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Original Article

Impaired granulosa cells promote self-damage by regulating the generation of macrophage in polycystic ovary syndrome

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Abstract: Granulosa cell is one of the most important somatic cell in ovary, abnormality of its proliferation or apoptosis plays key role in the development of polycystic ovary syndrome (PCOS). However, the molecular mechanism of it in PCOS is still unclear. In this study, we find that many inflammation genes including CD163, Fas are upregulated in PCOS granulosa cells by comparing the mRNA profile data in normal and PCOS granulosa cells. Further experiments demonstrate that granulosa cells have a faint CD163 expression, while supernatant from granulosa cells especially impaired granulosa cells can elevate the expression of CD163 in monocyte which represents the generation of macrophage. Besides, monocyte can markedly promote cellular apoptosis, reactive oxygen production and Fas protein expression. This alteration is also observed in KGN cells treated with THP1 derived macrophage. This study proposes a new mechanism of macrophage and granulosa cell impairment in PCOS which will help us better understand the formation of PCOS.

Keywords: Polycystic ovary syndrome, granulosa cells, CD163, macrophage

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine-metabolic disorders, with a prevalence of up to 10% in women of reproductive age [1]. It is characterized by ovarian dysfunction, hyperandrogenism and polycystic ovary morphology [2]. Many hereditary factors including early age of sexual maturation, premature fetal development and family history of PCOS are involved in the development of PCOS [3]. Besides, environmental factors such as devoid physical exercise, obesity, diet, environment toxins, medication and infection also play key roles in the generation of PCOS [4].

Ovary granulosa cell is a somatic cell of the sex cord and associated with the development of female oocyte by producing estradiol and various growth factors [5]. Study has demonstrated that the rate of cell death and proliferation in granulosa cell populations in PCOS patients are significantly altered [6]. The expression of some important protein molecular such as PPARγ and kindlin 2 in the granulosa cell of PCOS are often abnormal [7, 8]. Moreover upregulated catechol-O-methyltransferase (COMT) in the follicles and ovarian stroma increases the level of 2-methoxyestradiol which can decrease granulosa cell proliferation and steroidogenesis, raising the risk of PCOS [9]. Study also demonstrates anti-epileptic drug valproic acid (VPA) can increase the incidence of PCOS by inhibiting steroidogenesis in theca and granulosa cells [10].

During the last decades, researchers have validated that women with diabetes, obesity and insulin resistance often exhibit a high incidence of infertility and PCOS [11]. Chronic inflammation which is clearly associated with obesity, diabetes and insulin resistance is also often accompanied with women PCOS [12]. Many studies have confirmed that multiple markers of inflammation including C reactive protein,

interleukin, monocyte chemoattractant protein and white blood cell count are elevated in PCOS patients [13]. Recently, researcher finds that administration of traditional herbal prescription Kyung-Ok-Ko (KOK) can effectively prevent and improve dehydroepiandrosterone-induced PCOS via anti-inflammatory action [14].

CD163, also known as macrophage-associated antigen, is a member of the hemoglobin scavenger receptor cysteine-rich superfamily. It is exclusively expressed in monocytes and macrophages and plays a key role in the clearance and endocytosis of hemoglobin/haptoglobin complex by macrophage. Enhanced expression of macrophage marker CD163 has been found in joint inflammation [15], acute respiratory distress syndrome [16], obesity [17] and inflammatory bowel [18]. Moreover, soluble form of CD163 (sCD163) can be released into to serum and may be a valuable diagnostic parameter for monitoring macrophage activation status in inflammatory conditions [19]. Recent study shows that serum solute CD163 is elevated in PCOS and is associated with total testosterone [20].

In this study, we aim to investigate the difference of gene expression profile between normal and PCOS granulosa cells. We also try to elucidate the regulatory mechanism of CD163 in PCOS progress.

Materials and methods

Affymetrix microarray data

The microarray data GSE34526 used in this study was obtained from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). This dataset contained 3 normal women individuals and 7 PCOS patients. Differentially expressed genes between them was analyzed with R and Array tools software.

Protein interaction network and gene ontology consortium analysis

Top 50 differentially expressed genes were subjected to enrichment analysis using Gene Ontology Consortium webtool (http://www.geneontology.org). In order to map the protein interaction network, genes were analyzed by String webtool (http://www.string-db.org) and Cytoscape software.

Cell culture and treatment

Human granulosa tumor-derived cell line KGN was maintained in DMEM/F12 medium (Invitrogen, USA) supplemented with 10% FBS (Gibco, USA), penicillin (100 U/ml) and streptomycin (100 U/ml) in 5% CO_2 atmosphere at 37°C. Human THP1 cell was maintained in RPMI-1640 medium (Invitrogen, USA) with 10% FBS. Human monocyte in the peripheral blood was purified with Ficoll-Paque™ PLUS (GE Healthcare Life Sciences, USA) and maintained in RPMI-1640 medium with 10% FBS. In order to obtain macrophage, THP1 cells were seeded into 6-well plate and add 100 ng/ml PMA (Sigma, USA) for 24 h, medium is refreshed until all cells were adherent. For inhibitor treatment, KGN were seeded into 6-well plates and added different inhibitors for 12 h as the following concentration, 0.1 μM U0126 (MEK1/2), 0.05 μM SP600125 (JNK), 0.5 μM SB203580 (p38) and 10 μM JSH-23 (NF-KB). All the inhibitors were purchased from Beyotime Biotechnology (China).

Isolation of cell supernatant

KGN and human peripheral blood monocyte were seeded into 6-well plate with 2 ml proper medium. After 24 h, cell supernatant was collected and centrifuged to discard cellular debris. In order to obtain impaired KGN cell supernatant, KGN cell line was seeded into a 6-well plate and treated with 1 μg/ml doxorubicin for 12 h, then refreshed the medium for further 24 h and collected cellular supernatant. Before treating with cellular supernatant, cells were firstly cultured with normal medium. After 12 h medium was refreshed with supernatant or normal culture medium and cells were cultured for further 24 h.

Protein isolation and western blot analysis

Total protein was isolated with RIPA lysis buffer containing protease inhibitor (Roche, USA). Total protein concentration was measured using BCA protein assay kit (Beyotime Biotechnology, China). 20 μg total protein was separated with 8% SDS-PAGE, and electro-transferred to PVDF membrane. Then the membrane was blocked with 5% nonfat milk in TBST buffer for 1 h at room temperature, and probed with primary antibody against to CD163 (1:1000, Abcam, USA), Fas (1:1000, Abcam, USA) and GAPDH

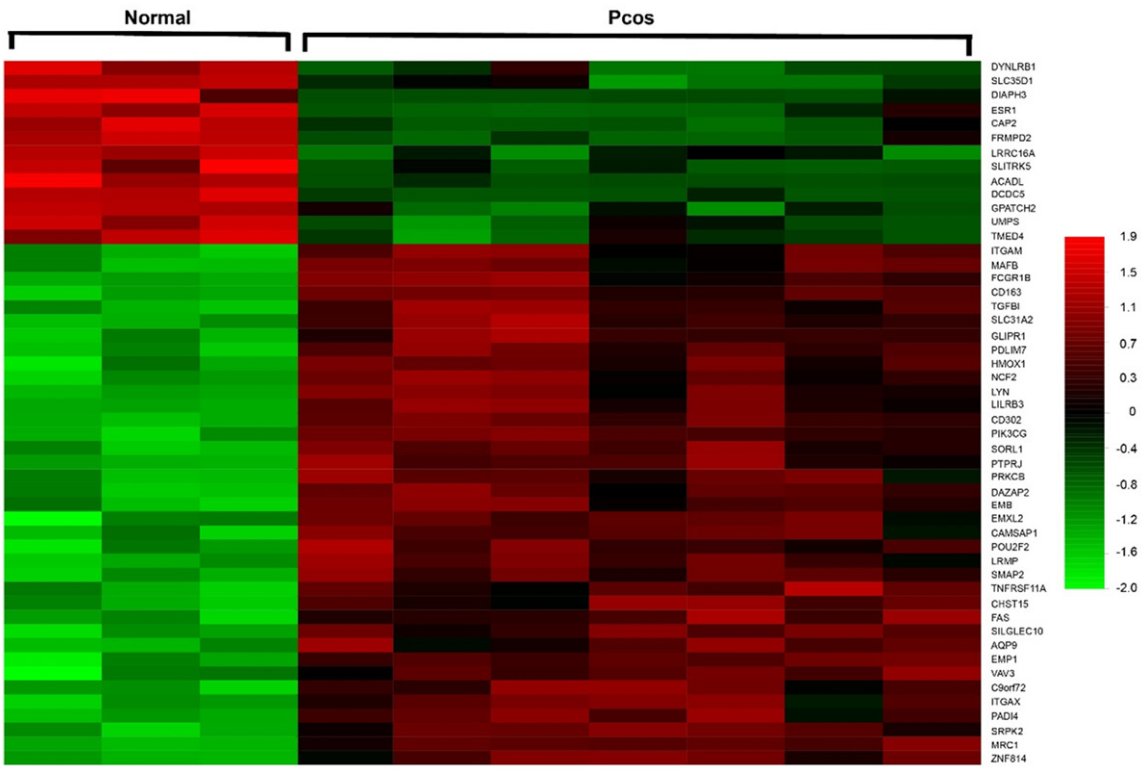

Figure 1. Differentially expressed genes between PCOS and normal granulosa cells. Data are analyzed with paired-samples t test.

Table 1. The major Gene Oncology (GO) analysis terms enriched by the top 50 significant expressed genes

GO analysis terms	Gene number	*P* value
GO biological process		
Immune system progress	21	1.01E-04
Endocytosis	11	3.08E-04
Immune response	16	2.05E-03
GO Cellular component		
Cell periphery	29	7.06E-04
Plasma membrane	28	1.82E-03

(1:2000, ZSGB-BIO, China). Subsequently, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or mouse IgG secondary antibody. After washing with TBST, proteins level were detected by ECL Western blotting substrate kit (Thermo Fisher, USA).

Apoptosis assay with Hoechst 33258 staining

1×10^4 KGN cells were seeded into 6-well cells and cultured overnight, then the medium was removed and refreshed with proper control medium or monocyte (or macrophage) supernatant. After 24 h, cells were stained with 1 μg/ml Hoechst 33258 (Sigma, USA) at room temperature in the dark for 20 min. Apoptosis cells were photographed and counted with fluorescence microscopy at a stimulation of 350 nm and an emission of 460 nm.

Intracellular ROS measurement

The production of intracellular reactive oxygen species (ROS) was measured with 2', 7'-dichlorogluorescin diacetate (DCFH-DA) (Sigma, USA). Cells which were treated with monocyte or macrophage supernatant for 24 h were stained with 20 μM ACFH-DA working solution diluted with medium in 37°C incubator for 30 min. Cells were washed three times with PBS and the positive cells were photographed and counted with fluorescence microscopy at a stimulation of 485 nm and an emission of 520 nm.

Statistics

Statistical analysis was performed using SPSS 13.0 software. All data in this study were

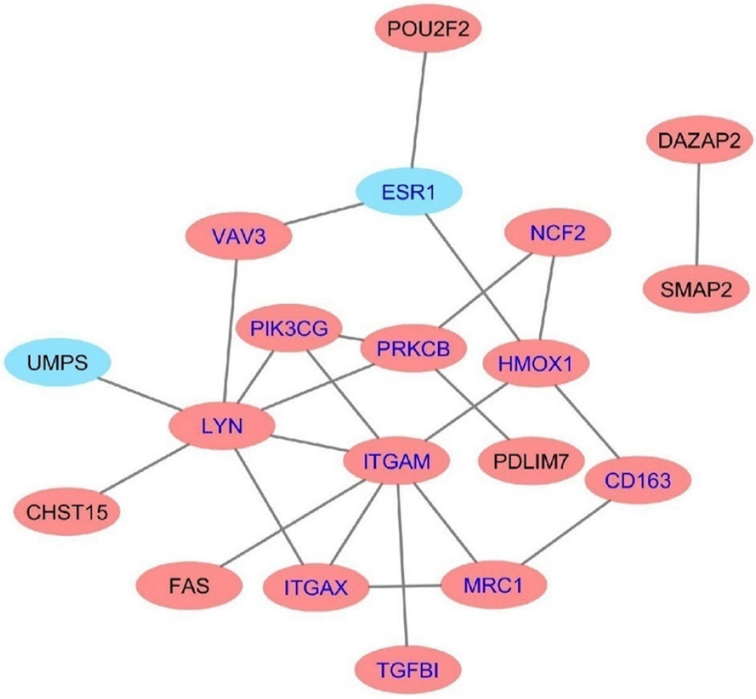

Figure 2. Protein interaction network among differentially expressed genes.

expressed as the mean ± SD of triplicate determinations. Two-tailed paired Student's t-test was used to analyze the data, values of $p < 0.05$ were considered as statistically significant.

Results

Genes differentially expressed between normal and PCOS granulosa cells

Differentially expressed genes between normal and PCOS granulosa cells were identified with R and array tools software. **Figure 1** showed that there were 13 downregulated genes and 37 upregulated genes among the top 50 genes.

Gene ontology consortium and protein interaction network analysis

In order to better understand the difference between normal and PCOS granulosa cells, the top 50 genes were subjected into AmiGO 2software. As shown in **Table 1**, we could find that the differentially expressed genes were mainly enriched in three GO biological process, Immune system progress, endocytosis and immune response. Besides, these genes were mostly enriched in two GO cellular component, cell periphery and plasma membrane, indicating expression of these genes in serum may be potential diagnostic markers for PCOS. Subsequently, the protein interaction networks were analyzed by String and Cytoscape tools. Results in **Figure 2** displayed that there were 19 genes could form interacted network, including 2 downregulated genes (blue background) and 17 elevated genes (pink background). Strikingly, up to 12 genes which were associated with inflammation were found in this network (Blue font).

Expression of CD163 in granulosa and monocyte

Recent study found that soluble serum CD163 was significantly elevated in PCOS patients, while the exact mechanism was not elucidated [20]. Result of microarray analysis displayed that CD163 was significantly upregulated in PCOS granulosa cells. So we tried to quest the upstream regulator of CD163 by treating KGN cells with several pathway inhibitors, U0126 (MEK1/2), SP600125 (JNK), SB203580 (p38) and JSH-23 (NF-KB). However, Western blot assay showed that KGN hardly expressed CD163 (**Figure 3A**). Considering so many inflammatory genes were altered in PCOS granulosa cells, we wondered whether monocyte was able to promote CD163 expression in KGN cell. So we purified human peripheral blood monocyte and stimulated KGN cell with monocyte supernatant. While, there was still no strong CD163 elevated was observed (**Figure 3B**). Surprisingly, when monocyte was stimulated with KGN supernatant, the expression of macrophage marker CD163 in monocyte was significantly upregulated, suggesting KGN may participate in the transformation of monocyte to macrophage (**Figure 3C**, **3D**).

Monocyte promotes the apoptosis and oxidative stress of KGN cell

We next set out to detect the function of monocyte on KGN cell. Hoechst 33258 was used to

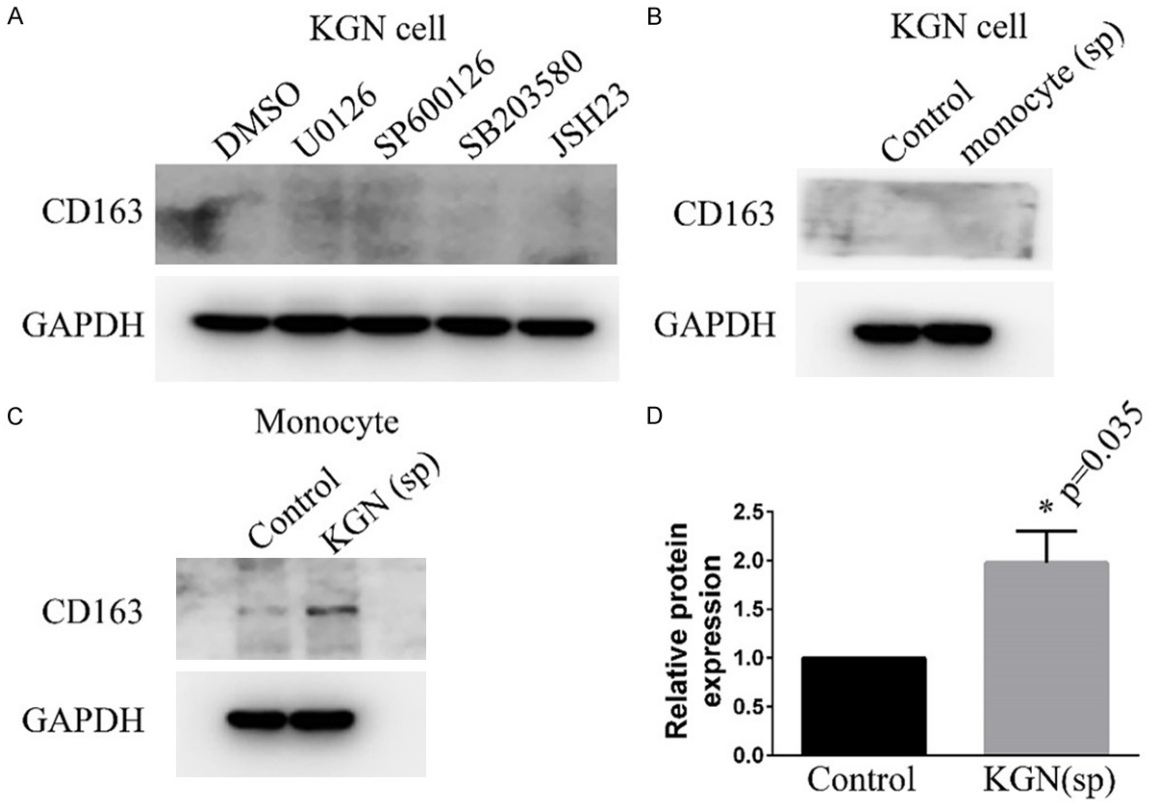

Figure 3. Expression of CD163 in granulosa cell and monocyte. A. The effect of several signaling inhibitors on the expression of CD163 in KGN is detected by Western Blot. B. The effect of monocyte on the expression of CD163 in KGN is examined by Western Blot. C. The effect of granulosa cell on the expression of CD163 in monocyte is examined by Western blot. D. Relative expression of CD163 in monocyte treated with KGN supernatant. Sp means cell supernatant medium, the *p* value is calculated by paired-samples t test.

examine the effect of monocyte supernatant on KGN cell apoptosis. Results showed that monocyte supernatant could significantly increase the amount of apoptotic cell (**Figure 4A**, **4B**). To further confirm the enhanced apoptosis effect, we also evaluated the expression of pro-apoptotic protein Fas by Western blot assay. The expression of Fas was obviously upregulated in monocyte derived supernatant stimulated group (**Figure 4C**, **4D**). This result was also consistent with the forward result of microarray analysis in which Fas was significantly elevated in PCOS granulosa cells. Studies had proved inflammation could accelerate the progress of PCOS by increasing cellular oxidative stress [13, 21]. To further investigate the effect of monocyte on the production of ROS in granulosa cell, we stained KGN cell with DCFH-DA fluorescence probe. As expected, we found ROS positive cells in monocyte supernatant group were significantly elevated compared with control medium group (**Figure 4E**, **4F**).

Impaired granulosa cells boost macrophage transformation

Previous studies had demonstrated that apoptotic cells were able to activate macrophage and increase macrophage's population [22, 23]. To examine whether apoptotic granulosa cell could boost macrophage transformation, we treated KGN cell with doxorubicin and obtained impaired granulosa cell supernatant. Subsequently, monocyte was stimulated with this supernatant and CD163 marker protein expression was detected. As shown in **Figure 5**, we found the level of CD163 was obviously increased compared with control medium or normal KGN supernatant.

THP1 derived macrophage facilitate cellular apoptosis and oxidative stress

To further prove the function of macrophage on KGN impairment, we obtained macrophage by

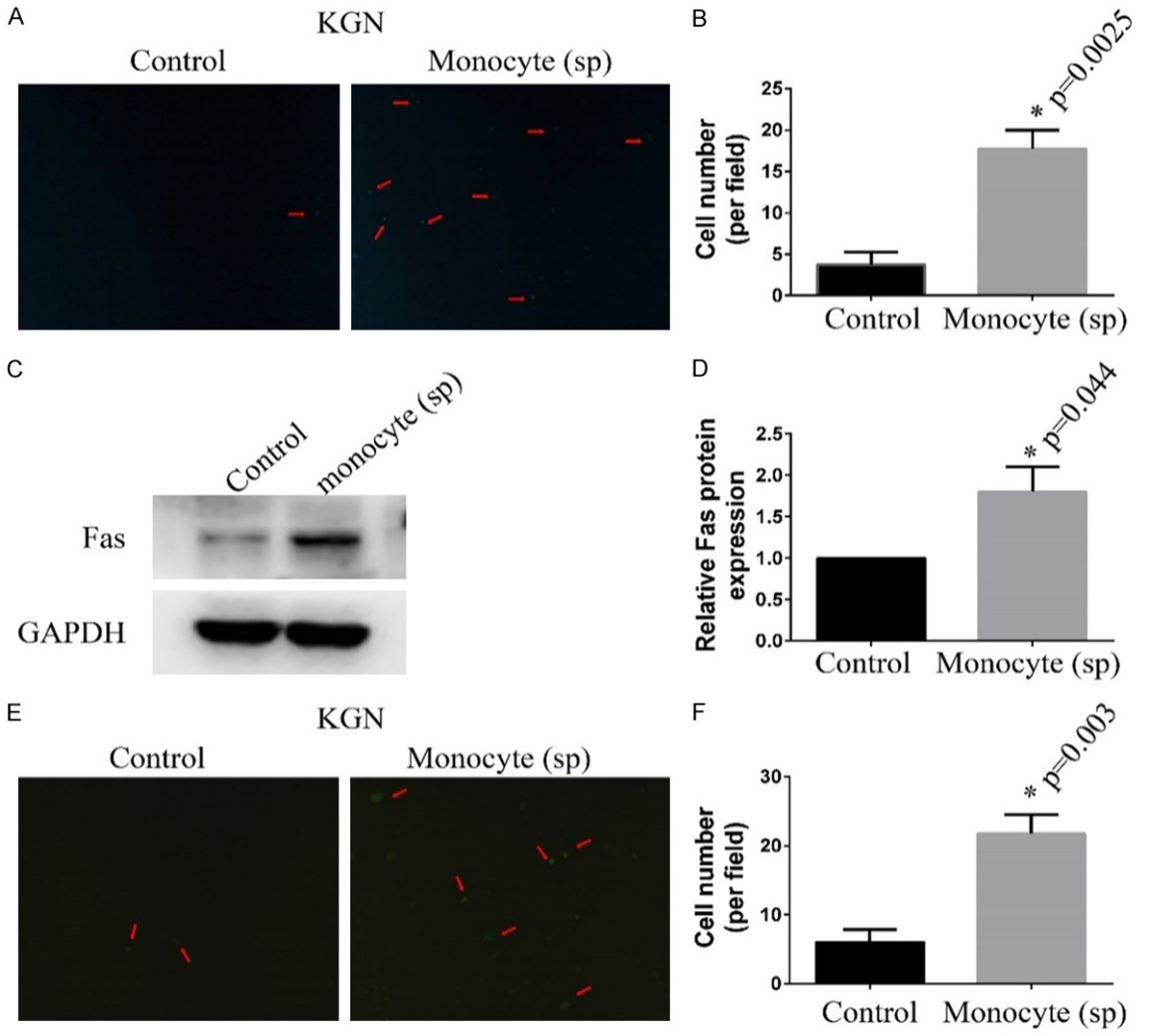

Figure 4. Effect of monocyte on the apoptosis and ROS generation of KGN cell. A. The effect of monocyte supernatant on KGN apoptosis is examined by Hoechst 33258 staining. B. Apoptotic cell number in KGN cell treated with or without monocyte supernatant. C. The expression of Fas in KGN cell is detected by Western Blot assay. D. Relative expression of Fas in KGN cell treated with monocyte supernatant. E. The effect of monocyte supernatant on KGN ROS is examined by DCFH-DA staining. F. ROS positive cell number in KGN cell treated with or without monocyte supernatant. Sp means cell supernatant medium and all data are analyzed with paired-samples t test.

stimulating THP1 cell with PMA. After incubation PMA for 24 h, suspended THP1 anchored to the 6-well plate and stretched out pseudopodia (**Figure 6A**). Hoechst 33258 staining assay demonstrated macrophage significantly promoted the apoptosis of KGN cell (**Figure 6B**, **6C**). This effect was also confirmed by elevated Fas protein expression in group treated with macrophage supernatant (**Figure 6D**, **6E**). We also performed DCFH-DA staining to assess the intracellular ROS in KGN cell. As expected, ROS positive cell had an obviously increase in macrophage group (**Figure 6E**, **6F**).

Discussion

Polycystic ovary syndrome (PCOS) is one of the most common endocrine metabolic disorder diseases in women of reproductive age. Granulosa cell is a key endocrine cell of woman and plays an important role in the maturation of oocyte by converting androgens to estradiol by aromatase. The expression of BMP system proteins including BMP ligands and their receptors are often abnormal in the granulosa cells of PCOS patients [24]. High level of nerve growth factor (NGF) in ovary and granulosa cell is associated with cystic morphology [25]. Recently,

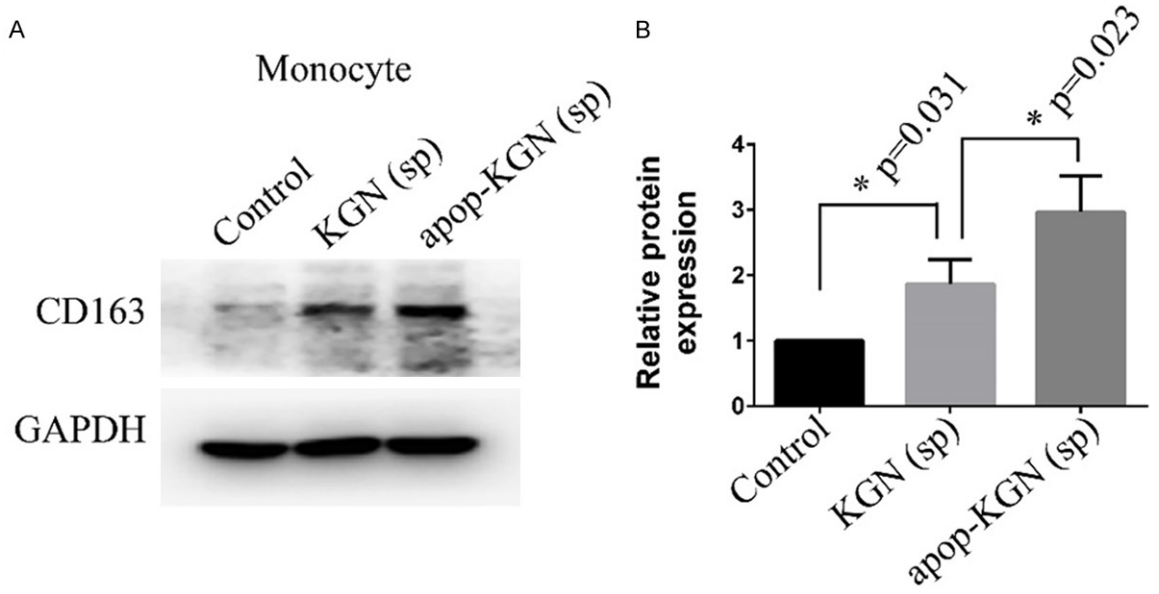

Figure 5. Impaired KGN cell promotes the transformation of monocyte to macrophage. A. The expression of CD163 in monocyte stimulated with impaired KGN supernatant is detected by Western blot assay. B. Relative expression of CD163 in monocyte stimulated with impaired KGN supernatant. Sp means cell supernatant medium, data are analyzed with paired-samples t test.

researchers demonstrate that AR alternative splice variants in granulosa cell disturb androgen metabolism and follicle growth, leading to PCOS by impairing transcription factor function [26]. In this paper, we identify many differentially expressed genes in PCOS granulosa cell. Moreover, these genes are mainly associated with immune response and endocytosis. We also find that many differentially expressed genes are cell periphery and plasma membrane proteins, indicating these proteins might be diagnostic biomarkers of PCOS. Protein interaction analysis display some inflammatory genes such as CD163, ITGAM, TGFBI which are significantly upregulated in PCOS granulosa cells can interact with each other.

CD163 is a macrophage specific protein and its elevated expression is one of the major change in the activated phenotypes of macrophage [27]. The ectodomain of CD163 can be cleaved by inflammation-responsive protease ADAM17 at CD163 (1044) Arg-Ser-Arg motif, leading to release of soluble CD163 (sCD163) [28]. Expression of soluble CD163 is increase in inflammatory condition. Recently, serum sCD163 have been proved to be a potential biomarker of disease including primary progressive multiple sclerosis (PPMS) [19], HIV infection [29, 30], perivascular fat and insulin resistance [31]. Furthermore, latest research demonstrate high expression of serum CD163 is associated with PCOS [20]. By analyzing microarray profiles we find the expression of CD163 is significantly upregulated in PCOS granulosa cells compared with healthy group. However, further experiments reveal CD163 has a faint level in human KGN granulosa cells, and its level has no visible change even if we stimulate KGN cell with various inhibitors or monocyte supernatant. Considering CD163 is a marker of macrophage, we also examine the expression of CD163 in monocyte after stimulating with KGN cells. Strikingly, our data displays that CD163 is significantly upregulated in monocyte which is stimulated by KGN supernatant. Moreover, this elevation is enhanced when monocyte is treated with impaired KGN supernatant, indicating impaired KGN cells can promote the transformation of monocyte to macrophage.

Clinical research has shown chronic inflammation is associated with PCOS. Patients with PCOS display significantly high lymphocytes, monocyte and inflammatory factors including TNF-α, IL-6 [32]. Research also demonstrates inflammation can increase the release of resistin into circulation, resulting in the insulin resis-

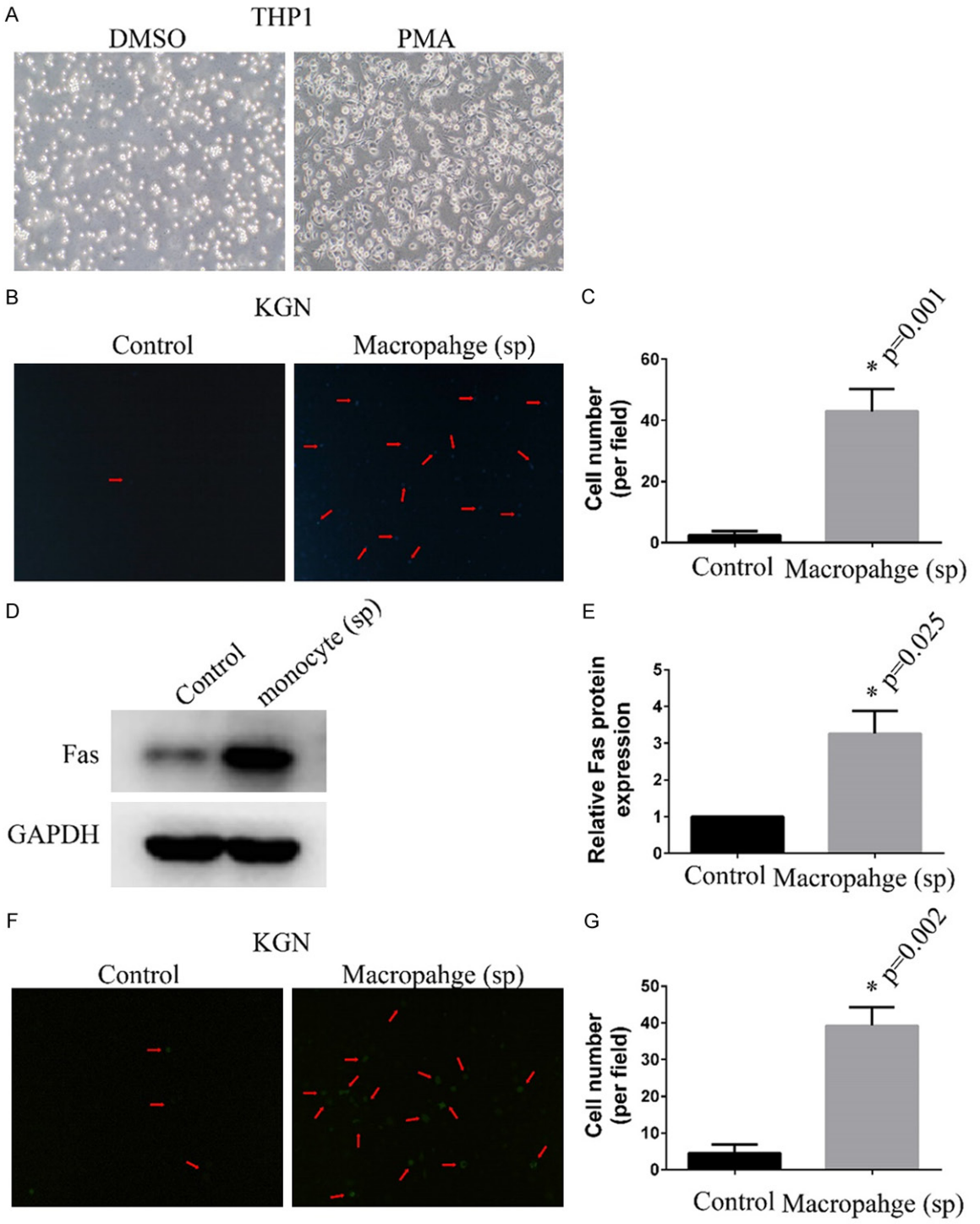

Figure 6. Effect of macrophage on the apoptosis and ROS generation of KGN cell. A. Cell morphology of THP1 cell and THP1 derived macrophage. B. The effect of macrophage supernatant on KGN apoptosis is examined by Hoechst 33258 staining. C. Apoptotic cell number in KGN cell treated with or without macrophage supernatant. D. The expression of Fas in macrophage supernatant treated KGN cell is detected by Western blot assay. E. Relative expression of Fas in macrophage supernatant treated KGN cell. F. The effect of macrophage supernatant on the generation of ROS in KGN is examined by DCFH-DA staining. G. ROS positive cell number in macrophage supernatant treated KGN cell. Sp means cell supernatant medium and all data are analyzed with paired-samples t test.

tance and PCOS [33]. Besides, hyperandrogenism which is a character of PCOS can induce the release of pro-inflammation factor TNF-α [34]. Macrophage, as a crucial inflammatory cell, promotes insulin resistance and PCOS by releasing chitotriosidase [35]. Traditional herbal prescription Kyung-Ok-Ko (KOK) can improve the symptom of PCOS by affecting T cells and macrophages [36]. In this paper, we find that incubation with monocyte supernatant promotes the apoptotic progress of KGN cells. This alteration is also confirmed by stimulating KGN cells with THP1 derived macrophage. To verify monocyte and macrophage can enhance granulosa cell apoptosis, we also detect the protein expression of Fas which plays a central role in the physiological regulation of cell apoptosis. Western blot assay proves monocyte and macrophage supernatant significantly promote the expression of Fas. Result of microarray analysis also reveals that Fas is obviously upregulated in PCOS granulosa cells, indicating the elevation of Fas may be caused by macrophage infiltration. Our finds can be also proved by another study, in which researchers prove that Fas and Fas ligand (FasL) are significantly upregulate in dehydroepiandrosterone-induced PCOS rats [37].

Reactive oxygen species (ROS) are a kind of chemically reactive molecular containing oxygen such as peroxides, superoxide, hydroxyl radical and singlet oxygen. Numerous studies have confirmed that ROS play crucial roles in human PCOS [38, 39]. Besides, elevated ROS are often associated with inflammatory response [40]. In this study, we prove supernatant derived from monocyte and macrophage are able to increase the ROS in granulosa cells, indicating monocyte and macrophage are key cause for the production of ROS in PCOS.

In summary, this study shows that many inflammatory genes are often upregulated in PCOS granulosa cells. Impaired granulosa cells participate in transformation of monocyte to macrophage. In turn, monocyte and macrophage can also promote the apoptosis and ROS generation of granulosa cells. This study also suggests that inflammation may be a key cause for the upregulation of Fas in PCOS granulosa cells, and the elevation of CD163 in PCOS may mainly be produced by activated macrophage. Our findings help better understand the genesis and molecular mechanism of PCOS.

Disclosure of conflict of interest

None.

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