Autophagy is activated in the ovarian tissue of polycystic ovary syndrome

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Abstract

The importance of autophagy in polycystic ovary syndrome (PCOS)-related metabolic disorders is increasingly being recognized, but few studies have investigated the role of autophagy in PCOS. Here, transmission electron microscopy demonstrated that autophagy was enhanced in the ovarian tissue from both humans and rats with PCOS. Consistent with this, ovarian granulosa cells from PCOS rats showed increases in the autophagy marker protein light chain 3B (LC3B), whereas levels of the autophagy substrate SQSTM1/p62 were decreased. In addition, the ratio of LC3-II/LC3-I was markedly elevated in human PCOS ovarian tissue compared with normal ovarian tissue. Real-time PCR arrays indicated that 7 and 34 autophagy-related genes were down- and up-regulated in human PCOS, Signal-Net, and regression analysis suggested that there are a wide range of interactions among these 41 genes, and a potential network based on *EGFR*, *ERBB2*, *FOXO1*, *MAPK1*, *NFKB1*, *IGF1*, *TP53* and *MAPK9* may be responsible for autophagy activation in PCOS. Systematic functional analysis of 41 differential autophagy-related genes indicated that these genes are highly involved in specific cellular processes such as response to stress and stimulus, and are linked to four significant pathways, including the insulin, ERBB, mTOR signaling pathways and protein processing in the endoplasmic reticulum. This study provides evidence for a potential role of autophagy disorders in PCOS in which autophagy may be an important molecular event in the pathogenesis of PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting approximately 10% of women of reproductive age (Goodarzi *et al.* 2011), with significant and diverse reproductive, metabolic (obesity, insulin resistance, type 2 diabetes, adverse cardiovascular risk profiles) and psychological features (Teede *et al.* 2010, Chan *et al.* 2017). In addition to a genetic predisposition, environmental and lifestyle factors contribute to the pathogenesis of PCOS (Insenser *et al.* 2013, Chan *et al.* 2017).

Autophagy is a major degradation pathway responsive to energy stress and is necessary for cellular homeostasis to maintain energy production and synthesis of new macromolecules (Delbridge *et al.* 2017, Jacob *et al.* 2017). Notably, the importance of autophagy in PCOS-related metabolic disorders is increasingly being recognized. For example, autophagy has an important role in the control of inflammasome activation in response to metabolic stress (Lee *et al.* 2016). In obesity,

autophagy genes are upregulated in human adipose tissue and autophagy is activated and associated with adipose dysfunction (Maixner *et al.* 2016). Furthermore, macrophage autophagy has a crucial role in the regulation of systemic insulin sensitivity in obesity (Kang *et al.* 2016). Several autophagic markers are inhibited in skeletal muscle in severely insulin-resistant patients with type 2 diabetes, and long-term high doses of insulin may generate a vicious cycle of insulin resistance through inhibiting autophagy (Moller *et al.* 2017). In addition, dysregulation of autophagy is frequently associated with cardiomyopathy and cardiac dysfunction during obesity and diabetes (Wang *et al.* 2017).

However, to date, the precise biological effects underlying mechanisms of autophagy in PCOS development remain largely unknown. To further elucidate the relationship between autophagy and PCOS, the present study investigated the autophagy and autophagy-related molecular network in human PCOS ovarian tissue and a PCOS rat model.

Materials and methods

Ethical statement

The study was conducted in accordance with ethical standards and the Helsinki Declaration of 1975.

Patients and tissue collection

This study was approved by the Institutional Review Board at China Medical University, and informed written consent was obtained from all patients prior to the initiation of the study. From October 2014 to September 2016, normal ovarian tissue ($n=10$) was obtained during the follicular phase from women with regular menstrual cycles who were younger than 40 years of age and underwent operative laparoscopy or laparotomy (as part of investigation and/or treatment of infertility or other gynecological disease). PCOS ovarian tissue ($n=10$) was obtained during the follicular phase from women with irregular menstrual cycles who were younger than 40 years of age and underwent laparoscopic wedge resection. PCOS was diagnosed according to the 2003 Rotterdam criteria (Rotterdam 2004).

Animal model and sample collection

All experiments were conducted according to the NIH Guide for Care and Use of Laboratory Animals and all experimental procedures involving animals were approved by the Animal Care and Use Committee of China Medical University. Female Sprague Dawley rats (3 weeks old) were purchased from the Beijing HFK Bioscience Co. Ltd. The rats were housed under standard laboratory conditions ($22 \pm 2^\circ\text{C}$, 12/12 h light/darkness cycle with lights on from 6:00 h to 18:00 h) and given *ad libitum* access to food and water. After one week of acclimatization, the rats were randomly divided into two groups: a control group and a dehydroepiandrosterone (DHEA) group (each group, $n=6$). The PCOS rat model was established by injection of DHEA dissolved in 0.2 mL sesame oil (60 mg/kg body weight, sc) with a high-fat diet (nutrients comprised 45% kcal fat, 35% kcal carbohydrate and 20% kcal protein), and the control group was subcutaneously injected with an equivalent amount of sesame oil (Zhang *et al.* 2016). Body weight was recorded weekly and fasting blood was collected from the abdominal aorta under anesthesia at the end of week 12. Histological samples of the ovary were placed in 4% paraformaldehyde for haematoxylin-eosin (H&E) staining and immunohistochemistry, and 2.5% glutaraldehyde for transmission electron microscopy (TEM), respectively.

Real-time PCR array

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. DNA contamination was removed by adding DNase I (Invitrogen). For the quantification of 224 key genes involved in autophagy, total RNA was then reverse-transcribed from 2 μg of RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa) and amplified by GoTaq qPCR Master Mix (Promega) in the ABI ViiA 7 Real-time PCR system (Applied Biosystems). The specific primer sequences are listed in [Supplementary Table 1](#)

(see section on [supplementary data](#) given at the end of this article). GAPDH mRNA was amplified as an internal control for the normalization of each sample. All samples were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method.

Immunohistochemistry for LC3B and SQSTM1/p62

The autophagy markers protein light chain 3B (LC3B) and autophagy substrate SQSTM1/p62 in the ovarian tissue of the control and PCOS rats were determined by immunohistochemical staining. Briefly, serial 4- μm sections were obtained from each paraffin-embedded tissue block. Following deparaffinization and rehydration, sections were subjected to microwave antigen retrieval. The primary antibodies were an anti-LC3B antibody (ab48394) (1: 200; Abcam) and an anti-SQSTM1/p62 antibody (ab91526) (1: 200; Abcam), and the sections were incubated overnight at 4°C with this antibody. 3,3'-diaminobenzidine was used as the chromogen. Nuclei were counterstained with haematoxylin, and slides were dried and mounted. Negative controls were incubated with PBS instead of the antibody. The ovarian tissue was treated with rapamycin (500 nM) for 6 h, which served as a positive control. The immunostaining was evaluated by two independent pathologists, who were blinded to the identity of the subject groups. Area quantification was performed with a light microscope at a magnification of 400 $\times$ and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). The immunoreactivity of LC3B and SQSTM1/p62 was evaluated according to the intensity and percentage of positively stained cells. The percentage of positively stained cells was graded as follows: negative, 0–29%; weak, 30–59%; strong, >60%. Any borderline values were classified into the higher category.

Western blotting for LC3-II/LC3-I and beclin-1

The autophagy markers LC3-II/LC3-I and essential autophagy gene beclin-1 in human normal and PCOS ovarian tissues were measured by western blotting. Briefly, 50 μg protein was separated on 12% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in TBS containing 0.1% Tween-20 and 5% non-fat dry milk for 60 min at room temperature and incubated with antibodies against LC3A/B (#12741) (1:1000; Cell Signaling Technology) and beclin-1 (ab207612) (1:2000; Abcam) overnight at 4°C . Next, the membranes were washed in PBS-Tween followed by 1 h incubation at room temperature with horseradish peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) and detected using enhanced chemiluminescence.

TEM

Ovarian tissue was cut into 1 mm³ pieces and then treated with 2.5% glutaraldehyde in PBS overnight at 4°C , followed by post-fixation in 1% buffered OsO₄ for 3 h, washed thrice in PBS and then dehydrated with a series of ethanol concentrations (50, 70 and 90%) at 15-min intervals. The samples were subsequently incubated in a mixture of alcohol and acetone (v:v 1:1) for 20 min, followed by incubation with pure acetone for 20 min at 4°C . Ovarian tissue sections were then embedded in resin and polymerized. Ultrathin (70 nm) sections were post-stained

with uranyl acetate and lead citrate, and examined under a Jeol 1230 electron microscope (Jeol, Akishima, Tokyo, Japan).

Molecular network analysis

STRING analysis (<https://www.string-db.org>) was used to identify direct and indirect interactions. STRING is a database that provides associations from computational prediction and other databases for interaction analysis. The STRING score was calculated from the combination of all predictions and network analysis was set at a medium confidence (0.400).

Data visualization and statistical analysis

The STRING database was used to identify the functional enrichments in the network. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations were also used. R packages pheatmap and ggplot2 were used for data visualization. Kolmogorov–Smirnov was used to test normality. A Bonferroni correction was used to minimize the false positive rate. Regression analysis was used to examine the possible relationship between the differentially expressed genes. The data are presented as means $\pm$ s.d. Statistical differences in the data were evaluated by Student's *t*-test or one-way ANOVA as appropriate, and were considered significant at $P < 0.05$.

Results

Active autophagy was observed in the ovarian tissue of PCOS rats

LC3 and LC3-II/LC3-I are reliable markers of autophagy. Autophagosomes can be observed using a transmission electron microscope and are considered the gold standards of autophagy activation. The rat model of PCOS exhibited polycystic ovaries (Fig. 1A). Ultrastructural observation using TEM demonstrated that autophagy was induced in the ovarian cortex of PCOS rats. In contrast, autophagosomes were rarely observed and autolysosomes were never observed in control rats (Fig. 1B). Consistent with this, ovarian granulosa cells of PCOS rats showed an increase in the autophagy marker LC3B (Fig. 1Ci and Cii), whereas levels of autophagy substrate SQSTM1/p62 were decreased (Fig. 1Di and Dii).

Active autophagy was observed in human PCOS ovarian tissue

TEM demonstrated that autophagy was enhanced in the ovarian tissue of patients with PCOS (Fig. 2A). Similarly, the ratio of LC3-II/LC3-I was markedly elevated in the PCOS group compared with the normal group (Fig. 2B), and there was a slightly increasing trend in expression of the essential autophagy protein beclin-1 (Fig. 2B).

A potent autophagy-related gene network was activated in human PCOS ovarian tissue

To further confirm the potential molecular basis of autophagy in PCOS, the expressions of 224 key genes

involved in autophagy were detected by a real-time PCR array applied to the human ovarian cortex of normal and PCOS samples. The results indicated that 41 genes were differentially expressed between normal and PCOS groups (Fig. 3A and B). STRING analysis indicated a wide range of interactions among these 41 genes (Fig. 3C and Supplementary Table 2). Interestingly, both STRING and Signal-Net analyses indicated that a potential network based on *EGFR*, *ERBB2*, *FOXO1*, *MAPK1*, *NFKB1*, *IGF1*, *TP53* and *MAPK9* may be responsible for autophagy activation in PCOS (Fig. 3C and Supplementary Fig. 1). In addition, our data showed a highly significant correlation ($R > 0.95$, $P < 0.000001$) between *ATG16L* and *SIRT2/NBR1* levels (Fig. 3D and E), and *HDAC1* and *NBR1/MBTPS2* levels (Fig. 3F and D). A significant correlation was also observed between the expressions of another 36 pairs of genes ($R > 0.90$, $P < 0.000001$) (Supplementary Table 3). Therefore, these results suggest that a potent autophagy-related gene network is widely activated in human PCOS ovarian tissue.

Systematic functional analysis of 41 active autophagy-related genes in human PCOS ovarian tissue

To define the functional significance of the extensive changes in autophagy-related genes in PCOS, GO analysis was performed, which revealed distinct functional categories for the PCOS-associated autophagy gene candidates. As shown in Fig. 4Ai and Supplementary Table 4, 41 differentially expressed genes were found to be highly involved in specific cellular processes, such as response to stress, external stimulus, endogenous stimulus and extracellular stimulus. STRING analysis indicated that 29 autophagy-related proteins were widely involved in response to stress, and identified *EGFR*, *TP53*, *IGF1*, *ERBB2* and *C12orf44* as the most important nodes in the stress response network (interaction node > 5) (Fig. 4Aii and Supplementary Table 5). Notably, protein binding, macromolecular complex binding, enzyme binding and purine ribonucleotide binding were the major molecular functions of these differentially expressed genes (Fig. 4Bi and Supplementary Table 6), and *ERBB2*, *IGF1*, *NFKB1*, *TP53*, *ATIC*, *MAPK1* and *EGFR* were the key regulators for protein binding (interaction node > 5) (Fig. 4Bii and Supplementary Table 7). In addition, *TP53*, *NFKB1* and *MAPK1* have a crucial role in the membrane structure of the organelles in the cytosol (Fig. 4Ci and Cii, Supplementary Tables 8 and 9). To investigate key pathways linked to these distinct autophagy-related genes, the interaction of the significant pathways associated with PCOS was built according to the KEGG database (Fig. 4D and Supplementary Table 10). Our analysis showed that these differentially expressed genes are linked to four significant pathways, including the insulin, ERBB, mTOR signaling pathways and protein processing in the endoplasmic reticulum.

Figure 1 Ovarian morphology and autophagy levels in the ovarian tissue of control rats and PCOS rats. (A) Representative sections of H&E staining from the ovarian tissue of rats in the control group and PCOS group, respectively. Magnification is 40× and 100×. (B) The ultrastructure was analyzed using transmission electron microscopy. Asterisks point to autophagosomes (1 μm) and autolysosomes (0.5 μm). (Ci and Di) Sections were subjected to immunostaining for LC3B and SQSTM1/p62, respectively. Each group had $n=6$. (Cii and Dii) Summary of the percentage of positively stained cells from the measurements shown in Ci and Di, respectively. (E and F) Positive control. H&E, haematoxylin-eosin; TEM, transmission electron microscopy.

Discussion

In this study, we reported for the first time that autophagy was activated in the ovarian tissue of humans and rats with PCOS. A potential autophagy-related gene network based on *EGFR*, *ERBB2*, *FOXO1*, *MAPK1*, *NFKB1*, *IGF1*, *TP53* and *MAPK9* may be responsible for autophagy activation in PCOS. To date, although there is no direct evidence for a role of autophagy in PCOS, some insight was gained by a previous study

showing that (i) *EGFR* was differentially expressed and is a potential cause of PCOS oocytes and cumulus cell disorder at early stages (Liu *et al.* 2016); (ii) variants in/near *ERBB2/HER2* are associated with PCOS at or near genome-wide significance (Day *et al.* 2015); (iii) *FOXO1* was significantly increased in PCOS cumulus cells (Shi *et al.* 2015) and phospho-ERK1/2 were decreased in PCOS granulosa cells (Lan *et al.* 2015), and alteration of ERK1/2 signaling is important for follicle assembly,

Figure 2 Ovarian autophagy levels in human normal and PCOS ovarian tissues. (A) Ultrastructure was analyzed using transmission electron microscopy. Asterisks point to autolysosomes. (B) Relative protein levels of beclin-1 and LC3-II/LC3-I were detected by western blotting ($n=9$ for the normal group, and $n=10$ for the PCOS group). Bar graphs show mean $\pm$ s.d. TEM, transmission electron microscopy.

further changing the rate of assembly of primordial follicles (Nilsson *et al.* 2013); (iv) Serum levels of NFkB1 in the PCOS groups were significantly higher than in the control group (Liu *et al.* 2015); (v) IGF1 is an important intermediate for follicular development and the pathogenesis of PCOS (Roche *et al.* 2016), cumulus cells from women with PCOS were characterized by abnormal expression of EGFR and IGF-like family proteins (IGF1R, IGF2R, IGF2BP2 and IGFBP2) (Haouzi *et al.* 2012) and a supraphysiological IGF1 concentration can decrease TP53 protein expression in the trophoblast (Velazquez *et al.* 2011). However, to the best of our knowledge, there is still no report regarding MAPK9 in PCOS.

Notably, these observations further correlate autophagy-related molecules and processes with physiological and pathological features of PCOS. For example, activation of EGFR on granulosa cells is a key event involved in ovulation (El-Hayek *et al.* 2014).

PI3K/AKT/FOXO1/mTOR signaling plays a critical role in regulating the interaction among androgens, insulin, insulin-like growth factors and hyperglycemic diet in acne (Ju *et al.* 2017). Furthermore, conditional ablation of the IGF1 receptor leads to increased adiposity and insulin resistance (Riera *et al.* 2017).

There is also preliminary evidence showing changes in the expression of autophagy genes in endometria from women with PCOS (Sumarac-Dumanovic *et al.* 2017). This evidence, when combined with our observation of ovarian autophagy activation in human and rats with PCOS, suggests that multiple organ autophagy changes may be a possible factor involved in the development of PCOS and PCOS-related metabolic disorders.

This study provides preliminary evidence for a potential role of autophagy disorders in PCOS, and understanding that the autophagy mechanisms involved in PCOS may yield new insight into the pathophysiology of the disorder.

Figure 3 Intracellular levels and interactions of autophagy-related genes in human normal and PCOS ovarian tissues. (A and B) Differences in expression patterns of 41 autophagy-related genes in the human ovarian cortex of the normal and PCOS samples (each group, $n=10$). (C) STRING analysis displayed the interactions of these 41 autophagy-related genes. (D, E, F and G) Correlation between ATG16L and SIRT2 levels/ NBR1 levels, or HDAC1 and NBR1 levels/MBTPS2 levels, respectively (each group, $n=20$). Bar graphs show mean $\pm$ s.d.

Figure 4 GO, STRING and KEGG analyses of 41 differentially expressed autophagy-related genes in human PCOS ovarian tissue. (Ai, Aii, Bi, Bii and Ci) Biological Process, Molecular Function and Cellular Component analysis of 41 differentially expressed autophagy-related genes between human normal and PCOS ovarian tissues, respectively. The top 10 GO terms are shown. (Aii, Bi, Bii, Ci and Cii) STRING analysis displaying the interactions of the top functional enrichments in Biological Process, Molecular Function and Cellular Component, respectively. (D) KEGG analysis of 41 differentially expressed autophagy-related genes between human normal and PCOS ovarian tissues, respectively. The top 30 KEGG terms are shown.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-17-0499>.

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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