Higher *PDCD4* expression is associated with obesity, insulin resistance, lipid metabolism disorders, and granulosa cell apoptosis in polycystic ovary syndrome

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Objective: To investigate the expression and clinical significance of programmed cell death 4 (*PDCD4*), a novel metabolism-associated gene, during polycystic ovary syndrome (PCOS) pathogenesis.
Design: Case-control study.
Setting: University hospital.
Patient(s): A total of 77 PCOS patients and 67 healthy women as matched controls.
Intervention(s): *PDCD4* expression in peripheral blood mononuclear cells analyzed by quantitative real-time polymerase chain reaction, and apoptosis of granulosa cells (GCs) detected by flow cytometry, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and small-interfering RNA.
Main Outcome Measure(s): *PDCD4* expression, body mass index (BMI), insulin 0, insulin 120, glucose 120, homeostasis model assessment for insulin resistance (HOMA-IR), homeostasis model assessment for β-cell function (HOMA-β), triglycerides, high-density lipoprotein (HDL), and GC apoptosis.
Result(s): The PCOS patients had higher *PDCD4* expression, but BMI was similar as matched with the obese group, which positively correlated with BMI, insulin 0, insulin 120, glucose 120, HOMA-IR, HOMA-β, triglycerides and negatively correlated with HDL ($P<.05$). After metformin treatment, *PDCD4* expression was distinctly down-regulated for the obese women with PCOS with insulin resistance. Compared with the healthy controls, the apoptosis percentage of GCs was higher in the PCOS group and was decreased by knocking down *PDCD4*. Furthermore, expression of proapotosis factor Bax and the Bax/Bcl-2 ratio were lower, whereas the expression of antiapoptosis factor Bcl-2 was increased. In a multivariate logistic regression analysis, the level of *PDCD4* expression independently related to the odds of PCOS risk after controlling for estradiol and insulin 120 (odds ratio 1.318).

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Conclusion(s): Our study suggests for the first time that higher *PDCD4* expression might play an important role in PCOS pathogenesis by affecting obesity, insulin resistance, lipid metabolism disorders, and GC apoptosis. (Fertil Steril® 2016;105:1330–7. ©2016 by American Society for Reproductive Medicine.)
Key Words: Apoptosis, granulosa cells, insulin resistance, lipid metabolism, obesity, PDCD4 expression, polycystic ovary syndrome

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Polycystic ovary syndrome (PCOS) is one of the most common female endocrine disorders, affecting 5% to 10% of women of reproductive age (1, 2). The syndrome is a complex ovarian dysfunction involving multiple organs and systems. Most cardinal clinical features of the syndrome are polycystic ovary morphology and hyperandrogenism (3). Its clinical manifestations include menstrual irregularities (oligomenorrhea and amenorrhea), signs of androgen excess (hirsutism, acne, and androgenic alopecia), and ovulation disorders (oligoovulation or anovulation) (4). It has been reported that the ovarian granulosa cells (GC) of PCOS patients are relatively easily apoptotic (5). In addition, these manifestations are accompanied by characteristic metabolic disturbances, including abdominal adiposity, insulin resistance, glucose intolerance, and dyslipidemia (6).

Many factors are believed to play a critical role in pathogenesis and increasing risk of PCOS, particularly genetic background, environmental factors, and lifestyle (7). However, the mechanisms underlying these associations have yet to be fully investigated, especially the origins of the metabolic or apoptotic alterations and new biomarkers for PCOS.

Programmed cell death 4 (*PDCD4*) was initially identified as an apoptosis-related gene expressed ubiquitously in human normal tissues with highest levels in the liver (8–10). There is evidence that *PDCD4* could regulate gene expression by influencing the transcription as well as the translation of different signal transduction pathways. It has been shown that *PDCD4* expression could inhibit the formation of the translation initiation complex by binding with eukaryotic translation initiation factor 4A (eIF4A), in which the MA-3 domain competes with eIF4G to interact with eIF4A, sufficiently inhibiting translation initiation (11–13). More research has shown that *PDCD4* plays an increasingly crucial role in some metabolic diseases. *PDCD4*-deficient mice are resistant to metabolic diseases such as autoimmune diabetes. *PDCD4*-deficient mice fed a high-fat diet display an absolutely lean phenotype through improving insulin sensitivity, with higher energy expenditure, lower epididymal fat weight, and reduced macrophage infiltration and inflammatory cytokine secretion in white adipose tissue (14). These data demonstrate that *PDCD4* expression might serve as a potential treatment target for obesity-associated diseases.

Moreover, *PDCD4* expression plays an important role during embryogenesis. Ovulated eggs are destined to undergo cell death if they are not fertilized. *PDCD4* levels are abundant in oocytes, and its expression seems to be induced at the eight-cell stage in mouse embryos (15). These data point to obesity and proapoptosis effects of *PDCD4* expression under certain pathologic conditions.

However, the role of *PDCD4* expression in PCOS as related to obesity and apoptosis has yet to be established. Our study analyzed the expression of *PDCD4* in PCOS patients and normal healthy women to explore further its effect on the development of GC apoptosis and to examine the possible significance of *PDCD4* expression in PCOS pathogenesis.

MATERIALS AND METHODS

Clinical Samples

A total of 144 Chinese Han women were enrolled via the Center for Reproductive Medicine, Shandong Provincial Hospital Affiliated to Shandong University. The participants included 77 PCOS patients and 67 healthy women as the case and control groups, with the definition of PCOS following the 2001 criteria of the revised Rotterdam European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (1). Written informed consent was obtained from all women before participation, and the Center for Reproductive Medicine institutional review board completely approved the study protocol.

The PCOS participants needed to present at least two of the following criteria: oligoovulation and/or anovulation, clinical or biochemical hyperandrogenism, and polycystic ovarian morphology as demonstrated through ultrasonography after exclusion of other etiologies (e.g., congenital adrenal hyperplasia, Cushing's syndrome, and androgen-secreting tumors). All the fertile women controls had normal androgen levels and regular menstrual cycles; signs of polycystic ovaries were excluded by transvaginal ultrasound examination. The physical examinations we performed included weight, waist circumference, hip circumference, and modified Ferriman-Gallwey score (mFG). A body mass index (BMI) ≥ 25 kg/m^2 was identified as obese. Plasma thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), estradiol (E^2), testosterone (T), and luteinizing hormone (LH) concentrations were measured by a chemiluminescent method. Blood samples were collected in tubes containing EDTA as an anticlotting factor and were used for further experiment.

Isolation of Granulosa Cells

Granulosa cells (GCs) were isolated from 23 women with PCOS undergoing in vitro fertilization and embryo transfer (IVF-ET) and 30 women enrolled in the control group who were being

treated for tubal or male factor infertility. Volunteers were excluded for a history of other gynecologic or medical disorders. All women were injected with a gonadotropin-releasing hormone (GnRH) agonist at the beginning of midluteal phase, and an ultrasound scan and serum estradiol assays were performed for monitoring follicular size. When there were three or more follicles with mean diameter $\geq$1.8 cm, 8000–10,000 IU human chorionic gonadotropin (Profasi; Serono) was administered 36 hours before ultrasound-guided immature oocyte retrieval. After anesthetization, the oocyte retrieval was performed through a 17-gauge double-lumen aspiration needle (K-OPS-WOOD-1235; Cook Australia). The GCs around oocytes were collected and washed twice with Dulbecco's modified Eagle medium for further study after removal of the oocyte in follicular aspirates using a Pasteur pipette. Red blood cells were removed using lysis buffer.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted from isolated peripheral blood mononuclear cells (using TRIzol reagent; Invitrogen). The purity of the RNA was measured based on the absorbance at 260 nm. Total RNAs (2 μg) were reversely transcribed to cDNA through the Reverse-Transcribe Kit (Promega). We performed cDNA amplification using SYBR Selected Master Mix (Applied Biosystems) and specific primer pairs. The level of mRNA for *PDCD4* relative to β-actin was calculated using the $\Delta\Delta$Ct method. The sequences of sense and antisense primers were as follows: PDCD4: 5′-GGG AGT GAC GCC CTT AGA AG-3′ and 5′-ACC TTT CTT TGG TAG TCC CCT T-3′; β-actin: 5′-CAT GTA CGT TGC TAT CCA GGC-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT-3′. The samples were denatured at 95°C for 5 minutes, which was then followed by 40 cycles of 95°C for 30 seconds, 60°C for 15 seconds, and 72°C for 15 seconds to terminate the reaction. All samples were normalized to β-actin.

Western Blot Assay

Cells were lysed in sodium dodecyl sulfate buffer, and the concentration of protein was determined using the BCA Protein Assay (Beyotime Biotechnology). Equal amounts of protein were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. Membranes were then blocked with 5% skim milk in Tris-buffered saline and Tween 20 containing 0.1% Tween-20 for 1 hour, and then filters were incubated at 4°C overnight with rabbit monoclonal antibodies against Bax and Bcl-2 (Cell Signaling Technology) and rabbit anti-β-actin polyclonal antibody (Santa Cruz Biotechnology), respectively. Immunoblotting was performed by incubating the membranes followed by secondary antibody (goat anti rabbit IgG) conjugated with peroxidase for 1 hour at room temperature. After washing, the signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). Western blot analysis was performed at least three times for each sample.

Small Interfering RNA Transfection

To knock down endogenous *PDCD4* expression, small-interfering RNA (siRNA) interference was performed as described elsewhere (16). The GCs were transfected using siPORT NeoFX Transfection Agent (AM4510; Ambion) according to the manufacturer's protocol: for siRNA-*PDCD4*: 5′-GGC UGG AAU AAU UUC CAA ATT-3′ and 5′-UUU GGA AAU UAU UCC AGC CTT-3′. The siRNA interference was performed using Lipofectamine 2000 according to the manufacturer's directions. Negative control siRNA (GenePharma) was used as the transfection control.

Flow Cytometry Analysis

Ovarian GCs were stained with the annexin V-fluorescein isothiocyanate apoptosis detection kit (556547; BD Pharmingen) and/or propidium iodide (BD Biosciences) according to the manufacturer's protocols. We first diluted 10 $\times$ annexin V binding buffer to 1 $\times$ buffer by phosphate-buffered saline. Then, 10^5 cells were added to 100 μL 1 $\times$ buffer, 5 μL fluorescein isothiocyanate, and 5 μL propidium iodide in turn. After incubation for 15 minutes at room temperature in a dark place, 400 μL of binding buffer was added. The samples were analyzed immediately using BD FACS Calibur (Becton Dickinson).

Statistical Analysis

All calculations were performed using the SPSS statistical software package (IBM). Analysis of variance or Student's *t* test was performed to determine statistical significance. Spearman's correlation and multivariate regression analyses were used to examine the association between *PDCD4* expression and clinical characteristics. All statistical analyses were two-sided, and the values are presented as mean $\pm$ standard error of the mean (SEM). $P<.05$ was considered statistically significant. Statistical significance was evaluated with data from at least three independent experiments.

RESULTS

Patient Characteristics

The analysis of clinical and biochemical profiles in women with PCOS and normal controls is shown in Table 1. Patients with PCOS had greater E_2, total testosterone, LH, insulin 0, insulin 120, and HOMA-IR than the controls ($P<.05$). No statistically significant difference was found in comparing age, BMI, TSH, glucose 0, glucose 120, HOMA-β, triglycerides, or HDL between the women with PCOS women and the controls ($P>.05$).

PDCD4 Expression in Peripheral Blood Mononuclear Cells of Controls and Women with PCOS

As shown in Figure 1A and Table 1, the *PDCD4* levels were statistically significantly higher in the women with PCOS compared with the controls by quantitative real-time polymerase chain reaction in peripheral blood mononuclear cells

TABLE 1

Clinical and biochemical profiles in women with polycystic ovary syndrome and in normal controls.

Characteristic	PCOS (n = 77)	Controls (n = 67)	*P* value
Age (y)	27.65 ± 3.24	28.55 ± 4.15	NS
BMI (kg/m^2)	26.05 ± 4.81	25.87 ± 4.61	NS
TSH (μIU/mL)	2.72 ± 1.45	2.37 ± 1.40	NS
FSH levels (mIU/mL)	5.88 ± 1.55	6.86 ± 2.28	.003[a]
E_2 (pg/mL)	54.91 ± 47.06	33.01 ± 17.17	.000[b]
Total T (ng/mL)	44.76 ± 19.62	25.94 ± 11.31	.000[b]
LH levels (mIU/mL)	10.32 ± 6.79	4.93 ± 2.77	.000[b]
Insulin 0 (mU/L)	20.18 ± 11.94	16.12 ± 9.66	.028[c]
Insulin 120 (mU/L)	91.64 ± 84.46	60.46 ± 41.66	.007[a]
Glucose 0 (mmol/L)	5.79 ± 1.20	5.46 ± 0.75	NS
Glucose 120 (mmol/L)	6.57 ± 2.51	6.12 ± 1.63	NS
HOMA-IR (mM, mU/L)	5.37 ± 3.89	4.10 ± 2.62	.025[c]
HOMA-β (mU/L, mM)	184.12 ± 107.28	156.22 ± 94.78	NS
Triglycerides (mmol/L)	1.39 ± 0.81	1.44 ± 2.01	NS
HDL (mmol/L)	1.20 ± 0.26	1.27 ± 0.29	NS
PDCD4/β-actin	2.47 ± 1.89	1.87 ± 1.53	.039[c]

Note: Numeric data are presented as mean ± standard deviation. BMI = body mass index; E_2 = estradiol; FSH = follicle-stimulating hormone; HDL = high-density lipoprotein; HOMA-β = homeostasis model assessment for β-cell function; HOMA-IR = homeostasis model assessment for insulin resistance; LH = luteinizing hormone; NS = not statistically significant; PCOS = polycystic ovary syndrome; T = testosterone; TSH = thyroid-stimulating hormone.

[a] $P<.01$.
[b] $P<.001$.
[c] $P<.05$.

Ding. PDCD4 and polycystic ovary syndrome. Fertil Steril 2016.

($P<.05$). According to their BMI, the women with PCOS and the controls could be further divided into obese and lean subgroups. After this classification, the *PDCD4* levels in the obese PCOS patients were statistically statistically different from those in the BMI-matched controls ($P<.01$; Fig. 1B and Table 2).

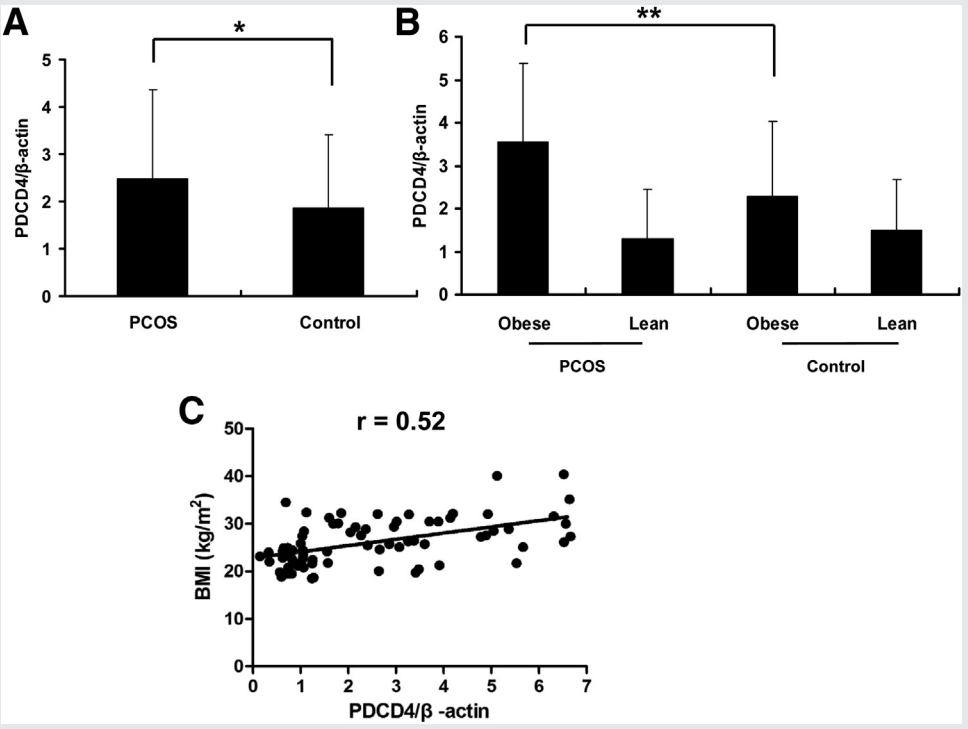
Relationship between *PDCD4* Expression and Obesity in PCOS Patients and Controls

To explore the significance of increased *PDCD4* levels in PCOS, we first analyzed the relationship between *PDCD4* expression and obesity. The results revealed no statistically significant correlation between *PDCD4* expression and BMI in the control group. However, *PDCD4* expression statistically significantly correlated with BMI ($r = 0.52$, $P<.0001$) in the PCOS group (Fig. 1C), which indicates that *PDCD4* expression is markedly associated with the progression of obesity in the PCOS patients.

Association between *PDCD4* Expression and Insulin Resistance and β-cell Function in Women with PCOS and Controls

It was known that HOMA-IR and HOMA-β were respectively most important indexes of homeostasis model assessment for

FIGURE 1



PDCD4 expression in peripheral blood mononuclear cells (PBMCs) of controls and women with PCOS detected by quantitative real-time polymerase chain reaction (qRT-PCR). (**A**) Expression levels of PDCD4 mRNA in 77 PCOS patients and 67 healthy women detected by qRT-PCR. Data were normalized to β-actin. (**B**) Normalized PDCD4 mRNA levels in different obese and lean subgroups. The difference was statistically significant (*$P<.05$, **$P<.01$). (**C**) Relationship between *PDCD4* expression and obesity in PCOS patients. *PDCD4* expression had a strong positive correlation with body mass index ($r = 0.52$, $P<.0001$).

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TABLE 2

Comparison of *PDCD4* expression and clinical features between women with polycystic ovary syndrome and body mass index–matched controls.

	PCOS (n = 77)		Controls (n = 67)		*P* value	
Characteristic	**Obese (n = 40)**	**Lean (n = 37)**	**Obese (n = 32)**	**Lean (n = 35)**	P_1	P_2
Age (y)	28.60 ± 3.33	26.62 ± 2.82	29.90 ± 4.20	27.28 ± 3.74	NS	NS
BMI (kg/m^2)	29.73 ± 3.57	22.07 ± 1.95	29.26 ± 2.60	21.24 ± 1.59	NS	NS
TSH (μIU/mL)	2.68 ± 1.49	2.76 ± 1.43	2.67 ± 1.63	2.01 ± 1.00	NS	.013[a]
FSH levels (mIU/mL)	5.87 ± 1.75	5.89 ± 1.32	7.01 ± 2.61	6.69 ± 1.85	.030[a]	.038[a]
E_2 (pg/mL)	43.79 ± 20.72	67.38 ± 63.16	31.63 ± 16.25	34.69 ± 18.48	.008[b]	.004[b]
Total T (ng/mL)	44.96 ± 17.50	44.54 ± 21.90	24.31 ± 10.52	27.95 ± 12.15	.000[c]	.000[c]
LH levels (mIU/mL)	8.16 ± 5.02	12.74 ± 7.73	4.73 ± 3.12	5.17 ± 2.31	.001[b]	.000[c]
Insulin 0 (mU/L)	26.35 ± 12.51	13.50 ± 6.56	18.69 ± 10.93	12.27 ± 5.70	.008[b]	NS
Insulin 120 (mU/L)	123.65 ± 98.24	56.96 ± 47.19	57.97 ± 41.37	64.21 ± 42.87	.001[b]	NS
Glucose 0 (mmol/L)	6.19 ± 1.41	5.34 ± 0.69	5.76 ± 0.72	5.18 ± 0.67	NS	NS
Glucose 120 (mmol/L)	7.49 ± 2.91	5.53 ± 1.41	6.13 ± 1.59	6.12 ± 1.74	.020[a]	NS
HOMA-IR (mM, mU/L)	7.15 ± 4.42	3.23 ± 1.65	4.50 ± 3.04	3.07 ± 1.71	.005[b]	NS
HOMA-β (mU/L, mM)	217.98 ± 115.72	136.55 ± 97.16	165.65 ± 114.77	125.50 ± 55.70	NS	NS
Triglycerides (mmol/L)	1.74 ± 0.92	1.02 ± 0.43	1.98 ± 2.70	0.90 ± 0.58	NS	NS
HDL (mmol/L)	1.09 ± 0.21	1.32 ± 0.26	1.17 ± 0.26	1.38 ± 0.29	NS	NS
PDCD4/β-actin	3.55 ± 1.82	1.31 ± 1.14	2.28 ± 1.76	1.49 ± 1.20	.004[b]	NS

Note: Numeric data are presented as mean ± standard deviation. P_1 is obese women with PCOS versus obese controls; P_2 is lean women with PCOS versus lean controls. BMI = body mass index; E_2 = estradiol; FSH = follicle-stimulating hormone; HDL = high-density lipoprotein; HOMA-β = homeostasis model assessment for β-cell function; HOMA-IR = homeostasis model assessment for insulin resistance; LH = luteinizing hormone; NS = not statistically significant; PCOS = polycystic ovary syndrome; T = testosterone; TSH = thyroid-stimulating hormone.

[a] $P<.05$.
[b] $P<.01$.
[c] $P<.001$.

Ding. PDCD4 and polycystic ovary syndrome. Fertil Steril 2016.

insulin resistance and β-cell function (17–19). The women with PCOS had statistically significantly higher insulin 0, insulin 120, and HOMA-IR compared with the controls ($P<.01$ or .05; Table 1). The results were similar with these indices with the addition of glucose 120 between the BMI-matched obese women with PCOS and the controls ($P<.01$ or .05; Table 2). There were no relation between *PDCD4* expression and these indexes in the control group. However, *PDCD4* expression positively correlated with insulin 0, insulin 120, glucose 120, HOMA-IR, and HOMA-β (r = 0.34, 0.31, 0.29, 0.36 and 0.25, separately; $P<.01$ or 0.05; Fig. 2A–2E) in the PCOS patients. It is interesting that after treatment with metformin, 500 mg twice daily for 2 or 3 months, *PDCD4* expression was distinctly down-regulated for obese women with PCOS and insulin resistance ($P<.05$; Fig. 2H). This suggests that *PDCD4* levels correlate with the insulin resistance or β-cell dysfunction of PCOS patients, which could be improved through oral metformin for decreasing the expression.

Correlation between *PDCD4* Levels and Lipid Metabolic Parameters

To determine effect of *PDCD4* expression on lipid metabolism for PCOS patients, we further analyzed the relationship between *PDCD4* expression and lipid metabolic parameters. Though there were no statistically statistical differences between the women with PCOS and the controls for triglycerides and HDL, *PDCD4* expression positively correlated with triglycerides (r = 0.39; $P<.001$; Fig. 2F) and negatively correlated with HDL (r = −0.41; $P<.001$; Fig. 2G) in the PCOS patients. These results indicate that *PDCD4* expression might greatly promote lipid accumulation in women with PCOS and might lead to lipid metabolic dysfunction.

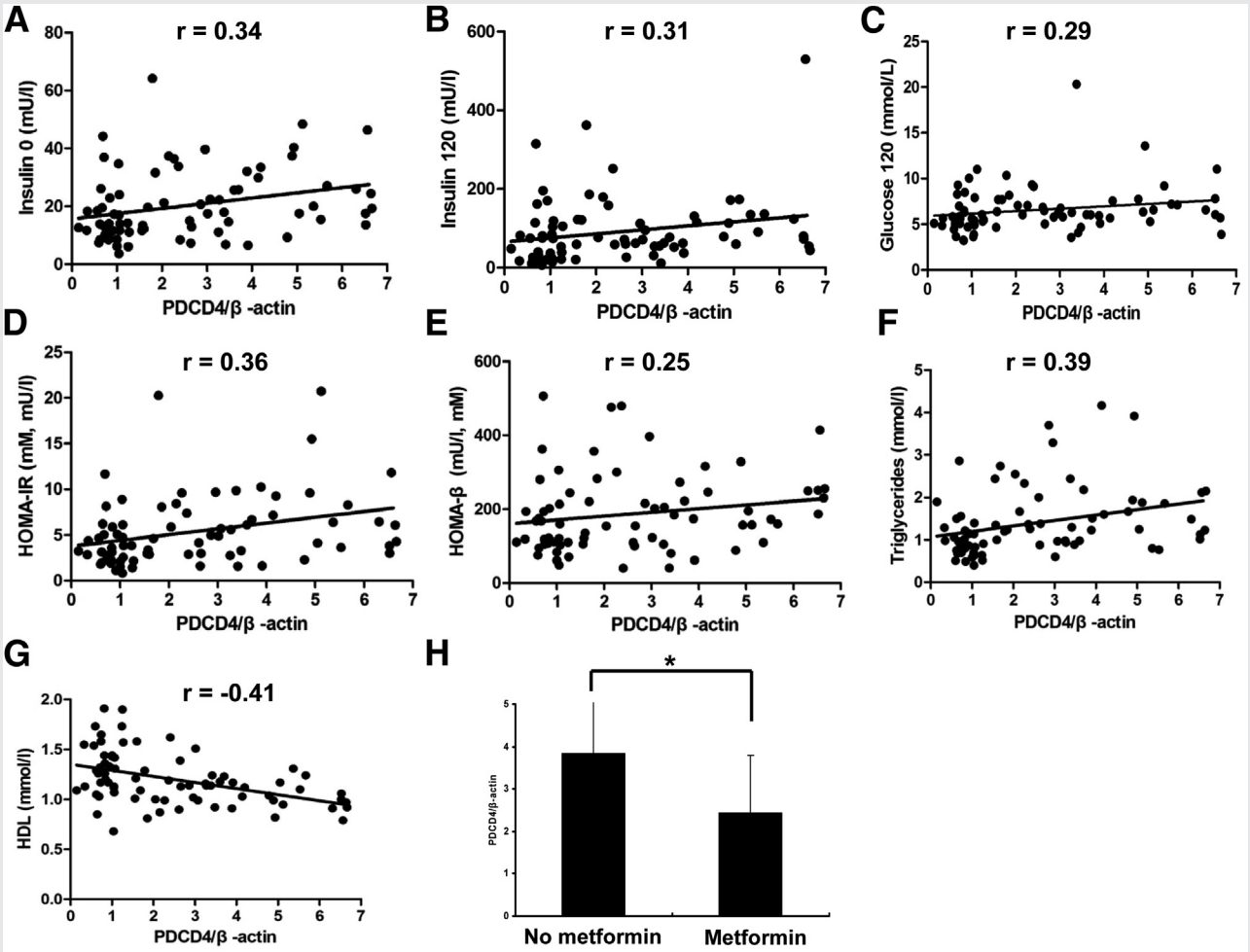
Apoptosis Suppression of GCs in Women with PCOS by Knocking Down *PDCD4*

It had been recognized that *PDCD4* expression could inhibit protein translation by binding with eIF4A and result in cell apoptosis. We found that there were more easily apoptotic cells among the ovarian GCs of PCOS patients. As shown in Supplemental Figure 1 (available online), flow cytometry analysis showed that the percentage of apoptotic GCs in the PCOS group was much higher than that of the controls ($P<.05$).

To confirm the important role of *PDCD4* expression in GCs apoptosis, we examined the effect of knockdown of *PDCD4* expression on GCs apoptosis via *PDCD4*-specific siRNA sequences. Compared with the negative control, the apoptosis percentage was statistically significantly diminished in the *PDCD4*-knockdown group through Annexin V-fluorescein isothiocyanate/propidium iodide analysis ($P<.05$; Supplemental Fig. 2A, available online). In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis further revealed that silencing of *PDCD4* expression statistically significantly decreased the number of apoptotic cells after 24 hours of siRNA transfection (see Supplemental Fig. 2B).

To determine whether silencing of *PDCD4* expression impacted the signal pathway of apoptosis, we detected both expression of proapotosis factor Bax and antiapoptosis factor Bcl-2. Western blot analysis showed that Bax expression was decreased, Bcl-2 expression was increased, and the Bax/Bcl-2 ratio was lower by inhibition of *PDCD4* expression (see Supplemental Fig. 2C).

FIGURE 2



Association between *PDCD4* expression and insulin resistance, β-cell function, and lipid metabolic parameters in women with PCOS. In PCOS patients, *PDCD4* expression positively correlated with (**A**) insulin 0, (**B**) insulin 120, (**C**) glucose 120, (**D**) HOMA-IR, and (**E**) HOMA-β (r = 0.34, 0.31, 0.29, 0.36, and 0.25, respectively; $P<.01$ or .05). Also in PCOS patients, *PDCD4* expression (**F**) positively correlated with triglycerides (r = 0.39; $P<.001$) and (**G**) negatively correlated with HDL (r = −0.41; $P<.001$). Normalized PDCD4 mRNA levels of obese women with PCOS and insulin resistance were analyzed after metformin, 500 mg twice daily for 2 or 3 months. The difference was statistically significant (*$P<.05$). (**H**) Contrast of *PDCD4* expression in a total 11 cases after metformin treatment.

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PDCD4 Expression Independently Related to the Odds of PCOS Risk

To examine whether *PDCD4* expression was an independent risk factor for PCOS patients, we performed multivariate logistic regression analysis to further explore the association between *PDCD4* expression and PCOS risk. Our model included potential confounders such as age, BMI, hormone concentrations, insulin resistance, and lipid metabolic indexes. The level of *PDCD4* expression could statistically significantly predict the risk of PCOS patients independent of other clinicopathologic variables after adjusting for E_2 and insulin 120 (odds ratio 1.318; 95% confidence interval, 1.021–1.703; $P<.05$) as shown in Supplemental Table 1 (available online). In our hospital, among the many PCOS patients those who were obese or nonobese had very high baseline estrogen levels within the normal range as shown in Table 2; this has also been discussed by other researchers (3). Therefore, the E_2 levels were further adjusted for exploring the risk association between *PDCD4* expression and PCOS.

DISCUSSION

In the present study, our results showed that higher *PDCD4* expression existed in PCOS patients and was associated with obesity, insulin resistance, lipid metabolism disorders, and apoptosis of ovarian GCs. By multivariate logistic regression analysis, we further concluded that *PDCD4* expression might serve as an independent risk factor in patients with PCOS. To our knowledge, this is the first study on the association of *PDCD4* expression and PCOS risk.

Obesity was highly frequent in the women with PCOS, particularly the abdominal phenotype, which could be detected in early stages of PCOS and was undoubtedly a useful clinical predictor of metabolic abnormalities (20). The prevention and treatment of obesity is extremely important for the management of PCOS (21–23). One study has shown that *PDCD4* deficiency remarkably inhibits adiposity and improves metabolic homeostasis (14). Consequently, we analyzed the association of *PDCD4* expression in peripheral blood mononuclear cells between the health controls and the women with PCOS; we found that the PCOS patients had higher *PDCD4* expression similar to the BMI-matched obese group, which positively correlated with BMI. In addition, we also discovered that *PDCD4* expression was statistically significantly increased in the obese subgroup compared with the lean subgroup in both the PCOS and control groups. However, the interclass analysis indicated that there was greater difference in the PCOS group ($P<.0001$) than the controls ($P<.05$), as shown in Figure 1B (data not shown). *PDCD4* expression may intensify the development of PCOS, though obesity was found in both groups. This further demonstrates that higher *PDCD4* expression is associated with obesity progression in PCOS patients.

Insulin resistance was prevalent in the women with PCOS and played a critical pathophysiologic role in both metabolic and reproductive complications. Both PCOS and obesity synergistically acted to impair insulin sensitivity, which strongly correlated with reproductive failure (24–29). We also found that the PCOS patients had higher insulin 0, insulin 120, and HOMA-IR. The BMI-matched obese group was statistically significantly different for these indexes as well as glucose 120.

The expression of *PDCD4* distinctly improved the insulin sensitivity of *PDCD4* gene knockout mice fed a high-fat diet (14), so we examined the relation between *PDCD4* expression and insulin resistance in women with PCOS. We found that *PDCD4* expression positively correlated with many related insulin resistance indexes in PCOS patients. These results indicate that higher *PDCD4* expression induces insulin resistance and β-cell dysfunction. Metformin is the most widely used insulin sensitizer drug to treat women with PCOS and insulin resistance (30, 31). Unexpectedly, after 2 to 3 months of metformin treatment, *PDCD4* expression was distinctly down-regulated in the obese PCOS women with insulin resistance, suggesting that metformin can restrain *PDCD4* expression to up-regulate insulin sensitivity. The possible mechanisms of metformin's effect on *PDCD4* expression remain to be further clarified.

Dyslipidemia, the most prevalent metabolic aberration in PCOS, presents in 70% of patients and is often represented by hypertriglyceridemia and lower HDL cholesterol levels (32–36). Metabolic abnormalities including dyslipidemia also seem to be transmitted to first-degree relatives of women with PCOS and further deterioration may occur with aging and obesity (37, 38). We found no statistically significant relation between the women with PCOS and the controls for the lipid metabolism parameters. Nevertheless, we observed that *PDCD4* expression positively correlated with triglycerides and negatively correlated with HDL. We speculate that in women with PCOS the elevated *PDCD4* expression contributes to dyslipidemia. Deficiency of *PDCD4* obviously improves lipid metabolic homeostasis in mice fed a high-fat diet (14). It is worth noting that *PDCD4* expression may influence target genes of the metabolism pathway to accelerate lipid accumulation, so this aspect needs further investigation in PCOS.

PDCD4 has been identified as a gene that is up-regulated during apoptosis and that plays an essential role in many biological processes of various cell lines, including embryonic development (15, 39). Any disruption/distortion of these processes could result in disease. Therefore, higher *PDCD4* expression might give rise to premature apoptosis in GCs of PCOS. Multiple studies have shown that GC apoptosis is increased in PCOS to induce follicular premature atresia (40), and this was also demonstrated by our results. It is interesting that the percentage was statistically significantly diminished with knockdown of *PDCD4* expression by specific siRNA. These results further reveal that *PDCD4* expression is involved in the premature apoptosis of GCs by means of inhibiting apoptotic pathway molecules.

In summary, our results suggest that up-regulation of *PDCD4* expression might be a key factor in PCOS progression. Therefore, inhibition of *PDCD4* expression might be an effective strategy for the treatment of PCOS. However, the precise mechanism by which *PDCD4* expression affects the pathophysiologic progress of PCOS requires further investigation.

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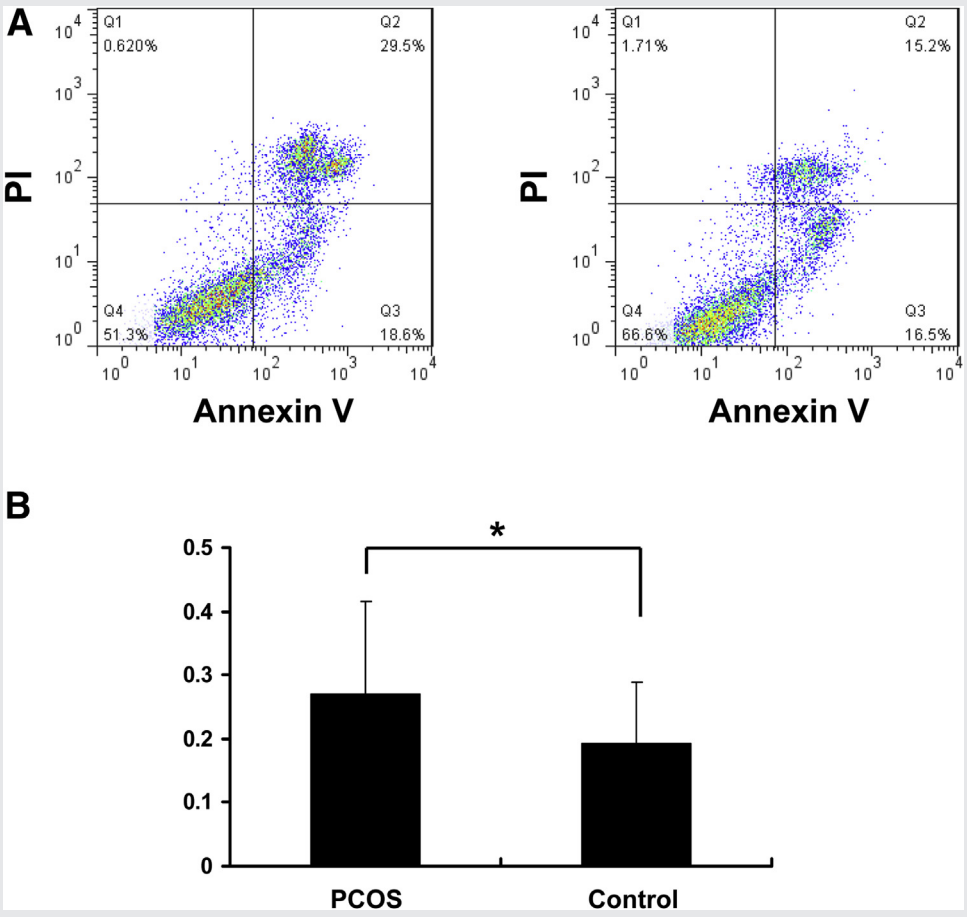
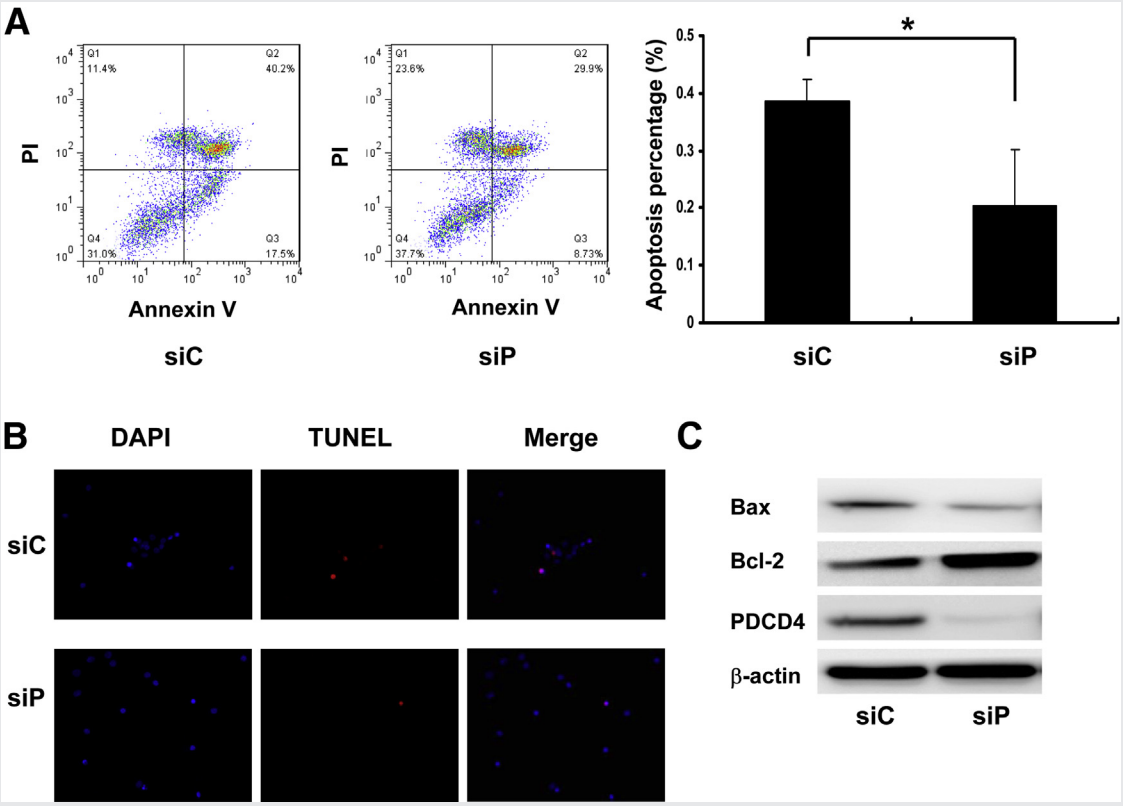
SUPPLEMENTAL FIGURE 1



Apoptosis detection of granulosa cells in PCOS women by flow cytometry analysis. (**A**) Apoptosis rate of annexin V (+) and propidium iodide (+) in one of the PCOS cases (29.5%) was higher when compared with healthy controls (15.2%). (**B**) The percentage of apoptotic granulosa cells in the PCOS group was statistically significantly higher than that of healthy controls (*$P<.05$).

Ding. PDCD4 and polycystic ovary syndrome. Fertil Steril 2016.

SUPPLEMENTAL FIGURE 2



Analysis of apoptosis suppression of granulosa cells (GCs) in women with PCOS by *PDCD4* small-interfering RNA (siRNA). (**A**) Apoptosis rate of annexin V (+) and propidium iodide (+) in one of the PCOS cases (40.2%) (siC) was decreased (29.9%) by knock down of *PDCD4* expression using specific siRNA (siP). The percentage of apoptotic GCs in five PCOS cases was statistically significantly decreased after transfection for 24 hours by *PDCD4* siRNA (*$P<.05$). (**B**) Apoptosis detection of GCs from PCOS patients after siRNA action for 24 hours was analyzed by TUNEL staining (laser confocal imaging, magnification ×20). (**C**) Bax and Bcl-2 expression was detected by Western blot analysis.

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SUPPLEMENTAL TABLE 1

Odds ratios (95% confidence interval) of polycystic ovary syndrome risk, by *PDCD4* expression levels.

Variable	*PDCD4* expression	*P* value
Adjusted OR (95% CI), Model 1	1.318 (1.021–1.703)	.034[a]
Adjusted OR (95% CI), Model 2	1.306 (1.009–1.691)	.043[a]
Adjusted OR (95% CI), Model 3	1.347 (1.014–1.791)	.040[a]

Note: Model 1, adjusted for estradiol, insulin 120; Model 2, adjusted for Model 1, follicle-stimulating hormone; Model 3, adjusted for Model 1, luteinizing hormone. CI = confidence interval; OR = odds ratio; PCOS = polycystic ovary syndrome.

[a] $P < .05$.

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