

Polysiphonia japonica extract suppresses the Wnt/ β -catenin pathway in colon cancer cells by activation of NF- κ B

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Abstract. Abnormal activation of the Wnt/ β -catenin pathway and subsequent up-regulation of β -catenin response transcription (CRT) are associated with the development of colon cancer. Thus, the Wnt/ β -catenin pathway is an attractive target for chemoprevention and treatment of this cancer. We used a cell-based screen to identify a methanol extract of *Polysiphonia japonica* (EPJ) that suppresses the Wnt/ β -catenin pathway without altering the level of β -catenin protein and reduces the expression of cyclin D1, which is a known β -catenin/T cell factor (TCF)-dependent gene. EPJ inhibited the growth of various colon cancer cells. In addition, EPJ induced the nuclear translocation of nuclear factor- κ B (NF- κ B) in SW480 colon cancer cells. Our findings suggest that EPJ attenuates Wnt/ β -catenin signaling via activation of NF- κ B and can potentially be used as a chemopreventive agent against colon cancer.

Introduction

The Wnt signaling pathway plays important roles in developmental processes and tumorigenesis (1-3). A key control of this pathway is the regulation of β -catenin activity, which depends on its intracellular protein level. In the presence of the Wnt signal, the signal is transduced the protein disheveled to negatively regulate glycogen synthase kinase-3 β (GSK-3 β), resulting in the accumulation of intracellular β -catenin (4-6).

In the absence of the Wnt signal, β -catenin is associated with a multiprotein complex that is composed of adenomatous polyposis coli (APC), Axin, and GSK-3 β (7-11) and leads to the degradation of β -catenin through a ubiquitin-dependent mechanism (12,13).

Inactive mutations of the APC tumor suppressor gene occur in the majority of sporadic colorectal cancers as well as in familial adenomatous polyposis (FAP) (9,10). In addition, mutations in the β -catenin gene sequences encoding the phosphorylation motifs in its N-terminal domain have been observed in colorectal cancer and melanoma (11). These mutations result in an excessive accumulation of β -catenin in the nucleus and stimulation of its target genes, including cyclin D1, myc, matrix metalloproteinase-7, and PPAR- δ , which play important roles in tumorigenesis (14-17). Thus, the constitutive activation of Wnt/ β -catenin signaling is a potential target for the chemoprevention and treatment of colorectal cancer.

We identified EPJ, which inhibits Wnt/ β -catenin signaling, through a cell-based screen in the present study. EPJ may suppress β -catenin response transcription (CRT) through cross-regulation of the Wnt/ β -catenin and NF- κ B pathways in colon cancer cells.

Materials and methods

Cell culture, plasmid, transfection, and luciferase assay. HEK293, HCT116, SW480, HCT15, DLD-1, and Wnt3a-secreting L cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 120 μ g/ml penicillin, and 200 μ g/ml streptomycin. For Wnt3a conditioned medium (Wnt3a CM), Wnt3a-secreting L cells were cultured in DMEM with 10% FBS for 4 days. The medium was harvested and sterilized using a 0.22-mm filter. Fresh medium was added, and the cells were cultured for another 3 days. The medium was collected and combined with the previous medium. Human Frizzled cDNA was cloned from SW480 cDNA (Clontech) by PCR and then subcloned into pCDNA3.1 (Invitrogen). The pTOPflash reporter plasmid was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Transfection was carried out with

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Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase assays were performed using the dual luciferase assay kit (Promega).

Preparation of extract. Species of marine algae (126) used in the study were gathered from the seashore around Jeju from February to April 2004 and identified botanically at the Faculty of Applied Marine Science in Cheju National University. The algae were cleaned, lyophilized and then ground into fine powder. The powdered sample (1 g) was extracted for 24 h with 80% methanol under continuous shaking at room temperature. The methanol extracts were then concentrated in vacuum at 40°C in a rotary evaporator and stored at 4°C.

Cell-based screening. The HEK293 reporter cell line was established by selecting HEK293 cells co-transfected with the plasmid expressing hFz-1 and TOPFlash, using media containing G418 (1 mg/ml). The cells were inoculated into 96-well plates at 15,000 cells per well in duplicate and grown for 24 h. Wnt3a CM was added, and the extracts were then added to the wells. After 15 h, the plates were assayed for firefly luciferase activity and cell viability.

Western blotting. The cytosolic and nuclear fraction was prepared as previously described (18). Proteins were separated by 4-12% gradient SDS-PAGE (Invitrogen) and transferred to a nitrocellulose membrane (Amersham Biosciences). The membranes were blocked with 5% nonfat milk and probed with anti- β -catenin (BD Transduction Laboratories), anti-p65 (Santa Cruz Biotechnology), anti-cyclin D1 (Santa Cruz Biotechnology), and anti-actin antibodies (Cell Signaling Technology). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology), and the bands were visualized using the ECL system (Santa Cruz Biotechnology).

Immunofluorescence analysis. SW480 cells were cultured on glass chamber slides and treated with either methanol or EPJ for 15 h. After treatment, the cells were washed with PBS, fixed with 4% formaldehyde, permeabilized in 0.3% Triton X-100, and blocked in 4% bovine serum albumin for 1 h. The cells were stained with anti-p65 antibody and then analyzed by confocal microscopy using a Zeiss LSM510 Meta microscope.

Cell viability assay. Cells were inoculated into 96-well plates and treated with EPJ for 48 h. The cell viability from each treated sample was measured in triplicate using Cell titer-Glo assay kit (Promega) according to the manufacturer's instructions.

Results

Identification of EPJ as an inhibitor of the Wnt/ β -catenin pathway. We established reporter cell lines, which were stably transfected with a TOPFlash reporter and the human Frizzled-1 (hFz-1) expression plasmid, to screen for extracts that could inhibit Wnt/ β -catenin signaling. When the HEK293 reporter cells were incubated with Wnt3a-conditioned medium (Wnt3a CM), TOPFlash reporter activity dramatically

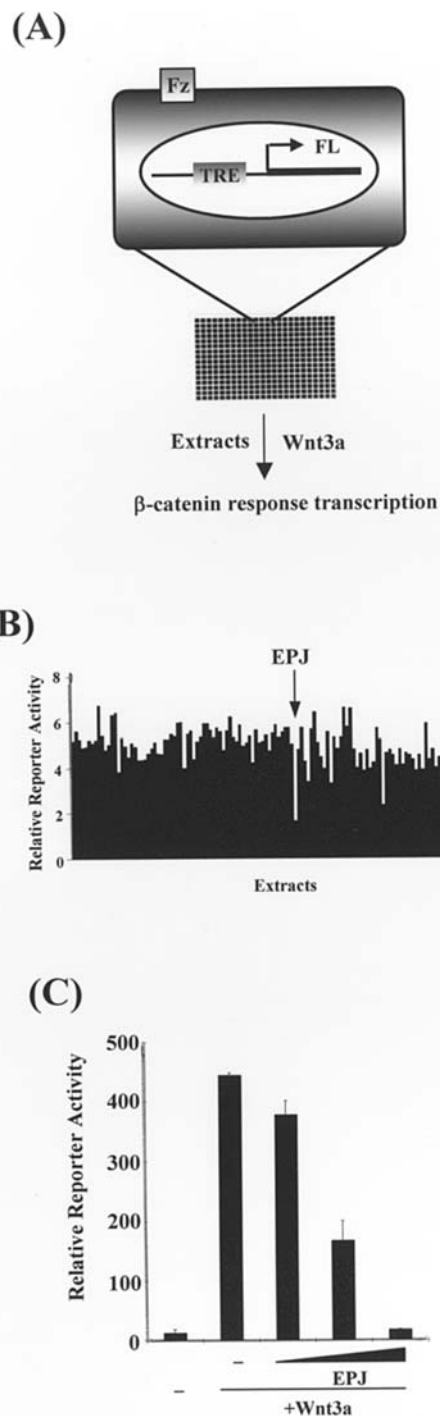


Figure 1. Identification of *Polysiphonia japonica* extract as an inhibitor of Wnt/ β -catenin signaling. (A) A schematic of the screening system. (B) Screening of extracts that inhibit Wnt/ β -catenin signaling. Extracts modulating TOPFlash reporter activity were screened using the HEK293 reporter cells. The controls were assayed in the presence or absence of Wnt3a CM. TOPFlash activities were normalized with Cell titer-Glo (Promega) activity. (C) HEK293 reporter cells were incubated with increasing concentrations of EPJ (10, 20 and 40 μ g/ml) in the presence of Wnt3a CM. After 15 h, luciferase activity was determined. The results are the average of three experiments, and the bars indicate standard deviations.

increased (data not shown). Using this system, we screened 126 methanol extracts from marine algae (Fig. 1A). One of the extracts identified from this screen was an extract of *Polysiphonia japonica* (EPJ) (Fig. 1B). As shown in Fig. 1C,

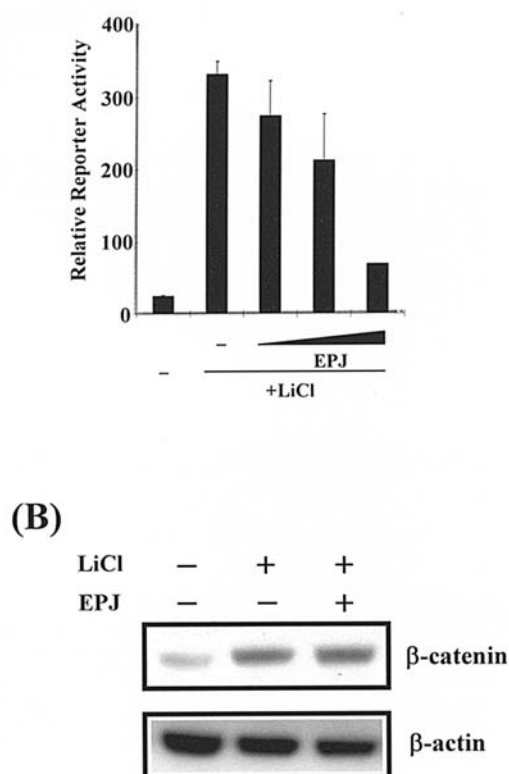


Figure 2. EPJ inhibits Wnt/ β -catenin signaling without altering β -catenin levels. (A) HEK293 reporter cells were incubated with increasing concentrations of EPJ (10, 20 and 40 μ g/ml) in the presence of 20 mM LiCl. After 15 h, luciferase activity was determined. The results are the average of three experiments, and the bars indicate standard deviations. (B) Cytosolic proteins were prepared from HEK293 reporter cells treated with either vehicle (methanol) or EPJ (20 and 40 μ g/ml) in the presence of 20 mM LiCl for 15 h and then subjected to Western blotting with β -catenin antibody. The blots were re-probed with anti-actin antibody as a loading control.

treatment with EPJ resulted in a concentration-dependent decrease in the CRT that had been induced by Wnt3a CM.

EPJ does not affect the level of β -catenin. HEK293 reporter cells were incubated with EPJ and LiCl, which specifically inhibits GSK-3 β , to investigate the molecular mechanism of the EPJ-induced Wnt/ β -catenin signaling inhibition. This procedure led to an accumulation of intracellular β -catenin and the activation of CRT (19). As shown in Fig. 2A, EPJ was able to inhibit LiCl-induced CRT. Therefore, EPJ inhibits Wnt/ β -catenin signaling by acting on β -catenin itself or its downstream components. In Wnt/ β -catenin signaling, CRT is dependent on the intracellular β -catenin level, which is regulated by ubiquitin-dependent degradation. To examine the possibility that EPJ affects the intracellular β -catenin level, we performed Western blotting with anti- β -catenin antibody to determine the amount of cytosolic β -catenin in response to EPJ. As shown in Fig. 2B, the level of β -catenin that accumulated in the presence of LiCl was not changed by EPJ treatment.

EPJ represses CRT in colon cancer cells. We tested the effect of EPJ on colon cancer cells because mutations that lead to the accumulation of β -catenin are frequently observed in colon

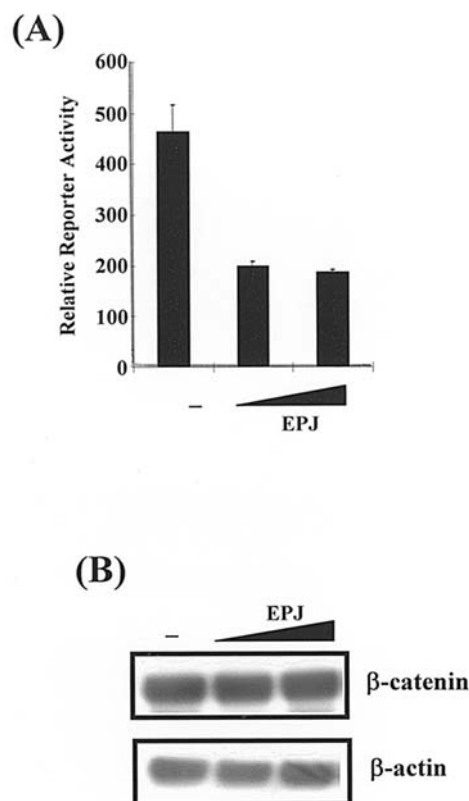


Figure 3. EPJ inhibits CRT in SW480 colon cancer cells. (A) SW480 cells were co-transfected with TOPFlash and pCMV-RL plasmids and incubated with increasing amounts of EPJ (20 and 40 μ g/ml) for 15 h. Luciferase activities were measured 39 h after transfection. Results are the average of three experiments, and the bars indicate standard deviations. (B) Cytosolic proteins were prepared from SW480 cells treated with the vehicle (methanol) or EPJ (20 and 40 μ g/ml) for 15 h and then subjected to Western blotting with β -catenin antibody. To confirm equal loading, the blot was re-probed with anti-actin antibody.

cancer (11). SW480 colon cancer cells, which contain mutant-type APC (20,21), were transfected with TOPFlash and then treated with EPJ. Consistent with the result in the reporter cells, incubation with EPJ resulted in a decrease of CRT in the SW480 cells (Fig. 3A). In addition, Western blot analysis using β -catenin antibody showed that EPJ did not affect the intracellular β -catenin level in the SW480 cells (Fig. 3B). These results suggest that EPJ inhibits Wnt/ β -catenin signaling without altering the intracellular β -catenin level.

EPJ down-regulates cyclin D1 and inhibits the proliferation of colon cancer cells. The cyclin D1 gene has been shown to be a β -catenin-dependent gene and to play a critical role in colon carcinogenesis (22,23). Thus, we investigated whether EPJ affects the expression of cyclin D1 in SW480 cells. SW480 cells were treated with EPJ for 15 h; we then determined the cyclin D1 protein level in response to EPJ treatment. Western blot analysis showed that the expression of cyclin D1 protein was down-regulated following the incubation with EPJ (Fig. 4A). Previous studies have demonstrated that disruption of the β -catenin function reduces the growth of human colon cancer cells (20,21). Because EPJ inhibits β -catenin activity, we postulated that EPJ would also inhibit the growth of colon cancer cells. To address this issue, we

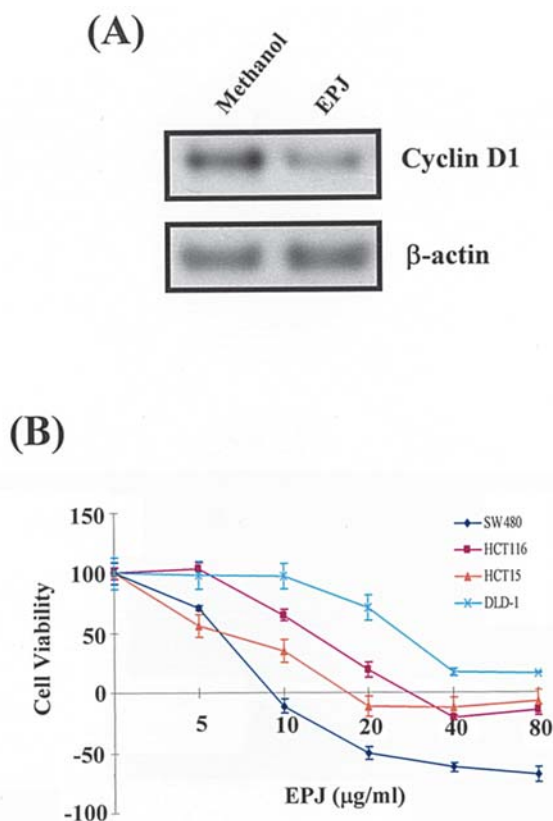


Figure 4. The effect of EPJ on colon cancer cells. (A) EPJ inhibits the expression of the TCF/ β -catenin target gene. SW480 cells were incubated with the vehicle (methanol) or EPJ ($40 \mu\text{g/ml}$) for 15 h. Nuclear extracts were prepared for Western blotting with anti-cyclin D1 antibody. To confirm equal loading, the blot was re-probed with anti-actin antibody. (B) Cytotoxic effect of EPJ on colon cancer cells. Cells were incubated with the indicated concentrations of EPJ for 48 h in 96-well plates, and cell viability was determined as described in Materials and methods. To calculate the inhibition of growth, the value at time 0 was first subtracted. The results shown are the average of three experiments, and the bars indicate standard deviations.

examined the effect of EPJ on the growth of human colon cancer cells, including SW480, HCT-15, HCT116, and DLD-1. The cells were incubated with various concentrations of EPJ, and cell viability was measured. As shown in Fig. 4B, EPJ efficiently inhibited the growth of the tested colon cancer cells. The IC_{50} values for the SW480, HCT-15, HCT116, and DLD-1 cells were 6.7, 6.5, 14.5, and $30.8 \mu\text{g/ml}$, respectively; indicating that EPJ is efficacious at inhibiting the proliferation of various colon cancer cells.

EPJ activates the NF- κ B pathway. In our previous study, we demonstrated that Wnt/ β -catenin signaling is attenuated by activation of the NF- κ B pathway without an alteration in the intracellular β -catenin level in colon cancer cells (24). Our data show that EPJ also suppresses Wnt/ β -catenin signaling without changing the β -catenin level. Thus, we asked whether EPJ induces NF- κ B signaling in SW480 colon cancer cells. Upon the activation of NF- κ B signaling, the NF- κ B that dissociates from I κ B, which is the NF- κ B inhibitor protein, is translocated into the nucleus; this process is followed by the induction of a wide variety of genes involved in inflammation, cell proliferation, and apoptosis (25-27). Therefore, we first examined

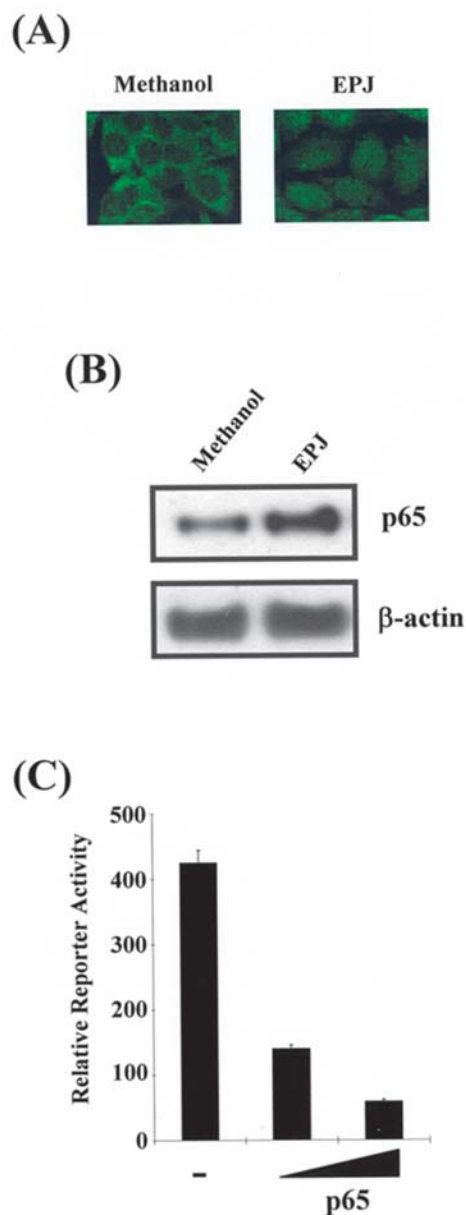



Figure 5. EPJ induces the degradation of I κ B α and the nuclear translocation of NF- κ B. (A) EPJ induces nuclear translocation of NF- κ B. The cellular location of NF- κ B in SW480 cells treated with methanol and EPJ ($40 \mu\text{g/ml}$) was determined by immunofluorescence analysis. After fixation, the cells were stained with anti-p65 antibody and observed at $\times 400$ magnification. (B) Nuclear proteins from SW480 cells treated with EPJ ($40 \mu\text{g/ml}$) were subjected to Western blotting with anti-p65 antibody. The blots were re-probed with anti-actin antibody as a loading control. (C) SW480 cells were co-transfected with TOPflash and pCMV-p65. At 48 h after transfection, the luciferase activity was measured. The results are shown as the average of three experiments; the bars indicate standard deviations.

the location of NF- κ B in the EPJ-treated SW480 colon cancer cells. Immunofluorescence analysis using anti-p65 antibody showed that p65, a subunit of NF- κ B, was primarily detected in the cytoplasm in vehicle (methanol)-treated cells (Fig. 5A). However, when SW480 cells were incubated with EPJ, the nuclear accumulation of p65 was observed (Fig. 5A). We also confirmed the EPJ-induced nuclear translocation of NF- κ B using Western blot analysis. In agreement with the immunofluorescence analysis, the level of p65 in the nuclear fraction

 SPANDIDOS[®] used in response to treatment with EPJ (Fig. 5B). The ectopic expression of p65 repressed CRT in SW480 cells (Fig. 5C). Taken together, these results indicate that treatment with EPJ inhibits the Wnt/ β -catenin pathway by stimulation of NF- κ B signaling in SW480 colon cancer cells.

Discussion

Previous studies have indicated that mutations in a component of the Wnt/ β -catenin pathway, leading to the up-regulation of β -catenin response transcription (CRT), are linked to the development of several cancers, including colon cancer and melanoma (10,28). In this study, we identified an extract from *P. japonica* (EPJ) that inhibits Wnt/ β -catenin signaling through cell-based screening. We also demonstrated that EPJ decreased CRT through a GSK-3 β -independent mechanism and without degradation of β -catenin. EPJ also suppressed the expression of cyclin D1, which is one of the β -catenin/TCF-dependent genes. In addition, EPJ had a growth inhibitory effect on various colorectal cancer cells. Most cancer cells that are resistant to chemotherapeutic agents contain mutant p53. EPJ showed effective cytotoxicity in cell lines containing wild-type p53 (HCT116) and mutant-type p53 (SW480). More importantly, EPJ efficiently inhibited the growth of multidrug-resistant (MDR) positive cells, which present serious problems for anticancer therapeutics.

NF- κ B is composed of two subunits, p65 and p50, and is normally retained in the cytoplasm by an inhibitory protein, I κ B α (29). Stimulation of the NF- κ B pathway leads to the phosphorylation and subsequent degradation of I κ B α , which then allows NF- κ B to translocate to the nucleus (30). Cross-regulation between the Wnt/ β -catenin and NF- κ B pathways has been demonstrated (31). In addition, ectopic expression of p65 (Rel A), which is a component of NF- κ B, was found to repress the CRT that had been induced by the overexpression of mutant β -catenin (32). Recently, a nonsteroidal anti-inflammatory drug (NSAID), diclofenac, was shown to inhibit Wnt/ β -catenin signaling through the activation of the NF- κ B pathway and without altering the level of the β -catenin protein (24). Therefore, the NF- κ B pathway may be a target mechanism for EPJ-mediated CRT inhibition. In the present study, EPJ induced the accumulation of p65 in the nucleus, suggesting that EPJ may suppress the Wnt/ β -catenin signaling via the activation of NF- κ B in colon cancer cells. Taken together, EPJ can be developed into a new chemopreventive agent against colorectal cancer.

Acknowledgements

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