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Loss of free fatty acid receptor 2 enhances colonic adenoma development and reduces the chemopreventive effects of black raspberries in Apc$^{Min/+}$ mice

Pan Pan, Chad W.Skaer, Hsin-Tzu Wang, Kiyoko Oshima[1], Yi-Wen Huang[2], Jianhua Yu[3], Jianying Zhang[4], Martha M.Yearsley[5], Kimberle A.Agle, William R.Drobyski, Xiao Chen and Li-Shu Wang*

Division of Hematology and Oncology, Department of Medicine, [1]Department of Pathology and [2]Department of Obstetrics and Gynecology, Medical College of Wisconsin, Milwaukee, WI, USA, [3]Division of Hematology, Department of Internal Medicine, College of Medicine, [4]Center for Biostatistics and [5]Department of Pathology, The Ohio State University, Columbus, OH, USA.

*To whom correspondence should be addressed. Tel/Fax: +414 955 2827; Email: liswang@mcw.edu

Abstract

We previously showed that black raspberries (BRBs) have beneficial effects in human colorectal cancer and a mouse model of colorectal cancer (Apc$^{Min/+}$). The current study investigated the role of free fatty acid receptor 2 (FFAR2) in colon carcinogenesis and whether the FFAR2 signaling pathway contributes to BRB-mediated chemoprevention in mice. FFAR2 (also named GPR43) is a member of the G-protein-coupled receptor family that is expressed in leukocytes and colon. Apc$^{Min/+}$ and Apc$^{Min/+}$-FFAR2$^{-/-}$ mice were given a control diet or the control diet supplemented with 5% BRBs for 8 weeks. FFAR2 deficiency promoted colonic polyp development, with 100% incidence and increased polyp number and size. The Apc$^{Min/+}$ mice developed colonic tubular adenoma, whereas the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice developed colonic tubular adenoma with high-grade dysplasia. FFAR2 deficiency also enhanced the cAMP-PKA-CREB-HDAC pathway, downstream of FFAR2 signaling, and increased activation of the Wnt pathway, and raised the percentage of GR-1^{+} neutrophils in colonic lamina propria (LP) and increased infiltration of GR-1^{+} neutrophils into colonic polyps. BRBs suppressed colonic polyp development and inhibited the cAMP-PKA-CREB-HDAC and Wnt pathways in the Apc$^{Min/+}$ mice but not the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice. They also increased the percentage of GR-1^{+} neutrophils and cytokine secretion in colonic LP and decreased the infiltration of GR-1^{+} neutrophils and IL-1β expression in colon polyps of Apc$^{Min/+}$ mice but not Apc$^{Min/+}$-FFAR2$^{-/-}$ mice. These results suggest that loss of FFAR2 drives colon tumorigenesis and that BRBs require functional FFAR2 to be chemopreventive. BRBs have the potential to modulate the host immune system, thereby enhancing the antitumor immune microenvironment.

Introduction

Colorectal cancer remains the second leading cause of cancer death in both men and women in the USA (1). It is estimated that 134490 new cases and 49190 deaths will be attributable to the disease in the USA in 2016, accounting for ~8.3% of all cancer deaths (1). Exposure to environmental chemical mutagens and carcinogens as well as to certain dietary factors contributes to the development of colorectal cancer (2). Due to the concern that a diet high in processed red meat might increase

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Abbreviations

BRBs	black raspberries
cAMP	cyclic adenosine monophosphate
CREB	cAMP response element binding protein
DSS	dextran sodium sulfate
FFAR2	free fatty acid receptor 2
HDACs	histone deacetylases
IHC	immunohistochemistry
LP	lamina propria
PKA	protein kinase A
SCFAs	short-chain fatty acids

colorectal cancer risk (3), the World Health Organization (WHO) recommended reduced consumption of red and processed meat (4). In contrast, a less understood, but widely recognized, negative association between dietary fiber and colorectal cancer has been reported (4).

Dietary fiber can be fermented by gut microbiota into short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate (5). SCFAs are directly absorbed by human colonic epithelial cells as a source of energy (5). In addition, they modulate various biological processes, such as electrolyte and water absorption, in the gastrointestinal (GI) tract by activating G-protein-coupled receptors (GPCRs) and inhibiting histone deacetylases (HDACs) (6). Free fatty acid receptor 2 (FFAR2, also named GPR43) is a member of the GPCR family and is expressed in neutrophils, monocytes (7), and colon epithelial cells (8,9). Activated FFAR2 couples to the $G\alpha_{i/o}$ pathway and inhibits adenylyl cyclase, resulting in lower concentrations of cyclic adenosine monophosphate (cAMP) (10,11). Suppressed adenylyl cyclase signaling then inhibits protein kinase A (PKA) and its substrate, cAMP response element binding protein (CREB), which leads to suppression of HDACs (12). Thus, SCFAs function as competitive inhibitors of class I HDACs (HDAC1, 2, 3 and 8) and class IIa HDACs (HDAC4, 5, 7 and 9) (13,14). SCFAs also can serve as anti-tumor agents to induce cell differentiation, growth arrest and apoptosis in colon cancer cells (15,16). Also, diets deficient or low in fiber enhance the development of dextran sodium sulfate (DSS)-induced ulcerative colitis, while a high-fiber diet or acetate protects against colitis by activating FFAR2 (17,18). Collectively, FFAR2 deficiency promotes DSS-induced colonic inflammation (18,19), suggesting that the FFAR2 signaling pathway is a negative regulator of inflammation.

Black raspberries (BRBs) contain abundant soluble fiber that colonic bacteria can ferment into SCFAs (20). Our previous studies have shown that BRBs protect against colon cancer in humans (21–23) and $Apc^{Min/+}$ mice (24). Therefore, the current study further investigated whether the FFAR2 signaling pathway contributes to colon carcinogenesis and studied the mechanisms underlying BRBs' protective effects. Our findings demonstrate a tumor suppressive role for FFAR2 in colon carcinogenesis and show that activation of the receptor is an indispensable mechanism of BRB-mediated chemoprevention. BRBs also have the potential to modulate the host immune system, which may enhance the antitumor immune microenvironment.

Materials and methods

Human specimens

Forty-two normal colon specimens from healthy donors, as well as 18 tubular adenomas, 17 advanced adenomas and 29 colorectal adenocarcinomas were obtained from the Cooperative Human Tissue Network (CHTN). The specimens were obtained and used in accordance with the dictates of the Institutional Review Board at the Medical College of Wisconsin (Milwaukee, WI).

Animals

All protocols were carried out in accordance with institutional guidelines for animal care dictated by the Medical College of Wisconsin Animal Care and Use Committee. Eight-week-old breeding pairs of $Apc^{Min/+}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Eight-week-old breeding pairs of FFAR2 heterozygous ($FFAR2^{+/-}$) mice were purchased from Deltagen, Inc. (San Mateo, CA). $Apc^{Min/+}$-$FFAR2^{-/-}$ mice were generated by first breeding the $Apc^{Min/+}$ mice with $FFAR2^{-/-}$ mice to generate $Apc^{Min/+}$-$FFAR2^{+/-}$ mice, and then breeding the $Apc^{Min/+}$-$FFAR2^{+/-}$ mice with $FFAR2^{-/-}$ mice.

Diets

The control diet was the AIN-76A diet (Dyets Inc., Bethlehem, PA). BRB powder was purchased from Berrihealth (Corvallis, OR) and stored at 4°C in vacuum-sealed plastic bags at the Medical College of Wisconsin.

Animal experiments

Four- to six-week-old $Apc^{Min/+}$ and $Apc^{Min/+}$-$FFAR2^{-/-}$ mice were fed either the control diet (Ctrl) or the control diet supplemented with 5% BRBs for 8 weeks. After the mice were euthanized by CO_2 asphyxiation, the number and size of their colon polyps were determined. The colons from these mice were then collected, fixed in formalin and embedded in paraffin (FFPE). The tissue sections were stained with hematoxylin and eosin (H&E) and examined by our pathologists.

Immunohistochemistry and computer-assisted image analysis

FFPE colon tissue blocks were cut into 4-μm sections and immunohistochemistry (IHC) was performed as previously described (24). A Dako Autostainer was used to stain the slides with primary antibodies to GPR43 (1:100, ab124272), PKA (phospho S99; 1:1000, ab32390), CREB (phospho S133; 1:500, ab32096), DKK3 (1:500, ab187532), β-catenin (1:500, ab32572), cMyc (1:50, ab32072), GR-1 (1:50, ab25377) and IL-1β (1:400, ab2105), all from Abcam (Cambridge, MA). Antibody to cAMP (1:250, LS-C121425) was obtained from LifeSpan BioSciences (Seattle, WA), antibody to HDAC2 (1:100, sc-7899) from Santa Cruz Biotechnology (Dallas, Texas), antibodies to HDAC4 (1:800, NBP2-22151) and SOX17 (1:1000, NBP2-24568) from Novus Biologicals (Littleton, CO) and antibodies to acetyl-Histone H3 (Lys9; 1:8000, 9649) and Ki67 (1:300, 12202) from Cell Signaling Technology (Danvers, MA). Stained slides were photographed at ×20 magnification, and positive staining only in the adenoma area was quantified as previously described (24).

Colonic lamina propria preparation

Lamina propria (LP) was prepared using a Dissociation Kit (130-097-410) from Miltenyi Biotec (San Diego, CA) according to the manufacturer's instructions. An independent cohort of $Apc^{Min/+}$ and $Apc^{Min/+}$-$FFAR2^{-/-}$ mice that had received either the control diet or the BRB diet were euthanized by CO_2. Colonic specimens were collected, feces were removed, and all the tissues were opened longitudinally and cut into short segments. All the segments were shaken in DMEM medium to remove remaining debris, transferred to predigestion solution (HBSS without calcium, 5 mM EDTA, 10 mM HEPES, 5% FBS, and 1 mM DTT), and incubated at 37°C for 20 min on a horizontal shaker at 200 rpm/min. After the incubation step above was repeated one more time with fresh predigestion solution, all the segments were transferred to HBSS without calcium and incubated at 37°C for 20 min on a horizontal shaker at 200 rpm/min. Then they were transferred to a gentleMACs C tube containing 2.5 mL of preheated (15 min at 37°C) Digestion Solution and Enzyme Mix (100 μL Enzyme D, 50 μL Enzyme R and 12.5 μL Enzyme A) and incubated at 37°C for 30 min on a horizontal shaker at 200 rpm/min. The gentleMACs C tube was then placed into the gentle MACs dissociator, and the m_intestine_01 program was run to further break down the tissue segments. Tissues in the gentleMACs C were collected and centrifuged to form a cell pellet. Supernatant was removed, and the pellet was resuspended in DMEM for flow cytometry.

Flow cytometry

LP samples were prepared for surface staining with mouse-specific CD45 and GR-1 antibodies (BD Biosciences, Franklin Lakes, NJ). The stained samples were then fixed and permeabilized by Cytofix/Cytoperm (BD Biosciences, Franklin Lakes, NJ) for staining with mouse-specific antibodies to the intracellular cytokines IL-1, IL-6 and TNF. The samples were analyzed on a LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ), and FlowJo software (Tree Star, Ashland, OR) was used to analyze the data.

Statistical analysis

Using *t*-tests, we compared FFAR2 expression between normal versus adenoma versus adenocarcinoma colon tissue samples. Two-way ANOVA analysis with post hoc tests was performed, using SigmaPlot (Systat Software, San Jose, CA), to determine the changes between genotypes (Apc$^{Min/+}$ vs. Apc$^{Min/+}$-FFAR2$^{-/-}$ mice) and the effects (control vs. berry diets). A $P < 0.05$ was considered statistically significant.

Results

FFAR2 protein expression in tubular adenoma, advanced adenoma and adenocarcinoma was lower than in normal human colon

Using IHC, we measured FFAR2 protein expression in paraffin-embedded tissues of true normal human colon (N, $n = 42$), tubular adenoma (TA, $n = 18$), advanced adenoma (AA, $n = 17$), and adenocarcinoma (AC, $n = 29$; Figure 1A). FFAR2 protein expression was significantly lower in tubular adenoma, advanced adenoma, and adenocarcinoma. Our data suggest a negative association between FFAR2 expression and colon cancer progression.

FFAR2 deficiency enhanced the development of colon adenoma in the Apc$^{Min/+}$ mice, and BRBs suppressed colon adenoma in the Apc$^{Min/+}$ mice but not the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice

Colons from 12- to 14-week-old Apc$^{Min/+}$ (A) and Apc$^{Min/+}$-FFAR2$^{-/-}$ (AF) male (M) and female (F) mice were examined for polyp incidence, number, and size. Consistent with previous findings (24), only 35% (7/20) of the Apc$^{Min/+}$ mice developed colon polyps (Figure 1B), whereas the incidence was 100% in the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice (Figure 1B). The Apc$^{Min/+}$-FFAR2$^{-/-}$ mice also developed significantly more and larger polyps than the Apc$^{Min/+}$ mice (Figure 1B). Histological examination of colon tissues indicated that all the polyps in the Apc$^{Min/+}$ mice were well-differentiated tubular adenomas, whereas most of the tubular adenomas in the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice exhibited high-grade dysplasia (Figure 1C). To determine if BRBs' ability to prevent the development of colon adenomas is FFAR2-dependent, we fed 4- to 6-week-old male and female Apc$^{Min/+}$ and Apc$^{Min/+}$-FFAR2$^{-/-}$ mice either a control AIN-76A diet or the AIN-76A diet supplemented with 5% BRBs for 8 weeks. In both genders, BRBs significantly decreased the number and size of the colon polyps in the Apc$^{Min/+}$ mice but not the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice (Figure 1B), suggesting that FFAR2 plays an indispensable role in BRB-mediated suppression of colonic adenoma development. FFAR2 deficiency also significantly enhanced adenoma development in the small intestine, and significantly decreased the number and size of the intestinal polyps in the Apc$^{Min/+}$ mice (Supplementary Figure 1).

FFAR2 deficiency enhanced the cAMP-PKA-CREB-HDAC pathway, and BRBs suppressed this pathway in the Apc$^{Min/+}$ mice but not the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice

We observed that FFAR2 expression was higher in the BRB-fed Apc$^{Min/+}$ mice (Figure 2). Given that BRBs contain abundant dietary fiber, which gut bacteria can ferment into SCFAs that activate FFAR2, our results provide direct evidence that feeding BRBs activate FFAR2 signaling. Activated FFAR2 couples to the G$\alpha_{i/o}$ pathway to suppress adenylyl cyclase, resulting in inhibition of the cAMP-PKA-CREB pathway (10,11). Loss of FFAR2 enhanced activation of the cAMP-PKA-CREB pathway in the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice compared with the Apc$^{Min/+}$ mice fed the

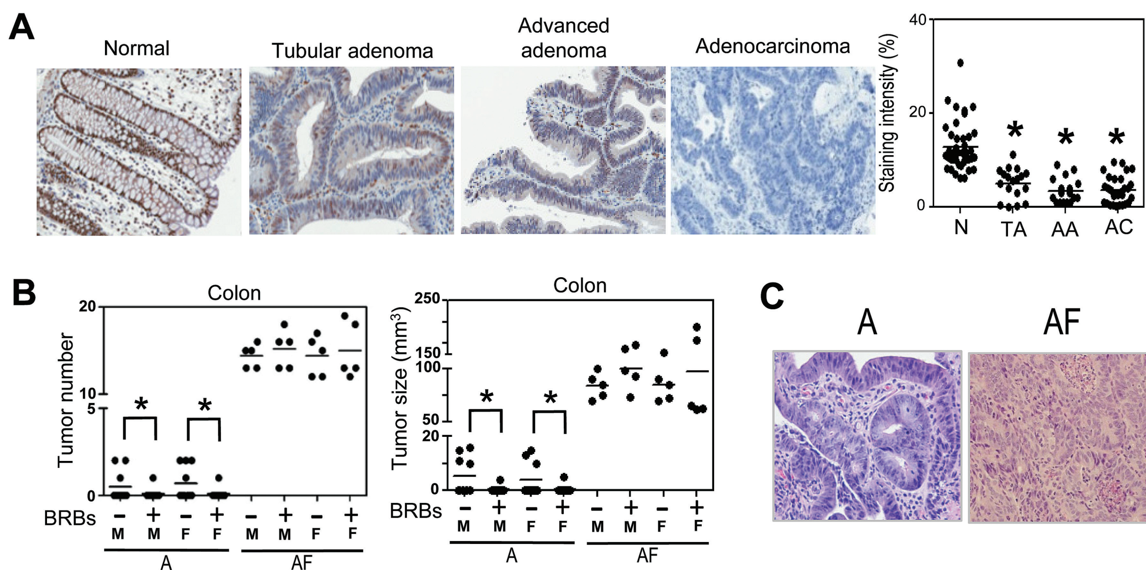

Figure 1. FFAR2 protein expression was lower in precancerous and cancerous human colon tissue, and FFAR2 deficiency enhanced colon adenoma formation in the Apc$^{Min/+}$ mice, and BRBs suppressed colon adenoma in the Apc$^{Min/+}$ mice but not the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice. (**A**) FFAR2 protein expression was significantly lower in human colorectal tubular adenoma (TA, $n = 18$), advanced adenoma (AA, $n = 17$) and adenocarcinoma (AC, $n = 29$) than in true normal colon (N, $n = 42$). (**B**) Colonic tumor number and size from 12- to 14-week-old male and female Apc$^{Min/+}$ and Apc$^{Min/+}$-FFAR2$^{-/-}$ mice were determined; a 5% BRB diet suppressed colon adenomas in both male and female Apc$^{Min/+}$ mice but not the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice ($n = 5$–10). (**C**) Representative H&E staining of colon (×20): the Apc$^{Min/+}$ mice developed well-differentiated colon tubular adenomas and the Apc$^{Min/+}$-FFAR2$^{-/-}$ mice developed colon tubular adenomas with high-grade dysplasia. A: Apc$^{Min/+}$; AF: Apc$^{Min/+}$-FFAR2$^{-/-}$; M: Male; F: Female. *$P < 0.05$.

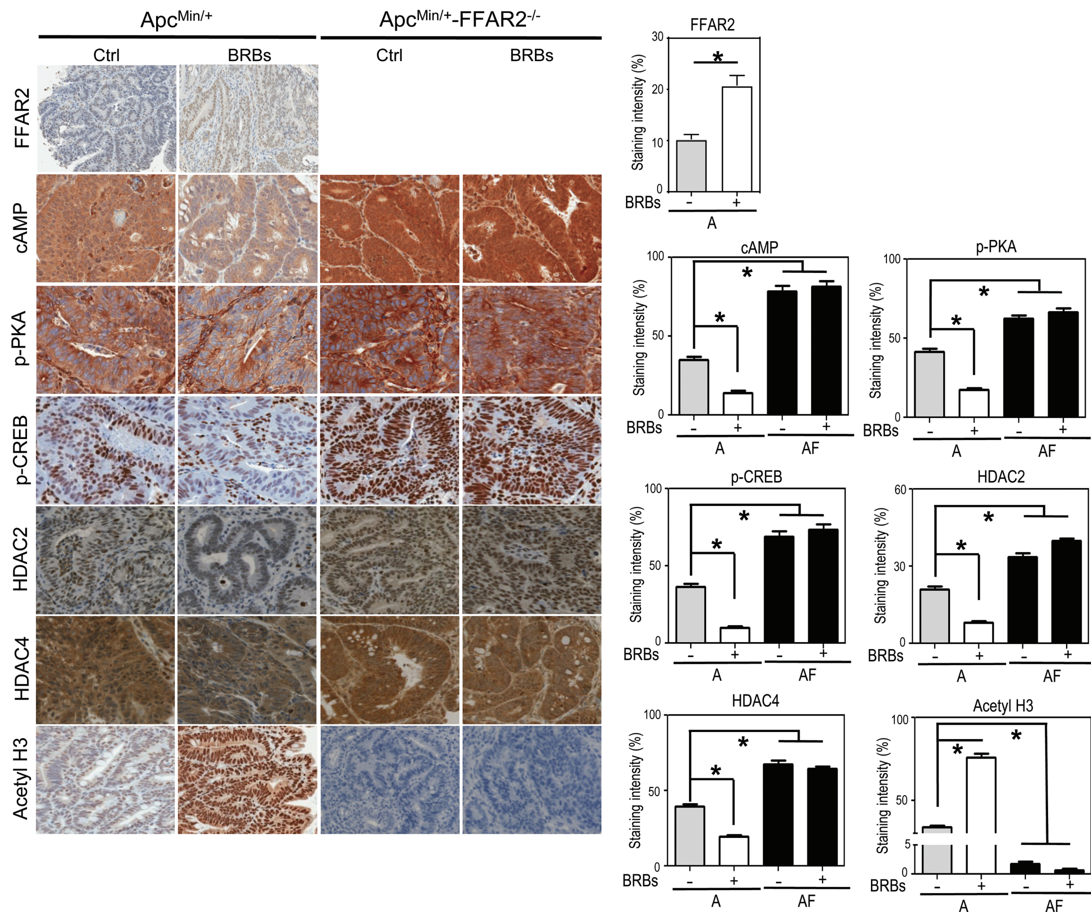

Figure 2. FFAR2 deficiency dysregulated the cAMP-PKA-CREB-HDAC pathway, and BRBs restored that pathway in the ApcMin/+ mice but not the ApcMin/+-FFAR2–/– mice. Representative immunohistochemical staining and quantitative results for cAMP, phosphor-PKA (p-PKA), phosphor-CREB (p-CREB), HDAC2, HDAC4 and acetyl H3 from the ApcMin/+ mice and ApcMin/+-FFAR2–/– mice fed a control diet or a BRB diet. Only staining in adenoma areas was quantified. n = 4–6. *P < 0.05.

control diet (Figure 2). BRBs suppressed cAMP-PKA-CREB levels in the ApcMin/+ mice but not the ApcMin/+-FFAR2–/– mice (Figure 2).

FFAR2 signaling has been reported to decrease HDAC protein expression (12), and we previously demonstrated that BRBs maintain HDAC2 protein expression at the control level in a mouse model of ulcerative colitis, a precursor to colorectal cancer (25). Therefore, we determined if BRBs can correct the increased HDAC protein expression seen in the ApcMin/+ and ApcMin/+-FFAR2–/– mice. We found that BRBs decreased the protein expression of HDAC2 (class I) and HDAC4 (class IIa) and increased acetyl H3 levels in the ApcMin/+ mice but not the ApcMin/+-FFAR2–/– mice (Figure 2). These results suggest that BRBs act through FFAR2 to suppress the cAMP-PKA-CREB-HDAC pathway.

FFAR2 deficiency enhanced the Wnt pathway, and BRBs suppressed the Wnt pathway in the ApcMin/+ mice but not the ApcMin/+-FFAR2–/– mice

In normal colonic tissues, the APC protein binds to β-catenin, inducing its proteasomal degradation. When the *apc* gene is mutated, however, β-catenin is overexpressed and translocated to the nucleus, leading to the transcription of several oncogenes, such as *c-myc* (26), a driver of cell proliferation as measured by Ki67 (27). In our previous studies, BRBs maintained β-catenin protein expression at normal levels; they also increased the transcription of negative regulators of the Wnt signaling pathway, such as *dkk3* and *sox17,* in DSS-induced ulcerative colitis in mice (25). In the current study, we observed increased expression of nuclear β-catenin, c-Myc and Ki67 and decreased expression of DKK3 and SOX17 when we compared the ApcMin/+-FFAR2–/– mice with the ApcMin/+ mice (Figure 3). Importantly, BRBs counteracted these changes by decreasing the expression of nuclear β-catenin, c-Myc and Ki67 and increasing the expression of DKK3 and SOX17 in the ApcMin/+ mice but not the ApcMin/+-FFAR2–/– mice (Figure 3).

FFAR2 deficiency increased GR-1+ neutrophil population in colonic LP and boosted GR-1+ neutrophil infiltration into polyps. BRBs significantly modulated the percentage of GR-1+ neutrophils and level of cytokine secretion in colonic LP and polyps of the ApcMin/+ mice but not the ApcMin/+-FFAR2–/– mice

FFAR2 is expressed in neutrophils (7), which secrete various cytokines that exhibit either protumor or antitumor effects depending on the microenvironment (28). Using GR-1 as a neutrophil marker, we determined infiltration of GR-1+ neutrophils into colonic polyps and measured IL-1β expression. We observed a significant increase in infiltrated GR-1+ neutrophils and IL-1β expression in the ApcMin/+-FFAR2–/– mice compared with the ApcMin/+mice (Figure 4). In contrast, BRBs significantly decreased the infiltration of GR-1+ cells and the expression of IL-1β in an FFAR2-dependent manner (Figure 4). In addition, we determined

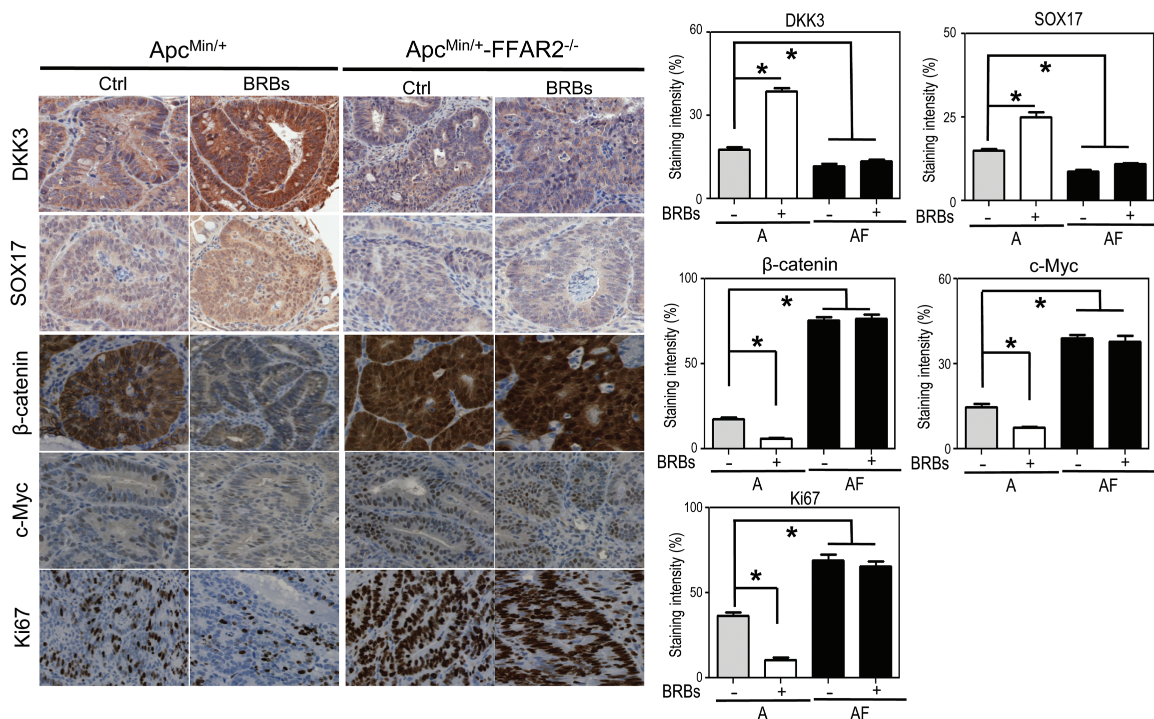

Figure 3. FFAR2 deficiency dysregulated the Wnt pathway, and BRBs corrected the Wnt pathway in the ApcMin/+ mice but not the ApcMin/+/FFAR2−/− mice. Representative immunohistochemical staining and quantitative results for DKK3, SOX17, β-catenin, cMyc and Ki67 from the ApcMin/+ mice and ApcMin/+-FFAR2−/− mice fed a control diet or BRB diet. Only staining in adenoma areas was quantified. n = 4–6. *P < 0.05.

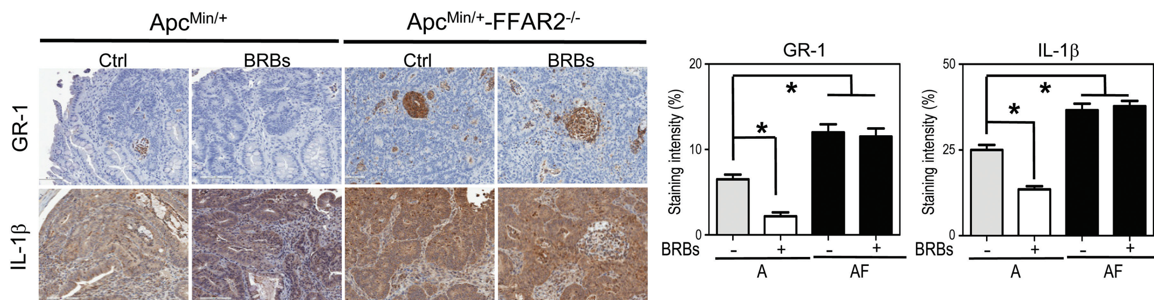

Figure 4. FFAR2 deficiency increased infiltration of GR-1+ neutrophils into colon polyps, and BRBs significantly decreased infiltrated GR-1+ neutrophils and IL-1β expression in colon polyps of the ApcMin/+ mice but not the ApcMin/+-FFAR2−/− mice. Representative immunohistochemical staining and quantitative results for GR-1 and IL-1β from the ApcMin/+ mice and ApcMin/+-FFAR2−/− mice fed a control diet or a BRB diet. Only staining in adenoma areas was quantified. n = 4–6. *P < 0.05.

the percentage of GR-1+ neutrophils and measured the secretion of IL-1β, IL-6 and TNF-α in colonic LP samples. The percentage of GR-1+ neutrophils was significantly higher in colonic LP from the ApcMin/+-FFAR2−/− mice than in colonic LP from the ApcMin/+ mice (Figure 5A). Interestingly, BRBs significantly increased the population of GR-1+ neutrophils (Figure 5A) and the secretion of IL-1β (Figure 5B), IL-6 (Figure 5C) and TNF-α (Figure 5D) in colonic LP of the ApcMin/+ mice but not in that of the ApcMin/+-FFAR2−/− mice.

Discussion

In the USA, colorectal cancer is the second most prevalent cause of cancer death in men and women after lung cancer (1). In recent years, natural compounds have drawn more attention for their chemopreventive effects in multiple organs, including the colon. Berries are enriched in many beneficial compounds, such as minerals, vitamins, dietary fiber and polyphenolic phytochemicals (29). Many different types of berries, including BRBs (24), strawberries (30), bilberries (31) and cloudberries (31), have been reported to be chemopreventive in mouse models of colorectal cancer. Our previous studies have demonstrated BRBs' protective effects in both mouse models of intestinal and colorectal cancer (24) and human colorectal cancer patients (21,22). Our current study demonstrates the importance of the FFAR2 signaling pathway in the development of colorectal cancer. We observed decreased FFAR2 protein expression in human colorectal adenomas and adenocarcinomas compared to normal colonic epithelium. In addition, FFAR2 deficiency promoted colon tumorigenesis in the ApcMin/+ mice through overexpression of the cAMP-PKA-CREB and Wnt pathways and increased HDAC protein expression. However, BRBs suppressed the cAMP-PKA-CREB-HDAC and Wnt pathways in the ApcMin/+ mice, and those protective effects were abolished in the ApcMin/+-FFAR2−/− mice. BRBs also increased the percentage of GR-1+ cells and the secretion of IL-1β, IL-6 and TNF-α cytokines in colonic LP. Finally, BRBs decreased the infiltration of GR-1+ cells into colonic polyps and lowered IL-1β expression in the ApcMin/+ mice, indicating that BRBs can modulate components of the host's immune system.

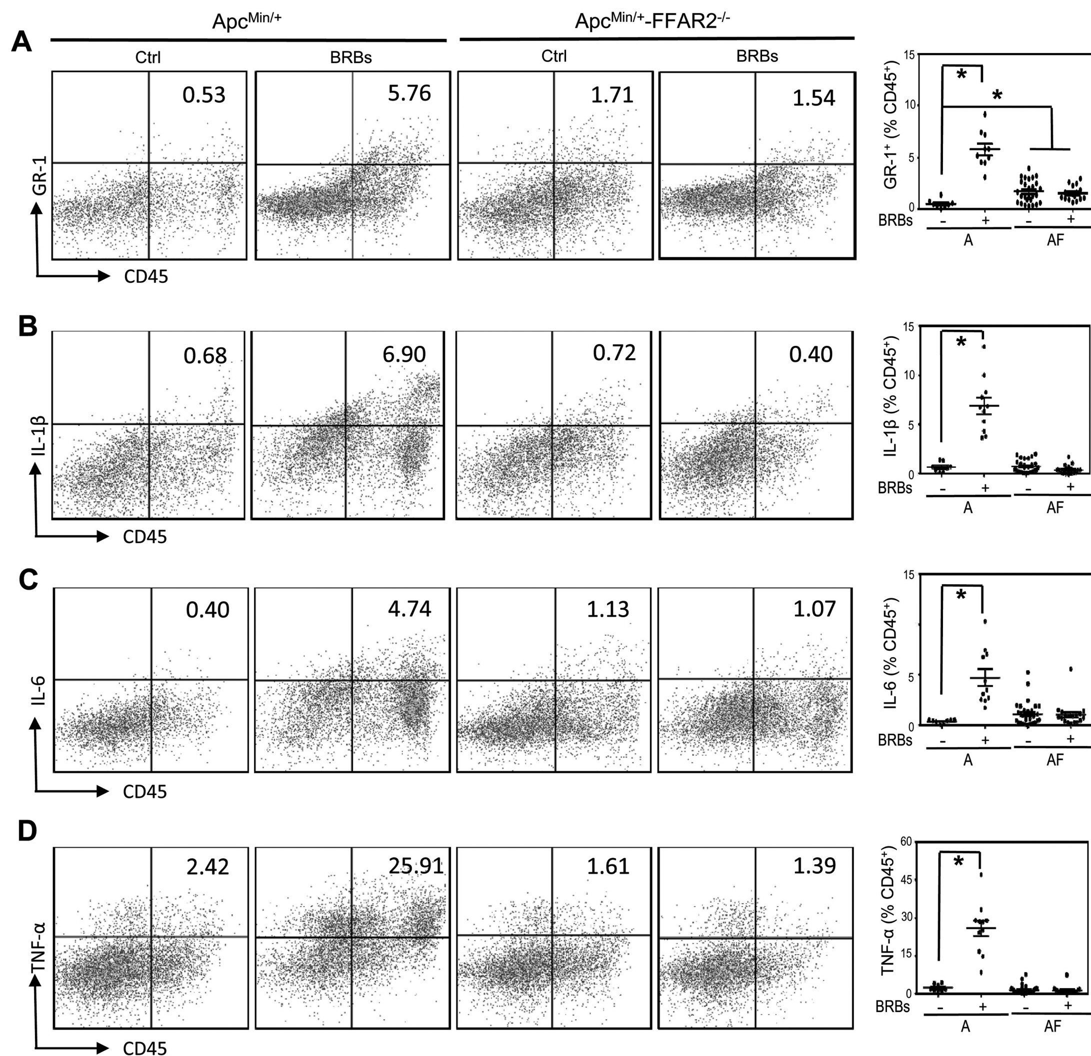

Figure 5. FFAR2 deficiency increased the percentage of GR-1+ neutrophils in colon LP, and BRBs significantly increased GR-1+ neutrophil population (**A**) and the secretion of IL-1β (**B**), IL-6 (**C**) and TNF-α (**D**) in colonic LP in the $Apc^{Min/+}$ mice but not the $Apc^{Min/+}$-$FFAR2^{-/-}$ mice. Representative flow cytometry images are presented. Data are expressed as percentages of CD45+ cells. n = 8–30. *$P < 0.05$.

In the past two decades, a family of fatty acid receptors has been discovered (32), including FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), GPR84 and GPR120. FFAR1 and GPR120 respond to long-chain fatty acids, while GPR84 is activated by medium-chain fatty acids (32). FFAR2 and FFAR3, which have 43% of their amino acids in common, are the two main receptors for SCFAs (33). Although FFAR3 is highly expressed in adipose tissue (33), FFAR2 is expressed in colonic neutrophils, monocytes (7,11,18) and colon epithelial cells (8,9). Thus, it has been suggested that FFAR2 is involved in pathophysiological events involving the immune system. For example, several studies have demonstrated the importance of FFAR2 signaling in gut inflammation (17,19,34,35). However, little is known about its role in colon carcinogenesis. In the current study, we demonstrated that FFAR2 protein expression was lower in human colorectal tubular adenomas, advanced adenomas and adenocarcinomas than in normal colon tissues. These data suggest that FFAR2 may function as a tumor suppressor in colon carcinogenesis.

We used $Apc^{Min/+}$ mice to study colon cancer progression, as the *apc* gene is mutated in most human colon cancers. We aimed to determine if FFAR2 signaling contributes to the pathophysiology of colon carcinogenesis in this mouse model. The incidence of colon polyps was 100% in the $Apc^{Min/+}$-$FFAR2^{-/-}$ mice but only 35% in the $Apc^{Min/+}$ mice. Also, FFAR2 deficiency significantly boosted both polyp number and size in the colon, as has been observed in other studies (36). After considering these observations alongside human tissue data, we speculated that loss of FFAR2 drives colon cancer progression. Furthermore, we fed both the $Apc^{Min/+}$ mice and $Apc^{Min/+}$-$FFAR2^{-/-}$ mice either a control diet or a BRB diet. We observed significant suppression of colon polyp development in the $Apc^{Min/+}$ mice fed BRBs, which is consistent with our previous study (24). BRBs were not protective in the $Apc^{Min/+}$-$FFAR2^{-/-}$ mice.

Inhibition of cAMP signaling by activated FFAR2 has been observed in polymorphonuclear cells (11), breast cancer cells (37), and colon cancer cells (38) *in vitro*. cAMP stimulates gene expression by PKA-mediated phosphorylation of CREB at Ser133 (39). Phosphorylated CREB promotes the recruitment of the co-activators CREB binding protein and p300 (39), which in turn competitively inhibits the binding of HDAC and chromatin (40). To the best of our knowledge, we are the first to show that FFAR2 negatively regulates the cAMP-PKA-CREB signaling pathway in a mouse model of colon cancer. In addition, BRBs restored the cAMP-PKA-CREB signaling pathway in an FFAR2-dependent manner. Consistent with our previous studies (25,41), we observed a

significant decrease in HDAC expression along with increased expression of acetyl-H3 and the negative regulators DKK3 and SOX17 in the mice fed BRBs. These mice also had lower levels of nuclear β-catenin and c-Myc expression. Therefore, we furthered our knowledge of BRBs' protective effects by demonstrating that BRBs depend largely on functional FFAR2 signaling.

As well as exerting epigenetic effects, BRBs might protect against colon cancer by modulating the immune system. Inflammation has been reported to be a major risk factor for the development of colitis-associated colorectal cancer as well as sporadic colon cancer and familial adenomatous polyposis (FAP)-promoted colon cancer (42–44). The literature shows that the complexity of the tumor microenvironment determines the functions of immune cells, especially those, such as neutrophils, with dual functions (45). As the first host defense against invading microorganisms, neutrophils can be attracted to the primary site and contribute to tissue repair (28). However, they can also infiltrate the tumor microenvironment to become tumor-associated immune-suppressive cells (28,46). In our study, we observed significantly increased infiltration of GR-1$^+$ neutrophils into colon adenoma and colon LP (as measured by IHC and flow cytometry) in FFAR2-deficient mice (Figures 4 and 5A). These tumor-infiltrated neutrophils could accelerate tumor cell growth and have immune-suppressive effects. More importantly, we observed that BRBs significantly decreased the infiltration of GR-1$^+$ neutrophils into colon adenoma of the Apc$^{Min/+}$ mice, as measured by IHC (Figure 4), suggesting that BRBs can prevent neutrophil infiltration. Interestingly, we observed a larger number of GR-1$^+$ neutrophils in colon LP of the Apc$^{Min/+}$ mice fed BRBs. As that group also had fewer neutrophils in its colon adenoma, it is possible that BRBs influence the tumor microenvironment and block neutrophil infiltration, causing them to accumulate in colon LP. Indeed, BRBs significantly increased the secretion of IL-1β, IL-6 and TNF-α in colon LP, and these cytokines could activate natural killer cells and cytotoxic T lymphocytes (47–50). Further investigation is warranted to examine if natural killer cells and cytotoxic T lymphocytes are involved in BRB-mediated protection.

In summary, the current study uncovers an important role for FFAR2 signaling in a mouse model of colorectal cancer and suggests an indispensable mechanism underlying BRB-mediated chemoprevention. Our findings imply that BRBs might modulate the immune system, which may contribute to their chemopreventive effects.

Supplementary data

Supplementary data are available at *Carcinogenesis* online.

Ethics approval

All protocols were carried out in accordance with institutional guidelines for animal care dictated by the Medical College of Wisconsin Animal Care and Use Committee.

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