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ORIGINAL ARTICLE

hCG activates Epac-Erk1/2 signaling regulating Progesterone Receptor expression and function in human endometrial stromal cells

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STUDY QUESTION: How does hCG signal in human endometrial stromal cells (ESCs) and what is its role in regulating ESC function?

SUMMARY ANSWER: hCG signaling in ESCs activates the extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) pathway through exchange protein activated by cyclic AMP (cAMP) (Epac) and transiently increases progesterone receptor (PR) transcript and protein expression and its transcriptional function.

WHAT IS KNOWN ALREADY: hCG is one of the earliest embryo-derived secreted signals in the endometrium, which abundantly expresses LH/hCG receptors. hCG signals through cAMP/protein kinase A (PKA) in gonadal cells, but in endometrial epithelial cells, hCG induces Erk1/2 activation independent of the cAMP/PKA pathway. Few data exist concerning the signal transduction pathways triggered by hCG in ESCs and their role in regulation of ESC function.

STUDY DESIGN, SIZE, DURATION: This is an *in vitro* study comprising patients undergoing benign gynecological surgery ($n = 46$).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Endometrial samples were collected from normal cycling women during the mid-secretory phase for ESCs isolation. The study conducted in an academic research laboratory within a tertiary-care hospital. The activation of the Erk1/2 signal transduction pathway elicited by hCG was evaluated in ESC. Signaling pathway inhibitors were used to examine the roles of PKA, PI3K, PKC, adenylyl cyclase and Epac on the hCG-stimulated up-regulation of phospho-Erk1/2 (pErk1/2). Erk1/2 phosphorylation was determined by immunoblot. siRNA targeting Epac was used to investigate the molecular mechanisms. To assess the role of Erk1/2 signaling induced by hCG on ESC function, gene expression regulation was examined by immunofluorescence and real-time quantitative PCR. The role of PR on the regulation of transcript levels was studied using progesterone and the PR antagonist RU486. All experiments were conducted using at least three different cell culture preparations in triplicate.

MAIN RESULTS AND THE ROLE OF CHANCE: Addition of hCG to ESCs *in vitro* induced the phosphorylation of Erk1/2 through cAMP accumulation. Such induction could not be blocked by inhibitors for PKA, PKC and PI3K. Epac inhibition and knockdown with siRNA prevented pErk1/2 induction by hCG. ESCs stimulated with hCG for up to 72 h showed a significant increase in PR mRNA and immunofluorescent label at 48 h only; an effect that was abrogated with the mitogen-activated protein kinase kinase inhibitor UO126. In addition, the hCG-activated Erk1/2 pathway significantly decreased the mRNA levels for secreted frizzled-related protein 4 (SFRP4) at 24 h, whereas it increased those for homeobox A10 (HOXA10) at 48 h ($P = 0.041$ and $P = 0.022$ versus control, respectively). Prolactin mRNA levels were not significantly modified. HOXA10 mRNA up-regulation by hCG was not enhanced by co-stimulation with progesterone; however, it was completely abolished in the presence of RU486 ($P = 0.036$ hCG versus hCG + RU486).

LARGE SCALE DATA: N/A.

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LIMITATIONS, REASONS FOR CAUTION: This is an *in vitro* study utilizing stromal cell cultures from human endometrial tissues. Furthermore, results obtained should also be confirmed *in vivo* in the context of the whole human endometrial tissue and hormonal milieu. The *in vitro* experiments using hCG have been conducted without other hormones/factors that may also modulate the ESCs response to hCG.

WIDER IMPLICATIONS OF THE FINDINGS: We have determined that hCG induces the PR through the Erk1/2 pathway in ESCs which may render them more sensitive to progesterone, increasing our understanding about the effects of hCG at the embryo–maternal interface. The activation of such a pathway in the context of the hormonal milieu during the window of implantation might contribute to a successful dialog between the embryo and the uterus, leading to appropriate endometrial function. Defective hCG signaling in the endometrial stromal tissue may lead to an incomplete uterine response, compromising embryo implantation and early pregnancy.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the National Fund for Scientific and Technological Development, Government of Chile (FONDECYT) grants 11100443 and 1140614 (A.T.-P.). The authors have no conflicts of interest to declare.

Key words: chorionic gonadotropin, hCG, endometrium / Epac, endometrial stromal cells / Erk1/2 / progesterone receptor

Introduction

The main factors determining a successful implantation are a receptive endometrium and a competent developing embryo with adequate cross-talk between them. Such molecular dialog is mediated by several molecules whose reciprocal signaling at the embryo–maternal interface regulates the implantation process. hCG is one of the earliest signals secreted by the embryo, the mRNA and secretion of which is detectable from Day 2 post-fertilization (Jurisicova *et al.*, 1999; Ramu *et al.*, 2011), and which has been measured in conditioned medium from pre-implantation blastocysts (Bonduelle *et al.*, 1988; Lopata and Oliva, 1993). hCG becomes the major pregnancy hormone and placental syncytiotrophoblasts synthesize large amounts of it (Hoshina *et al.*, 1984). The role of hCG in the establishment and maintenance of pregnancy is well known not only for stimulating progesterone synthesis *via* its luteotrophic effects but also for regulating uterine and trophoblastic function for embryo implantation (Shi *et al.*, 1993; Cronier *et al.*, 1994; Kraiem *et al.*, 1994; Kurtzman *et al.*, 2001; Keay *et al.*, 2004). Intrauterine infusion of hCG in women has been reported to improve endometrial receptivity markers such as leukemia inhibitory factor and vascular endothelial growth factor (Licht *et al.*, 1998) and has been associated with endometrial synchrony and reprograming of stromal development following ovarian stimulation (Strug *et al.*, 2016). These actions of hCG at the maternal side occur by paracrine signaling *via* the LH/CG receptor (LHCGR) (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989), which is present in endometrial epithelial cells (EECs) and endometrial stromal cells (ESCs) with the highest expression during the secretory phase of the endometrial cycle (Reshef *et al.*, 1990). The LHCGR is a member of the subfamily of glycoprotein hormone receptors within the superfamily of G protein-coupled receptors (GPCRs) and is associated with $G\alpha_s$ protein. LHCGR-mediated effects are mostly transduced by the $G\alpha_s$/adenylyl cyclase/cyclic AMP (cAMP)/protein kinase A (PKA) pathway, which is one of the classic activator systems for steroidogenesis in gonadal cells (Hirakawa *et al.*, 2002). In addition, LHCGR may act through the phospholipase C/$InsP_3$ pathway (Strauss *et al.*, 1992; Ascoli *et al.*, 2002). Both of these signaling routes have been shown to activate the mitogen-activated protein kinase (MAPK) pathway in many cell types (Steele and Leung, 1992; Ryu *et al.*, 1998).

Extracellular signal-regulated protein kinases 1/2 (Erk1/2) are isoforms of the MAPK pathway and the activated form of Erk1/2 (phospho-Erk1/2, pErk1/2) can subsequently phosphorylate several transcription factors that regulate cellular proliferation, differentiation and apoptosis (Roskoski, 2012). Erk1/2 can be activated by $G\alpha_s$-coupled receptors *via* a range of different signaling pathways (Lowes *et al.*, 2002). In EEC, hCG did not significantly alter adenylyl cyclase activity or increase intracellular cAMP, but it induced a PKA-independent phosphorylation of Erk1/2 which stimulated prostaglandin E_2 production (Srisuparp *et al.*, 2003; Banerjee *et al.*, 2009; Evans and Salamonsen, 2013). In human ESCs, hCG increases intracellular accumulation of cAMP (Chatterjee *et al.*, 1997), but the downstream signaling mechanism has not been determined. The Erk1/2 pathway has been shown to be important for ESC function (Chang *et al.*, 2007; Gentilini *et al.*, 2010) including decidualization (Lee *et al.*, 2013). On the other hand, aberrant levels of pErk1/2 have been described in ESCs derived from women with endometriosis which influences cAMP-dependent cell-cycle regulation (Klemmt *et al.*, 2006; Velarde *et al.*, 2009).

We investigated whether the Erk1/2 pathway is involved in hCG signaling in ESCs and its potential relevance in endometrial function related to embryo implantation. In this study, we report that pErk1/2 is induced upon hCG stimulation in ESCs in a PKA-independent manner, which increases expression of the progesterone receptor (PR) and regulates transcripts associated with endometrial receptivity.

Materials and Methods

Brefeldin-A (BFA, exchange protein activated by cAMP [Epac] inhibitor), 3-isobutyl-1-methylxanthine (IBMX, phosphodiesterase inhibitor), hCG, progesterone and RU486 (PR antagonist) were obtained from Sigma (B7651, I5879, CG10, P0130 and M8046, respectively; St. Louis, MO, USA). The Epac-selective cAMP analog 8-(4-chlorophenylthio)-2′-*O*-methyladenosine 3′,5′-cyclic monophosphate (8pCPT) was from Santa Cruz Biotechnologies (sc-257020, 200 µM; Santa Cruz, CA, USA). H-89 (PKA inhibitor, 371963), LY294002 (PI3K inhibitor, 440202), UO126 (MEK inhibitor, 662005), bisindolylmaleimide (BIM) I (PKC inhibitor, 203290) and SQ22536 (adenylyl cyclase inhibitor, 568500) were purchased from Calbiochem (EMD/Millipore, San Diego, CA, USA).

Study groups

Endometrial tissue samples were obtained at Hopital Clínico San Borja Arriarán (Santiago, Chile) from healthy women with proven fertility (35–45 years old) with indication of benign gynecological surgery (i.e.,

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laparoscopy for tubal sterilization), and no signs of endometriosis and inflammatory pelvic disease. Signed informed consent was obtained from participating women under the approval of the Ethics Review Boards from Servicio de Salud Metropolitano Central and University of Chile, Santiago, Chile; in accordance with the Declaration of Helsinki. None of the patients received hormonal treatment in the 3 months prior to participating in the study. Subjects had regular menstrual cycles (26–35 days), a normal BMI (20–25 kg/m^2) and no history of endometriosis. Endometrial samples were obtained during the mid-secretory phase using a Pipelle aspirator (Cooper Surgicals, Trumbull, CT, USA) for collecting the tissue from the uterine fundus under sterile conditions. A small fragment of tissue was fixed in 4% formalin for histological dating confirmation (Noyes *et al.*, 1951) and the remainder was used for isolation of ESCs.

Cell culture

Purified ESCs were prepared and maintained as previously described (Tapia-Pizarro *et al.*, 2013). Briefly, the endometrial tissue was washed twice with phosphate-buffered saline (PBS) solution, minced into small pieces and digested for 1 h at 37°C in 0.5% collagenase type 3 (45 U/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) and DNase I (Sigma). Subsequently, the suspension was filtered through a 100-µm nylon sieve (Falcon, BD, Franklin Lakes, NJ, USA) followed by a 40-µm nylon sieve (Falcon). Stromal cells that passed through the 40-µm sieve were thoroughly washed with PBS, resuspended in DMEM/F-12 (Gibco, Grand Island, NY, USA), 10% fetal bovine serum (FBS; Biological Industries, Beit-Haemek, Israel) and 1% Antibiotic–Antimycotic (Gibco). ESC preparations were individually grown (not pooled) in plastic flasks (Trueline, Salt Lake, UT, USA) up to the third passage. They were then grown to 90% confluence and FBS-starved for 12–14 h prior to treatment with hCG. In the experiments with H-89 (10 µM), LY294002 (20 µM), UO126 (10 µM), BIM (10 µM), SQ22536 (10 µM) and BFA (1 µg/ml) cells were grown on 100 mm dishes and pretreated with the corresponding inhibitor for 30 min prior to stimulation with hCG (stock solution prepared at 1 IU/µl in ultra-pure water) or vehicle (PBS solution). In the experiments for mRNA analysis by real-time quantitative PCR (qPCR), cells were grown on 6-well plates and incubated with some of the aforementioned reagents with or without progesterone (10^{-7} M) and/or RU486 (10^{-6} M) under FBS-starved conditions. Cell culture experiments were repeated at least three times, using different individual preparations of primary ESCs, and representative experiments are presented.

Measurement of intracellular cAMP levels

Intracellular cAMP levels were determined using the ELISA kit cAMP Direct Immunoassay (BioVison Research Products, Milpitas, CA, USA). Standards and samples were acetylated and assayed in duplicate according to the manufacturer's instructions. Absorbance was read at 450 nm using a BioRad Microplate reader 680 (BioRad, Hercules, CA, USA).

Immunofluorescence

ESCs grown on coverslips were fixed with cold methanol and then incubated with blocking serum for 15 min at room temperature (1:100 dilution of non-immune mouse serum). Then, the primary antibody detecting the A and B isoforms of the PR (6A1m, Cell Signaling Technologies, Danvers, MA, USA) or LHCGR (sc-25828, Santa Cruz Biotechnologies) was applied overnight at 4°C. After a 5-min wash in PBS, secondary antibody goat anti-mouse or goat anti-rabbit conjugated to fluorescein isothiocyanate was used for PR and LHGCR detection, respectively, and applied to the coverslips for 1 h at room temperature. Finally, coverslips were rinsed and mounted with DAPI. Photomicrographs were taken with an Olympus BX51 fluorescence microscope and images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Cell culture protein extraction and immunoblot detection

Following each of the respective treatments, cell homogenates were prepared with ProteoJET lysis buffer (Fermentas, Vilnius, Lithuania) supplemented with phosphatase inhibitors and a cocktail of protease inhibitors (Sigma). Protein concentration was determined using the bicinchoninic acid method (Pierce BCA Protein Assay kit; Thermo Fisher Scientific, Rockford, IL, USA) and 25 µg was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. Electrophoresed proteins were then transferred into polyvinylidene difluoride membranes (Hybond-P; Amersham/GE Healthcare, Buckinghamshire, UK). The membranes were then blocked and incubated with the phospho-specific antibodies for pErk1/2 (SC-7383, Santa Cruz Biotechnologies) or phospho-cAMP-responsive element binding protein (pCREB, D1G6; Cell Signaling Technologies) followed by incubation with their respective horse-radish peroxidase (HRP)-conjugated secondary antibody. Erk1/2 (SC-94; Santa Cruz Biotechnologies) or CREB (48H2, Cell Signaling Technologies) was detected in parallel or on the same blots that were stripped and reprobed with the respective antibodies. Immunodetection was developed using chemiluminescence (ECL, Thermo) and images were captured with the Discovery team 10gD (Ultralum, Claremont, CA, USA). The relative intensity of the pErk1/2: total Erk1/2 ratio was quantified by optical densitometry of the bands using the Carestream MI 5.0.6 software (Carestream Health Inc., Rochester, NY, USA).

Epac knock down with siRNA

ESCs grown in 100 mm dishes or 6-well culture plates to ~60% confluency were transfected with 80 pmol/dish or 30 pmol/well of a non-targeting negative control siRNA (siRNA-A) or Epac siRNA (sc-37007 and sc-41700, respectively, Santa Cruz Biotechnology). For this, Lipofectamine RNAiMAX transfection reagent was used (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After treatment for 24 h with the siRNA, the medium containing the transfection reagents was removed and replaced with fresh medium for the following 24 h.

Real-time qPCR

The RNeasy mini kit (Qiagen, Valencia, CA, USA) was used for total RNA extraction as per the manufacturer's instructions with on column DNAse I digestion (Qiagen) to eliminate genomic DNA contamination in the eluted RNA. Total RNA (1 µg) was used for cDNA synthesis using the SuperScript III kit (Invitrogen). Gene expression levels were assessed using the QuantiTect SYBR Green PCR Kit (Qiagen) in a StepOne Real-Time PCR thermocyler (Applied Biosystems, Foster City, CA, USA). GAPDH was used for normalization, and the primer pairs used for amplification of the LHCGR splice variants *a*, *b*, *c* and *d* were obtained from Dickinson *et al.* (2009). Primers for PR, secreted frizzled-related protein 4 (SFRP4), homeobox A10 (HOXA10) and prolactin (PRL) amplification are given in Supplementary Table 1. Primers for Epac were obtained from Santa Cruz Biotechnologies (sc-41700-PR).

Statistical analysis

All data were analyzed with the software Prism 5.0 (GraphPad Software, San Diego, CA, USA). To analyze the statistical differences between the data obtained from the responses of ESCs, we performed a Kruskal–Wallis test, followed by a Dunn's *post hoc* test. Levels for the LHCGR transcript variants a and b were compared with the Wilcoxon Rank-Sum (Mann-Whitney) test. *P*-values <0.05 were considered statistically significant.

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Results

LHCGR is expressed in ESCs

We confirmed the presence of the LHCGR in ESC cultures by immunofluorescence. The specific label was mainly localized to the cell membrane and perinuclear region (Fig. 1A). Semiquantitation of the fluorescent label showed a relative standard deviation of 18.9% among three ESC preparations (Fig. 1B). The presence of the four transcript variants (*a*, *b*, *c*, *d*) for the LHCGR was studied in ESC cultures by real-time qPCR analysis. Variants *c* and *d* were not detected, whereas transcript variants *a* and *b* were found to be present, with *b* being 2.3-fold higher than *a* (Fig. 1C).

hCG augments Erk1/2 phosphorylation in ESC

We studied the phosphorylation of Erk1/2 from total homogenates of cultured ESCs treated with increasing doses of hCG (0.1, 1, 10 and 100 IU/ml) in a temporal course from 2.5 to 30 min. hCG induced the phosphorylation of Erk1 and Erk2; however, activation of the Erk2 isoform (p42 MAPK) was dominant in most of the assays. Furthermore, due to the variation in the basal level of Erk1 phosphorylation, all data are presented as the increase in combined Erk1/2 phosphorylation. The doses of 0.1 and 1 IU/ml did not show a statistically significant increase in pErk1/2, whereas 10 and 100 IU/ml of hCG revealed an upward trend for Erk1/2 phosphorylation, with levels significantly higher at 10 min compared with the baseline conditions and the 0.1 IU/ml dose (Fig. 2A). All treatments had a reduction in pErk1/2 levels after 30 min of stimulation with hCG (Fig. 2A). From this result, we used hereinafter the lowest dose of hCG that showed a significant phosphorylation of Erk1/2 (10 IU/ml). When we expanded the temporal course for pErk1/2 detection up to 160 min with hCG 10 IU/ml we confirmed a significant increase in Erk1/2 phosphorylation at 10 min of treatment with no significant changes at longer incubation times (Fig. 2B).

Effect of hCG on cAMP accumulation in ESC

To determine the signal transduction pathways activated by hCG on ESCs leading to Erk1/2 activation, we assessed cAMP accumulation in ESCs over different times of hCG treatment. We confirmed that intracellular accumulation of cAMP increased and peaked at 10 min of stimulation, decreasing dramatically at 20 min (Fig. 2C). When hCG stimulation was preceded by a 30-min incubation with the phosphodiesterase inhibitor IBMX, the increase in cAMP intracellular levels was more rapid, being significantly different from the basal level at all time points studied (Fig. 2C).

Effect of PKA, PI3K, PKC and MEK inhibition on hCG-induced Erk1/2 phosphorylation in ESC

In gonadal cells, some LHCGR-dependent effects are mediated by activation of the cAMP/PKA pathway. Since Erk1/2 have been shown

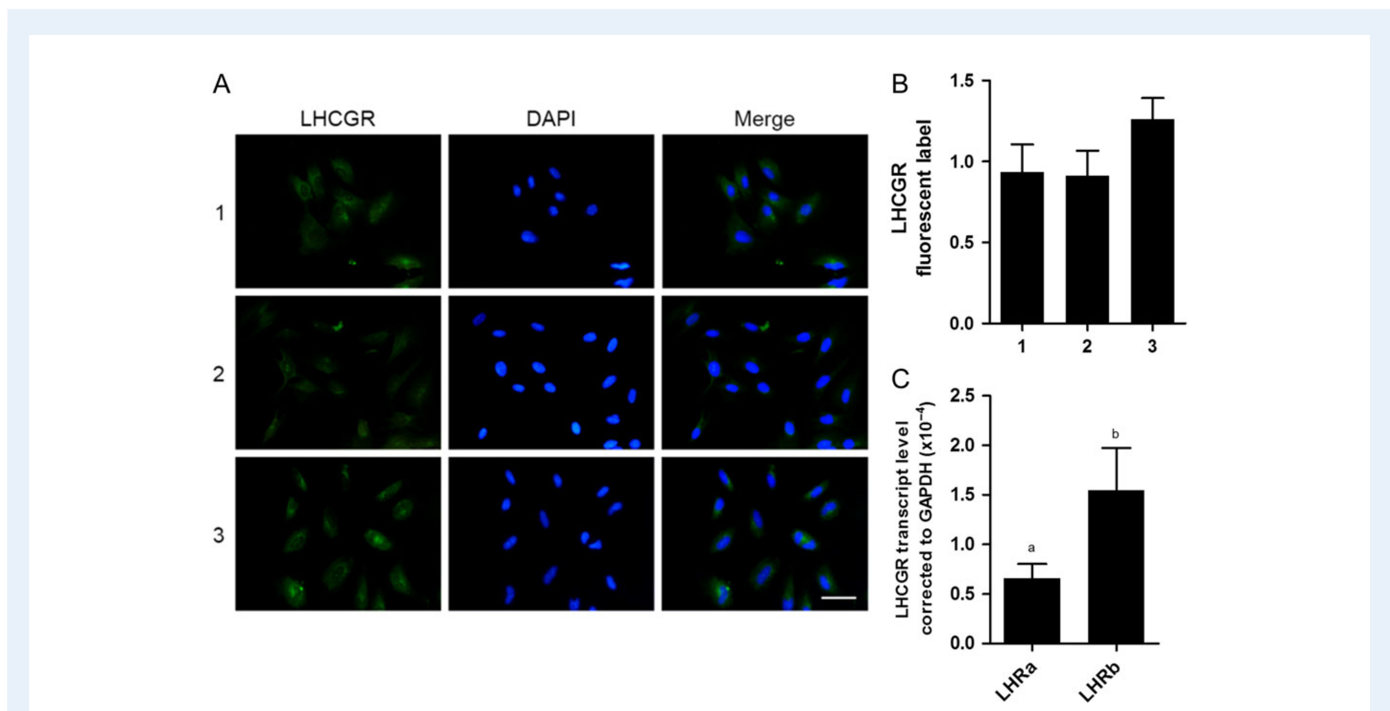

Figure 1 LH/CG receptor (LHCGR) is expressed in primary human endometrial stromal cells (ESCs) in culture. (**A**) Immunofluorescent detection for LHCGR in human ESCs obtained from different women during the secretory phase ($n = 3$). Positive labeling (green) at the plasma membrane and perinuclear region was observed. The nuclei were visualized with DAPI (blue). No staining for LHCGR was observed when the primary antibody was omitted. Scale bar in the lower right immunofluorescent image = 10 µm, applies to all images. (**B**) Semi-quantitative analysis of the fluorescence level in the three individual cultures shown in (**A**). Data are mean ± SD from 100 cells per sample. (**C**) Analysis of the transcript variants *a* and *b* of the LHCGR in ESCs by real-time quantitative PCR (qPCR). Data are mean ± SD; $n = 7$; $a \neq b$, $P < 0.05$, Wilcoxon test.

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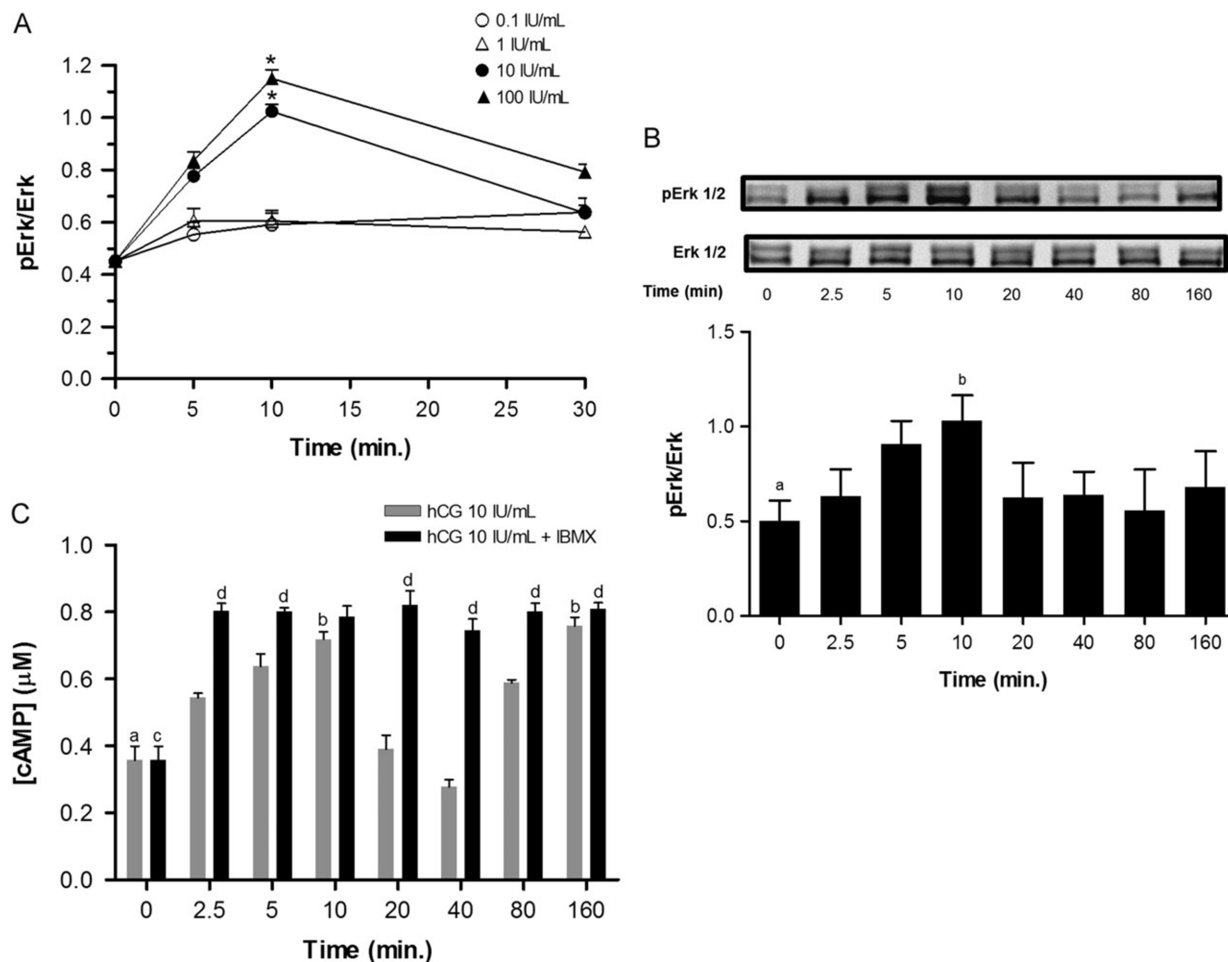

Figure 2 hCG stimulates Erk1/2 phosphorylation and cyclic AMP (cAMP) accumulation in human ESCs *in vitro*. (**A**) Dose–response curve for phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) in ESCs incubated in the presence of hCG 0.1 (○), 1 (Δ), 10 (●) or 100 (▲) IU/ml. ESCs were serum-starved overnight and then stimulated with hCG at 37°C for the indicated periods of time. Erk1/2 phosphorylation was detected by immunoblot and the relative intensities of pErk1/2:total Erk1/2 ratio quantitated by densitometry. Data are mean ± SD, $n = 3$. *$P < 0.05$ versus basal and versus 0.1 and 1 UI/ml hCG at 10 min. (**B**) Erk1/2 phosphorylation kinetics in ESCs stimulated with 10 IU/ml hCG from 0 to 160 min. pErk1/2:total Erk1/2 ratio from densitometric values of respective bands obtained by western blot is presented mean ± SD, $n = 3$; $a \neq b$, $P < 0.05$. The immunoblot shown is representative of the experiment summarized in the graph below it. (**C**) Time-course accumulation of intracellular cAMP in ESCs stimulated with hCG (10 IU/ml) with or without the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) , $a \neq b$, $c \neq d$, $P < 0.05$, Kruskal–Wallis test.

to be activated *via* PKA and other signaling routes including PI3K and PKC (Kolch *et al.*, 1993; Lowes *et al.*, 2002; El Haibi *et al.*, 2010), we investigated the possible contribution of these pathways to the phosphorylation of Erk1/2 by hCG in cultured ESC. We evaluated the participation of PKA by incubating the ESCs with H-89 prior to treatment with hCG. This failed to reverse the activating effect exerted by hCG on Erk1/2 after 10 min of treatment (Fig. 3A). We also investigated the involvement of PI3K and PKC-mediated pathways using the inhibitors LY294002 and BIM, respectively, and these also failed to block pErk1/2 induction by hCG (Fig. 3B and C). The co-incubation of hCG with H-89, LY294002 or BIM did not reach a pErk1/2 level that was statistically different from the control condition, suggesting that PKA, PI3K and PKC might also contribute to pErk1/2 induction. Finally, to confirm the canonical Ras-mediated activation pathway for Erk1/2 (Marshall, 1996), we employed a selective MEK1/2 inhibitor. Pretreatment of ESCs with UO126 prior to 10 IU/ml of hCG stimulation prevented the stimulatory effect of the hormone on Erk1/2 phosphorylation ($P < 0.05$, Fig. 3D).

Intracellular cAMP accumulation is required for hCG-induced activation of Erk1/2

Since both cAMP accumulation and pErk1/2 peak after 10 min of hCG stimulation, we investigated whether the hCG-induced increase in cAMP intracellular levels was associated with Erk1/2 activation. The adenylyl cyclase/cAMP/PKA pathway typically results in CREB phosphorylation, which is one of the major nuclear targets of PKA. As readout for the cAMP response, we determined pCREB levels induced by hCG (Fig. 4A). The pre-incubation of ESCs with the adenylyl cyclase inhibitor SQ22536 prior to stimulation with hCG resulted in decreased

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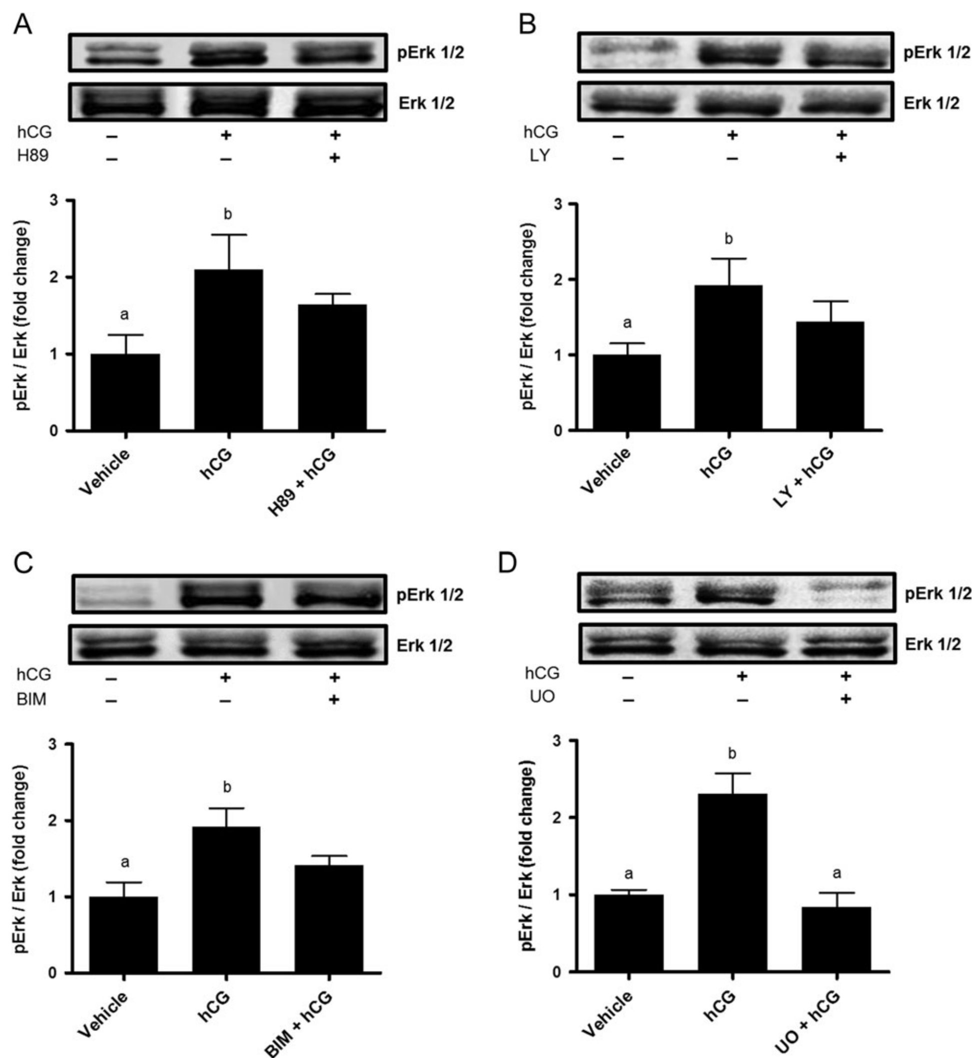

Figure 3 Effect of pretreatment with (**A**) H-89 (protein kinase A (PKA) inhibitor, 10 µM), (**B**) LY294002 (LY, PI3K inhibitor, 10 µM), (**C**) bisindolylmaleimide I (BIM, PKC inhibitor, 10 µM) and (**D**) UO126 (UO, mitogen-activated protein kinase kinase (MEK) inhibitor, 10 µM) on the phosphorylation of Erk1/2 by hCG in ESCs. Cells were pretreated for 30 min with the corresponding inhibitor and then stimulated for 10 min with hCG (10 IU/ml) or vehicle (phosphate-buffered saline, PBS). Values are mean ± SD, $n = 3$; $a \neq b$, $P < 0.05$, Kruskal–Wallis test.

CREB phosphorylation (Fig. 4A). Likewise, SQ22536 pre-incubation significantly reduced pErk1/2 (Fig. 4B).

Epac mediates hCG activation of Erk1/2 in ESC

Since PKA does not seem to play a major role in mediating Erk1/2 phosphorylation induced by hCG, we investigated whether cAMP guanine nucleotide exchange factor or Epac could result in Erk1/2 activation in ESCs upon hCG stimulation. The elements of the Epac-pathway mediating cAMP effects have been described in human ESCs and shown to be functional (Kusama *et al.*, 2013). Incubation with the Epac-specific cAMP analog 8pCPT (Enserink *et al.*, 2002) induced Erk1/2 phosphorylation in ESCs (Fig. 4C). BFA has been shown to antagonize the effects of 8pCPT at the crayfish neuromuscular junction, presumably by blocking Epac-mediated effects (Zhong and Zucker, 2005). Pretreatment of ESCs with BFA prior to 10 IU/ml of hCG stimulation completely reversed the stimulatory effect of the hormone on Erk1/2 phosphorylation (Fig. 5A). In addition, we induced a knockdown of Epac in ESCs with siRNA (Fig. 5C) and assayed the effects of hCG on Erk1/2 activation. hCG- and 8pCPT-induced Erk1/2 phosphorylation was completely abrogated when Epac was knocked down (Fig. 5B).

Effect of hCG-activated Erk1/2 on PR expression

We studied the regulation of the PR upon hCG stimulation in ESCs at 24, 48 and 72 h. PR transcript was detected in all the conditions studied (Fig. 6A–C) and showed an up-regulation in the hCG group at 48 h; an effect that was reversed in the presence of UO126 (Fig. 6B). The immunofluorescent detection of PR in ESCs showed that the label was

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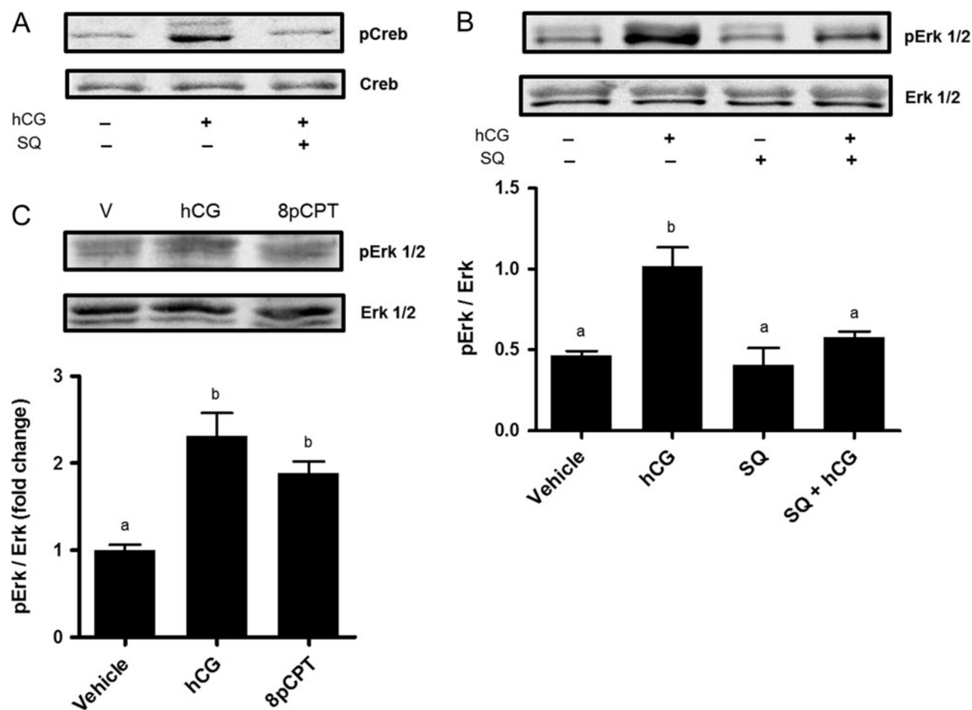

Figure 4 cAMP accumulation is required for hCG-induced Erk1/2 phosphorylation in ESC. The adenylyl cyclase inhibitor SQ22536 (SQ) reduced the hCG-induced phosphorylation of (**A**) the cAMP-responsive element binding protein (Creb, $n = 2$) and (**B**) Erk1/2; $n = 3$, $a \neq b$, $P < 0.05$. (**C**) The cAMP analog 8pCPT-2′-O-Me-cAMP (8pCPT), specific for the activation of exchange protein activated by cAMP (Epac), induced Erk1/2 phosphorylation in ESC; $n = 3$; $a \neq b$, $P < 0.05$, Kruskal–Wallis test.

present in the cytoplasm and nucleus (Fig. 6D and E). Immunoreactive PR levels increased significantly only at 48 h upon hCG stimulation and again, this increase was inhibited when Erk1/2 activation was blocked with UO126 (Fig. 6D and E). The fluorescence intensity also decreased below the basal level (set to 1.0) with hCG in the presence of UO126 at 72 h (Fig. 6E).

Effect of hCG-activated Erk1/2 on the transcript levels for PRL, HOXA10 and SFRP4 in ESC

We studied the effect of hCG on the mRNA expression levels of genes that have been described to be regulated by hCG and/or that have been associated with endometrial receptivity: PRL (Telgmann and Gellersen, 1998; Matsumoto *et al.*, 2008), HOXA10 (Taylor *et al.*, 1998; Lim *et al.*, 1999) and SFRP4 (Sherwin *et al.*, 2007; Tapia *et al.*, 2011). The transcript levels for PRL were not significantly changed by hCG at 24 or 48 h (Fig. 7A). UO126 decreased PRL mRNA significantly at 24 and 48 h, an effect that was also observed when it was co-incubated with hCG but only at 48 h (Fig. 7A). HOXA10 mRNA levels increased significantly at 48 h with hCG, an effect that was reversed in the presence of UO126 (Fig. 7B). On the other hand, transcript levels for SFRP4 significantly decreased at 24 h but not at 48 h, and co-incubation of hCG with UO126 showed no significant changes at 24 h (Fig. 7C).

Effect of progesterone and RU486 on HOXA10 transcript regulation by hCG in ESC

The up-regulation of the PR by hCG in ESCs suggests that the responsiveness of the cells to progesterone might be increased. HOXA10 transcript levels have been shown to be regulated in human endometrial tissue by both hCG and progesterone (Gui *et al.*, 1999; Fogle *et al.*, 2010). We studied the transcript levels for HOXA10 in ESCs under the effects of hCG in combination with progesterone and/or RU486. HOXA10 mRNA levels increased significantly at 48 h with hCG or a combination of hCG and progesterone (Fig. 8), an effect that was reversed in the presence of RU486 (Fig. 8). No additive or synergistic effect in HOXA10 mRNA up-regulation was observed with the incubation of hCG and progesterone (Fig. 8).

Discussion

In the present study, we have shown that hCG activates the phosphorylation of Erk1/2 through the adenylyl cyclase/cAMP/PKA pathway in human ESC, regulating gene expression and progesterone responsiveness. We confirmed the presence of the LHCGR in ESCs (Reshef *et al.*, 1990; Han *et al.*, 1997) and determined that splice variants *a* and *b* are the most abundant. Both LH and hCG signal in gonadal tissue in several species including human through the

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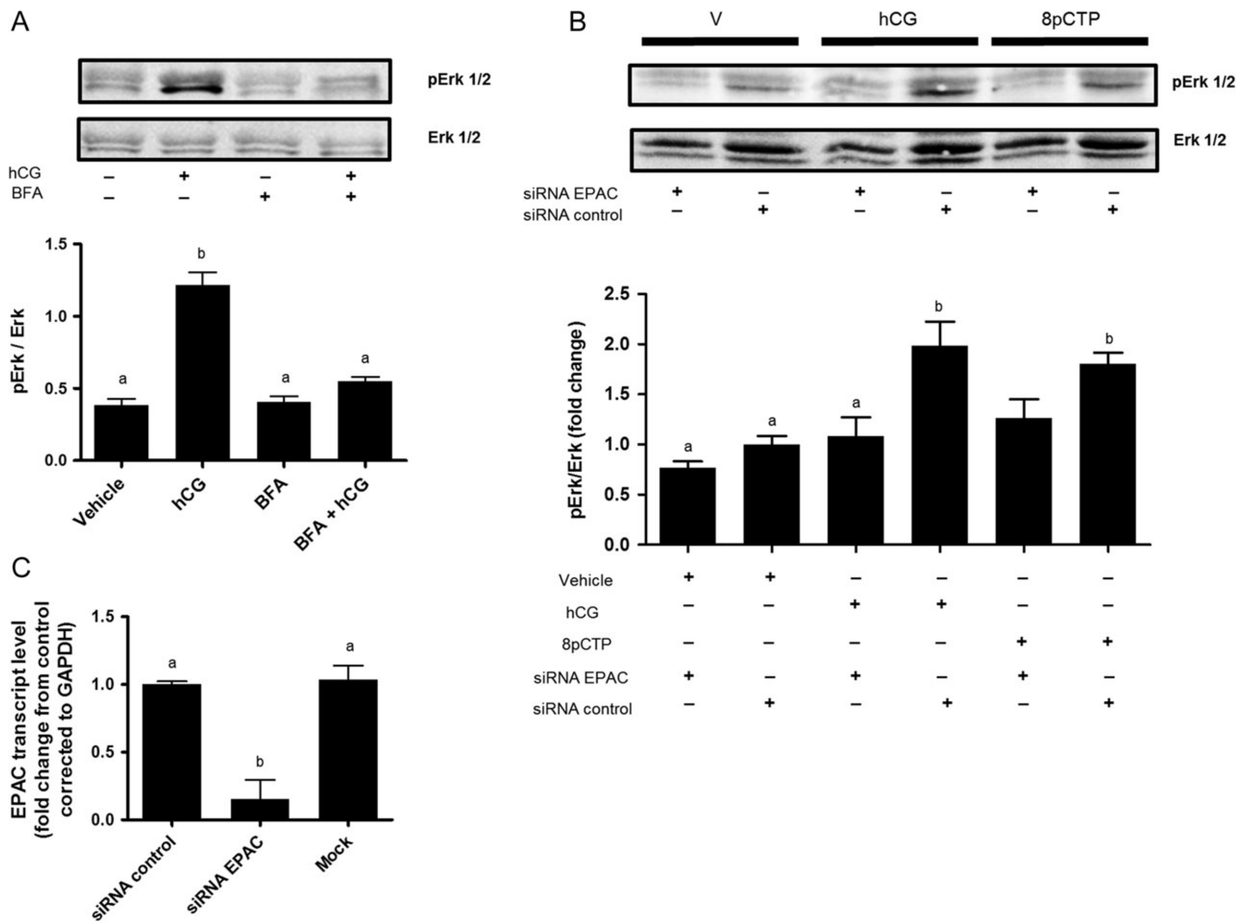

Figure 5 Effect of brefeldin-A (BFA) on Erk1/2 phosphorylation in ESCs with or without Epac knockdown with siRNA. (**A**) ESCs were pretreated with BFA and then stimulated for 10 min with hCG 10 IU/ml for pErk1/2 evaluation; $n = 3$, $a \neq b$, $P < 0.05$. (**B**) Epac knockdown with siRNA significantly reduced pErk1/2 induced by hCG in ESC; $n = 3$, $a \neq b$, $P < 0.05$. (**C**) Knockdown of Epac with siRNA efficiently reduced its mRNA transcript levels in ESCs analyzed by qPCR; $n = 3$, $a \neq b$, $P < 0.05$, Kruskal–Wallis test. A nonsilencing siRNA-transfected (siRNA control), mock-transfected (Mock, all reagents except for siRNA) and cells without treatment (PBS vehicle) were used as controls.

LHCGR, activating the Gs/adenylyl cyclase/cAMP/PKA and inositol phosphate/PKC pathways (Ascoli *et al.*, 2002). However, in human and baboon EEC, hCG does not signal through the adenylyl cyclase/cAMP/PKA but instead it activates the phosphorylation of Erk1/2 leading to an increase in prostaglandin E_2 secretion (Srisuparp *et al.*, 2003; Banerjee *et al.*, 2009). Here we show that unlike in EECs, intracellular cAMP levels increased upon hCG stimulation in ESCs, and this increase seems to occur by adenylyl cyclase activation and not through inhibition of phosphodiesterases, as reported previously (Chatterjee *et al.*, 1997). In addition, we demonstrated that pErk1/2 induced by hCG was dependent on increasing cAMP but independent of PKA activation. Intracellular changes in cAMP can also activate other proteins besides PKA such as Epac/Rap (Kiermayer *et al.*, 2005). This family of cAMP effector proteins is composed of two members, which are guanine exchange factors for the monomeric G-proteins Rap1 and Rap2 (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998a, b). Epac knockdown significantly reduced pErk1/2 induced by hCG in ESC.

We also found that hCG transiently increased PR expression in ESCs and this was dependent on Erk1/2 phosphorylation. Likewise, the hCG-induced up- and down-regulation of mRNA levels for HOXA10 and SFRP4, respectively, was also mediated by Erk1/2. The transient increase in PR expression in response to hCG may increase the effects of progesterone in ESCs and thus contribute to the described potentiation that hCG exerts on progesterone signaling in the endometrium (Sherwin *et al.*, 2007; Sherwin *et al.*, 2010). Intrauterine administration of hCG in women 3 days after oocyte retrieval during a stimulation cycle for oocyte donation increased the expression of the endometrial PR, evaluated 48 h later (Strug *et al.*, 2016), which is in line with the PR regulation we observed by immunofluorescence. Other morphological and molecular changes have been reported in the human and baboon endometrium after *in vivo* infusion of hCG to the uterine cavity (Fazleabas *et al.*, 1999; Sherwin *et al.*, 2007; Strug *et al.*, 2016). Such responses to hCG are compromised when progesterone effects are blunted by either a PR antagonist (Banaszak *et al.*, 2000) or a pathologic condition (Sherwin *et al.*, 2010).

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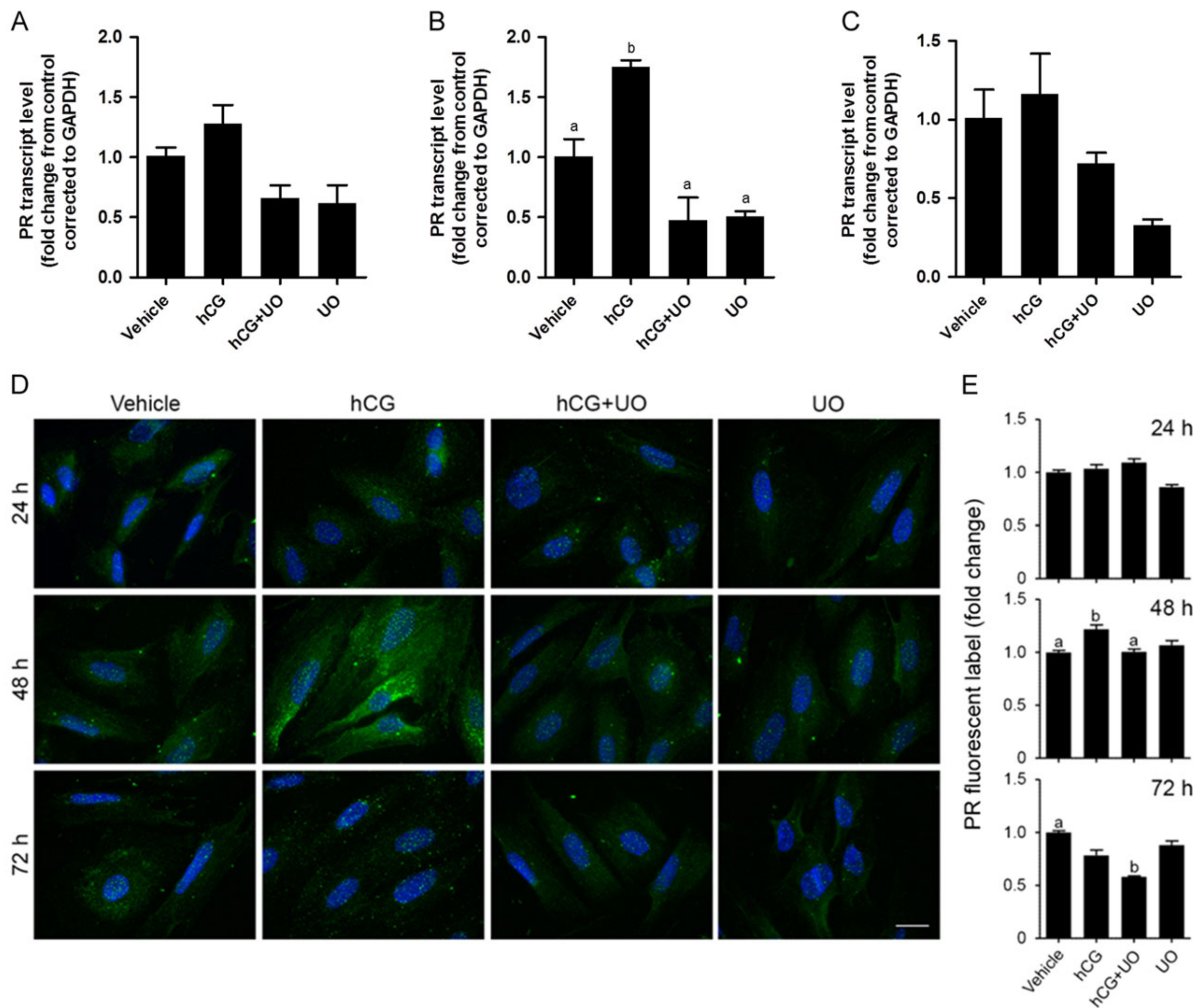

Figure 6 hCG modulates progesterone receptor (PR) expression through Erk1/2 in ESC. mRNA transcript level for PR in ESCs stimulated with hCG (10 IU/ml) and/or UO126 (10 μM) after 24 (**A**), 48 (**B**) and 72 h (**C**); data are mean ± SD, $n = 3$, $a \neq b$, $P < 0.05$. (**D**) Representative images for the immunofluorescent detection of PR in ESCs stimulated with hCG and/or UO126 after 24, 48 and 72 h. Positive labeling (green) at the cytoplasm and nucleus was observed. Nuclei were counterstained with DAPI (blue). Scale bar in the lower right immunofluorescent image = 10 μm, applies to all images. (**E**) Semiquantitation of the fluorescent label for PR at the respective incubation times and conditions, data are mean ± SE, $n = 4$, $a \neq b$, $P <$ 0.05, Kruskal–Wallis test.

In addition, several genes are co-regulated in the endometrium by hCG and progesterone (Sherwin *et al.*, 2007), supporting the co-operative effects of these hormones in the endometrium. The non-sustained increase of PR upon hCG stimulation in ESCs we observed *in vitro* may suggest that the gonadotropin is insufficient to induce endometrial functional differentiation, requiring the presence of ovarian steroids and/or supplementary stimulating effects from other cells (Pawar *et al.*, 2015).

In women, HOXA10 is up-regulated in the receptive endometrium and in ESCs stimulated with progesterone *in vitro* (Taylor *et al.*, 1998). In the mouse, *Hoxa10* regulates ESCs responsiveness to progesterone leading to adequate decidualization and successful pregnancy (Benson *et al.*, 1996; Lim *et al.*, 1999), which could be another level of regulation of the progesterone response induced by hCG. SFRP4 belongs to the SFRP family and is a glycoprotein that contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzled proteins. SFRPs act as a soluble antagonist of Wnt ligands, thereby attenuating the canonical/beta-catenin-Wnt signaling pathway. SFRP4 has been implicated in cell proliferation, differentiation and apoptosis (Goldsmith and Dhanasekaran, 2007). Endometrial SFRP4 expression is regulated by the ovarian steroids and may regulate uterine morphology and function (Jasinska *et al.*, 2006; Yotova *et al.*, 2011). In human endometrium, SFRP4 is restricted to the stroma and its expression has been shown to peak during the proliferative phase and decreases in the secretory phase (Abu-Jawdeh *et al.*, 1999; Tulac *et al.*, 2003; Tapia *et al.*, 2011) and decidualized endometrium (Duncan *et al.*, 2011), suggesting that progesterone induces its down-regulation. We have shown that SFRP4 transcript is also diminished in response to hCG in

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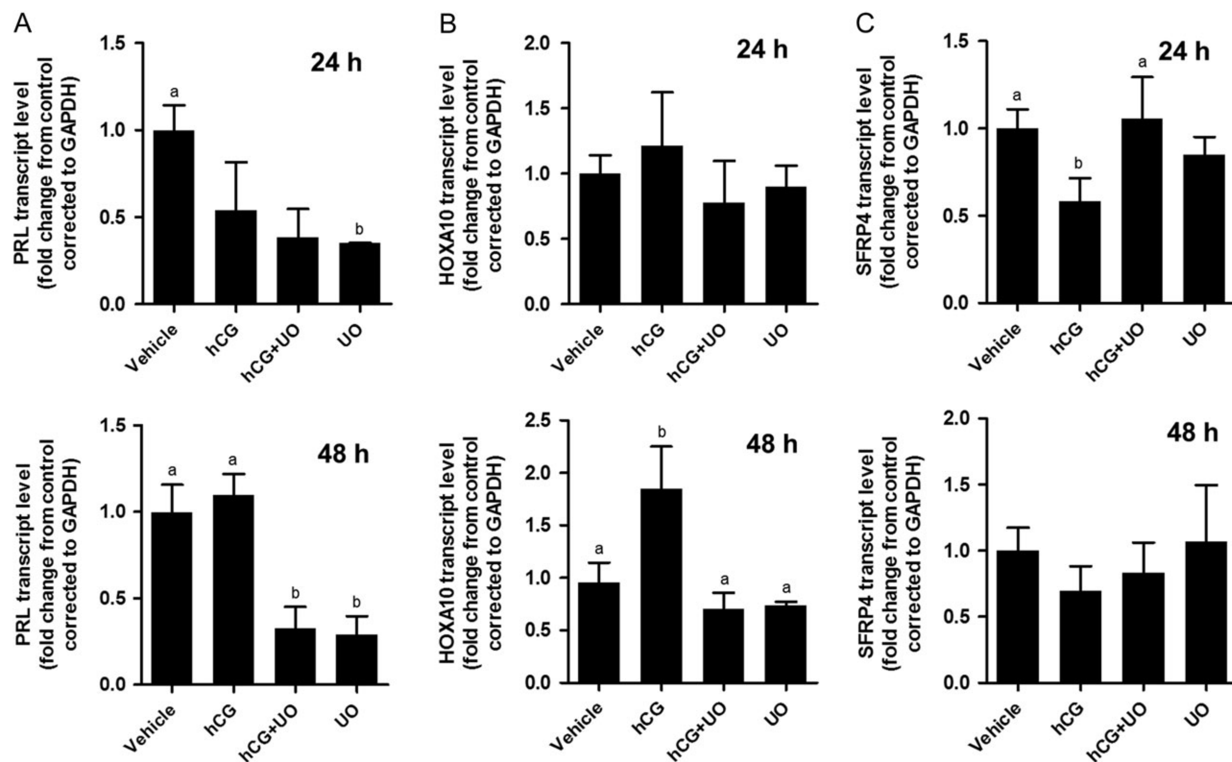

Figure 7 hCG regulates gene expression through Erk1/2 in ESC. mRNA levels for (**A**) prolactin (PRL), (**B**) Homeobox A10 (HOXA10) and (**C**) Secreted frizzled-related protein 4 (SFRP4) in ESCs stimulated with hCG (10 IU/ml) and/or UO126 after 24 or 48 h of incubation; data are mean ± SD, $n = 3$, $a \neq b$, $P < 0.05$, Kruskal–Wallis test.

ESCs through the Erk1/2 signaling pathway, which is in line with the decreased expression of SFRP4 induced by hCG in baboon endometrium *in vivo* (Sherwin *et al.*, 2007, 2010). hCG and progesterone, both physiologically essential hormones during embryo implantation and also used as luteal phase support in ART cycles, have been shown to reduce endometrial apoptosis *in vitro* and *in vivo* (Lovely *et al.*, 2005; Jasinska *et al.*, 2006). The observed decrease of SFRP4 expression upon hCG stimulation in ESCs has been proposed as a possible mechanism by which hCG could prevent endometrial apoptosis in the presence of an implanting embryo (Sherwin *et al.*, 2007) that otherwise will take place during the late secretory phase of a non-conceptional cycle (Vaskivuo *et al.*, 2000).

In ESC, hCG signaling induces an increase in intracellular cAMP as well as many other peptide hormones and prostanoids that promote decidualization (Gellersen and Brosens, 2003). However, we did not detect significant increases in PRL transcript levels after 72 h of incubation with hCG. On the contrary, PRL mRNA levels tended to decrease at 24 h, although not significantly. The failure in PRL induction may reflect that progesterone or a sustained PKA activation with cAMP analogs is required for initiating decidualization. When ESCs are stimulated for 48 h with the Epac-selective cAMP analog 8pCPT, mRNA for PRL and insulin-like growth factor binding protein-1 are not induced; however, the expression of decidualization markers is potentiated in the presence of either a PKA-selective cAMP analog, or estrogen and progesterone (Kusama *et al.*, 2013). While studies have revealed conflicting data on the decidualizing potential of LH/hCG (Tang and Gurpide, 1993; Kasahara *et al.*, 2001), the *in vitro* evidence suggest that Epac activation is not enough to induce decidual differentiation, and this requires a continuous activation of the PKA pathway.

Erk1/2 signaling is important for ESC function (Lee *et al.*, 2013) and abnormally increased levels of pErk1/2 have been found in ESCs derived from women with endometriosis with a blunted response to cAMP in cell-cycle regulation and decidualization (Klemmt *et al.*, 2006; Velarde *et al.*, 2009; Yotova *et al.*, 2011). Immunoreactive pErk1/2 increased in the endometrial epithelium but not the stromal compartment after 48 h of intrauterine infusion of hCG in women under a stimulation cycle for oocyte donation 3 days after oocyte retrieval (Strug *et al.*, 2016). We did not evaluate pErk1/2 in ESCs after 48 h of stimulation with hCG but our temporal course experiments showed that pErk1/2 levels beyond 10 min and up to 160 min were not significantly different from the basal condition.

We found that hCG stimulates Erk1/2 phosphorylation through Epac in ESCs which, in turn, regulates the expression of genes relevant for endometrial function. Such phospho-Erk1/2 induction was not dependent on PKA activation as was shown for EEC (Srisuparp *et al.*, 2003). Also, the kinetics of Erk1/2 phosphorylation and the effects on PRL transcript regulation induced by hCG are not consistent with those observed in ESCs stimulated with cAMP or cAMP analogs (Yoon *et al.*, 2005; Kusama *et al.*, 2013). Such differences could be explained by the transient nature of the signaling induced by hCG or by

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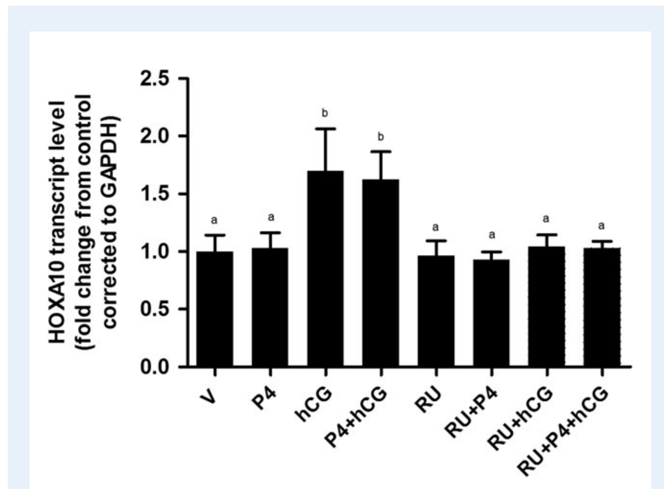

Figure 8 hCG-dependent induction of HOXA10 transcript levels in ESCs requires the PR function. mRNA levels for HOXA10 in ESCs stimulated with hCG (10 IU/ml) and/or progesterone (P4, 10^{-7} M) in the presence or absence of RU486 (RU, 10^{-6} M) after 48 h of incubation; data are mean ± SD, $n = 3$, $a \neq b$, $P < 0.05$, Kruskal–Wallis test. V: PBS vehicle.

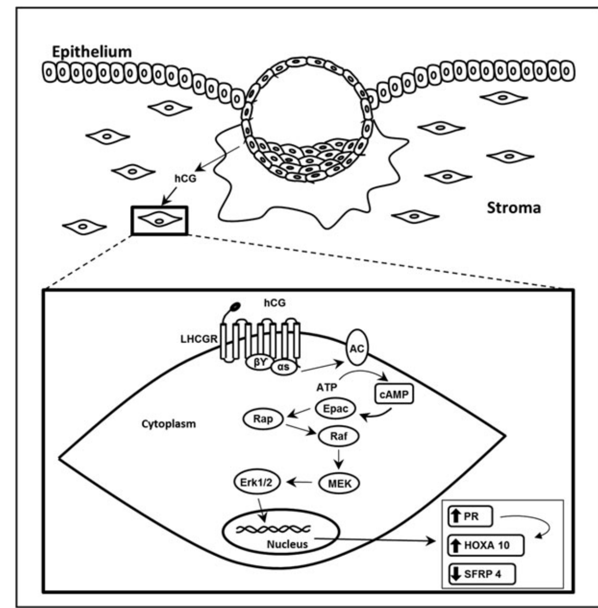

Figure 9 Schematic representation of hCG effects on human ESCs. hCG induces cAMP intracellular accumulation leading to Erk1/2 phosphorylation through Epac leading to a transient increase of PR and regulation of genes related to endometrial receptivity. AC, adenylyl cyclase; Rap, Ras-related GTP-binding protein; Raf, Rapidly accelerated fibrosarcoma protein.

compartmentalization of signaling molecules and enzymes. The functional confinement of signaling pathways has been shown to provide a mechanism by which unique responses can be attributed to the activation of distinct receptors (Ellisdon and Halls, 2016). As such, signal compartmentalization in ESCs could be a cellular determinant that may decide whether Epac and/or PKA will be activated by cAMP, resulting in differential responses in ESC. In addition, ligand-activated GPCR can associate with a diversity of other signal transduction proteins (Luttrell, 2008) and can cross-communicate (Natarajan and Berk, 2006), thereby diversifying the intracellular signaling pathways.

To investigate the functionality of the hCG-induced PR in ESC, we assessed how progesterone and the antagonist RU486 affected the transcript levels of HOXA10. Steady-state mRNA levels for HOXA10 increased significantly at 48 h with hCG but not with progesterone. Interestingly, such an increase was prevented with RU486, suggesting that the HOXA10 transcript levels are modulated by hCG through the PR. Ultimately, the gonadotropin might not only be inducing PR but also directly or indirectly recruiting it for gene expression regulation. The lack of an enhanced effect on HOXA10 transcript up-regulation at 48 h when hCG is combined with progesterone suggests that a longer period of stimulation or the presence of additional factors/hormones or cell context is required (Gui *et al.*, 1999; Fogle *et al.*, 2010; Palomino *et al.*, 2013).

In conclusion, our study showed that hCG activated the Erk1/2 signal transduction pathway in ESCs, which transiently modulates PR expression and function relevant to embryo implantation. A schematic of the possible pathways involved is shown in Fig. 9. In addition, this study demonstrates the possible roles of Epac-mediated cAMP signaling in human endometrium. The embryo-derived hCG might facilitate progesterone effects in ESCs by regulating gene targets of progesterone and PR signaling.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

A.T.-P. conceived the study, analyzed the data and wrote the manuscript. R.G.-R. assisted with sample collection and contributed with data analysis. S.A., F.A., C.V. and K.Z. performed the experiments and analyzed the data. M.C.J. and L.D. helped with the study design, provided critical comments and revised the manuscript.

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Conflict of interest

None declared.

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