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Epithelial Cell-specific Deletion of Microsomal Prostaglandin E Synthase-1 Does Not Influence Colon Tumor Development in Mice

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Activation of the COX-2/microsomal prostaglandin E synthase-1 (mPGES-1)/prostaglandin E_2 (PGE_2) signaling axis is a hallmark of many cancers, including colorectal cancer, prompting the implementation of prevention strategies targeting COX-2 activity. We have previously shown that targeting the downstream terminal PGE_2 synthase, mPGES-1 (*Ptges*), specifically reduces inducible PGE_2 formation without disrupting synthesis of other essential prostanoids, thereby conferring dramatic cancer protection against colon carcinogenesis in multiple mouse models. In order to accelerate its development as a viable drug target, and to better understand the mechanisms by which PGE_2 influences colon carcinogenesis, 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 PGE_2 formation that drives tumor formation in the colon.

Key Words PGE_2, mPGES-1, Colonic neoplasms, Azoxymethane, Dextran sulfate sodium

INTRODUCTION

Increased production of prostaglandin E_2 (PGE_2) is often associated with the pathogenesis of inflammation and cancer [1]. PGE_2 is generated from the arachidonic acid-COX pathway, and non-steroidal anti-inflammatory drugs have been widely used to suppress PGE_2 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*). The following study has incorporated this new model and was designed to evaluate the functional role of mPGES-1 directly within the colonic epithelia. *cKO* mice were crossed with *carbonic anhydrase 1* (*Car1*)-*Cre* mice (*cKO.Car1*) to inactivate *Ptges* directly within the epithelial compartment of the colon [7]. As detailed in this report, epithelial-specific blockade of *Ptges* in *cKO.Car1* mice did not protect mice against azoxymethane/dextran sodium sulfate (AOM/DSS)-induced colon tumor development. While this study provides new information about mPGES-1 activity in the colon, further studies using the *cKO* model will be necessary to define the cell type-specific location and functional role of mPGES-1 in epithelial homeostasis and how this critical terminal synthase contributes to tumor development in the colon.

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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, CACAGTAATCCTCCTGCCTCA and PTGESgtLXP-Rev, GGCTCCCTCAGATTCCCTTA. Cre-mediated recombination of *Ptges* was detected by PTGESgtLXP-Fwd and PTGESgtFRT-Rev, GAAACCCCTAATTCTCCTGTCTC, 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 (mm^3), V = (4/3) * *pi* * r^3.

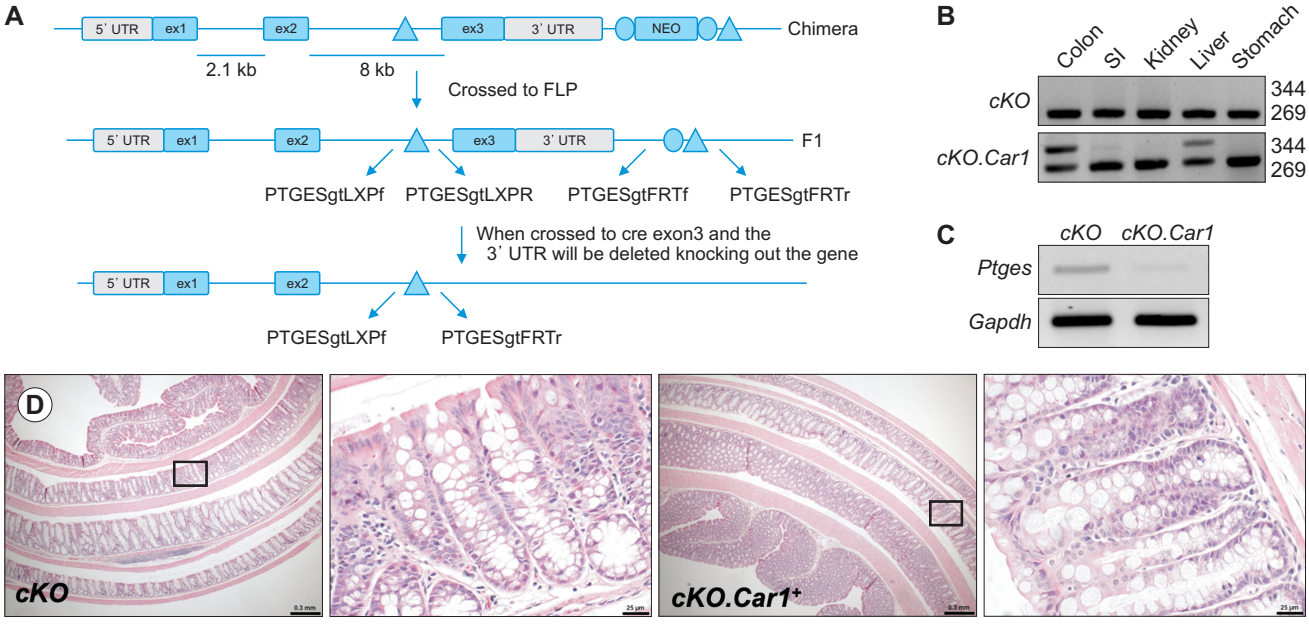

Figure 1. Generation of *Ptges* conditional knockout mouse model. (A) Design of *Ptges* targeted allele and primer locations. (B) Genomic DNA of different organs after the Cre recombination (344 bp). (C) Colonocytes isolated from *cKO.Car1* mice show reduced *Ptges* expression. (D) Representative H&E-stained colons of *cKO* and *cKO.Car1* mice showing normal appearance of crypt structures (40×). Boxed area is shown at high-power magnification (400×). UTR, untrasnlated regions; NEO, neomycin; F1, the first generation; SI, small intestine; *cKO, conditional Ptges* knockout mouse model*; Car1, carbonic anhydrase 1*.

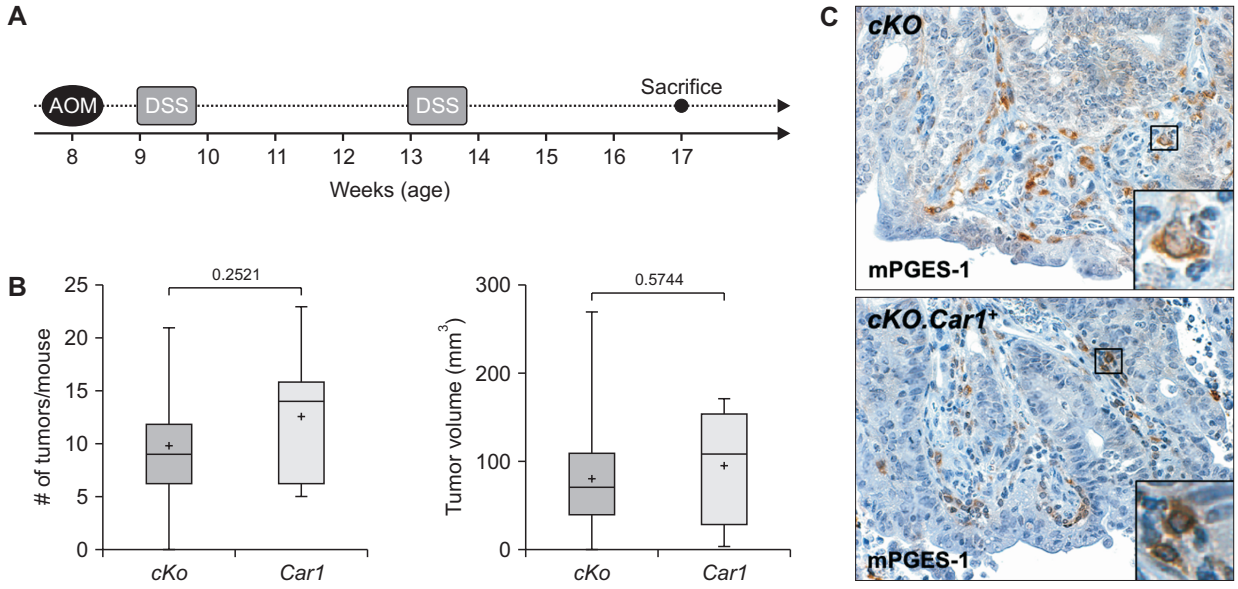

Figure 2. Epithelial-specific deletion of *Ptges* do not suppress AOM/DSS-induced colon tumor development. (A) Study design. (B) Quantification of colon tumors and tumor load between *wild-type* (*cKO*) and *cKO.Car1*. (C) Colon tissue sections were stained with the primary antibody against mPGES-1 and the anti-rabbit secondary antibody, followed by the DAB staining. Hematoxylin was used for a counterstainImmun ohistochemistry of mPGES-1 showing positive staining within the tumors of both *cKO* and *cKO.Car1* colon (200×), and the boxed area is shown at high-power magnification (600×). '+' in the boxplots indicates the mean value for each group. Groups were compared by Student's *t*-test. Numbers above the plots indicate *P*-values. AOM, azoxymethane; DSS, dextran sulfate sodium; *cKO*, conditional *Ptges* knockout mouse model; *Car1*, *carbonic anhydrase 1*; mPGES-1, Microsomal prostaglandin E synthase-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 colons of both *cKO* and *cKO.Car1* mice at equal intensity. However, as shown in the magnified im-

age of Figure 2C (lower box), mPGES-1 was predominantly localized to the peri-nuclear region of stromal cells within the apical region of the colon tumors. However, 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 PGE_2 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/PGE_2 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 PGE_2, 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 PGE_2 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 PGE_2 synthesis within the complex tissue architecture of the colonic mucosa. Further application of our conditional murine model will inform the functional role of inducible PGE_2 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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