of the ERK1/2 cascade occurs through a pathway that involves the intercellular activation of the EGFR. This experimental paradigm also confirms the existence of a previously described LHR-mediated activation of the ERK1/2 cascade that is intracellular and mediated by PKA but is Fyn-independent [5].

Materials and Methods

Plasmids and cells

The expression vector coding for the hLHR-wt modified with the myc-epitope at the N-terminus has been described [20]. Expression vectors for a dominant-negative mutant of human Fyn (i.e., kinase-deficient mutant, K229M) [21] and human Shc (i.e., mutation of tyrosine residues 239, 240 and 317 to phenylalanine) [22] were provided by Dr. Marilyn Resh of the Memorial Sloan Kettering Cancer Center and Dr. Kodi Ravichandran of the University of Virginia, respectively. The expression vector for rat cAMP phosphodiesterase 4D3 [23] was kindly donated by Dr. Marco Conti (Stanford University), and the expression vector for PKI [24] was donated by Dr. Richard Maurer (Oregon Health Sciences University). The PKI was modified with a myc epitope at the C-terminus to facilitate detection of the transfected product. Expression vectors for N-terminally tagged myc-ERK2 and C-terminally tagged EGFR-GFP and ERK2-GFP were donated by Drs. John Koland and Stefan Strack of this institution.

A siRNA duplex corresponding to the 2170-2188 region of the mouse EGFR mRNA (5'-GCAUUUGGCACAGUGUAUA-3') and a control siRNA duplex (5'-

CGUCUACUCCUAGGACGUA-3') that does not target any gene in the mouse genome were designed using the BLOCK-iT™ RNAi Designer Web site from Invitrogen (<https://rnaidesigner.invitrogen.com/sirna>). These synthetic oligonucleotide duplexes were purchased from Invitrogen. The sequence of the control siRNA duplex was derived by scrambling the sequence of a siRNA that targets the alpha subunit of Gs.

The origin and handling of MA-10 cells were as described earlier [25] with recent modifications [20]. I-10 cells (CCL-83), a clonal strain of Leydig tumor cells that lack LHR [26,27], were purchased from the American Type Culture Collection and maintained using the same growth medium used for MA-10 cells (RPMI-1640 medium supplemented with 15% horse serum, 20 mM Hepes and 50 µg/ml gentamicin, pH 7.4).

Transfections, co-cultures and transwell cultures

MA-10 cells and I-10 cells were plated individually in 100 mm dishes at a density of 3×10^6 cells/dish and transfected one day after plating. Transfections were done in 5 ml of OPTIMEM® supplemented with 700 µg/ml $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Each dish was transfected with a total of 10 µg of plasmid (see below) and 40 µl of Lipofectamine® [20]. After a three-hour incubation with the Lipofectamine/DNA mixture each dish received horse serum to give a final concentration of 15% (to stop the transfection) and the incubation was continued for another 16-24 hours. The transfection efficiency under these conditions is about 25% [20].

Routine transfections of MA-10 cells included the following amounts of plasmids: 5 µg of the myc-hLHR, 2.5 µg of myc-ERK2 and 2.5 µg of empty vector (EV = pEF-1, the expression vector used for the myc-hLR). Routine transfections of I-10 cells included the following amounts of plasmids: 3.75 µg of ERK2-GFP, 3.75 µg of EGFR-GFP and 2.5 µg of empty vector. Some transfection mixtures (see Figure 7) also included 3.75 µg of DN-Fyn, cAMP phosphodiesterase (PDE), the PKA inhibitor (PKI) or DN-Shc. When these plasmids were used in MA-10 cells we did not include an empty vector and we dropped the level of myc-ERK2 to 1.25 µg. When these plasmids were used in I-10 cells we did not include the empty vector and we dropped the level of ERK2-GFP to 2.5 µg.

The transfected MA-10 and I-10 cells were trypsinized and co-cultured in 6 well plates (35 mm wells) by plating them simultaneously in the same wells at a density of 2×10^5 and 3×10^5 cells/well, respectively¹. After plating the co-cultures were maintained in 2 ml of growth medium for 16-18 hours. The medium was then replaced with assay medium (RPMI-1640 medium supplemented with 1 mg/ml bovine serum albumin, 20 mM Hepes and 50 µg/ml gentamicin, pH 7.4) and the cells were incubated in this medium for another 6 hours. The medium was then replaced with 1 ml of fresh assay medium and hormones and other compounds were added as indicated in the figure legends.

For the transwell cultures (Figure 6) the MA-10 and I-10 cells were transfected as described above. The transfected I-10 cells were trypsinized and plated independently in 6 well plates (35 mm wells) at a density of 6×10^5 cells/well and the transfected MA-10 cells were trypsinized and plated in 24 mm collagen-coated microporous inserts with a 0.4 µm pore size at a density of 4×10^5 cells/insert. The two cell populations were cultured independently for 16-18 h each in a total volume of 2 ml of growth medium. The medium was then replaced with 2 ml of assay medium (see above) and the cells were incubated in this medium for another 6 hours. Finally the inserts (containing the transfected MA-10 cells) were placed in the wells containing the transfected I-10 cells and each well/insert pair received 2 ml of fresh assay medium just prior to the addition of hormones.

¹Changing the ratio of cells plated did not appreciably affect the results presented.

Experiments involving the siRNA silencing of the EGFR were done by transfecting cultures of MA-10 cells (in 6 well plates) in a total volume of 1 ml of OPTMEM® (see above) with a mixture of 1 µg of the expression vector for the hLHR and 1 µg (~50 pmol) of the control siRNA or EGFR-siRNA and 8 µl of Lipofectamine® as described above. One day after the transfection the cells were incubated in assay medium for 16-18 hours and the medium was replaced with 1 ml of assay medium just prior to hormone addition.

Western blots

At the end of the stimulation period the medium was aspirated from the wells containing the co-cultures and the cells were lysed with 100 µl of RIPA buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) supplemented with an EDTA-free protease inhibitor cocktail from Roche Applied Science, 1 mM NaF and 1 mM sodium orthovanadate. The resulting lysates were clarified by centrifugation and assayed for protein content using the BCA protein assay kit from Bio-Rad Laboratories. Equal amounts of protein from each lysate (10-30 µg) were then resolved on 7.5% or 10% SDS-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes [20]. Most experiments involved measuring only the phosphorylation of ERK1/2 or the EGFR. For ERK1/2 phosphorylation assays the blots were first incubated overnight at room temperature with a 1:5,000 dilution of a phosphoERK1/2 antibody (from Santa Cruz Biotechnology, catalog # 7383). After detection of the phospho ERK signal the blots were stripped and incubated with a 1:2,000 of a rabbit anti ERK antibody (from Santa Cruz Biotechnology, cat # sc 154) during an overnight incubation at room temperature. For EGFR phosphorylation assays the blots were first incubated for 1 h at room temperature with a 1:1,000 dilution of a rabbit antibody to phosphotyrosine 1068 of the EGFR (Cell Signaling, catalog # 2234). After detection of the phospho EGFR signal the blots were stripped and incubated with a 1:1,000 of a goat anti GFP antibody coupled to horseradish peroxidase (from Abcam, cat # ab6663-1000) during an overnight incubation at room temperature. This was possible because the EGFR expressed in the transfected cells is tagged with GFP (see above). The EGFR blot shown in Figure 8 was developed using an antibody to the intracellular domain of the EGFR as described earlier [7].

All primary antibody incubations were followed by a second 1 h incubation with a 1:3,000 dilution of a secondary antibody covalently coupled to horseradish peroxidase (from Bio-Rad Laboratories). All immune complexes in the Western blots were visualized using the Super Signal West Femto Maximum Sensitivity detection system (Pierce Chemical) and exposed to film or captured digitally with a Kodak Digital Imaging system (Eastman Kodak). The quantitative analysis presented was done with the Digital Imaging system. Quantitation of the digital images captured using this system is more accurate because of its wider dynamic range that makes signal saturation less likely. In addition, the software included with this imaging system can readily determine if an image is saturated thus preventing its quantitation. All phosphorylation data for ERK and the EGFR were corrected for the amount of ERK or EGFR present in the blots determined after stripping and reprobing the blots as described above.

In some experiments MA-10 or I-10 cells were transfected with DN-Fyn, Dn-Shc, PDE or PKI (Figures 7) prior to co-culture. In these experiments we ascertained that the transfected vectors were expressed in equivalent amounts in both cell types by Western blot analysis of individual cell lysates prepared just prior to co-culture (data not shown). The expression of the transfected DN-Fyn and Dn-Shc were detected as described earlier [7, 9]. The PKI construct is tagged with the myc-epitope at the C-terminus and it was detected using the 9E10 antibody as described earlier for the myc-tagged hLHR [28]. Since we do not have an antibody to PDE the expression of this construct was ascertained using cholera toxin-stimulated cAMP accumulation as an index. For these assays the MA-10 or I-10 cells transfected with or without the PDE expression vector were incubated with cholera toxin for 4 hours (in the absence of phosphodiesterase

inhibitors) as described earlier [5]. The cholera toxin-induced cAMP response in both cell types was reduced by ~50% when either cell type was transfected with PDE (data not shown).

Lastly, it should be noted that co-transfection of multiple constructs tagged with the myc epitope such as hLHR, (Mr ~ 85,000) ERK2 (Mr ~ 42,000) and PKI (Mr ~ 9,000) or two constructs tagged with GFP such as ERK2 (Mr ~ 72,000) and the EGFR (Mr ~ 210,000) does not complicate detection of the expression of the individual constructs because they have different molecular weights and can be readily separated on the gels prior to the Western blot analysis.

Hormone binding and cAMP accumulation assays

Binding assays were done during a one hour incubation at room temperature with 100 ng/ml of ¹²⁵I-hCG with or without an excess of non-radioactive hCG (to correct for non-specific binding) as described elsewhere [20]. Cyclic AMP was measured by radioimmunoassay using reagents prepared in our own laboratory. For these assays the cells were incubated with or without hCG (100 ng/ml) or cholera toxin (500 ng/ml) for 4 hours in the presence of 1 mM isobutylmethylxanthine (to inhibit cAMP phosphodiesterases) as described elsewhere [20]. The concentrations of hCG and cholera toxin chosen are known to be maximally effective in increasing cAMP accumulation.

Hormones and supplies

Purified hCG (CR-127, ~13,000 IU/mg) was purchased from Dr. A. Parlow and the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases and purified recombinant hCG² was provided by Ares Serono (Randolph, MA). Cell culture media was obtained from Invitrogen. Other cell culture supplies and reagents were obtained from Corning. Recombinant EGF was from Sigma. All other chemicals were obtained from commonly used suppliers.

Results

The use of a MA-10/I-10 co-culture system allows for detection of hCG-induced soluble, diffusible factors that act on neighboring cells to activate the ERK1/2 cascade

We modified a co-culture system initially described by Lefkowitz and colleagues to characterize the role of EGF-like factors in the adrenergic receptor-mediated phosphorylation of ERK1/2 in COS7 cells [12]. Our modification (Figure 1) involves the use of MA-10 cells, a well characterized and popular mouse Leydig tumor cell line that express the endogenous LHR [20,25,27] and I-10 cells, another mouse Leydig tumor cell line that do not express the LHR [27,29].

MA-10 cells express low levels of endogenous LHR (1,000-2,000 receptors/cell) that activate all classical LHR responses [20,25,27]. In the current experiments we used MA-10 cells transiently transfected with the hLHR to a density of ~100,000 receptors/cell to amplify the magnitude of the LHR-mediated responses as previously described [20]. These cells were also transiently transfected with myc-ERK2. Similarly, I-10 cells were transiently transfected with ERK2-GFP and EGFR-GFP. The individually transfected cell lines were then co-cultured, stimulated with hCG and Western blots of the co-cultures were analyzed for phosphorylation of myc-ERK2 (present only in MA-10 cells), ERK2-GFP and EGFR-GFP (present only in I-10 cells). Under these conditions any hCG-induced changes in the phosphorylation of ERK2-GFP

²Both preparations were used in this study and were found to be indistinguishable

and EGFR-GFP detected in the co-cultures would have to be indirectly mediated by the actions of hCG on MA-10 cells because I-10 cells do not express the LHR (Figure 1).

We first tested the responsiveness of the cells that were not co-cultured (henceforth referred to as monocultures) to ensure that they displayed the expected phenotypes (Figure 2). MA-10 cell monocultures bound ^{125}I -hCG and responded to hCG with a robust increase in cAMP accumulation [a classical LHR-mediated response, see ref. 1] but I-10 cell monocultures did not. Both cell types have an active Gs/adenylyl cyclase system, however, as documented by the ability of cholera toxin to stimulate cAMP accumulation in each of the monocultures.

We next characterized the phosphorylation of ERK1/2 and the EGFR in monocultures of MA-10 and I-10 cells as shown in Figures 3 and 4. The endogenous ERK1/2, the transfected myc-ERK2 and the endogenous EGFR all became phosphorylated when MA-10 cell monocultures were stimulated with hCG or EGF [Figure 3A and B and refs. 5, 7]. At maximally effective concentrations, EGF and hCG induced a 6- to 10-fold increase³ in the phosphorylation of the endogenous ERK1/2 or the transfected myc-ERK2 (Figure 3A). In contrast, the endogenous EGFR expressed in MA-10 cells could be only weakly phosphorylated in response to hCG stimulation (1.5- to 2-fold increase) and strongly (50- to 100-fold increase) phosphorylated in response to EGF stimulation [Figure 3B and ref. 7]. Monocultures of I-10 cells were unresponsive to hCG stimulation but they readily responded to EGF with an increase in the phosphorylation of the endogenous ERK1/2, as well as the transfected ERK2-GFP and EGFR-GFP (Figure 3C-E). The extent of phosphorylation of the transfected products was higher than the endogenous proteins in I-10 monocultures stimulated with EGF (Figures 3C and 3D). In fact, the EGF-induced phosphorylation of the endogenous EGFR in I-10 cells transfected with EGFR-GFP was difficult to detect because of the intensity of the signal generated by the EGFR-GFP (Figure 3D). Longer exposure of these blots more clearly showed that both proteins become phosphorylated, however (Figure 3E).

Lastly it is important to note that although the antibodies used in these and subsequent experiments could not distinguish between the endogenous and transfected proteins, the transfected myc-ERK2, ERK2-GFP and EGFR-GFP were initially identified by detection with myc or GFP antibodies (data not shown). Since all transfected constructs could be readily separated from the endogenous products the quantitation of the phosphorylation of the transfected products was routinely done using simple Western blots such as those shown in Figures 3 and 4.

Cyclic AMP analogs can also induce ERK1/2 phosphorylation in Leydig cells [5,7,8]. Although this phenomenon has been documented in MA-10 cells or primary cultures of Leydig cells [5-8] it was important to ensure that ERK phosphorylation in the LHR negative Leydig cells (I-10) is also sensitive to cAMP analogs. The results presented in Figure 4 show that addition of 8-CPT-cAMP to monocultures of MA-10 or I-10 cells resulted in robust phosphorylation of the endogenous and transfected ERKs. In Figure 4 we also show that the transfected and endogenous ERKs are expressed at similar levels and that the total levels of transfected or endogenous ERKs do not change during the course of the experiments.

When considered together these results document that MA-10 and I-10 cells display similar ERK phosphorylation responses to EGF and to cAMP analogs and a similar EGFR phosphorylation response to EGF. In contrast to MA-10 cells, however, I-10 cells do not respond to hCG with an increase in EGFR or ERK phosphorylation.

³All data on ERK or EGFR phosphorylation included here were quantitated following normalization for the levels of total ERK or EGFR as described in Materials and Methods and in the figure legends.

The results of a typical experiment testing for ERK and EGFR phosphorylation in co-cultures of MA-10 and I-10 cells stimulated with hCG are shown in Figure 5. These data show that addition of hCG quickly stimulated the phosphorylation of myc-ERK2 (which is present only in MA-10 cells) as well as the phosphorylation of ERK2-GFP and EGFR-GFP (which are present only in I-10 cells). Addition of an optimal concentration of hCG (100 ng/ml) to the co-cultures induced maximal phosphorylation of the EGFR in I-10 cells within 5 min. Maximal phosphorylation of ERK in I-10 and MA-10 cells occurred within 5-15 min of addition of hCG. Since hCG cannot directly act on I-10 cells these results show that hCG-stimulated MA-10 cells produce a factor (or factors) that stimulates the phosphorylation of the EGFR and ERK in I-10 cells.

The LHR-mediated phosphorylation of the EGFR and ERK1/2 in the I-10 cells present in the co-cultures could be due to the release of soluble, diffusible factors acting in a paracrine fashion [10-13,15] or the actions of membrane bound factors acting in a juxtacrine fashion [15,30]. To differentiate between these two models we also performed a more traditional experiment in which the two cell types were cultured in transwell plates that separate them into two physically distinct compartments but allow for the diffusion of soluble molecules (Figure 6). Under these conditions any effect of hCG on I-10 cells would have to be mediated by soluble factors because physical contact between the two cell types is not possible.

The results of a representative transwell experiment are presented in Figure 6 and they nicely recapitulate the data obtained in the simplified co-culture system in both magnitude and time course of the responses (c.f. Figure 5). In the transwell experiment the two cell types do not need to be transfected with epitope-tagged markers because they are separated physically and can be independently collected and analyzed. We used transfected cells for these experiments only because we wanted to compare the magnitude and time course of phosphorylation detected in the two assay systems

Together these results show that the LHR-mediated transactivation of the EGFR and ERK1/2 in I-10 cells involves the release of soluble, diffusible factors.

Transfection of interfering constructs into MA-10 or I-10 cells documents the existence of two different pathways for ERK1/2 activation

In the experiments summarized in Figure 7 we transfected MA-10 cells (panels A, B and C) or I-10 cells (panels D, E and F) with several vectors that were previously shown to interfere with the actions of hCG on ERK1/2 phosphorylation in MA-10 cell monocultures [5,7]. We then co-cultured the cells, added hCG and examined the phosphorylation of the EGFR in I-10 cells (panels A and D) as well the phosphorylation of ERK1/2 in I-10 (panels B and E) or MA-10 cells (panels C and F).

Addition of hCG to the co-cultures provoked a ~1.6-fold increase in the phosphorylation of the EGFR in I-10 cells (Figure 7A and D). Expression of DN-Fyn in MA-10 cells inhibited the ability of hCG to enhance the phosphorylation of the EGFR in I-10 cells whereas expression of PDE or PKI in MA-10 cells has no effect on the hCG-provoked phosphorylation of the EGFR in I-10 cells (Figure 7A). Expression of the same vectors in I-10 cells had no effect on the ability of hCG to enhance the phosphorylation of the EGFR in I-10 cells (Figure 7D).

Addition of hCG to the co-cultures provoked a ~3-fold increase in the phosphorylation of ERK in I-10 cells (Figure 7B and E). Expression of DN-Fyn, in MA-10 cells inhibited the hCG-induced phosphorylation of ERK in I-10 cells but expression of PDE, PKI or DN-Shc in MA-10 cells did not affect this response (Figure 7B). Expression of DN-Fyn, PDE or PKI in I-10 cells did not affect this response either, but expression of DN-Shc had an inhibitory effect (Figure 7E).

Addition of hCG to the co-cultures provoked a ~6-fold increase in the phosphorylation of ERK in MA-10 cells (Figure 7C and F). Expression of DN-Fyn, PDE, PKI or DN-Shc in MA-10 cells inhibited the hCG-induced phosphorylation of ERK in MA-10 cells (Figure 7C). Expression of the same vectors in I-10 cells did not affect the hCG-induced phosphorylation of ERK in MA-10 cells (Figure 7F).

These data show that manipulations of the MA-10 cell phenotype (prior to co-culture) can affect the hCG-induced responses in MA-10 and/or I-10 cells in the co-cultures but manipulation of the I-10 cell phenotype (prior to co-culture) can affect only the I-10 cell responses in the co-cultures. The effects of expression of the different interfering constructs in the two cell types indicate that (a) Fyn, but not cAMP/PKA is a mediator of the hCG-dependent production of soluble factors by MA-10 cells that act on I-10 cells to phosphorylate the EGFR and ERK; (b) the hCG-dependent phosphorylation of ERK in MA-10 cells depends on Fyn as well as cAMP/PKA; and (c) Shc is an intermediate in the hCG-induced phosphorylation of ERK in both MA-10 and I-10 cells.

Silencing the expression of the endogenous EGFR documents the involvement of EGF-like growth factors as mediators of the hCG-induced ERK phosphorylation in MA-10 cells

A causal relationship between the hCG-induced phosphorylation of the EGFR and ERK is suggested by the finding that AG1478 inhibits both of these phenomena in mono-cultures of MA-10 cells or primary cultures of rat Leydig cells [7,8]. In addition, AG1478 inhibits the hCG-induced phosphorylation of the EGFR and ERK in I-10 cells when added to MA-10/I-10 co-cultures stimulated with hCG (data not shown). In this set of experiments we sought to more rigorously test for the involvement of the EGFR on the hCG-induced ERK response using a genetic approach.

Monocultures of MA-10 cells were co-transfected with the hLHR plus a siRNA that targets the EGFR or with the hLHR plus a control siRNA. Transfection of MA-10 cells with the EGFR siRNA reduced the expression of the endogenous EGFR by ~70% but had no effect on the expression of endogenous ERK1/2 (Figure 8). We then stimulated ERK1/2 phosphorylation in these monocultures with a maximally effective concentration of hCG (100 ng/ml) or a concentration of EGF (0.1 ng/ml) chosen to match the magnitude of the response elicited by hCG. The data shown in Figure 8 show that transfection with the EGFR siRNA reduced the hCG- and EGF-induced phosphorylation of ERK1/2 by ~25% and ~40%, respectively.

Discussion

Previous studies on the effects of hCG on ERK1/2 phosphorylation in primary cultures of rat Leydig cells and MA-10 Leydig tumor cells have identified the involvement of several prominent signaling molecules such as PKA, Fyn, Ras, Shc and the EGFR as intermediates in this pathway [5-9]. Until now, however, it has been difficult to determine if the PKA and tyrosine phosphorylation cascades are independent or interdependent components in the actions of hCG. To reconcile the involvement of these two pathways we hypothesized that the hCG-induced phosphorylation of ERK1/2 involves intracellular pathways that require cAMP/PKA and intercellular pathways that activate the EGFR.

To examine the existence of an intercellular pathway that involves activation of the EGFR we first attempted to use conditioned media and/or immunological methods to detect EGF-like molecules that may be released from Leydig cells in response to hCG activation. Since these attempts were unsuccessful (presumably because of the low levels of soluble factors released), we turned to the co-culture system described herein. Our Leydig cell co-culture system (Figures 1 and 9) is based on a heterologous co-culture system previously used by Lefkowitz and colleagues who examined the mechanisms by which adrenergic receptors activate ERK1/2

[12]. Our modifications of this system involved the use of an EGFR positive, LHR-positive, hCG-responsive Leydig cell line (MA-10) and an EGFR positive, LHR-negative, hCG-unresponsive Leydig cell line (I-10) that have been individually transfected with different epitope-tagged versions of ERK2 and the EGFR. Upon co-culture this system allows for the simultaneous detection of the phosphorylation of these kinases using simple Western blots. Since I-10 cells do not express the LHR any hCG-induced phosphorylation of ERK or the EGFR in the I-10 cells must arise from intercellular signals generated by MA-10 cells in response to hCG stimulation.

All the results presented here support the hypothesis that hCG induces the phosphorylation of ERK1/2 in Leydig cells by two distinct and independent pathways (Figure 9). One pathway is entirely intracellular and it relies on cAMP and PKA, which are the traditional mediators of the actions of hCG on the differentiated functions of Leydig cells. PKA has been shown to activate Ras (the classical switch of the kinase cascade that ends with the phosphorylation of ERK1/2) in monocultures of MA-10 cells [dashed arrow #1 in Figure 9 and ref. 5] but the molecular basis of this activation step are not understood. In the co-culture system this pathway is operative only in MA-10 cells because it is entirely intracellular and directly downstream of the LHR. The second pathway is independent of cAMP/PKA but it involves the LHR-provoked activation of Fyn and the subsequent release of soluble factors that act on the originating cell and in neighboring cells. We have already shown that the LHR activates Fyn but we do not know the molecular details of this step [dashed arrow # 2 in Figure 9 and ref. 9]. Since others have shown that the Src family of kinases can stimulate the release of soluble forms of EGF-like growth factors [10-13] we propose that Fyn activates a similar pathway in MA-10 cells (dashed arrow # 3 in Figure 9) and that the released soluble factors activate the EGFR in MA-10 and I-10 cells leading to the phosphorylation of ERK/12 in both cell types. In other words in the co-culture system the LHR-provoked ERK phosphorylation in MA-10 cells depends on the intracellular cAMP/PKA-dependent pathway and the intercellular Fyn-dependent pathway whereas the LHR-provoked ERK phosphorylation in I-10 cells depends entirely on the intercellular Fyn-dependent pathway.

Addition of hCG to the co-cultures engages the LHR in MA-10 cells and the resulting activation of the cAMP/PKA pathway leads to the phosphorylation of ERK1/2 in MA-10 cells by the intracellular pathway that involves Ras activation. This pathway has been previously documented in monocultures of MA-10 cells or primary cultures of rat Leydig cells [5,7]. Its existence is confirmed here by preventing the rise in cAMP levels by expression of a cAMP phosphodiesterase or by preventing PKA activation by expression of PKI. Expression of either of these constructs in MA-10 cells prior to co-culture impairs the hCG provoked phosphorylation of ERK in MA-10 cells but not in I-10 cells. Conversely, expression of either of these two constructs in I-10 cells has no effect. We conclude from this data that the cAMP/PKA pathway is entirely intracellular and it participates only in the hCG-induced phosphorylation of ERK in MA-10 cells. We also conclude that cAMP/PKA are not mediators of the hCG-induced intercellular pathway leading to the phosphorylation of the EGFR and ERK in I-10 cells.

Using mono-cultures of MA-10 cells we have already shown that hCG activates Fyn in a cAMP-independent fashion [9] and that DN-Fyn expression or pharmacological inhibitors of the Src family of kinases interfere with the hCG-provoked ERK1/2 phosphorylation [7]. Here we confirm that expression of DN-Fyn in MA-10 cells prior to co-culture interferes with the ability of hCG to induce the phosphorylation of ERK in MA-10 cells but we also show that expression of DN-Fyn in MA-10 cells inhibits the ability of hCG to induce the phosphorylation of the EGFR and ERK in I-10 cells. Expression of DN-Fyn in I-10 cells has no effect on any of these events. Based on these data we conclude that the LHR-provoked activation of Fyn in MA-10 cells is a mediator of the intercellular pathway that results in the release of soluble

factors from MA-10 cells that phosphorylate the EGFR and activate the ERK1/2 cascade in I-10 cells (Figure 9).

The involvement of soluble, diffusible factors as mediators of the LHR-induced activation of the ERK1/2 pathway is supported by the transwell experiments presented herein. Our data on the phosphorylation of the EGFR suggest that these diffusible factors may be members of the EGF family of growth factors. If this is the case we also need to consider the possibility of an additional pathway involving juxtracrine signaling by growth factor precursors present at the cell membrane [14,15] as illustrated in Figure 9. Although this possibility is not excluded by the transwell experiments, it is difficult to envision how this pathway may be regulated by hCG. In this respect it should be noted that we have previously reported that GM6001, a broad metalloprotease inhibitor that blocks the release of the heparin-bound form of EGF does not inhibit the actions of hCG on the phosphorylation of the EGFR or ERK1/2 in monocultures of MA-10 cells [7]. This lack of effect of GM6001 is not necessarily in conflict with the data presented here because there are several EGF-like growth factors that may mediate the actions of hCG and several enzymes that can catalyze their processing [14,15,31,32]. Not all of these enzymes are sensitive to GM6001. We also tested the effects of GM6001 in the co-culture system but they are not included here because we found the results to be rather variable and dependent on the batch of GM6001 used.

In summary our studies on the actions of the LHR in Leydig cells show that the ability of this receptor to stimulate the ERK1/2 cascade involves at least two distinct pathways and they implicate the EGFR and the EGF family of growth factors as mediators of this response. These studies nicely complement the studies of Conti and co-workers who have clearly shown that the ovulatory response is mediated by a LHR-provoked increases in the synthesis and release of one or more members of the EGF-like family of growth factors [33-35].

Acknowledgements

We thank Dr. Colette Galet for help with the design of the siRNA against the EGFR and the control siRNA. We also thank Drs. Stefan Strack and John Koland for the myc-ERK2, ERK2-GFP and EGFR-GFP constructs.

*Supported by a grant from the National Cancer Institute (CA-40629).

References

1. Ascoli M, Fanelli F, Segaloff DL. The lutropin/choriogonadotropin receptor. A 2002 perspective. *Endocr Rev* 2002;23:141–174. [PubMed: 11943741]
2. Kumar TR. What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? *Reproduction* 2005;130:293–302. [PubMed: 16123236]
3. Themmen APN. An update of the pathophysiology of human gonadotrophin subunit and receptor gene mutations and polymorphisms. *Reproduction* 2005;130:263–274. [PubMed: 16123233]
4. Hirakawa T, Ascoli M. A constitutively active somatic mutation of the human lutropin receptor found in Leydig cell tumors activates the same families of G proteins as germ line mutations associated with Leydig cell hyperplasia. *Endocrinology* 2003;144:3872–3878. [PubMed: 12933660]
5. Hirakawa T, Ascoli M. The lutropin/choriogonadotropin receptor (LHR)-induced phosphorylation of the extracellular signal regulated kinases (ERKs) in Leydig cells is mediated by a protein kinase A-dependent activation of Ras. *Mol Endocrinol* 2003;17:2189–2200. [PubMed: 12920236]
6. Martinelle N, Holst M, Soder O, Svechnikov K. Extracellular Signal-Regulated Kinases Are Involved in the Acute Activation of Steroidogenesis in Immature Rat Leydig Cells by Human Chorionic Gonadotropin. *Endocrinology* 2004;145:4629–4634. [PubMed: 15242988]
7. Shiraishi K, Ascoli M. Activation of the lutropin/choriogonadotropin receptor (LHR) in MA-10 cells stimulates tyrosine kinase cascades that activate Ras and the extracellular signal regulated kinases (ERK1/2). *Endocrinology* 2006;147:3419–3427. [PubMed: 16614081]

8. Shiraishi K, Ascoli M. Lutropin/choriogonadotropin (LH/CG) stimulate the proliferation of primary cultures of rat Leydig cells through a pathway that involves activation of the ERK1/2 cascade. *Endocrinology* 2007;en.2007–0160.
9. Mizutani T, Shiraishi K, Welsh T, Ascoli M. Activation of the lutropin/choriogonadotropin receptor (LHR) in MA-10 cells leads to the tyrosine phosphorylation of the focal adhesion kinase (FAK) by a pathway that involves Src family kinases. *Mol Endocrinol* 2006;20:619–630. [PubMed: 16293639]
10. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 1999;402:884–888. [PubMed: 10622253]
11. Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol* 1999;11:184–189. [PubMed: 10209149]
12. Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ. Epidermal Growth Factor (EGF) Receptor-dependent ERK Activation by G Protein-coupled Receptors. A coculture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* 2001;276:23155–23160. [PubMed: 11290747]
13. Hsieh M, Conti M. G-protein-coupled receptor signaling and the EGF network in endocrine systems. *Trends Endocrinol Metabol* 2005;16:320–326.
14. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127–137. [PubMed: 11252954]
15. Blobel CP. ADAMS: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* 2005;6:32–43. [PubMed: 15688065]
16. van der Geer P, Wiley S, Gish GD, Pawson T. The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein-protein interactions. *Curr Biol* 1996;6:1435–1444. [PubMed: 8939605]
17. Luttrell DK, Luttrell LM. Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene* 2004;23:7969–7978. [PubMed: 15489914]
18. Gavi S, Shumay E, Wang H, Malbon CC. G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. *Trends Endocrinol Metabol* 2006;17
19. Bromann PA, Korkaya H, Courtneidge SA. The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* 2004;23:7957–7968. [PubMed: 15489913]
20. Hirakawa T, Galet C, Ascoli M. MA-10 cells transfected with the human lutropin/choriogonadotropin receptor (hLHR). A novel experimental paradigm to study the functional properties of the hLHR. *Endocrinology* 2002;143:1026–1035. [PubMed: 11861529]
21. Resh MD. Fyn, a Src family tyrosine kinase. *Int J Biochem Cell Biol* 1998;30:1159–1162. [PubMed: 9839441]
22. Pratt JC, van den Brink MRM, Igras VE, Walk SF, Ravichandran KS, Burakoff SJ. Requirement for Shc in TCR-Mediated Activation of a T Cell Hybridoma. *J Immunol* 1999;163:2586–2591. [PubMed: 10452997]
23. Swinnen JV, Joseph DR, Conti M. The mRNA encoding a high-affinity cAMP phosphodiesterase is regulated by hormones and cAMP. *Proc Natl Acad Sci (USA)* 1989;86:8197–8201. [PubMed: 2554303]
24. Day RN, Walders JA, Maurer RA. A protein kinase inhibitor gene reduces both basal and multihormone-stimulated prolactin gene transcription. *J Biol Chem* 1989;264:431–436. [PubMed: 2535842]
25. Ascoli M. Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology* 1981;108:88–95. [PubMed: 6257492]
26. Shin SI. Studies on interstitial cells in tissue culture: steroid biosynthesis in monolayers of mouse testicular interstitial cells. *Endocrinology* 1967;81:440–448. [PubMed: 4382264]
27. Ascoli M. Immortalized Leydig cell lines as models for studying Leydig cell physiology. In: Payne, AH.; Hardy, MP., editors. *The Leydig Cell in Health and Disease*. Humana Press; New York: 2007. Vol. In press, p. In press
28. Fabritz J, Ryan S, Ascoli M. Transfected cells express mostly the intracellular precursor of the lutropin/choriogonadotropin receptor but this precursor binds choriogonadotropin with high affinity. *Biochemistry* 1998;37:664–672. [PubMed: 9425089]

29. Shin S, Yasumura Y, Sato GH. Studies on interstitial cells in tissue culture. II. Steroid biosynthesis by a clonal line of rat testicular interstitial cells. *Endocrinology* 1968;82:614–616. [PubMed: 4868022]
30. Iwamoto R, Handa K, Mekada E. Contact-dependent growth inhibition and apoptosis of epidermal growth factor (EGF) receptor-expressing cells by the membrane-anchored form of heparin-binding EGF-like growth factor. *J Biol Chem* 1999;274:25906–25912. [PubMed: 10464334]
31. Gschwind A, Hart S, Fischer OM, Ullrich A. TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *EMBO J* 2003;22:2411–2421. [PubMed: 12743035]
32. Lautrette A, Li S, Alili R, Sunnarborg SW, Burtin M, Lee DC, Friedlander G, Terzi F. Angiotensin II and EGF receptor cross-talk in chronic kidney diseases: a new therapeutic approach. *Nat Med* 2005;11:867–874. [PubMed: 16041383]
33. Park JY, Su YQ, Ariga M, Law E, Jin SLC, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 2004;303:682–684. [PubMed: 14726596]
34. Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, Tsafiriri A. Epidermal Growth Factor Family Members: Endogenous Mediators of the Ovulatory Response. *Endocrinology* 2005;146:77–84. [PubMed: 15459120]
35. Hsieh M, Lee D, Panigone S, Horner K, Chen R, Theologis A, Lee DC, Threadgill DW, Conti M. Luteinizing Hormone-Dependent Activation of the Epidermal Growth Factor Network Is Essential for Ovulation. *Mol Cell Biol* 2007;27:1914–1924. [PubMed: 17194751]

Figure 1. Leydig cell co-cultures used to measure the simultaneous phosphorylation of signaling molecules in LHR positive and LHR negative cells

LHR positive MA-10 cells expressing the myc-hLHR and myc-ERK2 and LHR negative I-10 cells expressing ERK2-GFP and EGFR-GFP were co-cultured as indicated. Human CG binds only to the LHR in MA-10 cells (arrow labeled A) and any changes in the phosphorylation of ERK2-GFP or EGFR-GFP must occur through an indirect effect that involves an hCG-dependent increase in the release of MA-10 cell soluble, diffusible factors that act on I-10 cells and/or an hCG-dependent increases in MA-10/I-10 cell contacts (arrow labeled B). In contrast the phosphorylation of myc-ERK2 can be mediated by an intracellular pathway directly stimulated by hCG in MA-10 cells (arrow labeled A) or by the indirect pathway involving increased cell contact or release of diffusible factors acting on the originating MA-10 cells (arrow labeled C).

This figure is modified from a similar figure first published in reference [12].

Figure 2. I-10 cells do not bind hCG or respond to it with an increase in cAMP accumulation Monocultures of MA-10 cells transfected with the myc-hLHR, designated MA-10 (hLHR), or I-10 cells were used to measure ^{125}I -hCG binding (panel A) or cAMP accumulation (panel B) as described in Materials and Methods. Results are the mean $\pm$ SEM of three independent experiments.

MA-10 = LHR⁺, EGFR⁺, ERK⁺
[Transfected with myc-hLHR and myc-ERK2]

I-10 = LHR⁻, EGFR⁺, ERK⁺
[Transfected with ERK2-GFP and EGFR-GFP]

Figure 3. I-10 cells do not respond to hCG with an increase in ERK or EGFR phosphorylation
 The phosphorylation of ERK (panel A) or the EGFR (panel B) were measured in monocultures of MA-10 cells transfected with the myc-hLHR and myc-ERK2 and incubated at 37°C with buffer only (C), 100 ng/ml hCG for 15 min or 100 ng/ml EGF for 5 min as indicated. The phosphorylation of ERK (panel C) or the EGFR phosphorylation (panels D and E) were measured in monocultures of I-10 cells transfected with ERK2-GFP and EGFR-GFP and incubated at 37°C with buffer only (C), 100 ng/ml hCG for 15 min or 100 ng/ml EGF for 5 min as indicated. Panels D and E show a shorter (panel D) and a longer (panel E) exposure of the same blot. The results of a representative experiment are shown in each panel. The same results were obtained in 3 additional experiments. WB = Western blot, P-EGFR = phosphorylated EGFR, P-ERK = phosphorylated ERK.

Figure 4. A cAMP analog stimulates ERK phosphorylation in monocultures of MA-10 or I-10 cells
 Monocultures of MA-10 cells transfected with the myc-hLHR and myc-ERK2 (panel A) or monocultures of I-10 cells transfected with ERK2-GFP and EGFR-GFP (panel B) were incubated with 0.5 mM 8-CPT-cAMP at 37°C for the times indicated. Western blots (WB) of the lysates were developed with antibodies to phosphorylated ERK (P-ERK) or total ERK (ERK) as indicated.

Each panel shows the results of a representative experiment. The same results were obtained in 2 additional experiments.

Figure 5. Human CG stimulates the phosphorylation of the EGFR and ERK in I-10 cells when they are co-cultured with MA-10 cells

MA-10 cells transfected with the myc-hLHR and myc-ERK2 and I-10 cells transfected with ERK2-GFP and EGFR-GFP were co-cultured and incubated with hCG (100 ng/ml, 37°C) as indicated. Western blots (WB) of the lysates were developed with antibodies to phosphorylated ERK (P-ERK), total ERK (ERK), the phosphorylated EGFR (P-EGFR) or GFP (EGFR) as shown.

The results of a representative experiment are shown. The same results were obtained in 2 additional experiments.

Figure 6. Human CG stimulates the phosphorylation of the EGFR and ERK in I-10 cells when they are cultured with MA-10 cells in transwell plates

MA-10 cells transfected with the myc-hLHR and myc-ERK2 and I-10 cells transfected with ERK2-GFP and EGFR-GFP were cultured in plates containing transwells as shown (also see Materials and Methods). Human CG (100 ng/ml) was added to the cultures and they were incubated at 37°C as indicated. Lysates of I-10 cells were prepared after removal of the inserts and used for Western blots (WB) with antibodies to phosphorylated ERK (P-ERK), total ERK (ERK), the phosphorylated EGFR (P-EGFR) or GFP (EGFR) as shown.

The results of a representative experiment are shown. The same results were obtained in 2 additional experiments.

Figure 7. Effect of exclusive cellular expression of several interfering vectors on the ability of hCG to stimulate the phosphorylation of the EGFR and ERK in MA10 and I-10 cells in co-culture

Panels A, B and C: MA-10 cells transfected with the myc-hLHR, myc-ERK2 and the additional vectors indicated were co-cultured with I-10 cells transfected with ERK2-GFP and EGFR-GFP.

Panels D, E, F: I-10 cells transfected with ERK2-GFP, EGFR-GFP and the additional vectors indicated were co-cultured with MA-10 cells transfected with the myc-hLHR and myc-ERK2. In panels A and D the co-cultures were incubated with buffer only or hCG (100 ng/ml) for 5 min at 37°C. Western blots of the lysates were analyzed for the phospho-EGFR-GFP and total EGFR-GFP (i.e., the phosphorylated and total EGFR present in I-10 cells). The signals were quantitated as described in Materials and Methods and the phosphorylation data were normalized to total levels of EGFR-GFP. The normalized phosphorylation data were then expressed as fold-over-basal by comparison to the normalized phosphorylation data from the experiment done using I-10 cells co-transfected with an empty vector (EV) and incubated with buffer. This basal level of phospho-EGFR is shown by the horizontal line drawn in each panel. In panels B-F the co-cultures were incubated with buffer only or hCG (100 ng/ml) for 10 min at 37°C. Western blots of the lysates were analyzed for the phosphorylated and total myc-ERK2 (i.e., the ERK2 present in MA-10 cells) or phosphorylated and total ERK2-GFP (i.e., the ERK2 present in I-10 cells). The signals were quantitated as described in Materials and Methods and the phosphorylation data were normalized to total levels of myc-ERK2 or ERK2-GFP. The normalized phosphorylation data were then expressed as fold-over-basal by comparison to the normalized phosphorylation data from the experiment done using I-10 cells co-transfected with an empty vector (EV) and incubated with buffer (panels B and E) or the data from the experiment done using MA-10 cells co-transfected with an empty vector (EV) and incubated with buffer (panels C and F). These basal levels of phospho-ERK are shown by the horizontal line drawn in each panel.

Each bar shows the mean $\pm$ SEM of four independent co-cultures. The asterisks denote significant differences ($p < 0.05$, ANOVA with Dunnet's post test) when compared to the I-10 cells transfected with an empty vector and stimulated with hCG (i.e., the first bar in each panel).

Figure 8. Silencing the endogenous EGFR in MA-10 cell monocultures impairs the effects of hCG and EGF on the ERK1/2 response

Monocultures of MA-10 cells were co-transfected with the hLHR and a control siRNA (empty bars) or the hLHR and a siRNA that targets the EGFR (filled bars). The cells were then incubated with hCG (100 ng/ml) for 15 min or EGF (0.1 ng/ml) for 5 min prior to measuring the phosphorylation of the endogenous ERK1/2. The phospho-ERK1/2 and total ERK1/2 signals were quantitated as described in Materials and Methods and the phosphorylation data were normalized to the total ERK1/2 data. All normalized data were then expressed as fold-over basal by comparison to the normalized phospho-ERK1/2 signal detected in similarly treated cells incubated without hCG or EGF. Each bar shows the mean $\pm$ SEM of four independent transfections. The asterisks denote significant differences ($p < 0.05$, two tailed t-test) when compared to cells transfected with the control siRNA and incubated with the same stimulus (i.e., hCG or EGF). The basal level of phospho-ERK is shown by the horizontal line. Representative blots illustrating the levels of endogenous EGFR or ERK1/2 are also shown. The numbers under the blots show the quantitation of the EGFR or ERK1/2 signals in the cells transfected with the EGFR siRNA when expressed relative to that in cells transfected with the control siRNA. The results presented are the mean $\pm$ SEM of four independent transfections. The asterisk denotes a significant difference ($p < 0.05$, two tailed t-test) when compared to cells transfected with the control siRNA.

Figure 9. A model depicting the two pathways that are involved in the hCG-provoked phosphorylation of ERK1/2 in co-cultures of MA-10 and I-10 cells

The two cell types are shown in the dashed rectangles as indicated. The ERK1/2 pathway shown in both cells is the classical pathway.

When added to the co-cultures hCG binds to the LHR in MA-10 cells and stimulates cAMP accumulation using the classical Gs/adenylyl cyclase cascade. Cyclic AMP, acting through PKA, activates Ras (and the ERK1/2 cascade) by mechanisms that remain to be investigated (dashed line #1). The involvement of this pathway in the MA-10 cell ERK response and its lack of involvement in the I-10 cell ERK response is documented by the ability of PDE and PKI to inhibit only the hCG-induced ERK1/2 response when expressed in MA-10 cells and also by their inability to do so when expressed in I-10 cells.

The binding of hCG to the LHR in MA-10 cells also activates Fyn by a pathway that remains to be investigated (dashed line #2). We propose that Fyn activates the proteolysis of the precursor forms of a growth factor (Pro-X, dashed line #3) leading to the release of soluble, diffusible forms of this factor (X) that then stimulates the EGFR in MA-10 cells and in I-10 cells. The stimulated EGFR then activates Ras (and the ERK1/2 cascade) using the classical pathway involving the tyrosine phosphorylation of Shc. The involvement of Shc in both cell types is documented by the ability of DN-Shc to inhibit the hCG-induced ERK1/2 response when expressed in MA-10 cells or I-10 cells. The involvement of Fyn as an upstream activator of the release of soluble factors is documented by the ability of Dn-Fyn to inhibit the hCG-induced ERK1/2 response and the phosphorylation of the EGFR in I-10 cells only when it is expressed in MA-10 cells but not when it is expressed in I-10 cells. The involvement of the EGFR as a mediator of the hCG-induced ERK1/2 response is best documented by the inhibitory effect of the EGFR siRNA on the hCG-induced ERK1/2 response in MA-10 cells.

The hCG-induced activation of the EGFR (and the ERK1/2 cascade) in I-10 cells could also involve juxtacrine signaling as shown by the juxtaposition of "Pro-X" in MA-10 cells and the EGFR in I-10 cells at the bottom of the two cell types.