INTRODUCTION

Increased production of prostaglandin E2 (PGE2) is often associated with the pathogenesis of inflammation and cancer [1]. PGE2 is generated from the arachidonic acid-COX pathway, and non-steroidal anti-inflammatory drugs have been widely used to suppress PGE2 by inhibiting the functional activity of the COX enzymes [2]. Unfortunately, long-term treatment of patients with NSAIDs, and particularly the COX-2 specific inhibitors (Coxibs), are associated with toxicity, including stomach ulcerations, cardiovascular events and kidney damage [3]. Interest in this pathway, however, has been renewed by recent findings that inhibition of the terminal prostaglandin synthase, microsomal prostaglandin E synthase-1 (mPGES-1), is sufficient to achieve the same degree of cancer protection as direct COX-2 inhibition [4-6]. However, during the course of our preclinical studies in several mouse cancer models, we observed a range of mucosal alterations that may complicate mPGES-1 as an effective cancer chemoprevention target [4].

In order to accelerate its development as a viable drug target, and to better understand the underlying mechanisms that contribute to cancer prevention, we recently developed a conditional Ptges knockout mouse model (cKO). To evaluate the functional role of Ptges directly within the colonic epithelia, cKO mice were crossed with carbonic anhydrase 1 (Car1)-Cre mice (cKO.Car1), and colon tumors were induced using the azoxymethane/dextran sodium sulfate protocol. Unexpectedly, epithelial-specific blockade of Ptges failed to protect mice against colon tumor development. Further studies using the cKO mouse model will be necessary to pinpoint the cell type-specific location of mPGES-1 and its control of inducible PGE2 formation that drives tumor formation in the colon.

Key Words PGE2, mPGES-1, Colonic neoplasms, Azoxyemthane, Dextran sulfate sodium
MATERIALS AND METHODS

Animals
Ptges conditional knockout mice (cKO) were established in the Center for Mouse Genome Modification at the University of Connecticut Health Center (UCHC, Farmington, CT, USA) (Fig. 1). cKO mice were backcrossed to C57BL/6 mice 5 times to obtain relatively pure background. C57BL/6-Tg(Car1-cre)5Flt/J (Car1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Genotyping for cKO mice was performed using the following primers: PTGESgtLXP-Fwd, CACAGT AATCCTCCTGCCT-CA and PTGESgtLXP-Rev, GGCTCCCTCAGATTCCCT -TA. Cre-mediated recombination of Ptges was detected by PTGESgtLXP-Fwd and PTGESgtFRT-Rev, GAAACCCCTA-ATTCTCCTGTCTC, producing a 344-bp fragment for the floxed and 190-bp fragment for the wild-type allele. For the expression analysis, Ptges was detected by the primers: mPGES-1-Fwd, GGATGCGCTGAAACGTGGA and mPGES-1-Rev, CAGGAATGAGTACACGAAGCC. For validation analysis, organs (colon, small intestine, kidney, liver and stomach) were harvested from five-week-old cKO and cKO.Car1 mice, and genomic DNA and total RNA were extracted using AllPrep DNA/RNA Mini Kit (Qiagen, Germantown, MD, USA). For the colonocyte isolation, colons were harvested and cleaned by ice-cold PBS, followed by incubating in 1 mM EDTA for 60 minutes at 4°C with shaking. Total RNA was extracted from the colonocytes and cDNA was synthesized from 500 μg of RNA using RNeasy Mini Kit (Qiagen) and iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), respectively.

Animal treatment
Eight-week-old Ptges cKO (n = 27) and cKO.Car1 (n = 7) mice were injected with a single dose of 10 mg/kg of AOM (Sigma-Aldrich, St. Louis, MO, USA) or vehicle control (0.9% NaCl), followed by two cycles of DSS treatment in drinking water (1% for 5 days) as indicated in Figure 2A (Study design). Mice were sacrificed four weeks after withdrawal from the second round of DSS, and blood and colon tissues were collected for further analysis. Colons were flushed with ice-cold PBS and excised longitudinally. Specimens were fixed-flat in 10% neutral buffered formalin solution for overnight and stored in 70% ethanol thereafter. Both male and female mice were used in the study with access to maintenance diet (Teklad Global 19% Protein Extruded Rodent Diet) and drinking water ad libitum. All animal experiments were conducted with approval from the Center for Comparative Medicine (CCM) at UConn Health (AP-200208-0823).

Quantification of lesions
Fixed whole-mount colons were stained with 0.2% methylene blue and the number and size of tumors were scored under a dissecting microscope. Colon tumor load per mouse was determined using tumor diameter to calculate the spherical tumor volume (mm³), $V = \frac{4}{3} \pi r^3$.

Figure 1. Generation of Ptges conditional knockout mouse model. (A) Design of Ptges targeted allele and primer locations. (B) Genomic DNA of different organs after the Cre recombination (344 bp). (C) Colonocytes isolated from cKO.Car1 mice show reduced Ptges expression. (D) Representative H&E-stained colons of cKO and cKO.Car1 mice showing normal appearance of crypt structures (40×). Boxed area is shown at high-power magnification (400×). UTR, untranslated regions; NEO, neomycin; F1, the first generation; SI, small intestine; cKO, conditional Ptges knockout mouse model; Car1, carbonic anhydrase 1.
Immunohistochemistry
Fixed tissues were embedded in paraffin and sectioned at 5 μm thickness. Tissue sections were deparaffinized and stained with H&E, or incubated overnight with primary antibody for mPGES-1 (1:4,000, Abnova, Taipei City, Taiwan). Sections were incubated with HRP-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA), then counter stained with hematoxylin. Images were captured using conventional microscope or confocal microscope using Q-capture Pro 7 (Tucson, AZ, USA).

Statistical analysis
Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the means ± SEM. P-values were calculated by Student’s t-test. A P-value less than 0.05 was considered statistically significant.

RESULTS
Generation of Ptges conditional knockout mice
To further validate the functional role of mPGES-1 in colon tumor development, we generated a conditional knockout mouse model (cKO), in which exon 3 and the 3’-UTR of Ptges gene was flanked by loxp sites (Fig. 1A). Ptges cKO mice were then crossed with Car1-Cre (cKO.Car1) mice to achieve the genetic deletion of Ptges within colonic epithelial cells [7]. Colon, small intestine, kidney, liver and stomach were harvested from five-week-old cKO and cKO.Car1 mice and genomic DNA was extracted to determine the extent of Cre-mediated recombination of Ptges gene using PCR analysis. As shown in Figure 1B, the floxed Ptges gene was present in the colon and liver (bp = 344) of cKO.Car1 mice and absent in the small intestine, kidney and stomach of the cKO mice. Furthermore, mRNA expression of Ptges was reduced in the colonocytes isolated from cKO.Car1 mice (Fig. 1C). Both the cKO and cKO.Car1 mice demonstrated no health concerns for up to 20 weeks and the colons were histologically normal compared to wild-type C57/BL6 mice (Fig. 1D).

Epithelial cell-specific deletion of Ptges does not protect mice from colon tumor development
After development and characterization of the cKO mouse model, colon tumors were induced by treatment with AOM/DSS in the cKO and cKO.Car1 mice, as described above in Figure 2A. As shown in Figure 2B, despite genetic inactivation of Ptges, cKO.Car1 mice were not protected from tumor formation (cKO; 9.9 ± 1.0 vs. Car1; 12.6 ± 2.4, P = 0.2521). Moreover, the epithelial cell-specific deletion of Ptges did not affect overall tumor volume (cKO; 80.4 ± 11.9, Car1; 95.4 ± 24.1, P = 0.5744). To further evaluate potential mechanisms that may explain these findings, we performed immunohistochemical analysis of mPGES-1 in colon tissue. As shown in Figure 2C, we found that the mPGES-1 protein was strongly expressed within the tumors of both cKO and cKO.Car1 mice at equal intensity. However, as shown in the magnified im-
Several studies indicate that mPGES-1 expression is primarily of non-epithelial origin. This enzyme is largely absent within the epithelial crypts. These results, combined with the genetic studies presented above that show no cancer protection in the cKO mice, provide strong evidence that mPGES-1 functional activity is limited almost exclusively to the stromal compartment of the colonic mucosa.

**DISCUSSION**

Elevated expression of COX-2 and the concomitant increase in PGE₂ formation occur in up to 85% of human colorectal cancers [8]. Despite growing evidence for a plethora of unwanted side-effects associated with long-term NSAID use in patient populations, COX inhibition still remains one of the most effective strategies for colon cancer prevention [9]. To minimize the gastrointestinal and cardiovascular toxicities that have been associated with long-term treatment with these drugs, alternative approaches that target the COX-2/PGE₂ signaling pathway have been considered. Our data and others (reviewed in [10]) have suggested that the inducible terminal synthase, mPGES-1, may provide a reasonable candidate for chemoprevention. In order to accelerate its development as a viable drug target, and to better understand the mechanisms by which mPGES-1 and its metabolic product, inducible PGE₂, contribute to colon tumor promotion, we have generated a conditional mouse model in which mPGES-1 activity can be abrogated directly within distinct cellular compartments of the colonic mucosa.

The present study is the first of its kind to evaluate the influence of cell type-specific inactivation of mPGES-1 on colon carcinogenesis. Using our newly created conditional mouse model, we have selectively inactivated mPGES-1 directly within the colonic epithelial lineage via genetic inactivation. Our rationale for targeting the epithelial compartment is based primarily on the overwhelming abundance of literature ascribing its functional activity to the epithelia [11-13]. These conclusions are based, in part, on evidence acquired in cancer cell lines that are typically derived from cells of epithelial origin [11-13] as well as the results obtained from several immunohistochemical studies [11,14,15]. However, our findings clearly demonstrate that colonocytes harvested from cKO mice maintain only limited Ptges mRNA expression (Fig. 1C). While this relatively low level of expression does not conclusively rule out its potential role in normal epithelial cell homeostasis, our findings in the AOM/DSS model provide evidence for its far less significant role in tumorigenesis. Moreover, immunostaining of tumor sections shows that mPGES-1 expression is almost exclusively localized to the tumor stroma (Fig. 2C). These observations clearly indicate that the cellular source of inducible PGE₂ during colon tumor promotion is primarily of non-epithelial origin.

Several studies indicate that mPGES-1 expression is largely confined to macrophages and dendritic cells [16,17]. In fact, Chen et al. [18-20] generated a Ptges-Cre-Loxp mouse model to specifically delete the Ptges gene in vascular smooth muscle cells, endothelial cells and myeloid cells in order to study its potential role in cardiovascular diseases. While vascular smooth muscle-specific deletion of Ptges did not impact atherogenesis [19], deletion in myeloid cells attenuated the vascular proliferative response to injury during high-fat diet-induced atherogenesis [19], and also showed beneficial effects during the healing of myocardial infarction [20]. These observations provide further evidence of the multifaceted role of mPGES-1 in a variety of experimental systems in cell type-specific manner, as comprehensively reviewed earlier [1]. Overall, our findings warrant further studies to more precisely define the cell-of-origin of inducible PGE₂ synthesis within the complex tissue architecture of the colonic mucosa. Further application of our conditional murine model will inform the functional role of inducible PGE₂ synthesis and its impact on key stages of tumorigenesis, and support efforts to develop safe and effective pharmacological inhibitors of mPGES-1.

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**CONFLICTS OF INTEREST**

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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**REFERENCES**

1. Nakanishi M, Rosenberg DW. Multifaceted roles of PGE2 in inflammation and cancer. Semin Immunopathol 2013;35:123-37.
2. Smalley WE, DuBois RN. Colorectal cancer and nonsteroidal anti-inflammatory drugs. Adv Pharmacol 1997;39:1-20.
3. Wang D, DuBois RN. The role of anti-inflammatory drugs in colorectal cancer. Annu Rev Med 2013;64:131-44.
4. Nakanishi M, Menoret A, Tanaka T, Miyamoto S, Montrose DC, Vella AT, et al. Selective PGE(2) suppression inhibits colon carcinogenesis and modifies local mucosal immunity. Cancer Prev Res (Phila) 2011;4:1198-208.
5. Nakanishi M, Montrose DC, Clark P, Nambari PR, Belinsky GS, Claffey KP, et al. Genetic deletion of mPGES-1 suppresses intestinal tumorigenesis. Cancer Res 2008;68:3251-9.
6. Nakanishi M, Perret C, Meuillet EJ, Rosenberg DW. Non-cell autonomous effects of targeting inducible PGE2 synthesis during inflammation-associated colon carcinogenesis. Carcinogenesis
7. Tetteh PW, Kretzschmar K, Begthel H, van den Born M, Korving J, Morsink F, et al. Generation of an inducible colon-specific Cre enzyme mouse line for colon cancer research. Proc Natl Acad Sci USA. 2016;113:11859-64.
8. Wang D, DuBois RN. An inflammatory mediator, prostaglandin E2, in colorectal cancer. Cancer J 2013;19:502-10.
9. Maniewska J, Ježewska D. Non-steroidal anti-inflammatory drugs in colorectal cancer chemoprevention. Cancers (Basel) 2021;13:594.
10. Bergqvist F, Morgenstern R, Jakobsson PJ. A review on mPGES-1 inhibitors: from preclinical studies to clinical applications. Prostaglandins Other Lipid Mediat 2020;147:106383.
11. Yoshimatsu K, Golijanin D, Paty PB, Soslow RA, Jakobsson PJ, DeLellis RA, et al. Inducible microsomal prostaglandin E synthase is overexpressed in colorectal adenomas and cancer. Clin Cancer Res 2001;7:3971-6.
12. Kamei D, Murakami M, Nakatani Y, Ishikawa Y, Ishii T, Kudo I. Potential role of microsomal prostaglandin E synthase-1 in tumorigenesis. J Biol Chem 2003;278:19396-405.
13. Parent J, Fortier MA. Expression and contribution of three different isoforms of prostaglandin E synthase in the bovine endometrium. Biol Reprod 2005;73:36-44.
14. Yoshimatsu K, Altorki NK, Golijanin D, Zhang F, Jakobsson PJ, Dannenberg AJ, et al. Inducible prostaglandin E synthase is overexpressed in non-small cell lung cancer. Clin Cancer Res 2001;7:2669-74.
15. Rask K, Zhu Y, Wang W, Hedin L, Sundfeldt K. Ovarian epithelial cancer: a role for PGE2-synthesis and signalling in malignant transformation and progression. Mol Cancer 2006;5:62.
16. Monrad SU, Kojima F, Kapoor M, Kuan EL, Sarkar S, Randolph GJ, et al. Genetic deletion of mPGES-1 abolishes PGE2 production in murine dendritic cells and alters the cytokine profile, but does not affect maturation or migration. Prostaglandins Leukot Essent Fatty Acids 2011;84:113-21.
17. Weigert A, Strack E, Snodgrass RG, Brüne B. mPGES-1 and ALOX5/-15 in tumor-associated macrophages. Cancer Metastasis Rev 2018;37:317-34.
18. Chen L, Yang G, Xu X, Grant G, Lawson JA, Bohlooly-Y M, et al. Cell selective cardiovascular biology of microsomal prostaglandin E synthase-1. Circulation 2013;127:233-43.
19. Chen L, Yang G, Monslow J, Todd L, Cormode DP, Tang J, et al. Myeloid cell microsomal prostaglandin E synthase-1 fosters atherogenesis in mice. Proc Natl Acad Sci USA 2014;111:6828-33.
20. Chen L, Yang G, Jiang T, Tang SY, Wang T, Wan Q, et al. Myeloid cell mPges-1 deletion attenuates mortality without affecting remodeling after acute myocardial infarction in mice. J Pharmacol Exp Ther 2019;370:18-24.