Defective CFTR-regulated granulosa cell proliferation in polycystic ovarian syndrome

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Abstract

Polycystic ovarian syndrome (PCOS) is one of the most frequent causes of female infertility, featured by abnormal hormone profile, chronic oligo/anovulation, and presence of multiple cystic follicles in the ovary. However, the mechanism underlying the abnormal folliculogenesis remains obscure. We have previously demonstrated that CFTR, a cAMP-dependent Cl^- and HCO_3^- conducting anion channel, is expressed in the granulosa cells and its expression is downregulated in PCOS rat models and human patients. In this study, we aimed to investigate the possible involvement of downregulation of CFTR in the impaired follicle development in PCOS using two rat PCOS models and primary culture of granulosa cells. Our results indicated that the downregulation of CFTR in the cystic follicles was accompanied by reduced expression of proliferating cell nuclear antigen (PCNA), in rat PCOS models. In addition, knockdown or inhibition of CFTR in granulosa cell culture resulted in reduced cell viability and downregulation of PCNA. We further demonstrated that CFTR regulated both basal and FSH-stimulated granulosa cell proliferation through the $\text{HCO}_3^-/\text{sAC}/\text{PKA}$ pathway leading to ERK phosphorylation and its downstream target cyclin D₂ (*Ccnd2*) upregulation. Reduced ERK phosphorylation and CCND2 were found in ovaries of rat PCOS model compared with the control. This study suggests that CFTR is required for normal follicle development and that its downregulation in PCOS may inhibit granulosa cell proliferation, resulting in abnormal follicle development in PCOS.

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Introduction

During an ovarian cycle, a set of resting follicles are recruited and enter growth phase upon the stimulation by a number of endocrine and paracrine factors (Edson *et al.* 2009, Oktem & Urman 2010). The recruited follicles will undergo a selection process, during which one (in humans) or some (in rodents) of the recruited follicles with the growth/survival factors overwhelm the apoptotic factor, therefore continue(s) to develop and become(s) the dominant follicle(s), while the others are suppressed and finally committed to atresia (Edson *et al.* 2009, Oktem & Urman 2010). Normal follicle development depends on the balance between proliferation and apoptosis process. Abnormal and dysregulated follicle growth is often observed in ovarian disorders and underlies female infertility.

Polycystic ovarian syndrome (PCOS) is known to be an endocrine disorder and the leading cause of female infertility (Teede & Norman 2006, Norman *et al.* 2007). Apart from anovulation/oligoovulation and abnormal androgen-to-estrogen ratio in serum, another characteristic and diagnostic criterion of PCOS is the presence of multiple

cystic follicles in the ovary. The cystic follicles are observed as ‘arrested’, as none of them can grow further to become a dominant follicle committed to ovulation as observed in a normal cycle. These follicles show signs of atresia and degeneration of the granulosa cell layers (Broekmans & Fauser 2006), suggesting abnormal proliferation and/or apoptosis in granulosa cells of these follicles, resulting in failure in further development. However, the cause of abnormal follicle development in PCOS is unclear.

While preantral follicle development depends on local growth factors, antral follicle growth critically depends on follicle-stimulating hormone (FSH) derived from the pituitary (Kumar *et al.* 1997, Edson *et al.* 2009, Oktem & Urman 2010). FSH induces granulosa cell proliferation by regulating cell cycle activator cyclin D₂ (CCND2) and cell cycle inhibitor p27^{kip} (Robker & Richards 1998a, Hunzicker-Dunn & Maizels 2006, Muniz *et al.* 2006) through the PI3K/Akt (Park *et al.* 2005, Han *et al.* 2013) or MAPK/ERK pathway (Kayampilly & Menon 2004). Although FSH levels in PCOS patients are usually in the normal range (Broekmans & Fauser 2006), the aberrant

antral follicle development observed in PCOS suggests that there may be defect in FSH signaling in PCOS, resulting in reduced proliferation of granulosa cells.

Women with cystic fibrosis (CF), the most common genetic disease in Caucasians caused by mutations of *CFTR* (Kerem *et al.* 1989, Riordan *et al.* 1989, Rommens *et al.* 1989), also exhibit delayed puberty, anovulation, oligoovulation, amenorrhea (Stead *et al.* 1987, Aswani *et al.* 2003), cystic ovaries, and abnormal hormone levels similar to that observed in PCOS women (Shawker *et al.* 1983). Although *CFTR* has been demonstrated to be expressed in the female reproductive tract, including the ovary (Trezise *et al.* 1993, Chan *et al.* 2002), and involved in uterine HCO_3^- secretion (Wang *et al.* 2003) and HCO_3^- transport that is required for sperm capacitation (Xu *et al.* 2007, Chen *et al.* 2012a), the role of *CFTR* in ovarian function remained unknown for a long time. Recently, we have demonstrated that *CFTR* is expressed in human and rodent granulosa cells and involved in the regulation of the FSH-stimulated aromatase expression and estrogen production through an $\text{HCO}_3^-/\text{sAC}$ -dependent pathway (Chen *et al.* 2012b). In addition to abnormal hormone levels, polycystic ovaries associated with PCOS have also been observed in some CF women, suggesting a possible role of *CFTR* in the development of polycystic ovaries in addition to abnormal estrogen production in PCOS (Chen *et al.* 2012b). As FSH signaling plays an important role in regulating granulosa cell proliferation/apoptosis (Robker & Richards 1998b, Hussein 2005, Edson *et al.* 2009, Oktem & Urman 2010) and *CFTR* is able to modulate the FSH signaling in granulosa cells (Chen *et al.* 2012b), we suspected that *CFTR* might also influence the FSH signaling in regulating the proliferation and apoptosis of granulosa cells. We undertook this study to test this hypothesis. The results indicate that *CFTR*-dependent signaling potentiates the FSH/MAPK/ERK signaling and that granulosa cell proliferation can be affected by *CFTR* expression/function. These findings provide a novel molecular mechanism that may contribute to the abnormal follicle growth observed in PCOS.

Materials and methods

Animals

Female Sprague–Dawley (SD) rats and female Institute for Cancer Research (ICR) mice were kept in the Laboratory Animal Service Center, the Chinese University of Hong Kong with 12 h light:12 h darkness cycle and provided food and water *ad libitum*. Animals were killed by CO_2 inhalation. All procedures were approved by the Animal Ethical Committee of the Chinese University of Hong Kong.

PCOS models

Rat PCOS models were established as described previously (Chen *et al.* 2012b). For testosterone-induced PCOS model,

9-day-old SD rats were injected s.c. with 1.25 mg testosterone dissolved in corn oil. For vehicle control, only corn oil was injected. Rats were killed at the age of 70 days. Ovaries were collected for further analyses. For human CG (hCG) plus L-norgestrel-induced PCOS model, a piece of 3 mm L-norgestrel silica gel was embedded s.c. in the SD rat at the age of 24 days. From the age of 27 days, rats were injected with 1.5 U hCG twice a day for a period of 9 days. For the control group, rats received sham operation and saline injection. Rats were killed at the age of 36 days. Ovaries were collected for further analyses.

Granulosa cell culture

Ovaries were collected from 25-day-old female ICR mice. Ovaries were washed once in Hank's balance salt solution, and transferred to DMEM/F12 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Large preantral follicles were punctured by 25-gauge needles. Granulosa cells were released into the medium by pressing the punctured follicles, and then collected by centrifugation at 500 g for 5 min. Cells were seeded into six-well culture plates at a density of 1×10^6 /well. For ERK phosphorylation assay, on day 4 of culture, granulosa cells were pretreated with HCO_3^- free DMEM/F12 medium without serum for 2 h, followed by treatment of HCO_3^- free medium with vehicle, or 25 mM HCO_3^- medium with vehicle, 10 μM *CFTR* inhibitor GlyH101, 10 μM sAC inhibitor KH7, or 20 μM PKA inhibitor H89 in the presence or absence of 50 ng/ml FSH for 15 min.

CFTR knockdown by siRNA

Granulosa cells were transfected on day 3 of the primary culture. Cells were transfected with 40 nM Stealth siRNA (siRNA1: 5'-UUGGAAAGAAGACUAACAAGUUGUC-3'; siRNA2: 5'-AUUGAGAGCAGAAUGAAACUCUCC-3') or the scrambled siRNA (low GC content) (Invitrogen, Cat. 12935-200) using lipofectamine 2000 (Invitrogen). At 48 h after transfection, whole-cell protein was extracted for western blot analysis.

Immunofluorescence

Ovaries from control or PCOS rats were fixed with 4% paraformaldehyde overnight, dehydrated, and embedded by paraffin. The tissues were cut into 5 μm paraffin sections. Before staining, the sections were rehydrated, followed by antigen retrieval in 10 mM citric acid buffer (pH 6.0). For immunofluorescence, the sections were blocked with 1% BSA for 1 h and incubated with a rabbit anti-*CFTR* antibody (Alomone, Jerusalem, Israel, ACL-006, 1:50 dilution) at 4 °C overnight. The sections were washed three times with PBST. Then, the sections were incubated with Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. The slides were mounted with Prolong(R) Gold Antifade Reagent (Invitrogen, Cat. P36934). Images were obtained at the same setting under the Carl Zeiss LSM5 PASCAL Confocal microscope.

Quantitative RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). Two micrograms of RNA were reverse transcribed into cDNA using MMLV reverse transcriptase. One microliter of cDNA was mixed with forward and reverse primers (*Ccnd2*: forward, 5'-ATGCTGCTCTTGACGGAAC-3' and reverse, 5'-CCTCAGACTTCATTGAGCA-3'; *Gapdh*: forward, 5'-GACCACAGTCATGACATCACTGC-3' and reverse, 5'-GCTGTTGAA-GTCGAGGAGACAAC-3') and 2× iTaq SYBR Green Universal Supermix (Bio-Rad, 172-5121). PCR was performed on Applied Biosystem 7500 fast real-time PCR system. The program was run at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s, followed by elevation to 95 °C by 1 °C/min, holding at 95 °C for 30 s, and 60 °C for 15 s. A final melting curve showed a single peak of each PCR. PCR products were sequenced and blasted to confirm the specificity of the primers. The relative expression level was calculated by the $\Delta\Delta C_t$ method.

Western blot

Protein lysate was prepared from ovaries of PCOS model and cultured granulosa cells with RIPA buffer containing 10 mM Tris-Cl pH 8.0, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche, 11697498001). Protein was denatured by Laemmli sample buffer and heated to 95 °C for 5 min. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Blots were blocked with 5% non-fat dry milk in TBS-T for 1 h at room temperature and incubated with primary antibodies at 4 °C overnight. Rabbit anti-CFTR antibody (Alomone, ACL-006) was diluted at 1:200; mouse anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz, sc-56), rabbit anti-ERK (Cell Signaling, 9102; Danvers, MA, USA), rabbit anti-phospho-ERK (Cell Signaling, 9101), and rabbit anti-cleaved caspase 3 (Cell Signaling, 9661) antibodies were diluted at 1:1000; rabbit anti-*Ccnd2* antibody (Santa Cruz, sc-181) was diluted at 1:200. After three washes with TBS-T, blots were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG. Signals were detected with ECL western blotting detection reagent (GE Healthcare, Pittsburgh, PA, USA). Band intensity was measured by Alphamager System (ProteinSimple, San Jose, CA, USA) and quantified by the average density value of the selected area with the same size. Relative protein expression was quantified by the ratio of target protein band intensity to β -tubulin band intensity. Ratio of phospho-ERK band intensity to total ERK band intensity was used for quantification of ERK phosphorylation.

Cell viability assay

Granulosa cells were seeded into a 96-well plates at a density of 2×10^5 . On the 2nd day of the culture, cells were treated with 0.1% DMSO (v/v) or 10 μ M GlyH101 in DMEM/F12 containing 10% FBS. For FSH treatment, cells were treated with 0.1% DMSO, 0.1% DMSO plus 50 ng/ml FSH or 10 μ M GlyH101 plus 50 ng/ml FSH in serum-free medium. For siRNA transfection, cells were seeded into 96-well plates. At 24 h after seeding, cells were transfected with 40 nM Stealth siRNA or scramble control as described in CFTR knockdown by siRNA section. In MTS assay,

20 μ l MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution (Promega) was diluted in 100 μ l medium in each well. After 2 h of incubation at 37 °C, absorbance was read at a wavelength of A490 nm.

Statistical analysis

Statistics analysis was performed with three to four animals per group or three to four independent cultures. Data are expressed as mean $\pm$ S.E.M. Two-tail unpaired Student's *t*-tests were used for two groups of comparison. One-way ANOVA and Tukey's *post hoc* test were used for comparison among three or more groups. $P < 0.05$ was considered statistically significant.

Results

Reduced CFTR expression in granulosa cells of tertiary/cystic follicles in PCOS rat models

Our previous study had revealed that CFTR was down-regulated in the ovaries of PCOS rats (Chen *et al.* 2012b). In this study, we further investigated CFTR expression profile in different types of follicles. As shown in Fig. 1, immunofluorescence showed that in L-norgestrel + hCG-induced PCOS rats, the granulosa cells of the tertiary and cystic follicles exhibited lower CFTR expression compared with the healthy tertiary follicles of the control rats, whereas the signal difference in secondary and early tertiary follicles between PCOS and control rats was less prominent (Fig. 1A). In testosterone-induced PCOS rats, CFTR-positive signal is also reduced in granulosa cells of the cystic follicles compared with that in the healthy tertiary follicles of the control rats, whereas the signals in both control and PCOS secondary follicles were similar (Fig. 1B). These results suggest that CFTR in granulosa cells may be important for tertiary follicle development and that its downregulation may contribute to the formation of cystic follicles in PCOS.

Downregulation of PCNA in PCOS ovaries

We further examined whether apoptosis and/or proliferation was altered in PCOS ovaries. Western blot results indicated that there was no significant change in cleaved caspase 3, which is the central executor of apoptosis, between the control groups and PCOS groups in L-norgestrel + hCG- or testosterone-treated model (Fig. 2A and B). However, PCNA, a protein required for DNA synthesis and cell proliferation, was significantly downregulated in the ovaries of both PCOS models, indicating reduced cell proliferation in the PCOS ovaries (Fig. 2A and B).

CFTR affects viability of granulosa cells

In order to investigate the role of CFTR in granulosa cell proliferation, we examined the effect of CFTR inhibitors on the viability of granulosa cells. MTS cell viability

Figure 1 Immunofluorescence of CFTR and H-E staining in the ovaries of rat PCOS models. (A) Immunofluorescence of CFTR and H-E staining in the ovaries of control (i, ii, iii, iv, v, and vi) and L-norgestrel + hCG-induced PCOS model (vii, viii, ix, x, xi, xii, xiii, xiv, xv, xvi, xvii, and xviii). CFTR signal is reduced in granulosa cells of tertiary (3 °F) (viii and ix) and cystic follicles (cystic F) (xiv and xv) of PCOS rats compared with that in the healthy tertiary follicles of control rats (ii and iii). CFTR signal in the granulosa cells of the secondary (2 °F) and early tertiary (early 3 °F) follicles is similar between control (iv and v) and PCOS (x and xi, xvi, and xvii) groups. H-E staining showed the morphology of ovaries of control (vi) and PCOS groups (xii). Scale bar in i, ii, iii, iv, and v, vii, viii, ix, x, xi, xiii, xiv, xv, xvi, xvii, and xviii: 50 μ m. Scale bar in vi and xii: 200 μ m. (B) Immunofluorescence of CFTR and H-E staining in the ovaries of control (i, ii, iii, iv, v, and vi), testosterone-induced PCOS rat model (vii, viii, ix, x, xi, and xii). Reduced CFTR signals are found in cystic follicles in PCOS group (vii and viii) compared with tertiary follicle in the control group (i and ii). CFTR signal in the granulosa cells of the secondary and early tertiary follicles is similar between control (iii and iv) and PCOS (ix and x) groups. H-E staining showed the morphology of ovaries of control (vi) and PCOS (xii) groups. Scale bar in i, ii, iii, iv, and v, vii, viii, ix, x, and xi: 50 μ m. Scale bar in vi and xii: 200 μ m.

assays revealed that CFTR inhibitor GlyH101, which had been shown to inhibit CFTR with a higher specificity compared with other Cl^- channel blockers (Muanprasat *et al.* 2004), could inhibit primary granulosa cell growth (Fig. 3A). We knocked down *CFTR* with two siRNAs specifically targeting *CFTR*. Quantitative RT-PCR showed significant downregulation of *Cftr* mRNA after transfection of siRNAs (Fig. 3B). Western blot also showed downregulation of CFTR protein by siRNAs

(Fig. 3C). Specificity of the antibody was confirmed by antibody pre-absorption by antigen, which showed disappearance of the CFTR bands (Fig. 3C). Consistent with the result from CFTR inhibitors, when *CFTR* was knocked down by siRNA, granulosa cell growth was also inhibited compared with the scramble control (Fig. 3C). We also found that in the presence of FSH, GlyH101 also inhibited FSH-stimulated granulosa cell growth (Fig. 3D), indicating possible involvement of CFTR in

Figure 2 Expression of proteins related to proliferation and apoptosis in ovaries of rat PCOS models. (A) Western blot results of cleaved caspase 3 and PCNA in L-norgestrel + hCG PCOS model ovaries. (B) Western blot results of cleaved caspase 3 and PCNA in testosterone PCOS model ovaries. $n=4$, t -test, ** $P<0.01$, *** $P<0.001$ compared with control.

modulating the FSH signaling in regulating granulosa cell proliferation.

CFTR regulates cell cycle and proliferation via the HCO_3^- /sAC/PKA and ERK pathway

We further investigated how CFTR regulates granulosa cell proliferation in the primary granulosa cell culture. Knockdown of CFTR by siRNAs led to reduced expression of PCNA in primary granulosa cells (Fig. 4A), consistent with the role of CFTR in promoting granulosa cell proliferation. Similarly, CFTR inhibitor GlyH101 also downregulated PCNA expression in granulosa cells (Fig. 4B). Our previous study had demonstrated that CFTR regulates basal and FSH-stimulated aromatase expression in an HCO_3^- /sAC-dependent manner (Chen *et al.* 2012b). We thus examined the effect of sAC inhibitor KH7 on PCNA expression and the western blot analysis indicated that KH7 (10 μM) treatment could also downregulate PCNA expression, suggesting the involvement of sAC in granulosa cell proliferation (Fig. 4B). As sAC is an HCO_3^- sensor and activator of cAMP/PKA (Chen *et al.* 2000), we also examined the effect of extracellular HCO_3^- and FSH on PCNA expression. Western blot results indicated that neither HCO_3^- nor FSH significantly increased PCNA expression; however, HCO_3^- plus FSH could significantly upregulate PCNA expression (Fig. 4C). The upregulation of PCNA could be inhibited by GlyH101 and KH7 (Fig. 4C), indicating the involvement of the CFTR-dependent HCO_3^- /sAC pathway in modulating the FSH-regulated PCNA expression and thus granulosa cell proliferation.

As FSH is known to activate the ERK pathway, one of the important signaling pathways that control granulosa cell proliferation, through a cAMP/PKA-dependent pathway (Cotton *et al.* 2003, Hunzicker-Dunn & Maizels 2006), we suspected that the demonstrated involvement

of the CFTR/ HCO_3^- /sAC pathway in potentiating the effect of FSH on PCNA expression might involve activation of ERK. Indeed, our western blot results indicated that HCO_3^- could slightly enhance ERK phosphorylation, which could be inhibited by GlyH101, KH7, and PKA inhibitor H89 (Fig. 5A). In the absence of HCO_3^- , FSH slightly but not significantly increased ERK phosphorylation. However, HCO_3^- could enhance the effect of FSH on ERK phosphorylation, which could also be inhibited by GlyH101, KH7, and H89 (Fig. 5A), consistent with the involvement of the CFTR/ HCO_3^- /sAC pathway.

Cyclin D₂ (CCND2) is a cell cycle regulator protein downstream of ERK. It regulates cell cycle progression by promoting G₁/S transition, and promotes proliferation in granulosa cells. Real-time PCR results indicated that HCO_3^- could increase *Ccnd2* mRNA expression (Fig. 5B). In the absence of HCO_3^- , FSH did not

Figure 3 Effect of CFTR inhibition on viability of granulosa cells. (A) MTS assays of granulosa cells treated with 10 μM GlyH101 or vehicle control. (B) Real-time PCR showed that CFTR was knocked down in granulosa cells by two designs of siRNA. (C) Western blot showed that CFTR was knocked down by siRNAs (upper panel). When the CFTR antibody was pre-absorbed by an antigen, the CFTR-specific bands disappeared, confirming the specificity of the antibody (low panel). (D) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay of granulosa cell transfected with scramble control or CFTR siRNAs. (E) MTS assays of granulosa cells treated with 10 μM GlyH101 or vehicle control in the presence or absence of 50 ng/ml FSH. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared with control and FSH+GlyH101.

Figure 4 Effect of *CFTR* knockdown and inhibition on PCNA expression in cultured granulosa cells. (A) Downregulation of PCNA in *CFTR* knockdown granulosa cells. *CFTR* was knocked down by two designs of siRNA in granulosa cells at 48 h after transfection. One-way ANOVA, * $P < 0.05$ compared with scramble control. (B) Downregulation of PCNA by *CFTR* inhibitor GlyH101 (10 μ M) and sAC inhibitor KH7 (10 μ M). (C) PCNA expression is upregulated by FSH in the presence of HCO_3^- , which is inhibited by GlyH101 and KH7. One-way ANOVA, * $P < 0.05$, *** $P < 0.001$.

significantly increase *Ccnd2* expression (Fig. 5B). However, in the presence of HCO_3^- , FSH significantly upregulated *Ccnd2*, which was inhibited by GlyH101, KH7, and H89, as well as MEK inhibitor U0126 (Fig. 5B), indicating the role of *CFTR* in granulosa cell proliferation by regulating the ERK pathway and its downstream target *Ccnd2* via the *CFTR*/ HCO_3^- /sAC pathway. We further examined the level of p-ERK and CCND2 in the rat PCOS model induced by testosterone. Western blot results indicated downregulation of both p-ERK and CCND2 in the ovaries of PCOS group compared with control (Fig. 5C). Together with the observed downregulation of *CFTR* in PCOS, it supports a role of *CFTR* in regulating granulosa cell proliferation through ERK and CCND2.

Discussion

This study on two rat models of PCOS indicates that granulosa cells in the cystic ovaries have decreased cell

proliferation, which may underlie the abnormal follicle development in PCOS. Our finding is supported by another report which used a rat PCOS model induced by disrupting light–darkness cycle and found reduced PCNA in the granulosa cell layers of cystic follicles of PCOS rat compared with normal tertiary follicles in control animals (Salveti *et al.* 2009). Decreased granulosa cell proliferation was also reported in bovine PCOS (Isobe & Yoshimura 2000). However, in studies carried out with PCOS women, either increased proliferation or no difference was found in granulosa cells of early antral follicles (4–8 mm diameter) in PCOS group compared with those in the control group (Takayama *et al.* 1996, Stubbs *et al.* 2007, Das *et al.* 2008). The inconsistent results obtained from the human and rat may be due to the difference in the stage of the follicles examined. The follicles examined in human PCOS studies were at earlier stages, whereas the cyst follicles examined in rat PCOS models were at a later stage, with degenerated granulosa cell layers, which matched the ovarian morphology of PCOS.

Although altered cell proliferation in PCOS granulosa cells has been documented by a number of studies (Takayama *et al.* 1996, Isobe & Yoshimura 2000, Stubbs *et al.* 2007, Das *et al.* 2008, Salveti *et al.* 2009), the underlying mechanism is not well understood. Herein, we have found that *CFTR* is downregulated in granulosa cells of PCOS rats, accompanied by downregulation of the pro-proliferative gene *Pcna*. Furthermore, *in vitro* studies demonstrate that knockdown or inhibition of *CFTR* led to downregulation of PCNA, as well as decreased cell growth, further supporting the possible involvement of downregulation of *CFTR* in the abnormal follicle development in PCOS. The involvement of *CFTR* in regulating granulosa cell proliferation suggests its possible role in normal folliculogenesis during the ovary development in early life and the cyclic wave of follicle growth in reproductive age, defect of which may result in impaired folliculogenesis as observed in PCOS.

As FSH is known to stimulate rapid proliferation of granulosa cells in antral follicles (Robker & Richards 1998b), the observed altered granulosa cell proliferation in cystic follicles of PCOS suggests a possible defect in FSH-dependent regulation. Our previous study has demonstrated the involvement of the *CFTR*/ HCO_3^- /sAC pathway in potentiating FSH-stimulated estrogen production, showing reduced *CFTR* and aromatase expression in PCOS rat models and PCOS patients (Chen *et al.* 2012b). The present results indicate that this pathway also regulates the basal and FSH-induced ERK activation in granulosa cells, which is a well-known pathway in the regulation of cell cycle and proliferation. Similar to the case of regulation of CREB phosphorylation by *CFTR* (Chen *et al.* 2012b), the *CFTR*/ HCO_3^- /sAC pathway may potentiate the effect of FSH on ERK phosphorylation through the *CFTR*-dependent HCO_3^- entry, activation of sAC, and thus cAMP/PKA cascade.

In fact, the FSH-induced ERK phosphorylation depends on cAMP and PKA. Unlike CREB, ERK is not directly phosphorylated by PKA, but rather by MEK. PKA is known to phosphorylate and inactivate a 100 kDa tyrosine phosphatase, which in turn inhibits the de-phosphorylation pathway of ERK (Cottom *et al.* 2003). Therefore, the involvement of the CFTR/HCO₃⁻/sAC pathway in modulating ERK phosphorylation may be

mediated by the PKA-activated inhibition of the tyrosine phosphatase-dependent de-phosphorylation. The ability of the CFTR/HCO₃⁻/sAC pathway to modulate both FSH-stimulated estrogen production, as demonstrated previously, and granulosa cell proliferation, as demonstrated presently, through the CREB and ERK pathways, respectively, suggests that CFTR plays an important role in ovarian functions.

Cleaved caspase 3, which plays a central role in apoptosis, was not significantly changed in PCOS ovaries, as shown by our western blot results (Fig. 2). However, we did not examine its level in follicles of different stages. Therefore, it is unknown whether apoptosis is changed in follicles at certain stages. Studies examining apoptosis in rat PCOS model and PCOS patients by other groups showed increased apoptosis in cystic follicles in rat PCOS model (Salveti *et al.* 2009), but reduced apoptosis in early-stage follicles in PCOS patients (Das *et al.* 2008). Therefore, the alteration in apoptosis status may vary at different stages of follicles in PCOS. It would be of interest to examine whether CFTR may also play a role in granulosa cell apoptosis at different stages of follicle development as CFTR has also been implicated in the regulation of apoptosis (Noe *et al.* 2009, l'Hoste *et al.* 2010, Yang *et al.* 2011).

In conclusion, this study has demonstrated a novel mechanism for regulation of granulosa cell proliferation involving CFTR and its potentiation of FSH effect on the ERK pathway. The present results suggest an important role of CFTR in normal folliculogenesis, mutation or downregulation of which may be responsible for the formation of polycystic follicles observed in both CF and PCOS. Further investigation into how CFTR is down-regulated in PCOS and exploration of possible way(s) to re-upregulate CFTR may help develop potential treatment strategy for PCOS.

Figure 5 Enhanced basal and FSH-stimulated ERK phosphorylation and *Ccnd2* expression by the CFTR/HCO₃⁻/sAC pathway and reduced ERK phosphorylation and CCND2 in PCOS. (A) Western blot results of ERK and phosphorylated ERK in the absence and presence of FSH. 25 mM HCO₃⁻ can enhance the effect of FSH on ERK phosphorylation, which can be inhibited by CFTR inhibitor GlyH101 (10 μM), sAC inhibitor KH7 (10 μM), and PKA inhibitor H89 (20 μM). One-way ANOVA, **P*<0.05, ***P*<0.01. (B) Enhanced *Ccnd2* mRNA expression by the CFTR/HCO₃⁻/sAC pathway. Real-time PCR shows that 25 mM HCO₃⁻ can upregulate *Ccnd2* expression in granulosa cells. ***P*<0.01 when HCO₃⁻+/FSH- group was compared with HCO₃⁻-/FSH- group. In the absence of HCO₃⁻, FSH did not significantly increase *Ccnd2* expression. In the presence of 25 mM HCO₃⁻, FSH could upregulate *Ccnd2*, which can be inhibited by CFTR inhibitor GlyH101 (10 μM), sAC inhibitor KH7 (10 μM), PKA inhibitor H89 (20 μM), and MEK inhibitor U0126 (10 μM). One-way ANOVA, ***P*<0.01, ****P*<0.001 compared with HCO₃⁻+/FSH+ group; ****P*<0.001 compared with HCO₃⁻-/FSH- group. (C) Western blot showed reduced ERK phosphorylation and CCND2 expression in testosterone-induced PCOS model.

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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