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Original article

17β-Estradiol, through activating the G protein-coupled estrogen receptor, exacerbates the complication of benign prostatic hyperplasia in type 2 diabetes mellitus patients by inducing prostate proliferation

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ABSTRACT

Benign prostatic hyperplasia (BPH) is one of the major chronic complications of type 2 diabetes mellitus (T2DM), and sex steroid hormones are common risk factors for the occurrence of T2DM and BPH. The profiles of sex steroid hormones are simultaneously quantified by LC–MS/MS in the clinical serum of patients, including simple BPH patients, newly diagnosed T2DM patients, T2DM complicated with BPH patients and matched healthy individuals. The G protein-coupled estrogen receptor (GPER) inhibitor G15, GPER knockdown lentivirus, the YAP1 inhibitor verteporfin, YAP1 knockdown/overexpression lentivirus, targeted metabolomics analysis, and Co-IP assays are used to investigate the molecular mechanisms of the disrupted sex steroid hormones homeostasis in the pathological process of T2DM complicated with BPH. The homeostasis of sex steroid hormone is disrupted in the serum of patients, accompanying with the proliferated prostatic epithelial cells (PECs). The sex steroid hormone metabolic profiles of T2DM patients complicated with BPH have the greatest degrees of separation from those of healthy individuals. Elevated 17β-estradiol (E2) is the key contributor to the disrupted sex steroid hormone homeostasis, and is significantly positively related to the clinical characteristics of T2DM patients complicated with BPH. Activating GPER by E2 via Hippo-YAP1 signaling exacerbates high glucose (HG)-induced PECs proliferation through the formation of the YAP1-TEAD4 heterodimer. Knockdown or inhibition of GPER-mediated Hippo-YAP1 signaling suppresses PECs proliferation in HG and E2 co-treated BPH-1 cells. The anti-proliferative effects of verteporfin, an inhibitor of YAP1, are blocked by YAP1 overexpression in HG and E2 co-treated BPH-1 cells. Inactivating E2/GPER/Hippo/YAP1 signaling may be effective at delaying the progression of T2DM complicated with BPH by inhibiting PECs proliferation.

1. Introduction

As a disease progress related to aging, type 2 diabetes mellitus (T2DM) is associated with severe endocrine and metabolic disorders and a variety of complications, among which benign prostatic hyperplasia (BPH) is an obvious complication in aging men [1]. The characteristic of BPH is abnormal proliferation of prostatic epithelial and stromal tissues, the emergence of which leads to obstructive lesions at the bladder outlet, resulting in urinary storage, urination, and so on [2,3]. Lower urinary tract symptoms (LUTS) dysfunction is

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one of the most common complications of diabetes and its occurrence could be resulted from bladder dysfunction, prostate enlargement and so on [4,5]. Clinical data showed that the incidence rate of BPH/LUTS is high in patients with metabolic disorders, and up to 50% of T2DM patients had voiding dysfunction [6,7]. Previous studies have demonstrated that the incidence of T2DM complicated with BPH becomes more prevalent during aging [8]. Although studies have confirmed that there are clinical connections between T2DM and BPH, and multiple metabolic risk factors are involved in the development of BPH (such as hypertension, obesity, and hyperinsulinemia), the etiology and mechanism of T2DM complicated with BPH have not been fully elucidated [9].

Sex steroid hormones significantly influence energy metabolism, and dysregulated sex steroid hormones have an important impact on T2DM [10–12]. It is reported that the rate of T2DM is higher in men than that in women, and estrogen treatment during menopause worsens glucose tolerance by lowering fasting glucose [13,14]. The developments of insulin resistance (IR) are associated with lower levels of total testosterone (T) and free T, and T replacement may improve survival in T2DM men [15]. However, high 17β-estradiol (E2) levels increase the risk of T2DM [16]. A population-based cohort study and meta-analysis verified that total T or bioavailable T had no association with T2DM incidence, while the increased risk of T2DM in women had a close relationship with total E2 [17]. Taken together, these findings indicate that sex steroid hormone profiles are disrupted in T2DM patients and the dysregulated homeostasis of sex steroid hormones may strongly promote the pathological process of T2DM and/or related complications.

The prostate actively reacts to sex steroid hormones [18]. A growing body of evidence indicates that sex steroid hormones are involved in the development of BPH via sex steroid receptors, leading to the proliferation of prostatic epithelial cells (PECs) [19,20]. Many studies have focused on the role of androgens in the occurrence of BPH, especially T and dihydrotestosterone (DHT) [21,22]. However, increasing evidence has suggested that estrogens play an irreplaceable role in the proliferation of PECs in BPH [23]. The homeostatic pattern of serum sex steroid hormones had been revealed by our research group in the simple BPH patients through quantitative analysis of androstenedione (A4), dehydroepiandrosterone (DHEA), and androsterone (A), T, DHT, estrone (E1) and 17β-E2 [24]. In addition, our other clinical study showed that homeostasis model assessment of insulin resistance (HOMA-IR) was the key factor between the simple BPH patients and T2DM patients complicated with BPH [25]. However, the characteristics of sex steroid hormone homeostasis are still unknown in patients with this comorbid disease. In addition, another important question remains: which estrogens or androgens affect prostate pathologies in patients with T2DM complicated with BPH, and what is the underlying molecular mechanism may be involved?

Therefore, the purpose of this study is twofold: (1) To reveal the characteristic of the disrupted sex steroid hormones in T2DM patients complicated with BPH; (2) To explore the role and mechanism of sex steroid hormones in PECs proliferation of patients with T2DM complicated with BPH. Our findings disclose the proliferative effect and underlying mechanism of disrupted sex steroid hormones in T2DM complicated with BPH, and this study demonstrates that targeting E2/G-protein-coupled estrogen receptor 1 (GPER) may be effective in delaying the progression of T2DM complicated with BPH by inhibiting Hippo-YAP1 signaling-mediated proliferation of PECs.

2. Materials and methods

2.1. Reagents

E2 (17β-estradiol, HY-B0141), OP-1074 (HY-125263), G15 (HY-103449) and verteporfin (VP, HY-B0146) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). For *in vitro* studies, E2, G15 and VP were dissolved in dimethyl sulfoxide (DMSO) to generate a primary stock solution. The solutions were stored at −80 °C and then diluted in medium before *in vitro* experimental study.

The information of androgens, estrogens and internal standard are listed in Table S1. The information of primary and secondary antibodies are listed on Tables S2 and S3, respectively.

2.2. Clinical study

The inclusion and exclusion criteria of clinical serum are provided in Fig. S1. And the tissues are described in Fig. S2. The detailed information of clinical sample collection is presented in Supplementary materialsdata.

2.3. Cell culture and transfection

To simulate the internal environment under normal and diseased conditions and replicate the findings from clinical sample analysis, we design seven cell groups. After serum starvation for 8 h, the cells are cultured in fresh medium containing 1% fetal bovine serum (FBS) supplemented with different concentrations of drugs for 24 h, including glucose and E2. And then, the yes-associated protein 1 (YAP1) inhibitor VP or GPER inhibitor G15 is added to the medium for another 24 h. The specific groupings are as follows: normal glucose (NG), no drugs added; NG + DMSO, 1 μL of DMSO; NG + 4 nM E2, 4 nM/L E2; high glucose (HG), 30 mM glucose; HG + DMSO, 30 mM glucose and 1 μL of DMSO; HG + 1 nM E2, 30 mM glucose and 1 nM E2; and HG + 4 nM E2, 30 mM glucose and 4 nM E2.

To investigate the role of GPER in the occurrence and development of T2DM complicated with BPH, we used the GPER inhibitor G15 or GPER-knockdown viruses to reduce the expression of GPER in BPH-1 cells. The lentiviral vectors for specific targeted human GPER expression and negative control sequences are designed and produced by Gene Chem (Shanghai, China). The shRNA sequences of GPER are shown in Table S4. The specific groupings are as follows: NG: normal glucose; NG + G15: normal glucose + G15 50 nM; HG: 30 mM glucose; HG + 4 nM E2: 30 mM glucose and 4 nM E2; HG + 4 nM E2 + G15 50 nM: 30 mM glucose, 4 nM E2 and 50 nM G15; HG + 4 nM E2 + GPER-shRNA-Veh: GPER knockdown virus vehicle, 30 mM glucose and 4 nM E2; and HG + 4 nM E2 + GPER-shRNA: GPER knockdown virus, 30 mM glucose and 4 nM E2.

OP-1074, a specific degradation agent of estrogen receptor α (ERα) and ERβ, is used to identify the key role of GPER in the promotion of cell proliferation in *vitro* [26]. The specific groupings are as follows: HG + 4 nM E2, 30 mM glucose and 4 nM E2; HG + 4 nME2 + 2 nM OP-1074, 30 mM glucose, 4 nM E2 and 2 nM OP-1074; HG + 4 nM E2 + GPER-shRNA, GPER knockdown virus, 30 mM glucose and 4 nM E2; and HG + 4 nM E2 + GPER-shRNA + 2 nM OP-1074, GPER knockdown virus, 30 mM glucose, 4 nM E2 and 2 nM OP-1074.

We also use the YAP1 inhibitor VP or a YAP1 knockdown virus to downregulate YAP1 [27]. The specific groupings are as follows: NG: no drugs added; HG: 30 mM glucose; HG + 4 nM E2: 30 mM glucose and 4 nM E2; HG + 4 nM E2 + VP: 30 mM glucose, 4 nM E2 and 5 μMVP; HG + 4 nM E2 + YAP1-shRNA-Veh: YAP1 knockdown virus vehicle, 30 mM glucose and 4 nM E2; and HG + 4 nM E2 + YAP1-shRNA: YAP1 knockdown virus, 30 mM glucose and 4 nM E2.

Finally, VP or YAP1 overexpression viruses are used to demonstrate that the anti-proliferative effect of VP is achieved by inhibiting YAP1. The specific groupings are as follows: HG + 4 nM E2, 30 mM glucose and 4 nM E2; HG + 4 nM E2 + VP, 5 μM, 30 mM glucose, 4 nM E2 and 5 μMVP; HG + 4 nM E2 + VP, 5 μM + YAP1-OE-Veh, YAP1-

overexpressing virus vehicle; 30 mM glucose, 4 nM E2 and 5 μM VP; and HG + 4 nM E2 + VP, 5 μM + YAP1-OE, YAP1-overexpressing virus; 30 mM glucose; 4 nM E2; and 5 μM VP.

2.4. Cell counting kit-8 (CCK-8) assay

BPH-1 cells are cultivated in 1640 culture medium containing different concentrations of G15 (0 nM, 10 nM, 20 nM, 50 nM, 100 nM) to investigate the safety and effectiveness of G15 at various concentrations. BPH-1 cells are cultivated in RPMI 1640 culture medium supplemented with different concentrations of VP (1 μM, 5 nM) to determine the effective concentration of VP. Cell viability is determined by a CCK-8 assay (DoJinDo, Shanghai, China) [28].

2.5. 5-Ethynyl-2′-deoxyuridine (EdU) assay

EdU staining is carried out as described previously using EdU labeling/detection kit (Ribobio, Guangzhou, China) [27].

2.6. Histology analysis

The image of hematoxylin and eosin (H&E) staining of clinical sample is gained using microscope (Olympus, Tokyo, Japan), and the protocol of H&E is designed as previously [27].

2.7. Immunohistochemistry (IHC) staining

The processing methods for biological and cellular samples are as described previously [29].

2.8. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA is extracted from BPH-1 cells using TRIzol and reversely transcribed into cDNA by RevertAid reverse transcriptase (Thermo Fisher Scientific, Rockford, IL, USA). Real-time PCR is performed on a Light Cycler 480II real-time PCR detection system (Roche, Basel, Switzerland) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). The expression of β-actin and GPER is quantified using qRT-PCR analysis. The primer sequences are shown in Table S5.

2.9. Western blotting analysis

The total proteins (100 mg) are resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as previously described [30–32]. The blots are scanned, and the target bands are analyzed using ImageJ software.

2.10. Immunofluorescence (IF) staining

IF staining of BPH-1 cells and clinical sample are performed as described previously [27]. Confocal microscopy (Leica Stellaris 5, Leica Microsystems, Wiesbaden, Germany) and Olympus BX43F fluorescence microscope (Olympus, Tokyo, Japan) are used to obtain the images of tissue and cells.

2.11. Coimmunoprecipitation (Co-IP) assay

Based on the previous studies, Co-IP analysis is carried out in this research [30–32].

2.12. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of estrogens and androgens

The abundances of seven steroid hormones (DHEA, A4, T, DHT, A, E1 and E2) are quantified via LC-MS/MS as established in our previous study [27].

2.13. Ethics

All clinical studies are performed according to the Declaration of Helsinki. This study is approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University (No. XYFY2018-KL093) and registered in the Chinese Clinical Trial Register (No. ChiCTR1800020339).

2.14. Statistical analysis

Principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA) analysis are carried out by using SIMCA 14.0 software. The key metabolites are defined by the variables with a *VIP* value greater than 1.0. The other statistical analyses are performed using GraphPad Prism 9.0 software. Wekemo BioinCloud (https://www.bioincloud.tech) and Wei Sheng Xin(https://www.bioinformatics.com.cn) are used to perform hierarchical clustered heatmap analysis. For better analysis, the first set of data is normalized and each subsequent set of data are divided by the first set of data. Results are expressed as the mean ± standard error of mean (SEM). Data is tested by Student's *t*-test and one-way analysis of variance (*ANOVA*). The statistical significant difference is considered when $P < 0.05$.

3. Results

3.1. Sex steroid hormone profiles are disrupted in BPH patients complicated with T2DM

LC-MS/MS is used to quantitatively analyze the levels of five androgens and two estrogens in the clinical serum of individuals with simple BPH (BPH group, $n = 30$), newly diagnosed with T2DM (T2DM group, $n = 30$), complicated with T2DM and BPH (T2DM + BPH group, $n = 30$), and matched healthy individuals (normal group, $n = 30$). The clinical characteristics of this study population are provided in Table S6. Hierarchical clustering heatmap analysis of clinical plasma sex steroid hormone levels reveals that each group has separate cluster (Fig. 1A). OPLS-DA analysis shows that there is a markedly separation of the sex steroid hormone profiles between disease groups and healthy people, and the T2DM + BPH group have the greatest degree of separation, followed by the T2DM group and the BPH group (Fig. 1B). The *VIP* values reveal that E2 (*VIP* value > 1, $P < 0.05$) is the most prominent one for the intergroup differentiation (Fig. 1C). The levels of E2 in the serum of patients are simultaneously quantified. Compared with those in healthy men, E2 levels are significantly greater in BPH patients, further elevated in T2DM patients, and reached their maximum in T2DM complicated with BPH patients (Fig. 1D). Further correlation analysis reveal positive correlations between the levels of E2 and fasting blood glucose (FBG), and hemoglobin A1c (Hb1Ac), but these correlations are not statistically significant (Fig. S3). In addition, E2 levels have remarkable positive relationship with the total prostate-specific antigen (TPSA) ($R^2 = 0.11$, $P = 0.482$) and prostate volume (PV) ($R^2 = 0.1267$, $P = 0.242$) in T2DM patients complicated with BPH (Fig. 1E).

Subsequently, further *in vitro* studies are carried out in BPH-1 cells. Both HG and E2 induce BPH-1 cell proliferation, and E2 dose-dependently exacerbate cell proliferation in BPH-1 cells

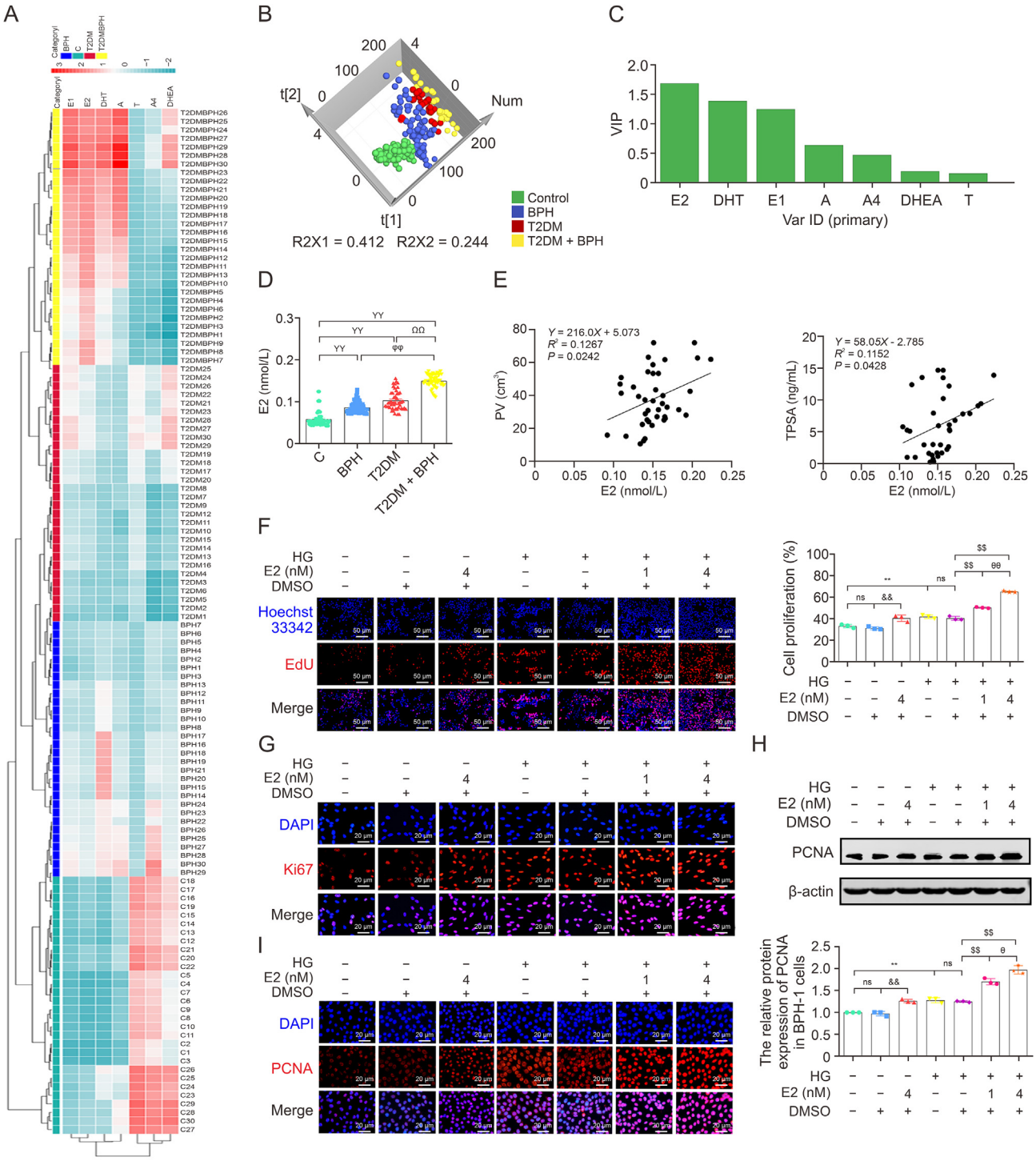

Fig. 1. Disruption of sex steroid hormone levels in the clinical serum of patients. (A) Hierarchical clustered heatmap of steroid hormone levels in the clinical serum of patients. ($n = 30$). (B) 3D scatterplot of steroid hormone levels in the clinical serum of patients. (C) *VIP* values of steroid hormones in the clinical serum of patients. (D) The concentration of estradiol (E2) in the clinical serum of patients. (E) Correlation analysis between the concentrations of E2 and the total prostate-specific antigen (TPSA) and prostate volume (PV) in the prostate of patients. (F) Proliferation of benign prostatic hyperplasia (BPH)-1 cells is determined by an 5-ethynyl-2′-deoxyuridine (EdU) assay. (G) The relative protein levels of Ki67 in BPH-1 cells are determined by immunofluorescence (IF) staining. (H) The relative protein levels of proliferating cell nuclear antigen (PCNA) in BPH-1 cells are determined by Western blot analysis. (I) The relative protein levels of PCNA in BPH-1 cells are determined by IF staining. Each bar represents the mean ± standard error of mean (SEM) for groups of six or three. $^{\gamma\gamma}P < 0.01$, compared to the control (C) group as indicated; $^{\varphi\varphi}P < 0.01$, compared to the BPH group as indicated; $^{\Omega\Omega}P < 0.01$, compared to the type 2 diabetes mellitus (T2DM) group as indicated; $^{**}P < 0.01$, compared to the normal glucose (NG) group as indicated; $^{\&\&}P < 0.01$, compared to the NG + dimethyl sulfoxide (DMSO) group as indicated; $^{\$\$}P < 0.01$, compared to high glucose (HG) + DMSO group as indicated; $^{\theta}P < 0.05$, $^{\theta\theta}P < 0.01$, compared to HG + E2 (1 nM) group as indicated. ns: not significance. DHT: dihydrotestosterone; E1: estrone; A: androsterone; A4: androstenedione; DHEA, dehydroepiandrosterone; T: testosterone; DAPI: 4′,6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide.

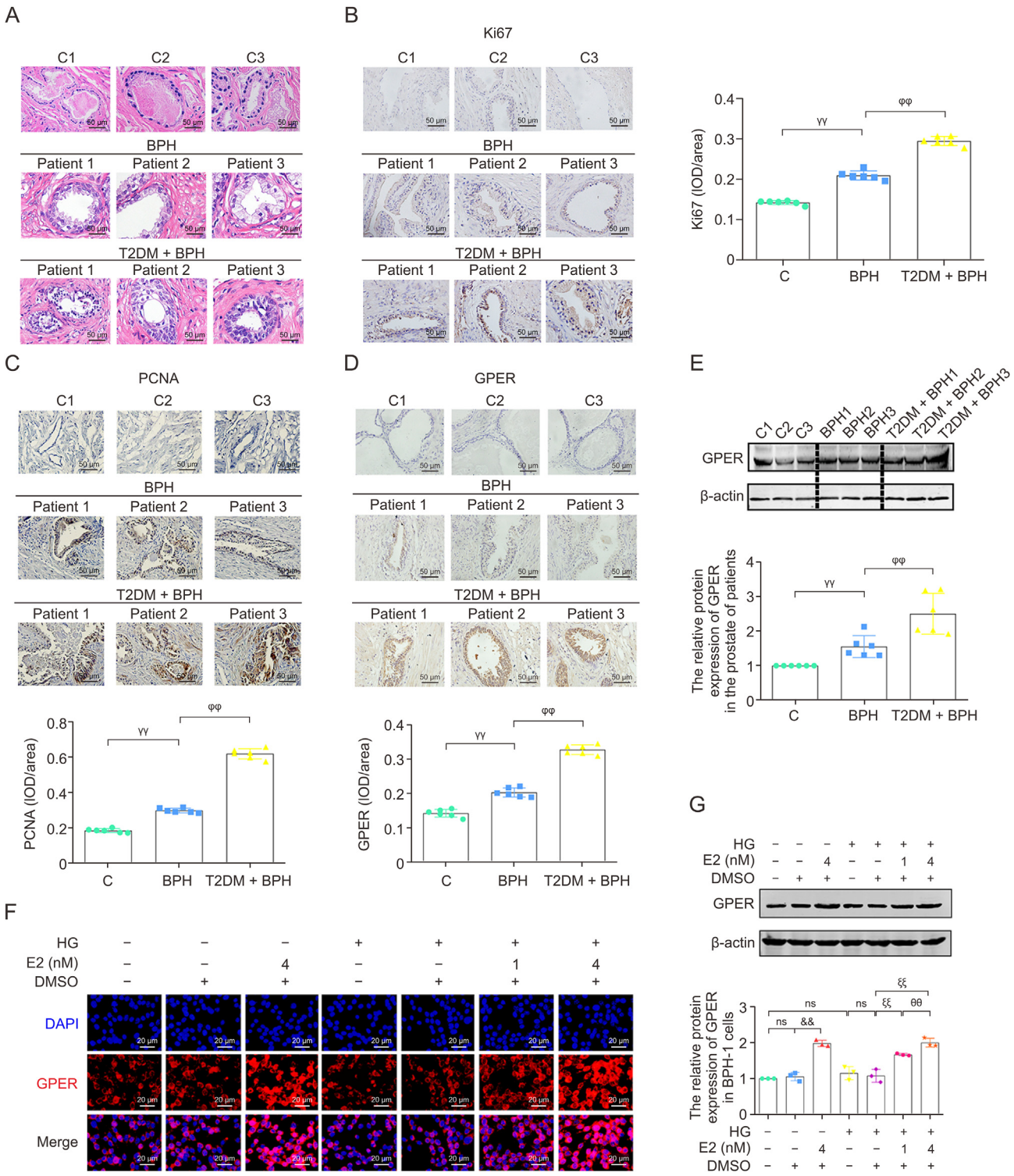

Fig. 2. The expression of estrogen receptors (ERs) in the prostate tissue of patients and high glucose (HG) and estradiol (E2) co-cultured benign prostatic hyperplasia (BPH)-1 cells. (A) Hematoxylin and eosin (H&E) staining of prostate tissue specimens. (B) The protein expressions of Ki67 in the different prostatic tissue specimens are determined by immunohistochemistry (IHC) staining. (C) The protein expressions of proliferating cell nuclear antigen (PCNA) in the different prostatic tissue specimens are determined by IHC staining. (D) The relative protein levels of G protein-coupled estrogen receptor (GPER) in different prostate tissue specimens are determined by IHC staining. (E) The relative protein levels of GPER in different prostate tissues are determined by Western blot analysis. (F) The relative protein levels of GPER in different prostate tissue specimens are determined by immunofluorescence (IF) staining. (G) The relative protein levels of GPER in BPH-1 cells are determined by Western blot analysis. Each bar represents the mean ± standard error of mean (SEM) for groups of six or three. $^{\gamma\gamma}P < 0.01$, compared to control (C) group as indicated; $^{\varphi\varphi}P < 0.01$, compared to BPH group as indicated; $^{\&\&}P < 0.01$, compared to normal glucose (NG) + dimethyl sulfoxide (DMSO) group, as indicated. $^{\xi\xi}P < 0.01$, compared to the high glucose (HG) + DMSO group as indicated; $^{\theta\theta}P < 0.01$, compared to the HG + DMSO + E2 group (1 nM) as indicated. ns: not significance. T2DM: type 2 diabetes mellitus.

stimulated with HG (Fig. 1F). The protein expressions of proliferating cell nuclear antigen (PCNA) and Ki67 are upregulated by HG or E2 and are further increased by HG and E2 co-stimulation in BPH-1 cells (Figs. 1G–I). Overall, these data indicate that sex steroid hormone homeostasis is disrupted in the serum of T2DM complicated with BPH patients, and E2 may be the main precipitating factor for the occurrence of T2DM complicated with BPH by enhancing PECs proliferation.

3.2. E2 exacerbates HG-induced PECs proliferation via GPER activation

Traditionally, E2 exerts physiological and pathological functions on cells mainly by activating two nuclear ERs, ERα and ERβ. H&E staining reveals significant thickening, hypertrophy, and hyperplasia with papillary projections of the lining epithelium in BPH patients, and these pathological changes are aggravated in T2DM patients complicated with BPH (Fig. 2A). When compared with those in the control group, the upregulated protein expressions of PCNA and Ki67 are further elevated in T2DM patients complicated with BPH, as demonstrated by IHC staining (Figs. 2B and C). However, the protein expressions of ERα or ERβ have no significantly changes in simple BPH patients or T2DM patients complicated with BPH (Fig. S4). Studies have revealed that E2 also mediates rapid signaling events via pathways that involve transmembrane ERs, such as GPER, formerly known as GPR30 [33]. Our results show that GPER was upregulated in simple BPH patients, and this change is further accelerated in T2DM patients complicated with BPH (Figs. 2D and E). Although the *in vitro* expression of GPER is not affected by HG, GPER expression is

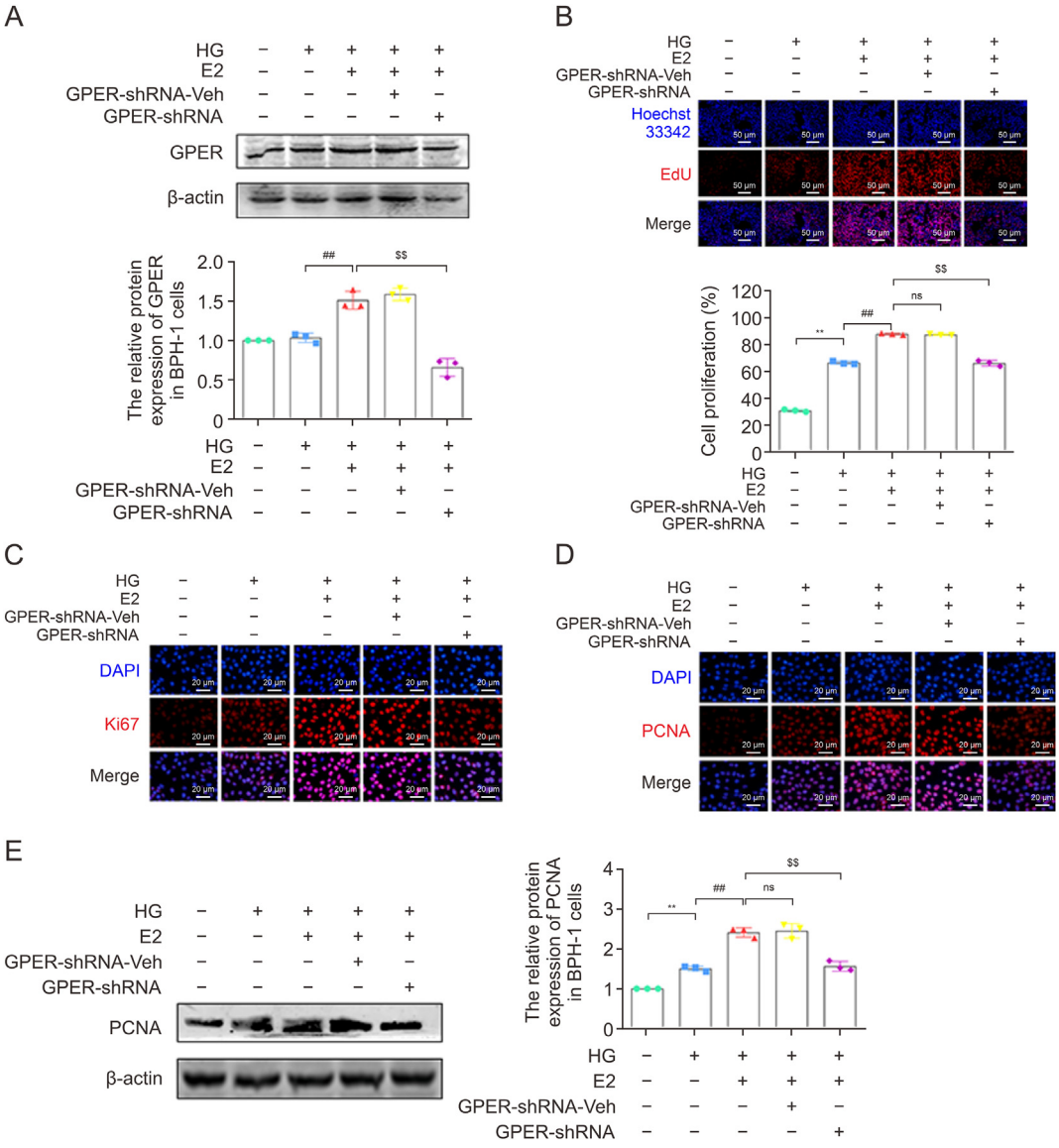

Fig. 3. Knocking down G protein-coupled estrogen receptor (GPER) inhibited proliferation in high glucose (HG) and estradiol (E2) co-cultured benign prostatic hyperplasia (BPH)-1 cells. (A) The relative protein levels of GPER in BPH-1 cells are determined by Western blot analysis. (B) Proliferation of BPH-1 cells is determined by a 5-ethynyl-2′-deoxyuridine (EdU) assay. (C) The relative protein levels of Ki67 in BPH-1 cells are determined by immunofluorescence (IF) analysis. (D) The relative protein levels of proliferating cell nuclear antigen (PCNA) in BPH-1 cells are determined by staining. (E) The relative protein levels of PCNA in BPH-1 cells are determined by Western blot analysis. Each bar represents the mean ± standard error of mean (SEM) for groups of three. $^{**}P < 0.01$, compared to the normal glucose (NG) group as indicated; $^{\#\#}P < 0.01$, compared to HG group as indicated; $^{\$\$}P < 0.01$, compared to the HG + E2 group (4 nM) as indicated. ns: not significance. DAPI: 4′,6-diamidino-2-phenylindole.

dose-dependently up-regulated by E2 in HG-cultured BPH-1 cells (Figs. 2F and G).

Then, GPER is knocked down to downregulate GPER expression in HG and E2 co-stimulated BPH-1 cells (Fig. S5 and 3. GPER knockdown not only inhibits proliferation, but also down-regulates PCNA and Ki67 protein levels in BPH-1 cells co-treated with HG and E2 (Figs. 3B–E). These results suggest that E2 enhanced PECs proliferation by activating GPER, resulting in accelerated complications in T2DM patients.

3.3. GPER knockdown inhibits the activation of Hippo-YAP1 signaling in vitro

The Hippo-YAP1 signaling pathway plays a critical role in cell proliferation, and studies have shown that estrogen, through GPER, promotes breast cancer cell proliferation by regulating the Hippo-YAP1 signaling pathway [34,35]. To investigate whether the Hippo-YAP1 signaling pathway is involved in the development of T2DM complicated with BPH, the Hippo-YAP1 signaling pathway is first examined in the prostate tissue of T2DM complicated with BPH patients. Mammalian sterile 20-like kinase (MST1) and large tumor suppressor 1 (LATS1) are two important core components of the Hippo signaling pathway. Although the phosphorylation of MST1 at Thr183 is not affected in BPH patients, it is significantly downregulated in T2DM patients complicated with BPH patients (Fig. 4A). LATS1 at Thr1079 is dramatically decreased in BPH patients, and this change trend becomes more pronounced in patients with T2DM complicated with BPH (Fig. 4B). Compared with that in the control group, upregulated total YAP1 is observed in BPH

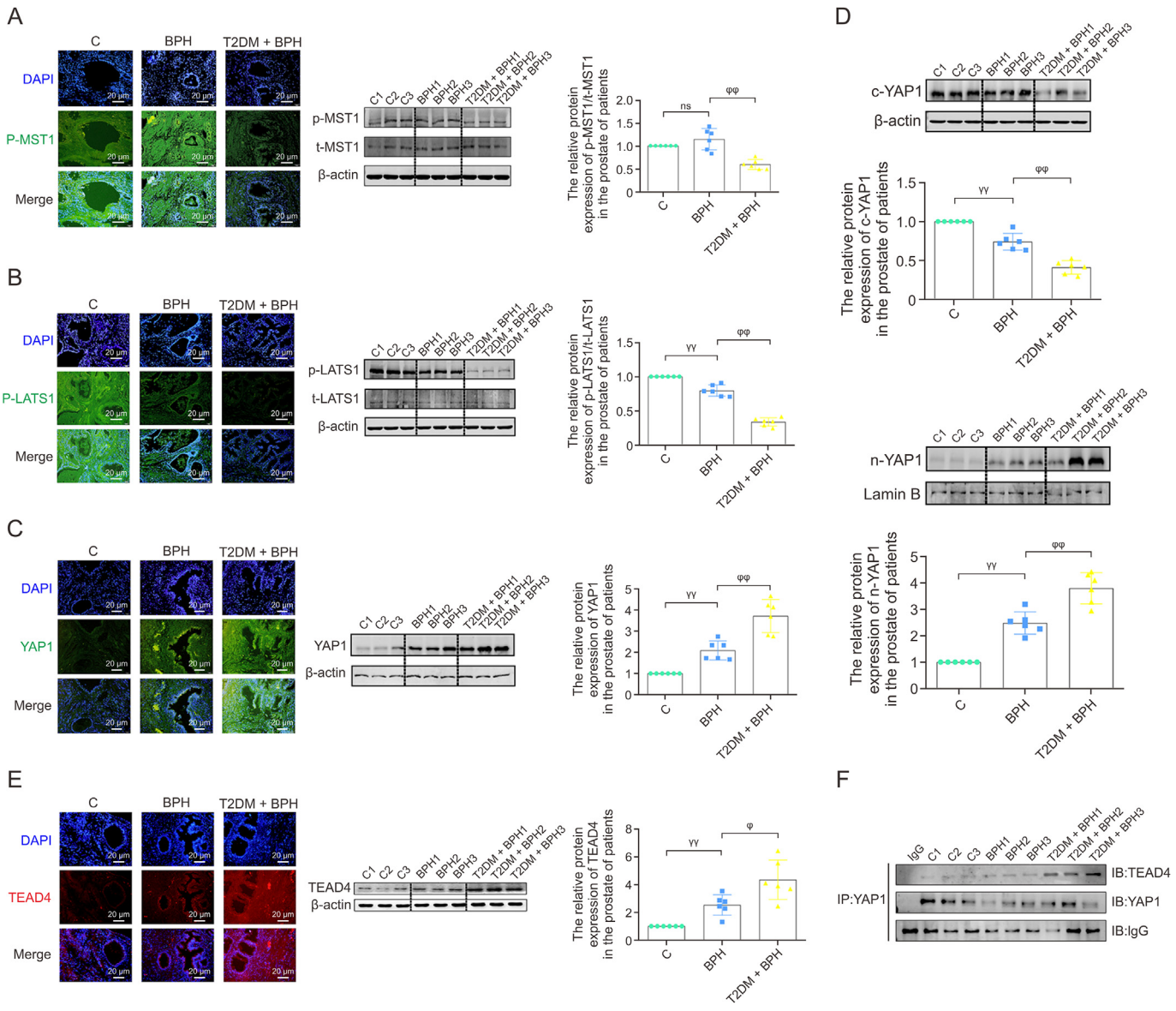

Fig. 4. The expression of Hippo-Yes-associated protein 1 (YAP1) signaling in the prostate tissue of patients. (A) The relative protein levels of phosphorylated mammalian sterile 20-like kinase (p-MST1) in different prostate tissue specimens are determined by Immunofluorescence (IF) staining and Western blot analysis. (B) The relative protein levels of phosphorylated large tumor suppressor 1 (p-LATS1) in different prostate tissue specimens are determined by IF staining and Western blot analysis. (C) The relative protein levels of YAP1 in different prostate tissue specimens are determined by IF staining and Western blot analysis. (D) The relative protein levels of cytoplasm (c)-YAP1 and nuclear (n)-YAP1 in different prostate tissues are determined by Western blot analysis. (E) The relative protein levels of TEA/ATS domain 4 (TEAD4) in prostate tissue specimens are obtained by IF staining and western blot analysis. (F) The interactions between YAP1 and TEAD4 are determined by coimmunoprecipitation (Co-IP) in different prostate tissues. $^{yy}P < 0.01$, compared to control (C) group as indicated; $^{\varphi}P < 0.05$, $^{\varphi\varphi}P < 0.01$, compared to benign prostatic hyperplasia (BPH) group as indicated. ns: not significance. DAPI: 4′,6-diamidino-2-phenylindole; t-LATS1: total large tumor suppressor 1; t-MST1: total mammalian sterile 20-like kinase; T2DM: type 2 diabetes mellitus.

patients, and this increase is greater in T2DM patients complicated with BPH patients (Fig. 4C). The increased nuclear translocation of YAP1 and TEAD4 expression are exacerbated in T2DM patients complicated with BPH (Figs. 4D and E). Furthermore, the Co-IP results show that the upregulated YAP1-TEAD4 heterodimer in BPH patients is further upregulated in T2DM patients complicated with BPH (Fig. 4F).

Next, we verify these changes *in vitro*. The decreased phosphorylation of MST1 induced by HG is not significantly different after co-culture with E2 (Fig. 5A). Although decreased phosphorylation of LATS1 can also be found in HG-cultured BPH-1 cells, E2 dose-dependently exacerbate this change trend *in vitro* (Fig. 5A). Regardless of whether HG or E2 can upregulate YAP1 expression, higher protein expression is observed in HG and E2 co-stimulated BPH-1 cells (Fig. 5B). The increased nuclear translocation of YAP1 and the protein levels of TEAD4 induced by HG or E2 are deteriorated by HG and E2 co-stimulation in *vitro* (Figs. 5C and D). In addition, HG or E2 increases the interaction between YAP1 and TEAD4, and HG and E2 co-stimulation exacerbates this change trend in BPH-1 cells (Fig. 5E).

To investigate whether GPER can regulate Hippo-YAP1 signaling in HG and E2 co-treated BPH-1 cells, small interfering RNA (siRNA) is used to reduce GPER expression in *vitro*. However, the decreased phosphorylation of MST1 induced by HG and E2 co-stimulation is not affected by GPER knockdown, which upregulated the phosphorylation of LATS1 in BPH-1 cells under both HG and E2 co-stimulation (Fig. 6A). Along with the decreased expression of YAP1 and subsequent nuclear translocation, GPER knockdown also downregulates the protein expression of TEAD4 in BPH-1 cells co-stimulated with HG and E2, in which the elevated interaction of the YAP1 and TEAD4 is also inhibited by GPER knockdown (Figs. 6B–E). Therefore, our results demonstrate that GPER knockdown

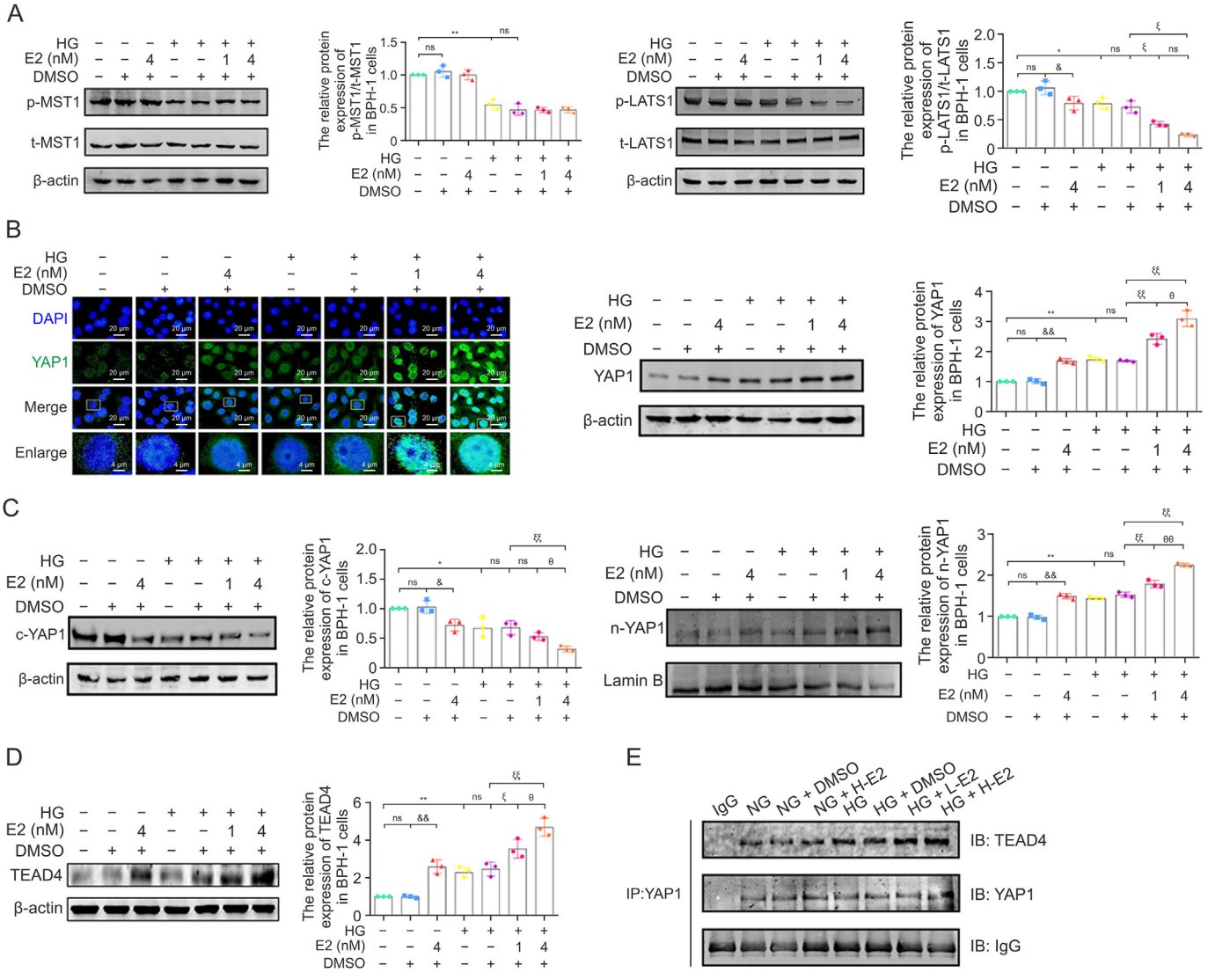

Fig. 5. The expression of Hippo-Yes-associated protein 1 (YAP1) signaling in high glucose (HG) and estradiol (E2) co-cultured benign prostatic hyperplasia (BPH)-1 cells. (A) The relative protein levels of phosphorylated mammalian sterile 20-like kinase (p-MST1) and phosphorylated large tumor suppressor 1 (p-LATS1) in BPH-1 cells are determined by Western blot analysis. (B) The relative protein levels of YAP1 in BPH-1 cells are determined by immunofluorescence (IF) staining and Western blot analysis. (C) The relative protein levels of cytoplasm (c)-YAP1 and nuclear (n)-YAP1 in BPH-1 cells are determined by Western blot analysis. (D) The relative protein levels of TEA/ATS domain 4 (TEAD4) in BPH-1 cells are determined by Western blot analysis. (E) The interactions between YAP1 and TEAD4 are determined by coimmunoprecipitation (Co-IP) in BPH-1 cells. Each bar represents the mean ± standard error of mean (SEM) for groups of three. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the normal glucose (NG) group as indicated; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, compared to the NG + dimethyl sulfoxide (DMSO) group as indicated; $^{\xi}P < 0.05$, $^{\xi\xi}P < 0.01$, compared to HG + DMSO group as indicated; $^{\theta}P < 0.05$, $^{\theta\theta}P < 0.01$, compared to HG + DMSO + E2 (1 nM) group as indicated. DAPI: 4′,6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; t-LATS1: total large tumor suppressor 1; t-MST1: total mammalian sterile 20-like kinase.

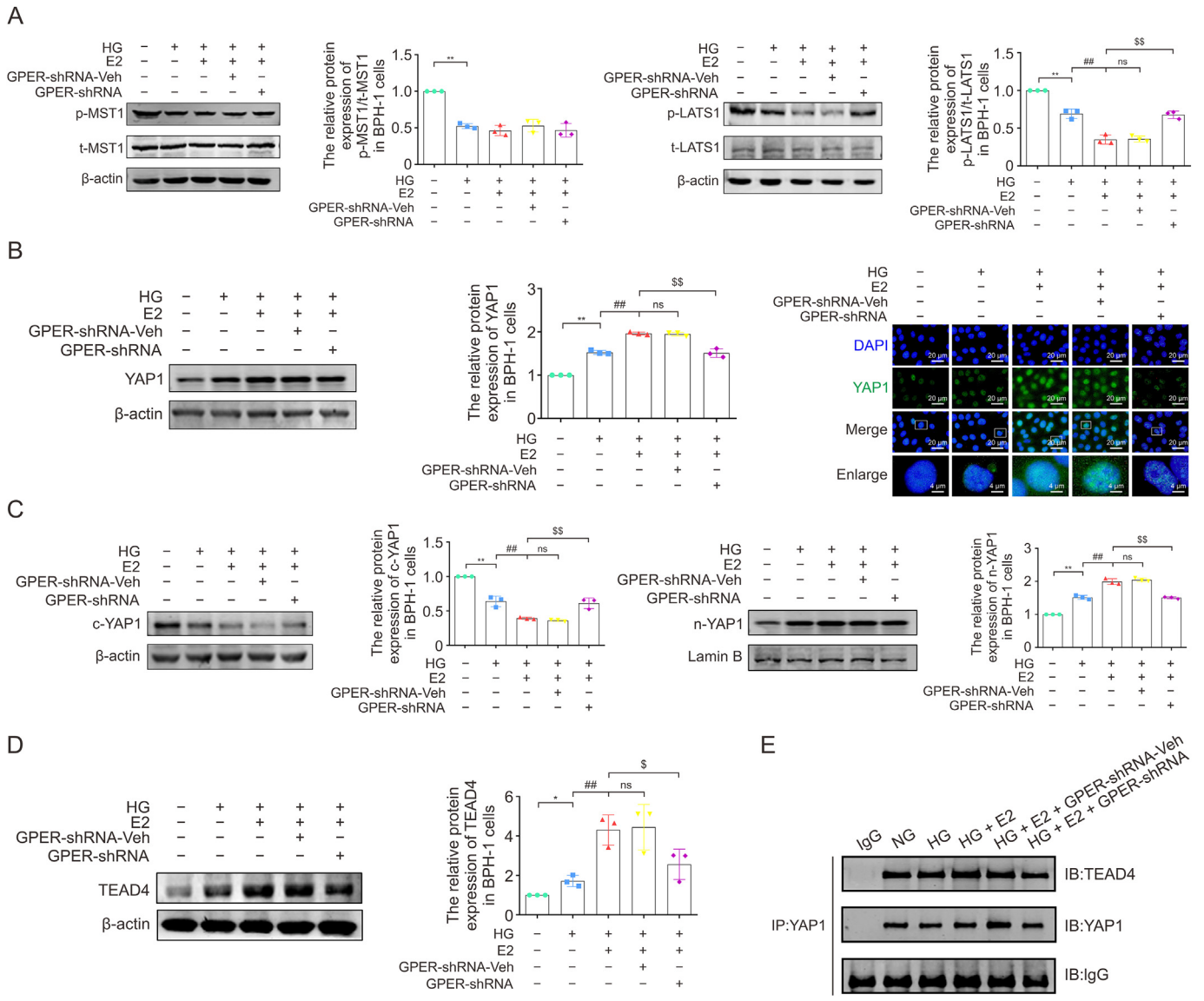

Fig. 6. Knocking down G protein-coupled estrogen receptor (GPER) inactivated Hippo-Yes-associated protein 1 (YAP1) signaling in high glucose (HG) and estradiol (E2) co-cultured benign prostatic hyperplasia (BPH)-1 cells. (A) The expressions of phosphorylated mammalian sterile 20-like kinase (p-MST1) and phosphorylated large tumor suppressors 1 (p-LATS1) in BPH-1 cells are determined by Western blot analysis. (B) The expression of YAP1 in BPH-1 cells is determined by Western blot analysis and immunofluorescence (IF) staining. (C) The expressions of cytoplasm (c)-YAP1 and nuclear (n)-YAP1 in BPH-1 cells are determined by Western blot analysis. (D) The expression of TEA/ATS domain 4 (TEAD4) in BPH-1 cells is determined by Western blot analysis. (E) The interactions between YAP1 and TEAD4 are determined by coimmunoprecipitation (Co-IP) in BPH-1 cells. Each bar represents the mean ± standard error of mean (SEM) for groups of three. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the normal glucose (NG) group as indicated; $^{\#\#}P < 0.01$, compared to HG group as indicated; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, compared to the HG + E2 group (4 nM) as indicated. ns: not significance. DAPI: 4′,6-diamidino-2-phenylindole; t-LATS1: total large tumor suppressors 1; t-MST1: total mammalian sterile 20-like kinase.

inhibits the activation of Hippo-YAP1 signaling induced by HG and E2 co-stimulation in BPH-1 cells.

3.4. Inactivation of GPER inhibits PECs proliferation via Hippo-YAP1 signaling in vitro

After confirming the regulatory effect of GPER on Hippo-YAP1 signaling in *vitro*, we probe the effect of inactivated GPER on the inhibition of PECs proliferation via Hippo-YAP1 signaling *in vitro*. G15, a GPER-specific inhibitor, is used in subsequent experiments. G15 treatment has no effect on the phosphorylation of MST1 in HG or E2 co-treated BPH-1 cells, but it elevates the phosphorylation of LATS1 compared with that in the HG and E2 co-treated groups (Fig. 7A). Moreover, the increases in the protein expression and nuclear translocation of YAP1 are inhibited by G15 treatment in HG and E2 co-treated BPH-1 cells (Figs. 7B and C). The protein levels of TEAD4 are down-regulated by G15, which also inhibits the interaction of YAP1 and TEAD4 in HG and E2 co-treated BPH-1 cells (Figs. 7D and E). Together with the inactivated Hippo-YAP1 signaling, G15 treatment inhibits the proliferation of PECs induced by HG and E2 co-stimulation, as demonstrated by EdU incorporation (Fig. 7F). In addition, the up-regulated expressions of Ki67 and PCNA are also decreased by G15 treatment in *vitro*, demonstrating that inactivation of GPER inhibits PECs proliferation via Hippo-YAP1 signaling *in vitro* (Figs. 7G and H).

3.5. Knockdown or inhibition of YAP1 inhibits PECs proliferation in vitro

Considering the participation of Hippo-YAP1 signaling in the proliferative effect of HG and E2 co-stimulation in BPH-1 cells, the significant role of YAP1 needs further be identified using a YAP1

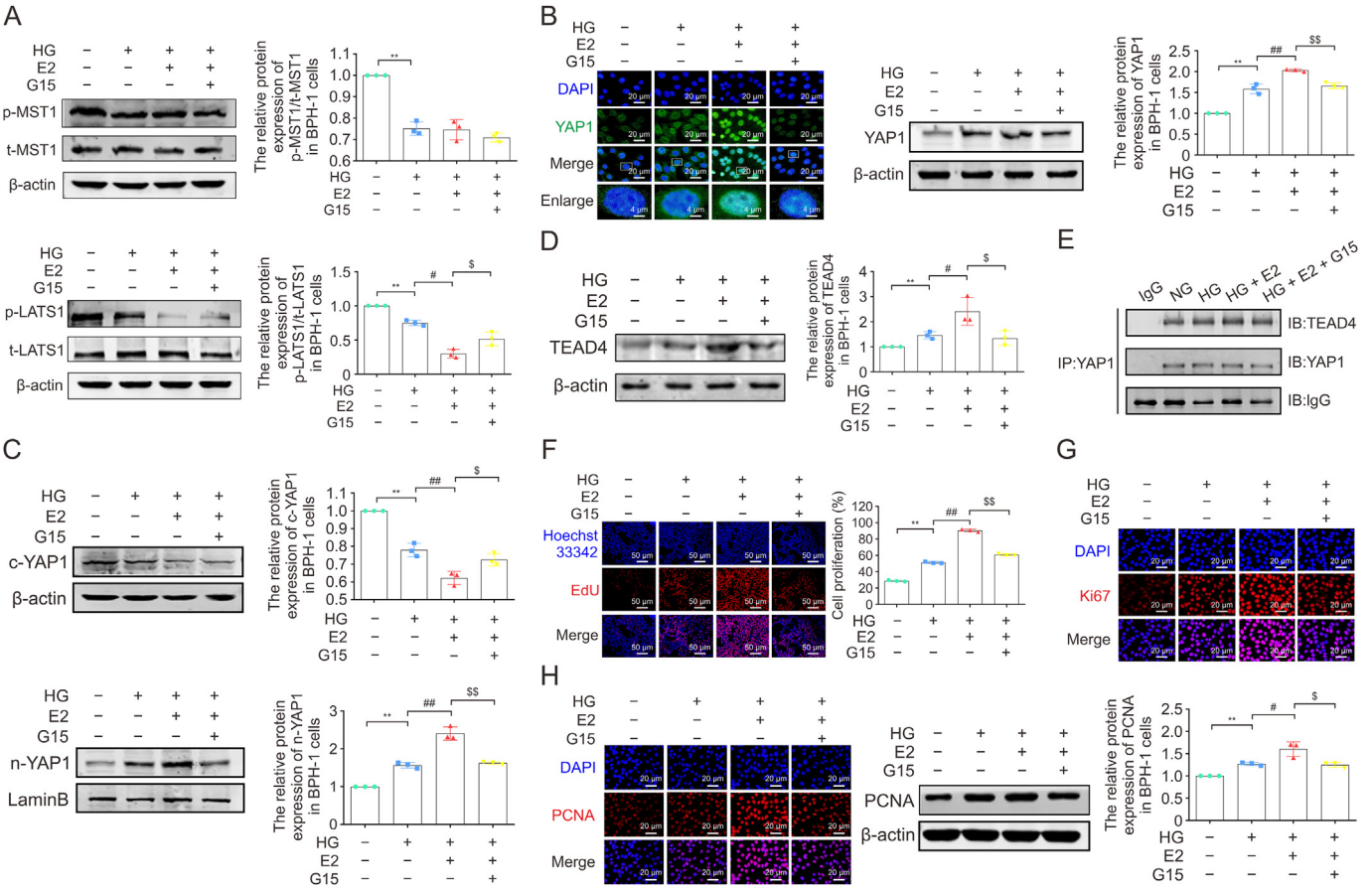

Fig. 7. Inhibition of G protein-coupled estrogen receptor (GPER) inhibited proliferation via Hippo-Yes-associated protein 1 (YAP1) signaling *in vitro*. (A) The expressions of phosphorylated mammalian sterile 20-like kinase (p-MST1) and phosphorylated large tumor suppressors 1 (p-LATS1) in benign prostatic hyperplasia (BPH)-1 cells are determined by Western blot analysis. (B) The expressions of p-LATS1 and p-MST1 in BPH-1 cells are assessed by immunofluorescence (IF) staining and western blot analysis. (C) The expressions of cytoplasm (c)-Yes-associated protein (YAP1) and nuclear (n)-YAP1 in BPH-1 cells are determined by Western blot analysis. (D) The expression of TEA/ATS domain 4 (TEAD4) in BPH-1 cells is determined by Western blot analysis. (E) The interactions between YAP1 and TEAD4 are determined by coimmunoprecipitation (Co-IP) in BPH-1 cells. (F) Proliferation of BPH-1 cells is determined by an 5-ethynyl-2′-deoxyuridine (EdU) assay. (G) The relative protein levels of Ki67 in BPH-1 cells are determined by IF staining. (H) The relative protein levels of proliferating cell nuclear antigen (PCNA) in BPH-1 cells are determined by IF staining and Western blot analysis. Each bar represents the mean ± standard error of mean (SEM) for groups of three. $^{**}P < 0.01$, compared to the normal glucose (NG) group as indicated; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to the high glucose (HG) group as indicated; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, compared to the HG + E2 group (4 nM) as indicated. DAPI: 4′,6-diamidino-2-phenylindole; t-LATS1: total large tumor suppressors 1; t-MST1: total mammalian sterile 20-like kinase.

small interfering RNA in *vitro* (Fig. S6). Western blot analysis of HG and E2 co-treated BPH-1 cells reveals that the protein expression of TEAD4 is decreased by YAP1 knockdown (Fig. 8A). The up-regulated YAP1-TEAD4 heterodimer induced by HG and E2 co-stimulation is reduced by YAP1 knockdown compared with that in the empty vector-transfected group (Fig. 8B). EdU staining reveals that YAP1 knockdown inhibits PECs proliferation, and the anti-proliferative effect is not affected by the virus vehicle (Fig. 8C). Moreover, the protein expression of Ki67 and PCNA are decreased by YAP1 knockdown (Figs. 8D and E).

Then, we used an inhibitor of YAP1, VP, to inhibit YAP1 expression. Our results show that in addition to the down-regulated YAP1, VP also blocks the nuclear translocation of YAP1 in HG and E2 co-treated BPH-1 cells (Fig. S7). The protein levels of TEAD4 are decreased by VP (Fig. 8F). VP treatment decreased the interaction between YAP1 and TEAD4 in HG and E2 co-treated BPH-1 cells (Fig. 8G). VP-mediated inhibition of YAP1 also inhibits the proliferation of BPH-1 cells co-treated with HG and E2 (Fig. 8H). Furthermore, the protein expression of Ki67 and PCNA are decreased by VP in *vitro* (Figs. 8I and J).

Although VP is an inhibitor of YAP1, it can also inhibit autophagy via a YAP1-independent pathway, leading to apoptosis evasion [36]. We select a YAP1-overexpressing lentiviral vector to prove that the anti-proliferative effect of VP is indeed achieved by inhibiting YAP1 in BPH-1 cells co-stimulated with HG and E2 (Fig. 9A). Along with the inhibited nuclear translocation of YAP1, YAP1 overexpression counteracts the effect of VP on TEAD4 levels in BPH-1 cells co-stimulated with HG and E2 (Figs. 9B and C). Compared with that in the HG + E2 + VP group, YAP1 overexpression increases YAP1-TEAD4 heterodimer in *vitro* (Fig. 9D). In addition, the anti-proliferative effect of VP is blocked by YAP1 overexpression, which also intercepts the regulation effect of VP on PCNA and Ki67 in HG and E2 co-treated BPH-1 cells (Figs. 9E–G). These results demonstrate that knocking down or inhibiting YAP1 suppresses the proliferation of BPH-1 cells co-treated with HG and E2.

4. Discussion

Dysregulated sex steroid hormones are key risk factors for T2DM. It has been well documented that the development and progression of BPH could be accelerated by the uncontrolled proliferation of PECs, which is also closely related to sex steroid hormones [37,38]. In view of these findings, we speculated that sex steroid hormone homeostasis may play a significant role in the

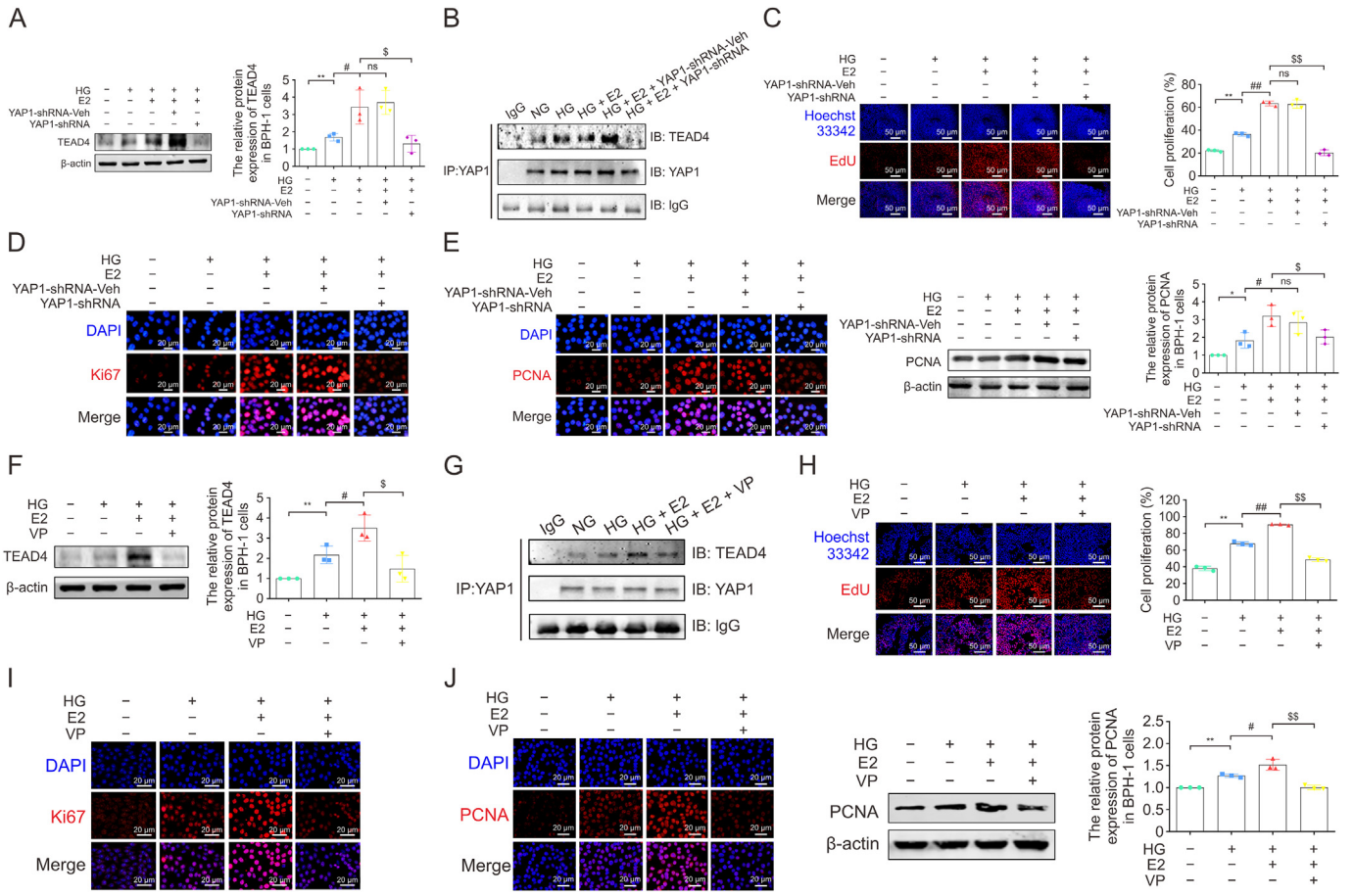

Fig. 8. Knocking down or inhibiting Yes-associated protein 1 (YAP1) inhibited proliferation in high glucose (HG) and estradiol (E2) co-cultured benign prostatic hyperplasia (BPH)-1 cells. (A) The expression of TEA/ATS domain 4 (TEAD4) in BPH-1 cells is determined by Western blot analysis. (B) The interactions between YAP1 and TEAD4 are determined by coimmunoprecipitation (Co-IP) in BPH-1 cells. (C) Proliferation of BPH-1 cells is determined by an 5-ethynyl-2′-deoxyuridine (EdU) assay. (D) The relative protein levels of Ki67 in BPH-1 cells are determined by immunofluorescence (IF) staining. (E) The relative protein levels of proliferating cell nuclear antigen (PCNA) in BPH-1 cells are determined by IF staining and western blot analysis. (F) The expression of TEAD4 in BPH-1 cells is determined by Western blot analysis. (G) The interactions between YAP1 and TEAD4 are determined by Co-IP in BPH-1 cells. (H) Proliferation of BPH-1 cells is determined by an EdU assay. (I) The relative protein levels of Ki67 in BPH-1 cells are determined by IF staining. (J) The relative protein levels of PCNA in BPH-1 cells are determined by IF staining and western blot analysis. Each bar represents the mean ± standard error of mean (SEM) for groups of three. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the normal glucose (NG) group as indicated; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to the HG group as indicated; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, compared to the HG + E2 group (4 nM) as indicated. ns: not significance. DAPI: 4′,6-diamidino-2-phenylindole; VP: verteporfin.

pathological process of simple disease in patients with T2DM complicated with BPH. With such a question in mind, we first evaluate the sex hormone metabolic profiles of different patient populations. The results of LC-MS/MS analysis, targeted metabonomic analysis and correlation analysis demonstrate that the disrupted sex hormones homeostasis (especially E2) may be involved in the emergency of BPH in T2DM. This hypothesis is further verified in HG-treated BPH-1 cells, and the data show that E2 decreases HG-induced PECs proliferation.

The physiological and pathological regulatory effects of estrogen are mediated by ERs, including the classical ERs (ERα and ERβ) and GPER [39]. The major function of the former is as ligand-activated transcription factors, and the latter activates a diverse array of signaling pathways [40]. ERs have been implicated in stimulating the development of prostate diseases, in addition to BPH and prostate cancer (PCa) [41,42]. Thus, we first compare the expression of ERα, ERβ and GPER between simple BPH patients and T2DM patients with BPH. A previous study confirmed that the expression of ERα and ERβ have no significantly changes in prostatic tissue specimens from early-stage BPH patients, elderly BPH patients or age-matched healthy men [43]. Activating GPER induces PEC apoptosis and prostatic stromal fibrosis and thereby contributes to BPH pathogenesis [43,44]. In this study, our results show that upregulated estrogen may function through GPER instead of ERα or ERβ in the progression of T2DM complicated with BPH, which is consistent with previous findings.

A recent study showed that sodium butyrate (NaB) inhibited the growth of BPH-1 cells via GPER [45]. Thus, short interfering RNAs, including shRNA-69526, shRNA-69527 and shRNA-69528, are used to confirm the important role of GPER in the proliferation of cells induced by E2 and HG co-culture. Green fluorescence in BPH-1 cells confirmed that the lentivirus invaded the cells, and the transfection efficiency of shRNA-69527 is the highest; thus, this strain is selected for subsequent experiments (Fig. S8). After confirming the anti-proliferative effect of GPER knockdown in HG and E2 co-treated BPH-1 cells, OP-1074, a specific degradation agent of ERα and ERβ, is used to verify the important role of GPER in promoting cell proliferation in *vitro*. Our results show that OP-1074 could not attenuate the proliferative effect of E2 in *vitro* and that it has no effect on the anti-proliferative effect of GPER knockdown on HG and E2 co-treated BPH-1 cells, confirming that E2 exacerbates HG-induced PEC proliferation by activating GPER, and rules out the involvement of ERα or ERβ-dependent signaling in this processes (Fig. S9).

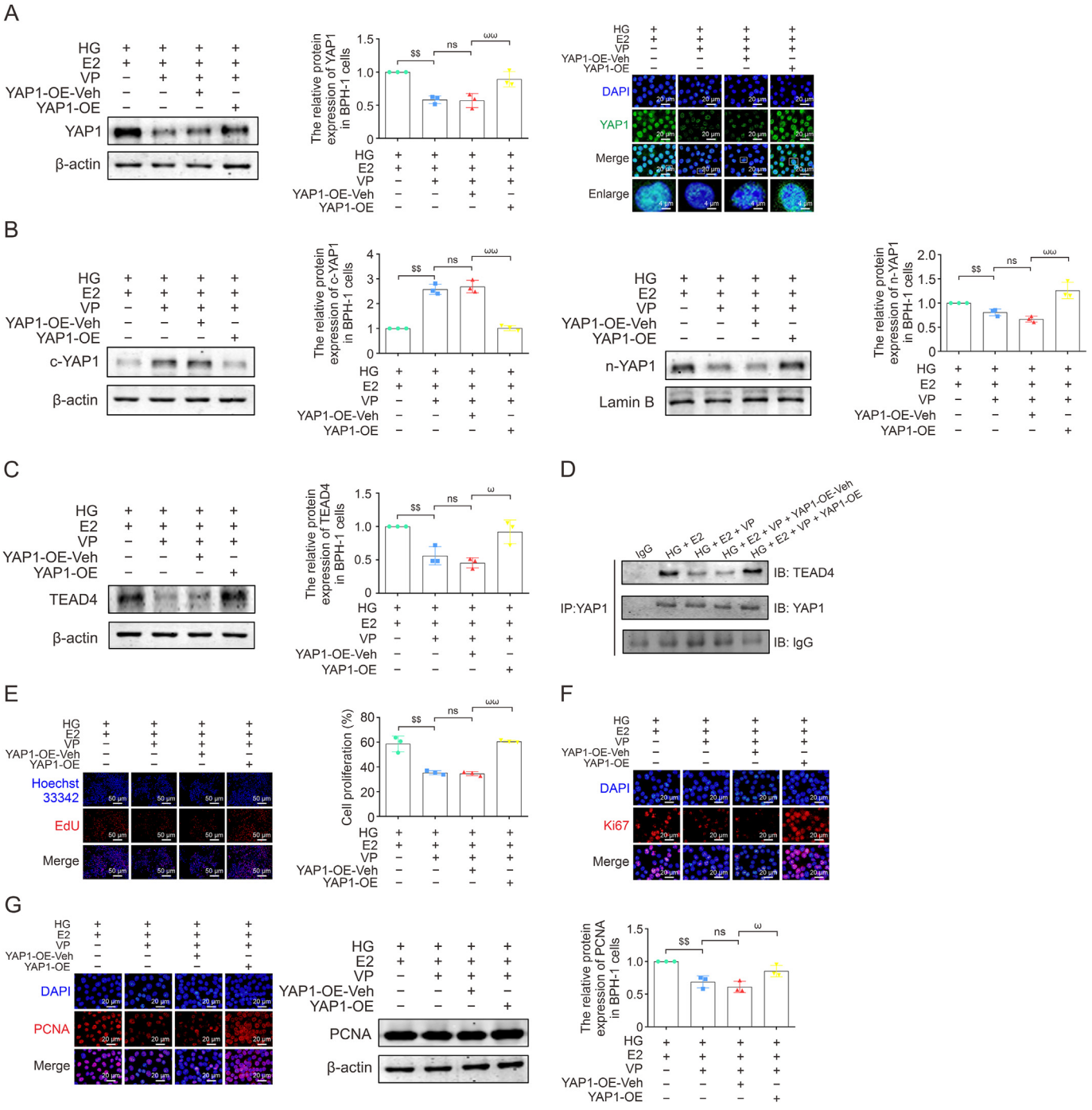

Fig. 9. Overexpression of Yes-associated protein 1 (YAP1) blocked the anti-proliferative effects of verteporfin (VP) *in vitro*. (A) The relative protein levels of YAP1 in BPH-1 cells are determined by immunofluorescence (IF) staining and western blot analysis. (B) The relative protein levels of cytoplasm (c)-YAP1 and nuclear (n)-YAP1 in BPH-1 cells are determined by Western blot analysis. (C) The relative protein levels of TEA/ATS domain 4 (TEAD4) in BPH-1 cells are determined by Western blot analysis. (D) The interactions between YAP1 and TEAD4 are determined by coimmunoprecipitation (Co-IP) in BPH-1 cells. (E) Proliferation of BPH-1 cells is determined by an 5-ethynyl-2′-deoxyuridine (EdU) assay. (F) The relative protein levels of Ki67 in BPH-1 cells are determined by IF staining. (G) The relative protein levels of proliferating cell nuclear antigen (PCNA) in BPH-1 cells are determined by IF staining and western blot analysis. Each bar represents the mean ± standard error of mean (SEM) for groups of three. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, compared to high glucose (HG) + E2 (4 nM) group as indicated. $^{\omega}P < 0.05$, $^{\omega\omega}P < 0.01$, compared to the HG + E2 (4 nM) + VP (5 μM) group as indicated. ns: not significance. DAPI: 4′,6-diamidino-2-phenylindole.

Numerous experimental studies have confirmed that the Hippo-YAP1 pathway regulates cell proliferation in a variety of diseases, including T2DM-associated disease and PCa [45–47]. As an important core component of Hippo signaling, activating MST1 stimulates the kinase LATS1 to phosphorylate downstream effectors. Our previous study of diabetic kidney disease (DKD) demonstrated that HG promoted the proliferation of mesangial cells via the Hippo-YAP1 pathway and that targeting MST1 could ameliorate DKD-associated renal fibrosis by forming a YAP1-TEAD4 heterodimer [29,32,48]. The Hippo-YAP1 pathway is a major downstream signal transduction pathway of the GPER signaling [34,35]. GPER stimulates human trophoblast invasion via YAP1, and targeted inhibition of GPER inhibits bisphenol S-induced migration of triple-negative breast cancer (TNBC) cells via the Hippo-YAP1

pathway [35,49]. Although studies have verified that the Hippo-YAP1 pathway is involved in the pathogenesis of PCa, its role in PECs proliferation is still unclear in T2DM complicated with BPH. Therefore, the Hippo-YAP1 pathway is first examined in the prostate tissue of patients in our study. On the basis of the results of clinical studies, we verify the findings of the Hippo-YAP1 pathway by using HG and EE co-treated BPH-1 cells, and our findings demonstrate that the Hippo-YAP1 pathway may participate in proliferation regulation of T2DM complicated with BPH.

GPER is a G-protein-coupled receptor (GPCR) of estrogen, and stimulation of GPCRs by glucagon or epinephrine activates LATS1 kinase activity, thereby inhibiting YAP1 function [50]. A study of human trophoblast cells show that activating GPER with E2 or G1 (a selective GPER agonist) decreases the phosphorylation level of LAST1 but have no effect on the phosphorylation of MST1 [49]. After finding that the LSTA1/YAP1 pathway is the major down-stream signaling branch of the E2/GPER pathway in PECs proliferation of patients with T2DM complicated with BPH, a GPER-specific inhibitor is used to further verify this speculation. G15, a specific inhibitor of GPER, suppress the estrogen-induced promotion of cell proliferation in goat mammary epithelial cells by inhibiting GPER [51]. Here, our data show that 50 nM G15 do not cause any obvious cytotoxic effects, whereas 100 nM G15 significantly decrease cell viability (Fig. S10A). G15 dose-dependently decrease the protein expression of GPER at 1 and 50 nM (Fig. S10B). Thus, 50 nM G15 is tested for its anti-proliferative effects on HG and E2 co-treated BPH-1 cells (Fig. S11). Here, our results verify that inhibition or knockdown of GPER exerts anti-proliferative role in HG and E2 co-treated BPH-1 cells via the LSTA1/YAP1 pathway.

Study showed that downregulation of YAP1 reduced the proliferation of prostate carcinoma cell [52]. Based on the above findings, we further propose that inhibiting YAP1 signaling could suppress the proliferation of HG and E2 co-treated BPH-1 cells. Short interfering RNAs (siRNAs) targeting YAP1 and VP, which is inhibitor of the YAP1-TEAD4 complex, are used to identify the role of YAP1 in PEC proliferation. Our data show that VP at a dose of 5 μM significantly decreases YAP1 expression in *vitro* (Fig. S12). As a result, 5 μM VP is selected for further study for its anti-proliferative effect. VP suppress the proliferation of multiple cancer cell types, and our results show that the promotion of PECs proliferation induced by HG and E2 co-culture is inhibited by YAP1 knockdown or VP [53–55]. A study reported that VP is also an autophagy inhibitor and that it has a YAP1/TEAD4-independent effect on autophagy and oxidative phosphorylation, enhancing its antitumor activity [56]. Therefore, a YAP1-overexpressing lentiviral vector is used to verify the important role of YAP1 in the anti-proliferative effect of VP on HG and E2 co-treated BPH-1 cells.

It is well known that the levels of E2 are closely related to energy metabolism in multiple organs, such as the liver, fat, muscle, and pancreas [57–60]. Being produced by adipose tissues, adipokines had a closely relationship with steroidogenesis, and E2 could also regulate the production of adipokines in adipose tissues [61,62]. There was a linkage between adipokines and IR, and the latter one is the key factor between the simple BPH patients and T2DM patients with BPH [25,63]. Besides, the acquirement of IR is associated with lower levels of T, indicating that IR may be the linkage between steroid hormones and energy metabolism in T2DM patients with BPH, which need to be verified by further research in the futher [15].

Here, we identify a new mechanism by which T2DM is complicated with BPH and proposed that targeting E2/GPER signaling could delay the pathology of T2DM complicated with BPH by suppressing Hippo-YAP1 pathway-mediated PECs proliferation. This study increased the understanding of the proliferative effect of the E2/GPER signaling-mediated Hippo-YAP1 pathway on HG and E2 co-treated BPH-1 cells. However, this study has several limitations. First, E2 not only participates in the regulation of energy metabolism in adulthood, but also plays a key role in the early stages of life, as well as in infancy and young adulthood [64,65]. A study demonstrated that E2 could regulate hepatic lipid metabolism through activating PKA, MAPK, and PI3K signaling via GPER [66]. Nevertheless, the regulatory effect of E2/GPER signaling on energy metabolism of T2DM complicated with BPH was not revealed in *vivo*. Second, even though we explore problems from clinical practice, and study the regulation mechanism by using BPH-1 cells, further *in vivo* evaluation was not carried out in present study. Third, short interfering RNAs (siRNAs) targeting GPER and specific inhibitors of GPER, were used to verify the important role of GPER in PTC proliferation *in vitro*, however, lentiviral-mediated GPER KO was not generated in BPH-1 cells, which need to be investigated in future studies. Fourth, androgen, especially DHT, also plays a key role in the pathology of BPH. Our study showed that even though the *VIP* value of E2 was the highest, the *VIP* value of DHT was greater than 1 and ranked second. Thus, another limitation was that we still unclear whether DHT was involved in the pathology of T2DM complicated with BPH, which was our other ongoing work.

5. Conclusions

Our data reveal that E2, the greatest contributor to the disrupted sex steroid hormone metabolic profiles in T2DM patients complicated with BPH, dose-dependently exacerbate the cell proliferation in HG treated BPH-1 cells via activating GPER. Further mechanistic studies show that E2 exacerbated PECs proliferation induced by HG through GPER-mediated Hippo-YAP1 signaling (Fig. 10). Our findings emphasize that targeting E2/GPER signaling through Hippo-YAP1 signaling may be a novel therapeutic approach for treating the T2DM patients complicated with BPH via the inhibition of PECs proliferation.

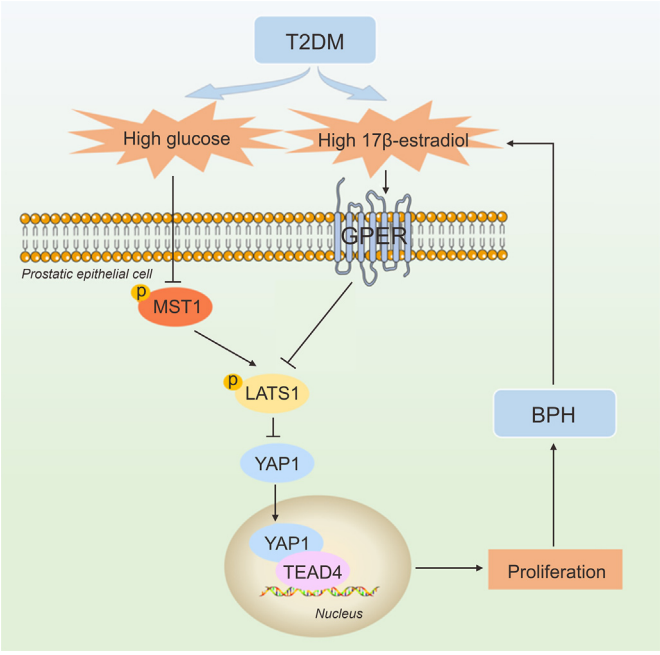

Fig. 10. The proposed model of estradiol (E2) was found to be the main promoter of type 2 diabetes mellitus (T2DM) complicated with benign prostatic hyperplasia (BPH). E2 may exacerbate high glucose (HG)-induced BPH-1 cell proliferation via the G protein-coupled estrogen receptor (GPER)-mediated Hippo–Yes-associated protein 1 (YAP1) signaling pathway. LATS1: large tumor suppressor 1; MST1: mammalian sterile 20-like kinase; TEAD4: TEA/ATS domain 4; YAP1: Yes-associated protein 1.

CRediT author statement

Tingting Yang: Conceptualization, Investigation, Funding acquisition, Supervision, Writing-original draft, Writing-review & editing; **Zhen Qiu**: Formal analysis, Data curation, Investigation, Project administration, Writing-original draft; **Jiaming Shen**: Software, Investigation, Writing- review & editing, Validation; **Yutian He**: Software, Investigation, Writing- review & editing, Validation; **Longxiang Yin**: Software, Investigation, Writing- review & editing, Validation; **Li Chen**: Writing- review & editing, Validation; **Jiayu Yuan**: Formal analysis, Data curation, Investigation, Project administration; **Junjie Liu**: Investigation, Project administration; **Tao Wang**: Methodology, Visualization; **Zhenzhou Jiang**: Methodology, Visualization; **Changjiang Ying**: Investigation, Project administration; **Sitong Qian**: Investigation, Project administration. **Jinfang Song**: Investigation, Project administration; **Xiaoxing Yin**: Conceptualization, Funding acquisition, Supervision, Writing-review & editing; **Qian Lu**: Conceptualization, Funding acquisition, Supervision, Writing-review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.03.003.

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