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Angelica sinensis polysaccharides ameliorate 5-flourouracil-induced bone marrow stromal cell proliferation inhibition via regulating Wnt/β-catenin signaling

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Abstract: Chemotherapy may cause cellular oxidative stress to bone marrow. Oxidative damage of bone marrow hematopoietic microenvironment is closely related to chronic myelosuppression after chemotherapeutic treatment. *Angelica sinensis* polysaccharides (ASP) are major effective ingredients of traditional Chinese medicine *Angelica* with multi-target anti-oxidative stress features. In the current study, we investigated the protective roles and mechanisms of ASP on chemotherapy-induced bone marrow stromal cell (BMSC) damage. The human bone marrow stromal cell line HS-5 cells were divided into control group, 5-FU group, 5-FU + ASP group, and 5-FU + LiCl group to investigate the mechanism of ASP to alleviate 5-FU-induced BMSC proliferation inhibition. The results showed that 5-FU inhibits the growth of HS-5 cells in a time and dose-dependent manner; however, ASP partially counteracted the 5-FU-induced decrease in cell viability, whereas Wnt signaling inhibitor Dkk1 antagonized the effect of ASP on HS-5 cells. ASP reversed the decrease in total cytoplasmic β-catenin, p-GSK-3β, and CyclinD1 following 5-FU treatment and modulated nuclear expression of β-catenin, Lef-1, and C-myc proteins. Furthermore, ASP also enhanced the antioxidant capacity of cells and reduced 5-FU-induced oxidative stress, attenuated FoxO1 expression, thus weakened its downstream apoptosis-related proteins and G0/G1 checkpoint-associated $p27^{Kip1}$ expression to alleviate 5-FU-induced apoptosis and to promote cell cycle progression. All the results above suggest that the protective role of ASP in 5-FU-treated BMSCs proliferation for the chemotherapy may be related to its activating Wnt/β-catenin signaling and keeping homeostasis between β-catenin and FoxO1 under oxidative stress. The study provides a potential therapeutic strategy for alleviating chemotherapeutic damage on BMSCs.

Introduction

Myelosuppression is the major side effect of chemotherapy (Chabner and Roberts, 2005; Papac, 2001) that may be caused even by minor doses of chemotherapeutic drugs, leading to hematopoietic dysfunction, hematopoietic reconstitution disorders, and other secondary adverse reactions (Dritschilo and Sherman, 1981; Marsh, 1976). The mechanisms of myelosuppression can be various, including direct cytotoxicity to marrow cells, inhibition of bone marrow stem cell or progenitor cell proliferation, or interference with hematopoietic growth factor and receptor signaling, subsequently affecting the downstream differentiation processes. Chemotherapy-induced cellular damage involved not only hematopoietic stem cells/progenitors but also stromal cells in the hematopoietic microenvironment, which may be the reason for chronic hematopoietic dysfunction (Galotto *et al.*, 1999; Kemp *et al.*, 2010; Li *et al.*, 2004; Nicolay *et al.*, 2016; Oliveira *et al.*, 2014; de Lima Prata *et al.*, 2010). As chemotherapy disrupts the steady-state function of hematopoietic and stromal cell, disruptions over time may cause severe bone marrow toxicity and the failure of cancer treatment. 5-FU, widely used in high-proliferative, tissue-derived cancers, particularly for colorectal cancer and breast cancer, exerts its anti-cancer effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA (Douillard *et al.*, 2000; Longley *et al.*, 2003; de Lima Prata *et al.*, 2010). It was reported that the mechanism of stromal cell proliferation inhibition and apoptosis after 5-FU treatment is oxidative damage (Somaiah *et al.*, 2018; Wang *et al.*, 2015). Our previous findings have confirmed that following oxidative damage of BMSCs 5-FU may alter bioactive substance and cause stress-induced premature senescence (SIPS) of hematopoietic cells

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(Xiao *et al.*, 2017). However, the specific underlying mechanism of 5-FU-induced BMSC proliferation inhibition remains unclear. Therefore, to explore its related mechanisms to reduce the side effects of chemotherapy drugs and to screen protective drugs during chemotherapy is of clinical guidance significance.

Wnt/β-catenin is an evolutionarily highly conserved signaling pathway that plays a key role in the development and is involved in cell proliferation, differentiation, apoptosis, and localization control (Nusse and Clevers, 2017; Petersen and Reddien, 2009). Particularly, the Wnt pathway involves various signal feedback that maintains the processes of stem cell proliferation, differentiation, and self-renewal (Luis *et al.*, 2011; Richter *et al.*, 2017). The properties of stem cells are conferred by the interaction of stem cells with their local microenvironment. Recent studies have evidenced that the Wnt/β-catenin signaling pathway is closely related to hematopoietic microenvironment affecting hematopoietic microenvironment function extensively, participate in BMSC proliferation, alleviate oxidative stress, and regulate hematopoietic stem cell self-renewal through stroma-dependent manner (Kim *et al.*, 2009; Oh, 2010; Schreck *et al.*, 2014). It is increasingly realized that the microenvironment keeps the threshold of Wnt signaling in stem cells at a physiological range. In the current work, it was clarified herein the roles of Wnt signaling in chemotherapy-induced stromal suppression and the ameliorative effects of ASP.

The Forkhead transcription factors family, including FoxO1 (or Fkhr), FoxO3a (or Fkhrl1), FoxO4 (or Afx), and FoxO6, are critically involved in the regulation of apoptosis, proliferation, and the control of oxidative stress (Eijkelenboom and Burgering, 2013). Stress conditions such as high ROS levels induce FoxO nuclear import and trigger the shifting of β-catenin from TCF/LEF to FoxO-mediated transcription (Behrens *et al.*, 1996; Essers *et al.*, 2005). In the hematopoietic system, activation of FoxO factor is sufficient to activate a variety of proapoptotic genes and to trigger apoptosis. Meanwhile, overexpression of FoxO factors cause a strong inhibition of cell proliferation (Burgering and Medema, 2003; Ma and Wang, 2012). As playing a critical role in proliferation and apoptosis, it has been aware that FoxO factors are closely related to chemotherapy-induced cell damage; nevertheless, studies are needed to clarify the relationship of FoxO factors and Wnt signaling in myelosuppression (Gomes *et al.*, 2008; Greer and Brunet, 2005).

Angelica of Chinese herb is a commonly used medicine to enrich the blood, promote blood circulation and treat menstrual disorders (Dietz *et al.*, 2016; Zhao *et al.*, 2003). *Angelica sinensis* polysaccharides (ASP) are major effective ingredients of *Angelica*, with significant bioactivities including anti-oxidation (Lei *et al.*, 2014; Zhuang *et al.*, 2018), anti-tumor (Tsai *et al.*, 2005; Zhang *et al.*, 2016), promoting hematopoiesis (Bradley *et al.*, 1999; Liu *et al.*, 2010a; Wang *et al.*, 2017), and delaying senescence (Lai and Liu, 2015; Mu *et al.*, 2017) effects. ASP shows antioxidant activity by suppressing the production of ROS and regulating several chemical substances associated with oxidative stress (Ai *et al.*, 2013; Wei *et al.*, 2016). Our previous work showed marked antioxidative role of ASP in BMSCs from 5-FU injury *in vitro*, thus protected hematopoietic cells against SIPS via alleviating oxidative stress, preventing oxidative DNA damage, promoting hematopoietic stimulating factors originated from BMSCs, and enhancing intercellular communication between stromal cells and hematopoietic cells (Xiao *et al.*, 2017). On this basis, we demonstrated herein that ASP alleviated 5-FU-induced stromal cell proliferation inhibition, apoptosis, and oxidative stress damage, and the underlying mechanism may be related to ASP activating Wnt/β-catenin signaling and keeping homeostasis between β-catenin and FoxO1 under oxidative stress.

Materials and Methods

Reagents

5-fluorouracil was purchased from Sigma-Aldrich Co., St. Louis, USA. *Angelica sinensis* polysaccharides are composed of long chains of several monosaccharide units including fucose, galactose, glucose, arabinose, rhamnose and xylose, and all the monosaccharide units are linked via various glycosidic bonds. In the study, ASP were purchased from Ci Yuan Biotechnology Co. Ltd., Shanxi, China. All standards were at least 98% pure, as confirmed by HPLC (Jin *et al.*, 2012; Wei *et al.*, 2016; Zhang *et al.*, 2014; Zhang *et al.*, 2016). LiCl (purity >95%) was purchased from Damao Chemical Reagent Factory, Tianjin, China. Fetal bovine serum (FBS) was purchased from MRC Company, Australia. Dulbecco's modified Eagle medium high-glucose(H-DMEM) was purchased from Gibco Co., NY, USA. Cell Counting Kit-8 was purchased from Dojindo Laboratories (Japan). EdU Cell Proliferation Assay Kit was purchased from RiboBio Co. Ltd., Guangzhou, China. β-catenin, GSK-3β, p-GSK-3β, Lef-1, Cyclin D1, C-myc, FoxO1, p-FoxO1, $p27^{Kip1}$, Bim, Bax, Bcl-2, and caspase-3 antibodies were purchased from Cell Signaling Technology, Danvers, USA. Dkk1 was purchased from R&D Systems (USA). Reactive Oxygen Species Assay Kit and Senescence β-Galactosidase Staining Kit were purchased from the Beyotime Institute of Biotechnology, Shanghai, China. Superoxide Dismutase (SOD) assay kit, Malondialdehyde (MDA) assay kit and Catalase (CAT) assay kit, were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

Cell culture and groups of experiment

Human bone marrow stromal cell line HS-5 was cultured in H-DMEM containing 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. Cells were divided into control group, 5-FU group, 5-FU + ASP group, and 5-FU+ LiCl group. The control group was routinely cultured; 5-FU group was treated with 5-FU on the concentration of 25 μg/mL; 5-FU+ ASP group was pretreated with ASP on the concentration of 100 μg/mL, and 25 μg/mL 5-FU was added after 6 h; 5-FU+ LiCl group was pretreated with LiCl on the concentration of 10 mmol/L, and 25 μg/mL 5-FU was added after 6 h, each group was cultured for 48 h.

CCK-8 cell viability assay

Cell viability assay and the screening of drug concentration were performed using the Cell Counting Kit-8. Cells were

plated in 96-well plates at a density of 5×10^3 cells per well. The optical density (OD) value at 450 nm was measured using a microplate reader (Massachusetts, USA). The cell viability of HS-5 was calculated according to the formula: Cell viability = [(OD experimental group − OD blank group)/(OD control group − OD blank group)] × 100%. Inhibition rate = [(OD control group − OD experimental group)/(OD control group − OD blank group)] × 100%.

EdU proliferation assay

The HS-5 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and treated as described in groups of experiment. After 48 h treatment, cells were exposed to 10 μmol/L EdU solution for 24 h. Cells were washed and fixed in 4% paraformaldehyde at room temperature for 30 min. After washing, cells were permeabilized in PBS containing 0.5% Triton X-100 for 20 min. Then, cells were washed and incubated with 1X Apollo® reaction cocktail for 30 min. Subsequently, cells were stained with Hoechst33342 for 30 min and observed under a fluorescence microscope (Olympus, Japan). Counting 200 cells at random, the proliferation rate of HS-5 cells was defined as the ratio of EdU-positive cells (green cells) to Hoechst33342-positive cells (blue cells).

Flow cytometry analysis

For cell apoptosis assay, the HS-5 cells were cultured then treated as described in groups of experiment. After 48 h treatment, cells were harvested and centrifuged at 1000 rpm for 5 min. Subsequently, cells were resuspended with 500 μL PBS solution for each tube. Cell apoptosis was detected by flow cytometry. For cell cycle assay, the HS-5 cells were cultured then treated as described in groups of experiment. After 48 h treatment, cells were harvested and fixed with pre-cooled 75% ethanol at 4°C for at least 5 h. After centrifugation, cells were incubated with propidium iodide (PI) and RNase A at 37°C for 30 min in the dark. Cell cycle was detected by the flow cytometry. The apoptosis and cell cycle were analyzed on a FAC-Scan laser flow cytometry (BD Biosciences, New Jersey, USA). The data were processed by Cell Quest software (BD Biosciences, New Jersey, USA).

Immunofluorescence staining

Sterile glass slides were put into 24-well plates; the HS-5 cells were cultured at a density of 5×10^4 cells per well in 24-well plates then treated as described in groups of experiment. After 48 h treatment, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing, cells were permeabilized in PBS containing 0.5% Triton X-100 for 20 min and then blocked with 10% goat serum for 1 h. Subsequently, cells were incubated with monoclonal antibody β-catenin (1:150) overnight at 4°C. After being washed thrice with PBS solution, cells were incubated with Cy3-labeled goat-anti-rabbit immunofluorescent secondary antibody (1:300) at 37°C for 2 h in the dark. The nuclei were stained with 4',6-diamidino-2-phenylindole (DIPI) for the last 5 min. The images were observed and acquired under the fluorescence microscope.

Immunoblot assay

The HS-5 cells were treated as described in groups of experiment. After 48 h treatment, cells were incubated with PIPA lysis buffer containing 1% protease inhibitor and phosphatase inhibitor for 30 min on ice, and proteins were isolated after centrifugation. The concentrations of proteins were detected by the BCA Protein Assay Kit (Beyotime, China). The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% skim milk for 1 h at room temperature and subsequently incubated overnight at 4°C with β-catenin, Cyclin D1, p-GSK-3β, GSK-3β, Lef-1, C-myc, FoxO1, p-FoxO1, $p27^{Kip1}$, Bim, Bcl-2, Bax, and caspase-3 primary antibodies (1:1000). After washing three times with Tris-Buffered Saline and Tween-20 (TBST), the membranes were incubated with secondary antibodies for 1 h at room temperature. The enhanced chemiluminescence (ECL) kit (Millipore, USA) was used for color development, and Image Lab 5.2.1 software was used for semi-quantitative analysis. The relative expression levels of the target proteins were determined by the ratio of the target protein gray value to the internal reference protein gray value.

Oxidation-associated biological indicators assay

For the detection of intracellular ROS, the HS-5 cells were seeded in 6-well plates at a density of 2×10^5 cells per well then treated as described in groups of experiment. After 48 h treatment, the cells were washed thrice by serum-free medium and then incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 20–30 min in the dark. The content of intracellular ROS was observed and acquired under the fluorescence microscope. The average optical density per unit area was analyzed using ImageJ software. For the detection of MDA content and SOD, CAT activity, the HS-5 cells were cultured and treated as described in groups of experiment. After 48 h treatment, cells were harvested, lysed, and centrifuged to collect the supernatant. MDA, SOD, and CAT were measured by the corresponding assay kits according to the manufacturer's instruction.

Statistical analysis

For all assays, the experiments were performed at least three times. All the results were analyzed by one-way analysis of variance (ANOVA) with SPSS 20. 0 statistical software. All the data were expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistical significance.

Results

5-FU inhibits the growth of HS-5 cells by down-regulating the Wnt/β-catenin signaling pathway

To assess the effect of 5-FU on proliferation, HS-5 cells were treated with 5-FU at different concentrations for 72 h. As shown in Fig. 1A, the inhibition ratio of HS-5 cells increased simultaneously with the increase of 5-FU concentration and the extension of treatment time, suggesting that 5-FU exerted the inhibitory role in HS-5 cell growth in a dose- and time-dependent manner. Treated with 25 μg/mL 5-FU for 48 h, the inhibition ratio of HS-5 cells dropped almost 50%, which meant half of the cells were suppressed to grow ($P < 0.05$). Thus, 25 μg/mL 5-FU

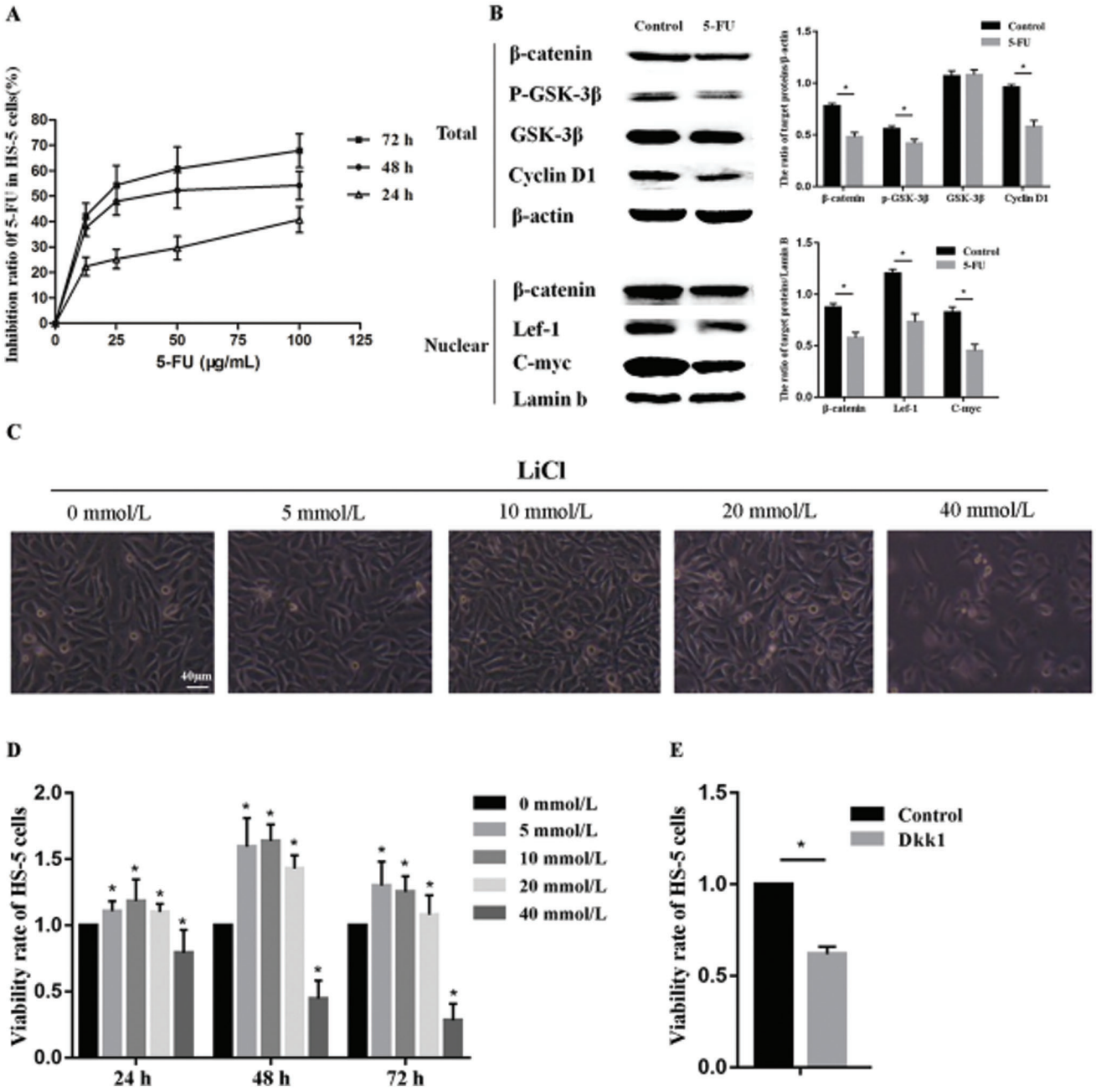

FIGURE 1. 5-FU inhibits HS-5 cells proliferation by regulating Wnt/β-catenin signaling pathway.
(A) Cell Counting Kit-8 (CCK-8) assay was performed to detect the inhibitory effect of 5-FU. (B) Western blot was performed to detect the effects of 5-FU on the expression of Wnt/β-catenin signaling pathway related proteins in HS-5 cells and the histograms of relative protein expression are presented. β-actin and Lamin B are probed as loading controls. Data are presented as means ± SD (N = 3/group) (C) Histologic feature of HS-5 cells treated with Wnt signaling agonist LiCl under inverted microscope (Scale bar = 40 μm). (D) Cell Counting Kit-8 assay showed the viability HS-5 cell treated with LiCl. HS-5 cell treated with 0 mmol/L LiCl were set as 1.0, and the results of HS-5 cell viability were normalized to the OD value of 0 mmol/L group (E) HS-5 cells were incubated with 50 ng/mL Wnt signaling inhibitor Dkk1 for 48 h and cell viability was detected by CCK-8. HS-5 cell treated without Dkk1 was used as a control, and the results of HS-5 cell viability were normalized to the OD value of control group (*$P < 0.05$ *vs.* control).

treating for 48 h was selected as an optional condition for the subsequent experiments. Interestingly, in the current study, we found that the inhibitory effect of 5-FU on HS-5 cell growth was relative to the Wnt/β-catenin signaling pathway. Western blot assay demonstrated that 5-FU downregulated the cytoplasmic levels of p-GSK-3β, total β-catenin, and Cyclin D1 in HS-5 cells, followed by nuclear protein expression of β-catenin, Lef-1, and C-myc down-regulation (Fig. 1B). These results hinted that 5-FU promoted the ubiquitination degradation of β-catenin mediated by GSK-3β-complex, inhibited the nuclear translocation of β-catenin, and downregulated the downstream target genes.

Then the activator and antagonist of the Wnt/β-catenin signaling pathway were tested to further illustrate the effect of 5-FU on Wnt/β-catenin signaling. Morphologically, 5, 10, 20 mmol/L of activator LiCl increased the number of HS-5 cells, however, the cellularity in the 40 mmol/L group significantly dropped concomitant with smaller and loosely

dispersed shape (Fig. 1C). Also, cell viability was tested by CCK-8 assay. As shown in Fig. 1D, cells treated with 5–20 mmol/L LiCl for 48 h showed different degrees of proliferation, among which the proliferation rate of cells peaked up to 150% of the control at the concentration of 10 mmol/L LiCl. In accord with the result by microscopy, 40 mmol/L LiCl also presented cytotoxicity to HS-5 cells even within 24 h ($P < 0.05$). Therefore, the pretreatment with 10 mmol/L LiCl was utilized as a positive control in the subsequent experiment. Furthermore, Dkk1, an antagonist for Wnt/β-catenin signaling, was used as a negative control to get more evidence for the Wnt/β-catenin signaling pathway on cell viability. 50 ng/mL Dkk1 treated for 48 h, the cells were dramatically inhibited compared with the control group revealed by the results of CCK-8 (Fig. 1E). All the results above indicate that the effect of 5-FU on inhibition of HS-5 cell growth correlates with the suppression of the Wnt signaling pathway.

Angelica sinensis polysaccharides antagonize growth inhibition of 5-FU-treated HS-5 cells through the Wnt/β-catenin signaling pathway

Tested by EdU the proportion of proliferating cells in 5-FU group was significantly lower than that of the control group; after pretreatment with ASP and LiCl, the proportion of EdU positive cells increased markedly compared with 5-FU group (Figs. 2A and 2B). The results of CCK-8 showed an obvious reduction after a 48 h-incubation with 5-FU compared with untreated control cells; however, ASP pretreatment partially reversed the reduction. Moreover, the ASP-induced increase in the viability was weakened by Dkk1 (Fig. 2C). Shown by immunofluorescence assay, the cytoplasmic and nuclear expression of β-catenin was decreased obviously after 5-FU treatment, however, ASP and LiCl pretreatment respectively rescued the expression of β-catenin and its nuclear translocation (Fig. 3A). Western blot revealed that ASP and LiCl pretreatment significantly reversed the 5-FU-induced decrease in cytoplasmic expression of total β-catenin, p-GSK-3β, and CyclinD1, meanwhile modulated nuclear expression of β-catenin, Lef-1, and C-myc proteins (Figs. 3B–3I) ($P < 0.05$). These data hint that ASP may activate Wnt signaling, which may be one mechanism that ASP counteract the inhibiting effect of 5-FU on HS-5 cell growth.

Angelica sinensis polysaccharides relieve 5-FU-induced intracellular oxidative stress

To elucidate the mechanism of 5-FU induced damage and ASP mediated protective effect on HS-5 cell growth, we

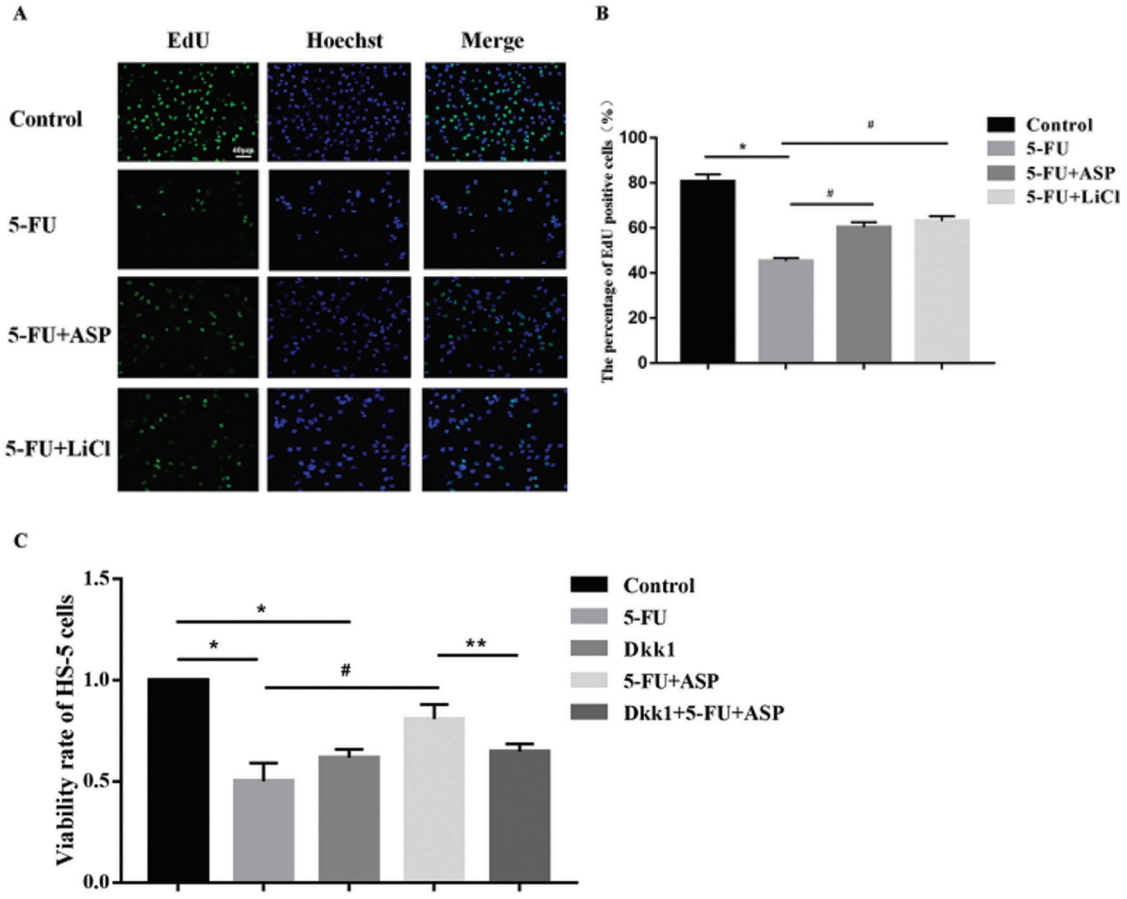

FIGURE 2. *Angelica sinensis* Polysaccharides antagonize the growth inhibitory effect of 5-FU on HS-5 cells via up-regulating Wnt/β-catenin signaling.

(A) The proliferative HS-5 cells were labeled by 5-ethynyl-20-deoxyuridine (EdU). The green fluorescence presents proliferative cells, the blue fluorescence presents nuclei (Scale bar = 40 μm). (B) The percentage of proliferating cells (EdU^+) was quantitated using ImageJ software. (C) The viability rate of HS-5 cell was measured by Cell Counting Kit-8. Control group was set as 1.0 (*$P < 0.05$ *vs.* control, #$P < 0.05$ *vs.* 5-FU, and **$P < 0.05$ *vs.* 5-FU+ASP group).

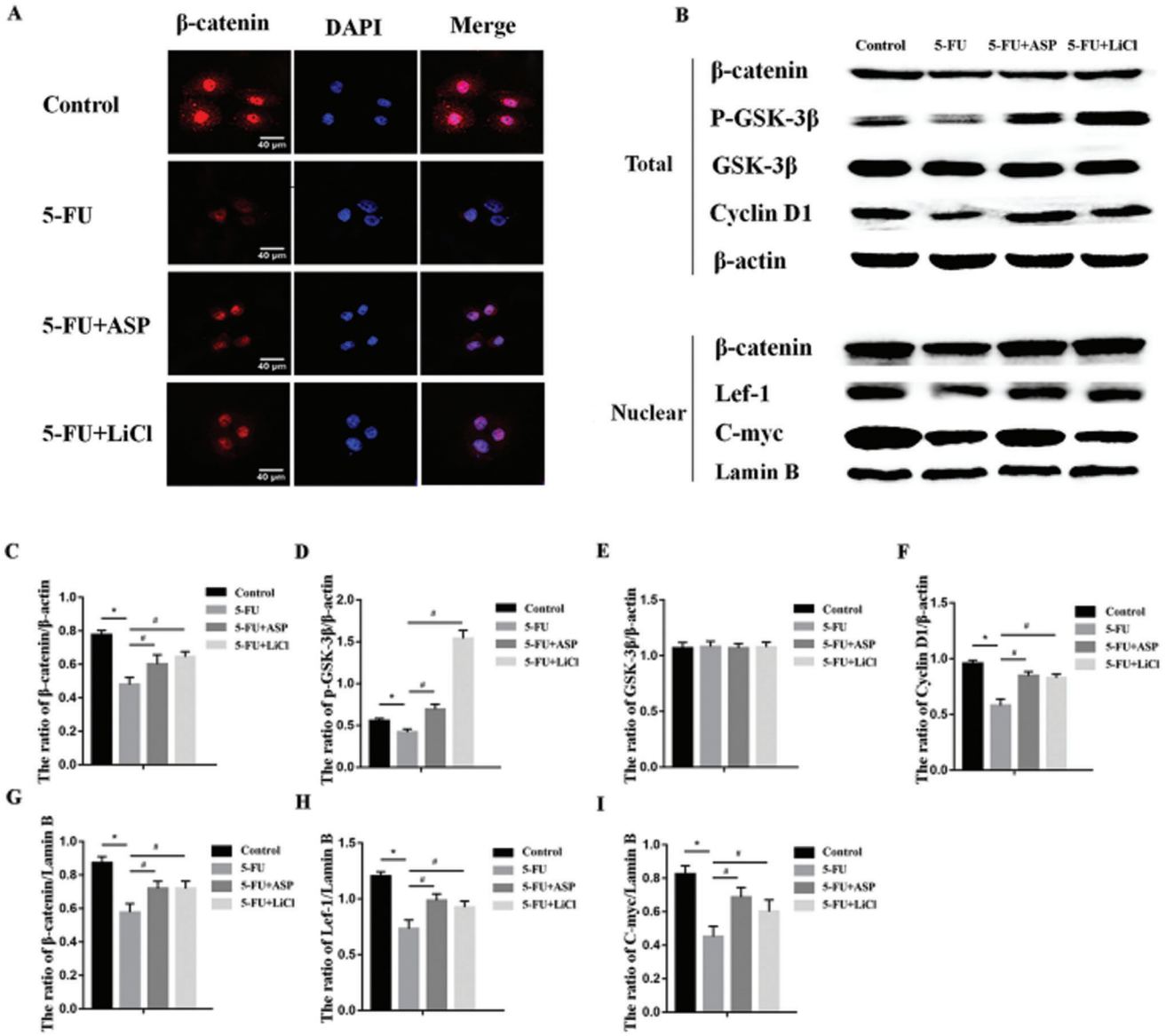

FIGURE 3. *Angelica sinensis* Polysaccharides activate Wnt/β-catenin signaling pathway.
(A) Expression of β-catenin in HS-5 cells was detected by the immunofluorescence method. The red fluorescence presents β-catenin protein expression, the blue fluorescence presents nuclei (Scale bar = 40 μm). (B) The Wnt signaling related protein expression in HS-5 cells were detected by the Western blot. β-actin and Lamin B were probed as loading controls. (C-I) The histograms of relative protein expression are presented. Data are presented as means ± SD (N = 3/group) (*$P < 0.05$ *vs.* control and #$P < 0.05$ *vs.* 5-FU).

assessed the indexes of oxidative damage. Increases in intracellular ROS and MDA were found in the 5-FU group compared with the control group, whereas single pretreatment of ASP or LiCl reversed the increase dramatically (Figs. 4A and 4C). On the contrary, ASP or LiCl administration protected the antioxidant enzymes, including SOD and CAT in HS-5 cells (Figs. 4D–4E) ($P < 0.05$). These results demonstrate that 5-FU cause oxidative stress to HS-5 cells, whereas ASP exert a significant anti-oxidative role to alleviate 5-FU-induced oxidative stress, which may be related to the activation of Wnt/β-catenin signaling.

Angelica sinensis polysaccharides ameliorate the activation of FoxO1 induced by 5-FU

FoxOs are transcriptional factors closely related to cellular survival and oxidative stress, Notably, activated FoxO1 may impair Wnt signaling via competitive combination with β-catenin in the nucleus. Western blot results demonstrated that compared with the control group FoxO1 expression in the 5-FU group rose concurrently with decreased p-FoxO1 expression. However, ASP or LiCl pretreatment significantly reduced FoxO1 expression via degradation of FoxO1 by an increased p-FoxO1 expression (Fig. 5A). It was inferred antioxidative properties of ASP may play a role in FoxO1 downregulation, which may be another possible mechanism for ASP upregulation of Wnt/β-catenin signaling. FoxOs may orchestrate apoptosis and cell cycle arrest. Here, in the context, the proteins correlating to apoptosis including Bcl-2, Bim, Bax, caspase-3 and cell cycle inhibitor as $p27^{Kip1}$ were detected via western blot assay. It revealed that ASP or LiCl abrogated 5-FU-induced increase in Bim, Bax caspase-3 and $p27^{Kip1}$ expression, however enhanced anti-apoptotic protein Bcl-2 expression (Figs. 5B–5F, and 5H) ($P < 0.05$). The results were in line with the data of flow cytometric

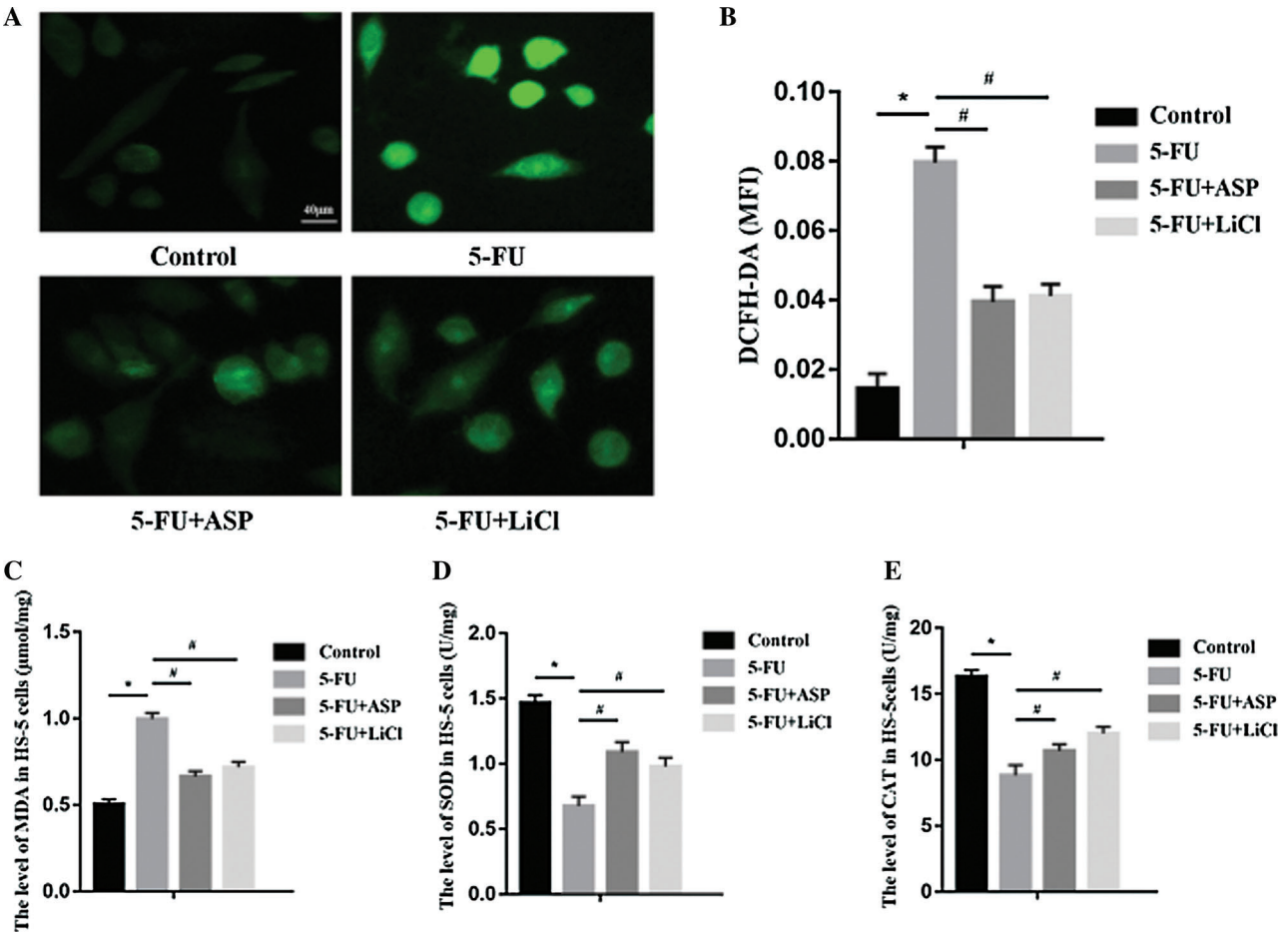

FIGURE 4. *Angelica sinensis* Polysaccharides reduce 5-FU-induced intracellular oxidative stress.
(A) The levels of intracellular reactive oxygen species (ROS) in HS-5 cells were measured by DCFH-DA assay under fluorescence microscope. (B) The mean fluorescence intensity of ROS is quantified and presented by histograms. (C) The results of MDA content in HS-5 cells are presented by histograms. (D) The content of SOD in HS-5 cells are presented by histograms. (E) The results of CAT content in HS-5 cells are presented by histograms. Data were presented as means ± SD (N = 3/group) (*$P < 0.05$ *vs.* control and #$P < 0.05$ *vs.* 5-FU).

analysis that a 2.2-fold increase in the apoptosis rate was found in the 5-FU group compared with the control group, whereas ASP or LiCl significantly decreased apoptotic cell percentage compared with that in 5-FU group (Fig. 5G) ($P < 0.05$). Meanwhile, ASP or LiCl pretreatment weakened 5-FU induced G0/G1 phase retard concurrent with S and G2/M phase recovery (Fig. 5I). All these data above hinted that the effects of 5-FU on HS-5 growth inhibition may relate to the activation of FoxO1 leading to apoptosis or cycle arrest. The anti-oxidative property of ASP exerts a protective effect against cycle arrest and apoptosis.

Discussion

Myelosuppression is one of the common side effects of chemotherapy, characterized by depletion of cells within the bone marrow (Ai *et al.*, 2013; Testa *et al.*, 1985). In general, myelosuppression is primarily attributable to the direct cytotoxicity to bone marrow cells, inhibition of bone marrow precursor or progenitor cell proliferation, the reduction in HSC reserves, and impairment in HSC self-renewal. Notably, because of the reduction of HM cellularity in varying degrees, the damaged-hematopoietic microenvironment may result in diminished or delayed hematopoiesis function, immune-related disorders, as well as long-term damage to the bone marrow recovery (Crawford *et al.*, 2004; Kuter, 2015). It has been shown that chemotherapeutic treatment damage the hematopoietic microenvironment *in vitro* and *in vivo* (Galotto *et al.*, 1999; Hu *et al.*, 2016; Kemp *et al.*, 2010; Li *et al.*, 2004; Oliveira *et al.*, 2014; de Lima Prata *et al.*, 2010). As chemotherapy disrupts the steady-state function of hematopoietic and stromal cells, disruptions over time may cause severe bone marrow toxicity and the failure of cancer treatment. To ensure this does not occur, finding appropriate agents to promote the recovery process following discontinuation of chemotherapy and to lessen the bone marrow damage has a profound significance.

Since it was first synthesized in 1957, 5-FU has remained one of the most widely used chemotherapeutic agents with broad-spectrum activity against many solid tumors (Wilson *et al.*, 2014). 5-FU exerts its anticancer effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA, leading to cytotoxicity and cell death (Longley *et al.*, 2003). Recent studies have indicated that 5-FU suppressed the proliferation of HSCs and induced the myelosuppression of mice by down-regulating the PI3K-AKT signaling pathway (Wang *et al.*, 2015;

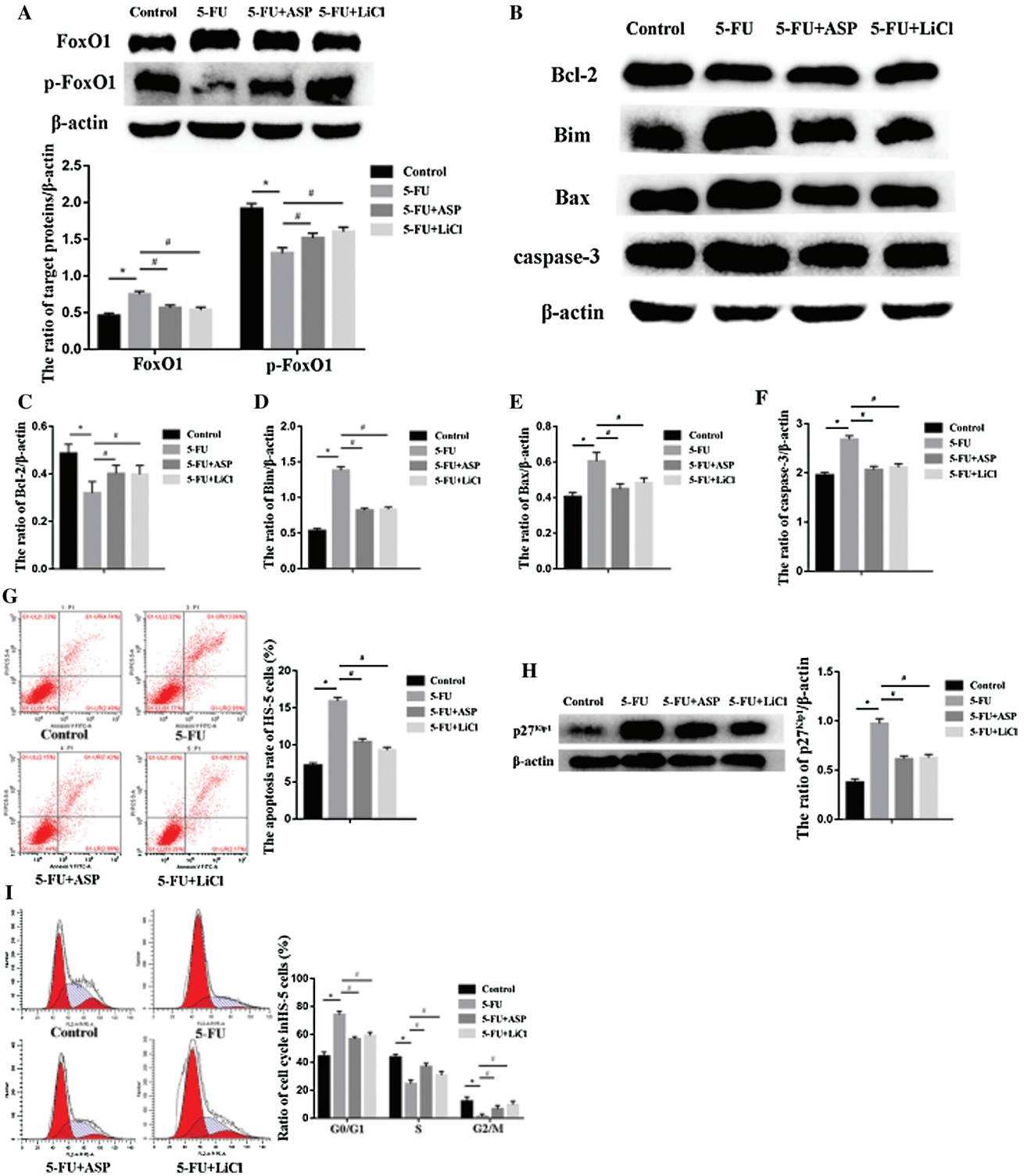

FIGURE 5. *Angelica sinensis* Polysaccharides ameliorate 5-FU-induced activation of FoxO1.
(A) The protein expression levels of FoxO1 and p-FoxO1 in HS-5 cells were detected by the Western blot. The relative protein expression is presented by histograms. β-actin is probed as loading control. (B) The protein expression levels of apoptosis in HS-5 cells were detected by the Western blot. (C–F) The histograms of relative protein expression are presented. β-actin is probed as loading control. (G) Annexin V-FITC/PI double staining was employed to detect cell apoptosis by flow cytometry and the histogram of apoptosis rate is presented. (H) The protein expression level of p27Kip1 in HS-5 cells was detected by the Western blot and the histogram of p27 Kip1 protein expression is presented. (I) Cell cycle was analyzed by flow cytometry and the histograms of phase distribution are presented. Data were presented as means ± SD (N = 3/group) (*$P < 0.05$ *vs.* control and #$P < 0.05$ *vs.* 5-FU).

Zhang *et al.*, 2019). However, the definite mechanism for 5-FU caused myelosuppression remains unclear. Focused on bone marrow stromal cells, we provided the evidence that 5-FU inhibited stromal cell growth and induced apoptosis, which was related to downregulation of Wnt/β-catenin signaling, also up-regulation of FoxO1 concomitant with an increase of cellular oxidative stress. Furthermore, the current work revealed that anti-oxidative property and role in Wnt signaling regulation

might be the key mechanisms of ASP to prevent 5-FU-induced stromal damage.

Stem cells display the defining capacity to self-renew, and their fate is primarily dictated by extrinsic, short-range signals, which typically emanated from the stem cell niche (Losick *et al.*, 2011). The non-hematopoietic cells in the hematopoietic microenvironment have a functional role in regulating hematopoiesis and the signaling pathways that regulate HM may be necessary for the development of functional niches that regulate hematopoietic stem cells and their progenitors (Morrison and Spradling, 2008; Zhang *et al.*, 2003). The Wnt signaling pathway exerts a variety of effects on target cell developmental processes, including cell proliferation, apoptosis, and differentiation. The canonical Wnt pathway affects cellular functions by accumulating β-catenin in the cytoplasm and eventually translocating into the nucleus. Within the nucleus, β-catenin binds to T cell factor (TCF) family/lymphoid enhancer factor (LEF) and regulates cell proliferation through Wnt downstream target genes (Clevers, 2006; Moon *et al.*, 2002; Nusse and Clevers, 2017). It was reported that Wnt/β-catenin signaling regulates HSCs function in a dosage-dependent manner (Fleming *et al.*, 2008; Huang *et al.*, 2012; Malhotra and Kincade, 2009; Mohammed *et al.*, 2016). Various degrees of activation of the pathway may cause different outcomes, leading to either enhanced repopulation capacity or exhaustion of the HSCs. A mild increase in Wnt signaling enhanced HSC function (Famili *et al.*, 2016; Luis *et al.*, 2011). However, a high Wnt level in HSCs eventually leads to stem cell exhaustion and impairment of reconstitution in irradiated recipients (Kirstetter *et al.*, 2006; Ming *et al.*, 2012; Scheller *et al.*, 2006). Most importantly, Wnt signaling regulates HSC reconstruction in a stromal-dependent manner. It was found that when hematopoietic cells were co-cultured with BMSCs supplemented with Wnt3a conditioned medium, the cellularity of $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ hematopoietic stem cells, was increased, and the hematopoietic transplantation and reconstruction capability were enhanced (Kim *et al.*, 2009; Nemeth *et al.*, 2009). Hence, in the current study, we focused on the Wnt signaling regulation on BMSCs following chemotherapy. It was found that 5-FU induced a decrease in cytoplasmic expression of total β-catenin, p-GSK-3β, and CyclinD1, meanwhile weakened nuclear expression of β-catenin, LEF-1, and C-myc proteins, causing HS-5 cells proliferation inhibition. The results herein are in line with the other data related to the relationship between canonical Wnt signaling and cell proliferation, which has confirmed that Wnt/β-catenin signaling positively stimulates cell growth via cell cycle regulation (Braunschweig *et al.*, 2015; Shtutman *et al.*, 1999).

Reactive oxygen species (ROS) are free radicals and active metabolites of oxygen containing unpaired electrons, which take a significant role in cell signal transduction and regulation (Owusu-Ansah and Banerjee, 2009). Chemical agents, as well as irradiation, can cause persistent ROS production. This accumulation of ROS may lead to excessive oxidative stress and DNA damage such as DSBs (double-strand breaks), which are considered to be the main potential mechanisms causing cellular damage (Meng *et al.*, 2003; Wang *et al.*, 2010). A previous study in our group has demonstrated that 5-FU weakened the antioxidant capacity of HS-5 cells and caused high sensitivity of cells to ROS, thus HS-5 cells underwent DSB which eventually resulted in either apoptosis or senescence (Xiao *et al.*, 2017). Oxidative stress is also related to cell cycle arrest. DSBs initiate DNA damage response through sequential stimulation of ATM, Chk2, and p53 (Sancar *et al.*, 2004). Activation of p53 and its downstream p21 may induce cell cycle arrest. Meanwhile, ROS can activate the p38 MAPK pathway (Ito *et al.*, 2006). Activation of p53 and p38 pathways can converge at p16 and augment of p16 expression may also lead to permanent cell cycle arrest (Beausejour *et al.*, 2003; Iwasa *et al.*, 2003). Interestingly, it is reported that β-catenin may be critical for antagonizing oxidative stress. Exposing β-catenin knockdown mice to chemotherapeutic agent or radiation caused a decreased expression of the hydrogen peroxide (H_2O_2) detoxifying enzyme catalase and led to the accumulation of ROS and superoxide (O_2^{-}) free radicals in cells and an inability to repair DNA damage (Lento *et al.*, 2014). On the opposite, effector molecules generated from oxidative DNA damage may also down-regulate the Wnt pathway by inhibiting transcriptional activity or participating in post-translational modifications to enhance ubiquitination degradation (Lin *et al.*, 2008). The evidence above hints that Wnt signaling is also closely correlated with oxidative stress. Therefore, in the current study, increased oxidative stress may be one of the reasons for the downregulation of Wnt signaling induced by 5-FU treatment. Whereas, a decrease in β-catenin protein accompanying reduction of antioxidase SOD and CAT induced by 5-FU treatment may be another mechanism of cell proliferation inhibition.

Forkhead box O (FOXO) family are transcription factors, which promote cell survival by regulating the cell cycle, apoptosis, and the response to oxidative stress (Eijkelenboom and Burgering, 2013). The accumulation of ROS may interrupt 14-3-3 combine to FoxO via JNK (c-Jun N terminal kinase), permit FoxO entrance into the nucleus, and induce its transcriptional activation (Morrison, 2009; Nakae *et al.*, 2008). FoxO can be phosphorylated by the phosphatidylinositol 3-kinase-Akt pathway (Han *et al.*, 2015; Zhang *et al.*, 2020). It is of note that FoxO-mediated transcription requires the binding of β-catenin. FoxOs can compete with TCF/LEF by directly binding β-catenin, thereby inhibit Wnt/β-catenin downstream signaling (Almeida *et al.*, 2007; Behrens *et al.*, 1996; Hoogeboom *et al.*, 2008; Iyer *et al.*, 2013). It was demonstrated herein, compared with the control group FoxO1 expression in 5-FU treated HS-5 cells rose dramatically concurrent with decreased p-FoxO1 expression. The reason for up-regulation of FoxO1 may be related to 5-FU triggered oxidative stress, whereas FoxO1 up-regulation may be another reason for 5-FU induced decrease in Wnt signaling (Burgering and Medema, 2003; Danciu *et al.*, 2004; Ma and Wang, 2012). FoxO transcription factor family regulates the proteins that are crucial for apoptosis, as well as the proteins involved in the proliferative status of a cell. FoxO factors may regulate antiapoptotic and proapoptotic proteins at multiple levels, finally trigger activation of the effector caspases. Bim promotes apoptosis by inhibition of antiapoptotic Bcl-2

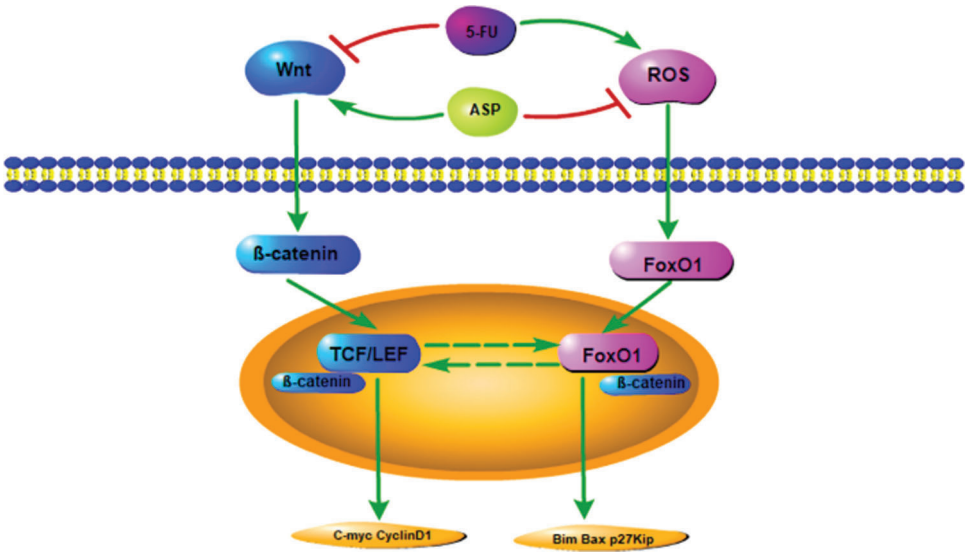

FIGURE 6. Model of Wnt signaling cascade.

family members or through direct activation of Bax-like molecules. FoxO factors may regulate Bim protein expression to cause cell death due to cytokine deprivation. FoxO factors may also repress transcription of Bcl-XL through the induction of the transcriptional repressor (Dijkers *et al.*, 2000; Stahl *et al.*, 2002; Tang *et al.*, 2002). Caspase-3 is an important effector protease, when it is cleaved, it acts as the final executor during apoptosis. In the current study, it was found that in 5-FU treated HS-5 cells, FoxO1 targeted apoptosis-related proteins to cause an increase in Bim, Bax, and caspase-3, whereas a decrease in Bcl-2. FoxO1 targeted apoptosis to disturb the dynamic balance of the cellularity of HS-5 cells, which may be one of the reasons for cell growth inhibition. Moreover, the cyclin kinase inhibitor $p27^{Kip1}$, a downstream target of FoxO1, acting as a potent inhibitor of cyclin/CDK complexes in the S-phase of cell cycle progression was also tested (Collado *et al.*, 2000; Kops *et al.*, 2002; Medema *et al.*, 2000; Nakamura *et al.*, 2000). It was found herein that 5-FU increased the expression of $p27^{kip1}$. In addition, 5-FU simultaneously reduced the expression of Cyclin D1. It is of note that transcriptional repression of D-type cyclins is vital to the FoxO-induced cell-cycle arrest, which is evidenced by transcriptional profiling and mRNA analysis. D-type cyclins are required for phosphorylation and inactivation of the retinoblastoma tumor suppressor protein (pRb), an essential determinant of cell-cycle progression in G1 (Ramaswamy *et al.*, 2002; Schmidt *et al.*, 2002). To sum up, 5-FU-induced HS-5 cell growth inhibition is probably associated with FoxO1 targeted apoptosis or cell cycle arrest.

The traditional Chinese medicine *Angelica sinensis*, which is commonly used to enrich the blood, promote blood circulation (Wei *et al.*, 2016). The active constituents of *Angelica sinensis* include polysaccharides, organic acid sand phthalides, among which *Angelica sinensis* polysaccharides (ASP) are regarded as the main biological activity ingredient responsible for pharmacological effects with multi-target property (Deng *et al.*, 2006). ASP have attracted more and more attention to its beneficial effects, such as hematopoietic effects (Liu *et al.*, 2010b), immunologic enhancement (Yang *et al.*, 2006), anti-tumor activity (Cao *et al.*, 2010; Shang *et al.*, 2003), and anti-radiation damage (Zhao *et al.*, 2012). The antioxidant properties of ASP suppress the production of ROS and protected the endothelial progenitor cells, hepatocytes, myocardial cells, and nerve cells from oxidative damage (Ai *et al.*, 2013; Ji *et al.*, 2014; Zhang *et al.*, 2010). Moreover, the evidence demonstrated that ASP promote cell proliferation, including in total spleen cells, macrophages (Yang *et al.*, 2006), and gastric epithelial cells (Xie *et al.*, 2019). Our previous studies suggested that ASP reduced oxidative stress and oxidative DNA damage, boosted direct cell-cell contact between stromal cells and hematopoietic cells through Cx43 junctions, regulated cytokines, growth factors and chemokines such as CXCL12, SCF, GM-CSF, RANTES and thus provided a homeostatic microenvironment for hematopoietic stem/progenitor cells to regenerate following chemotherapeutic myelosuppression. In the present study, it was further demonstrated that ASP protected HS-5 cells from 5-FU-induced proliferation inhibition and ameliorated cellular oxidative stress via the mechanism of up-regulation of Wnt/β-catenin signaling. Most importantly, it was first evidenced herein that ASP balanced the relationship between FoxO-mediated transcription and Wnt signaling in BMSCs under oxidative stress, which might be promising for the clinical therapeutic use of ASP to myelosuppression.

Conclusions

In conclusion, the present study has reported that ASP protect stromal cells against 5-FU-induced proliferation inhibition and apoptosis via activating the Wnt/β-catenin signaling pathway directly or the indirect effects on Wnt/β-catenin signaling by down-regulation of its antagonizing FoxO1 (Fig. 6), suggesting a broad role for ASP as a potential antioxidant protective agent for chemoradiation therapeutic, preventive agents.

Author Contribution: For research articles, LW conceptualized and designed the experiments; HXZX performed the experiments; RJQ, ZLW and MHX, YX, YPW contributed

reagents/materials/analysis tools; HXZX analyzed the data and wrote the paper; LW revised the paper.

Availability of Data and Materials: The datasets used in this study are available from the corresponding author upon reasonable request.

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