PCSK6 regulated by LH inhibits the apoptosis of human granulosa cells via activin A and TGF β 2

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Abstract

Mammalian proprotein convertases (PCs) play an important role in folliculogenesis, as they proteolytically activate a variety of substrates such as the transforming growth factor beta (TGF β) superfamily. PC subtilisin/kexin 6 (PCSK6) is a member of the PC family and is ubiquitously expressed and implicated in many physiological and pathological processes. However, in human granulosa cells, the expression of the PC family members, their hormonal regulation, and the function of PCs are not clear. In this study, we found that PCSK6 is the most highly expressed PC family member in granulosa cells. LH increased *PCSK6* mRNA level and PCSK6 played an anti-apoptosis function in KGN cells. Knockdown of *PCSK6* not only increased the secretion of activin A and TGF β 2 but also decreased the secretion of follistatin, estrogen, and the mRNA levels of FSH receptor (*FSHR*) and *P450AROM* (*CYP19A1*). We also found that, in the KGN human granulosa cell line, TGF β 2 and activin A could promote the apoptosis of KGN cells and LH could regulate the follistatin level. These data indicate that PCSK6, which is regulated by LH, is highly expressed in human primary granulosa cells of pre-ovulatory follicles and plays important roles in regulating a series of downstream molecules and apoptosis of KGN cells.

Key Words

- PCSK6
- LH
- apoptosis
- granulosa cells
- TGF β 2
- activin A

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Introduction

Mammalian proprotein convertases (PCs) are a family of serine endoproteases that activate a variety of protein precursors by catalytic cleavage, promoting their maturation. Nine PC family members have been identified: PCSK1, PCSK2, PCSK3 (FURIN), PCSK4, PCSK5, PCSK6, PCSK7, PCSK9, and SKI1/S1P (MBTPS1). The first seven are structurally and functionally similar to each other and are typical members of the family, while PCSK9 and SKI1/S1P are atypical (Artenstein & Opal 2011). The expression of PC family members varies in different species, organs,

and cells. PCSK1 and PCSK2 are expressed mainly in neuroendocrine tissues and the pancreas (Feng *et al.* 2002). PCSK4 is specific to the testes and ovaries (Tadros *et al.* 2001). By contrast, PCSK3, PCSK5, PCSK6, and PCSK7 are more widely expressed. Among the wide variety of PCs, substrates are precursors of hormones, enzymes, growth factors, receptors, cell membrane proteins, plasma proteins, viral glycoproteins, and bacterial toxins, all of which share the same basic cleavage target motifs (K/R)–(X)*n*–(K/R)_↓, with *n*=0, 2, 4, or 6. For instance,

beta nerve growth factor (β -NGF) is the substrate of furin and processed β -NGF is a mediator for neuron survival while secreted, unprocessed pro- β -NGF mediates apoptosis of neuron (Lee *et al.* 2001). PCs have been shown to be involved in a wide range of processes including embryogenesis, homeostasis, and in diseases, such as Alzheimer's disease, tumorigenesis, and infections (Artenstein & Opal 2011).

In the reproductive system, PCs participate in follicle and embryo development. Male *Pcsk4*-null mice exhibited severely impaired fertility and female *Pcsk4*-null mice exhibited delayed folliculogenesis in the ovaries (Mbikay *et al.* 1997). PCSK5 is suggested to play an important role in the processes of stromal cell decidualization, embryo implantation (Okada *et al.* 2005), cumulus expansion, and follicular rupture (Bae *et al.* 2008) in the mouse, and deletion of the *Pcsk5* gene causes early embryonic lethality (Essalmani *et al.* 2006). *Pcsk5* mutant mice exhibit progressive loss of ovarian function, altered gene expression, and the formation of ovarian pathology (Mujoomdar *et al.* 2011).

Granulosa cells play important roles in follicle development. In granulosa cells of mouse follicles at different stages of development, PC family members showed differential expression (Bae *et al.* 2008). Diaz *et al.* (2008) found that PCSK6 mRNA and protein were expressed in preantral granulosa cells, but not in cumulus or mural cells of the pre-ovulatory follicles, in the mouse ovary. However, very little is known about PC expression and functions in human granulosa cells. Only one recent study (Akiyama *et al.* 2012) has shown that, in humans, PCSK6 is expressed in both oocytes and granulosa cells (GCs) in growing follicles up to the antral stage with the expression level of PCSK6 in GCs increasing during follicle development.

In this study, we first examined the expression of the PC family members in human granulosa cells and then further studied PCSK6 as it was the most highly expressed family member in human granulosa cells. We studied the hormone regulation of PCSK6 in primary luteinizing GCs and in the KGN human granulosa tumor cell line. We also found that PCSK6 could inhibit apoptosis of KGN cells and influence a series of downstream molecules including local hormones, important receptors, and enzymes, some of which are involved in cell apoptosis.

Materials and methods

Human samples

Primary human GCs were obtained with the approval of the Human Investigation Committee of Hospital of Obstetrics and Gynecology, Fudan University, and informed consent

was obtained from all participants. Follicle aspirates were collected during oocyte retrieval from women with tubal blockage undergoing *in vitro* fertilization (IVF) and granulosa cells were isolated as described previously (Sasson *et al.* 2004). The IVF stimulation protocol was the standard long gonadotropin-releasing hormone (GnRH) agonist protocol and, before intra-cytoplasmic sperm injection (ICSI), we isolated granulosa cells from oocyte–corona–cumulus complex.

The culture and treatment of GCs or cell line

Human GCs were cultured in a humidified atmosphere of 5% CO₂–95% air at 37 °C in phenol-red-free RPMI-1640 (Sigma) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (DCC–FBS; Hyclone Laboratories, Logan, UT, USA) and 100 µg/ml Primocin (InvivoGen, San Diego, CA, USA). The KGN granulosa tumor cells were cultured in a humidified atmosphere of 5% CO₂–95% air at 37 °C in DMEM/F12 (Sigma) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate (Invitrogen). After culturing for 3 days, primary human GCs were stimulated with different concentrations of recombinant human luteinizing hormone (LH; 0, 1, 10, 100, and 1000 IU/l; Sigma) for different time periods (0, 1, 2, 3, or 6 h). KGN cells were treated with 100 IU/l human LH for 3 h before extraction of mRNA or with different concentrations of activin A (0.1, 1, and 10 ng/ml; R&D System, Minneapolis, MN, USA) or transforming growth factor beta 2 (TGFβ2; 0.1, 1, and 10 ng/ml; R&D System) for 24 h before detecting the apoptosis rate by flow cytometry.

Immunocytochemistry

GCs were seeded in tissue culture-treated plastic dishes that are 35 mm in diameter and cultured for 3 days. After washing twice with PBS, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized for 20 min at room temperature using 0.3% Triton/PBS. Cells were blocked with 10% fetal bovine serum at room temperature for 60 min and incubated overnight at 4 °C with either primary rabbit anti-human follicle-stimulating hormone receptor (FSHR) polyclonal antibodies (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or PBS, as the negative control. After washing twice with PBS, cells were incubated with a biotinylated secondary anti-rabbit antibody for 20 min at room temperature, washed with PBS, incubated with streptavidin–horseradish peroxidase

(HRP) for 20 min at room temperature, and then stained with DAB (Zhongshan Cambridge Company, Beijing, China) and counterstained with hematoxylin.

RNA extraction, RT, and PCR

After culture and experimental treatment, the cell culture medium was removed, cells were washed with PBS, and RNA was extracted using TRizol (Invitrogen). RNA concentration was measured based on the absorbance at 260 nm, and RNA was reverse transcribed into cDNA using a RT Kit (Fermentas, Pittsburgh, PA, USA). The primers used for qualitative PCR or SYBR Green quantitative PCR were designed by Shenggong Company (Shanghai, China) and are shown in Table 1. Qualitative PCR was performed according to the instruction and DNA products were detected by gel electrophoresis. The values of the band intensities were determined using the Image J Software (National Institute of Mental Health, Bethesda, MD, USA). Quantitative PCR was performed using the ABI PRISM 7300 Sequence Detection System according to the manufacturer's protocol (Applied Biosystems). Relative quantification of the mRNA levels of *PCSK* genes, *FSHR*, *LHR*, and *P450AROM* (*CYP19A1*) was performed using the $\Delta\Delta Ct$ method with *GAPDH* as the reference gene.

siRNA transfection

KGN cells were cultured in a medium as described above to achieve ~30% confluence at the time of transfection. Cells were transfected using a non-targeting negative control

siRNA (Dharmacon, Chicago, IL, USA) or siRNAs against human *PCSK6* (ON-TARGET plus SMART pool, Dharmacon) at a final concentration of 50 nM using Lipofectamine 2000 (InvitroGen). The group in which only Lipofectamine 2000 was used was treated as the blank control. Following transfection, cells were collected for mRNA and protein extraction after 24, 48, and 72 h or re-seeded into new plates after 48 h for further experiments (24 h after re-seeding).

Western blotting

After experimental treatment, cells were washed twice with PBS, lysed in ice-cold RIPA buffer with 1% phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 12 000 *g* at 4 °C for 30 min and protein concentration was determined using the Bradford protein assay (Beyotime Company, Shanghai, China). Proteins (50 µg) were separated on 10% SDS–polyacrylamide gels and transferred onto PVDF membranes. After blocking in PBS with 5% non-fat dry milk, the membranes were incubated with an anti-PCSK6 polyclonal antibody (1:2000, Abcam, Cambridge, UK) overnight at 4 °C, washed with PBS, and incubated with an HRP-labeled secondary antibody (1:5000, Santa Cruz Biotechnology). Blots were developed using the Amersham ECL detection kit (Amersham Pharmacia Biotech).

Enzyme-linked immunosorbent assay (ELISA)

After the knockdown of *PCSK6* or stimulation of LH for 24 h in KGN cells, the levels of activin A, follistatin, TGFβ2 (all from R&D System), and estrogen (CUSOBIO,

Table 1 PCR primer sequences for proprotein convertase, cytochrome P450 aromatase, *FSHR*, *LHR*, and *GAPDH* genes

Gene	Sequence (qualitative PCR)	Sequence 2 (quantitative PCR)
<i>PCSK1</i>	Forward: 5'-TCTTACAGCAGCGGAGATTACA-3' Reverse: 5'-CCAGGGACTTGATAGCATTTTC-3'	Forward: 5'-TCACCAAAGAAGTCCCCAAG-3' Reverse: 5'-AGGTCGTCTCTGTGCTTGTA-3'
<i>PCSK2</i>	Forward: 5'-GGTGAATGCTAAAGACTGG-3' Reverse: 5'-TCATGTTGATGTTCAAGTCTCC-3'	Forward: 5'-GAGAAGACGCAGCCTACACC-3' Reverse: 5'-TTTCCGTCAAATCCTTCCTG-3'
<i>PCSK3</i>	Forward: 5'-CAGAGGGAGCCTCAAGTACAGT-3' Reverse: 5'-GCCTGTTGTCATTCTGTGT-3'	Forward: 5'-TATGGCTACGGGCTTTTGGA-3' Reverse: 5'-GCCGTTTCCCGATGTCTTT-3'
<i>PCSK4</i>	Forward: 5'-CTACATCAGGGAAAACGTATCG-3' Reverse: 5'-GTCCCCGTGTTGAAATAGTAGC-3'	Forward: 5'-CAACAATGGCTTCTGTGGTG-3' Reverse: 5'-GCTGTAAATGTGGATGTGCTG-3'
<i>PCSK5</i>	Forward: 5'-CCATAGCAGGACGATTAAGAGG-3' Reverse: 5'-GAGCATCGTCGTAGTTTGCATCAG-3'	Forward: 5'-ATGACTGGAAAACCAATGCTG-3' Reverse: 5'-ACTGTTAGGGCGGATTGTCTT-3'
<i>PCSK6</i>	Forward: 5'-TGTAAGAAAGTGCCTGGATGAAC-3' Reverse: 5'-GTCGTGGAAGTGGAAAGTTTTC-3'	Forward: 5'-ACGAAGGGTGAAGAGACAGGT-3' Reverse: 5'-TTTTCTGTGTAGCCCCCTAT-3'
<i>PCSK7</i>	Forward: 5'-GCAGTGACAGTCTCCATCACT-3' Reverse: 5'-GGCACTCTCTAACAGCCTTGT-3'	Forward: 5'-TAGTGGATGACGGAGTGGAAC-3' Reverse: 5'-ATGGGGTCAGGGTCATTAGAG-3'
<i>FSHR</i>	Forward: 5'-ACAGGGTTTTCTCTGCCAAG-3'	Reverse: 5'-TCTGCCTCTATCACTCCAAG-3'
<i>LHR</i>	Forward: 5'-CTACACCTCACGTCATCA-3'	Reverse: 5'-AGAAAAGAGCCATCCTCCAAG-3'
<i>P450AROM</i>	Forward: 5'-GAAATGCTGAACCCGATACATT-3'	Reverse: 5'-AAGAGAAAAGGCCAGTGAGG-3'

Wuhan, China) in the cell culture supernatant were detected using ELISA. The minimal detectable concentrations for activin A, follistatin, TGF β 2, and estrogen were 3.67, 29, 7.0, and 25 pg/ml respectively.

Flow cytometry

For the apoptosis assay, cells were detached from the tissue culture plates with 0.25% Trypsin without EDTA, centrifuged at 1000 *g* for 5 min, re-suspended in PBS, fixed and permeabilized, and labeled with an FITC anti-human active Caspase 3 antibody (BD Bioscience, Heidelberg, Germany), or labeled directly with APC anti-human CD95 (Fas) or PE anti-human CD178 (FasL) (both from Biolegend, San Diego, CA, USA) for 30 min, or labeled with Annexin V and PI as described by the manufacturer (Merck), and then washed and detected by flow cytometry.

Statistical analysis

The SPSS Software package version 18.0 was used for statistical data analysis using one-way ANOVA. $P < 0.05$ was considered statistically significant. All experimental data are expressed as mean $\pm$ S.E.M. of at least three independent experiments.

Results

The expression of PCSK6 in human primary granulosa cells

As human GCs are the only cell type in the ovary that expresses FSHR, we used immunocytochemistry to investigate the purity of isolated human GCs. Over 95% of the isolated cells expressed FSHR (Fig. 1A), indicating good purity of isolated primary human GCs.

We analyzed human GCs obtained from six patients by qualitative PCR for the expression of *PCSK1*, *PCSK2*, *PCSK3*, *PCSK4*, *PCSK5*, *PCSK6*, and *PCSK7*. Of these seven PC family members, *PCSK3*, *PCSK4*, *PCSK5*, *PCSK6*, and *PCSK7* were expressed at detectable levels, while *PCSK1* and *PCSK2* expression were not detectable (Fig. 1B and C). To compare mRNA expression levels in human GCs, we designed additional primers for quantitative real-time PCR (qRT-PCR) to carry out these qualitative findings. In human GCs, qRT-PCR revealed that *PCSK6* was most highly expressed, followed by *PCSK3*, *PCSK5*, and *PCSK7* with *PCSK4* being lower. In addition, qRT-PCR indicated low, but detectable, expression of *PCSK1* and *PCSK2* (Fig. 1D).

Figure 1

The expression of PCSK6 in human primary granulosa cells. (A) Granulosa cells were stained with PBS (as control, left) or an antibody against follicle-stimulating hormone receptor (FSHR; right) to identify the purity of hGCs. (B and C) RT-PCR results of proprotein convertase family members. (D) Real-time PCR results using different pairs of primers. Each experiment was repeated at least six times.

PCSK6 inhibits apoptosis of KGN cells

To determine whether PCSK6 is involved in the biological functions of granulosa cells, we reduced PCSK6 expression by transfection of KGN cells with *PCSK6*-specific siRNAs. RT-PCR and western blot were used to detect the silencing effect. As shown in Fig. 2A and B, transfection reagent control without (blank) or with a non-specific siRNA (si control) did not affect PCSK6 expression, as expected.

Figure 2

PCSK6 inhibits apoptosis of KGN cells. KGN cells were transiently transfected with Lipofectamine 2000 (blank), Lipofectamine 2000 plus siRNAs against human *PCSK6* (*PCSK6* siRNA), or Lipofectamine 2000 plus a non-targeting negative control siRNA (si control), and then the knockdown effect was verified at the mRNA level by PCR (A) or the protein level by

western blotting (B). (C) After transfections of KGN cells for 72 h, cells were collected and flow cytometric assay was performed to compare the apoptosis rate in the three groups. Each experiment was repeated at least four times. * $P < 0.05$ compared with blank, ** $P < 0.01$, a and b represent different *PCSK6* siRNA concentrations, 25 and 50 nM.

By contrast, siRNAs targeting *PCSK6* reduced *PCSK6* mRNA and protein levels by over 85%, indicating specific and efficient knockdown of *PCSK6*. KGN cells with knockdown of *PCSK6* showed an increase in apoptosis compared with controls (Fig. 2C), indicating that *PCSK6* has an anti-apoptotic role.

PCSK6 regulates the secretion of local hormones, gonadotropin receptors, and enzyme in KGN cells

To investigate how *PCSK6* might inhibit apoptosis in KGN cells, we used ELISA to determine the effect of *PCSK6* knockdown on the expression of TGF β 2 (TGF β 2), activin,

and follistatin, all of which are *PCSK6* substrates that have been reported to be involved in the induction or repression of apoptosis (Patella *et al.* 2006, Quezada *et al.* 2012, Yu *et al.* 2012). Compared with blank or si control group, knockdown of *PCSK6* increased the levels of secreted activin A and TGF β 2 (Fig. 3A) and decreased the level of secreted follistatin (Fig. 3B).

The FSH and LH receptors and P450arom enzyme participate in the synthesis of sex hormones in granulosa cells and have been reported to inhibit apoptosis (Uchida *et al.* 2004, Fester *et al.* 2006, Xia *et al.* 2013). As shown in Fig. 3C, mRNA levels of *FSHR* and *P450AROM* were reduced in KGN cells after *PCSK6* knockdown, but *LHR*

Figure 3

PCSK6 regulates the secretion of local hormones, gonadotropin receptors and enzyme in KGN cells. After 72 h of transfection, the supernatant of KGN cells was collected and local hormones such as activin A (A), TGF β 2 (A), follistatin (B), and estrogen (C) were detected. ELISA was performed in

triplicate and repeated three times. After 48 h of transfection, changes in *P450AROM* and *FSHR* mRNAs were detected by real-time PCR (D). * $P < 0.05$ compared with blank and ** $P < 0.01$. Each experiment was repeated at least four times.

(*LHCGR*) mRNA level was not changed (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Moreover, knockdown of *PCSK6* reduced the estrogen level (Fig. 3D).

Activin A and TGF β 2 induce apoptosis of KGN cells

As *PCSK6* decreased the secretion of activin A and TGF β 2 and played an anti-apoptotic role, we speculated that activin A and TGF β 2 might be involved in apoptosis. We found that activin A and TGF β 2 could promote apoptosis of KGN cells in a dose-dependent manner (Fig. 4A and B).

LH up-regulates *PCSK6* in KGN cells

As *PCSK6* was expressed to the highest extent among PC family members in primary human granulosa cells, we investigated whether it was regulated by hormones. We found that LH up-regulated *PCSK6* mRNA levels in time- and dose-dependent manners both in primary human GCs and KGN cells (Fig. 5A, B and C).

LH inhibits the apoptosis of KGN cells and regulates expression of local hormones and receptors

Previous studies have demonstrated that LH can influence apoptosis in several different cell types (Porter *et al.* 2001, Slot *et al.* 2006, Zhang *et al.* 2011). Thus, we used flow

cytometry to determine whether 24 h of LH exposure affected apoptosis of KGN cells. As shown in Fig. 6A, 10–1000 IU/l LH reduced apoptosis of KGN cells in a dose-dependent manner, with 100 IU/l LH being the most effective one. To confirm that LH inhibits apoptosis of KGN cells, we detected apoptosis-related markers and found that LH could decrease their expression (Fig. 6B).

We also detected whether LH regulated expression of local hormones, enzymes, and gonadotropin receptors. As shown in Fig. 6C, LH up-regulated the secretion of follistatin but had no effects on the secretion of activin A and TGF β 2. Moreover, LH could also increase mRNA levels of *FSHR* and *P450AROM* (Fig. 6D).

Discussion

Prior to this work, there had been no research on the expression of PCs in human GCs of pre-ovulatory follicles. In this study, we examined the transcription of seven PC family members and found that the mRNA level of *PCSK6* was the highest. The mRNA levels of *PCSK3*, *PCSK5*, and *PCSK7* were relatively moderate, and the level of *PCSK4* was low, with *PCSK1* and *PCSK2* mRNAs present only in pre-ovulatory GCs at very low, but detectable levels.

One possible role of this highly expressed level of *PCSK6* in PC family members may be in regulating the apoptosis of human GCs. The loss of ovarian follicles through atresia, which accounts for more than 99% of

Figure 4

Activin A and TGF β 2 induce apoptosis of KGN cells. KGN cells were treated with different concentrations of activin A (0, 0.1, 1, and 10 ng/ml) (A) or TGF β 2 (0, 0.1, 1, and 10 ng/ml) (B) for 48 h and then the apoptosis rate was

detected by FCM. Each experiment was repeated at least four times.

** $P < 0.01$ compared with control, NS represents no significant difference.

Figure 5

LH induces PCSK6 both in primary granulosa cells and KGN cells. (A) hGCs were cultured in the DMEM medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum for 72 h and then stimulated with 100 IU/l LH for different time periods (0, 1, 2, 3, and 6 h). (B) hGCs were stimulated with different concentrations of LH (0, 1, 10, 100, and 1000 IU/l) for 3 h and then the mRNA level of PCSK6 was detected by real-time PCR. (C) KGN cells were treated with 100 IU/l LH for 3 h and then the mRNA level of PCSK6 in it was measured by real-time PCR. Each experiment was repeated at least 4 times. * $P < 0.05$ compared with control.

postnatal female germ cell depletion, is mediated through apoptotic cell death (Tilly *et al.* 1995). Studies have demonstrated that apoptosis of GCs can be manipulated, and the molecular mechanisms responsible for it are of great interest.

In this study, we found that PCSK6 siRNA could promote apoptosis of KGN cells and regulate a series of downstream molecules such as activin A and TGF β 2. PCSK6 was found to be essential for maintaining a high proliferative state in prostate cancer cell lines (Couture *et al.* 2012). Activin A and TGF β 2 were believed to be pro-apoptosis factors in various tissue and cell types such as murine adrenal X-zone (Beuschlein *et al.* 2003), intestinal mucosa (Dunker *et al.* 2002), trophoblast cells (Yu *et al.* 2012), granulosa cells (Quezada *et al.* 2012), oligodendrocytes (Quezada *et al.* 2012), hepatocytes (Hughes & Evans 2003), and endometrial stromal cells (Moulton 1994). However, follistatin acted as an antagonist to activin and was considered as an anti-apoptosis factor in cells such as hepatocytes (Patella *et al.* 2006) and renal tubular cells (Maeshima *et al.* 2001). We believe that PCSK6 played its anti-apoptosis role to some extent by regulating these apoptosis-related factors in GCs. However, whether or not PCSK6 influenced the transcriptional levels of inhibin α or β substrates or impaired the post-translational process of these local hormones was not clear. We found that PCSK6 siRNA decreased the mRNA levels of *inhibin β B* and it had no effects on *inhibin α* and *β A* (data not shown). Moreover, follistatin has no PCSK6 recognition motif, and we believed that other hormones in the follicle fluid such as activin or inhibin influenced the secretion of follistatin.

Furthermore, in this study, we found that PCSK6 siRNA could significantly inhibit the ability to invade but had no obvious effect on the proliferation (Supplementary Fig. 2, see section on supplementary data given at the end of this article). Perhaps, in this process, the matrix metalloproteinases (MMPs) were involved as PCSK6 knockdown inhibited the activation of MMPs and then influenced the invasion of KGN cells, which deserves further study. Studies (Kelty & Curry 2010) have demonstrated that PCSK3 results in the activation of MMP2 associated with the breakdown of the follicular wall during ovulation. Studies have also demonstrated that PCSK6 could enhance mouse skin carcinoma progression by increasing tumor cell invasiveness and promoting the malignant conversion of non-invasive keratinocytes into malignant cells (Mahloogi *et al.* 2002).

We used the siRNA technique to knockdown the PCSK6 gene in KGN cells and found that the mRNA level of *FSHR* and a downstream target of FSH signaling

Figure 6

LH inhibits the apoptosis of KGN cells and regulates local hormones and receptors. (A) We used different concentrations of LH (0, 10, 100, and 1000 IU/l) to stimulate KGN cells for 24 h and then the apoptosis rate was detected by FCM. (B) After KGN cells were treated with 100 IU/l LH for 24 h, the percentages of p-caspase 3-, Fas-, and FasL-positive cells were detected by FCM. Simultaneously, the supernatant was collected to detect the

concentration of local hormones such as follistatin, activin A, and TGF β 2 (C). (D) The effects of LH on *FSHR* and *P450AROM* mRNA levels were detected by real-time PCR. Each experiment was repeated at least four times. * $P < 0.05$ compared with control, ** $P < 0.01$ and *** $P < 0.001$. NS, no significant difference; Ctrl, control.

P450AROM were obviously decreased similar to that in *Pcsk6* mutant mouse (Mujoomdar *et al.* 2011). However, there was no difference in *LHR* (Supplementary Fig. 2) although the *Lhr* level was elevated in the mutant mouse (Mujoomdar *et al.* 2011). Changes in these genes are related to the production of steroid hormones such as estrogen, as they were either essential receptors or enzymes. For instance, *P450arom* is a rate-limiting enzyme involved in the process of estrogen synthesis.

Some hormones are PC substrates and PCs themselves may also be regulated by hormones. Previous studies have demonstrated that BMP2, BMP6, BMP7, and BMP15, but not activin A or GDF9, decrease *PCSK6* gene expression in human GCs (Akiyama *et al.* 2012). Bae *et al.* (2008) found that treatment of pregnant mares serum gonadotropin (PMSG)-primed rats with human chorionic gonadotropin (hCG) induced the expression of *PCSK5* in GCs of pre-ovulatory follicles *in vivo* and treatment with LH

stimulated both *PCSK5* mRNA and protein levels in rat pre-ovulatory follicles cultured *in vitro*. In this study, we detected LH regulation of *PCSK6* in GCs of human pre-ovulatory follicles and this response was dependent on time and dose.

It is unknown whether LH is a pro-apoptosis or an anti-apoptosis factor. LH plays an important role in the development of ovarian cancer, and it has been shown to inhibit apoptosis in ovarian cancer cells (Slot *et al.* 2006, Zhang *et al.* 2011). However, LH played a pro-apoptosis function in the theca-interstitial cells (Spaczynski *et al.* 2005). In this study, we found that LH inhibited the apoptosis of GCs in a dose-dependent manner and decreased the level of apoptosis-related molecules. Previous research (Porter *et al.* 2001) studied GCs isolated from bovine pre-ovulatory follicles before and 14 h after injection of GnRH to induce an LH surge and found that GCs isolated before the LH surge were susceptible

to apoptosis while cells isolated after the LH surge were resistant to apoptosis. Studies (Ruvolo *et al.* 2007) also found a lower apoptosis rate in human cumulus cells after administration of recombinant LH to women undergoing ovarian stimulation for IVF procedures. Our results are in accordance with these results and supplied another direct evidence of LH as an anti-apoptosis factor in GCs.

In conclusion, PCSK6 was the main PC family member in human primary GCs of pre-ovulatory follicles. PCSK6 inhibited apoptosis of KGN cells and influenced expression of downstream molecules including local hormones in the follicle fluid, important enzymes, and hormone receptors, some of which were involved in the apoptosis of KGN cells. Moreover, LH could increase the mRNA level of PCSK6 and played an anti-apoptosis role. This study provides new insights into the function and molecular mechanism of PCSK6 in human follicle development.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0592>.

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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