REPRODUCTION
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Pigment epithelium-derived factor regulation by human chorionic gonadotropin in granulosa cells

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Abstract

Human chorionic gonadotropin (hCG) is a known trigger of ovarian hyperstimulation syndrome (OHSS), a potentially life-threatening complication of assisted reproduction. Administration of hCG results in the release of vascular endothelial growth factor (VEGF) from the ovary. We have previously shown that expression of pigment epithelium-derived factor (PEDF) in granulosa cell line is regulated by hCG, reciprocally to VEGF, and that the PEDF–VEGF balance is impaired in OHSS. Our aim was to explore the signaling network by which hCG downregulates the expression of PEDF mRNA and protein in granulosa cells. We applied specific chemical inhibitors and stimuli to human primary granulosa cells and rat granulosa cell line. We found that PKA and protein kinase C, as well as EGFR, ERK1/2 and PI3K, participate in the signaling network. The finding that hCG-induced PEDF downregulation and VEGF upregulation are mediated by similar signaling cascades emphasizes the delicate regulation of ovarian angiogenesis.

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Introduction

The physiological functions of the female ovaries are hormonally controlled, undergoing in each cycle tissue modifications, such as angiogenesis, that are regulated by steroid hormones (Reynolds & Redmer 1998). The ovary responds to the mid-cycle surge of luteinizing hormone (LH) by ovulation of mature oocytes followed by development of extensive microvasculature in the follicular wall and in the granulosa cells layer, leading to corpus luteum (CL) formation. If pregnancy does not occur, ovarian angiogenesis regresses (Reynolds & Redmer 1998). Vascular endothelial growth factor (VEGF) is a key regulator of ovarian vasculature (Mauro *et al.* 2014) upregulated by LH in granulosa cells (Herr *et al.* 2013).

Pigment epithelium-derived factor (PEDF) is a secreted 50-kDa glycoprotein that belongs to the non-inhibitory members of the serine protease inhibitors (serpin) and is defined as a natural angiogenesis inhibitor with neurotrophic and immune-modulating properties (Crawford *et al.* 2013). The anti-angiogenic effect of PEDF was extensively investigated, showing that it can inhibit the stimulatory activity of several strong pro-angiogenic factors, and that it can hinder endothelial cell activity, both directly and indirectly, by inhibiting VEGF (Becerra & Notario 2013). We reported that PEDF is expressed in human and rodent ovaries, produced and secreted by granulosa cells in a hormonally dependent manner. The secreted PEDF exerts an anti-angiogenic effect, as demonstrated by *in vitro* inhibition of proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVECs) (Ninio-Many *et al.* 2013). We have further demonstrated that human chorionic gonadotropin (hCG), an LH analog, causes a decrease in the expression of PEDF, both *in vivo* and *in vitro* and that like in other tissues, the expression of PEDF is inverse to that of VEGF (Ninio-Many *et al.* 2013). Our preliminary studies indicated that PEDF–VEGF homeostasis is significant for maintaining intact ovarian function. This dynamic balance was shown to be modulated, at least in part, by hCG (Chuderland *et al.* 2013).

Several reproductive disorders are associated with impaired ovarian angiogenesis; two distinct ones are: polycystic ovarian syndrome (PCOS) that is characterized by an excessive formation of blood vessels (Gomez *et al.* 2011) and ovarian hyperstimulation syndrome (OHSS; Soares *et al.* 2008). OHSS is triggered by administration of hCG and is associated with unrestrained formation of blood vessels, as well as by increased capillary permeability; VEGF is considered as the main factor that causes increased vascular

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permeability and as an OHSS hallmark in humans and rodents (Soares *et al*. 2008). We have previously shown an impaired angiogenic balance in OHSS, where ovarian VEGF expression was high and PEDF expression low (Chuderland *et al*. 2013).

Whereas the signal transduction that governs hCG-induced VEGF biosynthesis is well documented and is known to be mediated by several factors, such as protein kinase C (PKC; Sriraman *et al*. 2008), PKA (van den Driesche *et al*. 2008), and amphiregulin (AREG; Karakida *et al*. 2011), hCG-mediated PEDF down-regulation is not yet investigated. In the current study, we report the involvement of PKA and PKC as well as AREG and the downstream effectors ERK1/2 and PI3K, in the hCG-mediated PEDF downregulation.

Materials and methods

Study design

We used rat granulosa cell line (LH-15 cells) or primary human granulosa cells (PHGC) obtained from women undergoing IVF treatments.

Cell culture

LH-15 cells (a gift from Prof. A. Amsterdam, Weizmann Institute of Science, Israel; Suh *et al*. 1992) were cultured in hormone-free DMEM/Ham F12 1:1 (DMEM-F12) supplemented with penicillin and streptomycin (100 IU/ml and 100 mg/ml respectively; Biological Industries, Beit-Ha'emek, Israel) and 10% Charcoal-stripped fetal bovine serum (CS-FBS; Invitrogen; culture medium). Cells were serum-starved for 16 h (0 or 0.1% CS-FBS) prior to and during the various stimulations. PHGC (Helsinki IRB approval 167/09*1; AssafHarofeh Medical Centre, Israel) were obtained from women who fulfilled the following criteria: 22–38 years of age, infertility due to male factor, and retrieval of 4–15 oocytes per woman. Patients were treated according to the long protocol guidelines. PHGC were isolated from aspirated follicular fluids after oocyte retrieval. The follicular fluid was centrifuged at 300 ***g*** for 5 min at room temperature. The resulting pellets were re-suspended in 10 mM Tris, 0.84% NH_4Cl, pH 7.4, to cause lysis of blood cells (15 min shaking at 37 °C) and were washed several times in PBS to eliminate debris. Cells were counted before seeding in order to reach equal confluence and make sure there is no contamination by leukocytes, plated in culture medium and washed every 24 h. On the fourth day after isolation, cells were serum-starved (0.1% CS-FBS) for 16 h prior to and during the various stimulations (Ninio-Many *et al*. 2013).

Activators and inhibitors

Activators – serum-starved (0 and 0.1% CS-FBS) LH-15 cells and PHGC, respectively, were incubated with i) PKC activator – Phorbol 12-myristate 13-acetate (PMA; P1585, Sigma; dissolved in DMSO), for 1 h; ii) human amphiregulin (AREG; 10558-HNAE, Sino Biological, Inc., Beijing, People's Republic China; dissolved in DDW) that interacts with the epidermal growth factor receptor (EGFR) and promotes cell growth, for 4 (LH-15 cells) or 16 (PHGC) hours. PEDF level (protein and mRNA) was detected at the end of the incubation period. Inhibitors – serum-starved (0.1% CS-FBS) LH-15 cells were incubated for 30 min with i) selective PKC inhibitor GF 109203X (GF, G2911, Sigma); ii) potent cAMP-dependent protein kinase inhibitor H89 dihydrochloride hydrate (H89, B1427, Sigma); iii) MEK (MKK1; MAPK kinase) inhibitor PD184352 (PD, PZ0181 Sigma); iv) phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (W1628, Sigma) or v) EGFR inhibitor AG18 (AG; S8009, selleckchem.com, Houston, TX, USA). All inhibitors were dissolved in DMSO. Culture media were replaced after stimulation with fresh starvation media (0.1% CS-FBS), supplemented with hCG (1 IU) for additional 6 h of incubation, after which PEDF level (protein and mRNA) was detected.

Western blot analysis

Proteins of granulosa cells were separated by SDS–PAGE (10%; Bio-Rad) and transferred onto nitrocellulose membranes (Whatman GmbH, Wendlingen am Neckar, Germany) in a mini tank transfer unit (TE 22, Amersham, UK). Approximate molecular masses were determined by comparison with the migration of pre-stained protein standards (Bio-Rad). Blots were blocked for 1 h in TBST (10 mM Tris–HCl, pH = 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% skim-milk (Alba, NY, USA) followed by an overnight incubation at 4 °C with primary antibodies. Primary antibodies: anti-PEDF (sc-25594; Santa Cruz Biotechnology) and anti-actin (MAB1501, Millipore, Temecula, CA, USA). Blots were washed three times in TBST and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. Secondary antibodies: HRP-conjugated monoclonal and polyclonal antibodies (Jackson Immunoresearch, West Grove, PA, USA). Immunoreactive bands were visualized by ECL (Thermo Scientific, Waltham, MA, USA) according to manufacturer's guidelines. Intensity of the protein bands was quantified by the open source software - Image J Software (NIH).

RNA isolation, reverse transcription, PCR, and real-time PCR (qPCR)

Total RNA was isolated from granulosa cells, using Trizol reagent (Invitrogen) according to manufacturer's instructions, and quantified with the Nano-Drop spectrophotometer (ND-1000; Thermo Scientific). First-strand cDNA was created by RT (Maxima TM Reverse transcriptase, Thermo Scientific) from a total of 1 μg RNA, using oligo-dt primers (Thermo Scientific). DNA was amplified using 1 μl RT reaction and 50 pmol gene-specific primers in ReadyMix mixture (Sigma). PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Changes in the level of expression of mRNA were detected by SYBR green reagent (SYBR Green PCR Master Mix, ABI, Carlsbad, CA, USA) along with 15 ng cDNA and specific primers, on a StepOnePlus Real-Time PCR System (Applied Biosystems).

RT-PCR primers

HPRT1 rat: Forward-5′ CTCATGGACTGATTATGGACAGGA3′; Reverse-5′ GCAGGTCAGCAAAGAACTTATAGCC3′; PEDF rat:

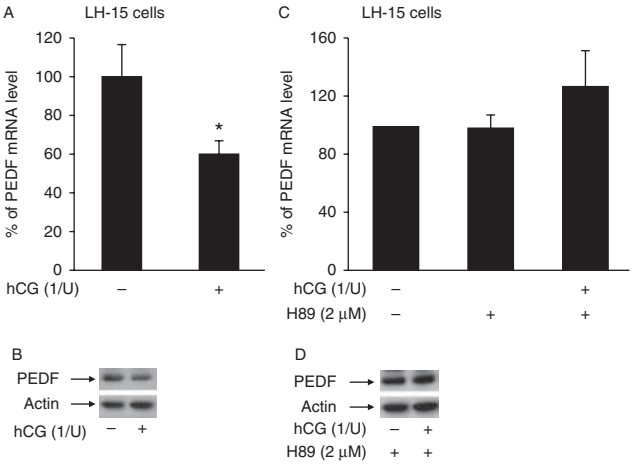

Figure 1 hCG mediates PEDF downregulation through PKA signaling pathway. Serum-starved LH-15 cells were exposed to (A and B) hCG (1 IU) for 6 h and (C and D) the PKA inhibitor H89 (2 μM) for 30 min. Culture medium was replaced with fresh 0.1% CS-FBS, supplemented with hCG (1IU); cells were incubated for 6 more hours. (A, B, and C) Changes in PEDF mRNA level were measured by qPCR analysis with specific primers for PEDF and calibrated with HPRT. Bars represent three independent experiments. The ratio between each treatment and control (DMSO) is plotted as Mean ± S.E.M., (*$P<0.05$) – significantly different from control value. (B and D) Cells were lysed and their proteins were analyzed by western blotting with anti-PEDF antibody, using actin as a loading control. Representative blots are presented.

Forward-5′ TTCACCCGGAGCAGTGAT3′; Reverse-5′ GCCTCCAGAATTGTGTTTGAG3′; HPRT1 human: Forward-5′ TGACACT GGCAAAACAATGCA3′; Reverse-5′GGTCCTTTTCACC AGCAAGCT′; PEDF human: Forward-5′CAACTTCGGCTATGA CCTGTA3′; Reverse-5′AGTAGAGAGCCCGGTGAAGA3′

Statistical analysis

Differences in the RT-PCR arbitrary units were assessed using *t*-test, with a significance of $P<0.05$. In all experiments, RT-PCR outcome of experimental groups was expressed as percent of the 'vehicle' RT-PCR outcome that was arbitrarily set as 100% (control). When inhibitors were used in an experiment, T-TEST was performed between the experimental group of 'inhibitor only' and 'inhibitor + hCG.' When activators were used in an experiment, *t*-test was performed between the control and the 'activator' experimental groups. Each experiment was repeated at least three times.

All participants signed written informed consent forms.

Results

The role of PKA in hCG-induced PEDF downregulation

We showed that PEDF level in hCG-stimulated LH-15 cells reached a nadir within 6 h (Fig. 1A and B; $P<0.05$). The major signaling cascade downstream to LH receptor is the cAMP-PKA pathway (van den Driesche *et al.* 2008). To establish the putative role of PKA in hCG-mediated PEDF downregulation, serum-starved LH-15 cells were stimulated with hCG in the presence or absence of a PKA inhibitor (H89; 2 μM). We found that inhibition of PKA abrogated hCG ability to downregulate the expression of PEDF at both mRNA and protein levels (Fig. 1C and D; $P>0.05$), implying that PKA participates in hCG-mediated PEDF downregulation.

The role of PKC in hCG-induced PEDF downregulation

There are considerable amounts of evidence indicating that LH can activate the PKC signaling pathway, though its function in this pathway is still controversial (Salvador *et al.* 2002). To evaluate whether PKC participates in hCG-mediated PEDF downregulation, serum-starved LH-15 cells were stimulated with hCG in the presence or absence of a PKC inhibitor (GF, 3 μM). We found that inhibition of PKC abrogated hCG-induced PEDF downregulation, at both mRNA and protein levels (Fig. 2A and B; $P>0.05$). To further establish the role of PKC in hCG-induced PEDF downregulation, serum-starved LH-15 cells were stimulated for 1 h with PMA (50 or 100 nM), a known PKC activator. Stimulation with PMA

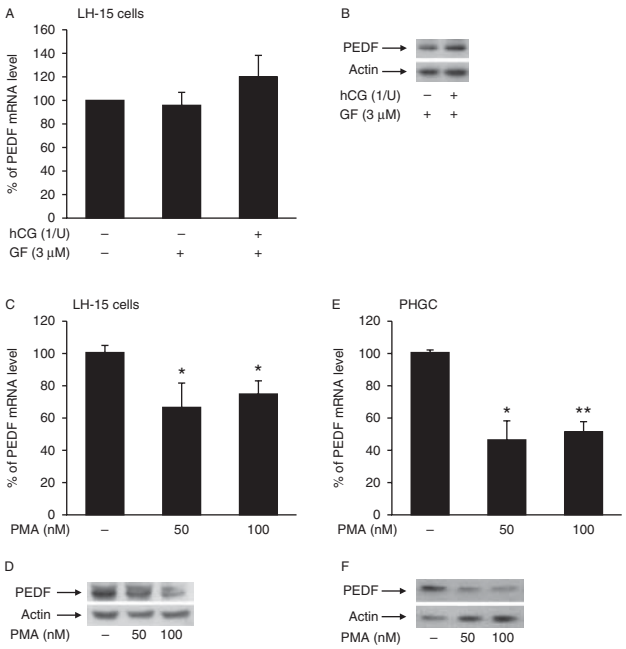

Figure 2 hCG mediates PEDF downregulation through PKC signaling pathway. (A and B) Serum starved LH-15 cells were exposed to the PKC inhibitor, GF (3μM), for 30 min. Culture medium was then replaced with fresh 0.1% CS-FBS, supplemented with hCG (1IU) for a 6-hour incubation period. (C, D, E, and F) Serum-starved (C and D) LH-15 cells and (E–F) PHGC were exposed for 1 h to increasing doses of PMA (50-100 nM). (A, C, and E) Changes in PEDF mRNA level were measured by qPCR analysis with specific primers for PEDF (rat or human) and calibrated with HPRT. Bars represent three independent experiments. The ratio between each treatment and control is plotted as Mean ± S.E.M., (*$P<0.05$; ** $P<0.01$) – significantly different from control value. (B, D, and F) Cells were lysed and their proteins were analyzed by western blotting with anti-PEDF antibody, using actin as a loading control. Representative blots are presented.

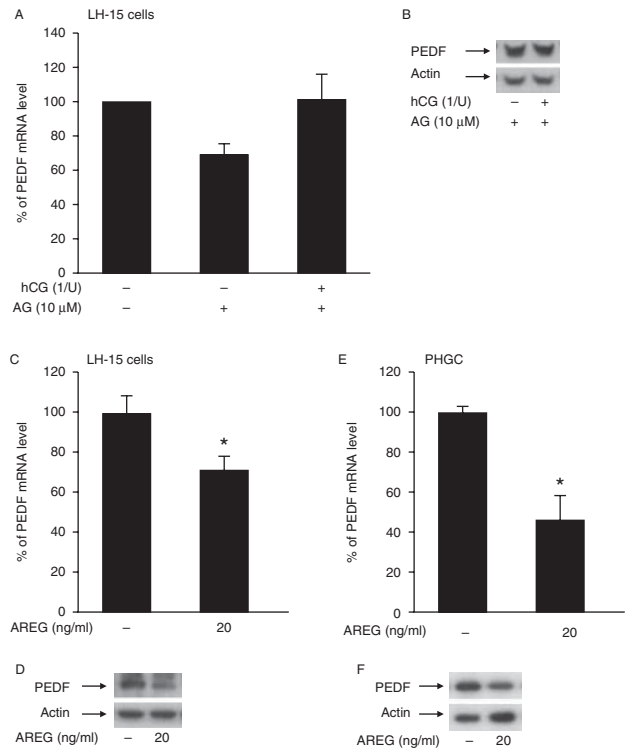

Figure 3 EGFR is involved in PEDF downregulation. (A and B) Serum-starved LH-15 cells were exposed to an EGFR inhibitor, AG (10 μM) for 30 min. Culture medium was then replaced with fresh 0.1% CS-FBS, supplemented with hCG (1 IU). Cells were incubated for 6 more hours. (C, D, E, and F) Serum-starved (C, D) LH-15 cells or (E and F) PHGC were exposed to AREG (20ng/ml) for 4 or 16 h respectively. (A, C, and E) Changes in PEDF mRNA level were measured by qPCR analysis with specific primers for PEDF (rat or human) and calibrated with HPRT. Bars represent three independent experiments. The ratio between each treatment and control is plotted as Mean ± S.E.M., (*$P<0.05$) – significantly different from control value. (B, D, and F) Cells were lysed and their proteins were analyzed by western blotting with anti-PEDF antibody, using actin as a loading control. Representative blots are presented.

significantly decreased the expression of PEDF mRNA and protein (Fig. 2 C and D; $P<0.05$). The findings of this experiment were supported by an *in vitro* model of PHGC, where PMA treatment caused a more prominent decrease in the expression of PEDF mRNA and protein (Fig. 2E and F; $P<0.05$). Thus, we suggest that hCG-induced PEDF downregulation occurs downstream to the PKC signaling pathway.

The role of EGFR in hCG-induced downregulation of PEDF

EGF-like growth factors are produced in response to LH stimulation both in human and rat granulosa cells (Park *et al.* 2004). In an attempt to evaluate whether EGF-like growth factors are part of the hCG-PEDF signaling cascade, we pre-treated serum-starved LH-15 cells with an EGF receptor inhibitor (AG; 10 μM). We found that AG abolished the ability of hCG to decrease the levels of PEDF mRNA and protein (Fig. 3A and B; $P>0.05$). Stimulation of LH-15 cells (Fig. 3C and D) or PHGC (Fig. 3E and F) with AREG for 4 or 16 h (respectively; 20 ng/ml), decreased the levels of PEDF mRNA and protein in both cell types although PHGC were more sensitive to the treatment ($P<0.05$). Thus, we suggest that EGF-like growth factors may participate in the signaling cascade that mediates hCG-induced PEDF downregulation.

It is accepted that transactivation of LH receptor is downstream of PKC cascade (Leserer *et al.* 2000, Cattaneo *et al.* 2014); it is also suggested that PKC can act downstream to EGF and that EGF-like growth factors can activate PKC directly (Li *et al.* 1991, Anne *et al.* 2013, Liu *et al.* 2014). To determine whether inhibition of PKC will hamper the ability of AREG to downregulate PEDF expression, we followed the expression of PEDF in AREG-stimulated LH-15 cells with or without pretreatment with a PKC inhibitor (GF; 10 μM). We found that the ability of AREG to downregulate the expression of PEDF mRNA and protein was not impaired (Fig. 4A and B; $P<0.05$). Therefore, we suggested that PKC is not involved in AERG-mediated PEDF downregulation.

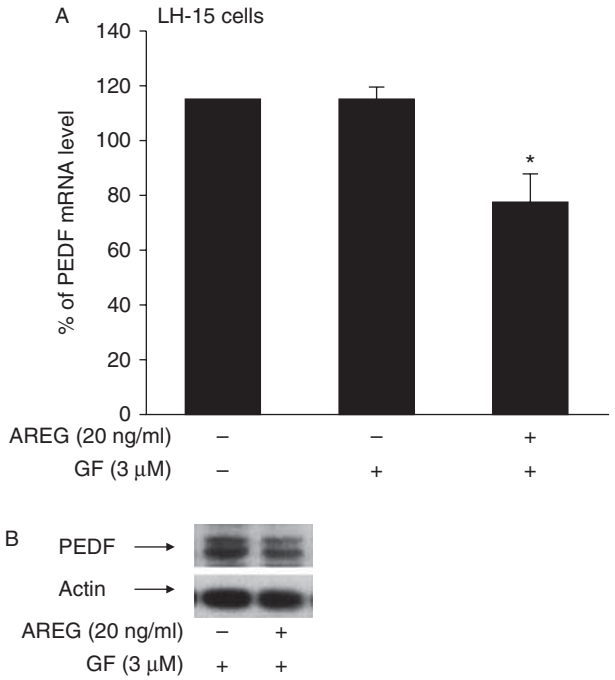

Figure 4 PKC is not involved in AREG-mediated PEDF downregulation. Serum-starved LH-15 cells were exposed to the PKC inhibitor, GF (3 μM), for 30 min. Culture medium was then replaced with a fresh 0.1% CS-FBS, supplemented with AREG (20 ng/ml) for 4 h incubation period. (A) Changes in PEDF mRNA level were measured by qPCR analysis with specific primers for PEDF and calibrated with HPRT. Bars represent three independent experiments. The ratio between the value of each treatment and control value is plotted as Mean ± S.E.M., (*$P<0.05$) – significantly different from control value. (B) Cells were lysed and their proteins were analyzed by western blotting with anti-PEDF antibody, using actin as a loading control. Representative blots are presented.

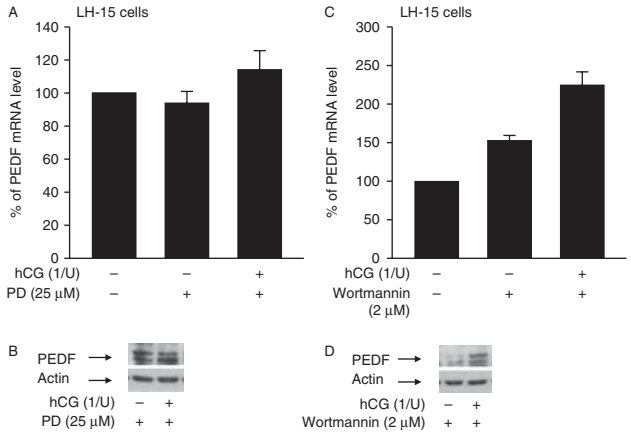

Figure 5 Inhibition of ERK or PI3K prevents hCG-induced PEDF downregulation. Serum-starved LH-15 cells were exposed for 30 min to (A and B) PD (an ERK inhibitor; 25 μM) or to (C and D) wortmannin (a PI3K inhibitor; 2 μM). Culture medium was replaced with fresh starvation medium (CS-FBS; 0.1%), supplemented with hCG (1 IU) for a 6-h incubation period. (A and C) Changes in PEDF mRNA level were measured by qPCR analysis with specific primers for PEDF; calibrated with HPRT. The ratio between each treatment and the control is plotted as Mean ± S.E.M., (*$P<0.05$) – significantly different from control value. (B,D) Cells were lysed and their proteins were analyzed by western blotting with anti-PEDF antibody, using actin as a loading control. Representative blots are presented.

The role of ERK and PI3K in hCG-induced PEDF downregulation

Our final aim was to evaluate whether ERK and PI3K, two signaling molecules located downstream to PKC and EGF, are part of the hCG-induced cascade of PEDF downregulation. LH-15 cells were pre-treated with a specific ERK inhibitor, PD (25 μM), or with a PI3K inhibitor, wortmannin (2 μM), before hCG stimulation. Both pathways were involved in hCG-induced PEDF downregulation (Fig. 5A, B, C, and D; $P>0.05$). Interestingly, inhibition of PI3K caused an elevation of PEDF mRNA basal level, which may point to the importance of PI3K as a PEDF regulator.

Discussion

Ovarian angiogenesis is a hormonally regulated physiological process. We have already shown in both *in vivo* and *in vitro* models that PEDF, one of the most potent anti-angiogenicendogenous factors (Becerra & Notario 2013), regulates the expression of ovarian VEGF and ovarian angiogenesis (Chuderland *et al.* 2013) and that its expression in the ovary is downregulated by LH, estrogen, and progesterone (Ninio-Many *et al.* 2013).

The signaling pathway of LH in granulosa cells is well documented; LH initiates ovulation through activation of the LH receptor (LH-R), a G-protein coupled receptor that activates downstream effectors as adenylate cyclase, PKA (Selvaraj *et al.* 1996), and PKC (Woods & Johnson 2007). Furthermore, stimulation by LH induces transactivation of EGFR by EGF-like growth factors, as AREG (Panigone *et al.* 2008), which is mediated, at least in part, by the cAMP/PKA (Freimann *et al.* 2004) and the PKC (Sun *et al.* 2009) cascades. In the current study, we report that downregulation of PEDF by hCG occurs downstream to PKA and PKC because inhibition of PKA and PKC prevents hCG-induced PEDF downregulation, and stimulation with PKC agonist leads to PEDF downregulation (Fig. 6). Finally, hCG-induced PEDF downregulation occurs downstream to ERK1/2 and PI3K because their inhibition prevents downregulation of PEDF (Fig. 6).

Angiogenesis is a tightly controlled process, executed by pro- and anti-angiogenic factors (Abdollahi & Folkman 2010). In the current study, we illustrate the delicate physiological angiogenic balance in the ovary. VEGF, a key regulator of ovarian vasculature (Mauro *et al.* 2014) is upregulated by LH/hCG in granulosa cells (Herr *et al.* 2013). The signal transduction that governs hCG-induced VEGF biosynthesis is well documented and is known to be mediated by several factors as PKC (Sriraman *et al.* 2008), PKA (van den Driesche *et al.* 2008) and AREG (Karakida *et al.* 2011)). In this paper, we focus on the signal transduction that governs hCG-induced PEDF downregulation. We found that similar signaling cascades participate in both hCG-induced VEGF upregulation and PEDF downregulation. These findings emphasize the importance of angiogenesis regulation around the time of ovulation that is required to facilitate the fast formation of the highly vascularized CL. Thus, our results point out the unique regulation of the orchestrated PEDF-VEGF balance, reflecting the cyclic angiogenic dynamics in the ovary.

Various pathologies of the female reproductive organs, like ovarian carcinoma, PCOS, and OHSS, are associated with unrestrained angiogenesis, attributed, at least in part, to high level of VEGF. We have shown that

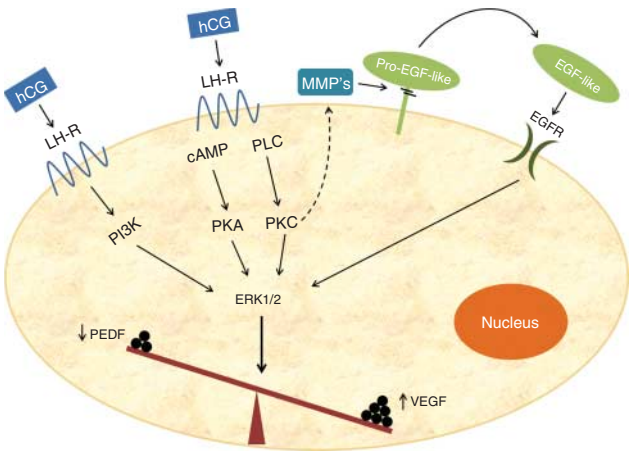

Figure 6 Schematic representation of hCG-mediated PEDF downregulation.

impaired PEDF-VEGF balance lies at the core of OHSS pathophysiology and that administration of recombinant PEDF reverts the pathological balance to normalcy (Chuderland *et al*. 2013). The current therapeutic armamentarium for OHSS consists mainly of symptomatic managing; there are no available drugs to treat this pathological situation effectively. The results obtained in this paper may imply the possible use of specific signaling modulators that would restore the angiogenic balance by affecting concomitantly the expression of both PEDF and VEGF.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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