Biochemical and Biophysical Research Communications 443 (2014) 132–137

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tussilagone suppresses colon cancer cell proliferation by promoting the degradation of β-catenin

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ARTICLE INFO

Article history:
Received 6 November 2013
Available online 22 November 2013

Keywords:
Tussilagone
Colon cancer
β-Catenin

ABSTRACT

Abnormal activation of the Wnt/β-catenin signaling pathway frequently induces colon cancer progression. In the present study, we identified tussilagone (TSL), a compound isolated from the flower buds of *Tussilago farfara*, as an inhibitor on β-catenin dependent Wnt pathway. TSL suppressed β-catenin/T-cell factor transcriptional activity and down-regulated β-catenin level both in cytoplasm and nuclei of HEK293 reporter cells when they were stimulated by Wnt3a or activated by an inhibitor of glycogen synthase kinase-3β. Since the mRNA level was not changed by TSL, proteasomal degradation might be responsible for the decreased level of β-catenin. In SW480 and HCT116 colon cancer cell lines, TSL suppressed the β-catenin activity and also decreased the expression of cyclin D1 and c-myc, representative target genes of the Wnt/β-catenin signaling pathway, and consequently inhibited the proliferation of colon cancer cells. Taken together, TSL might be a potential chemotherapeutic agent for the prevention and treatment of human colon cancer.

1. Introduction

The Wnt signaling pathway contains canonical or noncanonical types. The canonical Wnt pathway involves β-catenin, while the noncanonical pathways work independently of it. The well characterized Wnt/β-catenin pathway regulates cellular proliferation, differentiation, survival and death. Aberrant Wnt/β-catenin signaling pathway has been implicated in many human cancers including colorectal cancer [1]. Without Wnt signaling, the β-catenin can be degraded in the cytoplasm by a destruction complex machinery: Axin1, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). In the presence of Wnt proteins, the Wnt binds its receptor complex and the destruction complex is disrupted. This results in the accumulation of β-catenin in cytosol and its translocation into the nucleus. A complex of β-catenin/T-cell factor (Tcf) transcription factor induces the expression of target genes such as c-myc or cyclin D1, resulting in the proliferation and metastasis of tumor cells [2]. Therefore, modulators of Wnt/β-catenin pathway might be promising candidates for chemotherapeutic agents.

Colon cancer is the most common type of cancer. Mutations that cause the development and progression of colon cancer are well characterized compared to other human tumors [3]. Mutations in the APC gene are identified in familial adenomatous polyposis coli (FAP) and occur in majority of sporadic colorectal cancer [4]. N-terminal phosphorylation motif of the β-catenin is also frequently mutated in colorectal cancers [5]. These mutations lead to the excessive accumulation of cytosolic β-catenin, which is translocated into the nuclei and then activates the expression of its target genes, such as cyclin D1, myc, matrix metalloproteinase-7, and PPAR-δ, which play important roles in colorectal tumorigenesis. Thus, the promotion of β-catenin degradation is a potential therapeutic strategy for chemoprevention and treatment of colon cancers.

The flower buds of *Tussilago farfara* (Farfarae flos, Compositae) have been used as a traditional medicine for the treatment of bronchitic and asthmatic conditions [6]. It has been reported that the extracts of Farfarae flos exhibit the inhibition of arachidonic acid metabolism [7], inhibition of platelet activating factor receptor [8], cardiovascular and respiratory stimulation [9], antimicrobial activity [10], neuroprotective and antioxidative effects [11]. Tussilagone (TSL), a sesquiterpene isolated from the *T. farfara*, possess anti-inflammatory activity by the inhibition of nitric oxide production in lipopolysaccharide-activated microglia in our previous study [12]. As a part of our ongoing study to search for modulators of Wnt pathway from medicinal plants, we found that the extract of *T. farfara* showed the suppressive effect on the activated β-catenin/T-cell factor (Tcf) transcriptional activity. Herein, we report TSL as an inhibitor of Wnt/β-catenin pathway and disclosed the underlying mechanism.

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http://dx.doi.org/10.1016/j.bbrc.2013.11.062

2. Materials and methods

2.1. Isolation of tussilagonge from T. farfara

TSL was purified and the structure was identified as previously described [12].

2.2. Cell culture, transfection, and luciferase assay

SW480 and HCT116 colon cancer cells were plated in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Paisley, UK) containing 10% heat-inactivated FBS (Life Technologies, Paisley, UK), penicillin (100 U/mL) and streptomycin (10 μg/mL) (Life Technologies, Paisley, UK). Cells were maintained at 37 °C in 5% CO_2 air environment. Transfection was performed with Lipofectamine 2000 (Life Technologies, Paisley, UK) according to the manufacturer's instructions.

2.3. Preparation of Wnt3a-conditioned medium (Wnt3a-CM)

Wnt3a-secreting L cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/mL) and streptomycin (10 μg/mL). Wnt3a-conditioned medium (Wnt3a-CM) was prepared according to the manufacturer's instruction. In brief, Wnt3a-secreting L cells were cultured in DMEM with 10% FBS for 4 days. The medium was harvested and sterilized using a 0.22-μm filter. The cells were added fresh medium and cultured for another 3 days, and the medium was collected and combined with the previous medium.

2.4. Screening for a small-molecule inhibitor of Wnt/β-catenin signaling

The HEK293 (TOPFlash) and control (FOPFlash) cell lines were kindly gifted by Professor Sangtaek Oh (Kookmin University, Korea). The cells were inoculated into 96-well plates at 1000 cells/well in triplicate and grown for 24 h. Cells were treated by Wnt3a-CM and/or test compounds. After 15 h, the plates were assayed for firefly luciferase activity and cell viability.

2.5. Protein extraction and Western immunoblot

To prepare whole-cell lysate, cells were treated with TSL for 48 h. Harvested cells were washed twice with ice-cold PBS (pH 7.4), and gently lysed with cell lysis buffer (Cell Signaling Technologies, Beverly, MA). Cell lysates were then centrifuged at 10,000g for 20 min at 4 °C. Supernatants were collected and protein concentrations were determined using BCA protein assay kit.

The cytosolic and nuclear fractions were prepared as previously described with some modification [13]. Proteins were separated using 10% SDS–PAGE and transferred to PVDF membranes (GE healthcare, Piscataway, NJ) by a standard method. The membranes were blocked with 5% nonfat milk in phosphate buffered saline (PBS)–T (0.1% Tween20) and probed with primary antibodies (1:3000). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Cell Signaling, Beverly, MA) and visualized using the ECL chemiluminescence (GE healthcare, Piscataway, NJ). The antibody against β-catenin was purchased from BD Transduction Laboratories (San Jose, CA). The antibodies against c-myc, β-actin, laminA were purchased from Cell Signaling Technology (Beverly, MA). The antibody against cyclin D1 was purchased from Santa Cruz Biotechnology (Dallas, TX).

2.6. RT-PCR analysis

Cells (1×10^6 cells/60 mm dish) were treated for 15 h with or without TSL. After washing twice with phosphate buffered saline (PBS), total RNA was isolated from the cell pellet using a RNA-isolation Trizol reagent (Life Technologies, Paisley, UK). Two micrograms of RNA was reverse transcribed into cDNA using reverse transcriptase and random hexamer. The PCR samples, contained in the reaction mixture, comprised mixture buffer, dNTP, Taq DNA polymerase (Promega, Madison, WI) and primers (sense and antisense). The sense and antisense primers for β-catenin were 5′-GGGATGTTCACAACCGAATTGTTATC-3′ and 5′ACCAGAGTGAA AAGAACGATAGCTAGGA-3′, respectively. The sense and antisense primers for β-actin were 5′-TGTGATGGTGGGAATGGGTCAG-3′ and 5′-TTTGATGTCACGCACGATTTCC-3′, respectively. The PCR amplification was performed under the following conditions; 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, using a thermal cycler (Gene Amp PCR system 2400, Applied Biosystems, Foster City, CA). The amplified DNA was separated on 1.5% agarose gels and stained with ethidium bromide.

2.7. Cell viability

HCT116 and SW480 colon cancer cells were plated on 96-well plates at 5000 cells and treated with various concentrations of TSL for 24, 48 and 72 h. The cell viability from each treated sample was measured in triplicate using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

2.8. Immunofluorescence analysis

HCT116 and SW480 colon cancer cells were cultured on 12-well plate with cover glass and then treated with DMSO or TSL for 24 h. After treatment, the cells were washed with PBS, fixed with 4% formaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 4% bovine serum albumin for 1 h. The cells were stained with anti-β-catenin antibody and DAPI, then analyzed by confocal microscopy using a Zeiss AxioObserver Z1 microscope.

2.9. Statistics

The data were expressed as the mean ± S.D. of three experiments, and statistical analysis was performed by the Student's *t*-test, and a P value of <0.05 was considered to indicate a significant difference.

3. Results

3.1. Identification of TSL as an inhibitor of the Wnt/β-catenin pathway

To search for the inhibitors of Wnt/β-catenin pathway, we used a cell-based screening system. HEK293 reporter cells that are stably transfected with TOPFlash, a synthetic β-catenin/Tcf-dependent luciferase reporter, and human Frizzled-1 expression plasmids were plated on a 96-well plate. We purified four sesquiterpenoids and determined their structures as shown in Fig. 1A. After treatment of Wnt3a-CM and four sesquiterpenoids from *T. farfara* to the wells for 15 h, TOPFlash reporter activity was determined by the measurement of firefly luciferase activity. Among the compounds tested, TSL (Fig. 1A and B) was the most potent inhibitor of Wnt-3a-induced TOPflash luciferase activity. As shown in Fig. 1C, TSL dose dependently suppressed the Wnt3a-CM induced β-catenin response transcription. Meanwhile, the activity of FOPFlash, a negative control reporter with mutated β-catenin/Tcf

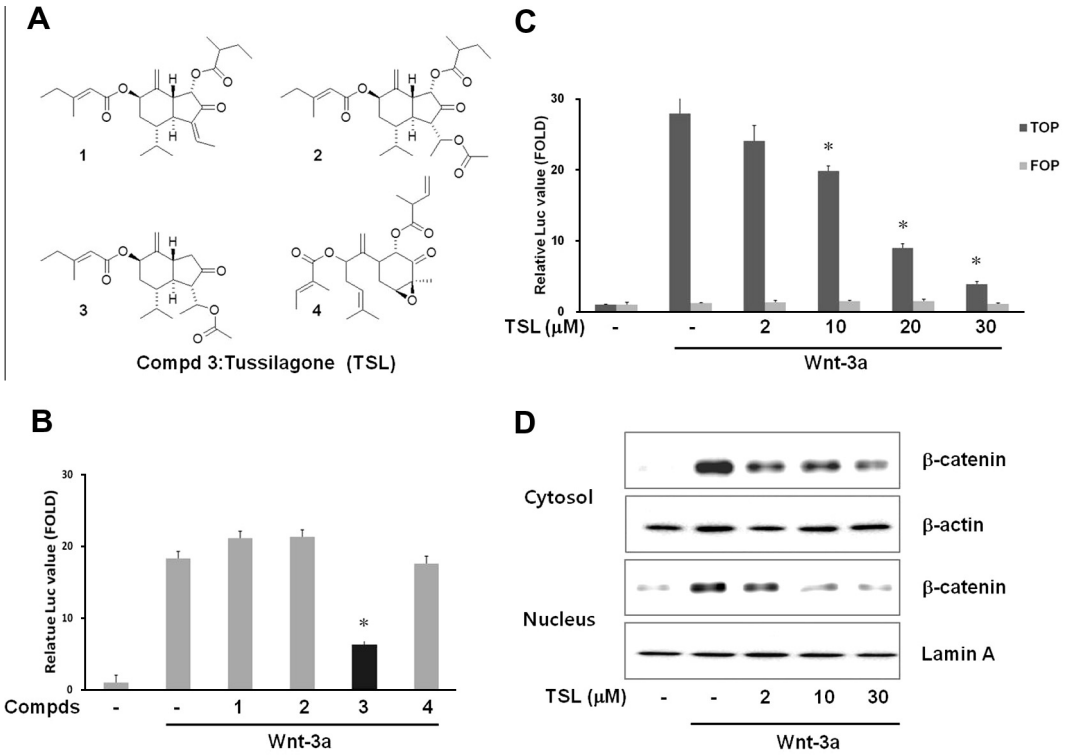

Fig. 1. Identification of TSL as an inhibitor of the Wnt/β-catenin pathway. (A) Chemical structures of sesquiterpenoids isolated from *Tussilago farfara*. (B) HEK293 reporter (TOPFlash) cells and (C) control (FOPFlash) cells were incubated with sesquiterpenoids in the presence of Wnt3a-CM. After 15 h, luciferase activity was determined. The results are the mean ± SD ($n = 3$). (D) Cytosolic and nuclear proteins were prepared from HEK293 reporter (TOPFlash) cells treated with either vehicle (DMSO) or TSL in the presence of Wnt3a-CM for 15 h and then subjected to Western blotting with β-catenin antibody.

binding elements, was not altered by the treatment of TSL and Wnt3a-CM. These results suggest that TSL specifically inhibits the Wnt/β-catenin pathway.

In the Wnt/β-catenin pathway, the level of intracellular β-catenin makes the important role in regulating the expression of several target genes. To determine whether TSL affects the intracellular level of β-catenin, the amount of cytosolic/nuclear β-catenin were determined in HEK293 reporter cells by Western blot analysis. As shown in Fig. 1D, incubation with TSL resulted in the down-regulation of β-catenin in cytosol and nucleus of Wnt3a-activated cell.

3.2. TSL inhibited the Wnt/β-catenin pathway independent of GSK-3β

Since GSK-3β activity is known to be contributed to the β-catenin degradation [14], we examined whether the GSK-3β was involved in the TSL-mediated β-catenin degradation. The treatment of HEK cells with LiCl, an inhibitor of GSK-3β [15], led to the stimulation of transcription activity (Fig. 2A). TSL abolished LiCl-mediated transcriptional activity in a dose-dependent manner (Fig. 2A). Furthermore, Western blot analysis showed that the amount of intracellular β-catenin induced by LiCl was decreased by the treatment with TSL at higher than 2 μM (Fig. 2B). These results suggest that the β-catenin degradation by TSL is independent of GSK-3β.

3.3. TSL promoted proteasome-mediated β-catenin degradation

We examined whether TSL reduces the mRNA expression of β-catenin in HEK293 reporter cells by using RT-PCR. In contrast to the protein level of β-catenin, mRNA expression of β-catenin was not changed by TSL, suggesting that TSL inhibits Wnt/β-catenin signaling by the post-transcriptional regulation of β-catenin (Fig. 3A). It was demonstrated previously that the intracellular level of β-catenin is regulated by ubiquitin-dependent proteolysis [16]. So we checked if TSL could modulate the proteasome-mediated degradation of β-catenin. As shown in Fig. 3B, the reduced cytosolic level of β-catenin by TSL was recovered by the treatment of MG132, a proteasome inhibitor. These results suggest that TSL repressed Wnt/β-catenin pathway by promoting the proteasome-mediated β-catenin degradation.

3.4. TSL inhibits proliferation of various colon cancer cells

Colon cancer cell lines of HCT116 and SW480 showed the elevated β-catenin expression and the increased β-catenin dependent transcriptional activity due to Ser45 deletion mutation in β-catenin and a truncated form of APC, respectively [17]. Colon cancer cells were transiently transfected with TOPFlash followed by the incubation with TSL. TSL repressed β-catenin dependent transcriptional activity in a dose dependent manner (Fig. 4A). The levels of cytosolic β-catenin were decreased by treatment with TSL in colon cancer cell lines of HCT116 and SW480 (Fig. 4B). And these results were also confirmed by the immunofluorescence analysis, suggesting that Wnt/β-catenin signaling is suppressed in response to TSL in colon cancer cells (Fig. 4C and D). We also evaluated the effects of TSL on the expression of β-catenin target genes such as cyclin D1 and c-myc. As shown in Fig. 4E, the expression of cyclin D1 and c-myc were suppressed in response to TSL. Several studies have reported that the disruption of β-catenin function specifically reduced the cell growth of human colon cancer cells [18–20]. HCT116 and SW480 colon cancer cells were incubated with TSL for 3 days and cell growth was measured by MTT assay. As shown in Fig. 4F, TSL efficiently inhibited the growth of colon cancer cells in a dose dependent manner.

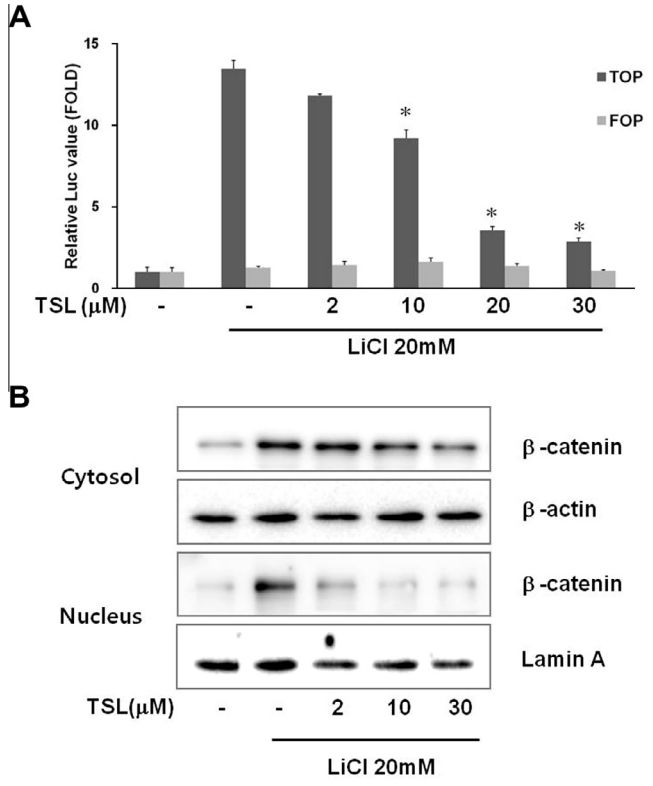

Fig. 2. TSL induces β-catenin degradation through a GSK-3β independent way. (A) HEK293 reporter cells were incubated with TSL in the presence of 20 mM LiCl. After 15 h, luciferase activity was determined. The results are the mean ± SD ($n = 3$). $^*P < 0.05$ compared with the vehicle control group. (B) Cytosolic and nuclear proteins were prepared from HEK293 reporter cells treated with either vehicle (DMSO) or TSL in the presence of 20 mM LiCl for 15 h and then subjected to Western blotting with β-catenin antibody. The blots were reprobed with anti-actin and lamin A antibody as a loading control.

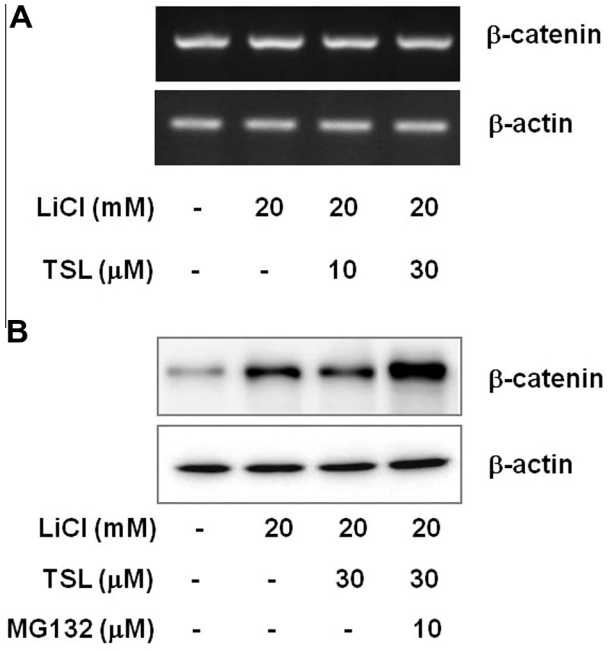

Fig. 3. TSL promotes the degradation of β-catenin via a proteasome. (A) Semiquantitative RT-PCR for β-catenin, and GAPDH was performed with total RNA prepared from HEK293 reporter (TOPFlash) cells either vehicle (DMSO) or TSL in the presence of Wnt3a-CM for 15 h. (B) Cytosolic proteins prepared from HEK293 reporter (TOPFlash) cells, which were incubated with vehicle (DMSO) or TSL in the presence or absence of Wnt3a-CM, exposed to MG-132 (10 μM) for 10 h, were subjected to Western blotting with anti- β-catenin antibody. The blots were reprobed with anti-actin antibody as a loading control.

4. Discussion

The β-catenin, a major transcriptional modulator in Wnt signaling, plays a key role in embryogenesis, stem cell renewal and organ regeneration [21–23]. Abnormal expression of β-catenin acts as an oncogene and modulates transcription of genes to drive cancer initiation, progression, survival and relapse. Therefore, the modulation of Wnt/β-catenin signaling pathway has been regarded as a guaranteed target for the treatment of cancer.

Many studies have demonstrated that inflammation is crucial for cancer development [24,25]. Cyclooxygenase-2, another hallmark of colon cancer, directly stimulates the Wnt/β-catenin pathway by prostaglandin E_2 (PGE_2) *via* PI3-kinase, inhibiting apoptosis in early stages of colon carcinogenesis [26,27]. Many kinds of nonsteroidal anti-inflammatory drug (NSAIDs) such as aspirin, diclofenac and sulinduc showed chemopreventive efficacy in colon cancer by blocking the Wnt/β-catenin pathway [28,29]. Traditional Chinese herbal medicine has used Kuandong Hua (*T. farfara* L.) for the treatment of various respiratory conditions. TSL, one of major constituents of this plant, was reported as a potent cardiovascular and respiratory stimulant [9]. In previous study, we reported its anti-inflammatory activity in activated microglia through the inhibition of NF-kappaB and nuclear translocation of p65 subunit of NF-kappa B [12]. In addition, other group demonstrated that TSL induced heme oxygenase-1 expression and indirectly inhibited the production of nitric oxide, tumor necrosis factor α, and PGE_2 as well as the expression of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide stimulated macrophages [30].

In this study, TSL is recognized as a potential inhibitor of Wnt/β-catenin pathway through the suppression of β-catenin dependent transcriptional activity (Fig. 1). TSL repressed the Wnt3a-CM or LiCl-activated Wnt/β-catenin signaling pathway in HEK293 reporter cell through the promotion of proteasome-mediated β-catenin degradation (Fig. 3). In addition, TSL suppressed the β-catenin levels in HCT116 and SW480 colon cancer cells in which Wnt/β-catenin signaling pathway is activated [31]. There are two pathways to regulate the degradation of intracellular β-catenin level, GSK-3β and Siah/APC pathways [32,33]. When co-treated with LiCl, an inhibitor of GSK-3β, TSL can still decrease the level of β-catenin, which suggests that TSL works as an inhibitor of Wnt/β-catenin signaling pathway in GSK-3β independent way. Siah-1, an E3 ubiquitin ligase, associates with β-catenin through APC and stimulates the degradation of β-catenin. Hexachlorophene, a Wnt/β-catenin pathway inhibitor by Siah-mediated β-catenin degradation, decreased the level of β-catenin in HCT116 but not in SW480 [34]. It seems that Siah/APC pathways may require a wild-type APC function. However, TSL decreased the level of β-catenin in SW480 colon cancer cells that accompany with truncated form of APC (Fig. 4A–D), suggesting that TSL-mediated β-catenin degradation is not by Siah/APC dependent way.

In the past decade, various pharmacological inhibitors of β-catenin have been identified from medicinal plants. Most of the plant-derived inhibitors are flavonoids such as quercetin, fisetin, silibinin and so on [35–37]. We demonstrated that TSL, a sesquiterpenoid from *T. farfara*, suppressed the Wnt/β-catenin signaling pathway through the promotion of β-catenin degradation. This is the first report that TSL suppressed the proliferation of colon cancer cells *via* the suppression of β-catenin target genes such as cyclin D1 and c-myc. Taken together, TSL, a sesquiterpenoid from *T. farfara* may be a new lead compound for the development of therapeutic agents against colon cancer through modulating Wnt/β-catenin signaling pathway.

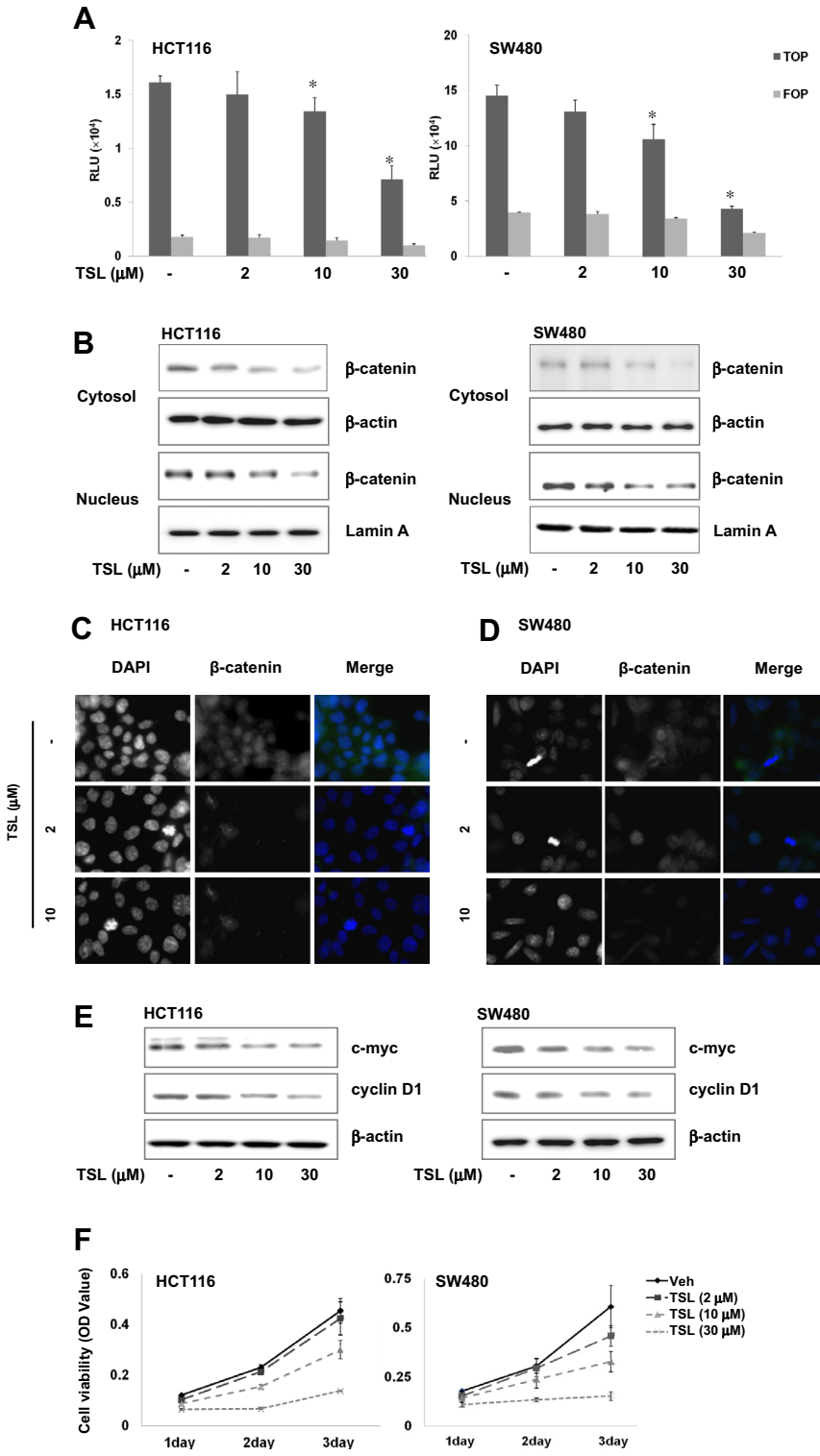

Fig. 4. TSL attenuated the Wnt/β-catenin pathway in colon cancer cells and inhibited the proliferation of colon cancer cells. (A) HCT116 and SW480 cells were transfected with TOPFlash and then incubated with indicated concentrations of TSL for 15 h. Luciferase activities were measured 21 h after transfection. TOPFlash activity is reported as relative light unit (RLU) normalized to β-galatosidase activity. (B) Cytosolic and nuclear proteins were prepared from cancer cells treated with either vehicle (DMSO) or TSL for 24 h and then subjected to Western blotting with β-catenin antibody. (C and D) Immunofluorescence analysis of HCT116 and SW480 cells incubated with either vehicle (DMSO) or TSL (2 and 10 μM). After fixation, the cells were stained with anti-β-catenin (green) antibody and observed at 400× magnification. DAPI (blue) was used to stain nuclei. (E) HCT116 and SW480 cells were incubated with the vehicle (DMSO) or TSL for 48 h, and then whole cell extracts were prepared for Western blotting with anti-cyclin D1 and anti-myc antibodies. To confirm equal loading, the blots were reprobed with anti-actin antibody. (F) HCT116 and SW480 cells were incubated with various concentrations of TSL for 24, 48 and 72 h, and cell viability was determined by MTT assay. The results are the mean ± SD ($n = 3$). *$P < 0.05$, compared with the vehicle control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korean government (MSIP) (2011-0030699) and the SRC Research Center for Women's Diseases of Sookmyung Women's University (2011).

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