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Targeted metabolomics identifies the cytochrome P450 monooxygenase eicosanoid pathway as a novel therapeutic target of colon tumorigenesis

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Disclosures

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Abstract

Colon cancer is the third most common cancer and the second leading cause of cancer-related death in the United States, emphasizing the need for discovery of new cellular targets. Using a metabolomics approach, we report here that epoxygenated fatty acids (EpFA), which are eicosanoid metabolites produced by cytochrome P450 (CYP) monooxygenases, were increased in both the plasma and colon of azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colon cancer mice. CYP monooxygenases were overexpressed in colon tumor tissues and colon cancer cells. Pharmacological inhibition or genetic ablation of CYP monooxygenases suppressed AOM/DSS-induced colon tumorigenesis in vivo. In addition, treatment with 12,13- epoxyoctadecenoic acid (EpOME), which is a metabolite of CYP monooxygenase produced from linoleic acid, increased cytokine production and JNK phosphorylation in vitro and exacerbated AOM/DSS-induced colon tumorigenesis in vivo. Together these results demonstrate that the previously unappreciated CYP monooxygenase pathway is upregulated in colon cancer, contributes to its pathogenesis, and could be therapeutically explored for preventing or treating colon cancer.

Keywords

Colon cancer; EpOMEs; cytochrome P450 monooxygenases; metabolomics

Introduction

Colon cancer is the third most common cancer and the second leading cause of cancer-related death in the United States (1). It is important to discover novel cellular targets which are crucial in the pathogenesis of colon cancer, which could facilitate development of mechanism-based strategies to reduce the risks of colon cancer. Eicosanoids, which are endogenous lipid signaling molecules produced from enzymatic metabolism of arachidonic acid (ARA, 20:4ω−6), play essential roles in inflammation and have recently been implicated in cancer (2,3). There are three major pathways involved in biosynthesis of eicosanoids: cyclooxygenases (COX) producing prostaglandins and thromboxanes, lipoxygenases (LOX) producing leukotrienes and hydroxyl fatty acids, and cytochrome P450 (CYP) monooxygenases producing epoxygenated fatty acids (EpFAs) (4,5). Besides ARA, other polyunsaturated fatty acids, such as linoleic acid (LA, 18:2ω−6), α-linolenic acid (ALA, 18:3ω−3), and docosahexaenoic acid (DHA, 22:6ω−3), are also efficient alternative substrates for these metabolizing enzymes, which convert them to the corresponding fatty acid metabolites (4,5). As a result, the enzymatic metabolism of polyunsaturated fatty acids leads to formation of a large array of eicosanoid metabolites, many of which act as autocrine or paracrine mediators to regulate inflammation and hemostasis (4,5).

Previous research has shown that certain eicosanoid metabolites are deregulated in colon cancer and contribute to its pathogenesis (6). Notably, the most prominent cancer-associated eicosanoids are prostaglandins, which are produced by COX-2 that is overexpressed in most human colon cancer samples (3). Guo et al. reported the increased ARA and COX-produced metabolite prostaglandin A_2 in the plasma of *Apc*$^{min/+}$ mice (7). Clinical and epidemiological studies support that pharmacological inhibitors of COX-2 are highly effective for preventing colon cancer (3), however, the gastrointestinal and cardiovascular toxicities induced by COX-2 inhibitors have limited their clinical applications (8,9). Besides COX-2, the roles of other eicosanoid pathways in colon tumorigenesis are not well understood (6). It is important to discover novel eicosanoid signaling pathways involved in colon tumorigenesis.

Most previous studies to investigate the roles of eicosanoids in colon tumorigenesis have only studied single or limited number of eicosanoid metabolite(s), few systematic analyses have been carried out, hampering our understanding of the roles of eicosanoid signaling in colon tumorigenesis (6). To discover novel eicosanoid metabolites and pathways involved in colon tumorigenesis, in this study we use a liquid chromatography tandem-mass spectrometry (LC-MS/MS)-based targeted metabolomics to systematically profile eicosanoids in a well-established azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colon cancer model in C57BL/6 mice, then use pharmacological and genetic approaches to validate the functional roles of identified metabolite in colon tumorigenesis.

Materials and Methods

Animal experiment

The animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst (protocol number: 2017–0019) and National Institutes of Health/National Institute of Environmental Health Sciences (protocol number: 05–18).

Animal Protocol 1: AOM/DSS-induced colon tumorigenesis in mice

C57BL/6 male mice (age = 6 weeks) were purchased from Charles River and maintained on a modified AIN-93G diet (containing 10% fat, corn oil was the only source of fat content, diet composition was described in Table S1) for 3 weeks, then the mice were divided into two groups: (1) the mice in the AOM/DSS group were treated with 10 mg/kg AOM (Sigma-Aldrich) via intraperitoneal injection, at week 1 post the AOM injection, they were given 2% DSS (36–50 kDa, MP Biomedicals) in drinking water for 1 week; and (2) the mice in control group were maintained on the same diet without AOM/DSS treatment. At week 9.5 post the AOM injection, the mice were sacrificed to harvest plasma and colon tissues for analysis. For analysis of colon tumor, colon tissues were cut open longitudinally, washed in PBS and inspected under a dissection microscope. The size of the tumor was determined by the following formula: tumor size = $\pi \times d^2/4$, where d is the diameter of each tumor. The methods for biochemical analyses are described in supplemental Materials and Methods, and the sequence of RT-PCR primers are listed in Table S2.

Animal Protocol 2: AOM/DSS-induced colon tumorigenesis in *Cyp2c*$^{+/+}$, *Cyp2c*$^{+/-}$ and *Cyp2c*$^{-/-}$mice

Littermate wildtype *Cyp2c*$^{+/+}$ mice, heterozygous *Cyp2c*$^{+/-}$ mice and knockout *Cyp2c*$^{-/-}$ mice (background on C57BL/6, age = 6 weeks) were treated with 10 mg/kg AOM via i.p. injection. At week 1 post the AOM injection, they were given 2% DSS in drinking water for 1 week. During the whole experiment, the mice were maintained on mouse chow. At week 9.5 post the AOM injection, the mice were sacrificed to collect blood and colon tissues for analysis.

Animal Protocol 3: Effects of 12,13- epoxyoctadecenoic acid (EpOME) on AOM/DSS-induced colon tumorigenesis

C57BL/6 male mice (age = 6 weeks) were treated with 10 mg/kg AOM via i.p. injection. At week 1 post the AOM injection, they were given 2% DSS in drinking water for 1 week. At week 5 post the AOM injection, the mice were subcutaneously implanted with osmotic mini-pumps (Durect, model 1004), which contained 12,13-EpOME (Cayman, dose = 2 mg/kg/day) or vehicle (a 1:1 vol/vol mixed solution of DMSO and PEG 400). During the whole experiment, the mice were maintained on mouse chow. At week 9 post the AOM injection, the mice were sacrificed to collect blood and colon tissues for analysis.

Animal Protocol 4: Effects of 12,13-EpOME on MC38 primary tumor growth in mice

C57BL/6 male mice (age = 6 weeks) were subcutaneously implanted with osmotic mini-pumps (Durect, model 1004), which contained vehicle (a 1:1 vol/vol mixed solution of DMSO and PEG 400) or 12,13-EpOMEs (dose = 2 mg/kg/day). After one week of mini-pump implantation, 4×10^5 MC38 colon cancer cells (purchased from Kerafast, catalog number ENH204, cell line authentication and mycoplasma testing have been verified by company) in 100 μL PBS were subcutaneously injected into each mouse to initiate primary tumor growth. Tumor size was measured using caliper, at the end of the experiment, the tumors were dissected, weighed and subjected to biochemical analysis.

Animal Protocol 5: Effects of CYP inhibitors (SKF-525A and clotrimazole) on AOM/DSS-induced colon tumorigenesis in mice

C57BL/6 male mice (age = 6 weeks, maintained on mouse chow) were treated with 10 mg/kg AOM via i.p. injection. At week 1 post the AOM injection, they were given 2% DSS in drinking water for 1 week. After the AOM/DSS stimulation, the mouse diet was changed to a modified AIN-93G diet containing 200 ppm SKF-525A or clotrimazole (Alfa Aesar) dissolved in polyethylene glycol 400 (PEG 400, Millipore) as vehicle (0.1% in diet, v/w), or vehicle alone for 6 weeks. The diets were freshly prepared and changed every 2–3 days. At week 8 post the AOM injection, the mice were sacrificed to collect blood and colon tissues for analysis.

Statistical analysis

Data are expressed as means ± SEM. Shapiro–Wilk test was used to verify the normality of data and Levene's mean test was used to assess equal variance of data. Statistical comparison of two groups was performed using either Student's t test or Wilcoxon-Mann-

Whitney test (when normality test is failed), comparison of three groups was analyzed by either parametric one-way ANOVA or nonparametric one-way ANOVA (Kruskal–Wallis test by ranks, used when normality test is failed) followed by Dunn's post hoc test. All of these data analysis was performed by using SigmaPlot software (Systat Software, Inc). P values less than 0.05 are reported as statistically significant. Gene expression data of The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma matched tumor and non-tumor were obtained from the Firebrowse (http://firebrowse.org/).

Results

CYP monooxygenase-produced eicosanoid metabolites are elevated in the plasma and colon of AOM/DSS-induced colon cancer mice

To our knowledge, a metabolomics-based approach to systematically profile eicosanoids in colon cancer has not been attempted (6). In an effort to better understand the roles of eicosanoids in colon tumorigenesis, we used a LC-MS/MS-based targeted metabolomics to compare the profiles of eicosanoids in the plasma and colon of control healthy mice (not treated with AOM/DSS) and colon cancer mice (treated with AOM/DSS) (Fig. 1A). In agreement with previous studies of the AOM/DSS model (10), 100% of the AOM/DSS-treated mice developed colon tumors (Fig. 1B), with increased expressions of pro-inflammatory genes (*Il-6*, *Tnf-α*, *Mcp-1*, and *Cox-2*) in the colon tissues (Fig. 1C).

LC-MS/MS detected 42 metabolites in the plasma and 56 metabolites in the colon (see complete LC-MS/MS result in Table S3–4). Among the detected metabolites, only CYP monooxygenase-produced EpFAs are significantly increased in both the plasma and colon tissues of the AOM/DSS-induced colon cancer mice (Fig. 1D-G). Indeed, the concentrations of an array of EpFAs, including 9,10- and 12,13-epoxyoctadecenoic acid (EpOME) produced from LA (Fig. 1D), 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EET) from ARA (Fig. 1E), 9,10- and 15,16-epoxyoctadecadienoic acid (EpODE) from ALA (Fig. 1F), and 19,20-epoxydocosapentaenoic acid (EDP) from DHA (Fig. 1G), are increased in the plasma and colon of the tumor-bearing mice.

CYP monooxygenases are overexpressed in the colon of AOM/DSS-induced colon cancer mice

To understand the underlying mechanisms behind elevated concentrations of EpFAs in colon cancer, we studied the expression of enzymes involved in EpFA production and removal. The biosynthesis and degradation of EpFAs involves three enzymatic steps: membrane-bound fatty acids are released by phospholipase A_2 (PLA_2) and related enzymes to generate intracellular free-form fatty acids, which are then metabolized by CYP monooxygenases (predominately CYP2C isoforms) to form EpFAs, which then undergo degradation by soluble epoxide hydrolase (sEH) (5,11). qRT-PCR showed that the expression of *Pla2g4a* (encoding cytosolic calcium-dependent PLA_2) was unchanged, the expression of several *CYP* monooxygenases (including mouse *Cyp2c38*, *Cyp2c39*, *Cyp2c65*, and *Cyp2c70*) was increased ($P < 0.05$), and the expression of *Ephx2* (encoding sEH) was decreased ($P < 0.05$), in colon tissue of mice with colon cancer (Fig. 1H). Together, these results support that there

is enhanced biosynthesis and reduced degradation of EpFAs in colon tumors of mice, which contributes to elevated EpFAs in colon cancer.

CYP monooxygenases are overexpressed in human colon cancer cells

To further validate that CYP monooxygenases are overexpressed in colon cancer, we studied their expressions in human colon cancer cells. Compared with normal human colon cells (CCD-18Co), the gene expression of *CYP* monooxygenases (human *CYP2C8*, *2C9*, *2C19*, and *2J2*) was significantly ($P < 0.05$) increased in human colon cancer cells (HCT116 and Caco-2) (Fig. S1A). Consistent with the qRT-PCR result, immunoblotting showed that the protein expression of CYP monooxygenase (using human CYP2C9 as a marker) was increased in human colon cancer cells (Fig. S1B). These results demonstrate that the CYP monooxygenases are overexpressed in human colon cancer cells, which is consistent with the results from animal experiments.

Genetic ablation of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis

To determine the roles of CYP monooxygenases in colon cancer, we tested whether genetic ablation of CYP monooxygenases modulates colon tumorigenesis. To this end, we performed the AOM/DSS-induced colon cancer model in a recently developed *Cyp2c* gene cluster knockout mouse, which has deletions of fourteen mouse *Cyp2c* genes, including *Cyp2c29*, *2c37*, *2c38*, *2c39*, *2c40*, *2c50*, *2c54*, *2c55*, *2c65*, *2c66*, *2c67*, *2c68*, *2c69*, and *2c70* (12) (Fig. 2A). Compared with $Cyp2c^{+/+}$ mice, the expression of *Cyp2e1* (encoding the enzyme Cyp2e1 to activate the mutagenic activity of AOM (13)) was not changed in $Cyp2c^{+/-}$ or $Cyp2c^{-/-}$ mice (Fig. S2), supporting that it is feasible to perform the AOM/DSS-induced colon tumorigenesis model in these mice.

Compared with AOM/DSS-induced $Cyp2c^{+/+}$ mice, the AOM/DSS-induced $Cyp2c^{+/-}$ mice had lower tumor number and total tumor burden (Fig. 2B), reduced expression of tumorigenic markers proliferating cell nuclear antigen (PCNA) and active β-catenin (Fig. 2C), and attenuated expression of pro-inflammatory and pro-tumorigenic genes (*Tnf-α*, *Il-1β*, *Axin2* and *C-myc*), in the colon tumor tissue (Fig. 2D), illustrating reduced colon tumorigenesis. In addition, we found that compared with AOM/DSS-induced $Cyp2c^{+/+}$ mice, the colon of AOM/DSS-induced $Cyp2c^{+/-}$ mice had lower colonic expression of *Cyp* monooxygenase (using mouse *Cyp2c38* as a marker, Fig. 2D) and reduced colonic concentrations of CYP monooxygenase-produced EpFAs (Fig. 2E, see complete LC-MS/MS result in Table S5), supporting the involvement of CYP monooxygenase pathway in colon tumorigenesis.

We should point out that compared with $Cyp2c^{+/+}$ mice, $Cyp2c^{+/-}$ mice showed minimal signs of basal inflammation, but $Cyp2c^{-/-}$ mice had severe basal liver inflammation, with increased expression of pro-inflammatory genes (*Tnf-α* and *Il-1β*, Fig. S3A) and enhanced infiltration of inflammatory cells (Fig. S3B) in the liver, as described previously (12). When $Cyp2c^{-/-}$ mice were stimulated with AOM/DSS, there was rapid death within 1–3 days post the DSS treatment (6 out of 8 $Cyp2c^{-/-}$ mice died during this period, Fig. S4), which could

be caused by exaggerated inflammatory responses induced by the DSS, making it difficult to use the *Cyp2c*$^{-/-}$ mice to study colon tumorigenesis.

Pharmacological inhibition of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis

We tested the effects of two different CYP monooxygenase inhibitors, SKF-525A and clotrimazole (14,15), on AOM/DSS-induced colon tumorigenesis in mice. Since the mutagenic activity of AOM requires metabolic activation by Cyp2e1 (13), we initiated treatment of SKF-525A and clotrimazole after the AOM and DSS treatment (see animal experiment scheme in Fig. S5A). We found that oral administration of these two inhibitors suppressed AOM/DSS-induced colon tumorigenesis in mice (Fig. S5B). Furthermore, treatment with these two inhibitors reduced expression of PCNA (a marker of cell proliferation), increased expression of cleaved caspase-3 (a marker of cell apoptosis), and decreased expression of pro-inflammatory and pro-tumorigenic genes (*Tnf-α*, *Mcp-1*, *Il-6*, *Il-1β*, *Ifn-γ*, *Axin2*, and *Cox-2*), in colon tumors (Fig. S5C-D). These results support that pharmacological inhibition of CYP monooxygenases suppressed AOM/DSS-induced colon tumorigenesis.

Treatment with EpOME, but not other CYP monooxygenase metabolites, increases inflammation and JNK phosphorylation in macrophage cells and colon cancer cells

To determine the specific metabolites involved in the colon cancer-enhancing effects of CYP monooxygenases, we studied the biological actions of CYP monooxygenase metabolites. The ω−6-series CYP metabolites, including EpOMEs produced from LA and EETs produced from ARA, are the most abundant EpFAs in the plasma and tissues (Fig. 1), therefore, we focused on these metabolites. Treatment with 9,10- and/or 12,13-EpOME (concentration = 100 nM) increased gene expression of pro-inflammatory cytokines in mouse macrophage RAW 264.7 cells and human colon cancer HCT-116 cells (Fig. 3A-C). In contrast, other types of CYP metabolites, including the down-stream metabolites of EpOMEs termed 9,10- and 12,13-dihydroxyoctadecenoic acid (DiHOME) or CYP metabolites derived from other fatty acids, such as 11,12- and 14,15-EET, had no such effects (Fig. 3A-C).

Since 12,13-EpOME showed that most potent effect to induce inflammation *in vitro*, we further studied this metabolite. Treatment with 12,13-EpOME (concentration = 1–100 nM) increased gene expression of *Il-6* and *Mcp-1* in a dose-dependent manner in RAW 264.7 cells (Fig. 3D). ELISA analysis further validated that 12,13-EpOME increased protein levels of IL-6 and MCP-1 in RAW 264.7 cells (Fig. 3E). Consistent with its enhancing effect on inflammation, 12,13-EpOME induced rapid phosphorylation of JNK (Fig. 3F). Similar results were also observed in colon cancer HCT-116 cells (Fig. 3G-H). Together, these results demonstrate that 12,13-EpOME had pro-inflammatory effects *in vitro*.

Treatment with EpOME exaggerates AOM/DSS-induced colon tumorigenesis *in vivo*

We determined the actions of 12,13-EpOME on colon tumorigenesis *in vivo*. To this end, we stimulated mice with AOM/DSS to induce colon tumors, then treated the mice with 12,13-EpOME or vehicle via Alzet® osmotic mini-pumps (Fig. 4A). Compared with vehicle-

treated AOM/DSS mice, the 12,13-EpOME-treated AOM/DSS mice had increased tumor number, tumor size, and total tumor burden, illustrating exacerbated colon tumorigenesis (Fig. 4B). Consistent with the increased colon tumorigenesis, treatment with 12,13-EpOME enhanced infiltration of $CD45^+$ and $CD45^+$ $F4/80^+$ immune cells, increased expression of pro-inflammatory and pro-tumorigenic genes (*Tnf-α*, *Il-1β*, and *Axin2*), and upregulated expression of tumorigenic markers (PCNA and active β-catenin), in the colon tumor (Fig. 4C-E). We further evaluated the effect of 12,13-EpOME on colon tumorigenesis in a second colon cancer mode, the MC38 xenograft model in C57BL/6 mice. We found that 12,13-EpOME had little effect on MC38 primary tumor growth (Fig. S6A-C), though it increased expressions of pro-inflammatory cytokines (*Mcp-1* and *Il-6*) in the tumors (Fig. S6D).

Discussion

Colon cancer is the third most common cancer and the second leading cause of cancer-related death in United States (1), emphasizing the need for discovery of novel cellular targets which are crucial in the pathogenesis of colon cancer. Using a LC-MS/MS-based targeted metabolomics, the central finding of our research is that EpFAs, which are eicosanoid metabolites produced by CYP monooxygenases, are significantly elevated in both the circulation and colon tissues of the AOM/DSS-induced colon cancer mice. On the basis of this finding, we further demonstrate that CYP monooxygenases are overexpressed in colon cancer and play critical roles in colon tumorigenesis. Together, our findings demonstrate that the previously unappreciated CYP/EpFA axis is upregulated in colon cancer, contributes to its pathogenesis, and could be therapeutically explored for preventing or treating colon cancer.

Here we show that CYP monooxygenase-produced EpFAs are increased in both plasma and colon of the AOM/DSS-induced colon cancer mice. A previous study showed that in DSS-induced colitis models, the circulating concentrations of EpFAs were not changed (16). Together, these results suggest that colon tumor, but not colonic inflammation, induces the CYP monooxygenase pathway. There could be many mechanisms by which the CYP monooxygenases are overexpressed in colon tumors. The expression of CYP monooxygenases has been shown to be elevated by hypoxia (5), which is a common feature of tumor tissues (17). Therefore, the hypoxic tumor microenvironment could contribute to the increased expression of CYP monooxygenases in tumor tissues. To date, the expression pattern of CYP monooxygenases in human colon tumor tissues are not well understood. We analyzed gene expression of *CYP monooxygenases* (*CYP2C8*, *CYP2C9*, *CYP2C19*, and *CYP2J2*) in TCGA database, and found that their expressions were not increased in colorectal adenocarcinoma (Fig. S7). However, we have to point out the functions of CYP enzymes are regulated by multiple mechanisms, including transcription, translation, and posttranslational modification (18). Previous research support that the protein expressions of CYP monooxygenases are upregulated in human colon cancer. Enayetallah et al. showed that CYP2C9 is detected in 13 out of 17 human colon tumor samples, while it is not detected in matched benign samples (19). In addition, recent studies have shown that CYP2W1 is highly expressed in embryonic colon and malignant colon tumors (20,21), and CYP2J2 is overexpressed in human colon tumors (22). More studies are needed to better understand the changes of CYP monooxygenase pathway in colon tumorigenesis.

Our results support that EpOMEs are critical regulators of colon tumorigenesis. We show that 9,10- and 12,13-EpOME are elevated in the circulation of mice with colon cancer; in addition, 12,13-EpOME has direct and potent effects to induce inflammation *in vitro*, and exacerbate colitis-associated colon tumorigenesis *in vivo*. A better understanding of the roles of EpOMEs in human colon cancer could help to develop EpOMEs as potential biomarkers of colon cancer, which could have important clinical implications. Treatment with 12,13-EpOME increased AOM/DSS-induced colon tumorigenesis, but had little effect on MC38 xenograft tumor growth. There could be multiple mechanisms for the observed results. Transplantation of cancer cells (such as MC38 cells) into immunocompetent hosts induce severe inflammation within the first few days (23), which could obscure the actions of 12,13-EpOME. In addition, the xenograft MC38 model is highly aggressive, which could also contribute to the lack of effects of 12,13-EpOME in this model. Previous studies, performed in other disease models, have shown that EpOMEs have an array of detrimental effects on human health. EpOMEs are elevated in the circulation of patients with severe burns, and are associated with multiple organ failure and adult respiratory distress syndrome in these patients (24–27). In animal studies, treatment with high-dose EpOMEs induced pulmonary edema, lung injury, and cardio-depression (28–30). Together, these results support that EpOMEs could contribute to the pathogenesis of colon cancer, as well as other human diseases.

The tissue concentrations of EpOMEs are in part mediated by the levels of LA in membrane phospholipids (5). The consumption of LA, which is highly abundant in vegetable oil products (such as corn, soybean, and canola oils, as well as fried food, salad dressing, and mayonnaise), is very high in western countries (31). Substantial animal experiments showed that a high dietary intake of LA is associated with increased AOM-induced colon tumorigenesis (32–36), but the underlying mechanisms are not well understood. Here our study showed that EpOMEs have potent effects to induce inflammation and colon tumorigenesis *in vitro* and *in vivo*, suggesting that EpOMEs could serve as a potential mechanistic linkage between overconsumption of LA and elevated risks of colon cancer. Validation of the roles of EpOMEs involved could help to design human studies to clarify the impact of LA consumption on colon tumorigenesis, which could lead to significant impact for public health.

Using both pharmacological and genetic approaches, our results support that CYP monooxygenases could be a potential therapeutic target of colon cancer. We showed that pharmacological inhibition or genetic ablation of CYP monooxygenases suppresses AOM/DSS-induced colon tumorigenesis in mice. The mutagenic activity of AOM requires metabolic activation by Cyp2e1 (13), in our experiments we used two strategies to minimize the potential impact of inhibition or deletion of CYP monooxygenases on AOM activation: (1) in the pharmacological inhibition experiment, we initiated the inhibitor treatment (SKF-525A and clotrimazole) 2 weeks post the AOM injection, and (2) in the genetically engineered mouse experiment, we checked gene expression of *Cyp2e1* and found that its expression was not altered $Cyp2c^{+/-}$ or $Cyp2c^{-/-}$ mice. Together, our results support that inhibition or deletion of CYP monooxygenases suppresses colon tumorigenesis, and this effect is not mediated by impaired AOM activation. This finding is in agreement with our previous report, which showed that compared with WT mice, genetically engineered mice

with endothelial overexpression of CYP2C8 monooxygenase (Tie2-CYP2C8 Tr mice) have enhanced xenograft tumor growth of B16F10 melanoma and T241 fibrosarcoma (37). In our experiment, we found that inhibition or deletion of CYP monooxygenases attenuated tumor inflammation, it remains to determine whether the attenuated inflammation is a direct consequence resulted from inhibition or deletion of CYP monooxygenases, or it is due to reduced colon tumorigenesis. Previous studies which have shown that CYP monooxygenases and their metabolites EpFAs have anti-inflammatory actions in many disease models (5), while the roles of this pathway in tumor inflammation are not well characterized. To better understand the roles of CYP monooxygenases in colon tumorigenesis, it is important to further study the extent to which inhibition or deletion of CYP monooxygenases affects tumorigenesis in other colon cancer models, such as *adenomatous polyposis coli* (*Apc*) mutation-induced colon tumorigenesis. Together, these results support that targeting CYP monooxygenases could be a potential strategy to inhibit colon cancer, as well as other types of cancer. Previous studies showed that some FDA-approved drugs are potent inhibitors of CYP monooxygenases (38), these drugs could be repurposed for preventing or treating colon cancer; and novel monooxygenase inhibitors could be developed for human translation. Furthermore, it is important to test how these CYP inhibitors would interact with standard colon cancer chemotherapy drugs to affect colon tumorigenesis.

In conclusion, our study demonstrates that the previously unappreciated CYP monooxygenase pathway is upregulated in colon cancer, contributes to its pathogenesis, and could be therapeutically explored for preventing or treating colon cancer. A better understanding of its roles in colon tumorigenesis could help to develop novel therapeutic targets or biomarkers of colon cancer, facilitating the development of mechanism-based strategies to reduce the risks of colon cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance: We find that the previously unappreciated CYP monooxygenase eicosanoid pathway is deregulated in colon cancer and contributes to colon tumorigenesis.

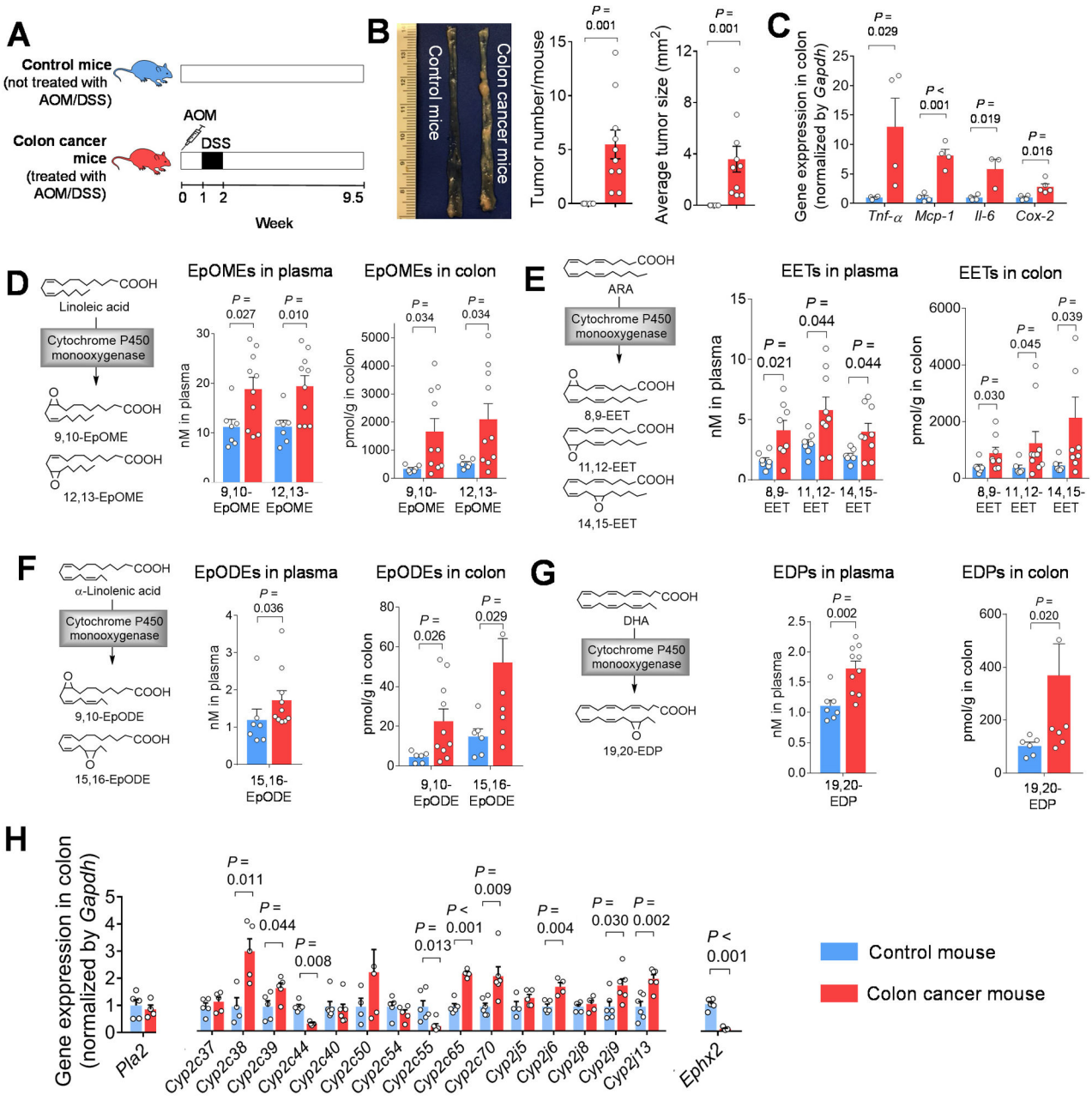

Figure 1. CYP monooxygenase-produced eicosanoid metabolites are increased in the plasma and colon of AOM/DSS-induced colon cancer mice.

(A) Scheme of animal experiment. (B) Quantification of colon tumor in mice (n = 6 in control group, and n = 10 in colon cancer group). (C) Gene expression of *Tnf-α, Mcp-1, Il-6* and *Cox-2* in colon (n = 4 per group). (D-G) Concentrations of CYP monooxygenase-produced EpFAs in plasma and colon (n = 6-10 per group). (H) Expression of *Pla2*, *Cyp monooxygenases*, and *Ephx2* (encoding sEH) in colon (n = 4-6 per group). The results are expressed as mean ± SEM. The statistical significance of two groups was determined using Student's *t* test or Wilcoxon-Mann test.

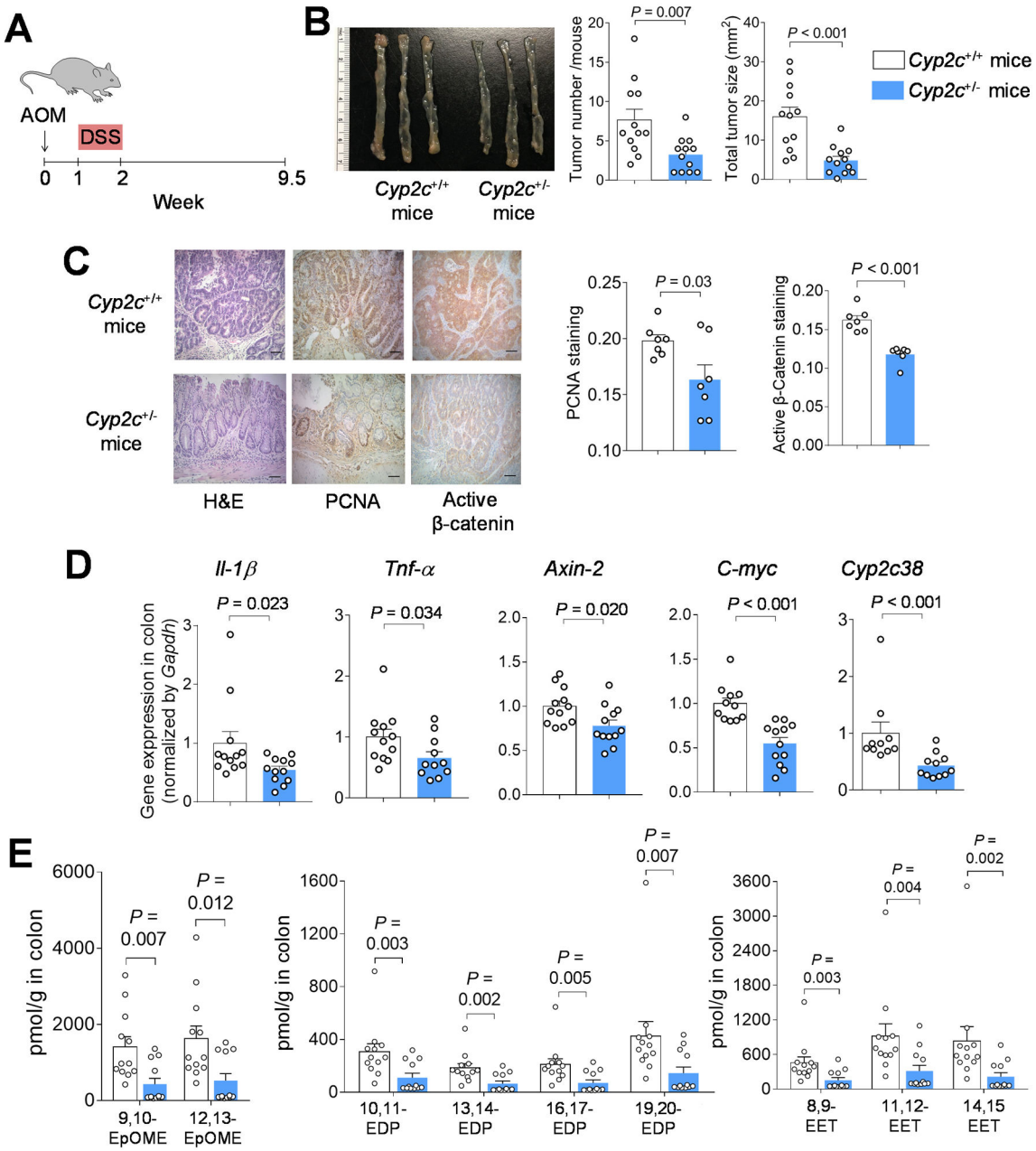

Figure 2. Compared with *Cyp2c*$^{+/+}$ mice, the AOM/DSS-induced colon tumorigenesis is reduced in *Cyp2c*$^{+/-}$ mice.

(A) Scheme of animal experiment. (B) Quantification of colon tumorigenesis (n = 12 per group). (C) H&E histology, and immunohistochemical staining of PCNA and β-catenin in colon (n = 7 per group, scale bar: 50 μm). (D) Expression of pro-inflammatory and pro-tumorigenic genes, and *Cyp monooxygenase* (using *Cyp2c38* as a marker) in colon (n = 10-12 per group). (E) Concentrations of CYP monooxygenase-produced metabolites in colon (n = 12 per group). The results are expressed as mean ± SEM. The statistical significance of two groups was determined using Student's *t* test or Wilcoxon-Mann test.

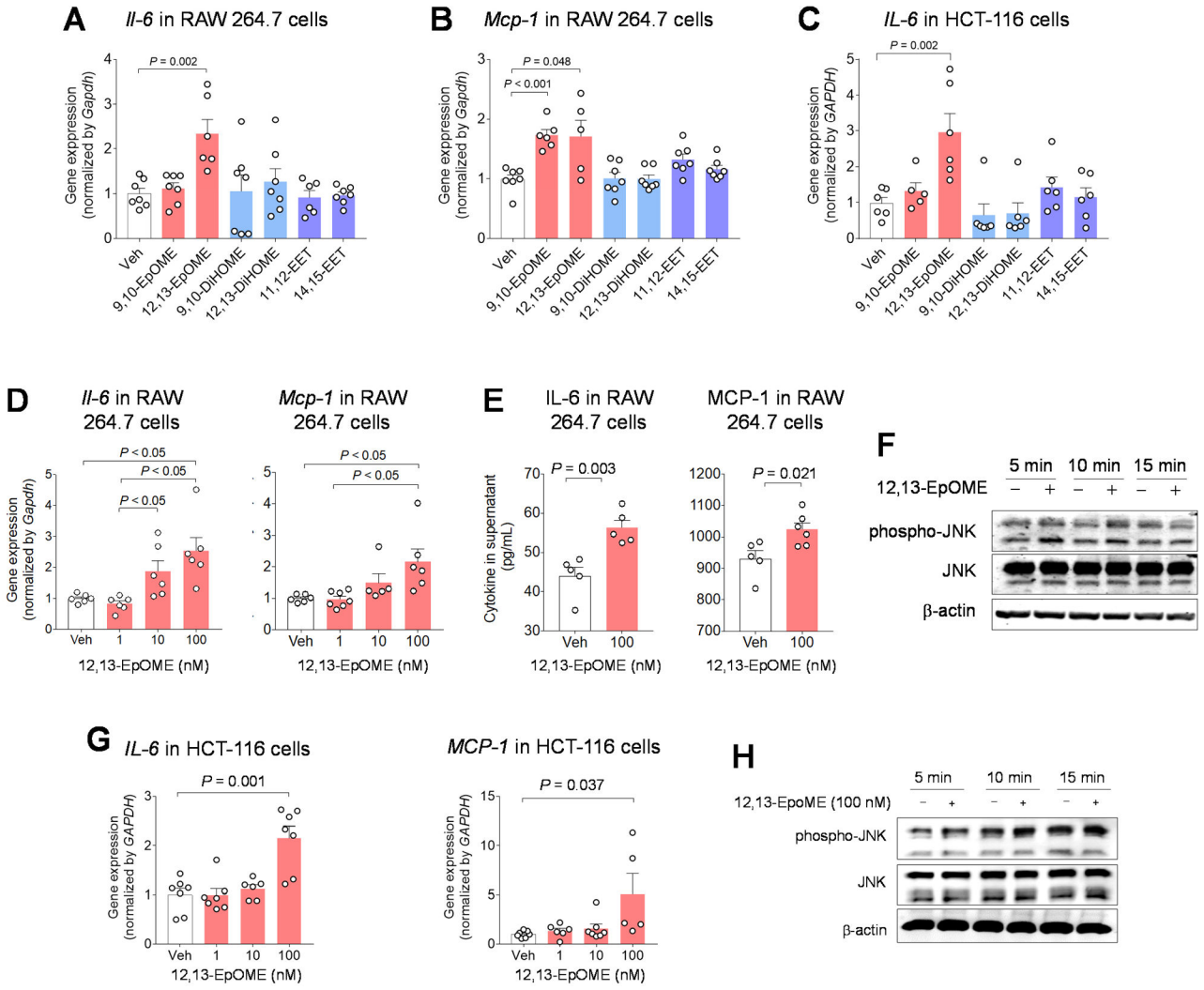

Figure 3. EpOME increases inflammation *in vitro*.

(A-B) Effect of EpOMEs, DiHOMEs and EETs (concentration = 100 nM) on gene expression of *Il-6* and *Mcp-1* in mouse macrophage RAW 264.7 cells (n = 5-7 per group). (C) Effect of EpOMEs, DiHOMEs and EETs (concentration = 100 nM) on gene expression of *IL-6* in human colon cancer HCT-116 cells (n = 5-6 per group). (D) Dose-response effect of 12,13-EpOME on gene expression of *Il-6* and *Mcp-1* in RAW 264.7 cells (n = 6-7 per group). (E) Effect of 12,13-EpOME on medium concentrations of IL-6 and MCP-1 in RAW 264.7 cells (n = 5-6 per group). (F) Effect of 12,13-EpOME on phosphorylation of JNK in RAW 264.7 cells. (G) Dose-response effect of 12,13-EpOME on gene expression of *IL-6* and *MCP-1* in HC-T116 cells (n = 5-7 per group). (H) Effect of 12,13-EpOME on phosphorylation of JNK in HCT116 cells. The results are expressed as mean ± SEM. The statistical significance of two groups was determined using Student's *t* test or Wilcoxon-Mann test, and comparison of three groups was determined using one-way ANOVA.

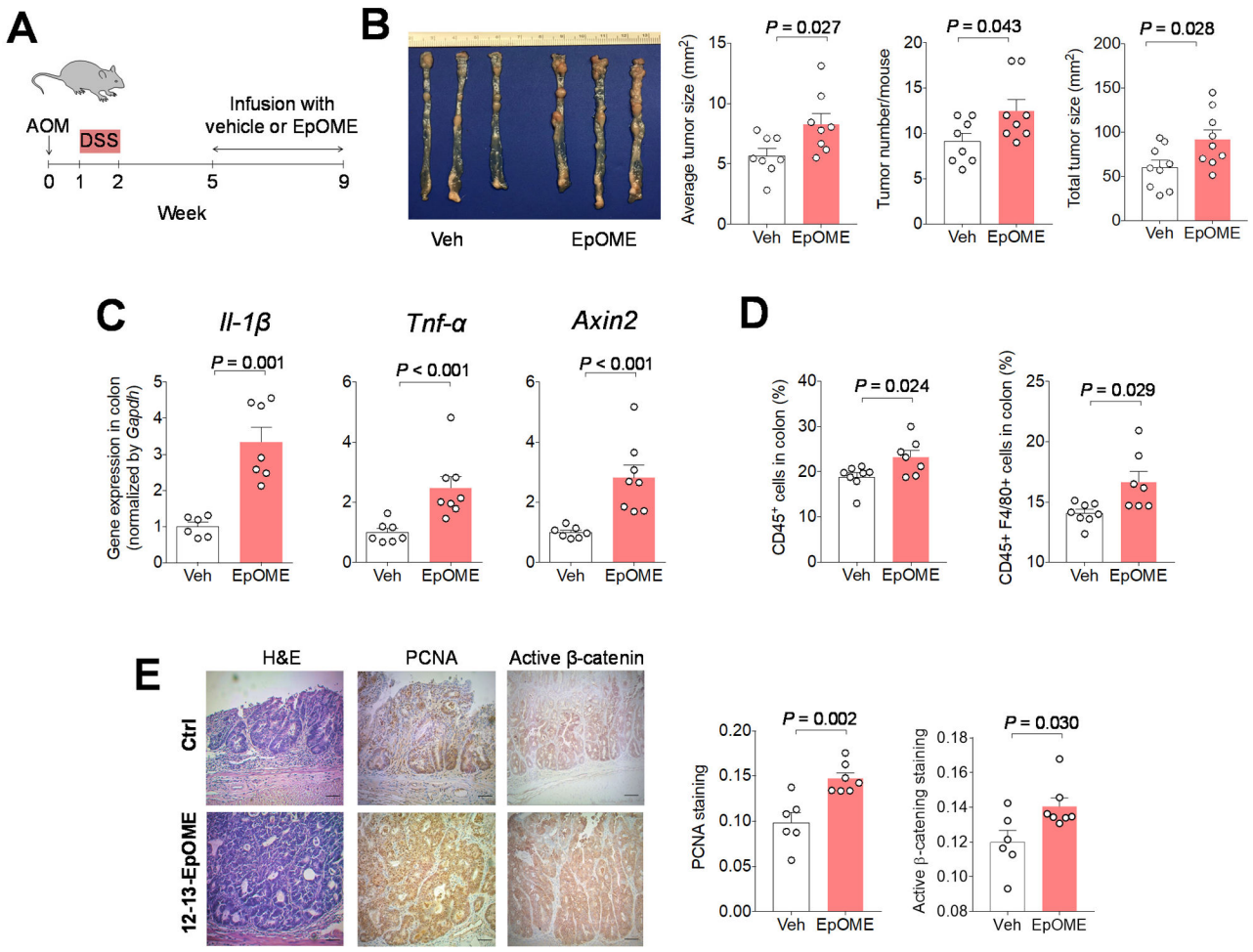

Figure 4. EpOME exaggerates AOM/DSS-induced colon tumorigenesis *in vivo*.

(A) Scheme of animal experiment to test the effect of 12,13-EpOME (dose = 2 mg/kg/day, administered via mini-pump) on colon tumorigenesis. (B) Quantification of colon tumorigenesis in mice (n = 8-9 per group). (C) Expression of pro-inflammatory and pro-tumorigenic genes in colon (n = 6-8 per group). (D) Quantification of $CD45^+$ and $CD45^+$ $F4/80^+$ immune cells in colon (n = 7-8 per group). (E) H&E histology, and immunohistochemical staining of PCNA and β-catenin in colon (n = 6-7 per group, scale bar: 50 μm). The results are expressed as mean ± SEM. The statistical significance of two groups was determined using Student's *t* test or Wilcoxon-Mann test.