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Cyclooxygenase 2 Promotes Proliferation and Invasion in Ovarian Cancer Cells via the PGE2/NF-κB Pathway

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Abstract

Ovarian cancer is the leading cause of death among gynecological malignancies. Cyclooxygenase 2 is widely expressed in various cancer cells and participates in the occurrence and development of tumors by regulating a variety of downstream signaling pathways. However, the function and molecular mechanisms of cyclooxygenase 2 remain unclear in ovarian cancer. Here, we demonstrated that cyclooxygenase 2 was highly expressed in ovarian cancer and the expression level was highly correlated with ovarian tumor grades. Further, ovarian cancer cells with high expression of cyclooxygenase 2 exhibit enhanced proliferation and invasion abilities. Specifically, cyclooxygenase 2 promoted the release of prostaglandin E2 upregulated the phosphorylation levels of phospho-nuclear factor-kappa B p65. Celecoxib, AH6809, and BAY11-7082 all can inhibit the promoting effect of cyclooxygenase 2 on SKOV3 and OVCAR3 cell proliferation and invasion. Besides, celecoxib inhibited SKOV3 cell growth in the xenograft tumor model. These data suggest that high expression of cyclooxygenase 2 promotes the proliferation and invasion of ovarian cancer cells through the prostaglandin E2/nuclear factor-kappa B signaling pathway. Cyclooxygenase 2 may be a potential therapeutic target for the treatment of ovarian cancer.

Keywords

Ovarian cancer, cyclooxygenase 2, proliferation, invasion, PGE2

Introduction

Ovarian cancer is one of the most common fatal tumors of the female reproductive tract and is the leading cause of death among gynecological malignancies[1]. Due to its asymptomatic development, the disease is often diagnosed at an advanced stage of incurability. Although ovarian cancer usually responds well to first-line chemotherapy based on platinum compounds and taxanes, most patients develop recurrence and chemical resistance[2]. The etiology of ovarian cancer remains largely unclear. Therefore, exploring the pathogenesis of ovarian cancer and finding new therapeutic targets is very important for improving the prognosis of patients with ovarian cancer.

Cyclooxygenases (COXs) are members of a family of myeloperoxidases that catalyze the biosynthesis of prostaglandins (PGs) from arachidonic acid. COX2 has low activity in normal tissue cells and can be transiently induced by stimuli such as cytokines, growth factors, mitogens, tumor promoters, and hormones. When cells are stimulated by these factors, the induced COX2 can promote the local synthesis of PGE2[3]. COX2 is also reported to regulate inflammation, differentiation, mitogenesis, and angiogenesis[3], which raises the likelihood that COX2 might be involved in tumorigenesis. Indeed, COX2 is highly expressed in tumor tissues; this high expression can affect the occurrence and development of tumors via various means by regulating downstream metabolites and stimulating tumor cell proliferation and invasion, thus playing an important role in disease progression[4]. In addition, both PGE2 and PGE2 Receptor 2 (PTGER2) have been shown

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to participate in cancer cell survival[5,6]. Therefore, the COX2/PGE2/PTGER2 pathway plays a crucial role in tumorigenesis.

COX2 is also found to be highly expressed in ovarian cancer tissues, and promotes the proliferation and invasion of ovarian cancer cells[7,8]. Uddin et al. proposed that COX2 plays an important role in ovarian tumor growth by regulating the key carcinogenic pathway of cancer cell dependence[9]. In addition, COX2 and its downstream gene PGE2 regulate the ovarian cancer cell invasion and metastasis by promoting the expression of matrix metalloproteinases (MMP2 and MMP9)[10]. However, some studies even have reversed effects of COX2 in ovarian cancer. Yoshida et al. proposed that cell proliferation activity in ovarian tumors is not related to COX2[11].

Nevertheless, the role of COX2 in proliferation and invasion of ovarian cancer and the corresponding molecular mechanisms remain unclear. The current study aims to characterize the expression pattern of COX2 in different ovarian tumor grades and evaluate the function of COX2-associated molecular pathway in ovarian cancer. Here, we provide the evidence that COX2 expression level is highly correlated with ovarian tumor grades. High expression of COX2 enhances the proliferation and invasion in ovarian cancer cells, which may attribute to the upregulation of PGE2 release and phosphorylation of nuclear factor-kappa B (NF-κB). This molecular pathway will shed light on unraveling the pathogenesis of ovarian cancer and provide clues for identifying new therapeutic targets.

Materials and Methods

Patients and Ovarian Tumor Specimens

We retrospectively analyzed 89 formalin-fixed paraffin-embedded ovarian tissue samples from patients diagnosed with different grades of ovarian tumors and subjected to ovariectomy between 2014 and 2016 at the China-Japan Friendship Hospital. The pathologic diagnosis of these specimens was evaluated by at least two pathologists according to the International Federation of Gynecology and Obstetrics 2013 criteria. The specimens included 17 cases of ovarian cystadenoma (a type of benign tumor that develops from ovarian tissue), 17 cases of borderline ovarian cancer, 27 cases of ovarian cancer, and 28 cases of metastatic ovarian cancer. The study protocol was approved by the Ethics Committee of the China-Japan Friendship Hospital. Specimens were handled and procedures were carried out in accordance with the approved guidelines.

Animals

A total of 25 5-week-old female BALB/c nude mice were purchased from Sibefu Biotechnology Co. (Beijing, China). The mice were bred under specific pathogen-free conditions with a 12 hour/12 hour light/dark cycle and fed food and water *ad libitum*. All procedures performed on animals were approved by the Institutional Animal Care and Use Committee of the China-Japan Friendship Hospital.

Immunohistochemical Staining and Evaluation of Immunoreactivity

Tumor tissue samples were fixed in 10% formalin, embedded, and cut into 5 μm slices. Briefly, slides were baked at 60°C for 30 minutes, dewaxed with xylene, and rehydrated in an ethanol gradient, followed by incubation in 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were immersed in citrate and microwaved for antigen retrieval. Then, sections were incubated at 4°C with specific primary antibodies against COX2 (1:500, Cell Signaling Technologies, Danvers, MA, USA, cat. no. 12282), p-NF-κB p65 (1:500, Cell Signaling Technologies, cat. no. 3031), CYP19 (1:100, Santa Cruz, Dallas, TX, USA, cat. no. sc-374176), Ki-67 (1:100, Abcam, Cambridge, MA, USA, cat. no. ab16667), MMP2 (1:100, Cell Signaling Technologies, cat. no. 4022), and MMP9 (1:200, Cell Signaling Technologies, cat. no. 3852), followed by incubation with a secondary antibody (ZSGB-BIO, Beijing, China). Then 3,3'-diaminobenzidine (ZSGB-BIO) was used to produce a brown stain. Finally, sections were counterstained with hematoxylin, dehydrated, and covered with coverslips for further analyses. Slides were scanned via a computerized imaging system comprising an Olympus CCD camera (Tokyo, Japan) connected to a Nikon Eclipse Ti-S microscope (Tokyo, Japan).

Immunoreactivity was semiquantitatively evaluated on the basis of staining intensity and distribution using an immunoreactivity score as intensity score × proportion score. The intensity score was defined as: 0, negative; 1, weak; 2, moderate; or 3, strong. The proportion score was defined as: 0, negative; 1, < 10%; 2, 11–50%; 3, 51–80%; or 4, > 80% positive cells. The total score ranged from 0 to 12. The stained cervical tissues were scored by two researchers who were blinded to the clinical data.

Cell Lines and Cell Culture

The human ovarian cancer cell lines SKOV3 and OVCAR3 were purchased from the American Type Culture Collection. McCoy's 5A medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen) was used to culture SKOV3 cells. OVCAR3 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 20% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2.

Cell Transfection

To generate cell lines overexpressing COX2, SKOV3 and OVCAR3 cells were transfected with the lentiviral vector Lenti-COX2-CMV-EGFP or with Lenti-EGFP as the

control. Lentivirus was obtained from GeneChem (Shanghai, China). SKOV3 and OVCAR3 cells were seeded in 24-well plates in the appropriate medium, grown to a confluence of approximately 50%, and transfected with lentivirus according to the manufacturer's protocol. Cells stably overexpressing COX2 were selected from the transfected cultures with 400 μg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA) for 8 days.

Cell Viability Assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in a 96-well flat-bottomed plate (2×10^3 cells in 100 μl per well), incubated overnight to allow cell attachment, and exposed to 100 mM celecoxib (Pfizer, New York, NY, USA), 10 μM AH6809 (ApexBio Technology, Houston, TX, USA) or 10 μM BAY11-7082 (ApexBio Technology) for 24, 48, 72, and 96 hour. Then 10 μl of CCK-8 solution was added to each well, the cells were incubated for another 2 hours, and the absorbance at 450 nm was measured with a microplate reader. All experiments were performed thrice.

Quantitative Real Time Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen). Next, 1 μg of total RNA was converted to cDNA using oligo-dT primers and Superscript III reverse transcriptase (Takara, Shiga, Japan). Quantitative polymerase chain reaction (qPCR) was performed using an iQ™ SYBR® Green Supermix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a CFX96 Touch™ real-time PCR instrument (Bio-Rad Laboratories, Inc.), using oligonucleotides specific for human COX2 (forward: 5'-TCAAGTCCCTGAGCATCTACGGTT-3', reverse: 3'-CTGTTGTGTTCCCGCAGCCAGATT-5'), PTGER2 (forward: 5'-GTCTGCTCCTTGCCTTTCAC-3', reverse: 3'-TGAACGCATTAGTCTCAGAACAG-5') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-CTTAGCACCCCTGGCCAAG-3', reverse: 3'-GATGTTCTGGAGAGCCCCG-5'). All experiments were performed thrice.

Western Blot Analysis

Total protein was extracted from cells and tumor tissues by adding radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) containing 1% phenylmethanesulfonyl fluoride (Beyotime) and incubated on ice. Equal amounts of protein were separated by gel electrophoresis and transferred onto polyvinylidene fluoride (Merck Millipore, Darmstadt, Germany) membranes. Membranes were blocked with 5% non-fat dry milk and were then incubated with primary antibodies specific for GAPDH (1:1000, Cell Signaling Technologies, cat. no. 2118), COX2 (1:1000, Cell Signaling Technologies, cat. no. 12282), p-NF-κB p65 (1:1000, Cell Signaling Technologies, cat. no. 3031), NF-κB p65 (1:1000, Cell Signaling Technologies, cat. no. 8242), CYP19 (1:1000, Santa Cruz, Dallas, TX, USA, cat. no. sc-374176), C-MYC (1:1000, Cell Signaling Technologies, cat. no. 5605), STAT3 (1:1000, Cell Signaling Technologies, cat. no. 9139), p-STAT3 (1:2000, Cell Signaling Technologies, cat. no. 9145), MMP2 (1:1000, Cell Signaling Technologies, cat. no. 4022), MMP9 (1:1000, Cell Signaling Technologies, cat. no. 3852), and PTGER2 (1:400, Bosterbio Technologies, Pleasanton, CA, USA, cat. no. BM5194) overnight at 4°C. Incubation with horseradish peroxidase (HRP)-linked anti-rabbit IgG (1:1000, Cell Signaling Technologies, cat. no. 7074) or HRP-linked anti-mouse IgG (1:1000, Cell Signaling Technologies, cat. no. 7076) secondary antibodies according to the species reactivity of the primary antibodies was performed at room temperature for 1 hour. Antibody binding was detected using enhanced chemiluminescence detection buffer and an Alpha Innotech imaging system (San Leandro, CA, USA). All experiments were performed in triplicate.

Transwell Assays

To assess cell invasion *in vitro*, we used 24-well Transwell chambers (Corning Inc., Newark, NJ, USA) coated with Matrigel (BD Biosciences, San Diego, CA, USA). Cells were trypsinized and seeded into the upper chamber at a density of 5×10^4 cells per well in 2 ml of fresh medium containing 1% FBS and 100 mM celecoxib, 10 μM AH6809, or 10 μM BAY11-7082. The lower chambers contained 2 ml of medium supplemented with 10% FBS. After incubation at 37°C for 24 hour, cells attached to the upper surface of the membrane were carefully removed with cotton swabs, whereas cells that invaded the underside of the membrane were fixed with 10% formalin, stained with crystal violet for 3 minutes at room temperature and counted. All experiments were performed thrice.

Enzyme-Linked Immunosorbent Assay

Cells were seeded in six-well plates. Then, 100 mM celecoxib was added to the medium of cells overexpressing COX2, and the cells were cultured for 24 hours. The supernatant was collected and PGE2 released into the culture medium was measured using commercially available enzyme-linked immunosorbent assay kits from Invitrogen. All experiments were performed thrice.

Tumor Xenograft Mouse Model

A total of 25 nude mice were randomized into five groups and either 1×10^7 SKOV3-Lenti-GFP or 1×10^7 SKOV3-Lenti-COX2 cells resuspended in 0.1 ml of serum-free phosphate buffered saline were injected into the left armpit of the mice. Then 10 days after this injection, the mice in the four groups injected with SKOV3-Lenti-COX2 cells were

administered an intraperitoneal injection of 5 mg/kg cisplatin (DDP, Abcam, Cambridge, MA, USA), 5 mg/kg celecoxib, 5 mg/kg DDP combined with 5 mg/kg celecoxib, or an equal volume of normal saline three times weekly for 28 days. The mice injected with SKOV3-Lenti-GFP cells were administered normal saline. The volume (V=[length × width^2]/2) of the subcutaneous tumors was measured three times weekly. After the end of treatment, we measured the green fluorescent protein (GFP) fluorescence signal intensity in the xenografted mice using a Multi-functional *In Vivo* Imaging System (Molecular Devices, Shanghai, China). Then, the mice were sacrificed and the tumor tissues were harvested, fixed in 10% formalin and embedded in paraffin for histological analyses.

Statistical Analysis

Data are expressed as the means ± SDs. Analysis of variance was used to evaluate the differences between groups using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) with Dunn's test as post hoc. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

COX2 Expression is Upregulated in Ovarian Cancer Tissues

We examined the COX2 expression in ovarian tissues from patients with ovarian cystadenoma, borderline ovarian cancer, ovarian cancer, and metastatic ovarian cancer to verify the expression level of COX2 in different grades of ovarian tumors. We retrospectively analyzed 89 samples from patients subjected to ovariectomy. The expression of COX2 and CYP19 in the cytoplasm of specimens from patients with ovarian cancer or metastatic ovarian cancer was significantly higher than that in specimens from patients with ovarian cystadenoma or borderline ovarian cancer, as was NF-κB in nucleus (Figure 1). We further calculated the relationship between the expression level of COX2, NF-κB, and CYP19 and the grade of ovarian tumor, and found expression levels of COX2, NF-κB, and CYP19 are positively correlated with ovarian cancer grades ($r = 0.757, 0.717, 0.649$ respectively; $p < 0.01$).

COX2 Promotes Ovarian Cancer Cell Proliferation and Invasion

To determine whether COX2 affects ovarian cancer cell proliferation and invasion, we first overexpressed COX2 in two ovarian cancer cell lines, SKOV3 and OVCAR3 (Figure 2(A)). When COX2 was overexpressed, the proliferation of SKOV3-Lenti-COX2 and OVCAR3-Lenti-COX2 cells was enhanced, and the differences were statistically significant at 72 hours and 96 hours compared with the corresponding Lenti-GFP cells. That proliferation was significantly decreased in both cell lines after COX2 was inhibited with celecoxib (Figure 2(B)). To verify the effect of COX2 on the proliferation of ovarian cancer cells, we then examined the expression of nuclear protein Ki67 associated with proliferation in SKOV3 and OVCAR3 cells. When COX2 was overexpressed, Ki67 expression was increased in SKOV3 and OVCAR3 cells and that Ki67 expression was decreased in both ovarian cancer cell lines after treatment with the COX2 inhibitor celecoxib (Figure 2(C) and 2(D)). These data indicate that COX2 can promote the proliferation of ovarian cancer cells. We further assessed the effect of COX2 on the invasion of these cells via Transwell assays. Overexpression of COX2 enhanced the invasion of SKOV3 and OVCAR3 cells. When celecoxib inhibited COX2, it prevented the invasion of these cells significantly (Figure 2(E) and 2(F)). These results indicate that COX2 can promote the invasion of ovarian cancer cells. Although COX2 was highly expressed, the levels of CYP19, C-MYC, p-STAT3, MMP2, and MMP9 in SKOV3 and OVCAR3 cells were upregulated. However, when COX2 was inhibited by celecoxib, the expression level of these proteins in SKOV3 and OVCAR3 cells was reduced (Figure 2(G) and S1).

Effect of COX2 on the Levels of PGE2, PTGER2 and NF-κB

To investigate how PGE2 and PTGER2 are regulated by COX2, we examined the effect of COX2 on their levels. We found that COX2 significantly increased the level of PGE2 in SKOV3 and OVCAR3 cell culture supernatants, indicating that COX2 promoted the synthesis and release of PGE2 by ovarian cancer cells (Figure 3(A)). Correspondingly, this effect was inhibited by celecoxib (Figure (A)). However, neither COX2 nor celecoxib affected the expression level of PTGER2 in SKOV3 and OVCAR3 cells (Figure 3(B) and 3(C)). These results suggest that COX2 affects ovarian cancer cells by increasing the PGE2 level but not PTGER2. We also examined the effect of COX2 on p-p65. We found that COX2 increased the level of p-p65 in SKOV3 and OVCAR3 cells, which could be inhibited by celecoxib (Figure 3(B) and 3(C)). These results indicate that COX2 may upregulate the phosphorylation of p65, which may promote the proliferation and invasion in ovarian cancer cells.

COX2 Promotes Ovarian Cancer Cell Proliferation and Invasion Through the PGE2/NF-κB Pathway

To further explore whether the proliferation and invasion of ovarian cancer cells promoted by COX2 is through the PGE2/NF-κB pathway, we treated SKOV3 and OVCAR3 ovarian cancer cells overexpressing COX2 with the PTGER2 inhibitor AH6809 and the NF-κB inhibitor BAY11-7082. After inhibition of PTGER2 or NF-κB, the proliferation of SKOV3 and OVCAR3 cells overexpressing COX2 was decreased significantly (Figure 4(A)). The expression of Ki67 in SKOV3-Lenti-COX2 and OVCAR3-Lenti-COX2 was also decreased significantly after the addition of PTGER

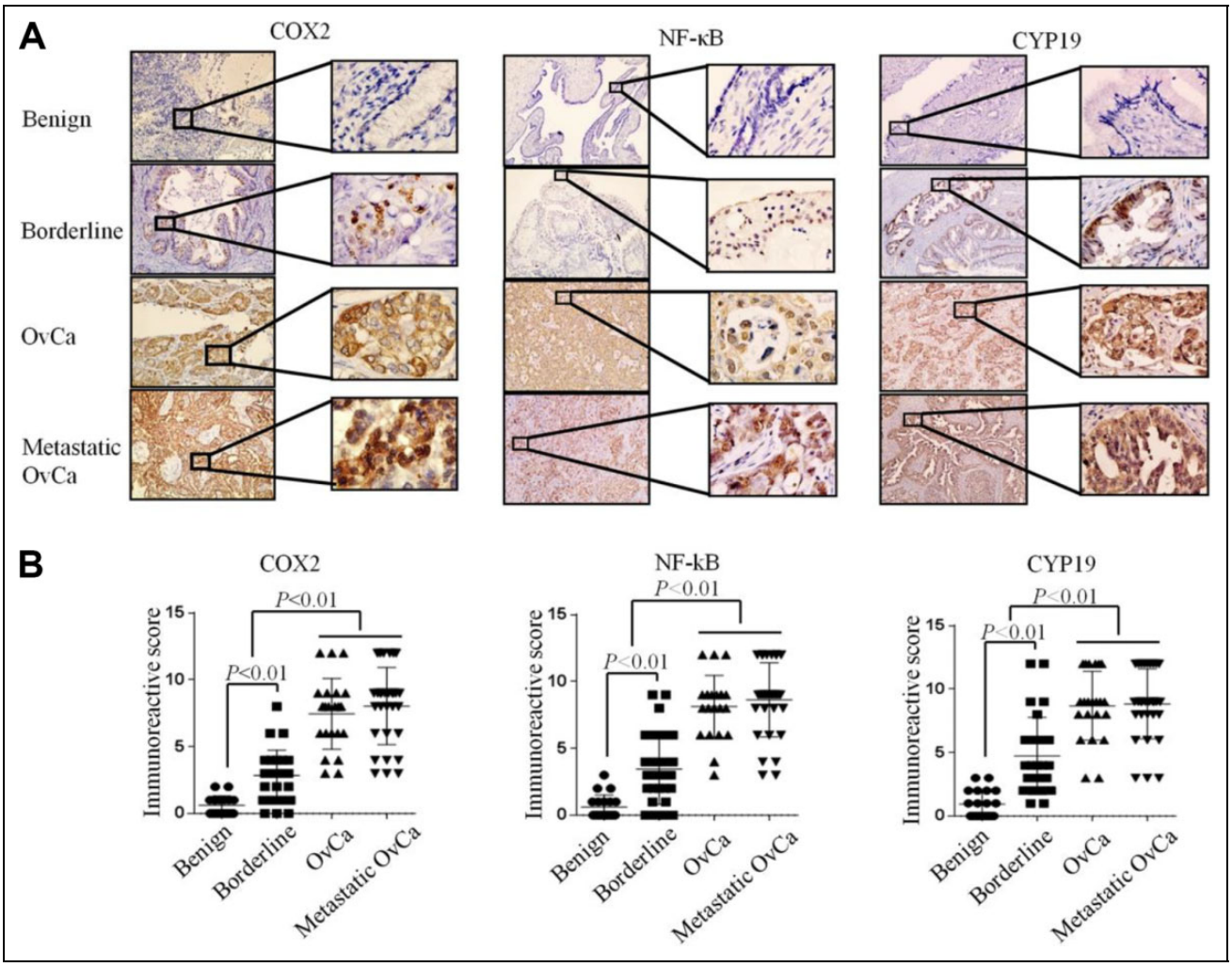

Figure 1. Characteristic cyclooxygenase 2 (COX2) expression levels in ovarian cancer. (A) COX2 expression was detected by immunohistochemical (IHC) staining in ovarian cystadenoma, borderline ovarian cancer, ovarian cancer, and metastatic ovarian cancer tissues. Magnification: ×400. (B) Immunoreaction score of COX2, nuclear factor-kappa B (NF-κB), and CYP19 staining in ovarian tissues. Analysis of variance was used to evaluate the differences between groups with Dunn's test as post hoc.

of NF-κB inhibitors (Figure 4(B) and 4(C)). These data indicate that COX2 may promote the proliferation of ovarian cancer cells through the PGE2/NF-κB signaling pathway. Then we assessed the effect of AH6809 and BAY11-7082 on the invasion of SKOV3-Lenti-COX2 and OVCAR3-Lenti-COX2 cells. The invasion of SKOV3-Lenti-COX2 and OVCAR3-Lenti-COX2 cells treated with AH6809 or BAY11-7082 was statistically decreased (Figure 4(D) and 4(E)), indicating that COX2 may promote the invasion of ovarian cancer cells through the PGE2/NF-κB signaling pathway. Finally, we evaluated the effect of AH6809 and BAY11-7082 on the levels of COX2, p-NF-κB p65, and proteins involved in proliferation and invasion via western blotting. We found that AH6809 and BAY11-7082 suppressed the levels of p-p65, CYP19, C-MYC, p-STAT3, MMP2, and MMP9 in SKOV3-Lenti-COX2 and OVCAR3-Lenti-COX2 cells. However, the expression of COX2 in both cells was not affected by AH6809 and BAY11-7082 (Figure 4(F) and S2). Thus, after the inhibition of PTGER2 or NF-κB phosphorylation, the promotive effect of COX2 on the proliferation and invasion in ovarian cancer cells was decreased.

Targeting COX2 Inhibits Xenograft Tumor Growth

After SKOV3-Lenti-GFP and SKOV3-Lenti-COX2 cells were implanted into 5-week-old nude mice, tumor growth was observed for approximately 10 days before treatment was initiated. During the 4-week treatment period, we measured the volume of subcutaneous tumors three times weekly. SKOV3-Lenti-COX2 cells formed tumors with a larger volume than those formed by SKOV3-Lenti-GFP cells. However, the volume of tumors in mice injected with SKOV3-Lenti-COX2 cells and treated with celecoxib or celecoxib combined with DDP increased slightly and even

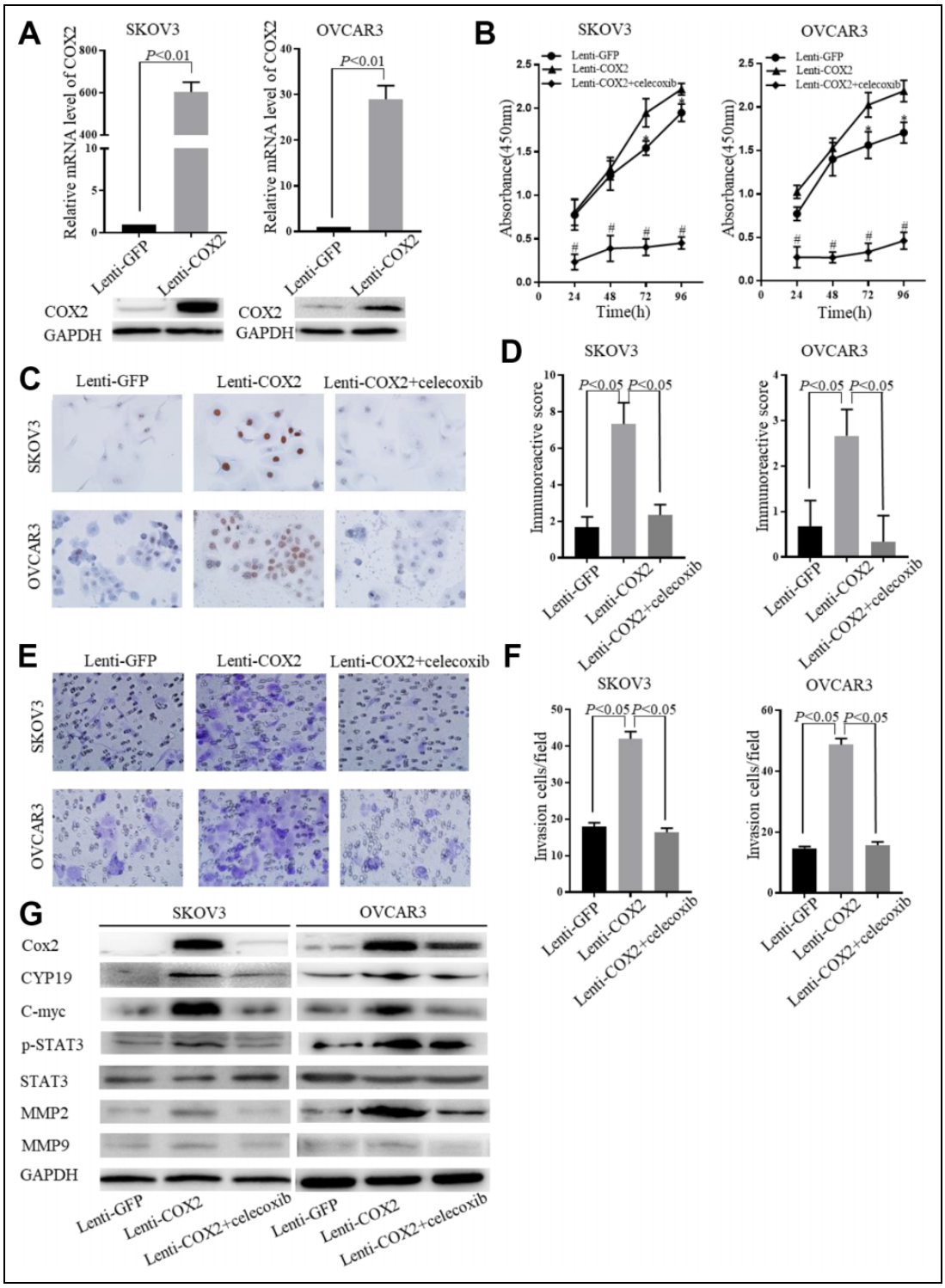

Figure 2. Cyclooxygenase 2 (COX2) promotes ovarian cancer cell proliferation and invasion. (A) The expression level of COX2 was measured by quantitative polymerase chain reaction (qPCR) and western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as the loading control. (B) The viability of SKOV3 and OVCAR3 cells was measured daily by a CCK-8 assay. [a]Compared Lenti-COX2 with Lenti-GFP, $p < 0.05$; [b]compared Lenti-COX2+celecoxib with Lenti-COX2, $p < 0.05$. (C) and (D) Representative immunohistochemical (IHC) staining of SKOV3 and OVCAR3 cells conducted with an antibody against Ki67. Magnification: ×400. (E) and (F) SKOV3 and OVCAR3 cells in growth media were seeded in 24-well Transwell chambers coated with Matrigel and cultured for 24 hours. Cell invasion was estimated. (G) The expression levels of CYP19, C-MYC, STAT3, p-STAT3, matrix metalloproteinase 2 (MMP2), and MMP9 were estimated by western blot analysis. The data are presented as the means ± SDs.

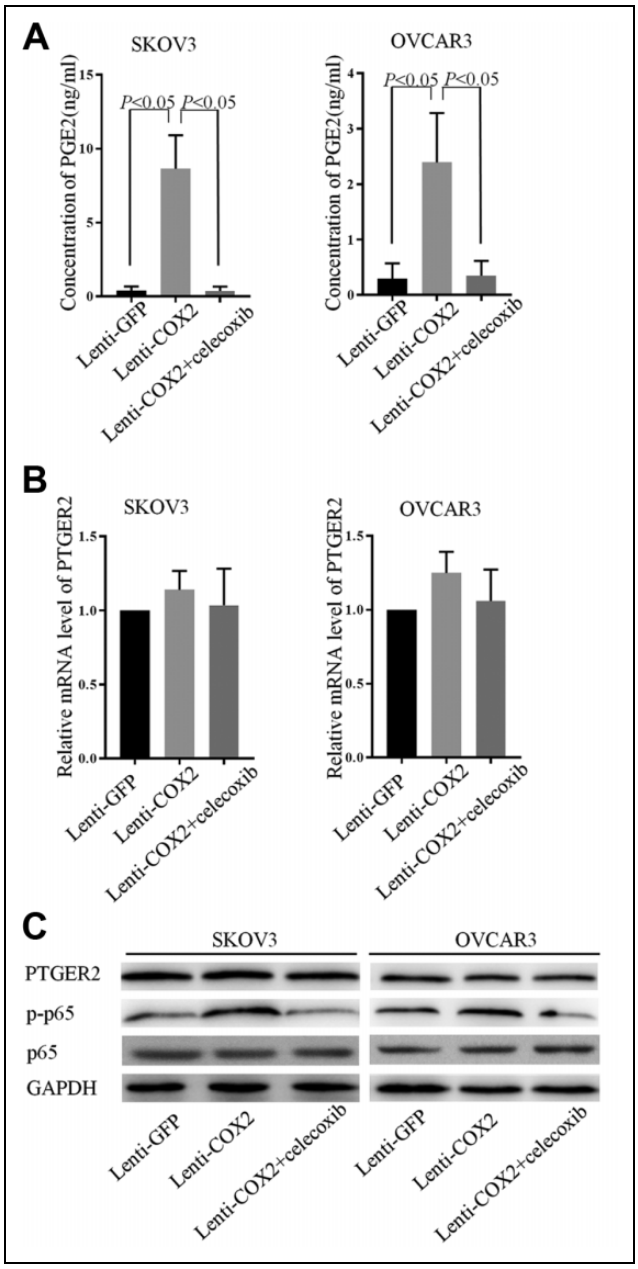

Figure 3. Effects of cyclooxygenase 2 (COX2) on prostaglandin E2 (PGE2), PGE2 Receptor 2 (PTGER2) and p-nuclear factor-kappa B (NF-κB) p65 expression. (A) PGE2 release into the culture supernatants was measured by enzyme-linked immunosorbent assay. (B) The expression level of PTGER2 was estimated by quantitative polymerase chain reaction (qPCR). (C) The expression level of PTGER2, NF-κB p65, and p-NF-κB p65 and western blotting. The data are presented as the means ± SDs.

decreased during treatment, whereas the volume of tumors in mice injected with SKOV3-Lenti-COX2 cells and treated with normal saline increased the most rapidly in all the groups (Figure 5(A) and 5(B)). These findings indicate that COX2 promotes the proliferation of SKOV3 cell xenografts, whereas celecoxib in combination with DDP significantly inhibits it. The expression of COX2 in tumors formed by SKOV3-Lenti-COX2 cells was higher than that in tumors formed by SKOV3-Lenti-GFP cells, as was the expression of NF-κB and CYP19 (Figure 5(C) and 5(D)), which corresponded to the immunohistochemical (IHC) results in ovarian tumor patients. When COX2 was overexpressed, the expression of Ki67, MMP2, and MMP9 was also upregulated (Figure 5(C) and 5(D)). After treatment with either DDP or celecoxib, the expression of NF-κB, CYP19, Ki67, MMP2, and MMP9 was significantly decreased, but this effect was most significant when these agents were used in combination (Figure 5(C) and 5(D)). These results indicate the COX2 inhibitor celecoxib could significantly inhibit the growth of SKOV3 xenografts.

Discussion

In recent decades, COX2 has been reported to be overexpressed in a variety of human tumors, including breast, lung, colon, skin, bone, cervical, esophageal, pancreatic, prostate, and bladder cancer[1,12–19].

High expression of COX2 and sustained release of PGE2 appear to play a major role in the initiation and promotion of cancer progression. The increase of PGE2 synthesis and related signaling are important for malignant transformation and progression in epithelial ovarian cancer[20]. Our data are consistent with those of a previous study showing that COX2 is highly expressed in ovarian cancer[7]. And we found the expression level of COX2 was positively correlated with ovarian tumor grades.

Studies have shown that COX2 overexpression can increase the expression of MMP2 and MMP9 and enhance the invasion of tumor cells[7]. Further studies have shown that PGE2 can promote cytoskeletal reorganization and increase tumor cell metastasis and invasion[21,22]. Thus, COX2 plays an important role in the invasion of tumor cells. Studies have found that COX2-derived PGE2 promotes ovarian cancer cell invasion[23]. Indeed, Gupta et al. identified COX2 as one of four key "metastatic progression" genes that collectively regulate the distant metastasis of tumor cells[24]. PGE2 mediates these effects through various signaling pathway-related mechanisms, including increasing the activation of NF-κB[25], promoting the activation of MMP2 and MMP9[26], facilitating STAT3 phosphorylation, and inhibiting IL-12, ultimately resulting in increased cell proliferation, metastasis, invasion, angiogenesis, and immunosuppression[27]. Moreover, PTGER2 was demonstrated to participate in PGE2-induced tumorigenesis as well as in PGE2-mediated cancer cell survival[5,6]. Qiu et al. indicated that PTGER2 inhibition reduced COX2 activity-driven tumor cell proliferation and invasion[28]. In our study, COX2 increased the PGE2 level but not PTGER2, thereby enhancing the binding of PGE2 to PTGER2, activating downstream signaling pathways, and affecting the biological function of ovarian cancer cells.

NF-κB is a nuclear transcription factor regulated by a proteolytic enzyme-dependent receptor signal transduction pathway. Numerous studies have shown that NF-κB is closely

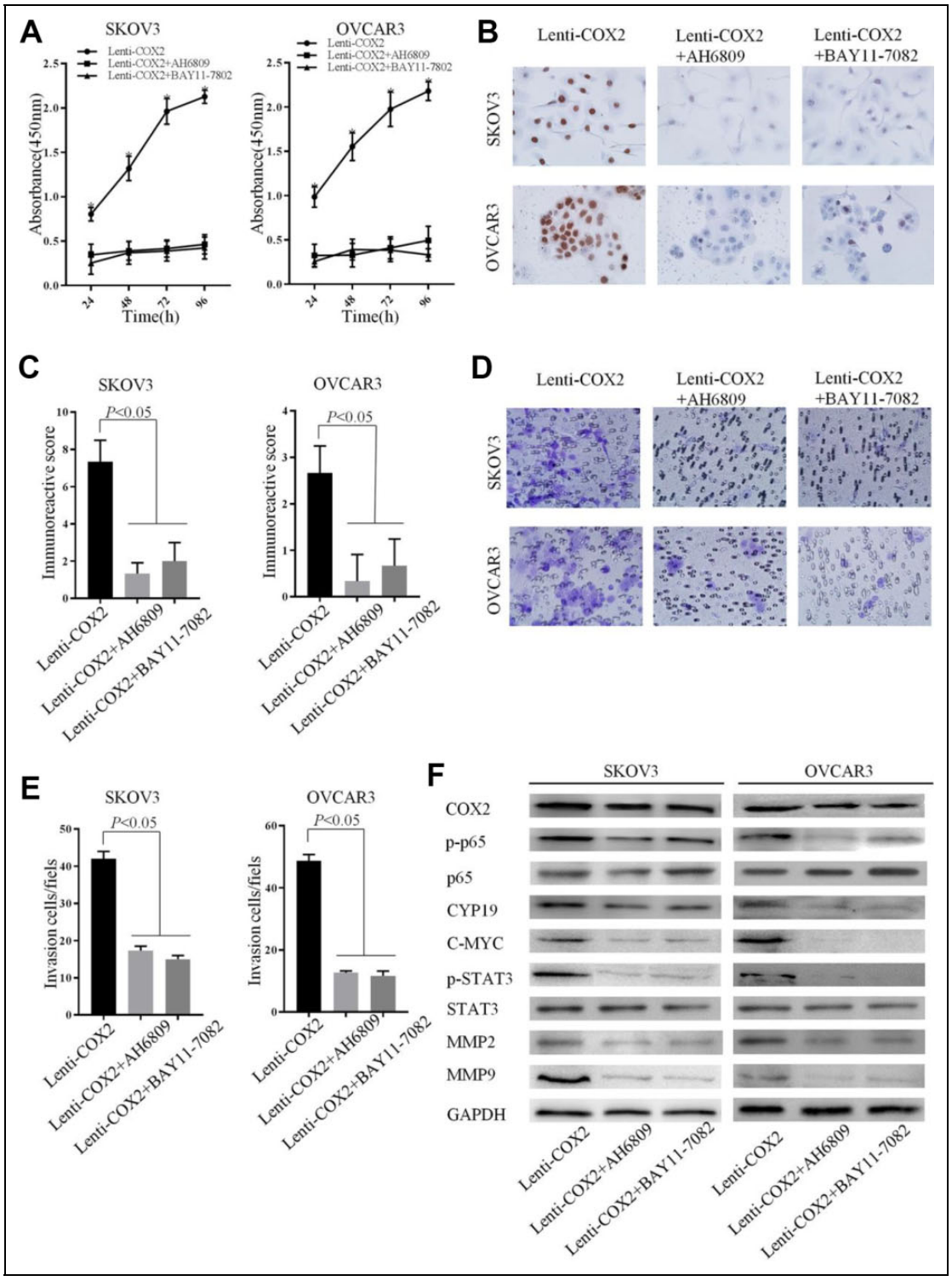

Figure 4. Prostaglandin E2 Receptor 2 (PTGER2) and nuclear factor-kappa B (NF-κB) inhibitors inhibit the cyclooxygenase 2 (COX2)-promoted proliferation and invasion of ovarian cancer cells. (A) Cell viability was measured by a CCK-8 assay. [a]Compared Lenti-COX2 with Lenti-COX2+AH6809 and compared Lenti-COX2 with Lenti-COX2+BAY11-7802 separately, $p < 0.05$. (B) and (C) Representative immunohistochemical (IHC) staining of SKOV3 and OVCAR3 cells conducted with an antibody against Ki67. Magnification: ×400. (D) and (E) SKOV3 and OVCAR3 cells were seeded in 24-well Transwell chambers coated with Matrigel and cultured for 24 hours. Cell invasion was estimated. (F) The expression levels of CYP19, C-MYC, STAT3, p-STAT3, matrix metalloproteinase 2 (MMP2), and MMP9 were estimated by western blot analysis. The data are presented as the means ± SDs.

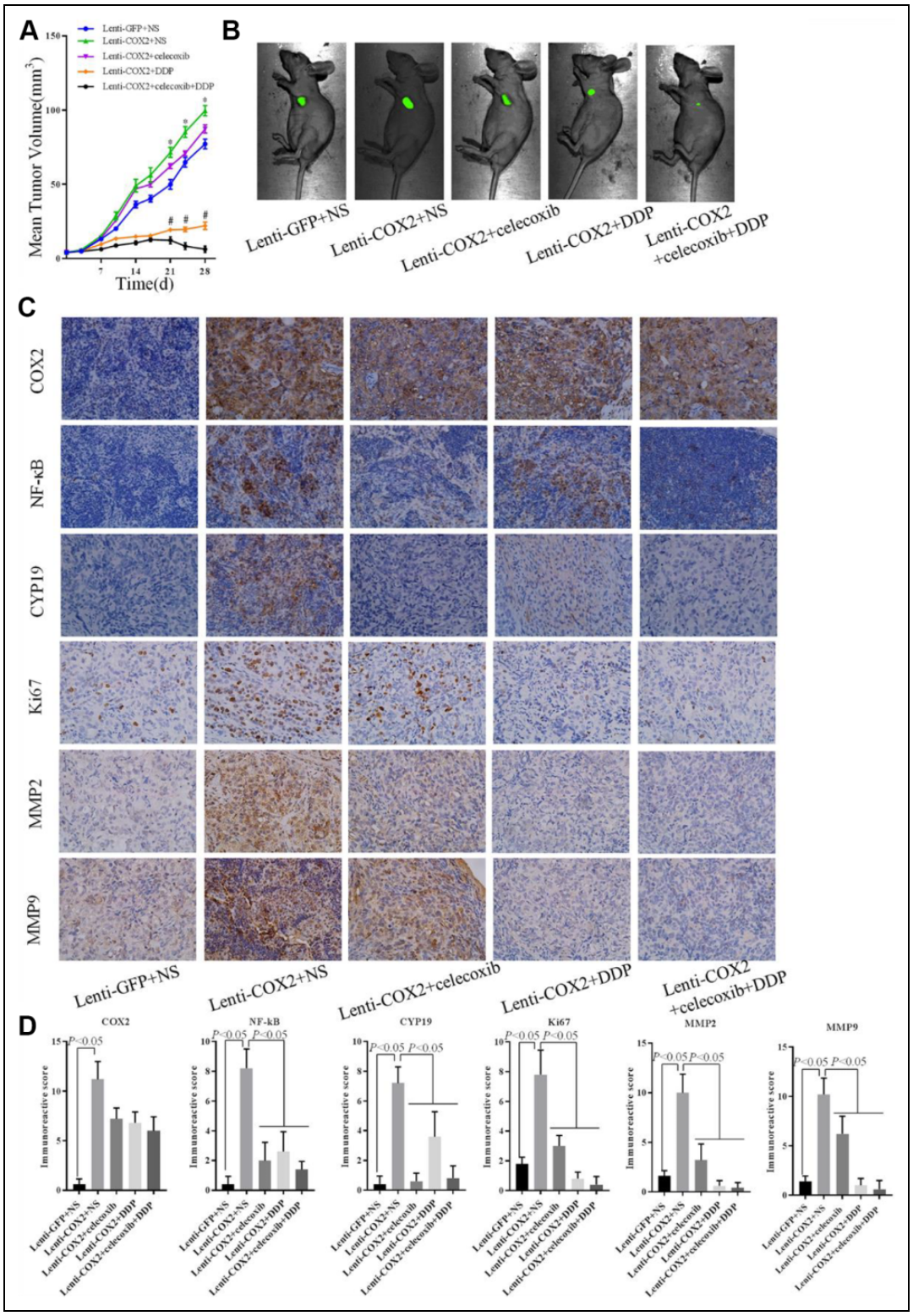

Figure 5. Targeting cyclooxygenase 2 (COX2) inhibits SKOV3 cell growth in a xenograft tumor model. (A) Xenograft tumor volumes were measured three times weekly during treatment. [a]Compared Lenti-COX2+NS with Lenti-GFP+NS, Lenti-COX2+celecoxib, Lenti-COX2+DDP, and Lenti-COX2+celecoxib+DDP separately, $p < 0.05$; [b]compared Lenti-COX2+DDP with Lenti-COX2+celecoxib+DDP, $p < 0.05$, $n = 5$. (B) The fluorescence signal intensity in xenografted mice was determined using the Multi-functional In Vivo Imaging System (MIIS) system. (C) and (D) Immunohistochemical (IHC) staining was conducted with antibodies against COX2, nuclear factor-kappa B (NF-κB), CYP19, Ki67, matrix metalloproteinase 2 (MMP2), and MMP9. Magnification: ×400. The data are presented as the means ± SDs.

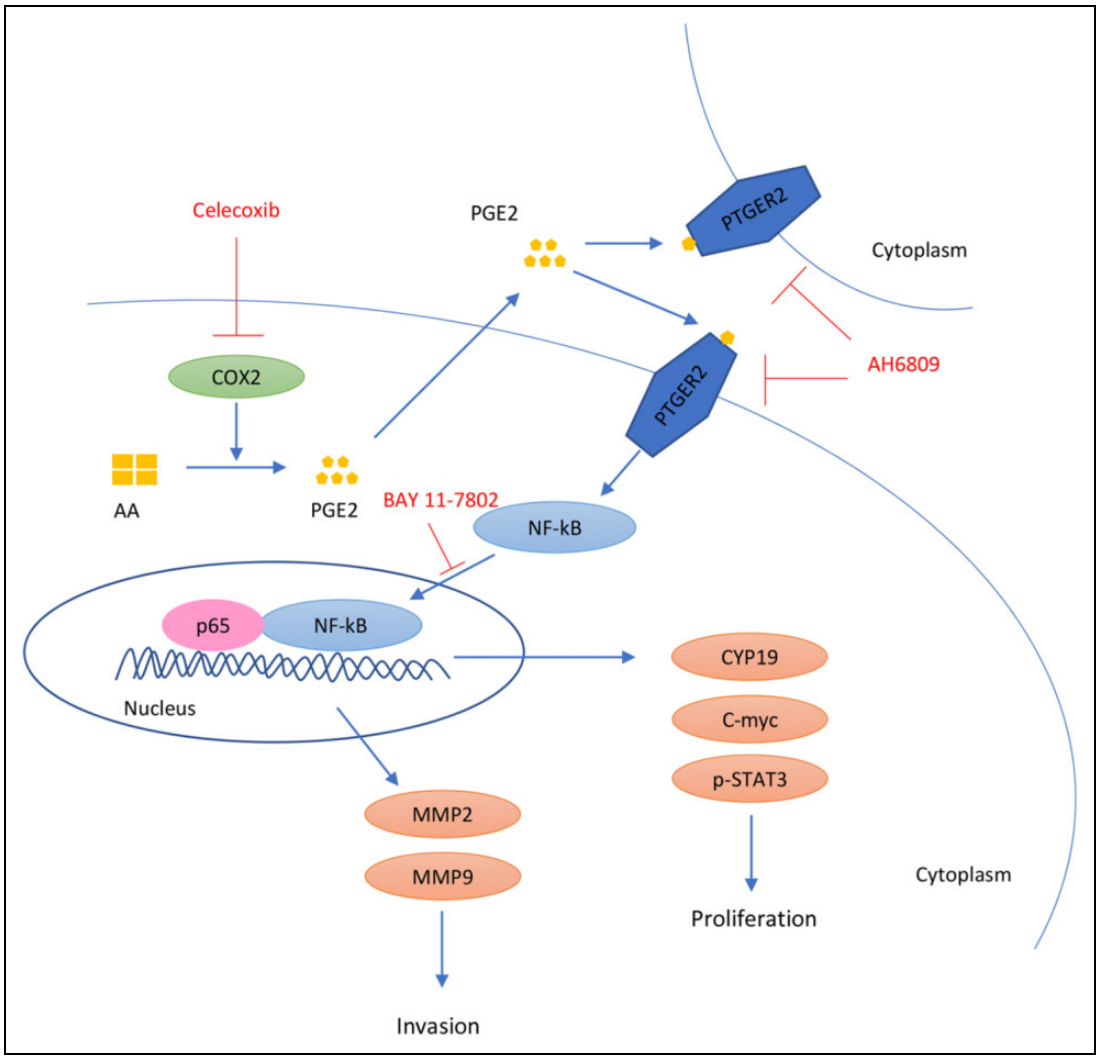

Figure 6. Cyclooxygenase 2 (COX2) promotes ovarian cancer cell proliferation and invasion via the prostaglandin E2 (PGE2)/ nuclear factor-kappa B (NF-κB) signaling pathway.

related to tumor occurrence and to the proliferation, invasion, apoptosis, and metastasis of tumor cells. In addition, studies have found that NF-κB is in a resting state in most normal cells but is constitutively activated in many tumor cells. Various cytokines and cytokine receptors are regulated by the NF-κB pathway or regulate the activation of the NF-κB pathway. For example, a study showed that the oncogene C-MYC is regulated by NF-κB and plays a role in tumorigenesis. Among the target genes regulated by NF-κB, most are cytokines belonging to the family of tumor cell growth factors. NF-κB has been reported to upregulate MMP2 expression, promoting migration and infiltration of the extracellular matrix by tumor cells[29]. Pidgen et al. found that NF-κB upregulates the expression of MMP2, thereby promoting tumor metastasis[30]. Studies have shown that inhibition of NF-κB pathway activity in epithelial tumor cells significantly reduces the proliferation ability[31]. Tumor growth in nude mice was also significantly inhibited. Moreover, CYP19 is the rate-limiting enzyme of estrogen synthesis and plays an important role in the proliferation of ovarian cancer cells. Previous studies have found that NF-κB also upregulates the transcription of CYP19[32]. Our data indicate that COX2 can promote the phosphorylation of NF-κB, thereby upregulating the expression of CYP19, C-MYC, and p-STAT, thus promoting the proliferation of ovarian cancer cells and upregulating the expression of MMP2 and MMP9, ultimately enhancing the invasive ability of ovarian cancer cells (Figure 6).

The important role of COX2 in tumorigenesis and development provides a new strategy for clinical treatment. Wang et al. believe that COX2 inhibitors play a therapeutic role by reducing the loss of the basement membrane and reducing the malignant degree of ovarian cancer cells[33]. Li et al. established xenografts of the human ovarian cancer cell line SKOV3 in mice and found the COX2-selective inhibitor nimesulide acted in a dose-dependent manner and that its inhibitory effect was related to a

decrease in the PGE2 level[34]. In our study, SKOV3 xenograft tumors were established in mice, and the COX2 inhibitor celecoxib was found to inhibit COX2 overexpression in these xenograft tumors. Combination of COX2 inhibitors and chemotherapy drugs can enhance the inhibition of ovarian cancer cells *in vivo*.

Studies showed PGE2 production is regulated by COX1, not COX2, in ovarian cancer cells[35]. But our previous research found that gonadotropins promoted ovarian cancer cell invasion via a COX2-dependent pathway. Follicle-stimulating hormone/luteinizing hormone treatment could rapidly regulate COX2 expression, whereas COX1 expression remained unchanged[7]. COX1 expression is intrinsically responsible for maintaining the basal prostaglandin levels required for tissue homeostasis[36], whereas COX2 expression is inducible, has a low activity in normal tissue cells, and can be induced by inflammatory factors. So we focused on COX2 instead of COX1 in this article. Their impact on ovarian cancer, as well as the potential value in treatment, needs more research to be confirmed.

In conclusion, we provide evidence that COX2 is highly expressed in ovarian cancer and that high expression of COX2 promotes the proliferation and invasion of ovarian cancer cells through the PGE2/NF-κB signaling pathway. COX2 may, therefore, be a potential therapeutic target for the treatment of ovarian cancer.

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Author Contributions

XZ contributed to the acquisition of data, the analysis and interpretation of data, and drafting the manuscript; KY and LD contributed to acquisition of data; JL and HL contributed to analysis and interpretation of data; DF and BL contributed to the conception and design; all authors revised the work critically for important intellectual content, approved the final manuscript to be published, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Availability of Data and Materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Ethical Approval

The study protocol was approved by the Ethics Committee of the China-Japan Friendship Hospital and the Institutional Animal Care and Use Committee of the China-Japan Friendship Hospital, Beijing, China.

Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with the Institutional Animal Care guidelines of the China-Japan Friendship Hospital, China and approved by the Institutional Animal Care and Use Committee of the China-Japan Friendship Hospital, Beijing, China.

Statement of Informed Consent

Informed consent for patient information to be published in this article was not obtained because the study of clinical samples was retrospective.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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