Abstract. Programmed cell death 4 (PDCD4) has been identified as a tumor-suppressor gene that inhibits neoplastic transformation, tumor progression, and protein translation. It has been reported that multiple factors participate in the regulation of PDCD4 mRNA and protein. The endometrium is regulated by changing concentrations of ovarian hormones, such as estrogen and progesterone, and shows periodical changes. However, whether ovarian hormones regulate PDCD4 expression remains unclear. This study aimed to explore the effect and mechanism of estrogen or progesterone on PDCD4 mRNA and protein expression in human endometrial cancer cells. The expression of PDCD4 mRNA and protein in Ishikawa and HEC-1-A cells was detected by quantitative real-time PCR and western blot analysis. The signaling pathway-related proteins were detected by western blot analysis. The results showed that PDCD4 mRNA levels exhibited no significant changes after treatment with estrogen or progesterone in both Ishikawa and HEC-1-A cell lines. Estrogen also had no obvious effect on PDCD4 protein expression. However, progesterone effectively decreased the expression of PDCD4 protein and the PI3K/AKT pathway may be involved in the downregulation of PDCD4 protein induced by progesterone. These results suggest that the downregulation of PDCD4 induced by progesterone affects the therapeutic efficacy of progesterone in human endometrial cancer or endometriosis, which may have important implications for progesterone treatment in the clinic.

Introduction

Programmed cell death 4 (PDCD4) is an apoptosis-related gene. It is currently identified as a tumor-suppressor gene that inhibits neoplastic transformation (1,2), tumor progression (3) and protein translation (4). PDCD4 is expressed ubiquitously in different normal tissues, especially the liver (5). However, the expression of PDCD4 in various types of cancers, such as liver cancer (6), lung cancer (7), breast cancer (8), is lost or decreased. We also found that the expression of PDCD4 is downregulated in ovarian cancer tissues compared with control ovarian epithelial tissues (9). It has been reported that multiple factors participate in the regulation of PDCD4 mRNA and protein. PDCD4 was first identified as an upregulated gene during apoptosis (5). Different pro-apoptotic substances, including ionomycin, phorbol-12-myristate-13-acetate (PMA) or dexamethasone, were found to induce increased levels of PDCD4 (10), but other inducers of apoptosis such as arabinosyl cytosine (ara-C), UV irradiation or topoisomerase inhibitors had no effect on the expression of PDCD4 (11,12). Similarly, the levels of PDCD4 were found to be induced by interleukin (IL)-12, but decreased by IL-2 and IL-15 treatment (13). These results suggest that the expression of PDCD4 may vary depending on different stimuli (11).

The endometrium is regulated by changing concentrations of ovarian hormones, such as estrogen and progesterone, and shows periodical changes, including proliferative phase, secretory phase and menstrual phase. Estrogen is increased in the proliferative phase of the endometrium; after ovulation progesterone is produced and drives the endometrium into a secretory phase. When the levels of estrogen and progesterone are decreased, the endometrium enters into a
menstrual phase. Therefore, the levels of ovarian hormones during the menstrual cycle are changed. It has been reported that ovarian hormones affect the expression of many genes, such as catalase (14) and IL-10R (15), and further induce gene expression variation during the cycle changes of the endometrium. Ovarian hormones not only regulate gene transcription by directly or indirectly binding to DNA, but also regulate gene expression by binding to corresponding membrane receptors and further initiate various signaling pathways, including mitogen-activated protein kinase/extracellular regulated protein kinase (MAPK/ERK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathways (16). In a previous study, we found that the expression of PDCD4 in the proliferative phase of the endometrium was higher than that in the secretory phase of the endometrium (17). This suggests that the expression of PDCD4 may be regulated by ovarian hormones. However, the effect and mechanism of ovarian hormones on the expression of PDCD4 remain unclear.

In the present study, we aimed to investigate whether PDCD4 expression is regulated by estrogen and progesterone via the MAPK/ERK or PI3K/AKT pathways, and it was confirmed that progesterone could downregulate the expression of PDCD4 protein via PI3K/AKT pathway.

Materials and methods

Primary antibodies. Rabbit polyclonal or monoclonal antibodies specific for PDCD4 (cat. no. cst-95355); MAPK signaling pathway-related molecules: Stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) (cat. no. cst-9252), p44/42MAPK (ERK1/2) (cat. no. cst-4695), p38MAPK (cat. no. cst-8690); PI3K/AKT signaling pathway-related molecules: AKT (cat. no. cst-4691), mammalian target of rapamycin (mTOR) (cat. no. cst-2983), as well as phospho-SAPK/JNK (phosphorylation site: Thr183/Tyr185) (cat. no. cst-4668), phospho-p44/42 MAPK (ERK1/2) (phosphorylation site: Thr202/Tyr204) (cat. no. cst-4370), phospho-p38MAPK (phosphorylation site: Thr180/Tyr182) (cat. no. cst-4511), phospho-AKT (phosphorylation site: Ser473) (cat. no. cst-4060), phospho-mTOR (phosphorylation site: Ser2448) (cat. no. cst-5536) were purchased from Cell Signaling Technology. Mouse monoclonal antibody specific for β-actin (TA-09; ZSGB-Bio, Beijing, China) was used as a loading control.

Cell culture. Well-differentiated human endometrial cancer Ishikawa cells were kindly provided by Qilu Hospital. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-High Glucose medium (Hyclone) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin and streptomycin (100X, MACGENE). Moderately differentiated endometrial cancer HEC-1-A cells were obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum, penicillin and streptomycin. All the cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Hormone treatments. The cells were respectively treated with 0, 0.1, 1, 10, 100 and 1,000 nM of 17β-estradiol (E2) (Sigma-Aldrich; Merck KGaA) or 0, 0.01, 0.1, 1, 10 and 20 µM of progesterone (P4) (Sigma-Aldrich; Merck KGaA). All treated cells were collected to detect the expression of PDCD4 mRNA or protein by quantitative real-time PCR (qPCR) or western blot analysis. The cells were treated with 10 µM of progesterone for 0, 6, 12, 24, 36 and 48 h to determine the time when progesterone begins to reduce the expression of PDCD4.

In order to investigate the signal transduction pathway which was involved in PDCD4 down-regulation induced by progesterone, the cells were firstly treated with 10 µM of progesterone for 0, 1, 2 and 4 h, and the expression of signaling molecules was detected by western blot analysis. In addition, the cells were respectively pretreated with dimethyl sulfoxide (DMSO) and 5 µM (18) of PI3K inhibitor LY294002 (Selleckchem) for 1 h or 100 nM (19) of AKT inhibitor MK2206 (MedChem Express) for 2 h, and then the cells were treated with 10 µM of progesterone for 4 h. All treated cells were collected to detect the expression of PDCD4 protein and signaling molecules.

RNA isolation and qPCR. Total RNA was extracted using RNAfast 200 (Fastagen) according to the manufacturer's protocol, and then reverse transcribed into complementary DNA using Reverse Transcription System (Takara). The expression levels of genes were assessed by qPCR using a 2X UltraSYBR Mixture and specific primers (CWBO) according to the manufacturer's instructions. qPCR was carried out using the Applied Biosystems STEP One Plus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers for PDCD4 were as follows: forward, CTTGGAAGATGTTGAGGATGAT and reverse, AACGGA TTTGGTCGTATTTGGA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for an internal control to analyze PDCD4 expression. The primers for GAPDH were as follows: Forward: ACAGGTGTATGTTGAGGA and reverse, TTCTCAAATGGCCTCTATCCAA. The qPCR protocol was as follows: Pre-denaturation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 1 min. The expression of PDCD4 was analyzed according to the comparative quantitative method (2^ΔΔCq) and the samples were examined in triplicate.

Total protein extraction and western blot analysis. The cells were lysed using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime) with protease inhibitor and phosphatase inhibitor (Bimake). After homogenization, the cells were centrifuged at 13,700 x g at 4°C for 30 min. The supernatant was quantified using the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Equal amounts of proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel, and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). After being blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in Tris-buffered saline Tween-20 (TBST) for 2-3 h. The membranes were respectively incubated overnight at 4°C with a 1:1,000 dilution of primary antibodies. Next day, the membranes were incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research) at room
temperature for 1 h. The signal was detected using the enhanced chemiluminescence kit (Millipore). β-actin was used as an internal reference for normalization of PDCD4. Total protein of mTOR, AKT, ERK1/2, P38, JNK1/2 were used as loading control for normalization of their phosphorylated protein levels.

Statistical analysis. Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad Software, Inc.). All data are presented as means ± SD. A Dunnett’s post hoc test after one way ANOVA was used to compared different test groups with one control group. The Student’s t-test was performed to evaluate the statistical significance between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of estrogen on the expression of PDCD4 in human endometrial cancer cell lines. After Ishikawa and HEC-1-A cells were treated with different concentrations of 17β-estradiol (E2) for 24 h, qPCR was used to detect the expression of PDCD4 mRNA (Fig. 1A and B). After the above two cell lines were treated with different concentrations of 17β-estradiol (E2) for 48 h, western blot analysis was performed to detect PDCD4 protein expression (Fig. 1C-F). The results showed that for any concentration of 17β-estradiol no significant difference on the expression of PDCD4 mRNA and protein in the Ishikawa and HEC-1-A cells was observed (P>0.05) (Fig. 1).

Figure 1. Effect of estrogen on the expression of PDCD4 in human endometrial cancer cell lines. The results of qPCR showed that 17β-estradiol (E2) had no effect on the expression of PDCD4 mRNA in (A) Ishikawa (P>0.05) and (B) HEC-1-A cells (P>0.05). (C and D) The level of PDCD4 protein was detected by western blot analysis after treatment with 17β-estradiol (E2) in Ishikawa cells and no significant difference in the expression of PDCD4 protein was noted (P>0.05). (E and F) The level of PDCD4 protein was detected by western blot analysis after treated with 17β-estradiol (E2) in HEC-1-A cells and no significant difference in the expression of PDCD4 protein was noted (P>0.05). PDCD4, programmed cell death 4.

Effect of progesterone on the expression of PDCD4 in human endometrial cancer cell lines. The effect of different concentrations of progesterone on PDCD4 mRNA expression was determined in Ishikawa and HEC-1-A cells. The results showed that at any concentration of progesterone no significant effect on the expression of PDCD4 mRNA was observed in the Ishikawa and HEC-1-A cells (Fig. 2A and B). Furthermore, an experiment of the time kinetic investigation on the PDCD4 mRNA level in Ishikawa and HEC-1-A cells was conducted. The results confirmed that progesterone failed to regulate the expression of
**PDCD4** at the mRNA level (data not shown). Then, the effect of different concentrations of progesterone on PDCD4 protein expression was investigated in Ishikawa and HEC-1-A cells. The results showed that different concentrations (0.01, 0.1, 1, 10 and 20 µM) of progesterone obviously downregulated the expression of PDCD4 protein in Ishikawa cells (P<0.0001 vs. 0 µM) (Fig. 2C and D); however, only 10 and 20 µM of progesterone effectively reduced the expression of PDCD4 protein in HEC-1-A cells (P<0.05 vs. 0 µM) (Fig. 2E and F). Next, we detected the effect of progesterone (10 µM) on the PDCD4 protein expression at different time points in Ishikawa and HEC-1-A cells. It was found that PDCD4 protein began to decrease after progesterone treatment for 6 h in Ishikawa cells (P<0.01 vs. 0 h) (Fig. 3A and B). In HEC-1-A cells, the expression of PDCD4 protein started to decrease after progesterone treatment for 12 h (P<0.05 vs. 0 h) (Fig. 3C and D).

**Progesterone activates the PI3K/AKT/mTOR pathway but not the MAPK pathway.** We further determined whether the PI3K/AKT/mTOR pathway participates in the effect of progesterone on PDCD4 protein expression using 10 µM of progesterone to treat Ishikawa and HEC-1-A cells for 0, 1, 2 and 4 h. It was found that the expression of p-mTOR (P<0.05 vs. 0 h) and p-AKT (P<0.01 vs. 0 h) was significantly higher after treatment with progesterone (P4) for 4 h (Fig. 4A-C). In order to investigate whether the MAPK signaling pathway participates in the downregulation of PDCD4 protein expression induced by progesterone (P4), the expression of p-ERK1/2, p-P38 and p-JNK was detected. It was found that the levels of p-ERK1/2, p-P38 and p-JNK had no significant changes following progesterone (P4) treatment (P>0.05) (Fig. 4D-F).

**The PI3K/AKT pathway participates in the reduction of PDCD4 protein expression induced by progesterone.** In order to confirm that progesterone (P4) downregulates the expression of PDCD4 protein via the PI3K/AKT pathway in Ishikawa and HEC-1-A cells, we used a PI3K inhibitor (LY294002) to pretreat Ishikawa and HEC-1-A cells for 1 h (Fig. 5A-C) or
Figure 3. Effect of progesterone on the expression of PDCD4 protein at different time points. (A) The expression of PDCD4 protein was detected by western blot analysis in Ishikawa cells after treatment with 10 µM of progesterone at different time points (0, 6, 12, 24, 36 and 48 h). (B) The expression of PDCD4 protein began to decrease after progesterone treatment for 6 h in Ishikawa cells (*P<0.05 vs. 0 h; **P<0.01 vs. 0 h; ***P<0.01 vs. 0 h). (C) The expression of PDCD4 protein was detected by western blot analysis in HEC-1-A cells after treatment with 10 µM of progesterone at different time points (0, 6, 12, 24, 36 and 48 h). (D) The expression of PDCD4 protein started to decrease after progesterone treatment for 12 h in HEC-1-A cells (*P<0.05 vs. 0 h; **P<0.01 vs. 0 h; ns, not significant). PDCD4, programmed cell death 4.

Figure 4. Effect of progesterone on the expression of signaling molecules in the PI3K/AKT/mTOR and MAPK pathways. (A) The expression of phosphorylated (p)-mTOR, mTOR, p-AKT, AKT and β-actin was detected by western blot analysis after treatment with 10 µM of progesterone (P4) at different time points (0, 1, 2 and 4 h). Quantitative analysis of the ratios of p-mTOR/mTOR and p-AKT/AKT by measuring the relative band density in (B) Ishikawa and (C) HEC-1-A cells. (D) The expression of p-ERK, ERK, p-P38, P38, p-JNK, JNK and β-actin was detected by western blot analysis after treatment with 10 µM of progesterone (P4) at different time points (0, 1, 2 and 4 h). (E) Quantitative analysis of the ratios of p-ERK/ERK, p-P38/P38 and p-JNK/JNK by measuring the relative band density in (E) Ishikawa and (F) HEC-1-A cells. *P<0.05 vs. 0 h; **P<0.01 vs. 0 h.
AKT inhibitor (MK2206) to pretreat Ishikawa and HEC-1-A cells for 2 h (Fig. 5D-F), respectively, and then progesterone (P4) was administered. The results showed that the expression of p-AKT was significantly lower (P<0.05) and the expression of PDCD4 was significantly increased (P<0.05) in both cell lines when compared with P4 treatment alone, while the expression of p-mTOR exhibited no obvious changes (P>0.05) in Ishikawa and HEC-1-A cells (Fig. 5).

**Discussion**

PDCD4, as a tumor suppressor, regulates the expression of various genes related to tumor development and progression at the transcriptional and translational levels. PDCD4 was found to be decreased or lost in different types of cancer, including liver and lung cancer. The expression of PDCD4 is regulated by various factors, such as apoptosis inducers and cytokines. We previously found that PDCD4 expression was significantly decreased in the progesterone-predominated secretory phase of the normal endometrium compared with that in estrogen-predominated proliferative phase (17), which suggests that PDCD4 expression is hormonally regulated in the human endometrium.

The human endometrium is composed of epithelial and stromal cells, and it is cyclically regenerated. The reproductive cycle is induced by neuroendocrine signaling (20). The endometrium is driven into the proliferative phase of the cycle by increased estrogen, and then progesterone is produced by the ovary. The progesterone inhibits proliferation of the endometrium and drives the endometrium into a secretory phase in anticipation of fertilization (20). However, if there is no fertilization, the endometrium is shed and hormone
levels are decreased, and then the cycle is reactivated (21,22). As well known, prolonged exposure to estrogen is a major endocrine risk factor for the establishment and progression of various diseases, such as endometriosis (23) and endometrial carcinoma (24). Progesterone counteracts estrogen-mediated action and exhibits anti-proliferative and anti-inflammatory roles (25-27). Therefore, the antagonistic nature of progesterone to estrogen in the endometrium empowers progesterone as the first-line of hormonal therapy for the clinical treatment of endometriosis (28-31) and endometrial carcinoma. However, the therapeutic efficacy and beneficial effect of progesterone on the pathogenesis of endometrial carcinoma remain debatable (32). In the present study, it was demonstrated that progesterone downregulates PDCD4 protein expression, which suggests that PDCD4 is a progesterone target gene. Considering that PDCD4 is a type of tumor suppressor, which inhibits tumor development and progression, downregulation of PDCD4 by progesterone may be one of the reasons that progesterone has limited therapeutic efficacy.

Classically, the actions of progesterone are attributed to the binding of progesterone and nuclear progesterone receptor (nPR) and subsequent activation of its downstream target genes (33). In addition, cell membrane hormonal receptors, such as the membrane progesterone receptor (mPR), family, have been identified and demonstrated to be functional (34-37). mPR is able to activate the MAPK/ERK and PI3K/AKT pathways, which also leads to regulation of gene expression (38). Mammalian target of rapamycin (mTOR) is a target gene of PI3K/AKT. It has been reported that miR-21 inhibits PDCD4 expression and activates the PI3K/AKT/mTOR signaling pathway (39). Dorrello et al (40) revealed a new signaling branch of the mTOR pathway that controls the degradation of PDCD4. In the present study, it was demonstrated that p-AKT and p-mTOR were increased and PDCD4 was decreased after treatment of progesterone. Inhibition of PI3K/AKT by LY294002/MK2206 reduced the expression of p-AKT and upregulated the expression of PDCD4 protein, while expression of p-mTOR exhibited no obvious changes. These results suggest that the PI3K/AKT pathway is involved in progesterone-induced PDCD4 protein downregulation.

In conclusion, it was demonstrated for the first time that progesterone effectively decreases the expression of PDCD4 protein, and the PI3K/AKT pathway may be involved in the downregulation of PDCD4 protein. These results suggest that the downregulation of PDCD4 induced by progesterone could affect the therapeutic efficacy of progesterone in human endometrial cancer or endometriosis, which may have important implications for progesterone treatment in the clinic.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

ZW designed the research project and conducted the experimental study. XiaW participated in designing the experiments, writing and reviewing the manuscript. XisW performed the experiments and wrote the manuscript. YueL was involved in performing various experiments. LW and YaL were involved in statistical analysis. YSu and YuQL participated in preparation of the manuscript. YSh and LZ were involved in revising it critically for important intellectual content. HZ and JW assisted in data analysis and reviewing the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

None of the authors has any potential financial conflicts of interests related to this manuscript.

References

10. Shibahara K, Asano M, Ishida Y, Aoki T, Koike T and Honjo T:
11. Lankat-Buttgereit B and Goke R: The tumour suppressor Pdcd4:
12. Onishi Y and Kizaki H: Molecular cloning of the genes