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Cisplatin induces chemoresistance through the PTGS2-mediated anti-apoptosis in gastric cancer

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ABSTRACT

It has been proposed that the aberrant expressions of the classical apoptosis-related genes and the subsequent decrease of apoptosis contribute to the development of cisplatin resistance in gastric cancer. However, little is known about the correlation and the molecular regulation mechanisms of cisplatin and the apoptosis-related gene expressions. Herein, we first identified the expressions of the anti-apoptotic BCL2 and the prostaglandin-endoperoxide synthase-2 (PTGS2) genes, which were abundant in the gastric carcinoma and associated with poor patient survival, were closely related with the resistance against cisplatin. Further investigations revealed that PTGS2 served as an essential mediator involved in the developing process of the resistance against cisplatin via mediating the inhibition effects of cisplatin on BCL2 expression. Mechanistically, cisplatin induced PTGS2 expression through ROS/NF-κB pathway. In addition, PTGS2 mediated cisplatin-induced BCL2 expression and subsequent resistance to apoptosis via PGE2/EP4/MAPKs (ERK1/2, P38) axis. Analysis of the clinical specimens demonstrated that PTGS2 and BCL2 were positively correlated in human gastric cancer. Moreover, in the xenograft models, inhibition of PTGS2 by celecoxib significantly augmented the cytotoxic efficacy of cisplatin in the resistant gastric cancer via suppression of PTGS2 and BCL2 expressions regulated by ERK1/2 and P38 signal axis, suggesting PTGS2 might be employed as an adjunctive therapeutic target for reversal of the chemoresistance in a subset of cisplatin resistant gastric cancer.

1. Introduction

Gastric cancer (GC) with only 20–30% of 5-year survival rate is the second leading cause of cancer-associated mortality worldwide (Rivera et al., 2007; Du et al., 2009; Forman and Burley, 2006; Waddell et al., 2014; Ferlay et al., 2010). Cisplatin is the effective chemotherapeutic agent widely applied for the treatment of GC (Rivera et al., 2007; Zhang et al., 2018; Cunningham et al., 2006; Cats et al., 2018; Guo et al., 2018; Xin et al., 2018; Pasini and Fraccon, 2011). However, the chemoresistance of the GC cells exposed to cisplatin treatment becomes a significant impediment to the therapeutic efficacy, leading to the failure of chemotherapy (Wang et al., 2018; Kurtova et al., 2015). Understanding the molecular basis underlying the developing process of cisplatin resistance is essential to acquire strategies for improving the

Abbreviations: PTGS2, Prostaglandin-endoperoxide synthase-2; BCL2, B cell lymphoma/lewkmia-2; BAX, BCL2 associated X, apoptosis regulator; BCL-XL, B cell lymphoma/lewkmia-2-like 1; XIAP, X-linked inhibitor of apoptosis protein; Survivin, Baculoviral IAP repeat-containing 5; PGE2, Prostaglandin E2; PGH2, Prostaglandin H2; EP, Prostaglandins EP receptors; IC_{50}, Half maximal inhibitory concentration; GC, Gastric cancer

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therapeutic benefits.

The intracellular cisplatin causes DNA damage by triggering interstrand and intrastrand crosslinks between purine bases, resulting in cell apoptosis (Cohen and Lippard, 2001; Mandic et al., 2003; Eastman, 1987; Kelland, 2007; Kelland et al., 1993; Ratzon et al., 2016; Sedletska et al., 2005). Tumor cells became resistance to cisplatin via developing unique mechanisms to interfere the apoptosis-induced process of the cisplatin (Zhu et al., 2015; Huang et al., 2016), among which alterations in levels of the apoptosis-related genes are generally considered to be the closely direct reasons affecting the apoptosis induced by cisplatin (Fodale et al., 2011; Kowalski et al., 2002; Nakamura et al., 2004; Han et al., 2003). However, the detailed mechanisms by which cisplatin changes the expressions of the apoptosis-related genes remain elusive.

In the present study, the apoptosis-related genes and the prostaglandin-endoperoxide synthase-2 (PTGS2) gene, which codes a major enzyme in the conversion of arachidonic acid to prostaglandins, associated with the survival of the GC patients were first identified as the particular genes possibly influenced by chemotherapy via analysis of multiple databases. Comparison of the expressions of genes possibly related to chemoresistance between the cisplatin-resistant GC cells and the corresponding parental cells were further carried out to confirm the selected BCL2 and PTGS2 were positively correlated with the resistance against cisplatin. Further studies provided evidence that cisplatin induced PTGS2 expression through ROS/NF-κB pathway and PTGS2 was important in mediating cisplatin-induced BCL2 expression and the subsequent resistance to apoptosis via PGE2/EP4/MAPKs (ERK1/2, P38) dependent mechanism. Alteration of PTGS2 expression triggered the change of BCL2 expression as well as the resistance to the cisplatin. Consistently, co-expression of PTGS2 and BCL2 were also observed in 26 out of 30 (87%) primary gastric tumors. Moreover, co-administration of celecoxib, a specific inhibitor of PTGS2, suppressed PTGS2 and BCL2 expression and increased the sensitivity of the resistant GC cells to cisplatin *in vivo*, suggesting inactivation of the apoptosis mediator PTGS2 induced by cisplatin may potentially serve as a novel therapeutic intervention for reversal of chemoresistance in a subset of GC.

2. Materials and Methods

2.1. Materials

2.1.1. Reagents

TRIzol® and the vector pcDNA3.1(-) were from Thermo Fisher Scientific, Inc (Waltham, MA, USA). First-stand cDNA synthesis kit and SYBR green q-PCR kit were purchased from Bio-Rad Laboratories, Inc (Hercules, CA, USA). LIPOFECTAMINE 3000 was the product of Invitrogen (Carlsbad, CA, USA). Celecoxib, P38 inhibitor (SB202190), and ERK1/2 inhibitor (U0126) were from Selleck Chemicals (Houston, Texas, United States). EP4 inhibitor (L-161982) was the product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). ROS assay kit and NAC were from Beyotime Biotechnology (Shanghai, China). PVDF and ECL were from Millipore (Billerica, MA, USA). ELISA kit of PGE2 was the product of Elabscience Biotechnology Co Ltd (Wuhan, Hubei, China). Antibodies used were from Cell Signaling Technology (Danvers, MA, USA).

2.1.2. Cell culture and tissue samples

SGC-7901 and SGC-7901/PTGS2 cells were kept in our laboratory and cultured in RPMI-1640 media supplemented with 10% FBS. SGC-7901/DDP cell line was purchased from KeyGEN Biotech. Co. Ltd. (Nanjing, Jiangsu, China) and cultured in 1640 media containing 10% FBS plus 500 ng/ml DDP. Cells were incubated at 37 °C in an atmosphere of 5% CO_2. A total of thirty pairs of matched primary GC and the corresponding adjacent non-tumoral (NT) gastric tissue samples were obtained from Jiangxi Provincial People's Hospital based on the approval of the Internal Review and Ethics Boards of the hospital.

2.2. Methods

2.2.1. Database

Gene expression profiles and the related clinic pathological data were obtained from ONCOMINE (https://www.oncomine.org/resource/login.html). Survival Curves were generated by Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service).

2.2.2. Cell viability assays

Cells were seeded in 96-well plates at a density of 6×10^3 per well for SGC-7901 and SGC-7901/PTGS2 cells, and 8×10^3cells per well for SGC-7901/DDP cells. The cells were attached overnight and treated with DDP for 48 h. For groups of co-treatment with celecoxib, cells were treated with celecoxib for 1 h prior to additional of DDP. The cell viability was determined by the methylthiazoletetrazolium (MTT) colorimetric assay.

2.2.3. Suppression of PTGS2 expression by small interfering RNA

A small interfering RNA (siRNA) targeting PTGS2 (PTGS2 siRNA) with the sequence of sense 5′-GAUUAUGUGCAACACUUGAdTdT-3′, and anti-sense 5′-UCAAGUGUUGCACAUAAUCdTdT-3′ was synthesized by BBI Life Sciences Corporation (Shanghai, China). A scrambled siRNA (sc siRNA) obtained from BBI was used as a negative control. For siRNA-mediated inhibition of PTGS2 gene expression, SGC-7901/PTGS2 cells were transfected with PTGS2 siRNA or sc siRNA at a final concentration of 50 nM using LIPOFECTAMINE 3000 (Invitrogen) according to the manufacturer's instructions. Silencing efficiency was estimated at protein levels by western blotting.

2.2.4. Quantitative PCR analysis

Total RNA was isolated with TRIzol® according to the manufacturer's instructions. A first-strand cDNA synthesis kit was applied to produce cDNA from total RNA, which served as templates for q-PCR amplification with the SYBR green q-PCR Kit. The primers were showed in Supplemental Table 1. GAPDH was amplified as an internal control. The PCR conditions were 94 °C for 5 min followed by 40 cycles of 95 °C for 5 s, 58 °C–59 °C for 10 s, and 72 °C for 20 s.

2.2.5. Western blot analysis

The protein samples were resolved by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked at room temperature for 1 h in TBST containing 5% non-fat dry milk, and subsequently incubated with various primary antibodies at 4 °C overnight followed by incubation with goat anti-rabbit HRP-linked antibody for 1 h at room temperature. The blots were visualized using an ECL detection kit, and analyzed by Quantity One software to determine the ratio relative to GAPDH, a-Tubulin or PCNA.

2.2.6. ROS detection

For removal of the ROS, cells were treated with NAC for 1 h before addition of cisplatin. The DCFH-DA (10 mM) was applied to determine the ROS levels. Briefly, cells were incubated with DCFH-DA for 20 min at 37 °C in the dark, washed with serum-free medium for three times, and subjected to flow cytometric assay. FCS Express Version 3 software and FlowJo 7.6.1 were used to analyze and determine the ROS levels.

2.2.7. PGE2 secretion assay by ELISA

The levels of prostaglandin E2 (PGE2) were determined using ELISA kits (Elabscience, Wuhan, Hubei, China) according to the manufacturer's procedure. Briefly, the cultured media were collected and immediately incubated with the biotinylated antibody working solution at 37 °C for 45 min, followed by treatment with enzyme working solution at 37 °C for 30 min. HRP substrate solution (TMB) was added to each well and incubated at 37 °C for 15–30 min prior to termination of the reaction using the stop solution. The OD value was immediately measured at the wavelength of 450 nm using the microplate reader.

2.2.8. In vivo animal experiments

The SGC-7901/DDP cells (5×10^6 cells) were injected into the flank of 5-week-old male BALB/c nude mice. When tumors reached around 100 mm^3, mice were randomly distributed into four groups. Celecoxib was intraperitoneally injected at the dose of 5 mg/kg once daily, and the cisplatin was intraperitoneally injected at the dose of 3 mg/kg once every three days. The control group received the equivalent volume of PBS buffer alone. Tumors were monitored every two days for 30 days and the volume was calculated using the following formula:

Volume = $0.5 \times D \times d^2 mm^3$ (D: Long diameter, d: short diameter). Animals experiments conducted in the Laboratory Animal Institute were under the supervision and assessment by the Laboratory Animal Ethics Committee of Jinan University.

For immunohistological analysis, tumors were dissected, fixed, and paraffin embedded before incubation with anti-PTGS2 and anti-BCL2 antibodies. Images acquired using an Inverted Fluorescence Microscope (Zeiss, Feldbach, Switzerland) were analyzed using Zen (blue edition) and Image-Pro® Plus v 6.0 (For Windows).

2.2.9. Statistical analysis

The statistical analyses were performed using GraphPad Prism software 5.01. The student's *t*-test was applied for comparisons between two groups, and one way ANOVA followed by Tukey's multiple comparison test was used for multiple comparison. Differences were considered significant at $P < 0.05$.

3. Results

3.1. PTGS2 and the classical apoptosis-related proteins are abundant in GC and predict poor survival in GC patients

PTGS2 initially considered as an inducible enzyme conversing arachidonic acid to prostaglandins during inflammation was found to be closely associated with cell apoptosis and stimulate cancer progression (Desai et al., 2018). Therefore, in order to identify the gene(s) which might influence apoptosis and contribute to development of the resistance against cisplatin in GC, we compared the mRNA expression levels of the classical apoptosis-related genes (BAX, BCL-XL, XIAP, Survivin and BCL2) as well as PTGS2 between normal and tumor tissues using ONCOMINE. As showed in Supplemental Fig. 1, PTGS2 (A), BAX (B), BCL-XL (C), XIAP (D), Survivin (E) and BCL2 (F) were abundant in GC samples. Further analysis of the relationship between the expression levels of the above proteins and the survival rate in the Kaplan-Meier plotter website (http://kmplot.com/gastric) demonstrated that high levels of PTGS2 (A) as well as BCL-XL (C), XIAP (D), Survivin (E) and BCL2 (F) were related to poor survival (Supplemental Fig. 2), implying that except BAX, the classical apoptosis-related proteins and PTGS2 enriched in tumor tissues might play essential roles in development of the chemoresistance via affecting the cisplatin-induced apoptosis in GC.

3.2. PTGS2 and BCL2 are up-regulated in cisplatin-resistant GC cells

In order to confirm whether the genes identified by database are associated with the cisplatin-resistant development, we compared the expression levels of the selected genes including PTGS2, BCL-XL, XIAP, Survivin and BCL2 between the GC cell line SGC-7901 and the corresponding cisplatin-resistant cell line SGC-7901/DDP, which exhibited 6-fold higher resistance to cisplatin than SGC-7901 cells (Supplemental Fig. 3). As shown in Fig. 1(A–B), significantly elevated expression levels of PTGS2 and BCL2 were observed in SGC-7901/DDP cells. In addition, over-expression of PTGS2 remarkably enhanced BCL2 expression in the GC cell, which was decreased by knocking down of PTGS2 (Fig. 1C). The results suggested that PTGS2 might play an essential role in the cisplatin resistance via regulation of the anti-apoptotic protein BCL2.

3.3. PTGS2 enhances the chemoresistance via suppression of the apoptosis induced by cisplatin

To clarify the roles of PTGS2 on the resistance of the GC cells against cisplatin, the IC_{50} (concentration leading to 50% inhibition of cell growth) of the cisplatin for SGC-7901 cells over-expressing PTGS2 was assessed by MTT method. The results showed that over-expression of PTGS2 increased resistance to the cisplatin, with 4-fold higher IC_{50} of SGC-7901/PTGS2 cells (2.914 ± 0.874 μg/ml) than that of SGC-7901/pCDH 510B cells (0.7349 ± 0.1024 μg/ml) transfected with the control vector, which was reversed by celecoxib with IC_{50} of 1.218 ± 0.1294 μg/ml (Fig. 2A).

Because PTGS2 positively modulated the expression of the anti-apoptotic protein BCL2, and the impairment of the apoptosis induced by cisplatin contributes to development of the resistance to cisplatin, we speculated that PTGS2 may inhibit cisplatin-induced apoptosis by up-regulation of BCL2 expression, resulting in the increased resistance of the GC cells to cisplatin. As shown in Fig. 2B, Annexin V-FITC/PI staining combined with the flow cytometric analysis indicated that treatment of 2 μg/ml cisplatin caused (27.35 ± 1.35)% of SGC-7901/pCDH 510B cells and (9.40 ± 0.6)% of SGC-7901/PTGS2 cells undergoing apoptosis respectively. Dual staining with PI and Hoechest 33342 also showed that over-expression of PTGS2 decreased the percentage of cisplatin-induced cell death rate from (34.18 ± 3.12)% to (3.27 ± 3.27)% (Fig. 2C). The results suggested that PTGS2 could increase the resistance via suppression of cell death induced by cisplatin.

3.4. PTGS2 regulates BCL2 expression through PGE2/EP4/MAPKs (ERK1/2, P38) axis

In order to explore the molecular mechanism underlying PTGS2 regulation of BCL2 expression in the process of the resistance development, we first compared the activation levels of MAPKs and PI3K/AKT signaling pathways between the resistant cells and the parental cells. The phosphorylation levels of ERK1/2 and P38, but not JNK and AKT in SGC-7901/DDP cells significantly increased compared with those in SGC-7901 cells. Accordingly, over-expression of PTGS2 could activate both ERK1/2 and P38 axis which were in the activation state in the resistant cells, while knocking down of PTGS2 suppressed the activations of ERK1/2 and P38 axis, implying that PTGS2 might enhance the resistant potentials of the GC cells against the cisplatin via boosting ERK1/2 and P38 signaling (Fig. 3A).

PTGS2 is a key enzyme involved in catalyzing the arachidonic acid to prostaglandin H2, which is then converted by prostaglandin E2 synthase into the prostaglandin E2 (PGE2). The secreted PGE2 stimulates cancer progression via activations of the multiple signaling pathways followed by binding to the specific G protein coupled receptor EPs (EP1-4). To clarify whether PTGS2 regulated the expression of BCL2 through activations of PGE2-mediated downstream signaling pathways, the effects of PTGS2 on the production of the secreted PGE2 were first detected by ELISA. As shown in Fig. 3B, over-expression of PTGS2 significantly promoted the extracellular levels of PGE2, which could be decreased by treatment with celecoxib, a specific inhibitor of PTGS2. Interestingly, the semi-quantitative PCR assay showed that over-expression of PTGS2 up-regulated the expressions of both EP3 and EP4, while celecoxib had only inhibitory effect on EP4 but not EP3 expressions at the transcriptional levels (Fig. 3C). Moreover, either celecoxib or EP4 inhibitor L-161982 suppressed the activations of ERK1/2 and P38 signaling pathways, and BCL2 expression induced by PTGS2 (Fig. 3D). Accordingly, inhibition of ERK1/2 and P38 pathways by U0126 and SB202190 could reduce the expression levels of BCL2 in SGC-7901/PTGS2 cells (Fig. 3E). The results suggested that PTGS2 enhanced the BCL2 expression through activation of the PGE2/EP4/MAPKs (ERK1/2, P38) axis.

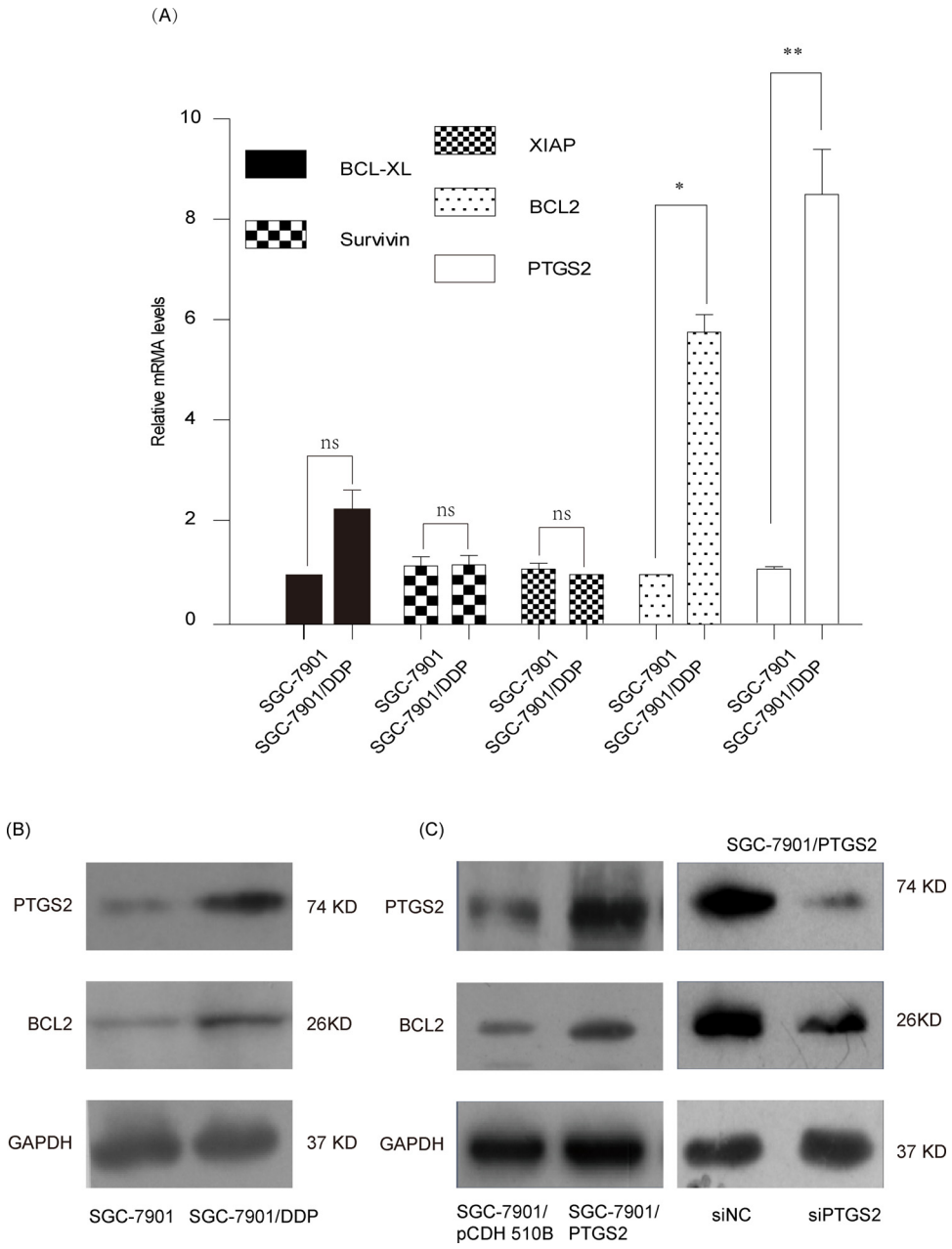

Fig. 1. PTGS2 and BCL2 are up-regulated in cisplatin-resistant GC cells. (A) RT-qPCR validation of the genes identified by database were executed on 3 independent RNA isolated from SGC-7901 as well as SGC-7901/DDP cells. Fold-change is calculated relative to SGC-7901. $^{*}P < 0.05$; $^{**}P < 0.01$, "ns" means "no significance". (B) The parental SGC-7901 cells and the cisplatin-resistant SGC-7901/DDP cells were seeded into 6-well plates overnight and then lysated by SDS. Whole cell lysates were subjected to immunoblot analysis with the indicated antibodies. (C) Ectopic over-expression or knocking down of PTGS2 altered BCL2 expression. The loading of each compartment in immunoblot analysis was indicated by GAPDH.

3.5. Cisplatin induced PTGS2 and BCL2 via ROS mediated NF-κB translocation

Since PTGS2 is an inducible enzyme greatly contributing to the resistant potentials of the GC cells against the cisplatin, we speculated that PTGS2 might be induced when the GC cells were exposed to the cisplatin. As expected, treatment with 500 ng/ml cisplatin enhanced PTGS2 and BCL2 expression in SGC-7901 cells (Fig. 4A). Further exploration found that the cisplatin increased the levels of the intracellular reactive oxygen species (ROS), which were also up-regulated in the resistant cells compared to the parental cells (Fig. 4B), and triggered the activations of ERK1/2 and P38 signal pathways (Fig. 4C). Scavenging of the ROS generation by the antioxidant agent, N-acetylcystein (NAC), suppressed the cisplatin-induced activations of ERK1/2 and P38 axis and the expressions of PTGS2 and BCL2 (Fig. 4D). Moreover, the cisplatin could promoted the translocation of nuclear factor-kappa B (NF-κB), an essential transcriptional factor of the PTGS2 and BCL2 genes (Appleby et al., 1994; Chen et al., 2005; Liu et al., 2017; Zhao et al., 2014), which was attenuated by scavenging of ROS (Fig. 4E). Inhibition of ERK1/2 or P38 also markedly suppressed cisplatin-induced NF-κB translocation as well as PTGS2 and BCL2 expressions (Fig. 4E-F). The results suggested that the axis composed of the cisplatin-stimulated ROS generation, the subsequent promotions of ERK1/2 and P38 axis, and NF-κB translocation is one of the mechanisms underlying PTGS2 and BCL2 inducement in GC cells exposed to the cisplatin.

3.6. PTGS2 and BCL2 are positively correlated in human GC

The above results showed that PTGS2 positively modulated the expression of the anti-apoptotic protein BCL2, leading to inhibition of cisplatin-induced apoptosis and increase of the resistance of the GC cells against cisplatin. In order to determine the potential association between PTGS2 and BCL2 in clinical GC, immunohistochemistry was carried out to detect the expressions of PTGS2 and BCL2 in specimens of human GC (n = 30). Higher expression levels of PTGS2 and BCL2 were

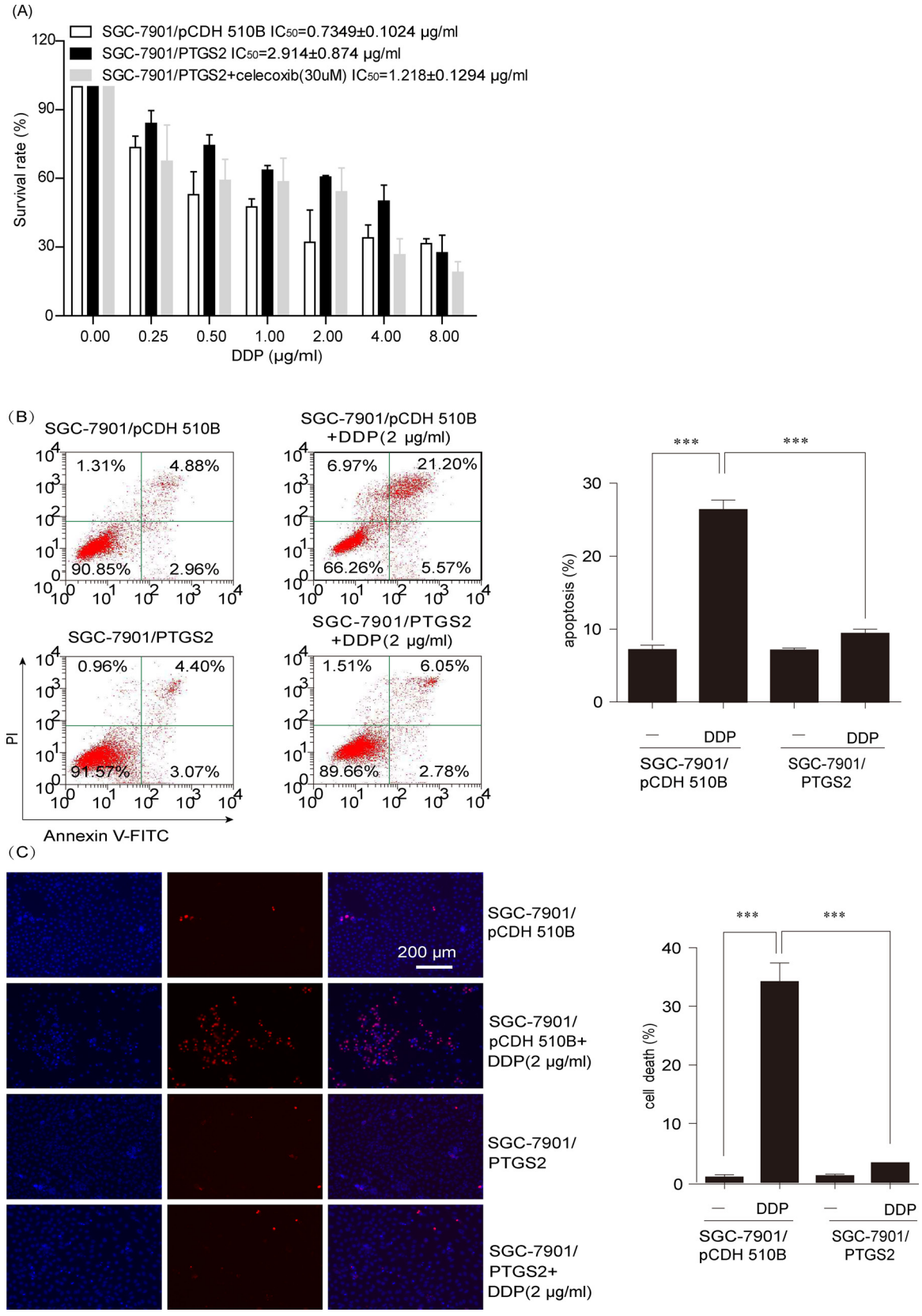

Fig. 2. PTGS2 enhances the chemoresistance via suppression of the apoptosis and cell death induced by cisplatin. (A) The IC_{50} of cisplatin were detected by MTT assay. For groups of co-treatment with celecoxib, cells were treated with celecoxib for 1 h prior to additional of cisplatin. (B–C) Cells were treated with 2 μg/ml cisplatin for 48 h. Cell apoptosis and cell death were quantified by Annexin V-FITC/PI and Hochest/PI staining, respectively. ***$P < 0.001$.

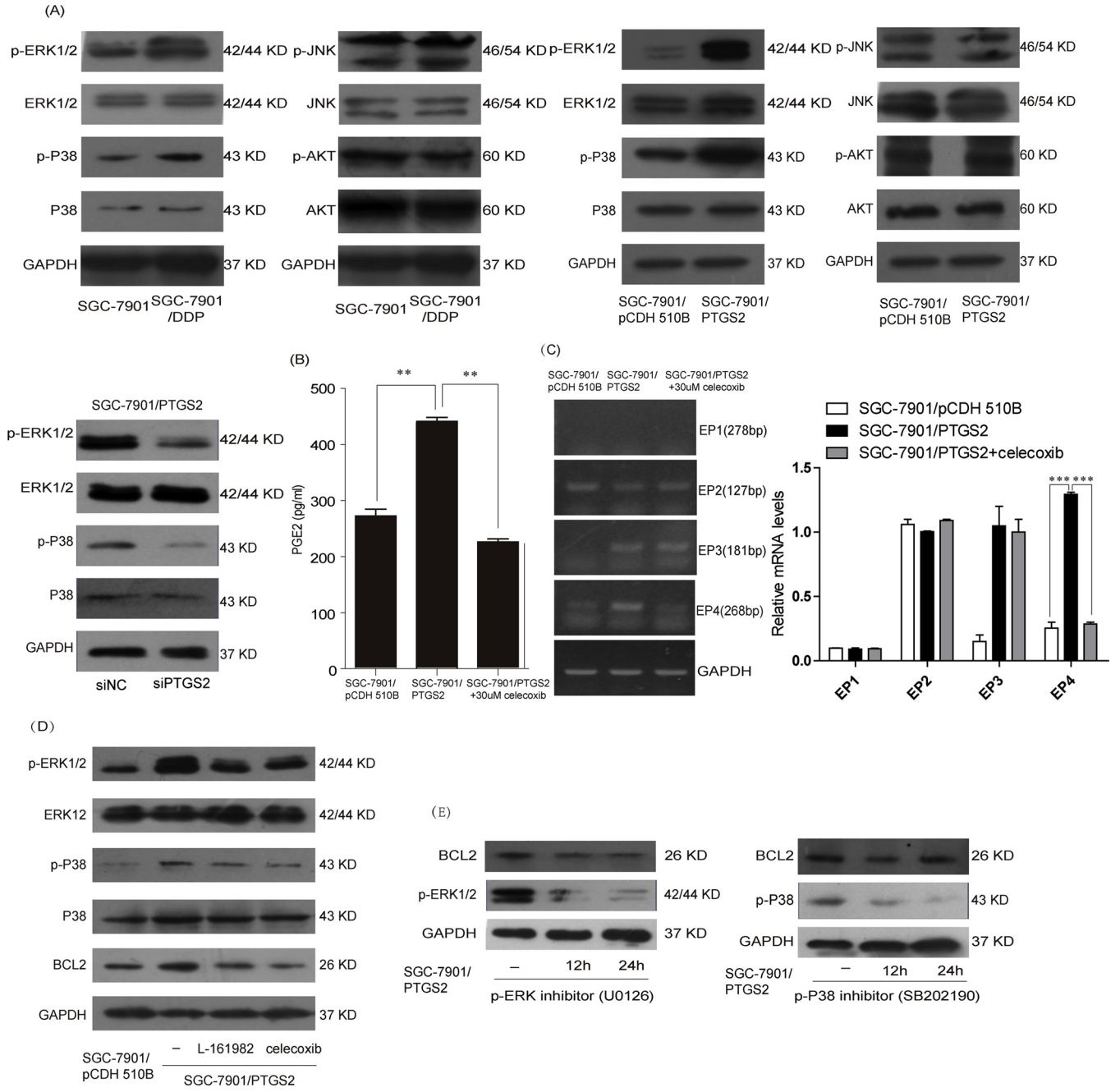

Fig. 3. PTGS2 regulates BCL2 expression through PGE2/EP4/MAPKs (ERK1/2, P38) axis. (A) Cells were lysed and subjected to immunoblot analysis with the indicated antibodies. (B–C) Cells were cultured with or without 30 μM celecoxib for 48 h. The supernatants were collected for analysis of the secreted PGE2 levels by ELISA (B), while cell lysates were used to detect the expressions of EP1-4 by semi-quantitative PCR (C). Values are expressed as the means ± SEM of three separate experiments. $^{**}P < 0.01$; $^{***}P < 0.001$. (D) Cells were treated with or without 10 μM L161982 and 30 μM celecoxib for 48 h, and subjected to western blot analysis. (E) SGC-7901/PTGS2 cells were treated with 10 μM U0126 and SB202190 followed by detection of BCL2 expression. GAPDH was used as the loading control.

detected in tumor regions than in adjacent regions as shown in the representative images (Fig. 5A). Further analysis of the Spearman's rank correlation coefficient revealed a statistically significant correlation between PTGS2 and BCL2 expressions among the clinical samples (Fig. 5B; Spearman's rank correlation coefficient $r_s = 0.6269$, $p = 0.0002$, $n = 30$).

3.7. PTGS2 inhibitor reverses the resistance to cisplatin via suppression of BCL2 expression in vivo

The *in vitro* study indicated that PTGS2 enhanced the chemoresistance by up-regulation of BCL2 and inhibition of the apoptosis, providing a possibility to reverse the resistance by targeting PTGS2. Therefore, we further explored the effects and the underlying mechanisms of PTGS2 inhibitor, celecoxib, on the anti-tumor activity of cisplatin using the resistant GC xenograft models. As shown in (Fig. 6A-C), compared with the control group, cisplatin treatment moderately inhibited tumor growth in SGC-7901/DDP xenograft. Although little inhibitory effect was found in celecoxib treatment alone, co-administration of celecoxib markedly augmented the anti-tumor effect of cisplatin in GC models with the acquisition of cisplatin resistance. Body weight was not affected at the dose of celecoxib examined in the

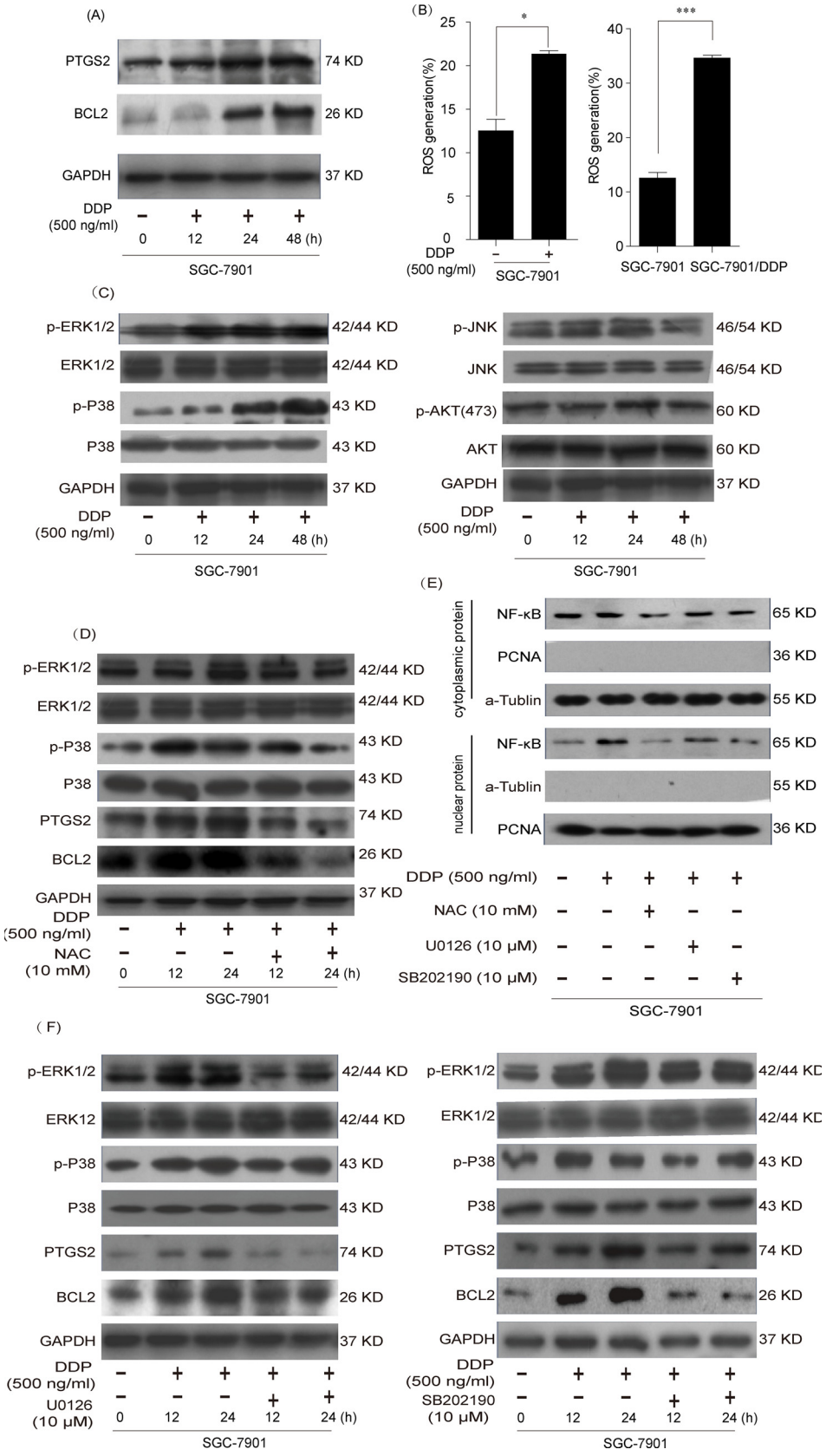

Fig. 4. Cisplatin induces PTGS2 and BCL2 via ROS mediated *NF-κB* translocation. (A) SGC-7901 cells were treated with 500 ng/ml cisplatin for 12, 24, and 48 h prior to immunoblot analysis of PTGS2 and BCL2. (B) Cells were treated with cisplatin for 48 h, and subjected to detection of the levels of reactive oxygen species (ROS) by flow cytometry. Values are expressed as the means ± SEM of three separate experiments. $^{*}P < 0.05$; $^{***}P < 0.001$. (C) SGC-7901 cells were treated with cisplatin for 12, 24, 48 h prior to western blot analysis of the activations of ERK1/2, P38, JNK, and AKT. (D) SGC-7901 cells were treated with 10 mM NAC for 1 h before stimulation with 500 ng/ml cisplatin. The effects of cisplatin-induced ROS on the activation of ERK1/2 and P38 signal pathways, and the expressions of PTGS2 and BCL2 were determined by western blot analysis. (E) The effects of 10 mM NAC, 10 μM U0126, or 10 μM SB202190 on cisplatin-induced NF-κB nuclear translocation were detected by western blot analysis. The loading of each compartment was indicated by α-Tublin (cytoplasmic) and PCNA (nucleus). (F) SGC-7901 cells were treated with 10 μM U0126 or 10 μM SB202190 for 24 h before stimulation with 500 ng/ml cisplatin. The effects of U0126 and SB202190 on the expressions of PTGS2 and BCL2 were analyzed by western blotting.

experiments (Data not show).

Further immunohistochemical results indicated that cisplatin alone increased the expression levels of both PTGS2 and BCL2 in the cisplatin-resistant xenograft models. Whereas compared with the cisplatin-treated group, significant decrease of PTGS2 and BCL2 expression was observed when celecoxib was co-administrated with cisplatin (Fig. 6D). Similar results were found in western blotting analysis of the tumor samples (Fig. 6E). In addition, celecoxib completely attenuated the

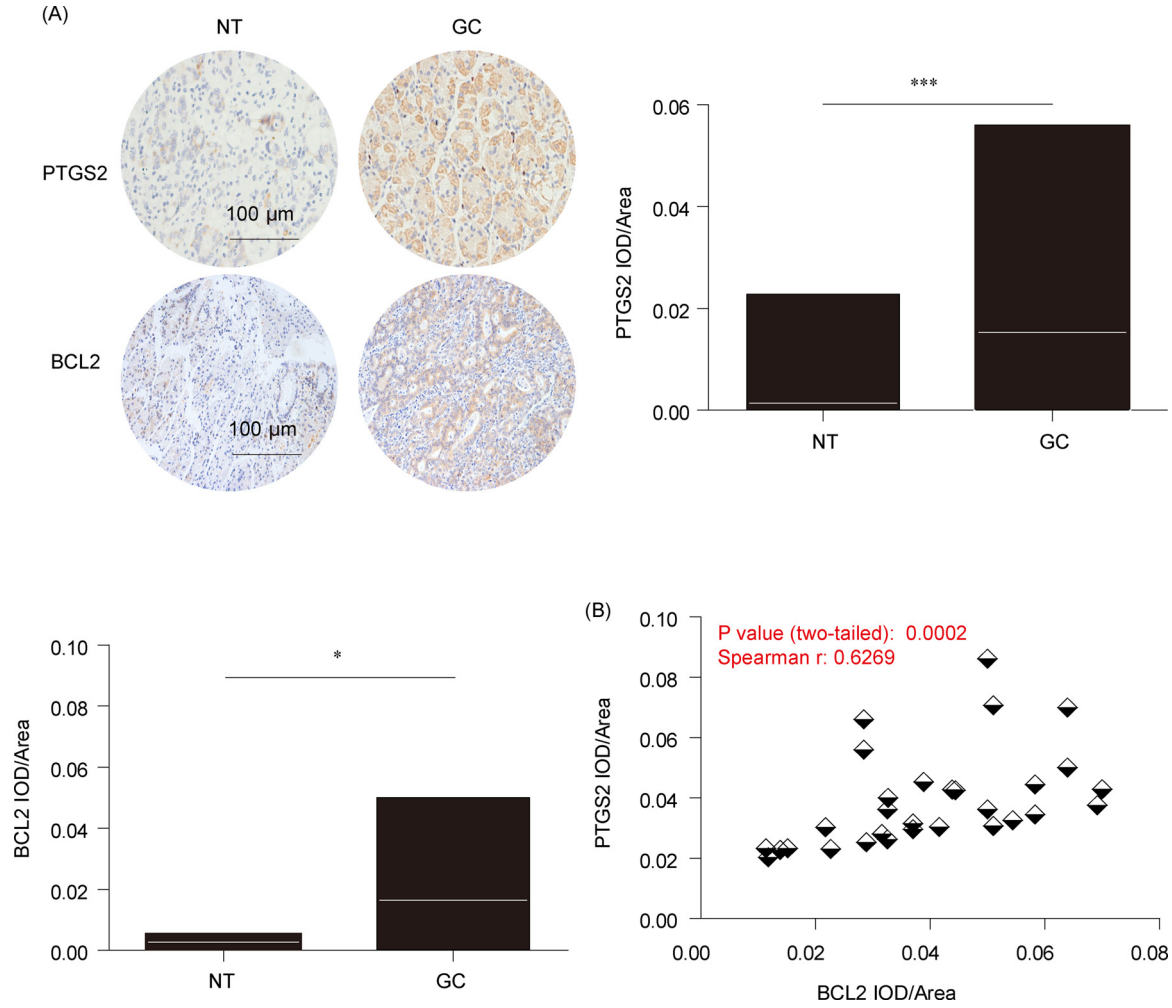

Fig. 5. PTGS2 and BCL2 are positively correlated in human GC. (A) IHC analysis of PTGS2 and BCL2 in clinical samples (n = 30). Shown at left top were the representative images. Images were analyzed using Image-Pro® Plus v 6.0 (For Windows) through calculating the average optical density (IOD) per unit (area). Scale bars: 100 μm. *$P < 0.05$; ***$P < 0.001$. (B) The association between PTGS2 and BCL2 was evaluated by Spearman's rank correlation coefficient.

activation of ERK1/2 and P38 induced by cisplatin (Fig. 6E). Taken together, these findings suggest inhibition of PTGS2 enhances the anti-tumor efficacy of cisplatin in the resistant GC in mouse models through suppression of PTGS2 and BCL2 expression regulated by ERK1/2 and P38 signal axis, which is coincided with the molecular mechanisms as revealed *in vitro*.

4. Discussion

Given exposed to the cisplatin is the root cause for tumor cells developing the acquired resistance against the cisplatin, which results in the failure of chemotherapy in clinic, understanding the mechanisms underlying the inducement process of cisplatin resistance, and development of novel therapies for enhancing the response to the cisplatin in resistant GC are urgently required. In this study, PTGS2 was found to be an essential mediator involved in the developing process of cisplatin-induced resistance of GC, and inhibition of PTGS2 activity with celecoxib markedly enhanced response of the resistant GC to cisplatin.

PTGS2 is an inducible enzyme, whose expression is triggered by a variety of external factors (Chen et al., 2001; Peppelenbosch et al., 1993). Previous studies mainly focused on the effects of PTGS2 on promoting tumorigenesis, angiogenesis and metastasis (Tsujii et al., 1997, 1998; Zhang et al., 2015). Recent investigations showed that PTGS2 was closely related with chemoresistance. For example, PTGS2 could increase the resistance to cisplatin in head and neck squamous cell carcinoma (Yang et al., 2016). Inhibition of PTGS2 expression by siRNA restored the sensitivity of esophageal cancer cells to 5-fluorouracil and cisplatin (Okamura et al., 2013). Our results demonstrated that PTGS2 enhanced the chemoresistance via mediating the augmentation effects of cisplatin on the expression of the anti-apoptotic protein BCL2, and attenuating the subsequent apoptosis via PGE2/EP4/MAPKs (ERK1/2, P38) dependent mechanism in GC, which provided a novel mechanism different from previous reports that PTGS2 might contribute to drug resistance through elevated expression of P-glycoprotein, an ABC transporter mediating multiple drug resistance by extruding drugs out of cells (Gu and Chen, 2012; Arunasree et al., 2008).

It has been reported that activations of MAPKs signaling pathways by various stimulants promote tumor progress and chemoresistance (Walczak et al., 2014; Wang et al., 2017; Galluzzi et al., 2012; Tong et al., 2018). Our results suggested that the MAPKs (ERK1/2, P38) signaling pathways on one hand are activated by cisplatin-induced ROS production and involved in the NF-κB translocation and PTGS2 expression, and on the other hand are activated by PGE2/EP4 and implicated in BCL2 expression and the apoptosis inhibition, leading to development of the cisplatin resistance in GC.

Given ROS mediated the regulation effects of cisplatin on PTGS2 expression, and involved in the development of drug resistance phenotype, we speculated that scavenging of ROS might inhibit the expressions of PTGS2 and BCL2, and thus promote cell apoptosis and reverse the cisplatin resistance. However, the results showed that

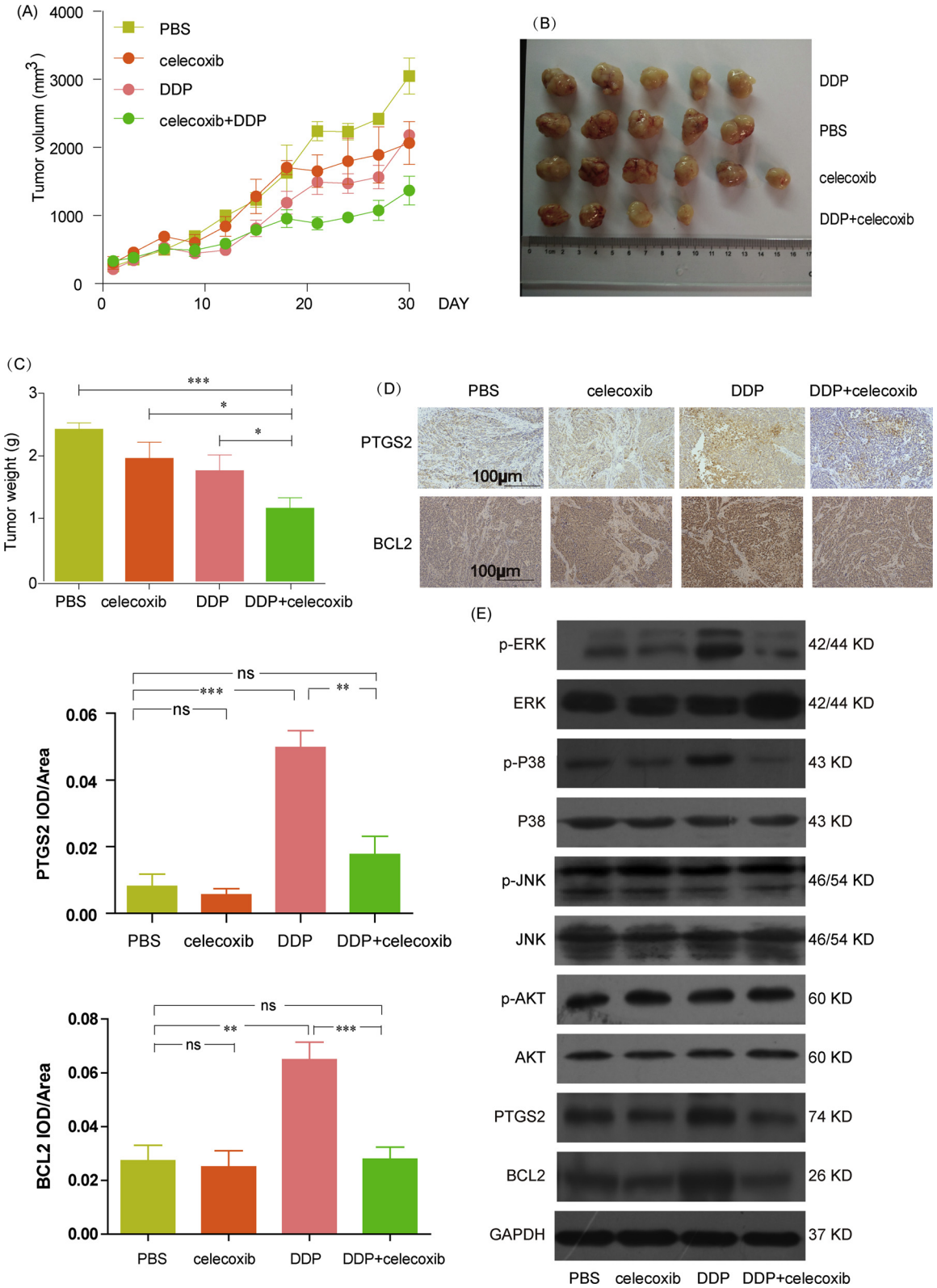

Fig. 6. PTGS2 inhibitor reverses the resistance to cisplatin via suppression of BCL2 expression *in vivo*. The SGC-7901/DDP cells (5×10^6 cells) were injected into the flank of 5-week-old male BALB/c nude mice (n = 20), which were randomly distributed into four groups as described in Materials and Methods. (A) Tumor growth curves were determined by calculation of the tumor volume every two days for 30 days. (B) Photographs of the tumors extirpated from the engrafted mice. (C) Comparison of tumor weights of the indicated groups. (D) Tumor sections were stained with anti-PTGS2 mAb (*upper*) and anti-BCL2 mAb (*lower*). Image-Pro® Plus v 6.0 (For Windows) was used to calculate the average optical density (IOD) per unit (area) of the images. (E) Western blotting was applied to detect the expressions of PTGS2 and BCL2, and the activations of ERK1/2, P38, JNK, and AKT in tumor tissues. GAPDH was used as the loading control. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

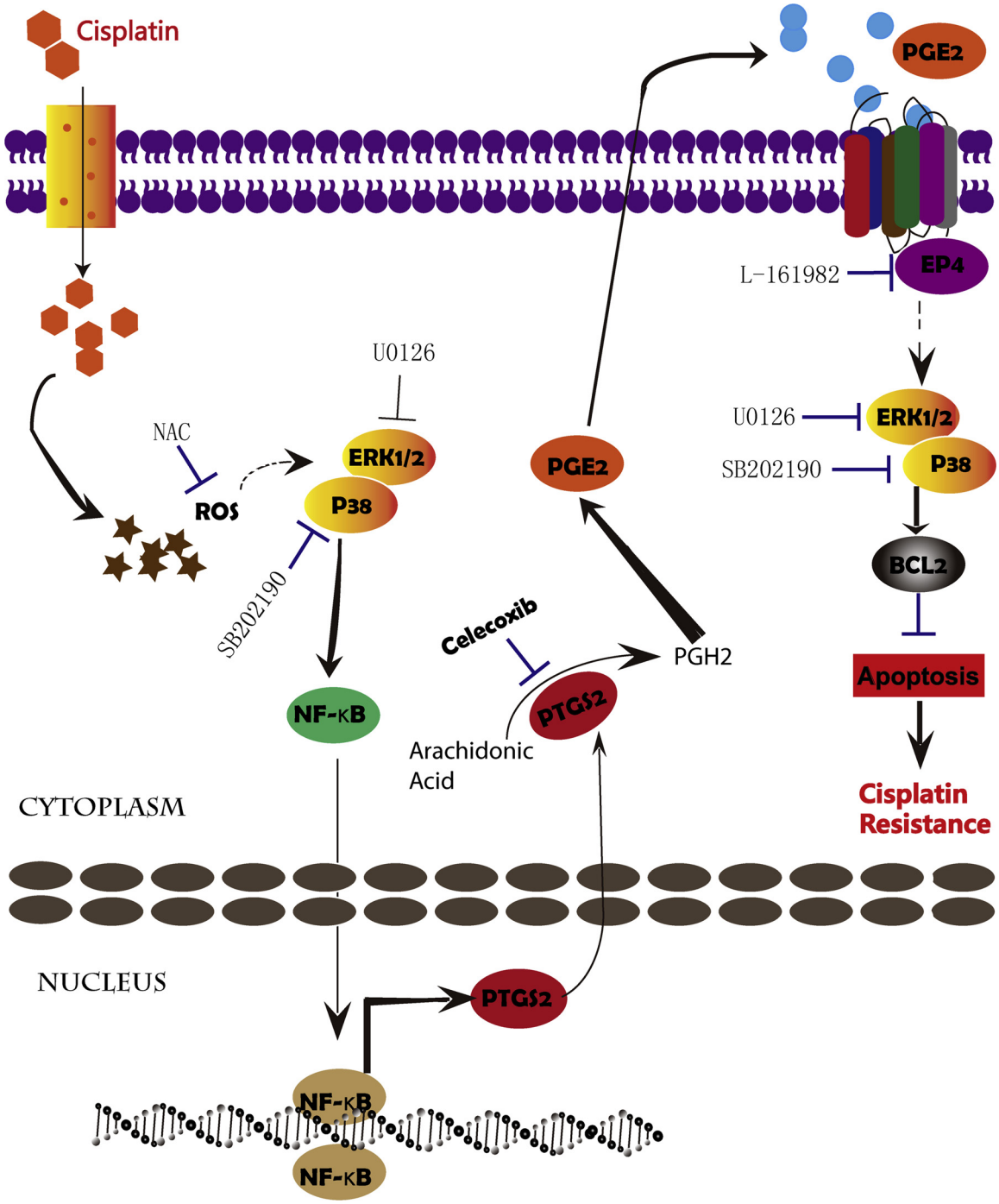

Fig. 7. Schematic representation of the Cisplatin/PTGS2/BCL2 axis mediating cisplatin-induced chemoresistance proposed in this study.

scavenging of ROS by antioxidant N-acetyl-cysteine (NAC) significantly reduced the 2 μg/ml cisplatin-induced apoptosis from (48.12 ± 1.61)% to (13.16 ± 2.64)% (Supplemental Fig. 4). The reason for the unexpected results may be due to the dual effects of ROS on the apoptosis induced by the cisplatin. The intracellular ROS generated by cisplatin on one hand promoted apoptosis by triggering oxidative stress, on the other hand initiated anti-apoptosis mechanisms at least partly via elevating the expressions of PTGS2 and anti-apoptotic protein BCL2. The effects of reduced ROS levels by NAC on promoting apoptosis via inhibiting expressions of PTGS2 and BCL2 were much less than that on suppression of apoptosis via decreasing the cellular oxidative stress, leading to in total reduced apoptosis by scavenging of ROS as observed. Therefore, although ROS served as an intermediator for cisplatin inducing resistance, the strategies of targeting ROS to reverse cisplatin resistance should be acted cautiously.

Our results identified PTGS2 as an essential mediator involved in development of resistance in the GC cells exposed to the cisplatin, implying that PTGS2 might be an effective target for reversal of the acquired resistance. As expected, inhibition of PTGS2 by celecoxib enhanced response of the resistant GC to cisplatin in the xenograft mouse models. However, one previous report indicated that celecoxib antagonized the cytotoxicity of cisplatin by reducing drug influx and the subsequent intracellular cisplatin accumulation (Chen et al., 2013). The opposite effect of celecoxib on the cisplatin efficacy may at least partially due to the different cell types used in two studies, as well as the corresponding individual mechanisms. Celecoxib exerted antagonizing

effect on cisplatin-induced cytotoxicity in GC MKN45 cells independent of its PTGS2 inhibitory activity as previously reported, whereas our results demonstrated that celecoxib potentiated the cytotoxic efficacy of cisplatin in the GC SGC-7901/DDP cells acquired the resistance against the cisplatin through suppression of PTGS2 activity. Further studies will be required to unambiguously define the reasons for this discrepancy. Nevertheless, the benefit of celecoxib in cisplatin-based therapy on anti-tumor effects might restrict to a subset of GC, especially the GC acquired the chemoresistance.

5. Conclusion

In summary, we revealed a novel molecular mechanism of PTGS2 in cisplatin-induced resistance and proposed a model for the regulation of PTGS2 in the process of developing resistance phenotype in GC cells. Our results demonstrated that cisplatin induced PTGS2 expression through ROS/NF-κB pathway and PTGS2 mediated cisplatin-induced BCL2 expression and the subsequent resistance to apoptosis via PGE2/EP4/MAPKs (ERK1/2, P38) dependent mechanism as annotated in Fig. 7. Targeting PTGS2 could be efficient as an adjunctive therapy for a subset of cisplatin resistant GC by improving the response to classic therapeutic agents.

Ethics approval and consent to participate

Animals experiments conducted in the Laboratory Animal Institute were under the supervision and assessment by the Laboratory Animal Ethics Committee of Jinan University.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105610.

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