RESEARCH ARTICLE

ATF4 assist in the expression of COX2/PGE2: A Potential Role of ATF4 in PCOS

Short title: Disturbed ATF4 action in PCOS lowers ovulation

Précis: ATF4 in hGCs ~~when~~ from PCOS women was decreased, which impeded hCG-induced COX2 expression and PGE2 production by reducing transcriptional activation and reduced the number of retrieved oocytes in rats.

Abbreviations

ATF4, activating transcription factor 4; ChIP, chromatin immunoprecipitation; CL, corpus luteum; COC, cumulus-oocyte complex; COX2, cyclooxygenase-2; DAB, diaminobenzidine; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FF, follicular fluid; hCG, human chorionic gonadotropin; hGCs, human granulosa cells; IVF-ET, *in vitro* fertilization-embryo transfer; LH, luteinizing hormone; OD260, optical density at 260 nm; PCOS, polycystic ovary syndrome; PGE2, prostaglandin E2; PTX3, pentraxin 3.

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Commented [A4]: Please include Disclosure. The author reports no conflicts of interest in this work. [Each manuscript needs to include a disclosure of financial interest or other conflict of interest statement. This is where these statements go].

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Abstract

Ovulatory disorder is common in patients with hyperprolactinemia or polycystic ovary syndrome (PCOS). Previous studies have shown ~~the critical roles~~ that ATF4 plays critical role in apoptosis and glucose homeostasis, but ~~the~~ its role ~~of ATF4~~ in regulating reproductive function ~~remains unknown~~was not explored. The ~~purpose of the~~ present study ~~was to~~ investigated the role of ATF4 in ovarian ovulatory function. Human granulosa cells (hGCs) from 45 women newly diagnosed with PCOS and 30 controls were used to determine ATF4 expression. *In vitro* cultured hGCs were used to detect the upstream and downstream genes of ATF4. A shRNA-ATF4 lentiviral vector (Sh*Atf4*) was injected into rat ovaries to establish an *in vivo* gene knockdown model to further ~~confirm the studies~~assess the *in vivo* relevance of the results from PCOS women ~~above~~. We ~~demonstrated for the first time~~found that *ATF4* mRNA expression ~~levels were~~was lower in hGCs from PCOS patients than in hGCs from non-PCOS women. Many pivotal transcripts involved in cumulus-oocyte complex ~~COCs~~ expansion, preovulatory tissue remodeling. The *in vivo* study showed that shRNA-lentivirus mediated *ATF4* knockdown in rat ovaries ~~via lentiviral infection obviously~~led to reduced ~~the~~ number of retrieved oocytes. ~~Taken together~~Collectively, these findings ~~may shed light~~suggested ~~on the~~previously unknown roles of ATF4 in regulating ovulation.

Keywords

ATF4, ~~ovulation~~Ovulation, COX2, PCOS

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1. Introduction

PCOS, also called Stein-Leventhal syndrome, is an endocrine disorder characterized by oligo-ovulation or continuous anovulation, insulin resistance, and hyperandrogenism. PCOS is common in adolescent and reproductive women and has a morbidity of 9 % - 18 % [1,2]. Previous studies have verified that multiple genes correlated with hormone biosynthesis and ovulation are significantly altered in hGCs [3,4]. For the release and transport of oocytes through the oviduct in fertilization, it is essential that the cumulus-oocyte complex ~~COC~~ (COC~~cumulus-oocyte complex~~) matrix is well composed and organized with proper expansion [5]. Diminished COC~~s~~ expansion may partially explain the anovulation in women with PCOS [6]. However, the molecular mechanism of reduced ~~PCOS in~~ ovulation in PCOS remains unclear.

Activating transcription factor 4 (ATF4), also known as CREB2, is constitutively expressed in a wide variety of tissues [7]. ATF4 is able to form heterodimers with members of C/EBP families, including C/EBP and C/EBPb, ~~of~~ which are essential for ovulation [8-10]. Several ~~researches~~ studies also identified the role of ATF4 in reproduction ~~field~~. Decreased~~s~~ ~~in~~ ATF4 expression in chorionic villus tissue ~~from women~~ could trigger miscarriage in pregnant women [11]. ~~Recently published studies revealed the role of ATF4 in regulating follicular function. For instance, a~~An altered expression~~ation~~ of ATF4 ~~was~~ closely correlated with the development of follicles [12-14]. Besides, ATF4 could be induced in the functional and early regression stages of the corpus luteum (CL) [15]. However, the specific regulatory mechanisms underlying the function of ATF4 in ovulation remain unclear. The abnormal gene expression profiles of the granulosa cells and ~~human~~ ovaries from PCOS patients have revealed many differentially expressed genes [4,16,17]. Utilizing bioinformatics methods (DAVID Bioinformatics Resources 6.7), we screened for transcription factors that can trigger differentially expressed genes ~~via DAVID Bioinformatics Resources 6.7~~ (data not shown). Among those transcript factors, ATF4 was ~~highlighted~~ singled-out due to ~~its~~ high frequency of its occurrence in the gene expression profiles. Therefore, based on the published microarray data, we hypothesized that ATF4 may play a critical role in PCOS patients.

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2. Materials and methods

A. Recruitment of patients

Forty-five female participants were randomly recruited from the Center for Reproductive Medicine, between September 2018 and September 2020. Forty of the participants, who were between 25 and 30 years old, were diagnosed with PCOS according to the Rotterdam criteria (oligo- and/or anovulation; clinical and/or biochemical signs of hyperandrogenism; and polycystic ovaries with the exclusion of other causes of hyperandrogenism, such as hyperprolactinemia, androgen-secreting tumors, Cushing's syndrome, and non-classical congenital adrenal hyperplasia) and received *in vitro* fertilization-embryo transfer (IVF-ET) [23]. The diagnosis of PCOS was satisfied when two or more of the three criteria were met. The remaining 37 participants in the non-PCOS group were healthy females with regular menstrual cycles (26–35 days) and normal ovarian morphology, and ~~who~~ were recruited during visits for routine physical examination, tubal factor infertility, or husband's infertility. Oocyte retrieval was performed at 30 hrs after hCG administration. Written informed consent was obtained from all participants. The clinical characteristics of the PCOS and non-PCOS groups are shown in Table 1.

B. Cell culture

hGCs were collected from the PCOS and non-PCOS subjects after their first IVF/intracytoplasmic sperm injection cycle at our center. At 30 hrs after triggering, COCs were retrieved via the transvaginal ultrasound-guided aspiration of follicles ≥ 10 mm. Follicular fluid (FF) from the patients was pooled. The protocol of GC isolation and culture was ~~moderately~~ slightly modified from the ~~method~~ previously described method [24]. In brief, the pellets were purified by density gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences) and then digested ~~in~~ with hyaluronidase (Sigma) at 37°C for 7 minutes. The dispersed cells were collected and ~~then~~ cultured in Dulbecco's modified Eagle medium /Ham's F12 with 10% fetal bovine serum (Gibco). The viable cells were seeded at 10^{-6} cells per well in a six-well culture plate, at 5×10^5 cells per well in a twelve-well culture plate or at 1×10^4 cells per well in a chamber slide.

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C. Transfection experiment

2×10^5 Cells were transfected with 50 nM ~~synthetic~~ siRNA in Opti-MEM (Invitrogen). The cells were electroporated at 170 V for 10 ms using an NEPA21 electroporator (Nepa Gene), for transfection. After dilution with Dulbecco's modified Eagle's medium/F12 containing 10 % fetal bovine serum and antibiotics, the cells were transferred to a six-well culture plate and were ready for treatment after 72 hrs of incubation. The knockdown efficiency was determined using quantitative real-time polymerase chain reaction (qRT-PCR) or W~~w~~estern blot assays.

D. qRT-PCR

Total RNA was extracted from hGCs using a total RNA isolation kit and the samples were then stored at -80 °C for subsequent analysis. ~~The~~ RNA concentration~~s~~ of each sample were determined by calculating the OD260/OD280 ratio using an ultramicro spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). A total of 200 ng of RNA was used for cDNA synthesis with the RT Master Mix Perfect RealTime Kit. Three separate experiments were performed, and each sample was assayed in triplicate. The mean value was used for the determination of mRNA levels by the comparative Ct ($2^{-\triangle\triangle Ct}$) method, with *GAPDH* as the reference gene. The primer sequences are shown in the ~~supplementary~~ Supplementary Table 1.

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E. Enzyme-linked Immunosorbent assay (ELISA) for PGE2

A human PGE2-specific ELISA kit was used in accordance with the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA). Culture medium was collected, and the PGE2 levels in the culture medium were determined by ELISA kit. The OD values ~~of~~ for PGE2 were normalized ~~to~~ with the protein concentrations of the corresponding cell lysates. The normalized PGE2 values obtained from the treated cells are relative to those of the control cells. The linear range of the PGE2 concentrations was 10 – 1000 pg/mL.

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K. Immunohistochemistry

The ovaries of rats were fixed in 4% paraformaldehyde and embedded in paraffin. Then, 5-μm sections were prepared, followed by deparaffinization and rehydration with a graded ethanol series. The tissue sections were blocked with rabbit serum for 1 hr at room temperature and incubated with an anti-ATF4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100) overnight at 4°C in a dark chamber. After ~~being~~ washinged with PBS, the sections were incubated with a secondary antibody (1:200) for 1.5 hr at room temperature. ~~:~~ Tthen, diaminobenzidine (DAB) was added to initiate the color reaction ~~was visualized by exposure to diaminobenzidine (DAB)~~ for 5 min~~s~~. To test the specificity of immunocytochemical staining, separate tissue sections were exposed to preimmune serum instead of the primary antibody (negative control).

3. Results

A. The expression of ATF4 in hGCs from women with PCOS was decreased

Immunofluorescence results showed strong ATF4 staining in hGCs (Figure 1A). We first determined whether *ATF4* expression was changed in clinical PCOS patients. As shown in Table 1, compared with the controls, PCOS patients were characterized by increased testing indexes, including basal LH, LH/FSH, T_0 and AMH levels. qRT-PCR was performed to measure *ATF4* mRNA in primary hGCs. As shown in Figure 1C, pentraxin 3 (*PTX3*) levels were lower in the PCOS group than in the non-PCOS group, in accordance with previous research results [27], and the mRNA abundance of *ATF4* was lower in the PCOS group than in the control group. Therefore, *in vitro* and *in vivo* experiments ~~should be~~were performed to explore the physiological role of ATF4.

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B. ATF4 regulated a variety of genes associated with ovulatory response in hGCs

To further illuminate the role of ATF4 in ovulation regulation, siRNA-mediated gene knockdown was used to down-regulate the endogenous expression of *ATF4* in hGCs. The qRT-PCR results showed a significant decrease in *ATF4* expression in hGCs (Figure 2A). More importantly, we found that several ovulation-related genes were significantly altered. The mRNA levels of genes associated with cumulus expansion in hGCs, including *COX2*, *PTX3*, *CD44*, *TNFAIP6*, and *HAS2*, were decreased when *ATF4* was deficient (Figure 2B). Other genes that play a pivotal role in extracellular matrix (ECM) remodeling were detected through qRT-PCR. The mRNA ~~transcriptional~~ expression of *MMP2*, *MMP9*, and *ADAMTS1* in hGCs was significantly ~~obviously~~ impeded when *ATF4* knockdown was achieved with ~~small interfering~~ RNAi (Figure 2C). The expression levels of *HSD3β* and *CYP11A1* were barely changed (Figure 2D). Altogether, these results indicated that ATF4 participates in regulating the expression of ovulatory genes.

C. Atf4 in rat ovaries regulates ovulation function

To further verify the *in vivo* function of *Atf4* ~~in vivo~~, immunohistochemical staining assay was ~~firstly~~ initially performed. *Atf4*-deficient ovarian tissues were established in rats via the intrabursal injection of lentivirus sh*Atf4* (Supplemental Figure 1D and 1E). Lentivirus sh*Atf4* successfully blocked nearly 40% of *Atf4* expression in rat ovaries without affecting ovary weights (Figure 5A-C). When sh*Atf4* rats were treated with PMSG and hCG, preovulatory follicular development was normal (Figure 5F (a) and (b)). However, COC expansion was impaired slightly (Figure 5F (c) and (d)). Furthermore, when COC expansion was ~~tested~~ examined *in vitro*, the physiological mediators of COX2 [29], was less effective in inducing expansion of COCs collected from sh*Atf4* compared with shNC (Figure 2E). To integrally verify the function of *Atf4* in regulating ovulation, rats were injected with PMSG, followed by ~~and then received an~~ hCG injection, 24 hrs later.

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4. Discussion

PCOS is a common endocrine disorder that causes dysfunctional follicular maturation and oligo-ovulation or anovulation, eventually resulting in infertility [30]. Ovulation, the ~~most~~ essential ~~process~~ prerequisite ~~of~~ for mammalian reproduction, is a strictly controlled event involving the ~~variation~~ participation of multiple genes. In the process of ovulation, the expression of ovulation-related genes is mainly affected by the luteinizing hormone (LH) surge [31]. The normal expansion of COCs is required for ovulation, which requires the abundant expression of transcripts, such as COX2, PTX3, CD44, TNFAIP6, and HAS2 [32]. Several recent studies have shown that some genes, including PTX3 and TNFAIP6, are down-regulated in PCOS [6,27]. Moreover, variations in the ECM secreted from cumulus cells and the follicular wall are also essential for oocyte release; several classes of proteinases have been reported to regulate ovarian ECM remodeling, including MMPs, the plasmin/plasminogen activator system, and ADAMTS [33].

Among these ovulation-related genes, COX2~~PGE2~~, a catalyst of PGE2 production ~~COX2~~, is recognized as a pivotal mediator [34]. The suppression of COX2 could inhibit multiple reproductive functions, including ovulation, while exogenous PGE2 could rescue the inhibitory effects of COX2 on ovulation [35,36]. These studies clearly support the concept that COX2-derived PGE2 plays a critical role in regulating ovulation. ATF4 is a critical determinant of diverse physiological events. In addition to its classical role in lipid and glucose metabolism and insulin secretion and sensitivity, ATF4 has been recently identified as an important controller of reproduction [37,38]. However, the role of ATF4 in PCOS pathogenesis and the underlying mechanisms remain to be described. Intriguingly, ATF4 can induce COX2 expression in the kidney, emphasizing the potential correlation between ATF4 and COX2. Based on the above study, the aim of our study is to elucidate the role of ATF4 in PCOS and to further investigate the underlying mechanism.

In the present study, we demonstrated that ATF4 was expressed in hGCs; this finding was indispensable ~~for determining~~to determine the physiological function of ATF4. Whether ATF4 expression was changed in ~~clinical~~ PCOS patients remained unclear. Thus, we further investigated the role of ATF4 in PCOS by analyzing clinical samples. First, we determined that *PTX3* expression was lower in PCOS, and we regarded this as a positive control result, and~~;~~ this finding was consistent with previous ~~research~~ reports ~~results~~ [27]. Consistently, we found that the mRNA expression levels of *ATF4* in primary hGCs

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from PCOS patients were decreased. These data implied that the aberrant expression of ATF4 might be correlated with PCOS. The absence of ATF4 in hGCs resulted in a~~n obvious~~ decrease in the expression of multiple genes involved in COCs expansion, preovulatory tissue remodeling, and progesterone production. hCG, a hormone secreted by the placenta, can induce progesterone and ovulation production in the ovaries, which is consequentially used for the treatment of infertility [12,39]. The mechanisms underlying the regulation of ATF4 by hCG ~~remained unclear~~are not known. ~~Herein~~In the present work, several signaling pathways that might be involved in ~~the~~ this ~~event~~ process were examined. We found that hCG-induced ATF4 up-regulation was related to activation of the PI3K/AKT pathway rather than the PKA/CREB and ERK1/2 signaling pathways. Previously, we identified the key role of ATF4 in modulating the expression of COX2 in hGCs (Figure 2B). Mechanistically, we found that ATF4 likely exerts its effects by ~~could~~ directly binding to the promoter of *COX2* in hGCs. Thus~~; as a result~~, ATF4 deficiency impaired the hCG-mediated induction of COX2 and PGE2 expression.

An *in vivo* study was also performed to ~~integrally~~ investigate the role of *Atf4* in ovulation. Due to the high efficacy and long-term stability of shRNA, we employed lentivirus sh*Atf4*, which could stably block the expression of *Atf4* in rat ovaries. Intrabursal injection is a promising method for ensuring the local knockdown of target genes with high efficacy. ~~All of T~~these results collectively support the pivotal role of *Atf4* in maintaining normal ovulation in rats.

Our results demonstrate that decreased *ATF4* expression in hGCs is correlated with PCOS. hCG could activate the PI3K/AKT signaling pathway and then stimulate ATF4 expression in hGCs. Through *in vivo* studies, we confirmed that the knockdown of *Atf4* disrupted ovulation.

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