HB-EGF induces mitochondrial dysfunction via estrogen hypersecretion in granulosa cells dependent on cAMP-PKA-JNK/ERK-Ca\(^{2+}\)-FOXO1 pathway

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Abstract

Polycystic ovarian syndrome (PCOS) is one of the most prevalent endocrinopathies and the leading cause of anovulatory infertility, but its pathogenesis remains elusive. Although HB-EGF is involved in ovarian cancer progression, there is still no clarity about its relevance with PCOS. The present study exhibited that abundant HB-EGF was noted in follicular fluid from PCOS women, where it might induce the granulosa cells (GCs) production of more estrogen via the elevation of CYP19A1 expression after binding to EGFR. Furthermore, HB-EGF transduced intracellular downstream cAMP-PKA signaling to promote the phosphorylation of JNK and ERK whose blockage impeded the induction of HB-EGF on estrogen secretion. Meanwhile, HB-EGF enhanced the accumulation of intracellular Ca\(^{2+}\) whose chelation by BAPTA-AM abrogated the stimulation of HB-EGF on FOXO1 along with an obvious diminishment for estrogen production. cAMP-PKA-JNK/ERK-Ca\(^{2+}\)-FOXO1 pathway played an important role in the crosstalk between HB-EGF and FOXO1. Treatment of GCs with HB-EGF resulted in mitochondrial dysfunction as evinced by the reduction of ATP content, mtDNA copy number and mitochondrial membrane potential. Additionally, HB-EGF facilitated the opening of mitochondrial permeability transition pore via targeting BAX and raised the release of cytochrome C from mitochondria into the cytosol to trigger the apoptosis of GCs, but this effectiveness was counteracted by estrogen receptor antagonist. Collectively, HB-EGF might induce mitochondrial dysfunction and GCs apoptosis through advancing estrogen hypersecretion dependent on cAMP-PKA-JNK/ERK-Ca\(^{2+}\)-FOXO1 pathway and act as a promising therapeutic target for PCOS.

Key words: HB-EGF; estrogen; cAMP-PKA-JNK/ERK-Ca\(^{2+}\)-FOXO1 pathway; mitochondrial dysfunction; granulosa cell

Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrinopathies with an incidence of 5 to 20% in women of childbearing age and the leading cause of anovulatory infertility followed by a heightened risk of type 2 diabetes as well as cardiovascular disease [1,2]. Accumulating evidence has revealed that dysfunction of ovarian granulosa cells (GCs) may contribute to plenty of PCOS symptoms, containing menstrual irregularity, arrested follicular development and anovulation [3,4]. Simultaneously, GCs from PCOS patients produced more estradiol which resulted in follicle growth arrest and its supplementation in female mice brought about anovulatory and follicular cysts [5-8]. But there is limited report about the underlying mechanism of GCs dysfunction in PCOS.

Heparin-binding EGF-like growth factor (HB-EGF), a ligand of epidermal growth factor receptor (EGFR), was originally identified as a secreted glycoprotein in human macrophage medium with high affinity for heparin and exhibited an important function in female reproduction such as early embryo development, blastocyst implantation, decidualization, etc. [9,10]. In rat ovary, HB-EGF was
highly expressed in GCs of primordial and primary follicles followed by an apparent weakness with follicular development, becoming absent in preovulatory follicles, implying that down-regulation of HB-EGF may be essential for follicular maturation [11]. Further analysis found that HB-EGF was increased in serum and peritoneal fluid of ovarian cancer patients and its blockage repressed tumour growth [12-15]. However, little information is available regarding the relevance between HB-EGF and PCOS.

The present study revealed that HB-EGF was abundant in follicular fluid of PCOS patients, where it might induce the hypersecretion of estrogen and bring about mitochondrial dysfunction and apoptosis of GCs through cAMP-PKA-JNK/ERK-Ca²⁺-FOXO1 pathway dependent on EGFR.

Materials and methods

Collection of follicular fluid

Follicular fluids were collected from PCOS and non-PCOS women undergoing in vitro fertilization at the Centre of Reproductive Medicine, Second Hospital of Jilin University and their use was approved by hospital Ethics Committee concomitant with the obtainment of informed consent from all participants. Twenty PCOS patients, whose mean age was 30.83 ± 3.29 years and mean body mass index (BMI) was 28.17 ± 3.59 kg/m², were diagnosed according to the Rotterdam criteria. Sixteen non-PCOS patients, who had normal ovarian morphology and regular menstrual cycles but were infertile because of tubal blockage or male factor along with mean age of 31.08 ± 3.39 years and mean BMI of 27.52 ± 2.33 kg/m², were referred as control.

GCs treatment

Human KGN ovarian GCs (Biobw) were incubated with recombinant human HB-EGF protein (rHB-EGF, 20 ng/ml, R&D Systems) for 12 h in the absence or presence of EGFR inhibitor PF299804 (500 nM, Selleck), protein kinase A (PKA) inhibitor H89 (10 μM, Selleck), c-Jun N-terminal kinase (JNK) inhibitor SP600125 (20 mM, Selleck), extracellular signal-regulated kinase (ERK) inhibitor GDC-0994 (10 μM, Selleck), intracellular calcium ion (Ca²⁺) chelator BAPTA-AM (20 μM, Selleck), forkhead box O 1 (FOXO1) inhibitor AS1842856 (10 μM, MCE), estrogen receptor antagonist ICI 182780 (0.1 μM, MCE) and mitochondrial permeability transition pore (mPTP) opening inhibitor ER-000444793 (2 μM, MCE). In addition, after treatment with rHB-EGF and EGFR inhibitor PF299804, cells were supplemented with cAMP analogue 8-bromoadenosine-cAMP (8-Br-cAMP, 500 μM, Sigma). PF299804, H89, SP600125, GDC-0994, BAPTA-AM, AS1842856, ICI 182780 and ER-000444793 were dissolved in DMSO, while rHB-EGF and 8-Br-cAMP were dissolved in PBS. Controls received the vehicle only.

ELISA

Concentration of HB-EGF protein in follicular fluids was measured using a commercial ELISA kit (Cusabio). Meanwhile, after KGN GCs were treated as mentioned above, culture supernatants were gathered and then applied to determine the levels of estrogen in the light of corresponding ELISA kit (Cusabio).

Real-time PCR

After total RNA extraction and cDNA synthetization, the expression levels of cytochrome P450 family 19 subfamily a member 1 (CYP19A1), FOXO1, Bcl2-associated X protein (BAX) and caspase 3 (CASP3) were measured by real-time PCR analysis using the corresponding primers (Table 1) as described previously [16].

Table 1. Primers used in this study

| Gene       | Primer Sequence                          | Accession number | Size |
|------------|------------------------------------------|------------------|------|
| CYP19A1    | GAGCCCTCACATCCTCEACGG                     | NM_000103        | 195 bp |
| CASP3      | CTGACGTTCGCGCTGAGAG                       | NM_004346        | 159 bp |
| BAX        | ACCGGCTCTCCCTCCACCTT                   | NM_138761        | 107 bp |
| FOXO1      | GAGAGGCGTGGCCTCTCACAAGGG                | NM_002015        | 149 bp |
| GAPDH      | ATTTGGCTACAGCAACAGC                      | NM_002046        | 256 bp |

Western blotting

After extraction of total and nuclear proteins, western blotting was performed with primary antibody against JNK (1:1000, Cell Signaling Technology), phospho-JNK (1:1000, Cell Signaling Technology), ERK1/2 (1:1000, Proteintech), phospho-ERK1/2 (1:1000, Cell Signaling Technology), FOXO1 (1:1000, Proteintech), BAX (1:1000, Proteintech), CASP3 (1:1000, Proteintech), histone H3 (1:5000, Proteintech) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000, Proteintech) as described previously [16].

Measurement of intracellular cAMP level

After various treatments, intracellular cyclic adenosine monophosphate (cAMP) level was detected by cAMP-Glo™ Assay (Promega). Briefly, KGN GCs were incubated with Induction Buffer followed by the supplementary of cAMP-Glo™ Lysis Buffer. After addition of cAMP Detection Solution, GCs were
replenished with Kinase-Glo® Reagent on the heels of the assessment of luminescence using plate-reading luminometer.

**Determination of intracellular Ca^{2+}**

After various treatments, KGN GCs were incubated with fluorescent probe Fluo-3 AM (5 μM, Beyotime), and then analyzed by flow cytometry to determine the level of intracellular Ca^{2+}.

**Measurement of ATP content**

After different treatments, KGN GCs were lysed and then supernatants were gathered to calculate the content of adenosine triphosphate (ATP) by the corresponding kit (Beyotime).

**Measurement of mitochondrial membrane potential (MMP)**

After different treatments, KGN GCs were incubated with JC-1 fluorescent probe (Beyotime) followed by the analysis of flow cytometry or another MMP indicator TMRM (1:1000, ThermoFisher Scientific) followed by the nuclear counterstaining of Hoechst 33342 prior to the visualization in fluorescence microscope.

**Determination of mitochondrial DNA (mtDNA) copy number**

After different treatments, DNA was isolated and then appraised the ratio of mtDNA/nuclear DNA (ncDNA) by real-time PCR to determine mitochondrial DNA (mtDNA) copy number as described previously [17].

**Opening of mitochondrial permeability transition pore (mPTP)**

After different treatments, KGN GCs were incubated with Calcein AM (Beyotime) together with the replenishment of cobalt chloride to quench intracellular green fluorescence. Finally, cells were analyzed by flow cytometry or visualized in fluorescence microscope behind the nuclear staining with Hoechst 33342.

**Assessment of cytochrome C release**

After introduction of pCytochrome C-GFP plasmid (Addgene), KGN GCs were treated as described above and then incubated with TMRM followed by the nuclear counterstaining of Hoechst 33342. Images were obtained in fluorescence microscope.

**Dual luciferase analysis**

CYP19A1 promoter sequence (-123 to +41) contained FOXO1 binding site was amplified by the following primer: 5’- CTCGAGCAGACAGACCGTGG-3 and 5’- AAGCTTCCTTCTGTTGCCTCCACG. After enzyme digestion, fragment was inserted into pGL6 luciferase reporter vector. Followed by the introduction of pGL6-CYP19A1 plasmid, GCs were treated with rHB-EGF in the absence or presence of FOXO1 inhibitor AS1842856. Afterwards, luciferase activity was measured by dual luciferase reporter gene assay kit (Beyotime). The pRL-SV40 plasmid (Beyotime) was used for data normalization.

**Cell apoptosis**

KGN GCs were resuspended after trypsinization and then incubated with Annexin V-FITC (Beyotime) and propidium iodide for 20 min followed by the analysis of flow cytometry. Meanwhile, cells were lysed and then supernatants were collected to calculate the activity of CASP3 by the corresponding assay kit (Beyotime).

**Statistical analysis**

All experiments were independently repeated at least three times. Significance of difference between two groups was compared by Independent-Samples T Test. The multiple comparisons were tested with one-way ANOVA with Tukey’s post hoc test. Data were shown as means ± SEM. P < 0.05 was considered statistically significant.

**Results**

**HB-EGF induced the hypersecretion of estrogen and GCs apoptosis via EGFR**

To clarify the association between HB-EGF and PCOS, we compared the difference of HB-EGF content in follicular fluid between PCOS and non-PCOS patients and found that elevated protein level of HB-EGF was noted in follicular fluid from PCOS women (Figure 1A). Further analysis evidenced that addition of rHB-EGF caused KGN GCs production of more estrogen, but this effectiveness was blocked by PF299804 (Figure 1B), an irreversible EGFR inhibitor. Simultaneously, HB-EGF induced the expression of CYP19A1 mRNA, which was an important rate-limiting enzyme in ovarian estrogen biosynthesis, whereas replenishment of PF299804 abrogated this induction (Figure 1C).

After treatment with rHB-EGF, apoptosis rate of GCs was obviously raised (Figure 1D). Furthermore, HB-EGF raised the mRNA and protein levels of BAX and CASP3, and induced the cleavage of CASP3 concomitant with an increase for CASP3 activity. But supplementation of EGFR inhibitor PF299804 ameliorated above effectiveness conferred by HB-EGF (Figure 1E-G).
**HB-EGF activates cAMP-PKA signaling through EGFR**

In KGN GCs, HB-EGF induced the accumulation of intracellular cAMP, which was an important second messenger and principally activated PKA, but this induction was blocked by EGFR inhibitor PF299804 (Figure 2A). Replenishment of 8-Br-cAMP reversed the blockade of PF299804 on estrogen production and CYP19A1 expression, counteracted the rescue of PF299804 on GCs apoptosis, and antagonized PF299804 regulation of BAX and CASP3 expression as well as CASP3 activity under the context of rHB-EGF (Figure 2B-G). Concurrently, addition of PKA inhibitor H89 abrogated the induction of HB-EGF on estrogen secretion and CYP19A1 expression, impeded the apoptosis of GCs by HB-EGF, and lessened the expression or activity of BAX and CASP3 (Figure 2B-G).

**cAMP-PKA signaling mediates the regulation of HB-EGF on JNK and ERK**

Treatment of GCs with rHB-EGF resulted in the dramatic up-regulation for JNK and ERK phospho-

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*Figure 1. HB-EGF induces the hypersecretion of estrogen and GCs apoptosis via EGFR. A, HB-EGF content in follicular fluid between PCOS and non-PCOS patients. B, Effect of HB-EGF on estrogen secretion in the absence or presence of EGFR inhibitor PF299804. PF, PF299804. C, Regulation of HB-EGF on CYP19A1 expression with/without PF299804. N = 6. D, Effect of HB-EGF on GCs apoptosis in the existence or not of PF299804. N = 3. E and F, Real-time PCR and western blot analyses of CASP3 and BAX expression after treatment with rHB-EGF in the absence or presence of PF299804. N = 3. G, Effect of HB-EGF on CASP3 activity with/without PF299804. N = 5. * P < 0.05 versus control, # P < 0.05 versus rHB-EGF treatment.*
phorylation, but this up-regulation was hampered by EGFR inhibitor PF299804 (Figure 3A). Further analysis evidenced that JNK inhibitor SP600125 and ERK inhibitor GDC-0994 hindered the stimulation of HB-EGF on estrogen level and CYP19A1 expression, alleviated the apoptosis of GCs conferred by HB-EGF, and restrained the expression or activity of BAX and CASP3 (Figure 3B-G). We next clarified whether cAMP-PKA signaling might mediate the regulation of HB-EGF on JNK and ERK. Replenishment of 8-Br-cAMP restored the induction of HB-EGF on JNK and ERK phosphorylation in the presence of PF299804, whereas PKA inhibitor H89 disrupted this induction of JNK and ERK phosphorylation by HB-EGF (Figure 3A).

**HB-EGF induces intracellular Ca\(^2+\) via cAMP-PKA-JNK/ERK pathway**

After exposure to rHB-EGF, intracellular Ca\(^2+\) content was obviously enhanced, but this enhancement was prevented by EGFR inhibitor PF299804 (Figure 3H). Administration of intracellular Ca\(^2+\) chelator BAPTA-AM retarded the elevation of estrogen level and cell apoptosis rate along with an apparent decline for the expression or activity of CYP19A1, BAX and CASP3 in rHB-EGF treated GCs (Figure 3B-G). We next dissected the involvement of cAMP-PKA-JNK/ERK pathway in the regulation of HB-EGF on Ca\(^2+\). Blockade of PKA, JNK and ERK by the corresponding inhibitor brought about an inability of HB-EGF in facilitating the increase of intracellular Ca\(^2+\) content, whereas 8-Br-cAMP neutralized the resistance of PF299804 to Ca\(^2+\) content elicited by rHB-EGF (Figure 3I and J).

**Figure 2.** cAMP-PKA signaling mediates the effects of HB-EGF on estrogen secretion and GCs apoptosis. A, HB-EGF induced the accumulation of intracellular cAMP via EGFR. B and C, cAMP-PKA signaling mediated the effect of HB-EGF on estrogen secretion (N = 9) and CYP19A1 expression (N = 4). D, cAMP-PKA signaling mediated the effect of HB-EGF on GCs apoptosis. N = 3. E-G, Regulation of HB-EGF on the expression or activity of CASP3 and BAX was mediated by cAMP-PKA signaling. N = 3. * P < 0.05 versus control, # P < 0.05 versus rHB-EGF treatment, & P < 0.05 versus rHB-EGF plus PF299804 treatment.
HB-EGF increases FOXO1 expression via cAMP-PKA-JNK/ERK-Ca²⁺ pathway

After addition of rHB-EGF, GCs exhibited an obvious increase for FOXO1 mRNA and total protein as well as nuclear protein, while EGFR inhibitor PF299804 attenuated this increase (Figure 3L and N). Repression of FOXO1 by AS1842856 resulted in the defective capability of HB-EGF in inducing estrogen production and GCs apoptosis, and renewing the
expression or activity of CYP19A1, BAX and CASP3 (Figure 3B-G). By bioinformatic analysis, CYP19A1 promoter region displayed the presence of FOXO1 binding site from +29 to +35. After transfection with CYP19A1-PGL6 plasmid, HB-EGF obviously enhanced luciferase activity, but this enhancement was abrogated by FOXO1 inhibitor AS1842856 (Figure 3K). We subsequently determined whether cAMP-PKA-JNK/ERK-Ca²⁺ pathway was implicated in the regulation of HB-EGF on FOXO1. Supplementation of corresponding inhibitor for PKA, JNK and ERK or addition of intracellular Ca²⁺ chelator antagonized the stimulation of HB-EGF on FOXO1 expression, while 8-Br-cAMP counteracted the improvement of PF299804 on FOXO1 expression in rHB-EGF-treated GCs (Figure 3L-N).

**HB-EGF causes mitochondrial dysfunction via cAMP-PKA-JNK/ERK-Ca²⁺-FOXO1 pathway**

In KGN GCs, HB-EGF brought about the obvious reduction for ATP content and mtDNA copy number, and weakened MMP as indicated by a significant decline for red/green fluorescence intensity ratio (Figure 4A-C). To visualize MMP change, another MMP indicator was used. After treatment with rHB-EGF, fluorescence intensity of TMRM was attenuated (Figure 4D). Further analysis evidenced that blockade of PKA, JNK, ERK and FOXO1 by corresponding inhibitor or replenishment of intracellular Ca²⁺ chelator antagonized the

![Figure 4](https://www.ijbs.com)

Figure 4. HB-EGF causes the aberration of ATP level, mtDNA copy number and MMP via cAMP-PKA-JNK/ERK-Ca²⁺-FOXO1 pathway. A and B, HB-EGF brought about the reduction of ATP content (N = 6) and mtDNA copy number (N = 5) via cAMP-PKA-JNK/ERK-Ca²⁺-FOXO1 pathway. C and D, HB-EGF attenuated the MMP via cAMP-PKA-JNK/ERK-Ca²⁺-FOXO1 pathway by flow cytometry analysis or visualization in fluorescence microscope. N = 3. Scale bar, 20 µm.
diminishment of ATP content, mtDNA copy number and MMP conferred by HB-EGF, while 8-Br-cAMP resisted the rescue of PF299804 on aforementioned mitochondrial parameters in the existence of rHB-EGF (Figure 4A-D).

To further assess the role of HB-EGF in maintaining mitochondrial function, we analyzed its effect on mPTP opening and cytochrome C release. In GCs, HB-EGF induced the opening of mPTP as evidenced by the diminished fluorescence intensity of mitochondrial calcein and provoked the release of cytochrome C from mitochondria into the cytosol, whereas addition of EGFR inhibitor PF299804 counteracted above effectiveness, but this counteraction was of no avail after supplementation of 8-Br-cAMP (Figure 5A-E). Impediment of PKA, JNK,
ERK and FOXO1 by corresponding inhibitor or adjunction of intracellular Ca\(^{2+}\) chelator opposed the promotion of HB-EGF on mPTP opening and cytochrome C release (Figure 5A-E). Further analysis demonstrated that after treatment with ER-000444793, a blocker of mPTP opening, release of cytochrome C was obstructed and GCs apoptosis rate was mitigated concomitant with the reduction for cleaved CASP3 expression and activity but not BAX (Figure 6A-E).

**HB-EGF induced GCs apoptosis and mitochondrial dysfunction via estrogen hypersecretion**

As described above, HB-EGF induced the hypersecretion of estrogen and enhanced GCs apoptosis. We next explored whether estrogen might mediate the effects of HB-EGF on GCs apoptosis. After exposure to estrogen receptor antagonist ICI 182780, HB-EGF presented the defective ability in inducing GCs apoptosis and enhancing the expression of CASP3 and BAX as well as CASP3 activity (Figure 6F-I). Furthermore, treatment of GCs with ICI 182780 brought about the apparent amelioration for aberrant ATP level, mtDNA copy number and MMP elicited by HB-EGF, hindered the opening of mPTP and impeded the release of cytochrome C from mitochondria into the cytosol (Figure 7A-G).

**Figure 6. HB-EGF induces GCs apoptosis through promoting mPTP opening and enhancing estrogen secretion.** A and B, mPTP opening inhibitor ER-000444793 hampered the induction of HB-EGF on cytochrome C release and GCs apoptosis. N = 3. C-E, Blockage of mPTP opening weakened the induction of HB-EGF on cleaved CASP3 expression and activity, while did not alter change its regulation on BAX. N = 4. F, ER antagonist ICI 182780 impeded GCs apoptosis by HB-EGF. N = 3. ICI, ICI 182780. G-I, ER antagonist ICI 182780 attenuated the facilitation of HB-EGF on the expression or activity of CASP3 and BAX. N = 3.
Figure 7. HB-EGF impairs mitochondrial function through enhancing estrogen secretion. A and B, Estrogen receptor antagonist ICI 182780 prevented the impairment of HB-EGF on ATP level and mtDNA copy number. N = 5. C and D, ICI 182780 resisted the regulation of HB-EGF on MMP. N = 3. E and F, ICI 182780 counteracted the induction of HB-EGF on mPTP opening. N = 3. G, ICI 182780 impeded the induction of HB-EGF on cytochrome C release. N = 3.

Discussion

HB-EGF is implicated in the regulation of ovarian cancer progression, but its relevance with PCOS remains unknown. The present study exhibited the elevation of HB-EGF level in follicular fluid from PCOS women. Meanwhile, aberrant HB-EGF expression was also noted in PCOS patient GCs [18]. Together these observations imply a potential involvement of HB-EGF in PCOS etiology. Ovarian GCs were required for folliculogenesis and ovulation, and its dysfunction was regarded as a predisposition of PCOS [3,4,19]. HB-EGF induced the excessive production of estrogen, which was also noted in GCs from PCOS patients [5-7]. Injection of estrogen into female mice brought about anovulatory and follicular cysts, while treatment with estrogen antagonist clomiphene citrate enhanced the ovulation rate of PCOS patients [8,20,21]. CYP19A1 was an important enzyme in converting testosterone to estrogen and its
inhibitor was used to treat anovulatory PCOS [21-23].
In GCs, HB-EGF induced the expression of CYP19A1,
confirming the importance of HB-EGF in the
regulation of estrogen synthesis. Further analysis
evidenced that HB-EGF might exert its biology function
via binding EGFR [24]. Blockade of EGFR by
PF299804 abrogated the induction of HB-EGF on
estrogen production and CYP19A1 expression,
indicating that EGFR was prerequisite for HB-EGF in
the induction of GCs dysfunction.

As an important second messenger, cAMP was
abundantly accumulated in GCs from PCOS women
[6,25]. In GCs, HB-EGF via EGFR induced the
shift of intracellular cAMP level which might
principally activate PKA and its accumulation
prevented oocytes from maturation [26,27]. Treatment
with PKA inhibitor H89 hampered the induction of
HB-EGF on the granulosa production of estrogen,
while replenishment of cAMP analogue 8-Br-cAMP
counteracted the improvement of PF299804 on
estrogen secretion under the context of rHB-EGF.
Together these observations indicate that HB-EGF
may transmit intracellular downstream signaling via
cAMP-PKA pathway after binding to EGFR. Further
analysis found that cAMP-PKA signaling mediated
the regulation of HB-EGF on JNK and ERK which
were important for folliculogenesis as well as
ovulation, and their aberrant expression was also
observed in PCOS [28-33]. Addition of corresponding
inhibitor for ERK and JNK disrupted the inducement
of HB-EGF on estrogen secretion. Consistently,
blockade of JNK attenuated the HB-EGF-induced
cytotrophoblast cell migration, while repression of
ERK weakened the DNA synthesis of vascular smooth
muscle cells and intestinal restitution conferred
by HB-EGF [34-36]. Collectively, these data state that
HB-EGF modulates the secretory function of GCs via
JNK and ERK dependent on cAMP-PKA signaling.

Ca^{2+} was a versatile messenger molecule that
operates numerous different cellular functions
including hormone secretion and was crucial for
oocyte maturation [37,38]. Addition of intracellular
Ca^{2+} chelator BAPTA-AM retarded the granulosa
production of estrogen after different stimulation [39].
In GCs, HB-EGF induced the elevation of intracellular
Ca^{2+} content which was noted in the serum from
PCOS patients [40] and its reduction by chelator
BAPTA-AM hampered the effect of HB-EGF on
estrogen secretion. Simultaneously, Ca^{2+} acted the
transcription of downstream target gene [41]. Under
the context of rHB-EGF, BAPTA-AM suppressed the
expression of FOXO1 which was an important
transcription factor in modulating follicular
development and also involved in the pathogenesis of
PCOS due to its effects on insulin resistance and
chronic inflammation that were important features for
PCOS [24-44]. Repression of FOXO1 by AS1842856
alleviated the induction of HB-EGF on estrogen
production. Taken together, these observations
suggest that FOXO1 may serve as a downstream
target of Ca^{2+} to mediate the regulation of HB-EGF on
estrogen. Further analysis evidenced that blockage of
JNK and ERK abrogated the stimulation of HB-EGF
on intracellular Ca^{2+} accumulation and FOXO1
expression, implying that JNK and ERK exerts an
important action in the crosstalk between HB-EGF
and Ca^{2+} as well as FOXO1.

Mitochondrial dysfunction was considered as a
crucial causative factor to PCOS aetiology and its
improvement by mitochondria-targeted antioxidant
MitoQ_{10} mitigated the symptoms of PCOS rats [45-47].
In GCs, HB-EGF induced mitochondrial dysfunction as
eviced by the reduction of ATP content, mtDNA
copy number and MMP, but this dysfunction was
ameliorated by estrogen receptor antagonist ICI
182780. Concurrently, mPTP definitely reflects the
integrity of mitochondrial function [48]. Treatment of
GCs with HB-EGF resulted in the opening of mPTP
which gave rise to the release of cytochrome C from
mitochondria into the cytosol [48,49]. BAX, an
important gatekeeper of mPTP, was required for
cytochrome C release [50]. HB-EGF promoted the
opening of mPTP and raised the expression of BAX,
whereas addition of ICI 182780 resisted this
effectiveness. Collectively, these evidences reveal that
HB-EGF may impair mitochondrial function through
enhancing estrogen secretion. Furthermore, following
the release of cytochrome C, caspase-dependent cell
death was triggered [49]. HB-EGF enhanced the
apoptosis rate of GCs, which might facilitate the
abnormalities of folliculogenesis and account for
anovulation and aberrant steroidogenesis in PCOS
[51,52], and promoted the cleavage and activity of
CASP3 that were the principal executor of apoptosis
[49], while blockage of mPTP opening by
ER-00044793 attenuated the induction of HB-EGF on
GCs apoptosis, implying that HB-EGF induced
apoptosis via the mitochondria-dependent pathway.

Conclusions

HB-EGF was abundantly noted in follicular fluid
of PCOS patients, where it might bind to EGFR and
induce the GCs production of more estrogen through
cAMP-PKA-JNK/ERK-Ca^{2+}-FOXO1 pathway, resulting
in mitochondrial dysfunction and GCs apoptosis
(Figure 8).

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Figure 8. Schematic depiction of HB-EGF regulation to GCs. Elevated HB-EGF was noted in follicular fluid of PCOS patients, where it might induce the GCs production of more estrogen through cAMP-PKA-JNK/ERK-Ca2+-FOXO1 pathway after binding to EGFR and brought about mitochondrial dysfunction, resulting in the release of cytochrome C from mitochondria into the cytosol to trigger GCs apoptosis.

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Author Contributions
JCH and CCD performed the experiments and analyzed the data. JCH, ZPY and BG wrote and edited the manuscript. SJ collected follicular fluids. SJ, CBS and YSW provided technical assistance. ZPY and BG designed and conducted the research. All authors read and approved the final manuscript.

Competing Interests
The authors have declared that no competing interest exists.

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