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Anthocyanin-containing purple-fleshed potatoes suppress colon tumorigenesis via elimination of colon cancer stem cells

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Abstract

Cancer stem cells (CSCs) are shown to be responsible for initiation and progression of tumors in a variety of cancers. We previously showed that anthocyanin-containing baked purple-fleshed potato (PP) extracts (PA) suppressed early and advanced human colon cancer cell proliferation and induced apoptosis, but their effect on colon CSCs is not known. Considering the evidence of bioactive compounds, such as anthocyanins, against cancers, there is a critical need to study anticancer activity of PP, a global food crop, against colon CSCs. Thus, isolated colon CSCs (positive for CD44, CD133 and ALDH1b1 markers) with functioning p53 and shRNA-attenuated p53 were treated with PA at 5.0 μg/ml. Effects of baked PP (20% wt/wt) against colon CSCs were also tested *in vivo* in mice with azoxymethane-induced colon tumorigenesis. Effects of PA/PP were compared to positive control sulindac. *In vitro*, PA suppressed proliferation and elevated apoptosis in a p53-independent manner in colon CSCs. PA, but not sulindac, suppressed levels of Wnt pathway effector β-catenin (a critical regulator of CSC proliferation) and its downstream proteins (c-Myc and cyclin D1) and elevated Bax and cytochrome *c*, proteins-mediating mitochondrial apoptosis. *In vivo*, PP reduced the number of crypts containing cells with nuclear β-catenin (an indicator of colon CSCs) via induction of apoptosis and suppressed tumor incidence similar to that of sulindac. Combined, our data suggest that PP may contribute to reduced colon CSCs number and tumor incidence *in vivo via* suppression of Wnt/β-catenin signaling and elevation of mitochondria-mediated apoptosis.

Keywords: Colon cancer stem cells; Purple-fleshed potatoes; Anthocyanins; β-Catenin; p53

1. Introduction

Colon cancer is the third leading cause of cancer related deaths in the United States [1]. There is mounting evidence that most cancers, including colon cancer, have a hierarchy of cells with cancer stem cells (CSCs) forming the core and sustaining the growth of the tumor [2]. Colon CSCs mimic the functionality of normal adult stem cells maintaining their un-differentiated state while dividing nonsymmetrically [3]. *In vivo* studies implicate Wnt/β-catenin signaling in the regulation of colon stem cell proliferation [4]. In the canonical Wnt pathway, mutations in *APC*, a tumor suppressor gene, leads to increased nuclear translocation of β-catenin and subsequent activation of Wnt transcriptional targets, ultimately causing adenoma [2,5]. Nuclear translocation of β-catenin is implicated in the transformation of stem cells to CSCs in the colon [6]. *P53*, a critical tumor suppressor gene called the guardian of the genome, is mutated in over 50% of cancers, including colon cancer [7]. Mutated p53 allows for uncontrolled proliferation and leads to progression from adenoma to carcinoma [8]. Thus, it is important to test whether strategies developed against colon CSCs work even in the absence of p53.

Sulindac, a nonsteroidal anti-inflammatory drug (NSAID) eliminated colon stem cells with nuclear β-catenin, an indicator of colon CSCs, and reduced polyp number in $APC^{Min/+}$ mice, a well-established model for colon cancer [9]. However, long-term use of NSAIDs, in particular sulindac, is associated with adverse gastrointestinal and renal toxicities [10,11]. Conversely, as colon cancer involves stepwise mutations in multiple genes, there is a long latency period [12,13] before it manifests, and thus, there is an opportunity to target colon cancer by suitable modification of diet. There is increasing evidence of preventive/protective role of bioactive components in the food against colon cancer. Purple-fleshed potatoes (PP) are a good source of anthocyanins and phenolic acids, compounds that have also demonstrated anti-colon cancer efficacy in different models [14–16].

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Potato is one of the largest consumed food crops in the United States. Indeed, consumption of color-fleshed potatoes increased by 17%, due to putative health benefits, while traditional potatoes decreased during the last 10 years [17]. We have previously shown that PP contains high levels of polyphenols such as anthocyanins compared to white-fleshed potatoes (WPs) and retains these levels even after baking [18]. Acetylation makes potato anthocyanins more stable and distinguishable from other food sources such as berries [19,20]. We also showed that anthocyanin-containing PP extracts, even after baking, suppressed proliferation and induced apoptosis similar to raw PP extracts, in early and advanced colon cancer cell lines HCT-116 and HT-29, respectively [18]. Colon CSCs *in vitro* have been shown to be targeted by dietary bioactive compounds such as curcumin [21]. However, there are no laboratory studies investigating the anticancer properties of dietary whole foods such as PP on colon CSCs. Given that the potato is the most consumed vegetable in the United States, the establishment of a link between anthocyanin-containing PP and inhibiting colon CSCs could be very impactful.

Colon CSCs (positive for CD44, CD133 and ALDH1b1 markers) isolated from primary human colon cancer tumors, are a useful model for *in vitro* experiments to screen anticancer compounds [22]. *In vivo* azoxymethane (AOM), a DNA alkylating agent, induced mouse colon cancer model has been shown to be the best model to predict chemopreventive efficacy [23]. AOM-induced tumors also exhibit aberrant *APC* expression and nuclear localization of β-catenin [24,25]. Thus, these *in vitro* and *in vivo* models were used to test the anticancer properties of the anthocyanin-containing PP. Furthermore, we examined the possible molecular mechanisms that underlie its anticancer activity.

2. Materials and methods

2.1. Chemicals

Ethanol and methanol were purchased from VWR International (Bristol, CT, USA). Antibodies for Bax, Bcl-2, β-actin (Actin), β-catenin, cyclin D1, c-Myc and topoisomerase-2 beta (TOP2B) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cytochrome *c* was obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2. Plant material

Uniform-sized PP tubers (Purple Majesty variety) were baked in a conventional oven preheated to 204°C for 1 h and 15 min. Before baking, each potato was washed, dried, wrapped in food-grade aluminum foil and pierced approximately 1.5 cm deep with a knife at 3-cm intervals. Baked potatoes were cooled for 15–20 min, diced with skin into pieces weighing 7±1 g, freeze dried and stored at −20°C. For *in vitro* experiments, ethanolic extracts of anthocyanin-containing baked PP were prepared as per our published protocols [18]. Equivalent doses of ethanol were used as solvent control for all *in* vitro experiments. Another batch of baked PP was freeze dried, powdered and stored at −20°C before incorporation into diets for the mice study.

2.3. Potato characterization

Ultra-performance liquid chromatography and mass spectrometry (UPLC-MS) analysis of WP (Atlantic variety) and PP extracts (2 μl) was done using a Waters Acquity UPLC system from Waters (Milford, MA, USA) with a Waters HSS T3 column (1.8 μm, 1.0×100 mm) and a gradient from solvent A (100% water, 0.1% formic acid) to solvent B (95% methanol, 5% water, 0.1% formic acid). Column eluent was infused into a Q-Tof Micro mass spectrometer (Waters) fitted with an electrospray source. Data were collected similar to our earlier published protocols [18]. Peak detection was performed using MarkerLynx software (Waters). To identify metabolite differences between potato varieties, we also carried out peak annotation using METLIN metabolite database (http://metlin.scripps.edu) using simple, fragment and neutral loss search elements. Phenolic metabolite differences between WP and PP are presented in Table 1.

2.4. Cancer stem cells

Colon CSCs, positive for CSC markers CD133, CD44 and ALDH1b1, were obtained from Celprogen (San Pedro, CA, USA). To maintain the cells in their undifferentiated state, colon CSCs growth media and specially coated cell culture flasks obtained from Celprogen were used. Cells were maintained in incubation at 37°C and 5% CO_2. Cell cultures at approximately 80% confluence were used for all *in vitro* experimental procedures. For all experiments, low passage number (less than 10) cells were used (not more than 3 weeks after resuscitation).

Table 1
Phenolic and anthocyanin composition of white vs purple-fleshed potatoes by UPLC/MS

Compound identity	Molecular ion M+ (*m/z*)	Retention time (min)	WP	PP
Phenolic acids				
p-Coumaric acid	165.1	5.55	527.5±41.9	544.5±23.2
Chlorogenic acid	355.1	6.08	6543.5±35.9	15,176.6±73.9
Anthocyanins				
Pet-3-rut-5-glc	787.3	5.79	0.0	1578.6±105.7
Mal-3-rut-5-glc	801.3	6.19	0.0	237.5±14.8
Cya-3-*O*(6-*O*-malonyl-β-D-glc)	535.1	6.37	0.0	691.1±3.2
Peo-3-(p-coum)-isophoro-5-glc	933.3	7.26	0.0	2729.2±275.7
Peo-3-rut-5-glc	771.3	7.80	0.0	2871.1±29.9
Pet-3-(p-coum)-rut-5-glc	933.3	7.92	0.0	28,748.5±235.7
Peo-3-caffeyl-rut-5-glc	933.3	8.03	0.0	27,215.4±2295.1
Pel-3-(p-coum)-rut-5-glc	887.3	8.11	0.0	80.8±5.4
Pel-3-(4-ferul-rut)-5-glc	917.3	8.15	0.0	1569.3±142.7
Peo-3-(p-coum)-rut-5-glc	917.3	8.21	0.0	1810.2±135.2
Mal-3-(p-coum)-rut-5-glc	947.3	8.31	0.0	2707.4±204.4

The compounds are reported as the area under the curve per gram dry weight. Values are presented as the means±S.E. of 6 replicates.

2.5. Lentiviral shRNA-mediated attenuation of p53 in colon CSCs

Colon CSCs were infected with lentiviral particles encoding shRNA targeting p53 obtained from Santa Cruz Biotechnology according to the manufacturer's protocol. Briefly, colon CSCs were infected at a multiplicity of infection of 10 in CSC growth medium containing 5 μg/ml of polybrene (for selection of cells with successful lentiviral induction) at 37°C and 5% CO_2. After 24 h, the spent media was replaced with fresh media and the cells were cultured for 2 days. The transduced cells were selected in the presence of puromycin (7.5 μg/ml) for 5 days.

2.6. Cell proliferation

Cell viability was assessed by BrdU (5-bromo-2′-deoxyuridine) assay kit from Cell Signaling Technology (Danvers, MA, USA). Briefly, cells were plated at a density of 1×10^5 per well in 12-well plates. Media was replaced after 24 h with colon CSCs media without serum (Celprogen) and dosed with PA or sulindac (for in vitro experiments, sulindac sulfide, the active form of sulindac was used). After 24 h, BrdU incorporation was assayed as per the manufacturer's protocol. The experiment was carried out in triplicate, and results were expressed as the means±S.E.

2.7. Terminal transferase dUTP nick end-labeling assay

Apoptosis was quantified by using fluorescein-labeled nucleotide and terminal deoxynucleotidyl transferase (TdT) to identify DNA fragmentation (characteristic of apoptosis). Briefly, cells (9×10^4) were seeded in four-chambered glass slides, and after treatment for 12 h, the *in situ* cell death detection kit from Roche Diagnostics (Indianapolis, IN, USA) was used for quantifying apoptosis according to the manufacturer's protocol. Slides incubated without TdT served as a negative control. The percentage of apoptotic cells (apoptotic index) was calculated by counting the stained cells in 12 fields, each containing at least 50 cells. The experiment was carried out in triplicate, and results were expressed as means±S.E.

2.8. Sphere formation assay

Briefly, colon CSCs (10,000 cells per well) were cultured in stem cell specific serum free media in an ultra-low attachment 6-well plates. The cells were maintained in similar conditions as mentioned earlier under the cancer stem cells section. PA or sulindac was added six hours after the cells were added to the 6-well plates. At the end of 10 days, the number of spheres was assayed using a phase contrast microscope. The experiment was carried out in triplicate, and results were expressed as the means±S.E.

2.9. Western blot

Cells were plated in 6-well plates at a concentration of 3.0×10^5 cells per well in colon CSCs media. After 24 h, cells were transferred to a serum-free medium for 18 h. Protein was extracted according to our previously published protocols [26–28]. The blots were incubated with primary antibodies overnight at 4°C at a dilution of 1:500. Subsequently, secondary antibodies incubation was for 2 h at room temperature at a dilution of 1:10,000. Blots were imaged and quantified using the Odyssey Infrared Imaging System and software (Lincoln, NE, USA) and normalized to β-actin, a loading control for cytoplasmic proteins and TOP2B as a loading control for nuclear proteins. Each treatment was carried out in triplicate, and results were expressed as means±S.E.

2.10. Animal study

A/J male mice (6 weeks old; n=13 per group) purchased from the Jackson Laboratories (Bar Harbor, ME, USA) were housed in stainless steel wire cages (3 or 4 per cage) with a 12-h light/dark cycle. Mice were allowed access to laboratory rodent chow and water *ad libitum*. After 2 weeks of acclimatization, all mice were randomly assigned to four groups and fed AIN-93G diets obtained from Harlan Laboratories (Indianapolis, IN, USA). The Institutional Animal Care and Use Committee at Colorado State University approved all experimental procedures involving the use of mice.

2.11. AOM carcinogen injection

All mice except saline controls received six weekly subcutaneous injections of AOM (Sigma Aldrich, St. Louis, MO, USA) in saline for aberrant crypt foci induction at 5 mg/kg starting at 8 weeks of age.

2.12. Experimental diets

At 16 weeks of age, the AOM-injected animals were fed the following diets — AIN-93G control, AIN-93G supplemented with baked PP (20% w/w) and AIN-93G supplemented with Sulindac (0.06% w/w).

2.13. Colon tissue collection

After 1 week of dietary intervention, five animals from each group were euthanized using isoflurane. The remaining animals (n=8/group) were euthanized after 4 weeks of dietary intervention. The colon was resected and washed with RNAse-free phosphate-buffered saline and observed under a dissection microscope for counting tumors. Tumors greater than 2 mm were recorded.

For immunohistochemistry and immunofluorescence analysis, about 1 cm of the colon tissue was collected and fixed with 10% buffered formalin. Specimens were then flattened, paraffin embedded and orthogonally sectioned. The tissue was sectioned at 4-μm thickness and mounted on positively charged slides.

2.14. Immunohistochemistry/Immunofluorescence staining

2.14.1. Pretreatment of slides

Prior to staining, the paraffin was softened and the tissue specimens fixed additionally by baking the slides in an oven at 55°C for 20 min. Deparaffinization was performed with Fisherbrand (Pittsburg, PA, USA) clearing agent citrisolv twice for 5 min and hydrated with decreasing concentrations of ethanol (100–100–95–70 vol/vol). For target retrieval, the slides were incubated in citrate buffer at pH 6 (9 mM citrate, 1 mM citric acid) at 95°C for 20 min. To quench auto fluorescence from formalin residues, slides were pretreated with sodium borohydride (1 mg/ml) for 5 min. Mouse sections were blocked with mouse IgG serum from the M.O.M. kit and avidin/biotin obtained from Vector Labs (Burlingame, CA, USA) as per manufacturer's protocol.

2.14.2. β-Catenin staining

β-Catenin staining was performed at 4°C overnight using a Abcam rabbit anti-β-catenin antibody (Cambridge, MA, USA). Biotinylated secondary antibody in combination with streptavidin fluorescein (Vector Labs) was used for visualization. Mounting media with DAPI (Vector Labs) was used as a counterstain. All images were taken in Olympus BX-63 microscope with the help of Cell Sens software from Olympus America (Center Valley, PA, USA). Nuclear β-catenin index was calculated as a percentage of total number of crypts with nuclear β-catenin accumulation as described previously [9]. At least 300 crypts were counted per animal.

2.14.3. Terminal transferase dUTP nick end-labeling staining (apoptosis)

Terminal transferase dUTP nick end labeling (TUNEL) staining was performed using a cell death detection kit from Roche Diagnostics according to the manufacturer's protocol for formalin-fixed, paraffin-embedded tissues. Apoptotic index was calculated as a percentage of total number of crypts with at least one TUNEL-positive cell. At least 300 crypts were counted per animal.

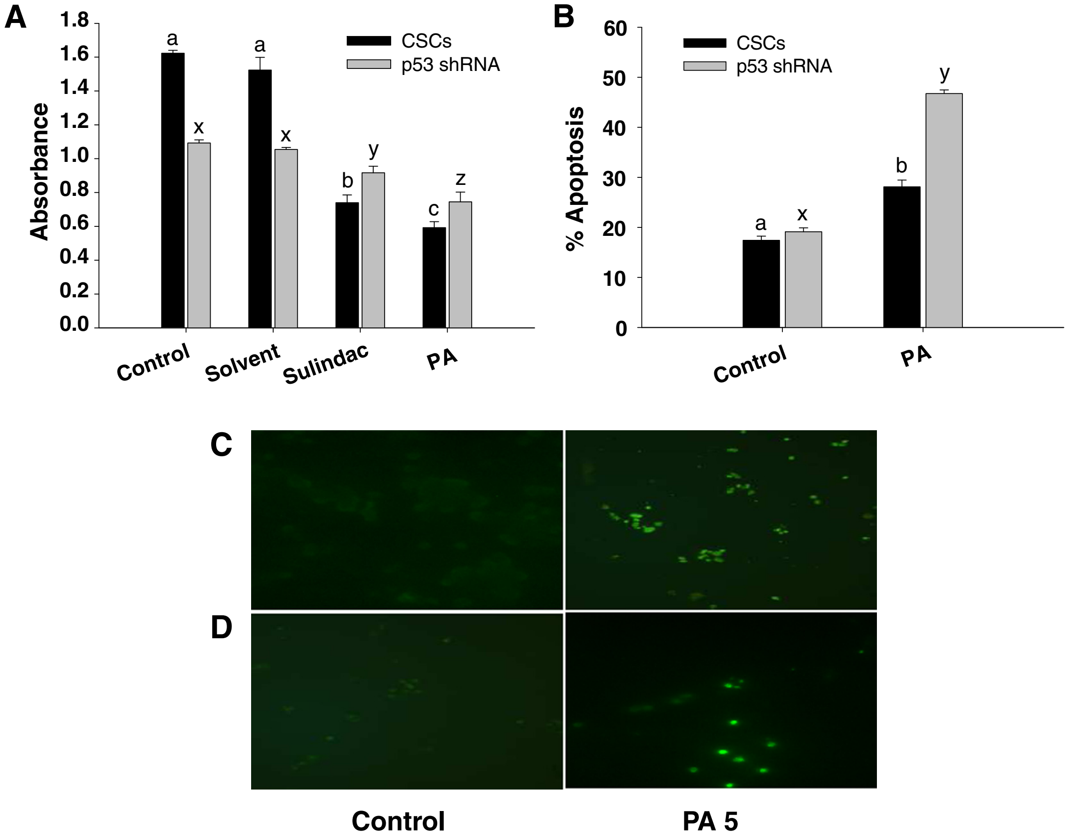

Fig. 1. PA suppressed proliferation and induced apoptosis in colon CSCs independent of p53. (A) Antiproliferative effect of PP anthocyanin extract (PA) in colon CSCs with functioning p53 and with attenuated p53. Cells were treated with PA (5 μg/ml) or sulindac (12.5 μg/ml) for 24 h and BrdU assay was performed as described in Materials and Methods. (B–D) PA induced apoptosis in colon CSCs with functioning p53 and attenuated p53. TUNEL assay was performed and the results are expressed as percentage apoptosis. Cells fluorescing bright green due to fragmented DNA indicate apoptotic cells. Pictures were taken on a fluorescence microscope at 20× magnification (12 fields per treatment and at least 500 cells were counted). Representative pictures are shown for control and PA at 5.0 μg/ml. PA=baked purple-fleshed potato extract. Values are in means±S.E. Means that differ by a common letter (a, b, c for CSCs and x, y, z for CSCs with shRNA-attenuated p53) differ (P<.05).

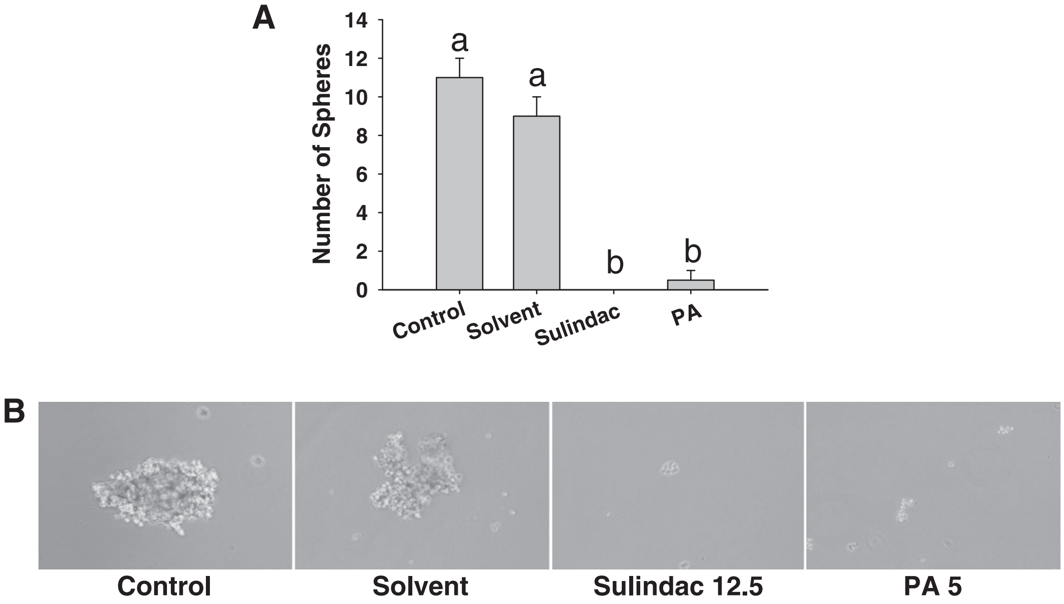

Fig. 2. PA suppressed sphere formation of colon CSCs similar to that of sulindac (A). Representative pictures taken at 100× magnification are shown for control, solvent, sulindac at 12.5 μg/ml and PA at 5.0 μg/ml (B). PA=baked purple-fleshed potato extract. Values are in means±S.E. Means that differ by a common letter (a, b, c) differ (P<.05).

2.15. Statistical design

Data are expressed as means±S.E. for *in vitro* data and as means±S.D. for *in vivo* data. Significance was determined by one-way analysis of variance with post hoc Tukey analysis using IBM SPSS software (Armonk, NY, USA) for *in vitro* data. For animal studies, analysis of data was done using mixed procedure in SAS v9.4 software (Cary, NC, USA). *P* values <.05 were considered significant.

3. Results

3.1. UPLC-MS profile of phenolic compounds in PP

Peak annotations using METLIN metabolite database are presented in Table 1. Phenolic acids (chlorogenic acid and p-Coumaric acid) were

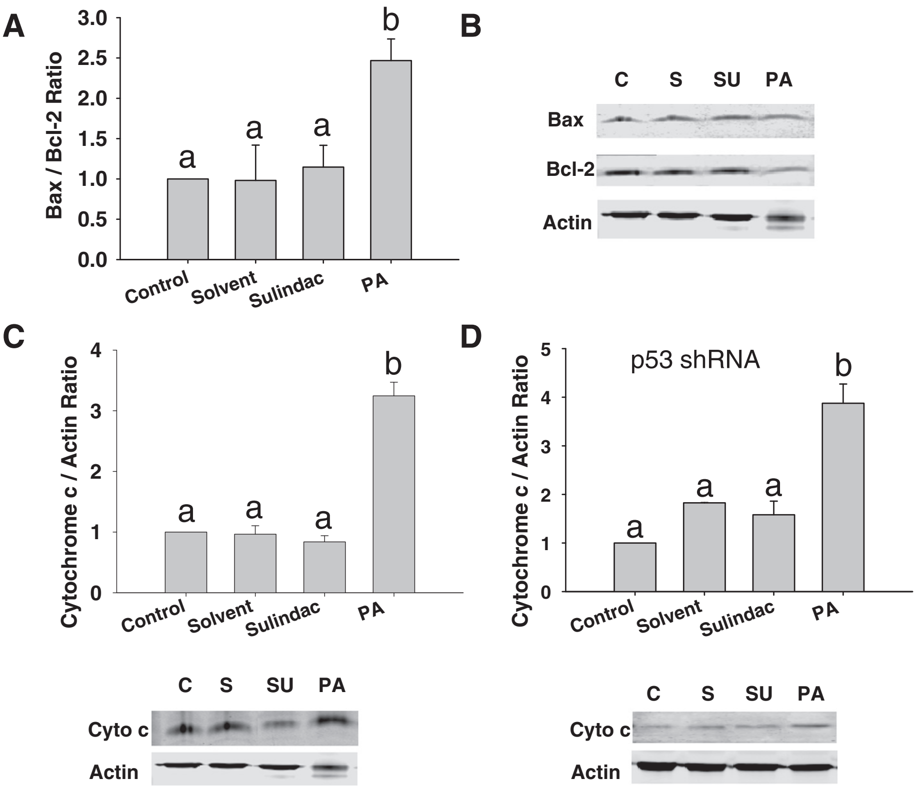

Fig. 3. PA elevated levels of mitochondria-mediated apoptosis pathway proteins. PA elevated Bax/Bcl-2 ratio (A, B) and cytochrome *c* levels in colon CSCs independent of p53 (C, D). Colon CSCs were treated with PA (5 μg/ml) or sulindac (12.5 μg/ml) for 24 h, and whole-cell lysates were analyzed for Bax (proapoptotic), Bcl-2 (antiapoptotic) and cytochrome *c* (proapoptotic) levels by western blotting. Actin was used as loading control. C=control; S=solvent; SU=sulindac; PA=baked purple-fleshed potato extract. Values are in means±S.E. Means that differ by a common letter (a, b) differ (P<.05).

detected in both WP and PP varieties; however, the relative abundance was higher in PP. Glycosylated anthocyanins were only detected in PP. We have previously shown that PP retains anthocyanins even after processing (baking) [18]. Baked PP extracts (PA) suppressed early (HCT-116) and advanced (HT-29) human colon cancer cell proliferation and induced apoptosis similar to that of raw PP extracts and were more potent compared to WP [18]. Hence, for our *in vitro* and *in vivo* experiments, we used baked PP.

3.2. PA suppressed proliferation and induced apoptosis in colon CSCs in a p53-independent manner

Proliferation was assayed by measuring BrdU incorporation and confirmed using cell counting. For all our experiments on colon CSCs with functioning p53 and shRNA-attenuated p53, we used a dose of 5.0 μg/ml PA extract and 12.5 μg/ml sulindac. PA at 5.0 μg/ml suppressed proliferation by 63% and 32% compared to control (Fig. 1A) in colon CSCs with functioning p53 and shRNA-attenuated p53, respectively. Sulindac treatment at 12.5 μg/ml resulted in suppression of proliferation by 55% in colon CSCs with functioning p53 (Fig. 1A). However, in colon CSCs with attenuated p53, suppression of proliferation by sulindac was modest (16%), indicating p53 dependency. Induction of apoptosis was analyzed using TUNEL assay. PA induced 28% and 46% apoptotic cell death in colon CSCs with functioning p53 and shRNA-attenuated p53 (Fig. 1B–D). These results suggest that PA inhibits the growth of colon CSCs independent of p53.

3.3. PA suppressed sphere formation ability of colon CSCs

Self-renewal is a key property of CSCs that is largely measured in functional assays that require proliferation, making it difficult to distinguish molecules that affect self-renewal vs. proliferation. Hence, to assess PA ability to target the self-renewal capability of CSCs, sphere formation assay was used as described previously [29]. We treated colon CSCs with PA or sulindac at 5.0 and 12.5 μg/ml, respectively. PA significantly suppressed sphere formation similar to that of sulindac (Fig. 2A). Fig. 2B shows representative images from the sphere formation assay demonstrating complete suppression in comparison to the control. This demonstrates that, in addition to the antiproliferative and proapoptotic activities, PA inhibits the colon CSCs self-renewal property.

3.4. PA elevated mitochondria-mediated apoptotic pathway proteins Bax/Bcl-2 and cytochrome c

Cytosolic cell lysates of colon CSCs with functioning p53 and shRNA-attenuated p53 treated with PA and sulindac were subjected to Western blot analysis. Bax/Bcl-2 ratio was elevated in PA treated colon CSCs with functioning p53 (Fig. 3A and B). Cytochrome *c* levels were also elevated

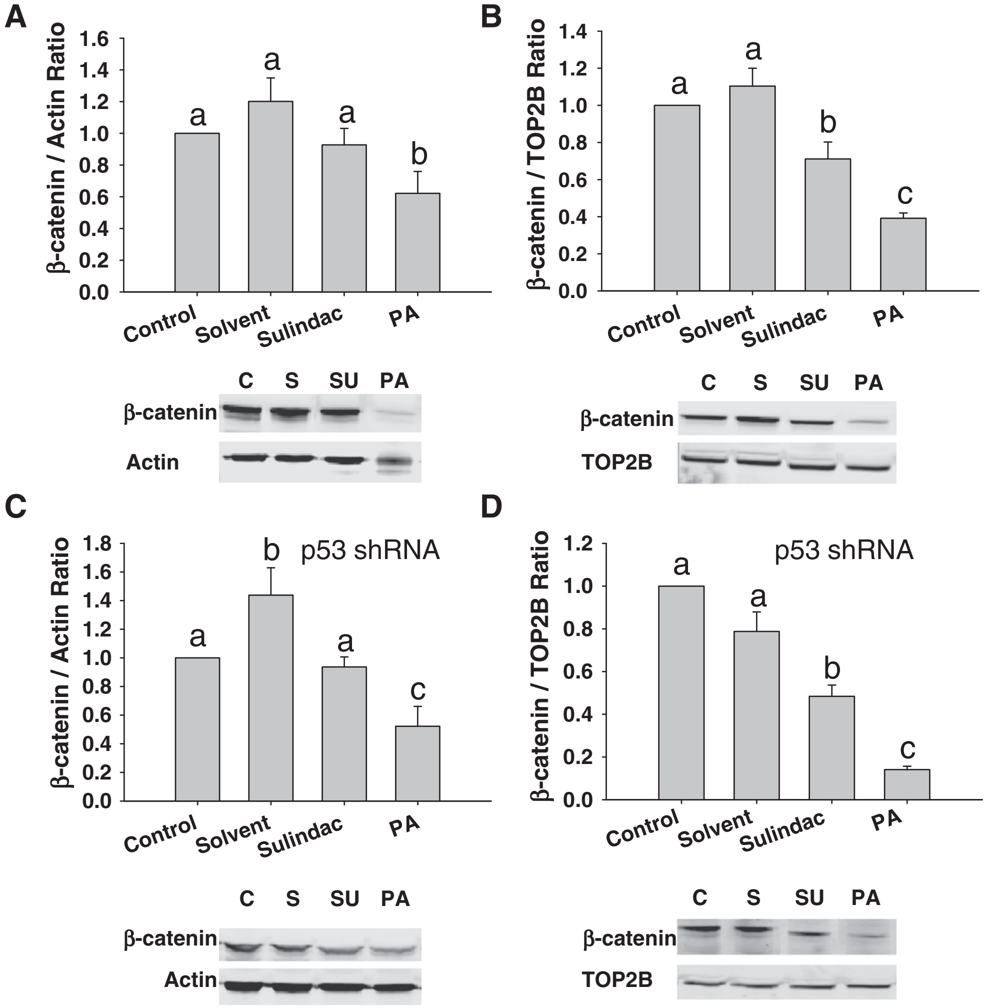

Fig. 4. PA suppressed cytosolic and nuclear β-catenin levels in colon CSCs with functioning p53 (A, B) and attenuated p53 (C, D). Colon CSCs were treated with PA (5 μg/ml) or sulindac (12.5 μg/ml) for 24 h, and cytosolic and nuclear lysates were analyzed for β-catenin by Western blotting. Actin and TOP2B were used as loading controls for cytosolic and nuclear lysates, respectively. C=control; S=solvent; SU=sulindac; PA=baked purple-fleshed potato extract. Values are in means±S.E. Means that differ by a common letter (a, b, c) differ ($P<.05$).

by PA treatment independent of p53 status (Fig. 3C and D), indicating that the induction of apoptosis might be via mitochondria-mediated apoptotic pathway [30]. Although sulindac induced apoptosis in colon CSCs, it did not result in elevation of Bax/Bcl-2 or cytochrome *c* levels.

3.5. PA suppressed Wnt pathway proteins

Western blot analysis was performed to investigate whether PA-induced inhibition of colon CSCs growth was associated with Wnt/β-catenin pathway. PA suppressed levels of cytoplasmic and nuclear β-catenin greater than that of sulindac in colon CSCs with functioning p53 (Fig. 4A and B) and shRNA-attenuated p53 (Fig. 4C and D). The Wnt/β-catenin pathway downstream targets c-Myc (Fig. 5A and C) and cyclin D1 (Fig. 5B and D) were suppressed by PA in colon CSCs with functioning p53 and shRNA-attenuated p53. These results confirm suppression of β-catenin nuclear translocation by PA, thus limiting colon CSC growth.

3.6. PP induced apoptosis and reduced number of crypts with nuclear β-catenin accumulated colon CSCs

Since PA was able to suppress nuclear translocation of β-catenin *in vitro*, we hypothesized that PP consumption will eliminate stem cells with nuclear β-catenin in mice with AOM-induced colon cancer. PP supplementation for 1 week markedly induced apoptosis detected by TUNEL staining, with 16% of crypts containing at least one TUNEL-positive cell, comparable to 18.5% in mice receiving sulindac (Fig. 6A). PA or sulindac treatment reduced crypts containing cells with nuclear β-catenin by 50% at week 1 (Fig. 6B and C). These results suggest that PP treatment rapidly removes intestinal stem cells or progenitors with aberrant activation of Wnt signaling.

3.7. PP suppressed AOM-induced colon cancer tumors

At week 4, all mice that received AOM injections developed tumors. PP treatment suppressed the incidence of tumors (greater than 2 mm) by 50% (Fig. 7) and could be due to elimination of colon CSCs via apoptosis as seen in animals euthanized at week 1 (Fig. 6A). Sulindac also showed potent suppression of tumor incidence (Fig. 7); however, unlike the PP group, sulindac consuming mice had significant gastrointestinal toxicity (stomach/intestinal ulcers) marked with loss of fat deposits (data not shown).

4. Discussion

Our results demonstrate that *in vitro* PA significantly suppressed proliferation in CSCs both with functioning p53 and with attenuated p53,

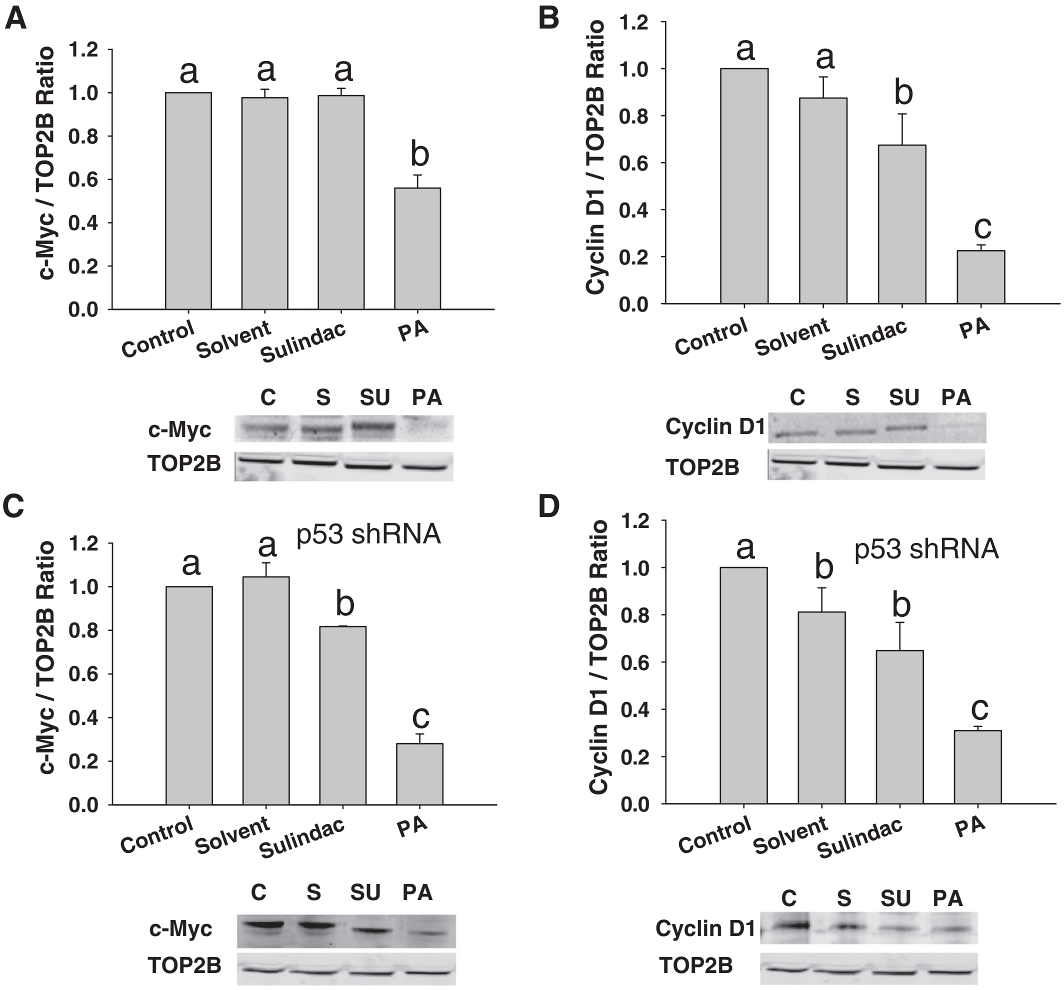

Fig. 5. β-Catenin targets c-Myc and cyclin D1 levels were suppressed by PA in colon CSCs with functioning p53 (A, B) and attenuated p53 (C, D). Colon CSCs were treated with PA (5 μg/ml) or sulindac (12.5 μg/ml) for 24 h, and nuclear lysates were analyzed for c-Myc and cyclin D1 by Western blotting. TOP2B was used as a loading control. C=control; S=solvent; SU=sulindac; PA=baked purple-fleshed potato extract. Values are in means±S.E. Means that differ by a common letter (a, b, c) differ (P<.05).

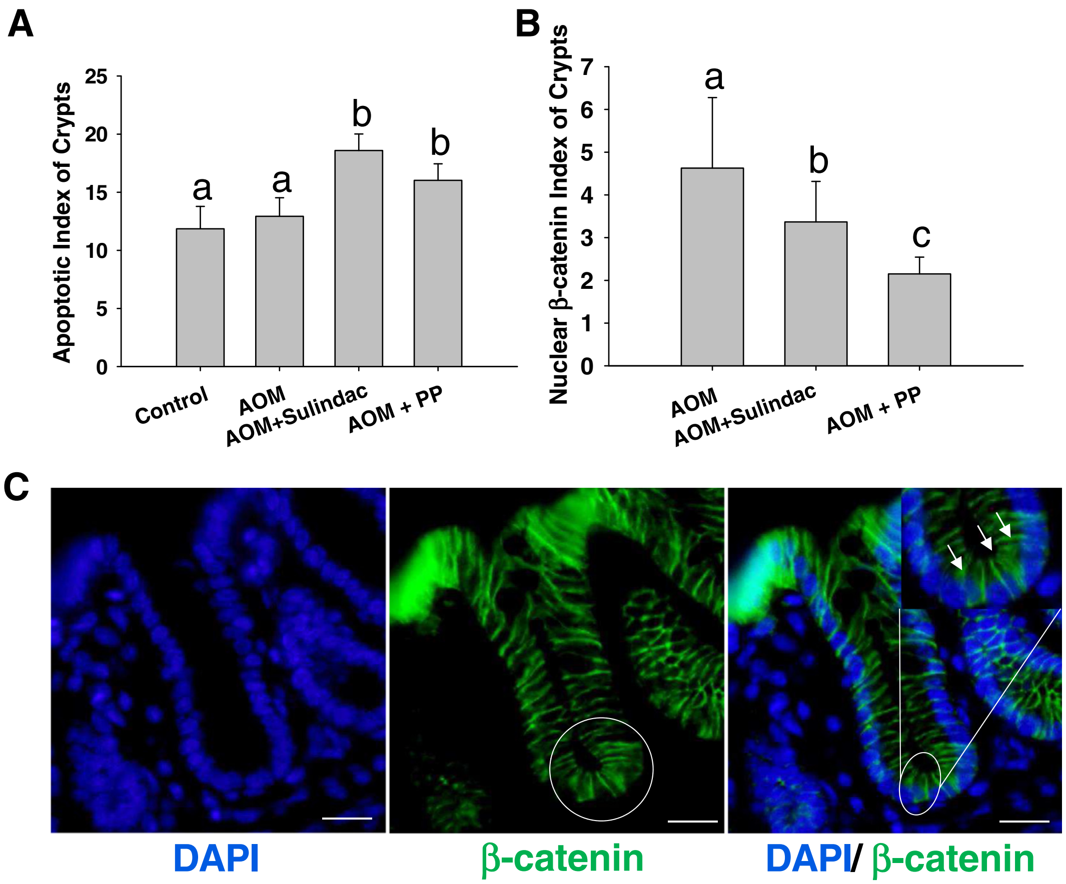

Fig. 6. PP treatment induced apoptosis (A) and reduced number of crypts with nuclear β-catenin accumulated intestinal stem cells similar to that of sulindac. Mice injected with AOM were fed with control, baked PP (20% w/w) or sulindac (0.06% w/w) supplemented diet for 1 week. Distal colon sections from the mice were analyzed for TUNEL-positive crypts and β-catenin localization by immunofluorescence. (A) The fractions of crypts containing at least one TUNEL-positive cell were determined. (B) Nuclear β-catenin index was calculated as a percentage of total number of crypts with nuclear β-catenin accumulation. (C) Staining of β-catenin and DAPI (blue; nuclear counterstain) in mice treated with AOM. Circles mark representative colon CSCs with nuclear β-catenin. Values are in means±S.D. (n=5 in each group). At least 300 crypts from each animal were analyzed. Means that differ by a common letter (a, b, c) differ (P<.05; scale bars: 15 μm).

suggesting that PA may work even in p53-independent cancers. PA also up-regulated proteins involved in mitochondria-mediated apoptotic pathway and down-regulated proteins involved in the Wnt/β-catenin signaling pathway. PP eliminated colon CSCs with nuclear β-catenin *in vivo* via induction of apoptosis and suppressed tumor incidence in mice with AOM-induced colon cancer lending support to the anticancer properties of PP, warranting further investigation using detailed studies.

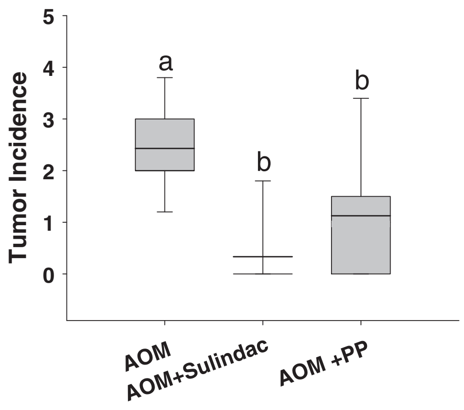

Fig. 7. PP suppressed tumor incidence in the colon similar to that of sulindac. Mice injected with AOM were fed with control, baked PP (20% w/w) or sulindac (0.06% w/w) supplemented diet for 4 weeks and euthanized. Whole colon tissue was resected and observed in a dissection microscope for visible tumors greater than 2 mm in size. Values are in means±S.D. (n=8 in each group). Means that differ by a common letter (a, b) differ (P<.05).

Polyphenolic compounds, especially anthocyanins derived from fruits and vegetables, demonstrate chemopreventive and chemotherapeutic activity through modulation of multiple molecular targets making them ideal for the prevention/treatment of cancer [31]. Potatoes are a rich source of phenolic acids, and color-fleshed potatoes also contain other bioactive compounds such as anthocyanins and carotenoids. UPLC-MS analysis comparing PP and WP showed that besides higher levels of phenolic acids, only PP contained anthocyanins (compared to WP; Table 1). We also showed previously that PP had more potent anticancer activity on early (HCT-116) and advanced (HT-29) colon cancer cell lines *in vitro* [18]. However, the effect against colon CSCs is not known, and for this purpose, we treated colon CSCs with PP and compared it with sulindac, a positive control.

PA at 5.0 μg/ml suppressed proliferation and induced apoptosis in colon CSCs with and without functioning p53; however, sulindac demonstrated p53 dependency (Fig. 1A). The p53 dependency of sulindac has been investigated previously in an AOM-induced mouse model with dysfunctional p53 [32]. Sulindac was not able to restore acute apoptosis response in p53−/− mice when compared to that of p53+/+ mice. This is particularly important because in late/metastatic stages of colon cancer, p53 is mutated [33]. PA-induced apoptosis

(Fig. 1B–D) was accompanied by elevated Bax/Bcl-2 ratio and cytochrome *c* (Fig. 3). Bax is a proapoptotic protein that binds Bcl-2 and aids in the release of cytochrome *c*, a key promoter in mitochondria-mediated apoptosis [34]. These results indicate that PP induces apoptosis through the mitochondria-mediated apoptotic pathway. We have also shown that PA suppressed sphere formation, since the formation of colonospheres is a measure of stemness. Our results provide the evidence that PA has the potential to target the self-renewal of colon CSCs.

PA treatment resulted in significant suppression of β-catenin at both nuclear and cytosolic levels in both colon CSCs with and without functioning p53 (Fig. 4) greater than that of sulindac. Stabilization of β-catenin and its subsequent accumulation in the nucleus are accompanied by increased transcriptional activation of proteins such as c-Myc and cyclin D1, which promote carcinogenesis by increasing cell proliferation [35,36]. Indeed, PA-treated colon CSCs had suppressed levels of c-Myc (Fig. 5A and C) and cyclin D1 (Fig. 5B and D) independent of p53.

Several characteristics of colon CSCs may explain the elimination by PP. Stem cells express high levels of "stemness" factors including the oncoprotein c-Myc [37], which is overexpressed in colon CSCs [38]. We have also shown *in vitro* that PA suppressed Wnt effector β-catenin and its downstream targets c-Myc and cyclin D1 levels in colon CSCs. Therefore, stem cells with oncogenic alterations, such as accumulation of β-catenin, may be more sensitive to PA-induced apoptosis, relative to differentiated cells with such alterations.

To further test whether PP can eliminate colon CSCs *in vivo*, we used an AOM-induced colon cancer mice model. Mice were fed with modified AIN 93G diet containing human relevant doses of PP (20% w/w) or sulindac (positive control; 0.06% w/w) for 1 or 4 weeks. Week 1 euthanized animals were used to study the early molecular mechanism of PP. Week 4 euthanized animals were used for endpoint analysis of tumor incidence. PP or sulindac fed mice had significant increase in the number of crypts with TUNEL-positive cells (indicator of apoptosis) compared to AOM control (Fig. 6A). Nuclear β-catenin localization is observed predominantly in colon CSCs but rarely in other cells of the crypt in $APC^{Min/+}$ mice [9] (Supplementary Fig. S1); hence, we looked at the number of crypts containing nuclear β-catenin. More than 50% of crypts with nuclear β-catenin accumulated intestinal stem cells were eliminated in mice fed with PP or sulindac for 1 week when compared to AOM control (Fig. 6B and C). In animals fed with PP or sulindac for 4 weeks, we observed very few stem cells with accumulated nuclear β-catenin. It has been previously reported that sulindac treatment eliminates colon CSCs with accumulated nuclear β-catenin via rapid apoptosis, which is not detected after week 1 [9]. At the end of week 4, PP significantly suppressed tumor incidence (Fig. 7) comparable to that of sulindac.

In summary, this study demonstrated anticancer mechanism of PP (vs. sulindac) against colon CSCs *in vitro* and *in vivo* involving the induction of mitochondria-mediated apoptosis and targeting the Wnt/β-catenin signaling. However, a more detailed understanding of this molecular mechanism and its effects in different types of cancer requires further research. In conclusion, we believe that this study reveals a new direction and strategy for future studies of PP bioactive compounds and the development and application of related natural compounds.

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