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Polymethoxyflavones prevent benzo[*a*]pyrene/dextran sodium sulfate-induced colorectal carcinogenesis through modulating xenobiotic metabolism and ameliorate autophagic defect in ICR mice

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Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental carcinogenic pollutants and they have become an important issue in food contamination. Dietary intake of PAHs has been recognized as a major route of human exposure. However, the mechanisms behind dietary PAH-induced colorectal cancer (CRC) remain unclear. Several studies have shown that polymethoxyflavones (PMFs) are effective in preventing carcinogen-induced CRC or colitis. In this study, we investigated the preventive effect of PMFs on benzo[*a*]pyrene/dextran sulfate sodium (B*a*P/DSS)-induced colorectal tumorigenesis in ICR mice. We found that PMFs significantly prevented B*a*P/DSS-induced colorectal tumor formation. B*a*P mutagenic metabolite and DNA adducts were found to be reduced in colonic tissue in the PMFs-treated groups through the modulation of B*a*P metabolism. At the molecular level, the results of RNA-sequencing indicated that PMFs ameliorated B*a*P/DSS-induced abnormal molecular mechanism change including activated inflammation, downregulated anti-oxidation targets, and induced metastasis genes. The autophagic defect caused by B*a*P/DSS-induced tumorigenesis was improved by pretreatment with PMFs. We found B*a*P/DSS-induced CRC may be a Wnt/β-catenin independent process. Additionally, consumption of PMFs extracts also altered the composition of gut microbiota and made it similar to that in the control group by increasing butyrate-producing probiotics and decreasing CRC-related bacteria. B*a*P in combination with DSS significantly induced colorectal tumorigenesis through induced DNA adduct formation, abnormal gene expression, and imbalanced gut microbiota composition. PMFs were a powerful preventive agent that suppressed B*a*P/DSS-induced CRC via modulating multiple pathways as well as ameliorating autophagic defect. These results demonstrated for the first time the chemopreventive efficacy and comprehensive mechanisms of dietary PMFs for preventing B*a*P/DSS-induced colorectal carcinogenesis.

Key words: polycyclic aromatic hydrocarbons, polymethoxyflavones, colorectal cancer, autophagic defect, microbiota

Abbreviations: AUC: Average area under curve; BPDE: B*a*P-7,8-dihydrodiol-9,10-epoxide; B*a*P: benzo[*a*]pyrene,; ; CRC: colorectal cancer; CX-43: conexin-43; CYPs: cytochrome P450s; DSS: dextran sulfate sodium; ESI: electrospray ionization source; H&E: hematoxylin and eosin; HPLC: high-performance liquid chromatography; LAMP1: lysosomal-associated membrane protein 1; LC3: microtubule-associated protein 1 light chain 3; Tris-EDTA: Tris (hydroxymethyl) aminomethane-ethylenediaminetetraacetic acid; PAHs: polycyclic aromatic hydrocarbons; PMFs: polymethoxyflavones; PCD: programmed cell death; PCDH17: protocadherins 17; TSG: tumor suppressor gene; XEMs: xenobiotic metabolizing enzymes

Additional Supporting Information may be found in the online version of this article.

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What's new?

Carcinogenic polyaromatic hydrocarbons (PAHs) are pervasive in the environment and are a potential source of food contamination, with their ingestion via dietary sources greatly increasing colorectal cancer (CRC) risk. This study examined the possibility of preventing CRC induced by intake of the PAH benzo[*a*]pyrene (B*a*P) via administration of polymethoxyflavones (PMFs), anticancer substances found in citrus peels. The results show that colon tumorigenesis induced by B*a*P and dextran sulfate sodium (DSS) in mice can be blocked by PMFs oral administration. PMFs were associated with reduced mutagenic metabolite levels and DNA adduct formation and with alleviation of a B*a*P/DSS-induced autophagic defect.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and second most commonly diagnosed cancer in females worldwide with an annual incidence of 1.4 million new cases and over 160,000 deaths.[1] Epidemiological studies demonstrated that environmental and lifestyle factors, including dietary habits and components, have been strongly associated with CRC development.[2,3] According to recent literature by Harrad and Ma, dietary intake has been the major vehicle of human exposure to polycyclic aromatic hydrocarbons (PAHs).[4] They are recognized as having hazardous mutagenic and carcinogenic potential in mammals and present an increasingly serious issue to food safety and food contamination.[4] One of the most significant current discussions in PAHs exposure focuses on an increased risk of CRC through environment, occupation and dietary intake.[5,6]

PAHs can contaminate foods through both natural and anthropogenic sources including cooking, smoke, barbeque and industrial food processing.[7] When PAHs enter the body, their bioactivation is thought to be catalyzed by cytochrome P450s (CYPs), also called phase I enzymes, to form the ultimate mutagenic metabolite such as benzo[*a*]pyrene (B*a*P) converted to mutagenetic B*a*P-7,8-dihydrodiol-9,10-epoxide (BPDE) that can react with DNA to lead BPDE-DNA adduct formation and consequent gene mutation, thus initiating tumorigenesis.[8] Nevertheless, previous study suggested that mice orally administrated B*a*P found the highest mutation frequency in the colon but it did not induce colorectal tumorigenesis. This limitation may be due to a lack of promotion/progression factors.[2,9] Several factors act as potential tumorigenesis promoters such as a polluted environment, intangible stress, western diet patterns and individual heredity.[10–12] Those factors significantly increase the risk of CRC development when followed by the ingestion of PAHs-contaminated foods.[13] However, the underlying molecular mechanisms behind dietary PAHs combined with promotion factor-induced CRC remain unclear. Therefore, investigation of dietary PAHs, particularly B*a*P that is a representative carcinogen species for PAHs, in inducing colorectal tumorigenesis is essential.

CRC development, a dynamic and long-term process, involves many complex factors such as DNA mutation, signaling pathway disorder, and colonic microbiota imbalance.[14,15] During colorectal tumorigenesis, chemicals or free radicals induce DNA damage/mutation that plays an initiation role affecting cell growth regulation, oncogene/tumor suppressor gene (TSG) expression and cell differentiation. Therefore, the strategy of a chemoprevention approach targeting the initiation stage is desirable in order to block tumor development. This is accomplished through modulation of the type and expression level of xenobiotic metabolizing enzymes (XEMs) to reduce the production of reactive metabolites or increase the ability of antioxidative stress.[16,17] Following initiation, promotion is a prolonged stage that contain several reversible mechanisms in the process of tumor promotion such as cell cycle dysregulation, uncontrolled cell proliferation, programmed cell death (PCD) disorder and excessive inflammatory response.[15,18] Therefore, intervention in the promotion stage is a suitable and useful strategy. Additionally, recent evidence demonstrated that gut microbiota is implicated in variety of chronic diseases including diabetes, bowel diseases and CRC.[19,20] Accumulating reports suggest that the composition of gut microbiota is closely correlated with colorectal tumorigenesis, which includes the initiation and promotion/progression stages.[21] For instance, Baxter *et al*. found an increase of *Parabacteroides* and *Akkermansia* in the feces of humans with a higher rate of colon tumorigenesis.[22] In contrast, an increased amount of *Lactobacillus salivarius* contributed to reduced colonic inflammatory responses and prevented colorectal carcinogenesis.[23,24]

In this study, we investigate how the B*a*P in combination with dextran sodium sulfate (DSS), the most common tumorigenic promoter for inducing colitis in the murine model, induced colorectal tumorigenesis and it was prevented by polymethoxyflavones (PMFs), which was isolated from citrus peels and have been shown to exhibit various biological activities including cancer prevention, anti-inflammatory activity and inhibition of metastasis.[15] We found that the oral administration of PMFs significantly prevented B*a*P/DSS-induced colorectal tumorigenesis through reduced BPDE and DNA adduct formation at the initiation stage. At the promotion/progression stage, RNA-sequencing results demonstrated that mice pretreated with PMFs blocked the B*a*P/DSS modulated CRC associated gene expression and inhibited the cell cycle regulator, activated inflammation and metastasis genes and raised cell proliferation biomarkers. Interestingly, we found B*a*P/DSS was able to cause an autophagic defect that may be related to CRC development. Additionally, the consumption of PMFs also altered the composition of gut microbiota and

made it similar to that in the control group by increasing the butyrate-producing probiotics and decreasing CRC-related bacteria. We hope this study will contribute to the explanation of PAHs-induced CRC development and prevention through dietary PMFs.

Material and Methods

Reagents

B*a*P was purchased from Sigma Chemical Co. DSS (molecular weight of 36,000–50,000 g/mol) was purchased from ICN Biomedicals. PMFs were obtained from Florida Flavors Company (Lakeland, FL, USA) and their composition was determined by high-performance liquid chromatography (HPLC) as shown in Supporting Information, Figure S1 and Table S1.

Animals and experimental procedure

Male ICR (Institute for Cancer Research) mice at 4–6 weeks of age were purchased from the BioLASCO experimental animal center (Taiwan Co., Taipei, Taiwan), housed in metal wire-bottomed cages and acclimatized under a controlled atmosphere (25 ± 1°C, 50% relative humidity, 12 hrs light/12 hrs dark cycle). All mice were fed standard AIN-76 diet and top water *ad libitum* at all times, acclimatized for one week, and then randomly distributed by body weight into control and experimental groups. All protocol used in these animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (project number: B201600115).

The animal experimental design for this study is shown in Figure 1*a*. Experimental animals were randomly allotment into five groups: control, PMFs alone, B*a*P/DSS, 0.5% PMFs + B*a*P/DSS and 1.0% PMFs + B*a*P/DSS. Mice in B*a*P/DSS treatment groups were administered B*a*P orally (125 mg/kg/day for 5 days), followed by 2% DSS in drinking water (supplied twice, with a two-week interval). The PMFs treatment groups were fed diets containing 0.5% or 1.0% PMFs, respectively, until the end of the study. In mice treated with B*a*P, we also collected feces for the analysis of B*a*P metabolites by LC/MSMS. At 8 weeks, all mice were euthanized by CO_2 asphyxiation for examination of molecular and histopathological changes in the colon tissues. Blood samples were collected and isolated in serum for biochemical analysis. The liver, spleen and kidneys were also immediately removed and weighted.

Immunohistochemical analysis (IHC)

Colon tissues were fixed overnight in 10% buffered formalin and embedded in paraffin. Sections of 3 μm thickness were incubated with 0.3% hydrogen peroxide to quench the endogenous peroxidase activity and then were heated in Tris (hydroxymethyl) aminomethane-ethylenediaminetetraacetic acid (Tris-EDTA) buffer (10 mM Tris, 1 mM EDTA, pH 9) in a microwave oven for antigen retrieval. After antigen retrieval, sections were incubated with the primary antibody to microtubule-associated protein 1 light chain 3II (LC3II), lysosomal-associated membrane protein 1 (LAMP1), and proliferating cell nuclear antigen (PCNA) for 1 hr at room temperature. Sections were then incubated with a biotin-conjugated horseradish peroxidase secondary antibody (3,3′-diaminobenzidine tetrahydrochloride was used as the substrate) or fluorescent-dye-conjugated secondary antibody.

BPDE–DNA adduct determination by ELISA kit

Genomic DNA was isolated from colonic mucosa as described previously.[25] The extracted genomic DNA was used for determining the concentration of BPDE-DNA adducts through use of a BPDE-DNA adduct kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instruction.

Detection of B*a*P metabolites from feces by UPLC/MSMS

Feces samples of the mice were collected during their initial oral dose of B*a*P to 6 days. 200 mg of the feces sample and 1 ml acetonitrile were placed in an Eppendorf with sonication for 10 min at room temperature. The supernatant was collected and filtrated with 0.22 μm PTFE membrane. B*a*P metabolites were determined by the Water UPLC system (Acquity, Waters, Mildford, MA, USA) interfaced to a triple quadrupole mass spectrometer (TQS, Waters Micromass, Manchester, UK) equipped with an electrospray ionization source (ESI). The separation of B*a*P metabolites was conducted using a Waters ACQUITY UPLC BEH phenyl column (1.7 μm; 2.1 mm × 150 mm) at 45°C. Ultrapure water (A) and acetonitrile (B) containing 0.1% formic acid were used as the mobile phase. The gradient of chromatographic separation was as follows: 0–10 min (75/25, A/B) to (5/95, A/B) and kept for 1 min; followed by an increase to 75% A during 0.5 min and held for 0.5 min to allow for equilibration. Positive ESI ions in a multiple reaction monitoring (MRM) model with collision energy of 35 eV, 35 eV, 50 eV and 45 eV were used to determine B*a*P (*m/z* 252 => 252), B*a*P-phenols (*m/z* 268 => 239), B*a*P-dihydrodiols (*m/z* 284 => 239) and B*a*P-quinones (*m/z* 283 => 226), respectively.

Quantification of mRNA expression by real-time PCR and targeted RNA sequencing

Total RNA from liver and colon tissues were isolated using the Trizol reagent (Invitrogen, Renfrewshire, UK) according to the supplier's protocol and then transcription to complementary DNA using SuperScript III RNA reverse transcriptase (Invitrogen, Renfrewshire, UK). In a real-time PCR analysis, specific primers were designed to clone XMEs as shown in Supporting Information, Table S2. For RNA-sequencing by next generation sequencing (NGS) technology, PCR primers were designed for 114 genes using the primer3 (http://bioinfo.ut.ee/primer3-0.4.0/), and necessary modification was made to span exon–exon junctions. The range of amplicon sizes were among 100–120 bp. The PCR products were purified for NGS library construction by performing end-repairing, adding a-overhang and adaptor ligation. Library preparation was carried out using illumine TruSeq

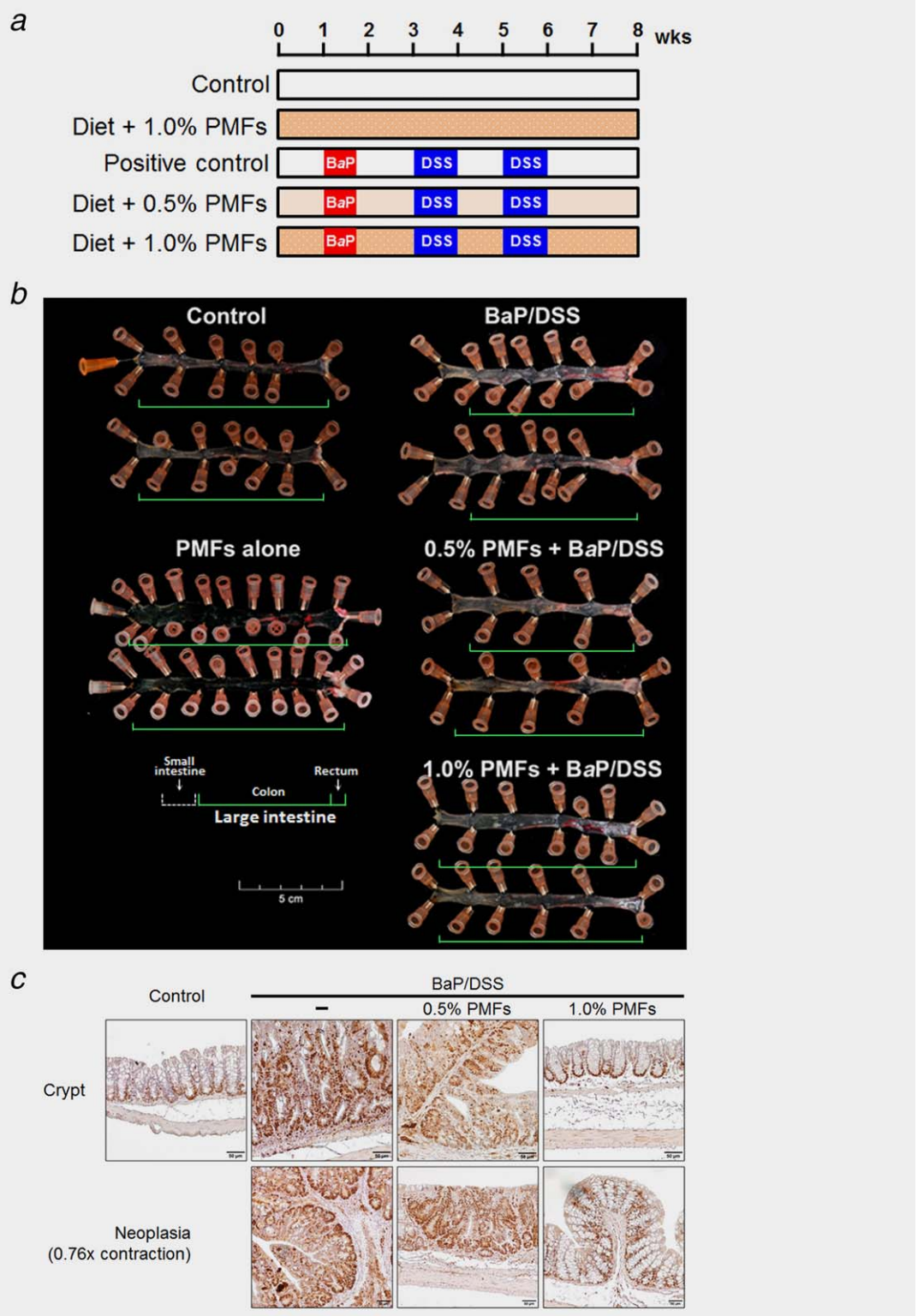

Figure 1. Oral administration of PMFs prevented the B*a*P/DSS-induced colorectal tumor formation. (*a*) Experimental design of B*a*P/DSS-induced colorectal tumorigenesis in ICR mice. (*b*) Macroscopic view of tumor formation in the colonic mucosal surface of B*a*P/DSS-treated mice and combined with oral administration of PMFs daily. (*c*) Distribution of cell proliferation biomarker in colonic tissue was visualized by IHC staining. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1. Chemopreventive efficiency of PMFs on the colon length and number of neoplastic lesions in B*a*P/DSS induced colorectal carcinogenesis model

		Number of polyps/colon (incidence, %)			
	Colon length (cm)	HP	AD	ADC	Total
Control	10.0 ± 0.6^{a}	0.2 ± 0.4 (20%)^{a}	0.0 ± 0.0 (0%)^{a}	0.0 ± 0.0 (0%)^{a}	0.2 ± 0.4 (20%)^{a}
1.0% PMFs	9.6 ± 0.6^{ab}	0.4 ± 0.9 (20%)^{a}	0.0 ± 0.0 (0%)^{a}	0.0 ± 0.0 (0%)^{a}	0.4 ± 0.9 (20%)^{a}
B*a*P/DSS	7.4 ± 0.5^{c}	7.0 ± 1.4 (100%)^{b}	3.4 ± 1.1 (100%)^{b}	1.6 ± 2.3 (40%)^{a}	12.0 ± 3.5 (100%)^{b}
0.5% PMFs + B*a*P/DSS	8.6 ± 0.7^{d}	4.0 ± 0.7 (100%)^{c}	1.2 ± 0.8 (100%)^{c}	0.6 ± 1.3 (20%)^{a}	5.8 ± 1.8 (100%)^{c}
1.0% PMFs + B*a*P/DSS	9.1 ± 0.3^{bd}	2.4 ± 1.3 (100%)^{d}	0.2 ± 0.4 (20%)^{a}	0.0 ± 0.0 (0%)^{a}	2.6 ± 1.1 (100%)^{a}

Within a group means that share the same superscript letter do not differ significantly at the 0.05 level (after applying Duncan's adjustment for multiple comparisons).
Abbreviations: HP, hyperplastic polyp; AD, adenoma; ADC, adenocarcinoma.

DNA Sample Prep Kit, and then the reads were pooled equally for sequencing. The total reads of each library ranged from 0.68 to 1 M. After trimming sequences based on quality values (Q30), the reads were mapped to mouse genome GRCm38/mm10 assembly from the UCSC database using Illumina local run manager. Only unimapped reads were used to count expression levels.

Western blot analysis

Scraped colon mucosa tissue was homogenized with gold lysis buffer (50 mM Tris-HCl, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40 and 10 μg/mL leupeptin) on ice for 30 min, followed by centrifugation at 12,000g for 30 min at 4°C to extract total protein. The protein concentration of supernatant fraction was measured using the Bio-Rad protein assay kit, resolved by SDS-polyacrylamide gel electrophoresis (50 μg protein per lane), and then electroblotted onto polyvinylidene difluoride-membranes. The membranes were blocked with 1% bovine serum albumin solution and immunoblotted overnight at 4°C with a primary antibody followed by a secondary antibody conjugated with horseradish peroxidase and visualized using enhanced chemiluminescence (ECL; Amersham Bioscience, Piscataway, NJ, USA). The densities of the bands were quantified with a computer densitometer (AlphaImager™ 2200 System Alpha densitometer, Innotech Corporation, San Leandro, CA, USA).

Taxonomic classification of gut microbiota by next generation sequencing

The genomic DNA of gut bacteria was isolated by the innuSPEED Stool DNA kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's instructions from intracolonic faeces samples collected from necropsy. The V4 region of bacterial 16S rDNA was amplified using PCR technology with universal primers that were obtained from a previous study.[26] The index-labeled libraries were constructed by used the Illumina DNA library preparation kit (Illumina, San Diego, CA, USA). The library DNA was sequenced by the IlluminaHiSeq2500 platform, and paired-end reads (2 × 150 bp) were generated.

Statistical Analysis

All data are expressed as mean values ± SD for the indicated number of independently performed experiments. Statistical differences were performed by Student's *t* tests and one-way ANOVA and Duncan's multiple range tests using SAS statistical software package (version 8.0). Differences with p values <0.05 were considered statistically significant.

Results

Orally administrated PMFs prevent B*a*P/DSS-induced colorectal tumorigenesis

To examine the preventive effect of PMFs in dietary intake on B*a*P in combination with DSS-induced colorectal tumorigenesis, we referred to a previous report published by Sonoda *et al.* to design this experimental model shown in Figure 1*a*.[27] After the end of the 8 weeks, all mice were sacrificed and the results of body and organ weight were shown in Supporting Information, Figure S2 and Table S3. We found that mice prefed with PMFs prevented any weight change caused by BaP/DSS. PMFs also abated B*a*P/DSS-induced liver and kidney toxicity through decreasing AST and ALT and restoring the level of ALP on serum biochemical parameters (Supporting Information, Table S4).

The types of polyps including hyperplastic polyp, adenoma and adenocarcinoma were identified by Hematoxylin and eosin (H&E) staining. The microscopic view and tumor counts of colonic tissues showed that PMFs significantly reduced the number of total polyps to 5.8 ± 1.8 and 2.6 ± 1.1 at 0.5% and 1.0%, respectively, compared with 12.0 ± 3.5 in the B*a*P/DSS-treated group (Fig. 1*b* and Table 1). IHC examination showed that PCNA, a typical cell proliferation biomarker, intensity on both crypt and neoplasia tissue was decreased in mice treated with PMFs compared to the B*a*P/DSS group (Fig. 1*c*). Together, those results suggested that oral administration of PMFs can prevent B*a*P/DSS-induced colorectal tumorigenesis.

PMFs decreased BPDE-DNA adduct formation in colon tissue through modulated B*a*P metabolism

It is well known that the carcinogenic ability of B*a*P depends on its mutagenic metabolite, BPDE, which reacts with DNA

to form covalently DNA adducts and initiates the processes of tumorigenicity.[28] We used IHC with BPDE-DNA adduct antibodies to determine the level and distribution of BPDE-DNA adducts in colonic tissue. The result was shown in Figure 2*a*. Microscopic views revealed that PMF-treated groups significantly reduced the BPDE-DNA adduct formation at colonic mucosa and submucosa. The BPDE-DNA adduct concentration in colonic tissue was also decreased by PMF treatment in a dose-dependent manner (Fig. 2*b*).

To confirm whether PMFs are able to affect B*a*P metabolism, we analyzed the type of B*a*P metabolites from feces samples and XEMs expression levels in the liver and colon by UPLC/MSMS and real-time PCR, respectively. We detected 3 main types of B*a*P metabolites including reactive metabolites such as B*a*P-quinones and nonreactive metabolites that contain B*a*P-phenols and B*a*P-dihydrodiols.[29] The analytic pattern of UPLC/MSMS was shown in Supporting Information, Figure S4. The concentration of B*a*P and its metabolites was shown from the average area under curve (AUC). This showed that dietary intake of PMFs contributes to the activation of B*a*P metabolism through increasing nonreactive metabolites and decreasing reactive metabolites (Fig. 2*c*). The XEMs expression level on the liver and colon was also regulated in mice treated with PMFs. PMFs exhibited a suitable inhibition of *CYP1As* and *CYP1Bs* on colonic mucosa and significantly induced liver phase II enzymes expression including *GSTAs*, *GSTMs*, *GSTTs*, *UGT1As*, *UGT2s* and *NOQ1* (Supporting Information, Fig. S5). Those data suggested that PMFs present a downregulation of B*a*P bioactivation and upregulation of B*a*P detoxification. Altogether, those results indicated that PMFs exhibit an efficiency in preventing B*a*P-induced DNA adduct formation via regulation of B*a*P metabolism and XMEs gene expression.

Comprehensive overview of how gene expression change from PMFs prevents B*a*P/DSS-induced colorectal carcinogenesis

To examine how the molecular mechanism of B*a*P/DSS-induced colorectal tumorigenesis is prevented by PMFs, we used RNA-sequencing technology to analyze a variety of CRC development-associated mRNA expressions. As shown in Figure 3*a* and Supporting Information, Table S5, when compared with the control group the B*a*P/DSS treated group markedly upregulated the inflammatory and metastasis gene and downregulated the antioxidation and cell cycle regulators gene. This finding confirms the ability of B*a*P in combination with DSS to induce colorectal tumor formation.

Meanwhile, the volcano plot was used to identify 111 differentially expressed genes of the 1.0% PMFs prevention groups compared to the B*a*P/DSS treatment only in colonic tissue (Fig. 3*b*). PMFs notably ameliorated B*a*P/DSS-induced inflammatory responses by inhibiting *IL-6, IL-10, TNF-α, COX-2, MCP1* and *CXCL-1* and activating antioxidation targets such as *HO-1, SODs* and *NQO1*. The fact that PMFs increase *TIMPs* and decrease *MMPs* suggests that PMFs possess antimetastasis properties. In particular, protocadherins 17 (PCDH17) exhibited anticancer cell growth and antimetastasis activities by inducing PCD and regulating the cell cycle.[30,31] In this study, we found that PMFs restored the substantially decreased *PCDH17* level in the B*a*P/DSS-induced tumorigenesis model. The total differentially expressed genes and hierarchical clustering between the control group, the PMFs prevention group, and the B*a*P/DSS treatment group are exhibited in Figure 3*c* and Supporting Information, Figure S6 by Venn diagram and heatmap, respectively.

Inducing defective autophagy may be an important role in B*a*P/DSS-induced colorectal carcinogenesis independent of the wnt/β-catenin pathway

The Wnt/β-catenin signaling pathway has an important role in modulating carcinogenesis in several tissues, including CRC.[32] During CRC development, most studies suggest that Wnt/β-catenin shows excessively abnormal activation and accumulation of β-catenin in cytoplasm, then subsequently induces aberrant cell proliferation by expressed cell proliferation biomarkers such as cyclin D1 and c-myc.[33,34] We used western blotting to determine the expression level of β-catenin in colonic tissue and used an individual colonic protein sample extracted from mice treated with azoxymethane for 23 weeks as an independent positive control. Unexpectedly, β-catenin, including total and active form, did not accumulate in cytoplasm in the B*a*P/DSS-induced CRC model, but the independent positive control did (Fig. 4*a*). Therefore, we roughly conjectured that B*a*P/DSS-induced colorectal tumor formation may be a Wnt/β-catenin independent carcinogenicity process. Nevertheless, results of RNA-sequencing indicated that B*a*P/DSS treatment significantly increased several non-canonical *Wnts* expression, including *Wnt4*, *Wnt5a* and *Wnt11*. Previously studies suggested that $Ca2^{+}$-dependently non-canonical Wnt/β-catenin pathway not only involved in cancer development but also inhibited TCF/LEF transcriptional activity.[35,36] To examine the role of non-canonical Wnt pathway in B*a*P/DSS-induced CRC, we used western blotting to examine the expression level of calmodulin as biomarkers in Ca^{2+}-dependently pathway. As shown in Figure 4*b*, oral administration of PMFs significantly suppressed B*a*P/DSS induced calmodulin expression. This result indicated that B*a*P/DSS-induced colorectal carcinogenesis may be related to activate Ca^{2+}-dependent pathway.

Phytochemical or drug-induced PCD, including apoptosis and autophagy, has been suggested as a potential strategy for the prevention and treatment of cancer.[37,38] Our previous studies found that inducing a completely autophagy process inhibited cancer cell growth *in vitro* and *in vivo* model by natural dietary compounds.[30] To examine the effect of PMFs on the induction of autophagy status in B*a*P/DSS-induced colorectal tumorigenesis in ICR mice, we used western blotting to determine the expression level of LC3 and Beclin-1 as autophagic initiation biomarkers and targets of autophagic degradation by lysosome including p62 and conexin-43 (CX-

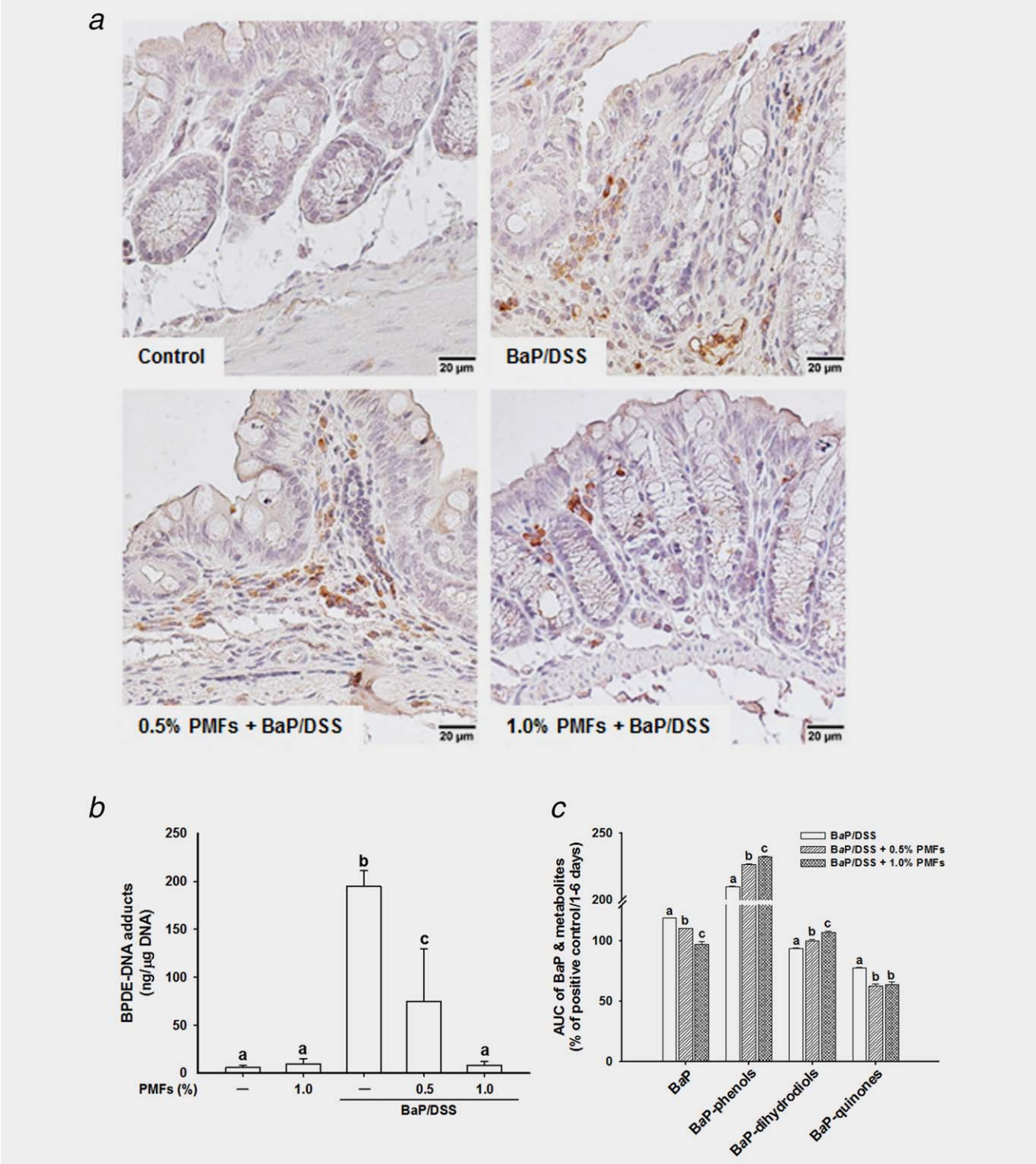

Figure 2. Oral administration of PMFs significantly reduced BPDE-DNA adduct formation through regulation of B*a*P metabolism in B*a*P/DSS-treated mice. (*a*) PMFs-treated groups have reduced BPDE-DNA adduct formation in colonic mucosal cells and were subjected to IHC staining with BPDE-DNA antibody. (400× magnification) (*b*) The BPDE-DNA adduct concentration of colonic mucosa tissue was detected using ELISA at the end of 8 weeks treatment. (*c*) The AUC for the concentration of B*a*P and its metabolites extracted from faeces following the oral administration of PMFs in B*a*P/DSS-treated mice, using UPLC/triple quadrupole mass spectrometer detection. Within groups, means that share the same superscript letter do not differ significantly at the 0.05. The feces were collected during initial oral treatment with B*a*P for six consecutive days. [Color figure can be viewed at wileyonlinelibrary.com]

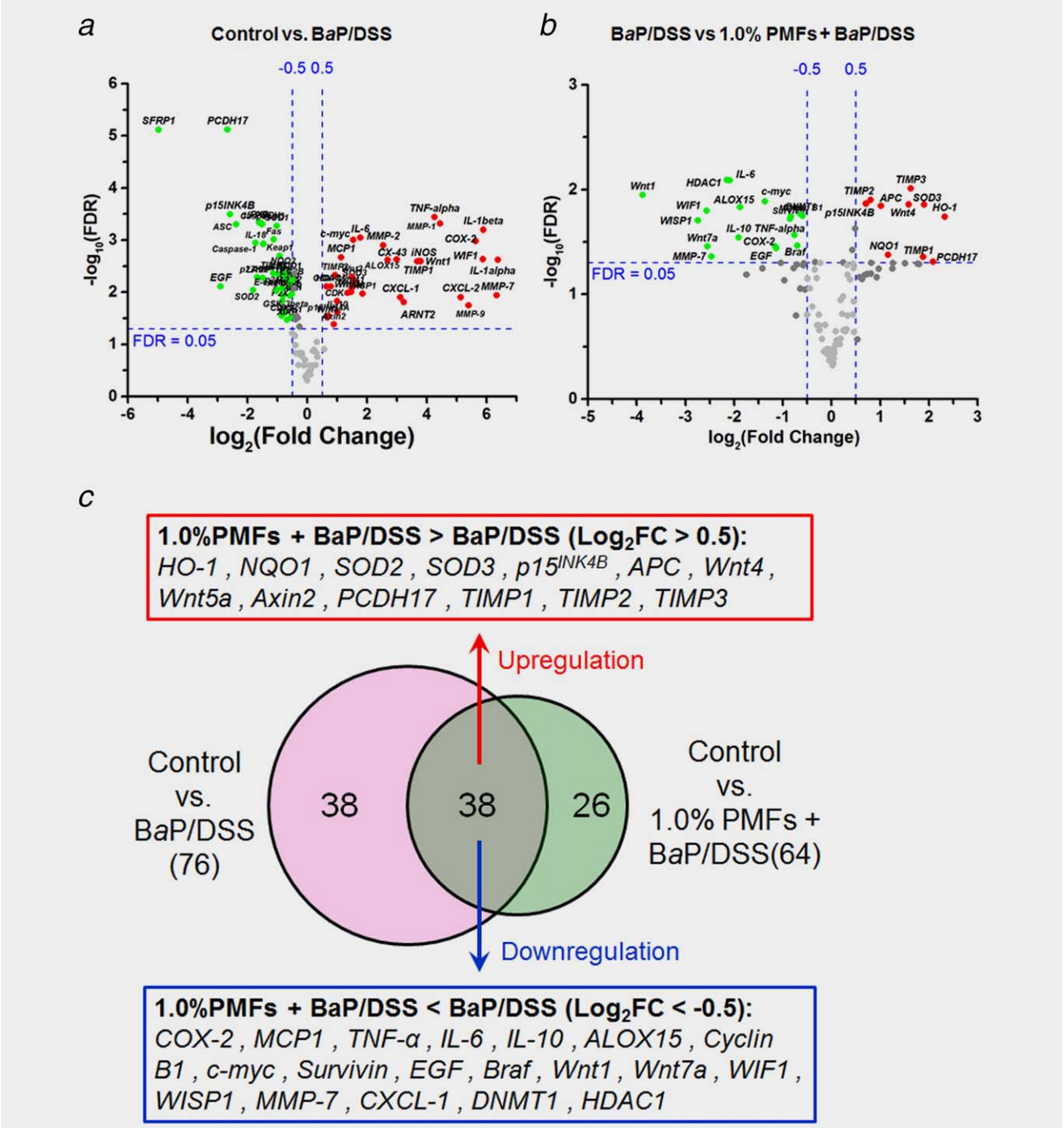

Figure 3. Overview of the colonic genes regulated by B*a*P/DSS treatment, or in combination with 1.0% PMFs. Volcano plot of RNA-sequencing data of genes between control vs. B*a*P/DSS group (*a*) and B*a*P/DSS vs 1.0% PMFs + B*a*P/DSS group (*b*). Downregulated and upregulated genes are marked in green and red, respectively, if they passed the threshold of false discovery rate (FDR) < 0.05 and $|\log_2 FC| > 0.5$. (C)The Venn diagram showing the number of significantly expressed genes in comparison with control group (p values <0.05 and FDR < 0.05). The overlapped area displays upregulated and downregulated genes in response to mice treated with 1.0% PMFs in B*a*P/DSS-induced tumorigenesis. The FDR was calculated by the Benjamin–Hochberg method. [Color figure can be viewed at wileyonlinelibrary.com]

43). As shown in Figure 4*c*, B*a*P/DSS significantly induced LC3 I to convert to LC3 II and Beclin-1 expression, but blocked degradation of p62 and CX-43 which contributed to defective autophagy. We also used the IHC with Beclin-1 antibody to confirm the phenomenon of autophagy. The formation of DAB puncta (brown) of Beclin-1 was only increased in the B*a*P/DSS-treatment group (Supporting Information, Fig. S7).

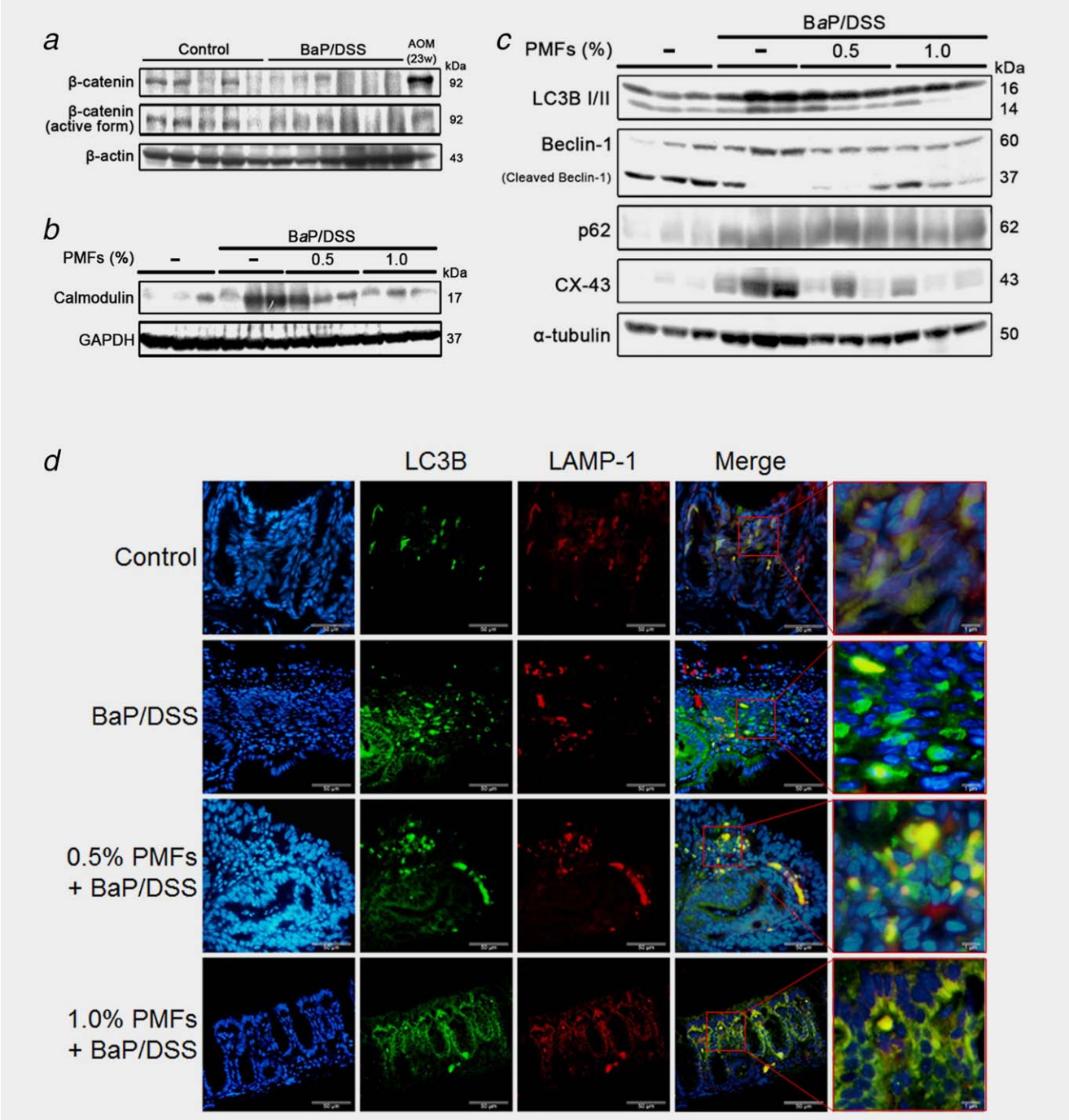

Figure 4. B*a*P/DSS-induced tumorigenesis through β-catenin-independent mechanism and caused autophagic defect. (*a*) Western blotting analyzed colonic mucosa protein expression level of total (GeneTex, #GTX22368) and active β-catenin (Millipore, #DAM1595540). Colonic mucosal protein extracted from ICR mice treated with AOM for 23 weeks as an independent positive control to confirm the specific and efficiency of β-catenin antibodies. (*b*) Expression level of calmodulin as a biomarker of $Ca2^{+}$-dependently noncanonical Wnt/β-catenin pathway was analyzed by western blot. (*c*) Expression level of autophagy related proteins were detected by western blot analysis. B*a*P/DSS treatment significantly blocked the p62 and CX-43 protein degradation, indices of autophagic degradation processes by induced autophagic defect which phenomenon was prevented by mice fed with PMFs. (*d*) Examination of the fusion status of autophagosomes and lysosome detected co-localization of LC3B and LAMP-1 by double-immunohistochemistry fluorescence staining with anti-LC3B (green) and anti-LAMP-1 (red) antibodies. PMFs treatment significantly improved defective autophagy by promoted autophagosomes and lysosomes fuse (yellow). [Color figure can be viewed at wileyonlinelibrary.com]

Park *et al.* and Galluzzi *et al.* found that partial autophagic defects support malignant cell transformation.[39,40] We used the fluorescent multiplex IHC with LC3B (green) and LAMP-1 (red) antibodies to confirm the phenomenon of autophagic defect. The formation of co-localization puncta (yellow) of LC3B and LAMP1 were inhibited in the BaP/DSS-treatment group by suppressed autophagosomes and lysosomes that fused to form autolysosomes (Fig. 4*d*). This was based on the fact that the pretreatment of mice with PMFs alleviated the BaP/DSS-induced autophagic defect.

PMFs altered the composition of gut microbiota similar to control group and increased butylate-producing probiotics

Gut microbiota composition is associated with a wide range of chronic human diseases as well as gastroenterologic diseases.[41,42] In this study, we found that BaP/DSS raised an abundance of *Sphingobacteriia* and *Gammaproteobacteria*. This was decreased in mice treated with PMFs (Fig. 5*a* and Supporting Information, Fig. S8A). Moreover, the CRC-related class *Bacilli* was lower in mice fed with 1.0% PMFs (1.0%) than the BaP/DSS Group (4.6%). In taxonomic order, the abundance of *Erysipelortrichales and Lactobacillales* was raised in DSS-induced colitis mice and in cancerous colonic tissue, respectively.[43,44] Similar results were found in this study, but this composition of microbiota was downregulated in the PMFs pre-treatment group (Fig. 5*b* and Supporting Information, Fig. S8b). A recent study by Kim *et al.* demonstrated that polyphenolic inhibited colorectal inflammation and colitis increases *Ruminococcaceae* which includes several butyrate-producing bacteria.[45] As shown in Figure 5*c* and Supporting Information, Figure S8c, the levels of Ruminococcaceae and *Ruminococcus* were increased in the 1.0% PMFs pretreatment group to 9.8% and 9.4%, compared with the BaP/DSS group which increased to 5.3% and 2.7%. Taxonomic bins at the genus level showed that the proportion of *Parabacteroides* significantly increased in BaP/DSS mice while oral administration of PMFs weakened this increase (Fig. 5*d* and Supporting Information, Fig. S8d). Additionally, consumption of PMFs also altered the composition of gut microbiota and made it similar to that in the control group by increasing the butyrate-producing probiotics, such as *Blautia* spp., and decreasing CRC-related bacteria, such as *Lactobacillus ruminis,* that is involved in inducing colon inflammation through activation of toll-like receptor 5 (Fig. 5*e*).

Discussion

In this study, the comprehensive tumorigenesis mechanisms induced by BaP/DSS were investigated, and we also explored how PMFs prevent BaP/DSS-induced tumor formation. At the tumor initiation stage, mice pretreated with PMFs can benefit from their protective effect on BPDE-DNA adduct generation through modulating XMEs expressed for decreasing reactive metabolites, such as BaP-quinones, and increasing detoxification metabolites in faeces.[29] The feces sample for analyzed BaP metabolites were collected during mice who were treated with BaP for 6 days, and those results explained the effects of PMFs on BaP metabolism. The expression level of XMEs was examined at the end of the treatment for 8 weeks. We will be determining the XMEs during BaP oral administration in a future study to present direct evidence for PMFs' effect on BaP metabolism via modulation XMEs.

To explain the effect of PMFs on the promotion/progression stage induced by BaP/DSS, 111 CRC-related gene expressions were determined by RNA-sequencing. As expected, BaP/DSS significantly activated inflammatory responses, proliferation and metastasis biomarkers and reduced cell cycle regulators and antioxidation proteins. Our findings demonstrate the beneficial effects of PMFs on preventing carcinogenesis in this model by the improvement in aberrant gene expression. We also found that *PCDH17*, a novel TSG and involved regulation autophagy,[30] notably downregulated during BaP/DSS treatment and may participate in the defective autophagy that serves cell malignant transformation as reported by Park *et al.*[39] However, treatment with PMFs acts as an autophagy regulation role by ameliorating BaP/DSS-induced autophagic defects through promoted autophagosomes that fuse with lysosomes and lead to the inhibition of cancer growth. Interestingly, we discovered that BaP/DSS did not induce the expression and accumulation of β-catenin which is highly expressed in most CRC models.[25] Similar to the results observed in this study, Zuo *et al.* showed a downregulated Wnt/β-catenin pathway in BaP-treated mice.[46] Inhibiting the β-catenin signaling pathway may contribute to BaP induced promotion of DNA mutation.[47]

Several studies suggested that the composition of gut microbiota has a close relationship with human diseases, including CRC.[42] This study is the first work to assess the effect of BaP/DSS-induced tumorigenesis on microbiota by examining the preventive effect of PMFs with oral administration. In this study, we found that BaP/DSS treatment group were significantly increased PAHs or carbon compounds degradation bacteria, such as *Sphingobacteria* and *Gammaproteobacteria*.[48] Those bacteria may be promoting BaP convert to reactive metabolites. Additionally, PAHs metabolites have been recognized to undergo enterohepatic circulation by reacted with gut microbiota contribute to intestine re-exposed reactive metabolites and increase DNA damage level.[49] The differential alterations of these gut microbiota in this study may directly lead to increasing butyrate-producing probiotics, such as *Ruminococcaceae* and *Oscillospira*, and decreasing CRC-related bacteria including Porphyromonadaceae and *Lactobacillus ruminis*.[50] Additionally, PMFs prevented BaP/DSS from changing the microbiota composition in that group and kept it similar to that in the healthy control group.

In conclusion, this study contributes new knowledge regarding the widespread mechanisms in BaP/DSS promoted colonic tumor formation. In addition to providing evidence towards the understanding of PMFs preventing colorectal tumorigenesis induced by BaP/DSS, this study supports PMFs as a powerful chemopreventive agent linked to modulating

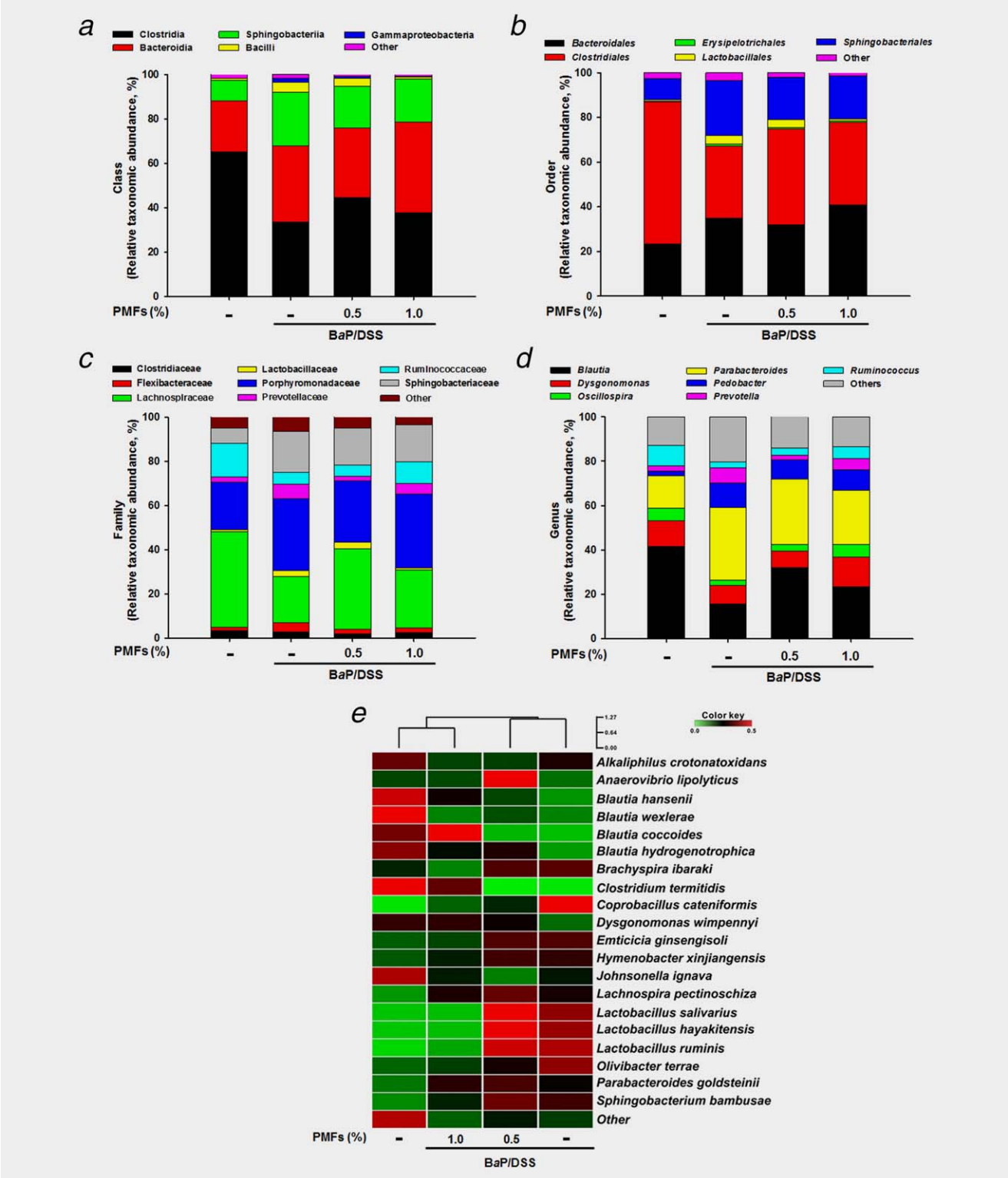

Figure 5. Effect of PMFs on colonic microbiota composition in B*a*P/DSS-induced colorectal tumorigenesis. The relative abundance of the predominant colonic bacteria according to (*a*) class (relative abundance above 1% of community); (*b*) order (relative abundance above 3% of community); (*c*) family (relative abundance above 3% of community); (*d*) genus (relative abundance above 5% of community). (*e*) Heat-map showing the similarity of different treatment groups with respect to the most abundant colonic bacterial species. Groups (columns) were clustered using the average linkage method based on correlation distance as the Euclidean distance metric. Colors indicate normalization row values relative to the mean. [Color figure can be viewed at wileyonlinelibrary.com]

PAHs metabolism, regulating CRC-related gene expression and improving complete autophagy and gut microbiota homeostasis (Supporting Information, Fig. S9).

Author Contributions

Jia-Ching Wu and Mei-Ling Tsai and Ching-Shu Lai designed experiments, carried out experiments, analyzed data and wrote the manuscript. Jia-Ching Wu and Mei-Ling Tsai did the bacterial 16 S rDNA sequencing and data analysis. Chi-Tang Ho and Ying-Jan Wang provided research insight and suggestions for modification of the manuscript. Min-Hsiung Pan conceived and designed experiments and reviewed the manuscript. Jia-Ching Wu and Chih-Yu Lo analyzed and interpreted data.

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