**Cellular senescence of granulosa cells in the pathogenesis of polycystic ovary syndrome**

**Running title:** Cellular senescence and senolytics in PCOS

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Abstract

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age, but its pathology has not been fully characterized and the optimal treatment strategy remains unclear. Cellular senescence is a permanent state of cell-cycle arrest that can be induced by multiple stresses. Senescent cells contribute to the pathogenesis of various diseases, owing to an alteration in secretory profile, termed ‘senescence-associated secretory phenotype’ (SASP), including with respect to pro-inflammatory cytokines. Senolytics, a class of drugs that selectively eliminate senescent cells, are now being used clinically, and a combination of dasatinib and quercetin (DQ) has been extensively used as a senolytic. We aimed to investigate whether cellular senescence is involved in the pathology of PCOS and whether DQ treatment has beneficial effects in patients with PCOS. We obtained ovaries from patients with or without PCOS, and established a mouse model of PCOS by injecting dehydroepiandrosterone. The expression of the senescence markers p16^{INK4a}, p21, p53, γH2AX, and senescence-associated β-galactosidase (SA-β-gal); and the SASP-related factor interleukin (IL)-6; were significantly higher in the ovaries of patients with PCOS and PCOS mice than in controls. To evaluate the effects of hyperandrogenism...
and DQ on cellular senescence *in vitro*, we stimulated cultured human granulosa cells (GCs) with testosterone and treated them with DQ. The expression of markers of senescence and a SASP-related factor was increased by testosterone, and DQ reduced this increase. DQ reduced the expression of markers of senescence and a SASP-related factor in the ovaries of PCOS mice and improved their morphology. These results indicate that cellular senescence occurs in PCOS. Hyperandrogenism causes cellular senescence in GCs in PCOS and senolytic treatment reduces the accumulation of senescent GCs and improves ovarian morphology under hyperandrogenism. Thus, DQ might represent a novel therapy for PCOS.

**Keywords:** cellular senescence, senolytic, polycystic ovary syndrome, dasatinib, quercetin, p16<sup>INK4a</sup>, p21, γH2AX, senescence-associated β-galactosidase, senescence-associated secretory phenotype
Polycystic ovary syndrome (PCOS) is the most common hormonal disorder in women, affecting 6%–10% of women of reproductive age (McCartney and Marshall, 2016). PCOS causes ~80% of cases of anovulatory infertility (Balen et al., 2016), and is also characterized by hyperandrogenemia, obesity, impaired glucose tolerance, and abnormal lipid metabolism. PCOS is a heterogeneous condition, and according to the Rotterdam criteria, is present if two of the three following features are present: hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004).

The characteristic morphological features of polycystic ovaries are the accumulation of antral follicles of 5–8 mm and follicular growth failure (Franks et al., 2000; Franks et al., 2008). Its pathophysiology has not been well characterized, but various mechanisms, including endoplasmic reticulum (ER) stress (Harada, 2022; Koike et al., 2023), oxidative stress (Zeber-Lubecka et al., 2023), and abnormal inflammation and immunity (Lima et al., 2018) have been implicated. Women with PCOS have high serum concentrations of pro-inflammatory molecules, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-18 (Kaya et al., 2010; Alissa et al., 2021; Mazloomi et al., 2023).
Although the pathogenesis of PCOS has not been fully characterized, androgen excess has been shown to be a key component (Walters et al., 2018), and findings made in a number of animal models created through the administration of androgen are consistent with this (Elia et al., 2006; Manneras et al., 2007; Corrie et al., 2021). For example, dehydroepiandrosterone (DHEA)-treated mice develop polycystic ovaries (Elia et al., 2006); an estrus cycle disorder; a high circulating luteinizing hormone (LH) concentration (Zhang H. et al., 2023); and a metabolic phenotype comprising insulin resistance, hypertriglyceridemia, and a high serum low-density lipoprotein-cholesterol concentration (Li et al., 2023). In addition, human and animal studies have shown that the etiology of PCOS involves genetic, epigenetic, and metabolic factors; inflammatory processes; and ER stress (Bednarska and Siejka, 2017; Harada, 2022; Koike et al., 2023).

Cellular senescence is defined as a cellular state that is induced by certain insults and physiological processes; and is characterized by prolonged and generally irreversible cell-cycle arrest, an altered secretory profile, macromolecular damage, and altered metabolism (Gorgoulis et al., 2019). Cellular senescence occurs in response to cellular damage or stress (Munoz-Espin and Serrano, 2014) associated with genotoxic drugs, oncogenic stress, oxidative stress, irradiation, or
ER stress (Gorgoulis *et al.*, 2019). Senescent cells secrete senescence-associated secretory phenotype (SASP)-related factors, which play a role in the crosstalk between senescent cells and their neighbors. SASP-related factors include many immune mediators, such as TNF-α, IL-1α/β, IL-6, IL-8, and tissue remodeling factors, including transforming growth factor (TGF)-β and matrix metalloproteinases (Tchkonia *et al.*, 2013; Gorgoulis *et al.*, 2019). The SASP is a hallmark of senescent cells and mediates many of their pathophysiological effects (Gorgoulis *et al.*, 2019; Harries, 2023), such as in aging and chronic diseases including lung fibrosis (Barnes *et al.*, 2019), osteoarthritis (Coryell *et al.*, 2021), age-related macular degeneration (Lee *et al.*, 2021), neurodegeneration (Carreno *et al.*, 2021), chronic kidney disease (Goligorsky, 2020), and diabetes (Palmer *et al.*, 2019). To date, no specific marker of cellular senescence has been identified (Hernandez-Segura *et al.*, 2017), and therefore cellular senescence can only be defined using a combination of multiple factors and features.

Two principal signaling pathways are responsible for the initiation and maintenance of senescence-associated cell-cycle arrest: the p53–p21–retinoblastoma protein (RB) and p16\(^{INK4a}\)–RB pathways (Childs *et al.*, 2017; Gorgoulis *et al.*, 2019). Markers of cellular senescence include high expression of p16\(^{INK4a}\) and p21; high SA-β-gal activity; high expression of SASP-related factors, such as IL-6, IL-8, IL-1α, and TGF-β-1 (Childs *et al.*, 2017; Gorgoulis *et al.*, 2019; Harries, 2023);
and DNA damage mediators, such as γH2AX (Hewitt et al., 2012; Kirkland and Tchkonia, 2017).

A senolytic is an agent that induces the death of senescent cells, thereby eliminating them. Some senolytics, including a combination of dasatinib and quercetin (DQ), are undergoing clinical trials as treatments for Alzheimer’s disease (Gonzales et al., 2023), idiopathic pulmonary fibrosis (Nambiar et al., 2023), and diabetic kidney disease (Hickson et al., 2019).

In the present study, we aimed to determine whether (a) granulosa cells (GCs) show a high level of senescence in PCOS; (b) hyperandrogenism induces cellular senescence in GCs; (c) hyperandrogenism induces the secretion of a SASP-related factor by GCs; and (d) DQ ameliorates PCOS in mice.

**Materials and Methods**

**Human samples**

Follicular fluid containing GCs was obtained from patients undergoing oocyte retrieval for IVF at the University of Tokyo Hospital, Matsumoto Ladies’ Clinic, and Phoenix ART Clinic. Informed written consent was obtained from all of the participants. PCOS was diagnosed according
to the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). The inclusion criteria for control patients were a normal ovulatory cycle, no endocrinological abnormalities, and normal ovarian morphology, confirmed ultrasonographically. The basal mRNA expression of markers of senescence in GCs was measured in samples obtained from 12 control patients and 12 patients with PCOS, the characteristics of whom are shown in Table 1. There were no significant differences between the groups with respect to age, BMI, serum basal FSH concentration, or the number of oocytes retrieved; whereas the serum basal LH concentration, LH/FSH ratio, and serum anti-Müllerian hormone (AMH) concentration of the patients with PCOS were significantly higher.

For immunohistochemical analysis, ovaries were obtained from women undergoing gynecological surgery. Informed written consent was obtained from all the participants. Normal ovaries were obtained from women with regular menstrual cycles, who were not undergoing hormonal treatment, and who underwent radical or extended hysterectomy as a treatment for carcinoma of the uterine cervix or endometrium. Ovaries were also obtained from patients with PCOS who were oligo- or anovulatory and who also underwent hysterectomy because of uterine cancer. The presence of polycystic ovaries was histologically confirmed. Four control ovaries and four PCOS ovaries were obtained from eight different patients for analysis. There was no
significant difference in the age of the two groups, but the BMI of the patients with PCOS was higher: the median (range) ages were 38.0 years (27–46 years) and 27.0 years (24–36 years) ($p = 0.1855$); and the median (range) BMIs were 18.77 kg/m$^2$ (16.80–20.96 kg/m$^2$) and 28.54 kg/m$^2$ (25.97–31.96 kg/m$^2$) ($p = 0.0007$) for the control and PCOS groups, respectively.

All the experimental procedures were approved by the relevant institutional review board (authorization number: 3594-11). All the participants provided their written informed consent, and the study was conducted in accordance with the principles of the Declaration of Helsinki.

**Animal model**

We used a well-established mouse model of DHEA-induced PCOS (Elia *et al.*, 2006; Koike *et al.*, 2022). Three-week-old female BALB/c mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). First to characterize the accumulation of senescent cells in the ovaries of PCOS mice, the animals were divided into two groups. The control group ($n = 5$) received daily subcutaneous (s.c.) injections of sesame oil for 20 days, whereas the PCOS group ($n = 5$) received daily s.c. injections of DHEA (60 mg/kg per day; Sigma-Aldrich, St. Louis, MO, USA) for 20 days. The animals were anaesthetized with isoflurane and sacrificed by decapitation, and their ovaries
were collected on day 21. Next, to determine the effects of DQ on the cellular senescence of GCs of the PCOS mice, other animals were obtained and divided into three groups: a PCOS group, a PCOS + vehicle group, and a PCOS + DQ group. All three groups received daily s.c. injections of DHEA (60 mg/kg per day) for 20 days. The PCOS group (n = 6) received no oral treatment, the PCOS + vehicle group (n = 6) was orally administered vehicle (10% polyethylene glycol 400; Wako, Osaka, Japan), and the PCOS + DQ group (n = 6) was orally administered dasatinib (5 mg/kg per day; Sigma-Aldrich) and quercetin (50 mg/kg per day; Sigma-Aldrich) on days 1, 6, 11, and 16. The ovaries of the mice were collected on day 21. Then, to determine the effect of DQ on the ovarian morphology of the PCOS mice, other mice were obtained and divided into four groups; a control group (n = 4), a PCOS group (n = 4), a PCOS+Vehicle group (n = 4) and a PCOS+DQ group (n = 4). All group mice were treated as described above and the ovaries of the mice were collected on day 21. All the animal procedures described were approved by the University of Tokyo Committee on the Use and Care of Animals (approval number: P21-005) and were performed in accordance with relevant guidelines and regulations, and the ARRIVE guideline 2.0 (Percie du Sert N et al., 2020).
RNA extraction, reverse transcription, and real-time quantitative PCR

RNA was extracted from the whole ovaries of mice using Isogen (Nippon Gene, Tokyo, Japan), then 1 μg of each preparation was reverse transcribed using ReverTra Ace qPCR RT Master Mix, with genomic DNA remover (Toyobo, Osaka, Japan), in a volume of 40 μL. The cDNA template was synthesized from human GCs using a SuperPrep Cell Lysis & RT Kit for qPCR (Toyobo). To measure the expression of target mRNAs, quantitative real-time PCR was performed on a Light Cycler system (Roche Diagnostics GmBH, Mannheim, Germany), and the mRNA expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the reference gene. The primer sequences are shown in Table 2. The PCR conditions were as follows: 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s.

Immunohistochemistry

Human and mouse ovaries were fixed in 10% v/v neutral-buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. Antigen retrieval was performed using Target Retrieval Solution (Dako, Tokyo, Japan). Non-specific binding was blocked using peroxidase-blocking solution (Dako) for 5 min at room temperature. The ovarian sections were then incubated
with anti-p16INK4a (1:1,000, RRID: AB_2737282; Abcam, Cambridge, UK), anti-p21 (1:1,600, RRID: AB_2077682; Proteintech Group, Tokyo, Japan), anti-p53 (1:4,000, RRID: AB_2881401; Proteintech Group), anti-γH2AX (1:400, RRID: AB_2118009; Cell Signaling Technology, Danvers, MA, USA), or anti-IL-6 (1:3,000, RRID: AB_2881543; Proteintech Group) antibodies, or isotype-specific IgG (RRID: AB_737182; Santa Cruz Biotechnology, Dallas, TX, USA) as a negative control, followed by secondary antibody (Real EnVision Detection System, RRID: AB_2888627; Dako). Immunohistochemistry was performed on at least three independent occasions using identical samples. The stained sections were examined using a BX50 microscope (Olympus, Tokyo, Japan), and ImageJ software (RRID: SCR_003073; National Institutes of Health, Bethesda, MD, USA) was used for their quantitative analysis of % of positive GCs area. (Schneider et al., 2012; Jensen EC, 2013; Varghese F et al., 2014).

Scoring of γH2AX immunoreactivity

To assess the protein expression of γH2AX we used the immunoreactive score (IS), since the intensity of γH2AX is considered to indicate the degree of DNA damage per cell (Mei et al., 2015). After immunohistochemical staining with an anti-γH2AX antibody, 10 follicles were
randomly selected from each of the control and PCOS groups. All of the GCs in a follicle were scored according to their staining intensity (0 (negative), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), or 3 (strong positive = brown or black)) independently by two authors. The proportions of cells with each score were multiplied by the scores, and all these were added together to obtain the IS of a follicle.

**Isolation and culture of human GCs**

GCs were isolated as previously reported (Takahashi et al., 2019; Kunitomi et al., 2021). The follicular fluid samples were centrifuged, and the pellets were resuspended in phosphate-buffered saline (PBS) containing 0.2% w/v hyaluronidase (Sigma-Aldrich) and incubated at 37 °C for 30 min. The suspension was then centrifuged again at 400 × g for 30 min after being layered over Ficoll-Paque (GE Healthcare, Amersham, UK). The GCs were collected from the interface and washed with PBS, then cultured in 6-, 12-, or 48-well plates at densities of $5 \times 10^4$, $5 \times 10^5$, or $2 \times 10^5$ cells/well, respectively, in Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% w/v fetal bovine serum (Sigma-Aldrich) and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 250 ng/mL...
amphotericin B; Thermo Fisher Scientific). Prior to each experiment, the GCs were cultured for 3–5 days at 37 °C in a humidified atmosphere containing 5% CO₂.

Treatment of human GCs

At the beginning of the study, to determine the most appropriate concentration of testosterone to use in the assessment of markers of senescence, human GCs were treated with 5, 10, or 15 μg/mL testosterone (Tokyo Chemical Industry Co., Tokyo, Japan) based on previous reports (Azhary et al., 2019) (Azhary et al., 2020) for 9 h, and then the protein expression of p21 was analyzed using western blotting (Supplementary Figure S1). To evaluate the effects of testosterone on the markers of senescence p16INK4a, p21, p53, γH2AX, and SA-β-gal; and the SASP-related factor IL-6; human GCs were treated with 15 μg/mL testosterone. To evaluate the effects of DQ on cellular senescence, human GCs were concurrently incubated with 1 nM dasatinib, 10 μM quercetin, and 15 μg/mL testosterone for 24 h. The optimal concentration of DQ was chosen on the basis of that used in a previous study (Du et al., 2022).

Western blotting
Human GCs were lysed in PhosphoSafe Extraction Reagent (Merck, Darmstadt, Germany) and then centrifuged at 16,000 \( \times g \) for 5 min. The supernatants were collected, and their protein concentrations was measured using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Ten \( \mu \text{g} \) of denatured protein were loaded per lane subjected to SDS-PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). After blocking with 5\% w/v skim milk prepared in Tris-buffered saline containing 0.1\% v/v Tween-20 at room temperature for 1 h, the membranes were incubated with anti-p16\(^{INK4a}\) (1:500, RRID: AB_628067; Santa Cruz Biotechnology), anti-p21 (1:1,000, RRID: AB_2077682; Proteintech Group), anti-p53 (1:5,000, RRID: AB_2881401; Proteintech Group), \( \gamma \text{H2AX} \) (1:400, RRID: AB_2118009; Cell Signaling Technology), or anti-\( \beta \)-actin (1:10,000, RRID: AB_476697; Sigma-Aldrich) antibodies overnight at 4 \( ^\circ \text{C} \), and then with a secondary antibody (anti-rabbit antibody, 1:2000, RRID: AB_2099233; or anti-mouse antibody, 1:2000, RRID: AB_330924; Cell Signaling Technology) at room temperature for 1 h. Bands were visualized using ECL Plus western blotting detection reagents (GE Healthcare) and imaged on an ImageQuant LAS 4000 Mini luminescent image analyzer (GE Healthcare). \( \beta \)-actin was used as the loading control. The intensities of the specific protein bands were quantified using ImageJ software (National Institutes of Health) (Schneider \textit{et al.}, 2012).
Staining for SA-β-gal activity was performed as previously described (Dimri et al., 1995; Serrano et al., 1997). After treatment, GCs cultured in 6-well plates were washed with PBS (pH 7.5), fixed in 3% w/v formaldehyde for 15 min, washed twice, and then stained with fresh SA-β-gal staining solution (PBS (pH 6.0) containing 1 mg/mL X-gal (5-bromo-4-chloro-3-indoly β-D-glucopyranoside; Wako), 1 mM MgCl₂, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide) for 3–4 h at 37 °C in the absence of CO₂. The stained cells were examined using an IX70 microscope (Olympus).

Measurement of IL-6 concentration

The concentrations of human IL-6 in follicular fluid samples and culture media were measured using ELISA kits (R & D systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. Absorbance was measured at 450 nm using a Synergy LX multi-mode reader (BioTek, Winooski, VT, USA). The sensitivity of the assay was 0.7 pg/mL, and the intra- and interassay coefficients of variation for the ELISA kit were 7.9 ± 0.8% and 6.5 ± 0.8%,
respectively.

**Histology**

Human and mouse ovaries were fixed in 10% v/v neutral-buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. The sections were stained with hematoxylin and eosin, and the number of atretic antral follicles were counted in every sixth section across the entire ovary, as previously described (Azhary et al., 2020). To ensure that each atretic antral follicle was only counted once, the adjacent sections were also analyzed. Atretic follicles were identified by the presence of shrunken granulosa cell walls and cumulus cells or their absence, the hyperplasia of theca cells, and the presence of macrophages in the antrum (Osman, 1985; Kauffman et al., 2015).

**Statistical analysis**

Statistical analyses were performed using JMP Pro 16 software (RRID: SCR_022199; SAS Institute Inc., Cary, NC, USA). All data are summarized as mean ± standard error of the mean (SEM) or median (range). Two groups were compared using Student’s t-test, and one-way ANOVA followed by Tukey-Kramer honest significant difference test was used for multiple comparisons. $p$
< 0.05 was considered to represent statistical significance.

Results

Senescent GCs accumulate in patients with PCOS

To determine whether senescence is upregulated in the GCs of patients with PCOS, we measured the mRNA expression of the markers of senescence p16\textsuperscript{INK4a}, p21, and p53 in the GCs of control patients and those with PCOS using quantitative real-time PCR. The mRNA expression of p16\textsuperscript{INK4a}, p21, and p53 in isolated GCs from patients with PCOS was 2.27-, 1.68-, and 3.52-fold higher (p = 0.0110, 0.0330, and 0.0012), respectively, than those in GCs from control patients (Figure 1A). Subsequently, we performed immunohistochemical analysis of the ovaries from the patients with or without PCOS to evaluate the protein expression of these markers. Two or three antral follicles were randomly selected from each ovarian section for quantitative analysis and the immunoreactivities of p16\textsuperscript{INK4a}, p21, and p53 in the GCs of ovaries from patients with PCOS were 1.72-, 1.96-, and 1.78-fold higher (p = 0.0067, 0.0021, and 0.0002), respectively, than those of controls (Figure 1B–E). These findings indicate that cellular senescence is induced in the GCs of
patients with PCOS.

Senescent cells accumulate in the ovaries of PCOS mice

Next, we measured the expression of markers of senescence in the ovaries of PCOS mice. Quantitative real-time PCR analysis demonstrated that the mRNA expression of \( p16^{INK4a} \), \( p21 \), and \( p53 \) in the ovaries of PCOS mice was 1.61-, 1.68-, and 1.44-fold higher (\( p = 0.0319, 0.0864, \) and 0.0439), respectively, than that of control mice (Figure 2A). Immunohistochemical analysis revealed that the protein expression of \( p16^{INK4a} \), \( p21 \), \( p53 \), and \( \gamma\text{H2AX} \) in the GCs of PCOS mice was 1.55-, 1.72-, 1.67-, and 1.43-fold higher (\( p = 0.0017, 0.0007, 0.0008, \) and 0.0003), respectively, than that of controls. (Figure 2B–F). These findings indicate that cellular senescence is upregulated in the GCs of PCOS mice, in accordance with the findings in women with PCOS.

Testosterone induces cellular senescence in cultured human GCs

To determine whether the accumulation of senescent cells in the GCs of patients with PCOS is associated with hyperandrogenism, we measured the protein expression of markers of senescence following the treatment of cultured human GCs with testosterone. To determine the most
appropriate concentration of testosterone for use in the assessment of senescence markers, human GCs were treated with 5, 10, or 15, μg/mL testosterone, and the protein expression of p21 was analyzed. The protein expression of p21 was increased more significantly by treatment with 15 μg/mL testosterone (by 1.33-fold, $p < 0.01$; Supplementary Figure S1). Therefore, we used 15 μg/mL testosterone treatment as a model of hyperandrogenism in cultured human GCs in subsequent experiments. Testosterone stimulation significantly increased the protein expression of p16\(^{\text{INK4a}}\), p21, p53, and γH2AX by 1.41-, 1.33-, 1.30-, and 1.43-fold ($p < 0.0001$, $= 0.0006$, 0.0003, and 0.0025), respectively (Figure 3A–D). We also conducted SA-β-gal staining to further evaluate the effect of testosterone on cellular senescence. As shown in Figure 3E, SA-β-gal activity was increased by testosterone treatment. These results indicate that local hyperandrogenism in the ovary causes the upregulation of senescence in human GCs.

**DQ reduces the testosterone-induced senescence of, and secretion of SASP-related substances by cultured human GCs**

Next, to determine the effects of DQ on testosterone-induced senescence, we concurrently incubated human GCs with testosterone and DQ for 24 h. DQ reduced the testosterone-induced
increase in p16\textsuperscript{INK4a} and p21 protein by 0.65- and 0.78-fold ($p = 0.0548$ and 0.0001), respectively (Figure 4A,B). To further investigate the effect of DQ on cellular senescence, we assessed the secretion of IL-6, which is known to be a pro-inflammatory SASP-related factor and to be upregulated in the ovaries of PCOS mice (Van Deursen, 2014; Shi \textit{et al.}, 2023). We measured the concentrations of IL-6 in conditioned medium using ELISA, and found that testosterone stimulation upregulates that by 1.99-fold ($p = 0.0451$), and concurrent incubation with DQ reduces the testosterone-induced upregulation of IL-6 by 0.35-fold ($p = 0.0344$) (Figure 4C). Pretreatment with DQ similarly reduced testosterone-induced senescence and IL-6 production of GCs (Supplementary Figure S2).

\textit{DQ reduces the senescence of, and secretion of SASP-related molecules by, GCs of PCOS mice; and restores their ovarian morphology}

To determine whether DQ treatment affects the senescence of GCs \textit{in vivo}, we administered DQ to PCOS mice and evaluated the protein expression of markers of senescence and a SASP-related factor in their ovaries by immunohistochemical analysis. The protein expression of p16\textsuperscript{INK4a}, p21, p53, and IL-6 in GCs from PCOS mice with DQ administration was reduced by 0.35-, 0.54-,
0.50-, and 0.49-fold \((p = 0.0012, 0.0002, 0.0001, \text{ and } 0.0018)\), respectively, vs. vehicle-treated mice; and those that were vehicle-treated did not significantly differ from those that were untreated \((p = 0.9900, 0.6148, 0.2260, \text{ and } 0.6293, \text{ respectively})\) (Figure 5A–E). Next, to determine the effect of DQ on the ovarian morphology of PCOS mice, the numbers of atretic follicles were counted. As shown in Figure 6, the number of atretic follicles in PCOS mice treated with DQ was 0.58-fold lower than that in PCOS+Vehicle mice \((p = 0.0128)\); and that of the PCOS mice was 1.57-fold higher than that of control mice \((p = 0.0135)\), which did not differ from that of the PCOS+Vehicle mice \((p = 0.9221)\). These findings suggest that DQ treatment reduces the cellular senescence of and IL-6 production by GCs, and restores the ovarian morphology of PCOS mice.

**Discussion**

In the present study, we have made the following findings. (a) In human GCs from patients with PCOS, the expression of \(p16^{\text{INK4a}}\), \(p21\), and \(p53\) is high at both the mRNA and protein levels. (b) In PCOS mice, the ovarian mRNA expression of \(p16^{\text{INK4a}}\), \(p21\), and \(p53\) and the protein expression of \(p16^{\text{INK4a}}\), \(p21\), \(p53\), and \(\gamma H2AX\) of GCs are high. (c) In isolated human GCs,
testosterone induces the protein expression of p16\textsuperscript{INK4a}, p21, p53, and γH2AX, and increases SA-β-gal staining. (d) In isolated human GCs, the senolytic combination DQ reduces the testosterone-induced protein expression of p16\textsuperscript{INK4a}, p21, and IL-6. (e) In PCOS mice, DQ treatment reduces the protein expression of p16\textsuperscript{INK4a}, p21, p53, and IL-6 in GCs and the number of atretic follicles. These results suggest that cellular senescence and the SASP-related factor IL-6 are involved in the pathogenesis of PCOS.

We found that the expression of the cellular senescence markers p16\textsuperscript{INK4a}, p21, p53, and γH2AX in GCs was high in patients with PCOS and PCOS mice. Cellular senescence has been reported to affect ovarian function; and in mice, insulin resistance reduces the ovarian reserve by causing the senescence of GCs (Gao \textit{et al.}, 2023). Furthermore, in one previous study, an association between cellular senescence and PCOS was identified; exosomal miR-424-5p derived from the follicular fluid of patients with PCOS induces the senescence of COV434 and KGN cells (Yuan \textit{et al.}, 2021). However, no previous studies have demonstrated the existence of cellular senescence in the ovaries of patients with PCOS or characterized the mechanism underpinning the increase in cellular senescence in PCOS. In the present study, we have demonstrated that cellular senescence is upregulated in the GCs of patients with PCOS or PCOS mice.
We found that local hyperandrogenism induces the protein expression of the cellular senescence markers p16\textsuperscript{INK4a}, p21, p53, and γH2AX in, and the SA-β-gal activity of, human GCs. These results suggest that local hyperandrogenism in PCOS induces the cellular senescence of GCs, but the underlying mechanism requires to be defined. Local hyperandrogenism in PCOS may directly induce the cellular senescence of GCs. It has been reported that androgen receptor activation is linked to the induction of cellular senescence in some cancer cells, including thyroid (Gupta et al., 2023) and prostate (Mirochnik et al., 2012) cancer cells. Alternatively, hyperandrogenism may induce the senescence of GCs \textit{via} the activation of ER stress. It has been reported that ER stress induces the senescence of several types of cells. In alveolar epithelial cells (AECs), epoxyeicosatrienoic acids, which are downstream metabolites of arachidonic acid, inhibit ER stress and alleviate AEC senescence (Zhang CY. et al., 2023); and in breast cancer cells, oroxin A induces cellular senescence through the activation of ER stress (He et al., 2016). ER stress pathways are activated in the GCs of women with PCOS and androgen-administered mice (Takahashi et al., 2017; Jin et al., 2020; Harada, 2022), and hyperandrogenism in the follicular microenvironment is an activator of ER stress in human (Azhary et al., 2019) and mice (Jin et al., 2020) GCs.

We found that the senolytic combination DQ ameliorated the androgen-induced increases in the
expression of cellular senescence markers and the SASP-related factor IL-6 in GCs \textit{in vitro} and \textit{in vivo}. Dasatinib, which is used for the treatment of chronic myeloid leukemia, is an inhibitor of several receptor tyrosine kinases (Montero \textit{et al}., 2011; Nieto \textit{et al}., 2023). Regarding its senolytic potential, dasatinib reduces the number of senescent preadipocytes following exposure to ionizing radiation (Zhu \textit{et al}., 2015) and reduces the cardiac expression of p16 in a mouse model of type 2 diabetes mellitus (Gu \textit{et al}., 2023). Quercetin is a flavonol of vegetable origin that has pharmacological effects, including antioxidant, anti-inflammatory, and anti-senescence effects. It has effects through the inhibition of the PI3K/AKT pathway (Olave \textit{et al}., 2010; Bruning, 2013). Regarding its senolytic effects, quercetin eliminates the senescence of HUVEC cells (Zhu \textit{et al}., 2015) and radioresistant HT500 cells (Russo \textit{et al}., 2023); inhibits the SASP-related production of IL-6, IL-8, and MMPs by nucleus pulposus cells (Shao \textit{et al}., 2021); and inhibits the production of IL-8 by radioresistant HT500 cells (Russo \textit{et al}., 2023). Interestingly, DQ treatment before testosterone also reduced the testosterone-induced expression of cellular senescence markers and the SASP-related factor IL-6 in GCs as shown in Supplementary Figure S2. Taken together, the senolytic combination DQ may ameliorate the androgen-induced cellular senescence both by eliminating cellular senescence induced by testosterone and by converting the function of testosterone in induction of cellular senescence.
Zhu reported that the DQ combination reduces cellular senescence in tissues involved in the etiology of multiple chronic disorders, because it targets multiple senescence-associated anti-apoptotic pathways (Zhu et al., 2015; Xu et al., 2018). Moreover, Xu reported that DQ reduces senescence; reduces the secretion of the pro-inflammatory cytokines IL-6, IL-8, MCP-1, PAI-1, and GM-CSF by human adipose tissue; alleviates physical dysfunction; and improves the survival of aged mice (Xu et al., 2018). Phase I clinical trials of this oral senolytic combination have been conducted in patients with Alzheimer’s disease (Gonzales et al., 2023) and idiopathic pulmonary fibrosis (Nambiar et al., 2023), and these showed that this approach is safe, well-tolerated, and feasible for patients. Another early clinical trial showed that treatment with DQ reduces senescence in the adipose tissue of patients with diabetic kidney disease (Hickson et al., 2020). Here, we have reported for the first time that DQ acts as senolytic with respect to the pathology of PCOS and reduces IL-6 secretion by GCs.

We have also demonstrated that the DQ treatment of PCOS mice improves their ovarian morphology. There have been some previous reports regarding the effects of quercetin on ovarian function and PCOS that did not evaluate cellular senescence. In a rat model of PCOS, quercetin ameliorated the abnormalities in ovulation through effects on C-type natriuretic peptide (CNP) and its receptor, natriuretic peptide receptor 2 (NPR2) (Zheng et al., 2022). In addition, two randomized,
double-blind, placebo-controlled clinical trials demonstrated that in patients with PCOS, oral quercetin supplementation ameliorates adiponectin-mediated insulin resistance and hormonal abnormalities (Rezvan et al., 2017), improves the oocyte and embryo grade, and improves the pregnancy rate (Vaez et al., 2023). However, there have been no previous studies of the use of quercetin as senolytic in PCOS. In the present study, the effects of DQ were determined in vitro in cultured GCs, and the findings suggest that the in vivo effects of DQ on the ovary may be at least in part direct and local. Therefore, cellular senescence may represent a novel therapeutic target in PCOS, and senolytics represent potential treatments for PCOS.

There were some limitations to the present study. First, quercetin may act as an antioxidant, and thereby reduce hyperandrogenism-induced IL-6 production through an effect on reactive oxygen species, rather than through a senolytic effect. Second, although our in vitro findings suggest that DQ has direct and local effects in the ovaries, it might also act indirectly, through systemic effects. Third, it is unclear whether DQ would be more effective than quercetin alone as a treatment for PCOS. Therefore, future studies should address this.

In conclusion, we have demonstrated that the senescence of GCs is greater in patients and PCOS mice, that testosterone induces senescence in cultured human GCs, and that DQ treatment
reduces the testosterone-induced cellular senescence and IL-6 production. In PCOS mice, DQ reduces the hyperandrogenism-induced senescence of GCs, reduces IL-6 expression, and improves ovarian morphology. IL-6 is thought to be a SASP-related factor in the pathophysiology of PCOS, and senolytics represent potential treatments for PCOS. However, further studies are required to clarify the mechanisms by which cellular senescence is induced and whereby DQ has its effects in PCOS.

**Data availability**

The data relating to this article are available within the article and the online supplementary material.

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Authors’ roles

T.T. designed and performed the experiments, analyzed and interpreted the data, and wrote the article. Y.U. and M.H. conceived the work, analyzed and interpreted the data, and wrote the article. C.K. conceived the work and performed experiments. A.K., H.K., Z.X., N.S., C.T., A.K., and A.T. performed experiments. N.T., O.W.-H., Y.H., and Y.O. contributed to the study design, data interpretation, and article revision.

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Conflict of interest

The authors declare no conflict of interest.

Figure legends

Figure 1. p16INK4a, p21, and p53 are upregulated in the granulosa cells (GCs) of patients with polycystic ovary syndrome (PCOS). (A) The expression of p16INK4a, p21, and p53 mRNAs in GCs from control patients (n = 12) and those with PCOS (n = 12) was measured by quantitative real-time PCR and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B–D) Immunohistochemical analysis was performed on GCs in the antral follicles of ovaries from control patients (n = 4) and those with PCOS (n = 4). Cross-sections of ovaries, stained with (B) anti-p16INK4a, (C) anti-p21, and (D) anti-p53 antibodies, and counterstained with hematoxylin. (E) Corresponding negative control. For the panels (c), two or three antral follicles were randomly selected from each ovary (total 8 antral follicles per group) for quantitative analysis of immunohistochemical staining. Representative images and data are shown. Scale bars: 50.0 µm. Values are mean ± SEM, and the mean differences are analyzed using Student’s t-test. *p < 0.05 and **p < 0.01. GC, granulosa cell layer; GCs, granulosa cells; NC, negative control.
Figure 2. p16\textsuperscript{INK4a}, p21, p53, and γH2AX are upregulated in the ovaries of polycystic ovary syndrome (PCOS) mice. (A) The mRNA expression of p16\textsuperscript{INK4a}, p21, and p53 in whole ovaries from control mice (n = 4) and those from PCOS mice (n = 4) was measured by quantitative real-time PCR and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (\textit{GAPDH}) expression. (B–E) Immunohistochemical analysis was performed on the GCs in antral follicles of ovaries from control mice (n = 5) and those from PCOS mice (n = 5). Cross-sections of ovaries were stained with (B) anti-p16\textsuperscript{INK4a}, (C) anti-p21, (D) anti-p53, and (E) anti-γH2AX antibodies, and counterstained with hematoxylin. (F) Corresponding negative control. Scale bars: 100 µm. (c) One or two antral follicles were selected from each ovary (total 8 antral follicles per group) for quantitative analysis of immunohistochemical staining. Values are mean ± SEM, and the mean differences are analyzed using Student’s t-test. *p < 0.05 and **p < 0.01. GCs, granulosa cells; NC, negative control. We repeated experiments three times using different mice, and representative data are shown.

Figure 3. Effects of testosterone on the expression of markers of senescence in cultured human
granulosa cells (GCs). Human GCs were treated with testosterone (15 µg/mL). (A–D) Protein expression levels of p16\textsuperscript{INK4a}, p21, p53, and γH2AX in cultured human GCs, measured using western blotting (n = 10). β-actin was used as a loading control. (b) Quantitative analysis of the western blots and (a) representative images are shown. All full-size, uncropped images of western blot used for analysis are shown in Supplementary Figure S3. Results are expressed relative to the mean control value. Values are mean ± SEM, the mean differences are analyzed using Student’s t-test. *p < 0.05 and **p < 0.01. (E) SA-β-gal staining of human GCs. Scale bars: 200 µm. (A–E) The experiments were performed ten times using ten different women’s GCs, and representative images are shown.

**Figure 4.** A combination of dasatinib and quercetin (DQ) reduces the testosterone-induced senescence of, and secretion of a SASP-related factor by, human granulosa cells (GCs). Human GCs were treated with testosterone (15 µg/mL), dasatinib (1 nM) and quercetin (20 µM), concurrently for 24 h. The protein expression of (A) p16\textsuperscript{INK4a} and (B) p21 in cultured human GCs was analyzed using western blotting (n=5). β-actin was used as a loading control. (b) Quantitative analysis of the western blots using five different women’s GCs are expressed relative to the mean
control value, and (a) representative images are shown. All full-size, uncropped images of western blot used for analysis are shown in Supplementary Figure S4. (C) Concentration of IL-6 in the culture medium, measured by ELISA using six different women’s GCs. Values are mean ± SEM, and the mean differences were analyzed using one-way ANOVA followed by Tukey-Kramer honest significant difference test. *p < 0.05 and **p < 0.01.

Figure 5. A combination of dasatinib and quercetin (DQ) treatment reduces the senescence of, and the secretion of a senescence-associated secretory phenotype (SASP)-related factor by, granulosa cells in the antral follicles of polycystic ovary syndrome (PCOS) mice. The mice were divided into three groups, which were subcutaneous (s.c.) injected with dehydroepiandrosterone (DHEA 60 mg/kg per day) (PCOS, n = 6), s.c. injected with DHEA and orally administered vehicle (10% polyethylene glycol 400) (PCOS + vehicle, n = 6), or s.c. injected with DHEA and orally administered DQ (D: dasatinib 5 mg/kg per day, Q: quercetin 50 mg/kg per day) (PCOS + DQ, n = 6). Subcutaneous injections were performed daily for 20 days, and oral administration was performed on days 1, 6, 11, and 16. Ovaries were collected on day 21. Cross-sections of ovaries were stained with (A) anti-p16\textsuperscript{INK4a}, (B) anti-p21, (C) anti-p53, and (D) anti-IL-6 antibodies and
counterstained with hematoxylin. (d) One or two antral follicles were randomly selected from each
ovary for quantitative analysis of the immunohistochemical staining (total 8 follicles per group).
Values are mean ± SEM, and the mean differences were analyzed using one-way ANOVA followed
by Tukey-Kramer honest significant difference test. **p < 0.01. (E) Corresponding negative control.
Scale bars: 100 µm. n.s., not significant; GCs, granulosa cells; NC, negative control. We repeated
experiments three times using different mice, and representative images and data are shown.

Figure 6. A combination of dasatinib and quercetin (DQ) treatment restores the ovarian
morphology of polycystic ovary syndrome (PCOS) mice. To evaluate the ovarian morphology, the
mice were divided into four groups; control (n = 4), PCOS (n = 4), PCOS + vehicle (n = 4) and
PCOS + DQ (n = 4). Control mice were subcutaneous (s.c.) injected with sesame oil, PCOS mice
were s.c. injected with dehydroepiandrosterone (DHEA 60 mg/1 kg per day), PCOS + vehicle mice
were s.c. injected with DHEA and orally administered vehicle (10% polyethylene glycol 400), and
PCOS + DQ mice were s.c. injected with DHEA and orally administered DQ (D: dasatinib 5 mg/kg
per day, Q: quercetin 50 mg/kg per day). Subcutaneous injection was performed daily for 20 days
and oral administration was performed on days 1, 6, 11, and 16. Ovaries were collected on day 21.
Cross-sections of ovaries were stained with hematoxylin and eosin. (A) ★ indicate atretic follicles. Scale bars: 500 µm. (B) The number of atretic follicles per ovary (four ovaries per group). Values are mean ± SEM, and the mean differences were analyzed using one-way ANOVA followed by Tukey-Kramer honest significant difference test. n.s., not significant; *p < 0.05. We repeated experiments three times using different mice, and representative images and data are shown.

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Figure 2

A

mRNA expression (GAPDH)

p16

p21

p63

Control

PCOS

B

(a)

(b)

C

(a)

(b)

D

(a)

(b)

E

(a)

(b)

F

NC

Control

PCOS

% of p16

positive DC area

% of p21

positive DC area

% of p63

positive DC area

190x254mm (300 x 300 DPI)
190x254mm (300 x 300 DPI)
Figure 5

A (a) (b) (c) (d) 

B (a) (b) (c) (d) 

C (a) (b) (c) (d) 

D (a) (b) (c) (d) 

E NC 

190x254mm (300 x 300 DPI)
Figure 6

A

(a) Control
(b) PCOS
(c) PCOS + Vehicle
(d) PCOS + DQ

B

The number of events/100

0 10 20 30 40

Control PCOS PCOS Vehicle PCOS DQ

190x254mm (300 x 300 DPI)
Table 1. Characteristics of the patients who provided follicular fluid samples

<table>
<thead>
<tr>
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<th>Control (n = 12)</th>
<th>PCOS (n = 12)</th>
<th>p-value</th>
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<tr>
<td>Age (years)</td>
<td>34.0 (26–40)</td>
<td>35.0 (24–39)</td>
<td>0.2534</td>
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<tr>
<td>BMI (kg/m$^2$)</td>
<td>22.2 (19.7–25.4)</td>
<td>21.5 (18.1–26.4)</td>
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<tr>
<td>LH (mIU/mL)</td>
<td>4.8 (2.1–5.6)</td>
<td>8.8 (5.9–15.4)</td>
<td>0.0003</td>
</tr>
<tr>
<td>FSH (mIU/mL)</td>
<td>7.2 (5.3–8.3)</td>
<td>6.5 (4.9–8.4)</td>
<td>0.0898</td>
</tr>
<tr>
<td>LH/FSH ratio</td>
<td>0.64 (0.32–0.91)</td>
<td>1.34 (0.87–2.23)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AMH (ng/mL)</td>
<td>2.56 (1.70–6.73)</td>
<td>8.27 (5.44–24.63)</td>
<td>0.0017</td>
</tr>
<tr>
<td>The number of oocytes retrieved</td>
<td>11.5 (6–28)</td>
<td>10.5 (1–22)</td>
<td>0.3072</td>
</tr>
</tbody>
</table>

Data are presented as median (range). All p-values are based on Student’s t-test. BMI, body mass index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone.
Table 2. List of primers used for quantitative real-time PCR

<table>
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<tr>
<th>Gene</th>
<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
<th>Accession No.</th>
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<tr>
<td>human p16</td>
<td>GGGTTTTCTGTGGTTCCATCC</td>
<td>CTAGACGCTGGCTCTCAGTA</td>
<td>NM_058195</td>
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<tr>
<td>human p21</td>
<td>TGTCCGTCAGACCGATGC</td>
<td>AAAGTCGAAGTTCCATGC</td>
<td>NM_078467</td>
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<tr>
<td>human p53</td>
<td>CCCAAGCAATGGATGATTGA</td>
<td>GGCATTCTGGGAGCTTCA</td>
<td>NM_000546</td>
</tr>
<tr>
<td>human GAPDH</td>
<td>TGGACCTGACCTGCCGCTTA</td>
<td>CTGCTTCACCACCTTCTTA</td>
<td>NM_002046</td>
</tr>
<tr>
<td>mouse p16</td>
<td>CGCAGGTTCTTGGTCAGGTG</td>
<td>TGTTACGAAAGCGAGCG</td>
<td>NM_009877</td>
</tr>
<tr>
<td>mouse p21</td>
<td>CACAGCTCATGGAGCTGGAA</td>
<td>ACCCTAGACCAAAATGCAG</td>
<td>NM_00111099</td>
</tr>
<tr>
<td>mouse p53</td>
<td>GCCAGACTTCTCCACCACA</td>
<td>CAGGCAAAACACGAGATTC</td>
<td>NM_011640</td>
</tr>
<tr>
<td>mouse Gapdh</td>
<td>TCCACCACCTGTGGCTGTA</td>
<td>GCCACAGTCCATGCCATC</td>
<td>NM_008084</td>
</tr>
</tbody>
</table>
C

Testosterone (−) (+)

β-actin (42kDa)

p53 (53kDa)

p53 (53kDa)

p53 (53kDa)

p53 (53kDa)

β-actin (42kDa)