Prevention and Epidemiology

Cancer Research

Chemoprevention of Intestinal Tumorigenesis in APC$^{min/+}$ Mice by Silibinin

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Abstract

Chemoprevention is a practical and translational approach to reduce the risk of various cancers including colorectal cancer (CRC), which is a major cause of cancer-related deaths in the United States. Accordingly, here we assessed chemopreventive efficacy and associated mechanisms of long-term silibinin feeding on spontaneous intestinal tumorigenesis in the *APC*$^{min/+}$ mice model. Six-week-old *APC*$^{min/+}$ mice were p.o. fed with vehicle control (0.5% carboxymethyl cellulose and 0.025% Tween 20 in distilled water) or 750 mg silibinin/kg body weight in vehicle for 5 d/wk for 13 weeks and then sacrificed. Silibinin feeding strongly prevented intestinal tumorigenesis in terms of polyp formation in proximal, middle, and distal portions of small intestine by 27% ($P < 0.001$), 34% ($P < 0.001$), and 49% ($P < 0.001$), respectively. In colon, we observed 55% ($P < 0.01$) reduction in number of polyps by silibinin treatment. In size distribution analysis, silibinin showed significant decrease in large-size polyps (>3 mm) by 66% ($P < 0.01$) and 88% ($P < 0.001$) in middle and distal portions of small intestine, respectively. More importantly, silibinin caused a complete suppression in >3 mm sized polyps and 92% reduction in >2 to 3 mm sized polyps in colon. Molecular analyses of polyps suggested that silibinin exerts its chemopreventive efficacy by inhibiting cell proliferation, inflammation, and angiogenesis; inducing apoptosis; decreasing β-catenin levels and transcriptional activity; and modulating the expression profile of cytokines. These results show for the first time the efficacy and associated mechanisms of long-term p.o. silibinin feeding against spontaneous intestinal tumorigenesis in the *APC*$^{min/+}$ mice model, suggesting its chemopreventive potential against intestinal cancers including CRC.
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Introduction

Chemoprevention has emerged as a pragmatic approach to reduce the risk of various cancers including colorectal cancer (CRC), one of the most common malignancies in the Western world (1). Familial adenomatous polyposis (FAP), a hereditary CRC predisposition syndrome (2), is caused by mutations in *adenomatous polyposis coli* (*APC*) gene and is characterized by progressive development of numerous adenomas in colon progressing to CRC during later stages (2, 3). Approximately 90% of FAP patients also develop small intestinal adenomas; overall, these patients are at ~330 times higher risk to develop small intestinal adenomas than normal population (4). Moreover, with increased survival seen in FAP patients following prophylactic colectomy, small intestinal adenomas are now a common cause of death in FAP patients (4, 5). Thus, animal models of intestinal tumorigenesis are needed to study the pathogenesis and to develop the strategies to control the malignancy including chemoprevention. *APC*$^{min/+}$ mouse, one of the most studied models of intestinal tumorigenesis, harbors a dominant germ line mutation at codon 850 of mouse homologue of human *APC* gene, which is similar to that in FAP patients (6, 7). *APC*$^{min/+}$ mice develop multiple adenomas in whole intestinal tract primarily in small intestine and fewer in colon (7). Thus, *APC*$^{min/+}$ mouse model is considered an analogue of human intestinal tumorigenesis and therefore is extensively used in both mechanistic and chemoprevention/intervention efficacy studies (7, 8).

Silibinin is used traditionally to treat various liver conditions and is largely nontoxic (9–11). In the last 20 years, we have shown silibinin efficacy in various *in vitro* and *in vivo* models of skin, prostate, bladder, and lung cancers (12–17). Regarding CRC, silibinin exhibits anticancer effects in HT29 cells in culture (11) and xenografts (18), and its dietary feeding inhibits azoxymethane-induced colonic aberrant crypt foci formation in rats without any adverse health effects (19). Together, these results suggested that silibinin could be an effective chemopreventive agent against CRC. Accordingly, for the first time, here we assessed the efficacy and associated mechanisms of long-term silibinin feeding on spontaneous intestinal tumorigenesis in *APC*$^{min/+}$ mice.

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Materials and Methods

Animals and chemoprevention study protocol. C57BL/6J-*APC*$^{min/+}$ and wild-type male mice (age, 5 wk) were from Jackson Laboratory. Silibinin was from Sigma with >98% purity (20). One week after acclimation, *APC*$^{min/+}$ mice (6 wk) were divided into two groups of 18 animals each and given 0.2 mL vehicle (0.5% w/v carboxy methyl cellulose and 0.025% Tween 20 in distilled water) or 750 mg silibinin/kg body weight by p.o. gavage in 0.2 mL vehicle for 5 d/wk for 13 wk. Negative controls (n = 9 per group) of wild-type mice were given vehicle or same silibinin treatment as for *APC*$^{min/+}$ mice. Selection of silibinin dose (750 mg/kg body weight) was based on published reports (14, 15, 17, 19, 21). Specifically, silibinin dose is extrapolated from the mice consuming up to 1% (w/w) silibinin in diet that did not show any apparent toxicity, which we have used in several animal studies (14, 15, 17, 19, 21). Mice in all groups received AIN-76A diet and water *ad libitum* throughout, and food consumption and mice body weights were recorded weekly. Animal care and treatments were in accordance with approved protocol and institutional guidelines. Experiment was terminated after 13 wk of treatment period (at 19-wk age) to minimize mortality risk caused by severe anemia and intestinal obstruction, which is more common in *APC*$^{min/+}$ mice at this age (22). Following euthanasia, small intestine and colon were removed, opened longitudinally, and rinsed with saline. Small intestine was divided by length into three equal sections (proximal, middle, and distal segments) and spread onto microscope slides. Polyps on intestinal segments including colon were counted, and their sizes were measured with digital caliper under a dissecting microscope.

Immunohistochemistry staining and quantification. Paraffin-embedded sections (5 μm thick) were deparaffinized and stained using specific antibodies followed by 3,3′-diaminobenzidine as described (14, 18). Primary antibodies used were mouse monoclonal anti–proliferating cell nuclear antigen (PCNA; 1:250 dilution, Dako), HIF-1α (1:100 dilution, Novus), and anti-nestin (1:100 dilution, Santa Cruz), and rabbit polyclonal anti–cleaved caspase-3 (CC3; 1:50 dilution, Cell Signaling), anti–vascular endothelial growth factor-A (VEGF-A; 1:100 dilution, Neomarkers), anti-eNOS (1:100 dilution, Abcam), anti–β-catenin (1:100 dilution, Santa Cruz), anti–cyclin D1 (1:100 dilution, Neomarkers), and anti–cyclooxygenase-2 (COX-2; 1:100 dilution, Cell Signaling). Secondary antibodies used were rabbit anti-mouse IgG (Dako) and goat anti-rabbit IgG (Santa Cruz). Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using Dead End Colorometric TUNEL System (Promega Corp.). PCNA, TUNEL, CC3, nuclear β-catenin, and cyclin D1–positive cells were quantified as described recently (14, 18). eNOS, VEGF, HIF-1α, and COX-2 were quantified by immunoreactivity (represented by intensity of brown staining) and scored as 0 (no staining), +1 (very weak), +2 (weak), +3 (moderate), and +4 (strong) at five randomly selected fields at 400× magnification in each sample. Newly formed nestin-positive microvessels were quantified as mean number of positive vessels at five randomly selected fields at 400× magnification in each sample. All microscopic histologic and immunohistochemical analyses were done by Zeiss Axioskop 2 microscope (Carl Zeiss, Inc.), and photomicrographs were captured by Carl Zeiss AxioCam MRC5 camera.

Western blot analysis. Tissue lysates of distal part of intestinal polyps from control and silibinin groups were analyzed by immunoblotting (14, 18). Densitometric analyses of bands are adjusted with β-actin as loading control.

Assay for prostaglandin E_2 levels. Small intestinal polyps were homogenized in 0.1 mol/L phosphate buffer (pH 7.4), containing 1 mmol/L EDTA and 10 μmol/L indomethacin with polytron-type homogenizer. Tissue extracts were acidified with HCl to pH 2.5, vortexed for 1 min after adding 1 mL of ethyl acetate, and centrifuged at 3,000 × g for 5 min. Organic layer was collected, evaporated under N_2, and stored at −80°C. Each sample was reconstituted in 1 mL enzyme immunoassay buffer (Cayman Chemical) and prostaglandin E_2 (PGE_2) levels measured using ELISA kit.

Mouse cytokine array. Tissue lysates of intestinal polyps from three randomly selected animals per group were applied to a mouse cytokine antibody array (RayBiotech, Inc.) to analyze the expression of various cytokine molecules. Expression of each protein was represented in duplicate on the membrane, which were scanned and quantified by Scion-Image Program, and densitometric data were analyzed using antibody array analysis tool (RayBiotech, Inc.).

Reporter gene assay. Transcriptional activity of β-catenin was measured using TOP/FOPFlash reporter activity assay. HT29 cells were plated to 35% confluency and then cotransfected with 1.2 μg of 8XTOPFLash/FOPFlash (from Dr. Randall Moon) and 250 ng of pRL-CMV for 12 h. Cells were then transfected with control or β-catenin siRNA and 12 h later treated with DMSO or 100 μmol/L silibinin for 48 h. Luciferase activity was measured using Promega's dual luciferase reporter assay system. Final reporter activity was normalized for transfection efficiency using *Renilla* luciferase activity.

Statistical analysis. Statistical analyses were done using SigmaStat software version 3.5 (Jandel Scientific). Quantitative data are presented as mean and SEM. Statistical significance of difference between *APC*$^{min/+}$ control and silibinin treatment groups was determined by unpaired Student's t test, and $P < 0.05$ was considered statistically significant.

Results

Silibinin feeding prevents intestinal tumorigenesis in APC$^{min/+}$ mice. Silibinin treatment of *APC*$^{min/+}$ mice resulted in a strong inhibition in intestinal tumorigenesis in terms of decreased polyp number, size, and appearance in small intestine (Fig. 1A–C). Specifically, control *APC*$^{min/+}$ mice developed 10, 20, and 33 polyps in proximal, middle, and distal portions of small intestine, respectively; however, silibinin feeding for 13 weeks significantly prevented polyps number in these portions of small intestine by 27% ($P < 0.001$), 34% ($P < 0.001$), and 49% ($P < 0.001$), respectively (Fig. 1A). Size distribution analysis of polyps in small intestine

showed differential silibinin efficacy depending on small intestine segment and polyp size. For example, silibinin reduced number of <1 mm size polyps by 30% ($P < 0.05$) in proximal and 32% ($P < 0.001$) in distal but no effect in middle segment; 1 to 2 mm size polyps by 19% ($P < 0.05$) in proximal, 41% ($P < 0.001$) in middle, and 45% ($P < 0.001$) in distal portions; and >2 to 3 mm size polyps by 32% ($P < 0.01$) in proximal, 47% ($P < 0.001$) in middle, and 48% ($P < 0.001$) in distal portions (Fig. 1B). Most prominent silibinin effect was observed on bigger-size polyps (>3 mm), which decreased by 66% ($P < 0.01$) and 88% ($P < 0.001$) in middle and distal portions, respectively (Fig. 1B).

Regarding its chemopreventive efficacy in colon, silibinin significantly decreased (55%, $P < 0.01$) number of colonic polyps in $APC^{min/+}$ mice (Fig. 1A). Silibinin effect was most profound on colonic polyps size, wherein it very strongly

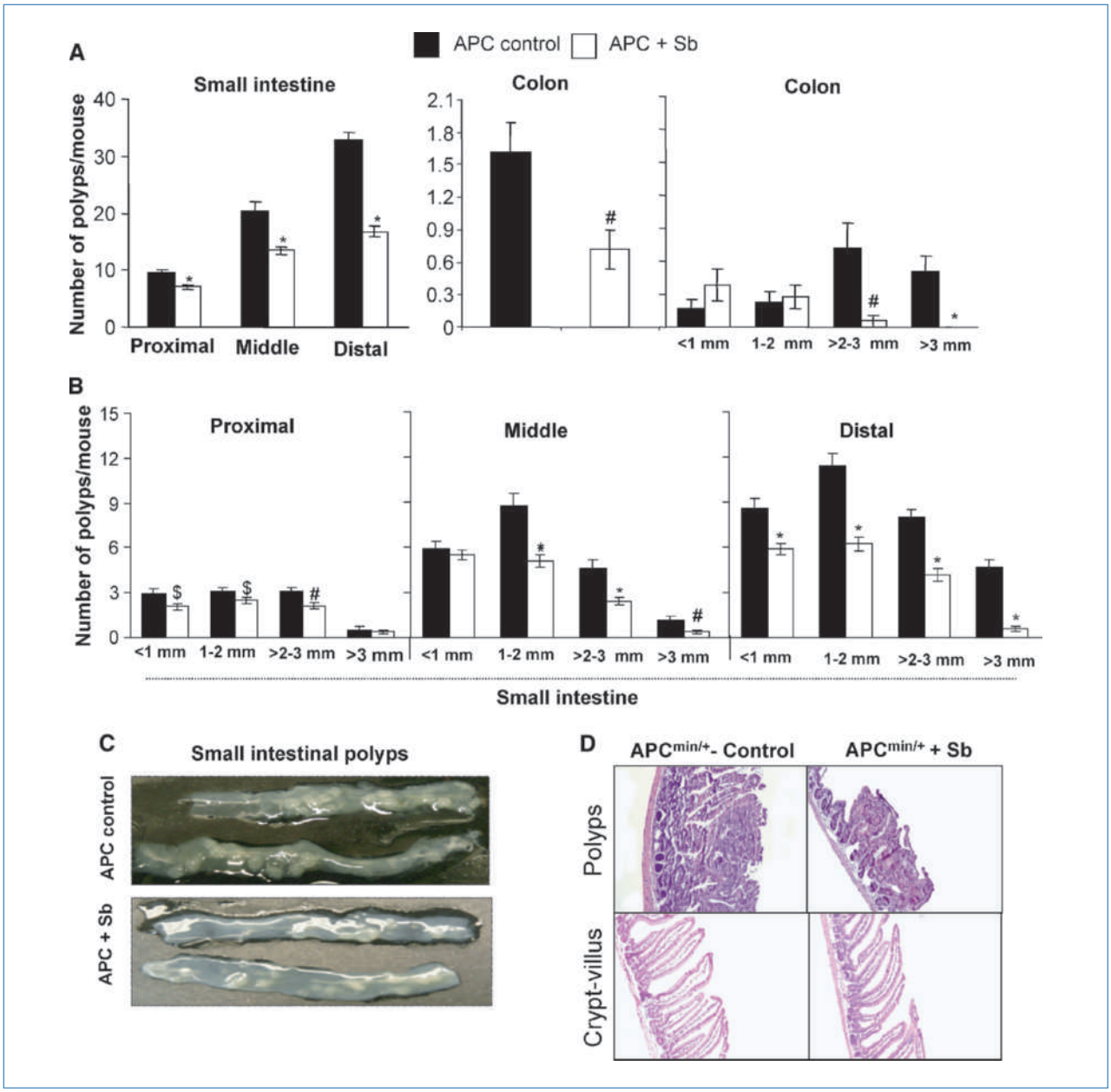

Figure 1. Silibinin feeding prevents spontaneous intestinal polyposis in APC^{min+} mice. At the end of the silibinin efficacy study, results are shown for the number of polyps/mouse in small intestine and colon and polyp size distribution in colon (A); the size distribution of polyps in proximal, middle, and distal portions of small intestine (B); the representative pictures of distal small intestinal polyps (C); and the H&E-stained sections from polyps and normally appearing crypt-villus axis from control and silibinin-treated $APC^{min/+}$ mice (D; 100×). Columns, mean from 18 animals in each group; bars, SEM. $, $P < 0.05$; #, $P < 0.01$; *, $P < 0.001$ versus control. Sb, silibinin.

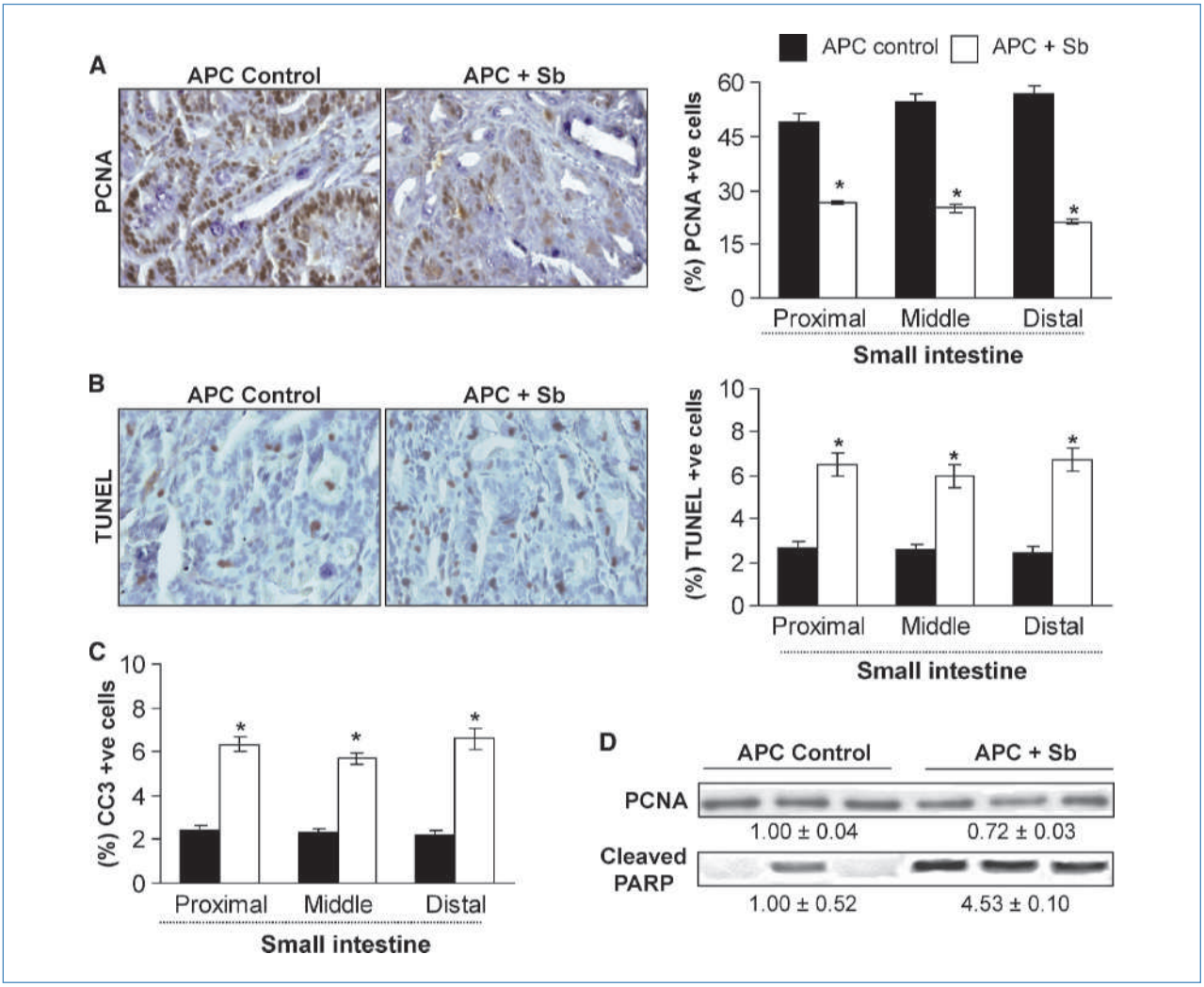

Figure 2. Silibinin feeding reduces proliferation but induces apoptosis selectively in small intestinal polyps of *APC*$^{min/+}$ mice. Small intestinal segments were processed for PCNA, TUNEL, and CC3 staining. Tissue sections from *APC*$^{min/+}$ control and silibinin-treated groups show brown-colored PCNA-positive (A) and TUNEL-positive (B) cells in polyps (400×). Quantitative data for proliferative (A) and apoptotic (B and C) indices were determined as number of PCNA-positive, TUNEL-positive, or CC3-positive cells × 100 / total number of cells, respectively, and represent mean ± SEM of six animals. *, $P < 0.001$ versus control. Polyps from distal portion of small intestine from each group were also analyzed by immunoblotting for PCNA and cleaved PARP levels. Values of band intensity adjusted with β-actin. Sb, silibinin.

arrested the growth of larger-size polyps (>2 mm) than smaller-size (<1 mm and 1-2 mm) polyps (Fig. 1A). Size distribution analysis of colonic polyps showed a strong decrease (92%, $P < 0.001$) in >2 to 3 mm size polyps in silibinin-fed group compared with controls (Fig. 1A). In terms of the incidence, eight mice in control group had >2 to 3 mm size polyps versus only one mouse in silibinin-fed group ($n = 18$; data not shown). More importantly, none of the mice showed >3 mm size polyps in silibinin-fed group accounting for a complete suppression, whereas 8 of 18 mice in control group showed colonic polyps >3 mm (up to 3.7 mm) in size (Fig. 1A). Importantly, observed decrease in larger-size polyps in silibinin-fed group was accompanied with almost 2-fold increase in <1 mm size polyps and a slight increase in 1 to 2 mm size polyps, suggesting that silibinin inhibits the growth and progression of smaller colonic polyps in to the larger ones.

In *APC*$^{min/+}$ mice, all polyps were histologically identified as adenomas (Fig. 1D); however, their number and diameter were significantly reduced in silibinin-fed mice. We did not observe any other histologic changes in crypt-villus axis between control and silibinin-fed *APC*$^{min/+}$ mice (Fig. 1D). In wild-type C57BL/6J mice, both control and silibinin-fed groups did not develop any polyps throughout the intestine, including colon, and showed normal intestinal histology (data not shown). Also, silibinin feeding did not show any considerable change in food consumption and gain in body weight compared with controls (data not shown) during entire treatment, which is consistent with previous studies (14, 15, 17–19).

Collectively, above efficacy study results clearly showed that silibinin feeding strongly prevents number of polyps in small intestine of *APC*$^{min/+}$ mice together with a significant decrease in polyp size. Notably, silibinin more strongly prevented incidence, multiplicity, and burden of colonic polyps. Whereas this effect of silibinin supports its translational potential, it limited our ability to perform mechanistic studies in colonic polyps because of their fewer numbers (an average of 1.6 polyps in control *APC*$^{min/+}$ group and 0.76 polyps in silibinin-fed group) and very small size specifically in silibinin-fed group. Accordingly, we selected only small intestinal polyps for mechanistic studies as reported by others (22, 23).

Silibinin feeding inhibits proliferation and induces apoptosis selectively in small intestinal polyps of* APC$^{min/+}$ *mice. PCNA, TUNEL, and CC3 are widely used markers/players for cell proliferation and apoptosis (24, 25). To assess whether silibinin efficacy is associated with its *in vivo* antiproliferative and proapoptotic effects, all three segments of small intestine were analyzed for PCNA, TUNEL, and CC3 immunostaining. Microscopic examination of tissue sections showed a decrease in PCNA (Fig. 2A) but an increase in TUNEL (Fig. 2B) and CC3-positive cells (immunohistochemical staining data not shown) in polyps from silibinin-fed compared with control *APC*$^{min/+}$ mice. Quantification of PCNA staining showed 46% to 63% ($P < 0.001$) decrease in proliferation indices; however, TUNEL and CC3-positive cells increased by 2.4-fold to 3-fold ($P < 0.001$) in proximal, middle, and distal segments of small intestinal polyps from silibinin-fed mice compared with controls (Fig. 2A–C). These results were further confirmed by immunoblotting (Fig. 2D), wherein densitometric analysis of bands (adjusted with β-actin as loading control) showed 28% ($P < 0.05$) decrease in PCNA expression and 4.5-fold ($P < 0.01$) increase in cleaved poly (ADP-ribose) polymerase (PARP) expression in intestinal polyps from silibinin-treated mice. We did not observe any difference in PCNA, TUNEL, and CC3-positive cells in crypt-villus regions throughout small intestine of control and silibinin-fed *APC*$^{min/+}$ as well as wild-type C57BL/6J mice (data not shown). Together, these results clearly suggest *in vivo* antiproliferative and proapoptotic effects of silibinin selectively in polyps during its chemopreventive efficacy against spontaneous intestinal tumorigenesis in *APC*$^{min/+}$ mice.

Silibinin feeding inhibits angiogenesis selectively in small intestinal polyps of* APC$^{min/+}$ *mice. Neoangiogenesis, formation of new blood vessels in tumor, plays a critical role in malignancy (26). Both hypoxia and VEGF are reported as most predominant and specific factors that stimulate neoangiogenesis. Hypoxia triggers induction of transcription factor HIF (27). Importantly, HIF-1α, which is induced under hypoxic condition, is considered a primary regulator of VEGF expression and angiogenesis (28). Thus, to determine whether silibinin affects polyp angiogenesis, we examined HIF-1α and VEGF expression by immunohistochemistry. Our immunostaining results showed intense immunoreactivity for HIF-1α and VEGF in small intestinal polyps of *APC*$^{min/+}$ mice but strongly decreased levels in silibinin-fed group (Fig. 3A and C). Quantitative data showed that silibinin decreases

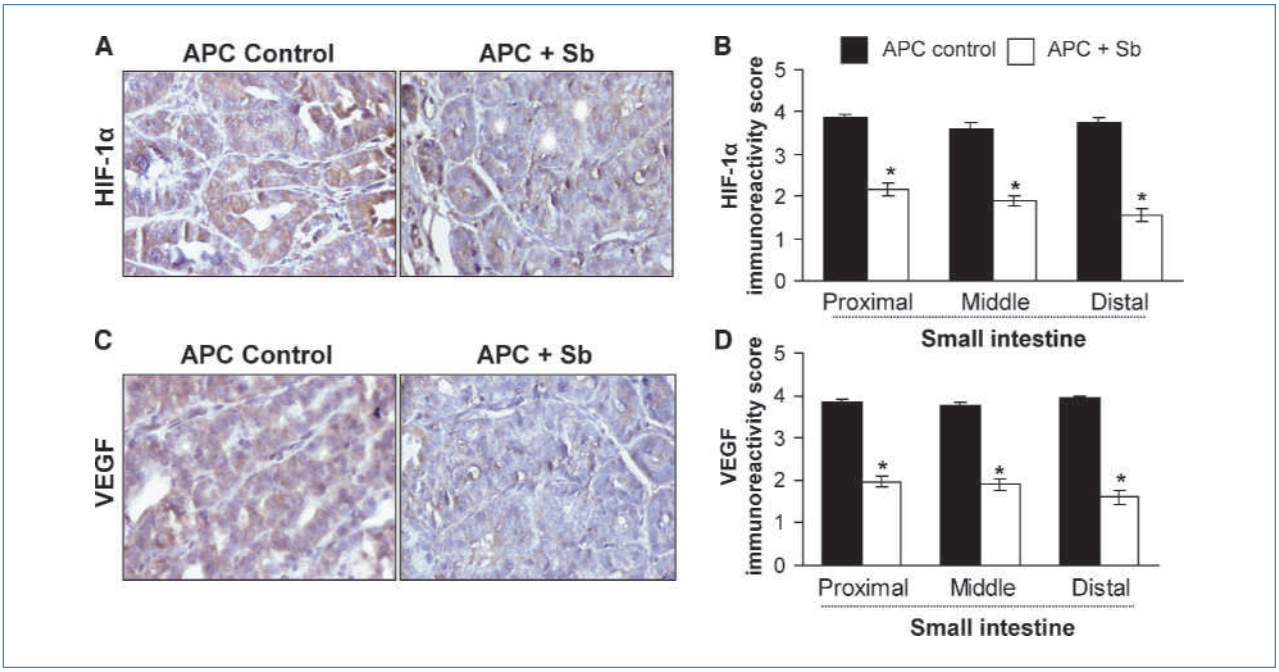

Figure 3. Silibinin feeding inhibits HIF-1α and VEGF expression selectively in small intestinal polyps of *APC*$^{min/+}$ mice. Small intestinal segments were processed for HIF-1α and VEGF staining. Tissue sections from *APC*$^{min/+}$ control and silibinin-treated groups show brown-colored HIF-1α–positive (A) and VEGF-positive (C) cells in polyps (400×). Quantitative data for HIF-1α (B) and VEGF (D) are shown based on their intensity of cytoplasmic brown staining and represent mean ± SEM of six animals. *, $P < 0.001$ versus control; Sb, silibinin.

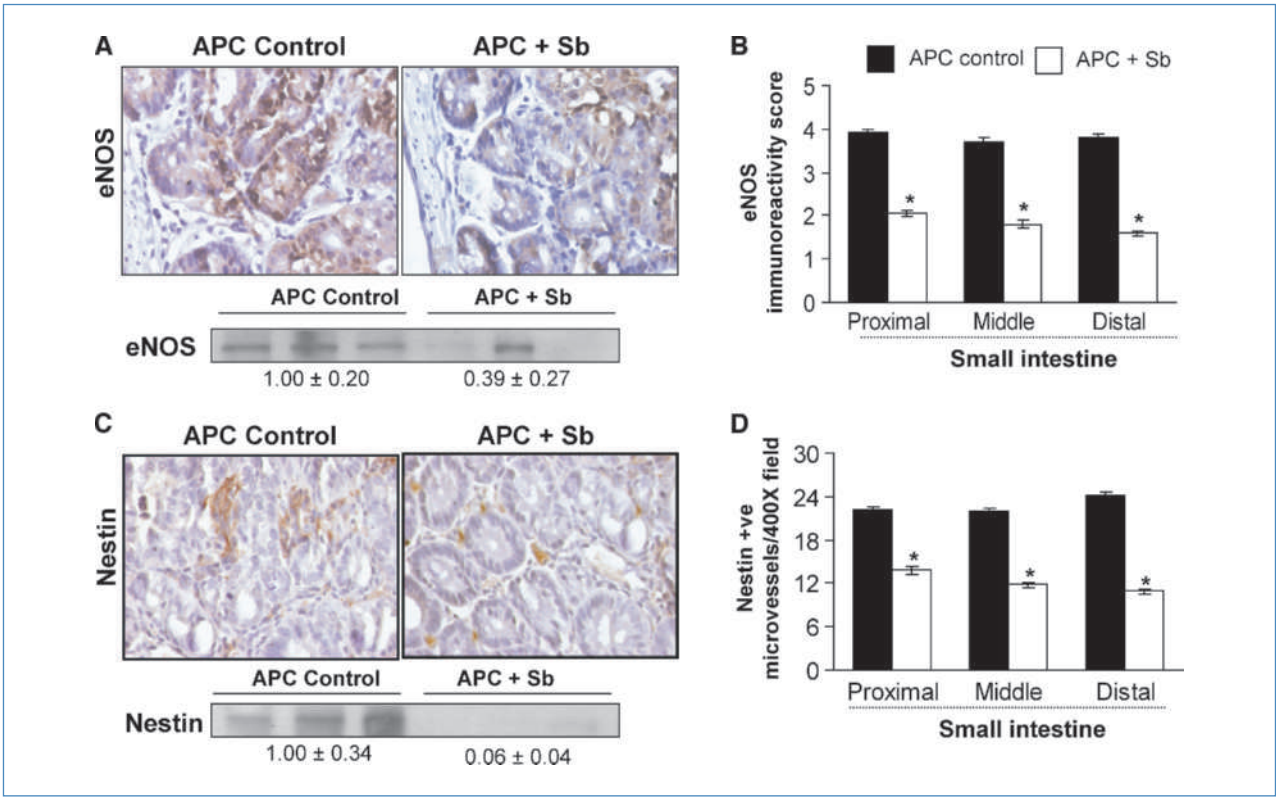

Figure 4. Silibinin feeding inhibits eNOS expression and nestin-positive microvessels selectively in small intestinal polyps of *APC*$^{min/+}$ mice. Small intestinal segments were processed for eNOS and nestin staining. Tissue sections from *APC*$^{min/+}$ control and silibinin-treated groups show brown-colored eNOS-positive (A) and nestin-positive (C) cells in polyps (400×). Quantification of eNOS was done based on its intensity of cytoplasmic brown staining (B), and microvessel numbers were quantified by measuring nestin-positive cells in five randomly selected fields at 400× magnification (D). Columns, mean of six animals; bars, SEM. *, $P < 0.001$ versus control. Polyps from distal portion of small intestine from each group were also analyzed by immunoblotting for eNOS and nestin levels. Values of band intensity adjusted with β-actin. Sb, silibinin.

HIF-1α and VEGF immunoreactivity scores in polyps by 44% ($P < 0.001$) and 49% ($P < 0.001$) in proximal, 47% ($P < 0.001$) and 49% ($P < 0.001$) in middle, and 58% ($P < 0.001$) and 59% ($P < 0.001$) in distal portions of small intestine (Fig. 3B and D).

Overexpression of eNOS, which catalyzes the production of nitric oxide (a key regulator of angiogenesis), is reported during carcinogenesis, although it is constitutively expressed in vascular endothelial cells (29); VEGF also activates eNOS expression (30). Nestin, a class VI intermediate filament protein and neuroepithelial stem cell marker, is expressed on newly formed microvessels (31) and is a novel angiogenic marker for newly formed blood vessels in CRC (32). To gain additional mechanistic insights regarding antiangiogenic effect of silibinin, expression of these two important angiogenic molecules, eNOS and nestin, was also examined by immunohistochemical staining. We observed intense immunoreactivity for eNOS in polyps from control *APC*$^{min/+}$ mice but strongly decreased expression in silibinin-fed group (Fig. 4A). Quantification showed that silibinin reduces eNOS immunoreactivity score in polyps from proximal, middle, and distal regions of small intestine by 48% ($P < 0.001$), 51% ($P < 0.001$), and 58% ($P < 0.001$), respectively (Fig. 4B). Regarding nestin, microscopic examination showed numerous nestin-positive microvessels in polyps from control *APC*$^{min/+}$ mice but a significant reduction in silibinin-fed group (Fig. 4C). Quantification of nestin-positive cells showed that silibinin also decreases nestin-positive microvessels by 37% ($P < 0.001$), 46% ($P < 0.001$), and 55% ($P < 0.001$) in the polyps from proximal, middle, and distal segments of small intestine, respectively (Fig. 4D). These results were further confirmed by immunoblotting, which showed that silibinin feeding decreases eNOS and nestin expression by 61% ($P < 0.05$) and 94% ($P < 0.01$) in intestinal polyps, respectively (Fig. 4A and C).

These results suggest that silibinin possibly targets neoangiogenesis through downregulation of HIF-1α, VEGF, eNOS, and nestin expression for its angiopreventive effects during spontaneous intestinal tumorigenesis. This antiangiogenic effect of silibinin was specific and limited to polyps, because we did not observe any considerable changes in the expression of these angiogenic factors in crypt-villus regions in small intestine of control and silibinin-treated *APC*$^{min/+}$ or wild-type C57BL/6J mice (data not shown). In these controls, we did not observe any changes in other molecular analyses described below, and therefore, these results are not mentioned hereafter.

Silibinin feeding modulates β-catenin levels and transcriptional activity and cyclin D1 expression. Alterations in β-catenin pathway due to loss of *APC* function is implicated in CRC initiation and progression (33). To assess silibinin effect on β-catenin pathway, expression of β-catenin and one of its downstream transcriptional targets, cyclin D1, was analyzed by immunohistochemistry, which showed strong staining for both nuclear β-catenin and cyclin D1–positive cells in polyps from $APC^{min/+}$ mice control but their decreased levels following silibinin treatment (immunohistochemical staining data not shown). Quantification of the staining showed that silibinin decreases nuclear β-catenin–positive cells by 40% ($P < 0.001$), 43% ($P < 0.001$), and 53% ($P < 0.001$; Fig. 5A) and cyclin D1–positive cells by 52% ($P < 0.001$), 53% ($P < 0.001$) and 54% ($P < 0.001$; Fig. 5B) in polyps from proximal, middle, and distal portions of small intestine, respectively. Immunoblot analysis further confirmed these results, showing that silibinin treatment decreases β-catenin and cyclin D1 expression by 64% ($P < 0.05$) and 44% ($P < 0.05$) in intestinal polyps, respectively (Fig. 5A and B). To further substantiate our findings that silibinin targets β-catenin in its efficacy, we also studied silibinin effect on β-catenin–mediated transcriptional activity using HT29 cells, wherein 100 μmol/L silibinin concentration reduced TOPFlash reporter activity by 45% compared with DMSO controls; however, silibinin did not show any effect in decreasing reporter activity when the expression of β-catenin was reduced by siRNA transfection of HT29 cells (Fig. 5C).

Silibinin feeding decreases COX-2 expression and PGE_2 levels in small intestinal polyps of $APC^{min/+}$ mice. A positive correlation between COX-2 and β-catenin pathway is reported during CRC development (34). COX-2 is a key

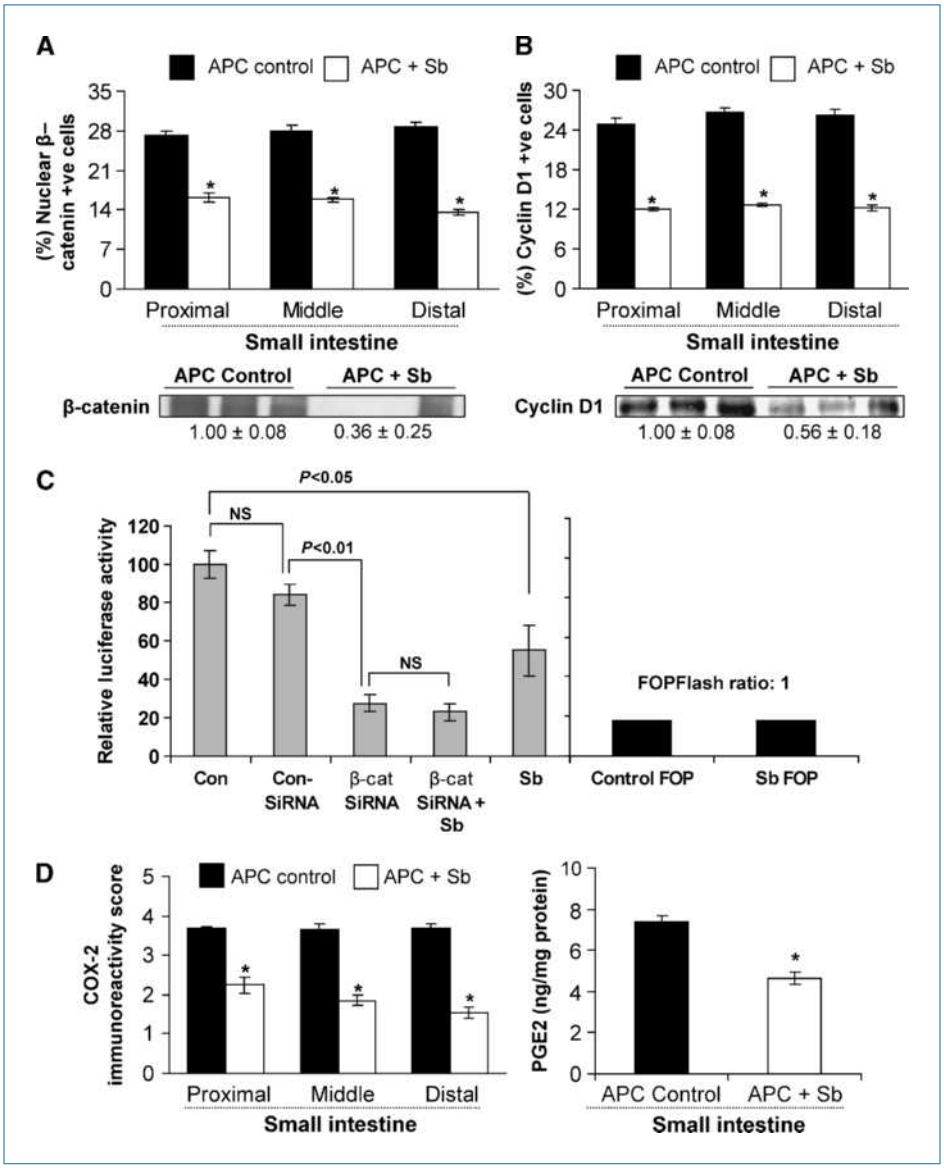

Figure 5. Silibinin feeding decreases β-catenin, cyclin D1, and COX-2 expression and PGE_2 levels selectively in small intestinal polyps of $APC^{min/+}$ mice and inhibits β-catenin–mediated transcriptional activity *in vitro*. Small intestinal segments or polyps were processed for immunohistochemical analyses or estimation for nuclear β-catenin (A), cyclin D1 (B), and COX-2 and PGE_2 levels (D), as detailed in Materials and Methods. Columns, mean of six animals; bars, SEM. *, $P < 0.001$ versus control. Polyps from distal portion of small intestine from each group were also analyzed by immunoblotting for β-catenin and cyclin D1 levels. Values of band intensity adjusted with β-actin. C, TOP/FOPFlash reporter activity was measured using dual luciferase assay kit from Promega, as described in detail in Materials and Methods. Columns, mean of three independent observations; bars, SEM. Sb, silibinin.

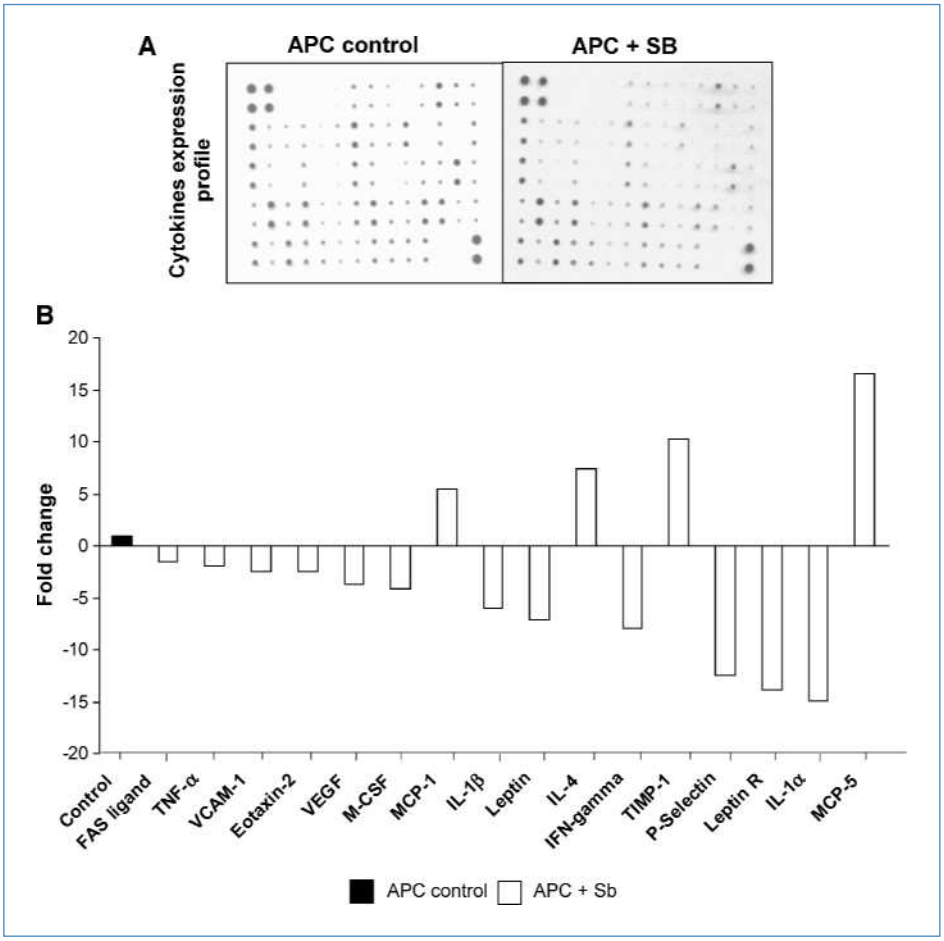

Figure 6. Silibinin feeding modulates cytokine profile in intestinal polyps of *APC*$^{min/+}$ mice. Lysates from small intestinal polyps were analyzed for various cytokines by cytokine antibody array kit. A, representative cytokine array blots from control and silibinin-treated *APC*$^{min/+}$ mice polyps. B, densitometric analysis data of cytokine levels from three samples in each group are shown as fold changes by silibinin treatment to that of control.

enzyme in biosynthetic pathway by which arachidonic acid is converted into five structurally related prostaglandins, including PGE_2 (35). Because both COX-2 and PGE_2 are involved in CRC development (36), we next studied silibinin effect on COX-2 expression by immunohistochemistry and PGE_2 levels by ELISA. Our results showed strong COX-2 immunoreactivity in small intestinal polyps of *APC*$^{min/+}$ mice, which decreased strongly by silibinin treatment (immunohistochemical staining data not shown). Quantification of immunostained cells showed that silibinin decreases COX-2 immunoreactivity by 39% ($P < 0.001$) in proximal, 47% ($P < 0.001$) in middle, and 57% ($P < 0.001$) in distal portions of small intestinal polyps (Fig. 5D). PGE_2 levels in small intestinal polyps were elevated (8-fold) compared with those in wild-type small intestinal tissue (data not shown). Silibinin treatment decreased PGE_2 levels in small intestinal polyps by 37% ($P < 0.001$; Fig. 5D). These results indicate that COX-2 could be a potential molecular target for the chemopreventive effects of silibinin against polyp growth.

Silibinin treatment modulates cytokine profile in intestinal polyps of* APC$^{min/+}$ *mice. Cytokines are diverse family of secreted proteins and act as immunomodulating agents (37). To examine silibinin effect on various cytokines, we used inflammatory cytokine antibody array to screen intestinal polyps from both control and silibinin-fed *APC*$^{min/+}$ mice (Fig. 6A). We found that among 62 cytokines tested, silibinin decreased the expression levels of 12 cytokines and increased the expression levels of four cytokines in small intestinal polyps compared with controls (Fig. 6B). Specifically, silibinin decreased the levels of Fas ligand (1.5-fold), tumor necrosis factor-α (TNF-α; 1.9-fold), vascular endothelial adhesion molecule-1 (VCAM-1; 2.4-fold), eotoxin-2 (2.4-fold), VEGF (3.7-fold), macrophage colony-stimulating factor (M-CSF; 4.1-fold), interleukin-1α (IL-1α; 14.9-fold), IL-β (6.0-fold), leptin (7.1-fold), IFN-γ (7.9-fold), P-selectin (12.5-fold), and leptin receptor (13.9-fold). However, the levels of monocyte chemoattractant protein-1 (MCP-1; 5.4-fold), MCP-5 (16.6-fold), IL-4 (7.3-fold), and tissue inhibitor of metalloproteinase-1 (TIMP-1; 10.3-fold) were increased in intestinal polyps from silibinin-fed compared with control *APC*$^{min/+}$ mice. These results indicate that, together with its angiopreventive effects, silibinin might also act as an immunomodulating agent, which could further strengthen its chemopreventive efficacy.

Discussion

This is the first study showing chemopreventive efficacy of long-term silibinin feeding on spontaneous intestinal tumorigenesis in $APC^{min/+}$ mice, a genetically predisposed animal model of human FAP. The key findings of this study are as follows: (*a*) silibinin significantly reduced the number as well as the growth of intestinal polyps and prominently decreased the incidence of larger colonic polyps in $APC^{min/+}$ mice without any adverse health effects; (*b*) chemopreventive effect of silibinin was associated with a decrease in proliferation and an increase in apoptosis indices in polyps; (*c*) silibinin inhibited neoangiogenesis as evidenced by reduced HIF-1α, VEGF, and eNOS immunostaining and a decrease in nestin-positive microvessels in polyps; and (*d*) silibinin decreased β-catenin levels and COX-2 pathways and modulated various cytokines in intestinal polyps in favor of its chemopreventive efficacy. These results, together with our earlier findings in CRC xenograft (18) and AOM-induced ACF (19) models, strongly support the chemopreventive efficacy of silibinin in three different preclinical animal models of CRC, highlighting their translational significance against human CRC.

Chemopreventive effect of silibinin observed in the present study was accompanied by its antiproliferative, proapoptotic, and angiopreventive mechanisms. Excessive cell proliferation and insufficient apoptosis are often associated with CRC development and progression (38), and the agents modulating them in neoplastic cells have immense potential in CRC chemoprevention and therapeutic intervention (1). In our study, silibinin inhibited cell proliferation and induced apoptosis as evidenced by decreased PCNA-positive and increased CC3-positive cells and increased cleaved PARP levels, respectively, in polyps from silibinin-fed animals. This antiproliferative and proapoptotic efficacy of silibinin in small intestinal polyps of $APC^{min/+}$ mice could be the underlying mechanisms for the strong decrease in the number as well as the size of polyps. Importantly, these effects of silibinin were specific and limited to polyps and not observed in normal crypt-villus regions in small intestine of control and silibinin-treated $APC^{min/+}$, as well as in wild-type C57BL/6J mice.

One of the most important observations in present study was that silibinin strikingly decreased the number of polyps, which were larger in size (>3 mm in middle and distal portions of small intestine and 2–3 mm and >3 mm in colon), suggesting that silibinin exerts strong antiangiogenic activity that possibly inhibited the progression of smaller polyps into larger ones. Neoangiogenesis is triggered when tumors grow beyond a minimal size (~2 mm) and agents that inhibit angiogenesis have significant implications for their clinical uses (26). Overexpression of HIF-1α, VEGF, and eNOS is well implicated in stimulating neoangiogenesis (39). Hypoxia and VEGF signaling play a key role in "angiogenic switch" (27, 30), and HIF-1α induction under hypoxic conditions directly activates a number of proangiogenic factors, including VEGF (28). Positive correlations between HIF-1α and VEGF expression, and VEGF and eNOS have also been documented (40). Overexpression of eNOS is reported during both carcinogenesis and neoangiogenesis, although it is constitutively expressed in endothelial cells (29). Consistent with these reports, overexpression of HIF-1α, VEGF, and eNOS and increased newly formed microvessel density were observed in intestinal polyps of control $APC^{min/+}$ mice, which were significantly decreased by silibinin treatment. Angiopreventive effect of silibinin observed in present study corroborates with previous reports showing that silibinin inhibits angiogenesis in various animal models of carcinogenesis (14, 15, 17, 18). Collectively, these antiangiogenic mechanisms of silibinin possibly played an important role in the observed suppression of vascular growth of polyps in $APC^{min/+}$ mice.

Aberrant β-catenin pathway is involved in CRC development (41), and a direct correlation between β-catenin signaling and regulation of angiogenesis is shown in CRC (33). In the present study, $APC^{min/+}$ mice showed increased level of β-catenin together with increased expression of cyclin D1, COX-2, and PGE_2 in polyps, which were significantly decreased by silibinin treatment. Overexpression of cyclin D1, a critical oncogene, is directly associated with increased proliferative index in CRC and results in a more aggressive cancer phenotype (42). Thus, downregulation of β-catenin and cyclin D 1 in polyps from silibinin-treated $APC^{min/+}$ mice also support their role in antiproliferative mechanism of silibinin against CRC. Because overexpression of COX-2 and increased PGE_2 levels induce endothelial cell proliferation by releasing various angiogenic factors (35, 36), the inhibition of COX-2 and PGE_2 by silibinin could be an additional mechanism for its angiopreventive effects. Moreover, VEGF and HIF-1α are also downstream targets of β-catenin (43, 44), indicating that downregulation of β-catenin signaling by silibinin could be an important mechanism for its antiproliferative as well as antiangiogenic effect in intestinal polyps of $APC^{min/+}$ mice.

Various cytokines are also associated with growth and development of CRC, which could be modulated by COX-2, PGE_2, and proangiogenic factors; some of them are also regulated by β-catenin pathway (45, 46). Tumor-promoting roles of Fas ligand, TNF-α, VCAM, eotaxin, M-CSF, leptin and its receptor, IFN-γ, IL-1α, IL-β, and P-selectin during cancer development are well documented (37). Therefore, we also examined the silibinin effect on the cytokine profile in polyps using cytokine array analysis, which showed that silibinin decreases the levels of all above-mentioned tumor-promoting cytokines in polyps of $APC^{min/+}$ mice. Conversely, silibinin increased MCP-1 and MCP-5 factors that activate tumoricidal activity of macrophages *in vivo*. TIMP-1 and IL-4 are known antiinflammatory cytokines (47–49), which were also increased by silibinin treatment. Together, these results suggest that immunomodulating effects of silibinin could be one of its chemopreventive mechanisms; however, additional studies are needed in the future to support it.

In summary, our results clearly show *in vivo* antiproliferative, proapoptotic, antiangiogenic, and antiinflammatory effects of silibinin in polyps, which collectively contribute to its strong chemopreventive efficacy against spontaneous intestinal tumorigenesis in $APC^{min/+}$ mice. Together with our previous reports on silibinin efficacy against CRC in

other preclinical models, present findings underscore the possibility that silibinin would be an effective agent in CRC prevention trials for patients with FAP.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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