Wogonin inhibits the proliferation and invasion, and induces the apoptosis of HepG2 and Bel7402 HCC cells through NF-κB/Bcl-2, EGFR and EGFR downstream ERK/AKT signaling

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Abstract. The anticancer effects of the natural flavonoid, wogonin, have been reported. However, its molecular mechanisms of action have not yet been fully explored. In the present study, we aimed to examine the molecular mechanisms of action of wogonin and its effects on the biological behavior of the HepG2 and Bel7402 hepatocellular carcinoma (HCC) cell lines. We also examined the effects of wogonin on nuclear factor-κB (NF-κB)/Bcl-2 and epidermal growth factor receptor (EGFR) signaling, as well as on downstream pathways of EGFR, namely extracellular signal-regulated kinase (ERK)/AKT signaling. We found that treatment with wogonin inhibited the proliferation and invasion, and induced the apoptosis of the HepG2 and Bel7402 cells. In addition, treatment with wogonin decreased cyclin D1, cyclin E, CDK4/6, Bcl-2 and matrix metalloproteinase 2 (MMP2) expression, and promoted the cleavage of caspase-3 and caspase-9 in a concentration-dependent manner. Further experiments revealed that wogonin inhibited NF-κB/Bcl-2 signaling by decreasing the IκB and p65 phosphorylation levels. Wogonin also inhibited the activation of the EGFR (Tyr845) signaling pathway, and that of downstream pathways of EGFR, namely ERK/AKT/MMP2 signaling. The depletion of EGFR by siRNA partly abolished the inhibitory effects of wogonin on cyclin D1, MMP2 expression. On the whole, our our findings demonstrate that wogonin effectively suppresses the proliferation, invasion and survival of HCC cells through the modulation of the NF-κB and EGFR signaling pathways.

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Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide and the incidence of HCC is increasing (1). Despite advances in surgical techniques and chemotherapeutic methods, the survival rate of patients with HCC remains poor. Recent studies have identified a number of molecules and signaling pathways that influence the malignant biological behavior of HCC (2-4). The identification of new molecules targeting these signaling pathways to inhibit cancer cell proliferation and metastasis may lead to the development of novel therapeutic strategies for HCC.

Wogonin belongs to the family of flavonoids, and is derived from the Chinese herb, *Scutellaria baicalensis* Georgi. It has been reported that wogonin exerts antioxidant, anti-thrombotic and anti-inflammatory effects (5-8). The inhibitory effects of wogonin on cancer cell growth and survival have been reported in several cancer cells, such as lung, cervical, leukemia and breast cancer cells (9-13). These studies also demonstrated some of the mechanisms through which wogonin exerts its inhibitory effects on cancer cell growth. For example, it was demonstrated that wogonin inhibited phorbol 12-myristate 13-acetate (PMA)-induced cyclooxygenase-2 (COX-2) protein and mRNA expression in human lung epithelial cancer cells (9). The authors also found that the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor, U0126, also inhibited PMA-induced COX-2 expression. In addition, the activity of the AP-1-driven promoter, but not that of nuclear factor-κB (NF-κB), was inhibited by U0126. In that study, the authors suggested that wogonin inhibited PMA-induced COX-2 mRNA expression by inhibiting c-Jun expression and AP-1 activation in lung cancer cells (9). Another study demonstrated that wogonin induced the apoptosis of lung cancer cells by promoting the generation of reactive oxygen species (ROS) (12). It has also been previously demonstrated that wogonin induces the apoptosis of breast cancer cells by modulating the PI3K/AKT pathway (13). Thus, these studies demonstrate that wogonin inhibits cell cycle progression, regulates the p21, p27 and p53 status, promotes the generation of ROS, and downregulates the expression of the anti-apoptotic protein, Bcl-2 (14).

EGFR, which is overexpressed in various malignancies, plays a central role in essential cellular functions, including proliferation, apoptosis and differentiation, making it an important target in cancer therapy (15,16). It has been demonstrated that the ERK pathway, which can induce pro-matrix metalloproteinase 2 (MMP-2) activation, is a downstream target of EGFR (30). EGFR also regulates AKT signaling. The PI3K/AKT pathway is a well-known signaling pathway, which plays an important role in cell growth, metabolism, proliferation, migration and apoptosis (17,18). In addition, EGFR is an effective target of anti-HCC drugs (19).

However, to date, the molecular mechanisms of action of wogonin in HCC are not yet fully understood. In this study, we examined the effects of wogonin on NF-κB and epidermal growth factor receptor (EGFR) signaling. Importantly, we confirmed that wogonin promoted HCC cell apoptosis through the inhibition of NF-κB-induced Bcl-2 expression, and suppressed HCC cell proliferation and invasion through the inhibition of the EGFR (Tyr845)/ERK/AKT-induced activation of cyclin D1 and MMP2.

Materials and methods

Cell culture and transfection with small interfering RNA (siRNA). The Bel7402 and HepG2 HCC cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum. Wogonin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and dissolved at a 500 mM concentration in dimethyl sulfoxide (DMSO) as a stock solution which was stored at -20˚C. The cells were treated with various concentrations of wogonin (0, 25, 50 and 100 μM) for 24 h. Cells not treated with wogonin were used as controls. In addition, the cells were treated with the NF-κB inhibitor, Bay 11-7082 (5 μM for 12 h; Sigma-Aldrich, St. Louis, MO, USA). siRNA targeting EGFR were purchased from Dharmacon (Thermo Fisher Scientific, Inc., Beijing, China). The sequence of the siRNA against EGFR was CACAGUGGAGCGAAUUCCU. The control non-targeting siRNA sequence was GGACUUGGAUGAAGAAAUC. The cells were transfected with the siRNA using DharmaFECT 1 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The transfection efficiency was determined by western blot analysis.

Cell counting kit-8 (CCK-8) cell proliferation assay. Cell proliferation assay was performed using CCK-8 solution (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Cell proliferation was examined on days 1, 2 and 3 following treatment with wogonin. The cells were seeded at approximately $5x10^3$ cells each well in 96-well plates and incubated with 10 μl CCK-8 solution for approximately 4 h. The optical density of the wells was measured at 450 nm using a Tecan F50 microplate reader (Tecan, Männedorf, Switzerland).

Quantitative (real-time) PCR (qPCR). qPCR was performed using the SYBR-Green master mix kit (Applied Biosystems, Foster City, CA, USA). PCR was performed using the 7500 Real-time PCR system (Applied Biosystems). β-actin

was used as the reference gene. The relative expression of target genes were calculated as $\Delta Ct = Ct$ gene - Ct reference, and the fold change of target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All PCR experiments in this study were repeated in triplicate. The sequences of the primers were as follows: cyclin D1 forward, 5'-GCTGGAGGTCT GCGAGGA-3' and reverse, 5'-ACAGGAAGCGGTCCA GGTAGT-3'; cyclin E forward, 5'-AGCCAGCCTTGGGA CAATAAT-3' and reverse, 5'-GAGCCTCTGGATGG TGCAAT-3'; Bcl-2 forward, 5'-ACGGTGGTGGAG GAGCTCTT-3' and reverse, 5'-CGGTTGACGCTCT CCACAC-3'; MMP2 forward, 5'-TGTGTTCTTTGCAGGG AATGAAT-3' and reverse, 5'-TGTCTTCTTGTTTTTGC TCCAGTTA-3'; β-actin forward, 5'-ATAGCACAGCCTGG ATAGCAACGTAC-3' and reverse, 5'-CACCTTCTACAAT GAGCTGCGTGTG-3'.

Western blot analysis. Whole cell extracts were prepared in cell lysis buffer (Pierce, Rockford, IL, USA) and quantified using the Bradford method. A total of 40 μg protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were transferred onto PVDF membranes (Millipore, Billerica, MA, USA) and blocked using non-fat milk. The membranes were incubated overnight at 4˚C with antibodies against p-ERK (4376), p-AKT (4060), p-EGFR (Tyr845; 6963), cyclin D1 (2978), MMP2 (4022), cyclin E (4129), Bcl-2 (15071), CDK4 (12790), CDK6 (3136), cleaved caspase-3 (9661) and cleaved caspase-9 (7237) at a 1:1,000 dilution (all from Cell Signaling Technology, Danvers, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 5174) at a 1:2,000 dilution (Cell Signaling Technology). This was followed by incubation with HRP-conjugated IgG antibody (1:2,000 dilution) (Cell Signaling Technology). All PVDF membranes were visualized using an enhanced chemiluminescence (ECL) kit (Pierce). Quantitative analysis of the western blots was performed using ImageJ software by assessing the grey value of the western blot bands.

Matrigel invasion assay and migration assay. Matrigel invasion assay was performed using a 24-well Transwell chamber (Costar, Cambridge, MA, USA). The inserts were coated with 20 μl Matrigel (1:5 dilution; BD Bioscience, San Jose, CA, USA). Following treatment, the HepG2 and Bel7402 cells were suspended in 100 μl of medium without serum and were transferred to the upper Transwell chambers. Approximately 600 μl of medium containing 10% fetal bovine serum (FBS) was added to the lower chamber. Following 16 h of incubation, the non-invaded cells on the upper membrane surface were removed using a cotton tip, and the cells that had passed through the filter were stained using hematoxylin (Sigma-Aldrich). The invading cell number was counted under a microscope (BX53; Olympus, Tokyo, Japan).

Cell cycle analysis and apoptosis by flow cytometry. Following incubation with wogonin, the cells were washed with phosphate-buffered saline (PBS) and suspended in a propidium iodide (PI) buffer (10 μg/ml PI, 0.5% Tween-20, 0.1% RNase in PBS). The cell cycle was analyzed using a FACS flow cytometer (Becton-Dickinson, San Jose, CA, USA).
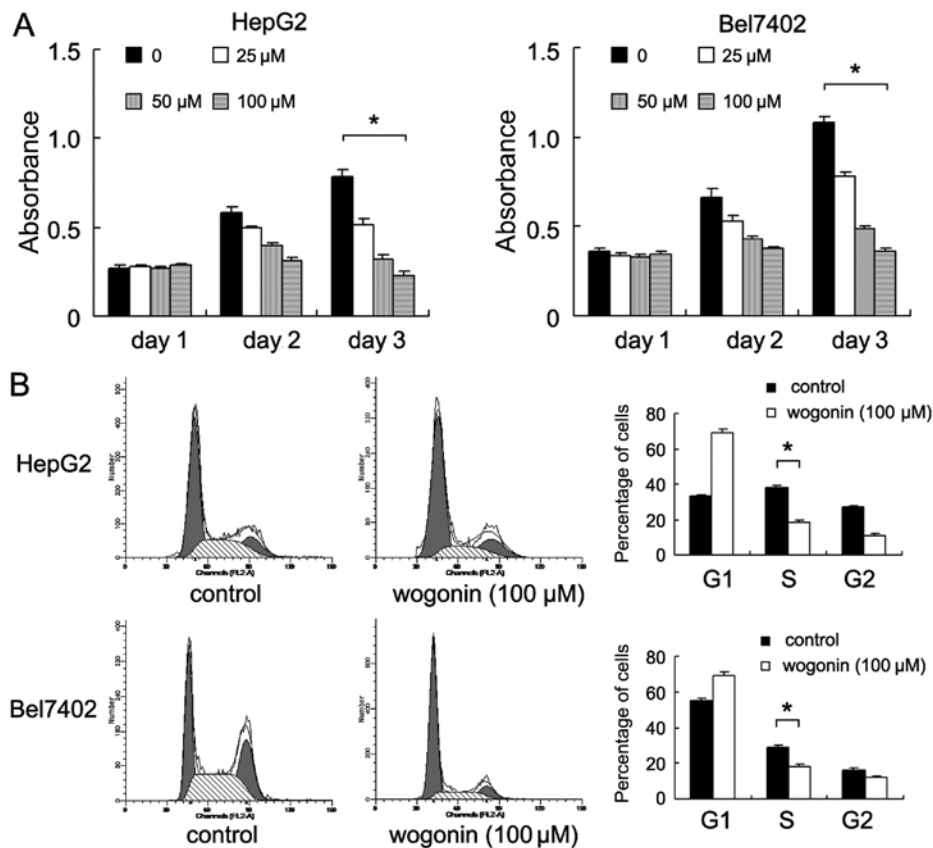
Figure 1. Wogonin inhibits the proliferation of HepG2 and Bel7402 cells. (A) Cell counting kit-8 (CCK-8) assay revealed that treatment with wogonin (0, 25, 50 and 100 μM, for 24 h) decreased the absorbance rate of HepG2 and Bel7402 cell lines in a concentration-dependent manner. (B) Cell cycle analysis revealed that treatment with wogonin decreased the percentage of HepG2 and Bel7402 cells in the S and G2 phase and increased that of cells in the G1 phase. *p<0.05.

For the detection of apoptosis, the Annexin V/PI apoptosis kit (Becton-Dickinson) was used. The cells were washed twice with PBS and suspended with binding buffer. Subsequently, 5 μl of Annexin V-FITC and 10 μl of PI were added followed by the incubation of the cells in the dark. The apoptotic rate was examined using a FACS flow cytometer (Becton-Dickinson).

Statistical analysis. SPSS version 11 for Windows was used for all analyses. ANOVA with a post-hoc test was applied to compare the differences between the control group and the wogonin-treated group. The Student's t-test was used to compare other data and a value of p<0.05 was considered to indicate a statistically significant difference.

Results

Wogonin inhibits the proliferation of HepG2 and Bel7402 cells by inhibiting the G1-S phase transition. The HepG2 and Bel7402 HCC cell lines were used to examine the growth inhibitory effects of wogonin (0, 25, 50 and 100 μM for 24 h). The results of CCK-8 assay revealed that treatment with wogonin inhibited the proliferation of both cell lines in a concentration-dependent manner (day 3: Bel7402, p<0.001; HepG2, p<0.001; ANOVA test) (Fig. 1A). Further analysis of the cell cycle revealed that wogonin significantly reduced the percentage of cells in the S phase and increased the percentage of cells in the G1 phase compared with the untreated control cells, thus indicating

that wogonin induced arrest cell cycle at the G1-S checkpoint (Bel7402, p<0.001; HepG2, p<0.001) (Fig. 1B). Wogonin also decreased the percentage of cells in the G2/M phase in both cell lines (p<0.05).

Cyclin D1, cyclin E and CDK4/6 are the key factors controlling cell cycle progression. Thus, we examined the expression levels of these proteins in the wogonin-treated HepG2 and Bel7402 cells. By performing western blot analysis, we found that treatment with wogonin markedly decreased the protein expression levels of cyclin D1, cyclin E and CDK4/6 in a concentration dependent manner (Fig. 2A). In addition, qPCR yielded similar results. The mRNA expression levels of cyclin D1 and cyclin E decreased in the cells following treatment with wogonin (Bel7402: cyclin D1 and cyclin E, p<0.001; HepG2: cyclin D1 and cyclin E, p<0.001; ANOVA test) (Fig. 2B).

Wogonin induces the apoptosis and suppresses the invasion of HCC cells. We detected apoptosis using Annexin V/PI staining. As shown in Fig. 3A, treatment with wogonin significantly increased the percentage of apoptotic cells compared with the untreated controls. We also examined the levels of cleaved caspase-3 and caspase-9. The results of western blot analysis revealed that wogonin increased the expression levels of cleaved caspase-3 and caspase-9. We also examined changes in the levels of apoptosis-regulating proteins and found that the expression of Bcl-2 was markedly decreased following treatment with wogonin (Fig. 4A). To examine the
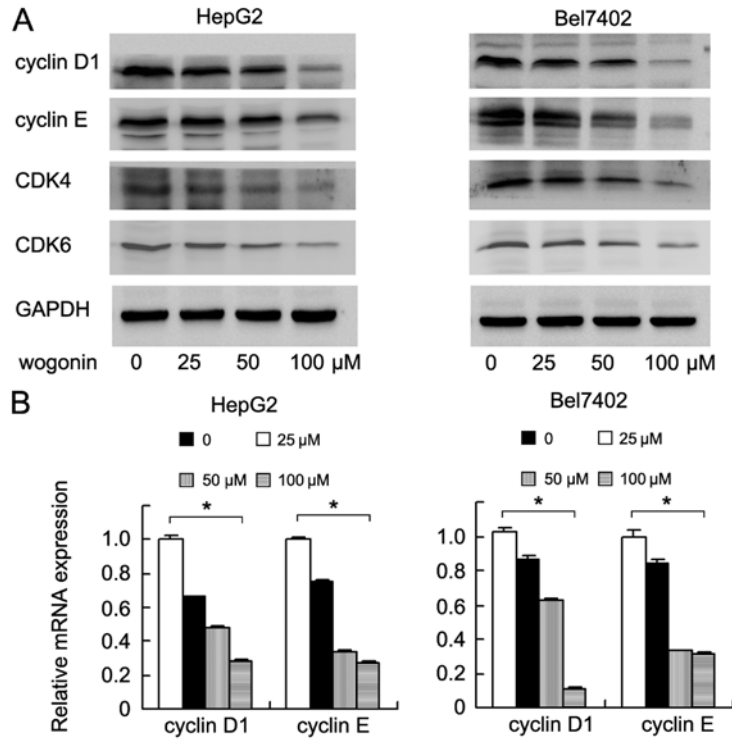
Figure 2. Wogonin inhibits the expression of cell cycle-related proteins. (A) Western blot analysis revealed that treatment with wogonin (0, 25, 50 and 100 μM, for 24 h) decreased the protein expression levels of cyclin D1, cyclin E, CDK4 and CDK6 in the HepG2 and Bel7402 cells in a concentration-dependent manner. (B) qPCR revealed that treatment with wogonin (0, 25, 50 and 100 μM, 24 h) decreased the mRNA expression of cyclin D1 and cyclin E in a concentration-dependent manner. *$p<0.05$.
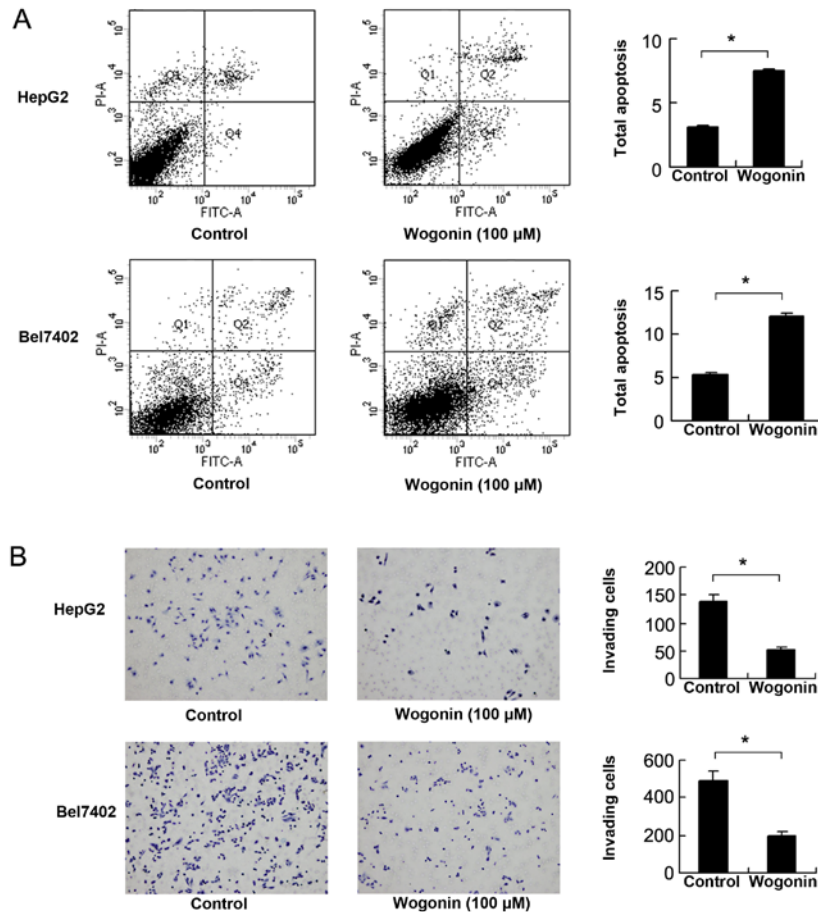

Figure 3. Wogonin induces the apoptosis and suppresses the invasion of HCC cells. (A) Annexin V/PI staining indicated that treatment with wogonin significantly increased the percentage of apoptotic HepG2 and Bel7402 cells. Q1, dead cells; Q2, late apoptotic cells; Q3, live cells; Q4, early apoptotic cells. (B) Matrigel invasion assay indicated that wogonin significantly decreased the invading number of HepG2 and Bel7402 cells. *$p<0.05$.
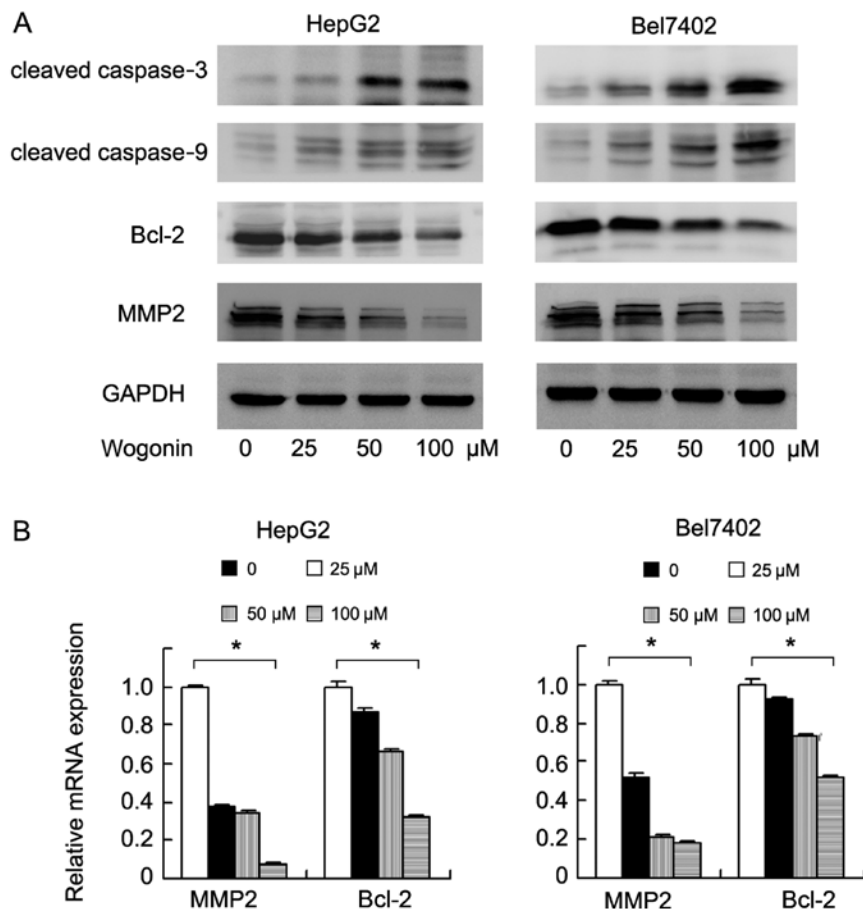
Figure 4. Wogonin regulates the expression of invasion- and apoptosis-related proteins. (A) Western blot analysis revealed that treatment with wogonin (0, 25, 50 and 100 μM, for 24 h) decreased the protein expression of Bcl-2 and matrix metalloproteinase 2 (MMP2) and upregulated the levels of cleaved caspase-3 and caspase-9 in a concentration-dependent manner. (B) qPCR revealed that treatment with wogonin (0, 25, 50 and 100 μM, for 24 h) decreased the mRNA expression of Bcl-2 and MMP2 in a concentration-dependent manner. *p<0.05.

effects of wogonin on the invasive ability of the HepG2 and Bel7402 cells, Matrigel invasion assay was carried out with the wogonin-treated cells using a Transwell chamber. As shown in Fig. 3B, treatment with wogonin for 24 h significantly decreased the number of invading HepG2 and Bel7402 cells (control vs. treatment: Bel7402, 492±31.5 vs. 198±16.5, p<0.001; HepG2, 137±10.5 vs. 51±6.5, p<0.001). In addition, we examined the levels of proteins associated with cell invasion and found that the expression levels of MMP2 and Bcl-2 were significantly downregulated at both the protein and mRNA levels (Bel7402: MMP2 and Bcl-2, p<0.001; HepG2: MMP2 and Bcl-2, p<0.001, ANOVA test) (Fig. 4B).

Wogonin induces apoptosis through NF-κB/Bcl-2 signaling. To explore the potential mechanisms of action of wogonin in the HepG2 and Bel7402 cell lines, we examined several signaling pathways which are related to cancer cell proliferation and invasion. We examined the effects of wogonin on NF-κB signaling. The results of western blot analysis revealed that the expression levels of p-IκB and p-p65 were significantly decreased following treatment with various concentrations of wogonin (Fig. 5A). Bcl-2 has been reported as a downstream target of NF-κB signaling (20). We demonstrated that treatment with the NF-κB inhibitor, Bay 11-7082 (5 μM for 12 h), decreased the expression of Bcl-2 in both cell lines. In addi-

tion, in the Bay 11-7082-treated cells, the suppressive effects of wogonin on Bcl-2 expression were not significant, suggesting that wogonin induced the apoptosis of HCC cells through the inhibition of NF-κB/Bcl-2 signaling; thus NF-κB signaling is required for the inhibitory effects of wogonin on Bcl-2 expression and for its promoting effects on apoptosis (Fig. 5B).

Wogonin suppresses HepG2 and Bel7402 cell proliferation and invasion through the inhibition of EGFR signaling and downstream ERK/AKT signaling. EGFR is a tyrosine kinase located at the cell membrane, which functions as an oncogene, mediating the malignant growth and invasion of various cancer cells (21-23). Our results revealed that wogonin inhibited EGFR (Tyr845) phosphorylation. The levels of downstream factors of EGFR signaling, including p-ERK and p-AKT were also downregulated following treatment with wogonin (Fig. 5A). To confirm the involvement of EGFR signaling in the wogonin-induced suppressive effects on MMP2 and cyclin D1 expression, we knocked down EGFR expression in these cell lines using siRNA. We found that, in the cells transfected with the siRNA targeting EGFR, the inhibitory effects of wogonin on cyclin D1 and MMP2 expression were not significant (Fig. 5C). These results suggested that wogonin inhibited HCC cell proliferation and invasion through the inhibition of EGFR (Tyr845) activity and that of
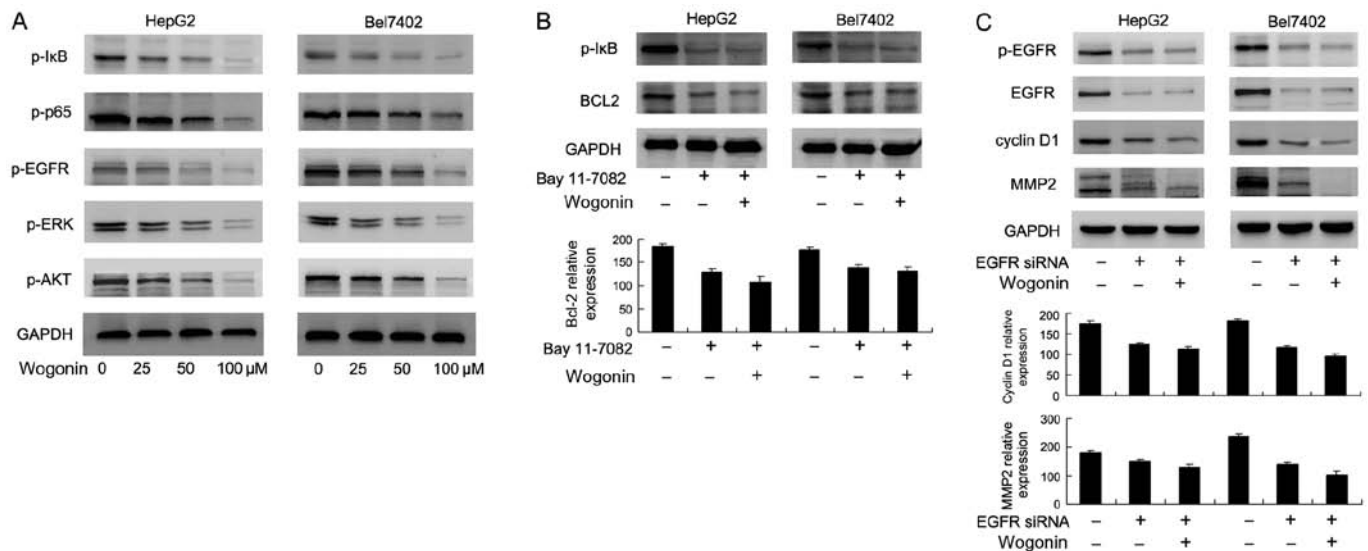
Figure 5. Wogonin regulates nuclear factor-κB (NF-κB)/Bcl-2 and epidermal growth factor receptor (EGFR)/ERK/AKT signaling. (A) Western blot analysis revealed that treatment with wogonin (0, 25, 50 and 100 μM, for 24 h) decreased the expression levels of p-IκB, p-p65, p-EGFR, p-ERK and p-AKT in a concentration-dependent manner. (B) Treatment with the NF-κB inhibitor, Bay 11-7082, decreased p-IκB and Bcl-2 expression. In the Bay 11-7082-treated cells, the effects of wogonin were not significant. (C) Transfection with EGFR siRNA significantly decreased the expression levels of p-EGFR, matrix metalloproteinase 2 (MMP2) and cyclin D1. In the cells transfected with EGFR siRNA, the effects of wogonin on these proteins were not significant.

its downstream factors, namely that of EGFR/ERK/MMP2, EGFR/AKT and EGFR/cyclin D1 signaling.

Discussion

In this study, we used the HCC cell lines, HepG2 and Bel7402, to examine the antitumor effects of wogonin. As shown by CCK-8 assay and Transwell assay, wogonin inhibited the proliferation and invasion of the HepG2 and Bel7402 cells in a concentration-dependent manner. In addition, cell cycle progression was arrested at the G1-S point, with the downregulation of cyclin family proteins, such as cyclin D1 and cyclin E. Invasion-related MMP2 expression was also downregulated. When examining the signaling pathways involved in the wogonin-mediated inhibitory effects on cell proliferation, we found that ERK and AKT signaling was significantly inhibited. Of note, we found that wogonin inactivated EGFR (Tyr845) phosphorylation, which is an upstream tyrosine kinase of ERK and AKT (24). To confirm the involvement of EGFR in the anticancer effects of wogonin, we knocked down endogenous EGFR expression in these cell lines and then examined the effects of wogonin on EGFR downstream factors, such as cyclin D1 and MMP2 (25,26). In the cells in which EGFR expression had been depleted, the effects of wogonin on cyclin D1 and MMP2 expression were not significant compared with those of the normal HepG2 and Bel7402 cells, suggesting wogonin exerts its anticancer effects through the inhibition of EGFR activity.

EGFR overexpression has been found in many types of cancer, and it plays important roles in cancer proliferation, invasion and metastasis, and is also associated with a poor survival rate (27,28). MMP2 plays a pivotal role in the invasion of many malignant cancers. EGFR upregulates its downstream molecule, MMP2, through the phosphorylation of the MEK/ERK/AP1 signaling pathway (29,30). In addition, EGFR activates AKT signaling, which plays a central role in cancer cell

proliferation and survival (31). Many studies have demonstrated that targeting EGFR activity inhibits tumor growth and improves survival (32-34). In this study, we demonstrated that wogonin targets EGFR phosphorylation to suppress HCC cell proliferation and invasion, suggesting that wogonin may be used as a chemotherapeutic agent in the treatment of HCC.

In addition to the inhibitory effects of wogonin on EGFR-related HCC cell proliferation and invasion, we demonstrated that wogonin promoted apoptosis, which was in parallel with the downregulation of Bcl-2 protein and the cleavage of caspase-3 and caspase-9, both of which contribute to the apoptosis-inducing effects of wogonin. We also found that wogonin inhibited NF-κB signaling, which has been reported to be involved in Bcl-2 and the regulation of apoptosis in various types of cancer (35-37). Our results also revealed that in the cells treated with the NF-κB inhibitor, the suppressive effects of wogonin on Bcl-2 were significantly reduced. In cancer cells, NF-κB is activated either due to mutations in genes encoding the NF-κB transcription factors themselves, or in genes that control NF-κB activity. Since the role of NF-κB activation in cancer cell growth and survival has been reported in HCC (38), we hypothesized that NF-κB activation may partly suppress the apoptosis-inducing effects of wogonin. In addition, EGFR has been reported to activate NF-κB signaling through the CARMA3/Bcl10 complex (39). Since there is a crosstalk between NF-κB and EGFR signaling, we hypothesized that the effects of wogonin on NF-κB are partly due to its effects on EGFR signaling.

In conclusion, in this study, we demonstrated that wogonin inhibited the malignant biological behavior of the HCC cell lines, HepG2 and Bel7402, by inhibiting the phosphorylation of EGFR (Tyr845) and its downstream EGFR/cyclin D1, EGFR/AKT and EGFR/ERK/MMP2 signaling pathways. Wogonin also inhibited the activation of the NF-κB/Bcl-2 pathway and induced apoptosis. Thus, wogonin may serve as a novel therapeutic agent for the treatment of HCC.

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