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Original Article

Protopanaxadiol and metformin synergistically inhibit estrogen-mediated proliferation and anti-autophagy effects in endometrial cancer cells

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Abstract: Metformin is commonly used for treating type II diabetes and has recently been reported to possess anti-proliferative properties that can be exploited for the prevention and treatment of a variety of cancers. Ginsenosides are the main effective biological components of ginseng. It has been reported that ginsenoside-Rb2 inhibit the invasiveness of endometrial cancer cells (ECC). The aim of this study was to investigate whether protopanaxadiol (PPD, a metabolite of ginsenosides) and metformin could synergistically regulate the biological behavior of ECC and analyze its possible mechanism. We here found that either metformin or PPD treatment led to a decreased viability and increased apoptosis and autophagy levels in ECC lines (Ishikawa and RL95-2 cells), and combination of PPD and metformin could enhance these effects induced by metformin or PPD *in vitro*. PPD and metformin significantly decreased the expression of estrogen receptor alpha (ERα) in Ishikawa and RL95-2 cells. Estrogen promoted the viability and restricted the apoptosis and autophagy of Ishikawa and RL95-2 cells, and PPD and metformin reversed these effects. *In vivo* trials showed that combination of PPD and metformin had the strongest activity of anti-tumor growth compared with PPD alone and metformin alone. These data suggest that PPD and metformin can be used together to play a more powerful anti-EC effect. Our study provides a scientific basis for the clinical application of PPD and metformin in the treatment of EC, especially in estrogen-dependent patients.

Keywords: Metformin, protopanaxadiol, estrogen, endometrial cancer cells, growth, autophagy

Introduction

As one of one of the most common gynecological malignancies, the incidence of endometrial cancer (EC) is increasing worldwide. This increase is attributed to the rising prevalence of nulliparity and obesity [1]. Although the prognosis of low-risk EC is generally favorable, chemotherapeutic options for high-risk EC patients are limited. Therefore, exploring novel therapeutic strategies is necessary to improve EC prognosis.

EC can be divided into two types: type I, estrogen-dependent EC; and type II, estrogen-independent EC [2, 3]. The type I EC usually has favorable prognosis while type II EC is more aggressive and presented poor prognosis [4]. The type I EC is hormone-sensitive and approximately occupied 80% cases, and those patients almost have risk of obesity, which is usually well-differentiated [5]. As the primary risk factor for development of EC, Estrogen can contribute to the growth of EC [6, 7]. In addition, obesity and diabetes are related-risk factors for type I EC [8]. With the increase of obese population, the levels of endogenous hormones are changed, the occurrence of EC are gradually increased [3, 9].

As one of the most commonly used hypoglycemic agents in the management of type II diabetes, metformin, has anti-tumor effects on breast, prostate, ovarian, and EC cells by alterations of glucose metabolism and inhibition of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and insulin/insulin-like growth factor (IGF)-I signaling pathways [10-12]. Metformin can prevent and inhibit ECs, especially patients with diabetes. It can also enhance the anti-tumors effects of chemotherapy drugs, such as carboplatin [13, 14]. A phase II/III study is currently underway to evaluate metformin vs. placebo in advanced or recurrent EC patients receiving paclitaxel and carboplatin (NCT02065687).

As a medicinal herb, ginseng is widely used in Asian countries and the North American. Ginsenosides are main components extracted from ginseng. Ginsenosides have been reported to exert anti-tumor activities for various types of cancer, such as lung cancer and prostate cancer [15-17]. It has reported that ginsenoside-Rb2 inhibits invasiveness to the basement membrane possibly via down-regulation of MMP-2 in EC [18]. Protopanaxadiol (PPD) is the metabolite of ginsenoside, also exhibit activity against a variety of cancer cells [17, 19, 20]. Ginsenosides structurally contain a steroidal backbone. They can mediate their cellular activities by binding to the active sites of steroid receptors [21], for example, PPD suppresses estrogen-stimulated gene expression and cell proliferation in ER-positive breast cancer cells [22]. Besides that, PPD synergistically enhances cytotoxicity of tamoxifen in an ER-independent manner, probably by down-regulating Akt activity [22]. However, it's still unclear whether PPD has anti-EC effects in a synergistic fashion with metformin.

Therefore, this study was performed to investigate the effects of PPD and metformin on the viability, apoptosis and autophagy levels, and estrogen receptor α (ERα) expression in ECC *in vitro* and *in vivo*.

Materials and methods

Reagents

The anti-human Beclin-1, P62, LC3B and Actin antibodies (Abs) were purchased from R&D Systems (USA); the Annexin V/7-AAD Apoptosis Detection Kit was purchased from BD Biosciences (San Jose, CA, USA); the anti-human ERα Abs were purchased from Abcam (USA); the Cell Counting Kit-8 (CCK-8) were purchased from Dojindo (Japan), the peridininchlorophylla protein cyanine 5.5 (PerCP-Cy™5.5)-conjugated anti-human Ki-67 and allophycocyanin (APC)-conjugated anti-human Bcl-2 Abs were purchased from BD Biosciences; PE-conjugated Bcl-xL Abs were purchased from Cell Signaling Technology; APC-conjugated Fas, PE-conjugated Fas Ligand (FasL) and fluorescein isothiocyanate (FITC)-conjugated anti-human Cytokeratin (CK7) Abs were purchased from Biolegend (San Diego, CA, USA); and 17β-estrogen (E_2), PPD and metformin were purchased from Sigma-Aldrich Co. LLC., (USA).

Cell culture

The human endometrial carcinoma cell lines Ishikawa and RL95-2 were obtained from the cell bank of Chinese Academy of Science (Shanghai, China) and grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were incubated under normoxic conditions (21% O_2, 5% CO_2, 74% N_2) at 37°C in a humidified incubator (Heal Force, HF 100, Shanghai, China).

CCK-8 assay

The Ishikawa cells and RL95-2 cells were seeded in 96-well plates (4×10^3 cells/well), and then treated with different concentration of PPD (0, 10, 20, 40, 80 or 160 uM), Metformin (0, 1, 5, 10, 20, 40 or 80 mM) or metformin (20 mM) plus PPD (0, 2.5, 5, 10, 20 or 40 uM) for 24 h. Subsequently, these cells were collected and detected the viability by the cell-counting Kit-8. According to the manufacturer's protocol, the CCK-8 regent was added to each well 10 ul and 90 ul culture solution then total cells was incubated at 37°C for 1 h. The absorbance (optical density) at 450 nm was measured and used to represent the viability of cells. Each experiment was performed in eight parallel wells, and all experiment repeated three times.

Annexin V/7-AAD apoptosis assay

The Ishikawa and RL95-2 cells were seeded in 24-well flat-bottom microplates at the density of 1×10^5 cells/well, and then treated with PPD (40 uM), metformin (20 mM), or PPD plus met-

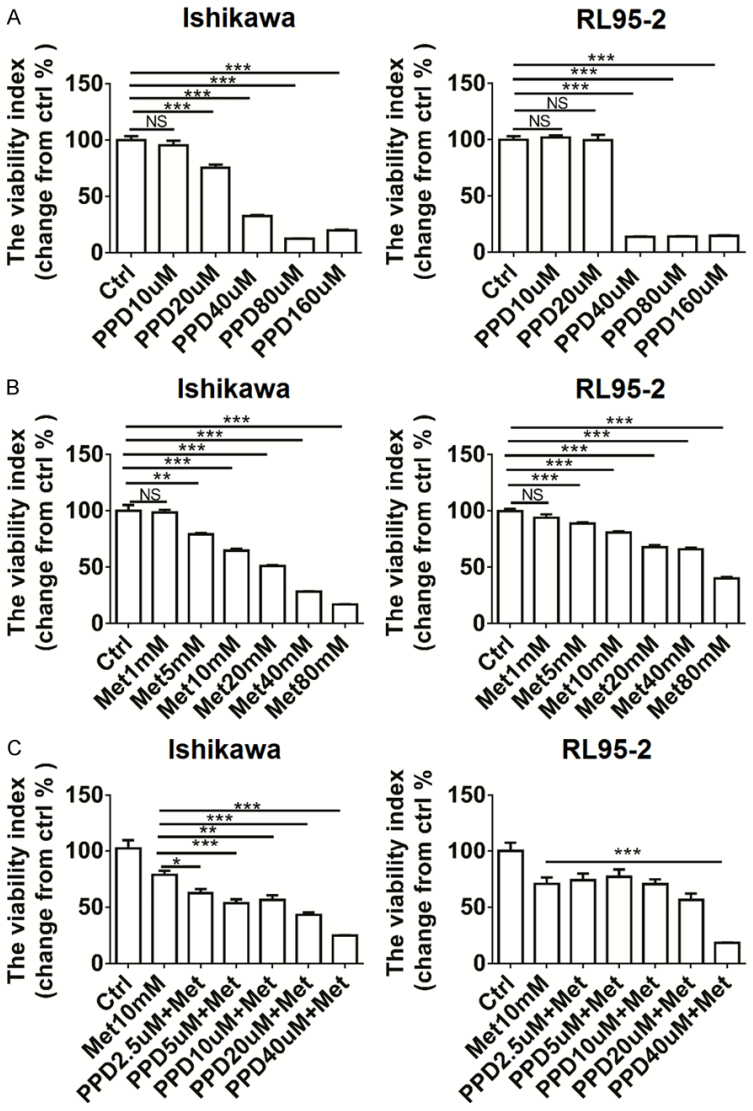

Figure 1. PPD enhances inhibitory effect of metformin on the viability of ECC. (A) Ishikawa and RL95-2 cells were treated with PPD at different concentration of 0, 10, 20, 40, 80 or 160 uM for 24 h, (B) Ishikawa and RL95-2 cells were treated with metformin at different concentration of 0, 1, 5, 10, 20, 40 or 80 mM for 24 h, (C) Ishikawa and RL95-2 cells were treated with metformin (20 mM) plus PPD (0, 2.5, 5, 10, 20 or 40 uM) for 24 h, then a CCK-8 assay was performed to detect the viability of Ishikawa and RL95-2 cells. Met: metformin. The data were expressed as means ± SEM. **P*<0.05, ***P*<0.01 or ****P*<0.001 (one-way ANOVA). NS: no significant difference.

formin for 24 h, then these cells were digested by 0.25% trypsin without EDTA, then centrifuged at 1200 rpm for 5 min, re-suspended with 200 ul binding buffer, and labeled by Annexin V and 7-AAD according to the protocol. Flow cytometry assay was performed to detect the percentage of early apoptotic cells and total apoptotic cells. The experiment were carried out triplicate, and repeated three times.

Protein extraction and western blotting

Cell were washed in phosphate buffered saline (PBS), detached with the cell scraper and centrifuged for 20 min at 12000 rpm at 4°C. The pellet was re-suspended in high efficiency cell tissue rapid lysis buffer (RIPA; Beyotime, Shanghai, China) containing 1% phenylmethanesulfonyl fluoride (PMSF; Beyotime) proteinase and 1% phosphatase inhibitors (Roche, USA). Cell lysates were boiled for 10 min at 95°C and then stored at -80°C. Protein concentrations were quantified using the BCA protein assay kit (Beyotime). Total proteins (20 ug) were electrophoresed in SDS-PAGE gels (EpiZyme scientific) on a Miniprotein III system (Bio-Rad, USA), and transferred into PVDF membrane (Millipore) at 2 h, overnight incubated with primary antibody against Beclin-1, p62, LC3b, Actin and ERα at 4°C, then the PVDF membrane washed three times by TBST solution, and incubated at room temperature for 1 h in peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (1:5000; Bioworld Technology, co. Ltd. USA). After then the membrane were washed three times and processed for chemiluminescence with Immobilon Western Chemiluminescent HRP Substrate Kit (Millipore).

Exposure with E_2

Ishikawa cells and RL95-2 cells were treated with E_2 (10^{-7} M), E_2 plus PPD (40 uM), E_2 plus metformin (20 mM), E_2 plus PPD and metformin for 24 h, we collected these cells, and analyzed the viability, apoptosis and autophagy levels by CCK8, apoptosis and western blotting assays, respectively.

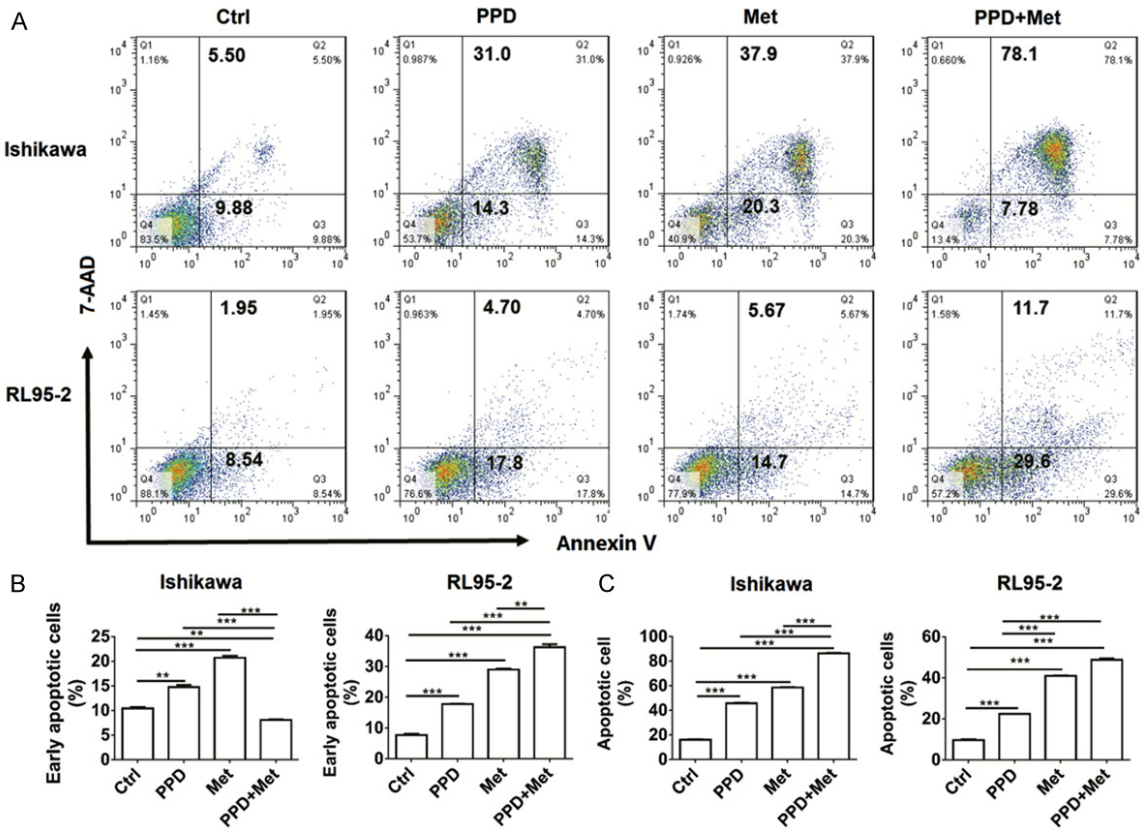

Figure 2. PPD and metformin cooperate in induction of ECC's apoptosis. A-C: Ishikawa and RL95-2 cells were treated with PPD (40 uM) and or metformin (20 mM) for 24 h, and then the Annexin-V/7-AAD assay was used to analyze the apoptosis of Ishikawa and RL95-2 cells. Early apoptotic cells: Annexin $V^+/7\text{-}AAD^-$ cells; late apoptotic or necrotic cells: Annexin $V^+/7\text{-}AAD^+$ cells. The data were expressed as means ± SEM. $^{**}P<0.01$ or $^{***}P<0.001$ (one-way ANOVA).

In vivo experiments

Nude mice of 4-5 weeks age were inoculated subcutaneously under the scruff on day 0 with 200 ul of 1×10^7 Ishikawa cells. PPD and or metformin were injected 100 mg/kg by intraperitoneal once a day after xentransplantation. Tumor growth was monitored by measuring the tumor volume every three day. Tumor volume was determined using the formula: volume (mm^3) =1/2(L×W×W). After 19 days, mice were euthanized, and the tumor tissues were collected.

Flow cytometry

The tumor tissues of nude mice were perfused thoroughly with cold PBS before cell collection, then tissues were minced on ice and digested with an enzyme mix of Liberase and Dispase (Invitrogen). Then we collected these cells and evaluated the expression of Ki-67, Fas, FasL, Bcl-xL and Bcl-2 by flow cytometry. The samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, USA) and Cellquest software (Becton Dickinson).

Statistics

All values were shown as the means ± SEM. The data were analyzed with GraphPad Prism version 5 by *t*-test or one-way ANOVA. Differences were considered statistically significant at $P<0.05$.

Results

PPD enhances inhibitory effect of metformin on the viability of ECC

To detect whether PPD and metformin regulate the viability of ECC, the CCK8 assay was performed to analyze the effect of PPD and metformin on the viability of ECC lines Ishikawa and RL95-2 cells. As shown in **Figure 1**, PPD significantly decreased the viability of Ishikawa and

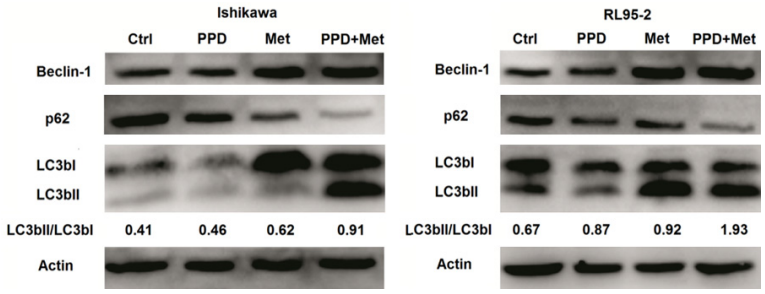

Figure 3. PPD and metformin corporately trigger ECC's autophagy. A, B: Ishikawa and RL95-2 cells were treated with PPD (40 uM) and or metformin (20 mM) for 24 h, and then the expression of autophagy-associated proteins Beclin-1, p62 and LC3b was analyzed by western blotting. In addition, the ratio of LC3b II to LC3B was counted by Quantity One analysis soft. Data were representative immune blots of assays.

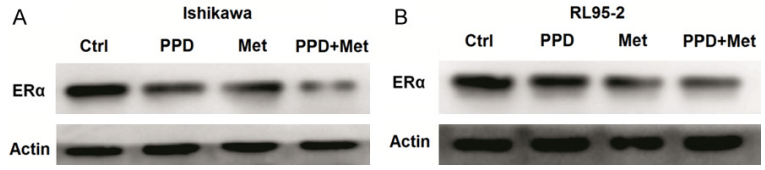

Figure 4. PPD and metformin down-regulates the expression of ERα in ECC. A, B: Ishikawa and RL95-2 cells were treated with PPD (40 uM) and or metformin (20 mM) for 24 h, and then the expression of ERα was analyzed by western blotting. Data were representative immune blots of assays.

RL95-2 cells in a dose-dependent manner, especially at concentration higher than 40 uM (P<0.001) (**Figure 1A**). Treatment with metformin also led to an obviously decreaseof the viability of Ishikawa and RL95-2 cells (P<0.01 or P<0.001) (**Figure 1B**). When the concentration was higher than 10 mM, the inhibition efficiency of metformin was the most significant (P<0.0001) (**Figure 1B**). In addition, PPD further amplified the inhibitory effect of metformin on the viability of Ishikawa and RL95-2 cells (P<0.05, P<0.01 or P<0.001) (**Figure 1C**).

PPD and metformin cooperate in induction of ECC's apoptosis and autophagy

Next, the results of apoptosis assay showed that both PPD and metformin promoted the early apoptosis (Annexin $V^+7\text{-}ADD^-$), late apoptosis or necrosis (Annexin $V^+7\text{-}ADD^+$) of Ishikawa and RL95-2 cells (P<0.01 or P<0.001) (**Figure 2A-C**). Compared with PPD alone and metformin alone, treatment with PPD plus metformin resulted in higher levels of early apoptotic, late apoptotic or necrotic Ishikawa and RL95-2 cells (P<0.01 or P<0.001) (**Figure 2A-C**).

Beclin-1 is essential in the induction process of autophagy [23], and considers as a "platform protein" that provides a framework for other autophagy-related (Atg) proteins and class III phosphoinositide 3-kinase (PI3K, Vps-34), which initiate macroautophagic activity together. The p62, also called sequestosome 1 (SQSTM1), is an autophagy adaptor protein [24]. The p62 protein is itself degraded by autophagy and may serve to link ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome. Therefore, p62 accumulates when autophagy is inhibited. The half-life of LCB3-II is very short and Atg4 may cleave LC3-II at the phosphatidylethanolamine (PE) and release back the LC3-I form. The enhanced LC3B-II/LC3B-I ratio indicates more intense formation of autophagy [25]. To explore the possible role of PPD and metformin in the autophagy of EEC, we analyzed the expression of autophagy-associated proteins (Beclin-1, p62 and LC3B) [26] in Ishikawa and RL95-2 cells after treatment with PPD and or metformin. As shown, either PPD or metformin led to higher levels of Beclin-1 and LC3B II/LC3B I, and low level of p62 expression in Ishikawa and RL95-2 cells compared with control group (**Figure 3A**, **3B**). Inaddition, PPD and metformin had synergistic effects on the expression of these autophagy-associated proteins (**Figure 3A**, **3B**). These data above indicate that there is a synergistic effect between PPD and metformin in promoting apoptosis and autophagy in ECC.

PPD and metformin down-regulates the expression of ERα in ECC

To investigate whether PPD and metformin regulate ERα expression in ECC, we detected the expression of ERα in Ishikawa and RL95-2 cells after treatment with or without PPD and metformin. The results of western blotting showed that exposure with PPD or metformin down-

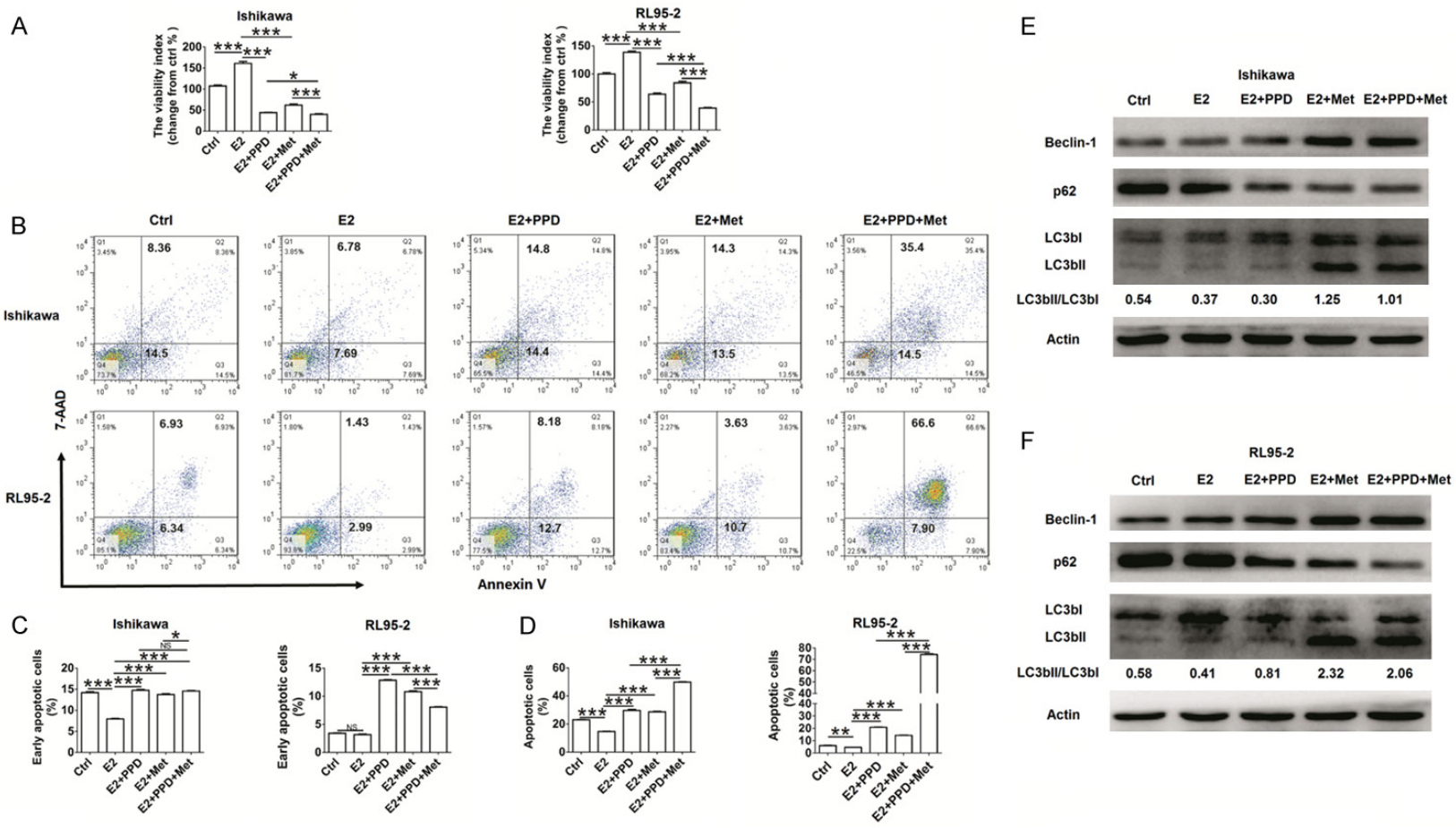

Figure 5. PPD and metformin restrict the effects of estrogen on ECC's growth and autophagy. Ishikawa cells and RL95-2 cells were treated with E_2 (10^{-7} M), E_2 plus PPD (40 uM), E_2 plus metformin (20 mM), E_2 plus PPD and metformin for 24 h, then we collected these cells, and analyzed the viability, apoptosis and autophagy levels by CCK8 (A), apoptosis (B-D) and western blotting (E, F) assays, respectively. E_2: 17β-estrogen. The data were expressed as means ± SEM. **P*<0.05, ***P*<0.01 or ****P*<0.001 (one-way ANOVA).

regulated the expression levels of ERα in Ishikawa and RL95-2 cells, especially exposure with PPD plus metformin (**Figure 4A**, **4B**). These results suggest that PPD and metformin may synergistically regulate the effect of estrogen on EEC by down-regulating the expression of ERα.

PPD and metformin restrict the effects of estrogen on ECC's growth and autophagy

Subsequently, we found that estrogen significantly promoted the viability of Ishikawa and RL95-2 cells ($P<0.001$) (**Figure 5A**), and these effects could be markedly reversed by PPD and or metformin ($P<0.001$) (**Figure 5A**). In contrast, exposure with estrogen obviously decreased the early apoptotic, late apoptotic or necrotic levels of Ishikawa and RL95-2 cells ($P<0.01$ or $P<0.001$) (**Figure 5B-D**), PPD and or metformin also suppressed these effects ($P<0.001$) (**Figure 5B-D**). Further analysis showed that PPD and or metformin could inhibit the role of estrogen on the expression of Beclin-1 and p62 and the ratio of LC3B II to LC3B I in Ishikawa and RL95-2 cells (**Figure 5E**, **5F**), especially combination of PPD and metformin. These data reveal that PPD and metformin remarkably antagonize the regulatory effect of estrogen on ECC's growth and autophagy, and this effect may be dependent on inhibition of ERα.

PPD and metformin synergistically inhibit EC growth in vivo

To probe into whether PPD and metformin play the anti-EC activity *in vivo*, Ishikawa cells were inoculated subcutaneously under the scruff of nude mice, and treated with PPD and or metformin. As shown, the tumor volumes of PPD and metformin groups were smaller than that of control group since 13d later ($P<0.001$) (**Figure 6A**). In addition, the tumor volume in combination group of PPD and metformin was minimal compared with PPD alone or metformin alone ($P<0.05$) (**Figure 6A**).

Both PPD and metformin promoted the expression of pro-apoptotic molecules Fas and FasL, and inhibited the expression of pro-proliferation molecule Ki-67, and anti-apoptotic molecules Bcl-2 and Bcl-xL in $CK7^+$ ECC from tumor tissues ($P<0.01$ or $P<0.001$) (**Figure 6B**). Moreover, the effects of combined application of PPD and metformin on these proliferation and apoptosis-related molecules were most significant ($P<0.05$ or $P<0.001$) (**Figure 6B**).

Discussion

Studies of EC have shown that it's the fourth common gynecological tumors in the developed countries [27]. In this study, we found that both PPD and metformin inhibited viability, and promoted apoptosis and autophagy of Ishikawa and RL95-2 cells. Moreover, PPD and metformin synergistically inhibited the stimulatory effect of estrogen on viability and the inhibitory effect of estrogen on apoptosis and autophagy in Ishikawa and RL95-2 cells, and this process may be associated with the down-regulation of ERα.

As a hormone-dependent disease, type I EC is sensitive to endogenous and exogenous estrogens. Estrogen binding to their receptors may stimulate MAPK/ERK1/2 pathways, which leads to ER phosphorylation and consecutive nuclear translocation to regulate gene transcription (e.g. cell proliferation, migration, differentiation) [28].

The potential role of metformin in treating EC has been uncovered in many studies [29, 30]. Metformin exerts its anti-tumorigenic effects possibly through indirect mechanisms by increasing insulin sensitivity, inhibiting liver gluconeogenesis, and reducing hyperglycemia and insulin levels [31], and direct mechanisms by activating AMP-activated protein kinase (AMPK), and further inhibiting the mammalian target of rapamycin (mTOR) pathway [32] and extracellular signal-regulated kinase (ERK) signaling [33]. In addition, metformin inhibits estrogen-dependent type I EC proliferation by activate AMPK-FOXO1 signal pathway [34]. Here, we found that metformin directly down-regulated the expression of ERα in Ishikawa and RL95-2 cells, and promoted the expression pro-apoptosis molecules Fas and FasL and decreased the expression of pro-proliferation molecule Ki-67 and anti-apoptosis molecules Bcl-2 and Bcl-xL. These data suggest the regulatory effects of metformin on these proliferation and apoptosis molecules may be associated with the inhibition of ERα and its downstream singling. The possible mechanism for this process needs to further research.

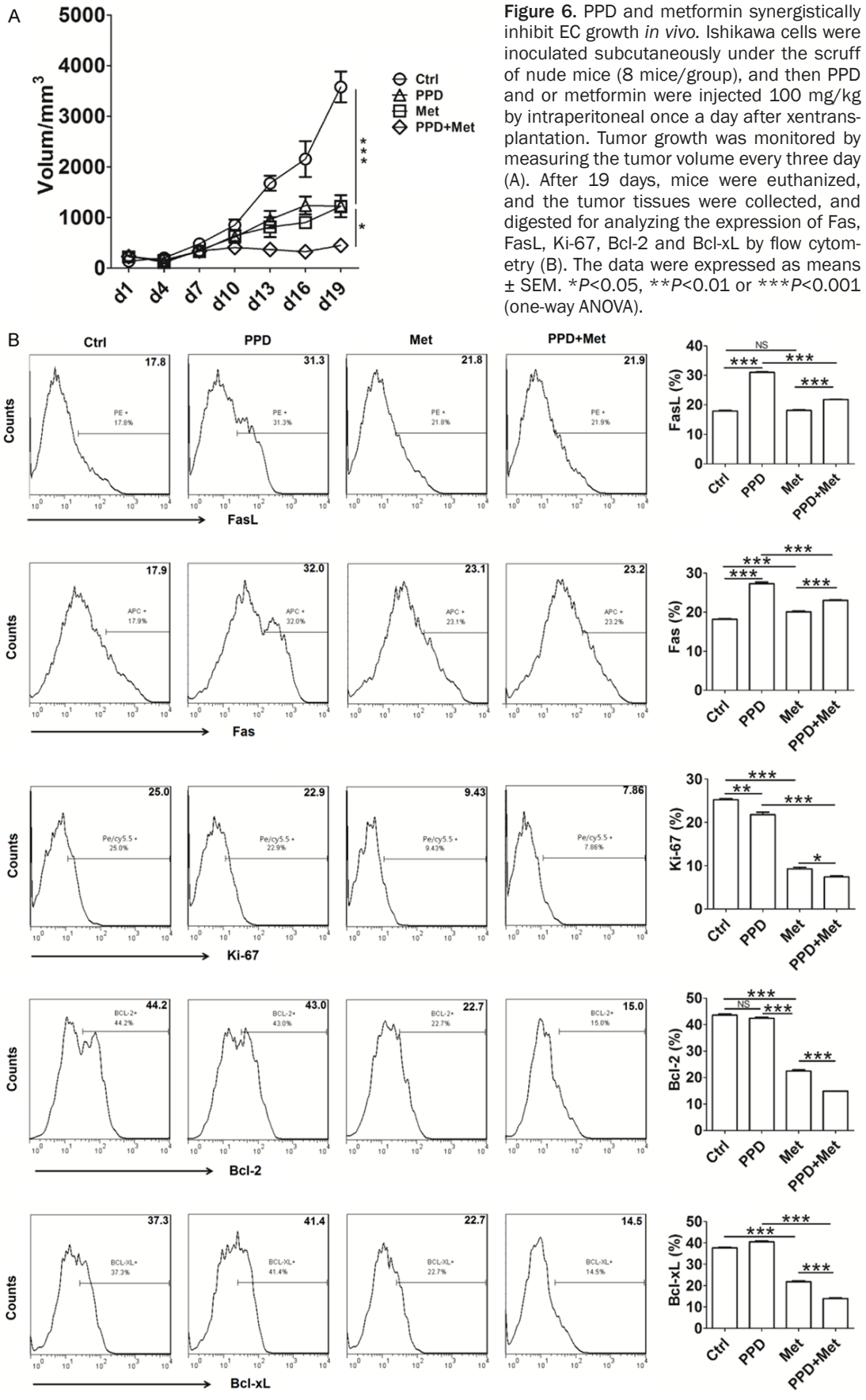

Figure 6. PPD and metformin synergistically inhibit EC growth *in vivo*. Ishikawa cells were inoculated subcutaneously under the scruff of nude mice (8 mice/group), and then PPD and or metformin were injected 100 mg/kg by intraperitoneal once a day after xentransplantation. Tumor growth was monitored by measuring the tumor volume every three day (A). After 19 days, mice were euthanized, and the tumor tissues were collected, and digested for analyzing the expression of Fas, FasL, Ki-67, Bcl-2 and Bcl-xL by flow cytometry (B). The data were expressed as means ± SEM. **P*<0.05, ***P*<0.01 or ****P*<0.001 (one-way ANOVA).

A lot of researches have reported that ginsenosides and its metabolites have a wide variety of antitumor activities [21]. PPD has anti-oxidative stress (e.g. up-regulation of ROS reproduction) [35, 36] anti-fatigue [37] properties. It also mediates mitochondrial apoptosis of tumor cell by cytochrome c/caspase-9 [38] or PI3K/AKT singling pathway [39, 40], stimulates acute lymphoblastic leukemia cells differentiate [41], and inhibits matrix remodeling and cell metastasis (e.g. down-regulation of MMP9 and MMP2 expression) [42, 43]. Furthermore, PPD are reported to change Ca^{2+} and NO levels in endothelia cells through glucocorticoid and ER for prevention and treatment of vascular diseases associated with endothelial cell dysfunction [44]. Here, we found PPD significantly inhibited the viability, promoted the apoptosis and autophagy of ECC. Moreover, it can cooperate with metformin to limit activities of estrogen on ECC's viability, apoptosis and autophagy. However, the exact mechanism for these processes remains to be further studied.

Some studies have revealed that metformin triggers autophagy of several tumor cells through CEBPD [45] or AMPKα [46]. However, little is reported about the regulation of PPD in cell autophagy. A recent study has showed that PPD induces autophagy and apoptosis in human melanoma via AMPK/JNK phosphorylation [47]. Therefore, in combination with our findings, we hypothesize that the synergistic inhibition of PPD and metformin on autophagy of EEC may be associated with regulation of AMPK signaling [48].

Autophagy takes place at the basal level but is also regulated developmentally and/or by environment stimuli, such as nutrient/energy availability, hypoxia and reactive oxygen species [48]. Our previous work also has confirmed that estrogen suppresses the autophagy of endometrial stromal cells from endometriosis by up-regulating CXCL12/CXCR4 signaling pathway [49]. In current study, both PPD and metformin could markedly down-regulated the expression of ERα in Ishikawa and RL95-2 cells, and restricted the anti-autophagy activation of estrogen, suggesting the stimulation activation of PPD and metformin may result from the inhibition of ERα and its downstream singling. However, we still cannot rule out the potential effects of PPD and metformin on autophagy by anti-oxidative stress. Further studies are needed to confirm these possible hypotheses.

Here, *in vivo* experiment further proved an evidence for the synergistic anti-EC activity of PPD and metformin. Therefore, it can be concluded that PPD and metformin have a synergistic potential of anti-EC, especially for the estrogen-sensitive and diabetes patients. In addition, it has been reported that metformin overcomes progesterone resistance in EC by targeting autophagy [50]. Thus, PPD and metformin may also have the potential to assist progesterone in the treatment of EC by creating an endocrine environment with antagonist of estrogen. However, the clinical value for the combination of PPD, metformin and progesterone in anti-EC still needs further study and evaluation.

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Disclosure of conflict of interest

None.

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