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Prostaglandin E₂ Promotes Colorectal Cancer Stem Cell Expansion and Metastasis in Mice

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Abstract

BACKGROUND & AIMS—Inflammation may contribute to formation, maintenance, and expansion of cancer stem cells (CSCs), which have the capacity for self-renewal, differentiation, and resistance to cytotoxic agents. We investigated the effects of the inflammatory mediator prostaglandin E₂ (PGE₂) on colorectal CSC development and metastasis in mice and the correlation between levels of PGE₂ and CSC markers in human colorectal cancer (CRC) specimens.

METHODS—Colorectal carcinoma specimens and matched normal tissues were collected from patients at the Mayo Clinic (Scottsdale, AZ) and analyzed by mass spectrometry and quantitative PCR. Human primary CRC cells and mouse tumor cells were isolated using microbeads and flow cytometry and analyzed in sphere-formation and flow cytometry assays. LS-174T cells were sorted by flow cytometry (for CD133⁺CD44⁺ and CD133[−]CD44[−] cells) and also used in these assays. NSG mice were given cecal or subcutaneous injections of LS-174T or human primary CRC cells. *Apc*^{Min/+} mice and NSG mice with orthotopic cecal tumors were given vehicle (controls), PGE₂, celecoxib, or/and Ono-AE3-208. PGE₂ downstream signaling pathways were knocked down with small hairpin RNAs, expressed from lentiviral vectors in LS-174T cells, or blocked with inhibitors in human primary CRC cells.

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RESULTS—Levels of PGE₂ correlated with colonic CSC markers (*CD133*, *CD44*, *LRG5*, and *SOX2* mRNAs) in human colorectal carcinoma samples. Administration of PGE₂ to *Apc*^{Min/+} mice increased tumor stem cells and tumor burden, compared with controls. NSG mice given PGE₂ had increased numbers of cecal CSCs and liver metastases, compared with controls, after intracecal injection of LS-174T or human primary CRC cells. Alternatively, celecoxib, an inhibitor of prostaglandin-endoperoxide synthase 2 (PTGS2 or COX2), reduced polyp numbers in *Apc*^{Min/+} mice, liver metastasis in NSG mice with orthotopic tumors, and numbers of CSCs in *Apc*^{Min/+} and NSG mice. Inhibitors or knockdown of PGE receptor 4 (PTGER4 or EP4), PI3K p85α, ERK1, or nuclear factor (NF)-κB reduced PGE₂-induced sphere formation by and expansion of LS-174T and/or human primary CRC cells. Knockdown of ERK1 or PI3K p85α also attenuated PGE₂-induced activation of NF-κB in LS-174T cells. An EP4 antagonist reduced the ability of PGE₂ to induce CSC expansion in orthotopic tumors and to accelerate formation of liver metastases. Knockdown experiments showed that NF-κB was required for PGE₂ induction of CSCs and metastasis in mice.

CONCLUSIONS—PGE₂ induces CSC expansion by activating NF-κB, via EP4–PI3K and EP4–MAPK signaling, and promotes formation of liver metastases in mice. The PGE₂ signaling pathway might therefore be targeted therapeutically to slow colorectal cancer progression.

Keywords

NSAIDs; cyclooxygenase; colon cancer; tumorigenesis

INTRODUCTION

Epidemiologic and experimental evidence strongly implicate chronic inflammation as a risk factor for the development of many cancers, including colorectal cancer (CRC). Nonsteroidal anti-inflammatory drugs (NSAIDs) represent one class of drugs found to have beneficial effects on reducing the risk of CRC. NSAIDs are known to exert certain anti-inflammatory effects by targeting prostaglandin-endoperoxide synthases (PTGS) that include PTGS1 (COX-1) and PTGS2 (COX-2). Previous work confirms that PTGS2 expression is elevated in CRC and is associated with a lower survival rate among CRC patients^{1, 2}. In addition to CRC, PTGS2 levels are also elevated in other premalignant and malignant solid tumors, including stomach, esophagus, liver, pancreas, head and neck, lung, breast, prostate, and bladder cancer and this elevation is associated with decreased survival among these cancer patients as well³. PTGS2 has been shown to play an important role in inflammation and cancer⁴. However, long-term use of the selective PTGS2 inhibitors and non-selective NSAIDs (except for aspirin) will not be recommended for cancer prevention because of the unacceptable cardiovascular side effects of these drugs, especially in those individuals with a history of atherosclerotic heart disease⁵. One potential way to avoid these undesired side effects is to only target PTGS-derived prostanoids that mediate the tumor-promoting effects of PTGS2.

PTGS2-derived prostaglandin E₂ (PGE₂), a pro-inflammatory mediator, is the most abundant prostaglandin found in various types of human malignancies including colorectal, lung, breast, head and neck cancer and is often associated with a poor prognosis^{6–9}. Emerging epidemiologic evidence and a phase II biomarker study showed that urinary PGE₂

metabolite (PGE-M) levels were associated with an increased risk of developing colorectal^{10–12}, gastric¹³, and breast cancer^{14, 15} and were correlated with disease progression in head and neck squamous cell carcinomas¹⁶. PGE₂ exerts its effects by binding to EP receptors (EP1, EP2, EP3, and EP4) that belong to the family of seven transmembrane G protein-coupled receptors. Each EP receptor activates its own downstream signaling pathways that transduce a particular biologic affect. PGE₂ has been shown to promote tumor epithelial cell proliferation, survival, and migration/invasion via multiple signaling pathways¹⁷. However, all of the mechanisms by which PGE₂ accelerates CRC formation, progression, and metastasis in complex living systems are not fully understood.

Undifferentiated cancer cells have been referred to as cancer stem cells (CSCs) or tumor-initiating cells because they possess the capacity for self-renewal, differentiation, and innate resistance to cytotoxic agents^{18, 19}. CSCs are thought to be responsible for tumor initiation, growth, metastatic spread, relapse, and recurrence. Several studies have described the presence of CSCs in human CRC and shown that they are capable of initiating tumor development^{19, 20}. However, very little is known about their biology or how they are formed, maintained, and expanded. Emerging evidence supports the hypothesis that chronic inflammation might regulate the development and function of CSCs²¹ or convert epithelial mesenchymal cells to CSCs²². The observation that NSAIDs can eliminate oncogenic intestinal stem cells via induction of apoptosis in *Apc*^{Min/+} mice²³ and reduce colonosphere formation in human colorectal carcinoma cell lines *in vitro*²⁴ prompted us to postulate that PGE₂ might accelerate tumor initiation, growth, and liver metastasis by inducing colorectal CSC expansion.

METHODS

Animal experiments

All animal experiments conform to our animal protocols that were approved by the Institutional Animal Care and Use Committee at Arizona State University. NSG and *Apc*^{Min/+} mice were obtained from Jackson Laboratory (Bar Harbor, Maine). For the subcutaneous (sub-Q) injection, indicated cell numbers were injected into the flanks of male NSG mice at age of 7 weeks old. For the orthotopic mouse model, 1×10^4 of cells derived from LS-174T, LS-174T/vector, LS-174T/shp65, or 5×10^5 of cells derived from human primary CRC specimen were injected into the cecal wall of male NSG mice at age of 7 weeks old. Additional information on animal treatments is provided in Supplemental Methods.

Isolation of tumor epithelial cells

This study was approved by the Institutional Review Board of the Mayo Clinic. Fresh or frozen tumor specimens were obtained from patients with primary CRC at the Mayo Clinic. Human CRC specimens and mouse cecal tumor tissues were minced and digested in Chang medium with supplement containing 2 mg/ml collagenase III (Worthington Biochemical Corp.) at 37°C, 5% CO₂ for 5 hours with occasional shaking. Human epithelial tumor cells were isolated by using human CD326 (EpCAM) MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. Mouse colonic adenomas were minced and digested with

PBS containing with 0.1% BSA and 12 mg/ml collagenase I (Gibco). Mouse epithelial tumor cells were purified by Flow cytometry sorting using CD326 (EpCAM)-PE antibody (Miltenyi Biotec). Isolated tumor epithelial cells were subjected to *in vitro* sphere-forming assays, cecal injection, sub-Q injection, or flow cytometry analysis.

Immunohistochemical staining

Paraffin-embedded tissue sections (5 μ m thick; n=5 per animal) were stained with anti-CD133 rabbit antibody (1:100, Biorbyt, Cambridge, UK) and anti-CD44v6 mouse monoclonal antibody (1:100, R&D System) in 4°C overnight. The immunohistochemical staining was completed by using a Zymed-Histostain-SP Kit (Zymed, South San Francisco, CA) as described previously²⁵.

Flow cytometry analysis and sorting

Single-cell suspensions in staining buffer (Biolegend) were incubated with anti-hCD44v6-PE (1:20, R&D System), anti-hCD133-FITC (1:10, Miltenyi Biotec), and/or anti-EpCAM (CD326)-APC-Vio770 (1:100, Miltenyi Biotec) antibodies for 30 min on ice and analyzed on a Gallios flow cytometer (Beckman Coulter). CD133⁺CD44⁺ and CD133⁻CD44⁻ LS-174T cells were sorted by flow cytometer and subjected to *in vitro* sphere-forming and Western blotting assays.

Cell culture

Detailed information about cell culture and treatments is provided in Supplemental Methods.

In vitro sphere-forming assay

For PGE₂ treatment, 30,000 cells were cultured in 6-well Ultra-low Attachment surface plate (Costar) with serum-free DMEM/F12 medium containing with indicated dose of PGE₂, 10 μ M ONO-AE-208 (a gift of Ono Pharmaceutical Co., Osaka, Japan), 50 μ M PD98059 (Calbiochem La Jolla, CA), 10 μ M LY294002 (Calbiochem), 10 μ M SC19220 (Cayman Chemical), 10 μ M AH6809 (Cayman Chemical) and/or vehicle without B27 supplement, EGF, and FGF for 3 weeks. The culture medium was replaced by fresh medium with fresh PGE₂, inhibitors, and/or vehicle everyday for three weeks. For regular sphere-forming assays, 30,000 cecal tumor cells isolated from indicated mouse, vehicle-treated cells, or PGE₂-treated cells were cultured in 6-well Ultra-low Attachment surface plate with serum-free DMEM/F12 medium containing B27 supplement, 20 ng/ml EGF, and 10 ng/ml FGF without PGE₂ for three weeks. The sphere numbers in each well were quantified.

Lentivirus production and stable transfection

ERK1, PI3K p85 α , and NF- κ B p65 shRNAs were purchased from Open Biosystems. EP1, EP2, EP3, and EP4 shRNAs (lentiviral particles) were obtained from Santa Cruze. Lentivirus production and stable transfection were performed according to the manufacturer's instructions.

Western blot analysis

Detailed information about Western blotting assay and antibodies is provided in Supplemental Methods.

Quantitative PCR

Total RNA was isolated from human CRC specimens by the TRIzol reagent (Life Technologies) and was reversely transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Real-time q-PCR was performed with TaqMan[®] Gene Expression Assay Mix and TaqMan[®] Universal PCR Master Mix (Life Technologies) using ViiATM 7 Real-time PCR System (Life Technologies). TaqMan[®] Gene Expression Assay Mix for CD133, CD44, LGR5, and SOX2 were obtained from Life Technologies. The relative expression of target gene is the averages of triplicates that are normalized against the transcription levels of 18s rRNA.

PGE₂ measurement

Tissues were homogenized in PBS with 10% 2,6-di-*tert*-butyl-*p*-cresol. PGE₂ level were measured using an Agilent 6460 tandem mass spectrometry configured with the Agilent 1200 liquid chromatography system (LC/MS/MS, Agilent Technologies) and normalized with protein concentration.

Statistical analysis

Each *in vitro* experiment was done at least 3 times and each *in vivo* experiment was conducted at least twice. Data are presented as mean $\pm$ SEM. Comparisons among multiple groups were performed by factorial analysis of variance, followed by Bonferroni test. Comparisons between two groups were performed with Student's *t*-test or Mann-Whitney U test where appropriate. Fischer's exact test was used for categorical variables. $p < 0.05$ was considered significant.

RESULTS

PGE₂ levels correlate with the expression of CSC markers in human CRC

CD133, CD44, CD44v6, LRG5, and SOX2 are informative markers for CSCs in CRC^{19, 20, 26, 27}. However, RT-PCR could not be used to measure CD44v6 mRNA because primers cannot be designed to detect only CD44v6. Therefore, we examined whether PGE₂ levels correlate with the expression of CD133, CD44, LRG5, and SOX2 in human colon carcinomas at grade II–III with the matched normal tissues. As shown in Figure 1, the PGE₂ levels are positively associated with the expression of CD133 (panel A), CD44 (panel B), LGR5 (panel C), and SOX2 (panel D) at mRNA levels. These results indicate that PGE₂ may promote CSC expansion in CRC.

PGE₂ increases CSCs and promotes liver metastasis *in vivo*

Apc^{Min/+} mice have been used as a model for human familial adenomatous polyposis and a pre-malignant model for human sporadic CRC. Since NSAIDs reduced oncogenic intestinal stem cells in *Apc*^{Min/+} mice²³ and the expression of CSC markers has been found in human

adenomas²⁸, we first tested whether PGE₂ treatment affected oncogenic colonic stem cells in adenomas of *Apc*^{Min/+} mice. Treatment of *Apc*^{Min/+} mice with PGE₂ resulted in an increase in tumor burden (Supplemental Figure S1A) with more CD133– and CD44-positive epithelial cells in colonic adenomas as compared to control mice (Figure 2A). Since no antibody against mouse CD44v6 was available, we utilized an antibody directed against mouse CD44 for IHC staining. Colonic tumor cells isolated from PGE₂-treated mice formed more spheres (an index of CSC numbers) than those taken from control mice in *in vitro* sphere-forming assays (Figure 2B), demonstrating that adenomas from PGE₂-treated mice contain more tumor stem cells than those from vehicle-treated mice.

We also examined whether PGE₂ was able to increase CSCs in human colorectal carcinomas. LS-174T cells or primary carcinoma cells isolated from human CRC specimens were injected into the cecal wall of NSG mice. After injection, the mice were treated with vehicle or PGE₂. Since both antibodies against human CD44 and CD44v6 are commercially available, we tested both antibodies and found that the CD44v6 antibody is more specific than CD44 antibody in IHC staining and Flow cytometry analysis. Analyses of IHC and flow cytometry revealed that PGE₂ increased the number of CD133– and CD44v6-positive epithelial cells found in cecal tumors (Figure 2C–D), although PGE₂ treatment did not alter cecal tumor weight (Supplemental Figure S1B). Cecal tumor cells isolated from PGE₂-treated mice formed more spheres than those from control mice (Figure 2E). We further evaluated the tumor-initiating ability of these cecal tumor cells. After limiting dilution transplantation, 3 of 6 NSG mice implanted with 2000 cecal tumor cells from PGE₂-treated mice developed tumors, whereas none of 6 mice with 2000 cecal tumor cells from vehicle-treated mice formed measurable tumors (Table 1). In addition, tumor cells isolated from PGE₂-treated mice exhibited higher growth rates than those from vehicle-treated mice (Supplemental Figure S1C). These results demonstrate that PGE₂ treatment results in more tumor-initiating cells compared to vehicle treatment. Moreover, PGE₂ treatment promoted metastatic lesions in the liver and increased liver tumor burden overall (Figure 2F). These results demonstrate that exogenous PGE₂ treatment promotes CSC expansion and liver metastasis.

We further determined whether reduction of PGE₂ levels by inhibiting PTGS2 (COX-2) activity decreased CSC numbers and inhibited liver metastasis. Treatment of *Apc*^{Min/+} mice with a PTGS2 (COX-2) selective inhibitor (celecoxib) resulted in reduction of tumor stem cell numbers (Figure 3A) and tumor burden (Figure 3B, left panel) with decreased PGE₂ levels (Figure 3C) in small intestinal adenomas in small intestines. Celecoxib treatment resulted in a trend toward reduction of colonic tumor numbers but did not reach significance (Figure 3B, right panel). After intracecal injection of cells from human CRC specimens into NSG mice, the mice were then treated with vehicle, celecoxib, PGE₂, or celecoxib plus PGE₂. Celecoxib also reduced cecal CSC numbers (Figure 3D) and the number of liver metastatic tumors (Figure 3E) with decreased cecal PGE₂ levels (Figure 3F). Most importantly, exogenous PGE₂ treatment reversed the effect of celecoxib on reduction of CSC expansion and liver metastasis (Figure 3D–E). The effect of these agents on cecal tumors was not significant (Supplemental Figure S1D). These results demonstrate that reduction of PGE₂ by inhibition of PTGS2 (COX-2) activity suppresses CSC expansion and

liver metastasis. Collectively, our results demonstrate that endogenous PGE₂ induces CSCs and promotes liver metastasis *in vivo*.

PGE₂ directly induces colonic CSCs

It was not clear whether PGE₂ could directly induce CSCs or indirectly regulate CSCs via non-epithelial cells. To address this question, *in vitro* sphere-forming assays were performed. Treatment of LS-174T cells with PGE₂ significantly increased the number of spheres and their size (an index of CSC expansion) (Figure 4A). Since 0.1 μM PGE₂ was most effective at inducing sphere formation, this dose was chosen for subsequent studies described below. Similarly, PGE₂ also induced sphere formation and expansion in colonic epithelial tumor cells isolated from *Apc*^{Min/+} mice and in primary carcinoma cells isolated from three individual CRC patient specimens (Figure 4B–C). To further determine potential mechanisms underlying PGE₂ induction of sphere formation and expansion, LS-174T cells were sorted into CD133[−]CD44[−] and CD133⁺CD44⁺ cells. CD133⁺CD44⁺ cells formed more spheres than CD133[−]CD44[−] cells without PGE₂ treatment (Fig. 4D). Moreover, PGE₂ was able to induce sphere formation and expansion (data not shown) in both CD133[−]CD44[−] and CD133⁺CD44⁺ cells, suggesting that PGE₂ may not only induce expansion or survival of existing CSCs but also promote conversion of non-CSC tumor cells to CSCs.

PGE₂ has been shown to protect intestinal cells from anoikis²⁹. To avoid the effect of PGE₂ on anoikis in above *in vitro* sphere-forming assays, attached LS-174T cells and attached primary carcinoma cells from human CRC specimens were cultured in regular tissue culture plates and then treated with vehicle or PGE₂. After treatment, these cells were subjected to *in vitro* sphere-forming assays without PGE₂ treatment. Prior PGE₂-treated cells formed more spheres than vehicle-treated cells (Figure 4E), demonstrating that PGE₂ induces formation and expansion of cells with intrinsic self-renewal property without involvement of anoikis. Limiting dilution transplantation assays in xenograft models are considered as the gold standard to assess CSCs. After pre-treatment of attached LS-174T cells with PGE₂ or vehicle, these cells were injected into the flanks of NSG mice. 8 of 8 mice implanted with as few as 50 PGE₂-treated LS-174T cells developed into tumors, whereas 3 of 8 mice with vehicle-treated cells formed any measurable tumor (Supplemental Table 1). Collectively, these results demonstrate that PGE₂ can directly induce colonic CSCs by targeting tumor epithelial cells without involvement of other types of cells.

PGE₂ induces CSC formation and expansion by activating NF-κB via EP4-PI3K and EP4-MAPK pathways *in vitro*

PGE₂ exerts its cellular effects by binding to cell surface receptors that activate multiple signaling pathways, including PI3K-AKT and MAPK^{17, 30}. Indeed, treatment of LS-174T or primary carcinoma cells from human CRC specimen with an EP4 antagonist (ONOAE-208), a MEK1 inhibitor (PD98059), or a PI3K inhibitor (Ly294002) reduced PGE₂-induced CSCs (Figure 5A). In contrast, treatment with an EP1 antagonist (SC19220) or an EP1-3 antagonist (AH6809) did not block the effect of PGE₂ on CSCs (Supplemental Fig. S2A). In addition, both CD133[−]CD44[−] and CD133⁺CD44⁺ LS-174T cells expressed similar levels of the EP4 receptor (Supplemental Figure S2B). Furthermore, knockdown of ERK1 and PI3K p85α also completely inhibited PGE₂ induction of CSCs in LS-174T cells (Figure 5B).

These results demonstrate that EP4-PI3K/MAPK signaling pathways are responsible for PGE₂ induction of CSCs.

Since PI3K and MAPK pathways have been shown to activate NF- κ B in many other types of cancer cells³¹ and another recent study revealed that NF- κ B activation promoted intestinal stem cell survival and transformation via effects on Wnt signaling³², we determined whether NF- κ B is a downstream target of PI3K and/or MAPK pathways following PGE₂ treatment. Indeed, PGE₂ enhanced NF- κ B p65 nuclear levels in LS-174T/ctl-sh (non-silencing control shRNA), but not in ERK1 and p85 α knockdown LS-174T cells (Figure 5C), demonstrating that MAPK and PI3K pathways mediate the effect of PGE₂ on NF- κ B activation. Moreover, knockdown EP4, but not EP1 and EP2, completely inhibited PGE₂-induced nuclear translocation of p65 (Supplemental Figure S2C), demonstrating that EP4 mediates the effect of PGE₂ on activation of NF- κ B. Moreover, knockdown of p65 attenuated PGE₂ induction of CSCs in LS-174T cells (Figure 5D). These results demonstrate that NF- κ B is a downstream target of MAPK and PI3K-Akt pathways and required for PGE₂ induction of CSCs. Collectively, these results demonstrate that PGE₂ induces CSCs by activating NF- κ B via both EP4-MAPK and EP4-PI3K-Akt pathways *in vitro*.

EP4 and NF- κ B are required for PGE₂ induction of CSCs and liver metastasis

We further examined whether EP4 and NF- κ B are required for PGE₂ induction of CSCs and liver metastasis *in vivo*. Analyses of IHC and flow cytometry show that knockdown of NF- κ B p65 reduces PGE₂ induction of CD133- and CD44v6-positive epithelial cells in cecal tumors as compared to the control (Figure 6A–B). Moreover, p65 knockdown cecal tumor cells isolated from PGE₂-treated mice formed similar sphere numbers as p65 knockdown cecal tumor cells isolated from vehicle-treated mice (Figure 6C), demonstrating that NF- κ B is required for PGE₂ induction of CSCs. Knockdown of p65 also completely inhibited PGE₂ promotion of liver metastasis (Figure 6D and Supplemental Figure S3A), but did not affect cecal tumor weight (Supplemental Figure S3B), suggesting that cecal CSCs contribute to liver metastasis. These results demonstrate that NF- κ B is required for PGE₂ induction of CSCs and liver metastasis *in vivo*. Moreover, the EP4 antagonist (Ono-AE3-208) attenuated the effect of PGE₂ on induction of cecal CSC expansion (Figure 6E) and liver metastasis (Fig. 6F), demonstrating that PGE₂ induces CSCs and promotes liver metastasis via EP4. Taken together, our results demonstrate that PGE₂ induces CSCs and promotes liver metastasis by activating NF- κ B via EP4-MAPK and EP4-PI3K-Akt pathways.

DISCUSSION

Despite recent advances in treatment and effective screening methods, today nearly 50% of patients who present with advanced CRC experience tumor recurrence die within 5 years following treatment. One explanation could be the presence of chemotherapy-resistant CSCs³³ that are not being properly targeted. Emerging evidence supports the hypothesis that inflammatory mediators regulate CSC formation and expansion. *In vitro* studies showed that IL-1 β and IL-8, pro-inflammatory cytokines, increased colonosphere and mammosphere formation, respectively^{34, 35} however, these studies did not demonstrate whether the cells from these spheres possessed properties of CSCs. A recent *in vivo* study showed that NF- κ B

p65 is required for crypt stem cell expansion³². It seems likely that other pro-inflammatory mediators could also contribute to CSC regulation. Considering the importance of the PTGS2-derived PGE₂ in inflammation and cancer, we postulated that PGE₂ could regulate CSCs. Indeed, our *in vivo* results show that exogenous PGE₂ increases mouse oncogenic colonic stem cells, whereas celecoxib decreases these cells with reduction of PGE₂ levels. Moreover, our *in vivo* results also reveal that exogenous PGE₂ induces human colonic CSCs and promotes liver metastasis, whereas reduction of endogenous PGE₂ levels by inhibiting PTGS2 activity reduces CSC expansion and suppresses liver metastasis, suggesting that PGE₂ induces liver metastasis via induction of CSCs. It is well established that both tumor cells and tumor-associated stromal cells, in particular macrophages, highly express PTGS2 and produce high levels of PGE₂. A recent report showed that mesenchymal stem cells (MSCs) that were co-cultured with cancer cells also expressed high PTGS2 and produced PGE₂. These MSCs have been reported to contribute to a cancer stem cell niche via their production of PGE₂³⁶. Here we provide direct evidence demonstrating that PGE₂ induces CSC expansion by directly targeting on colorectal adenoma and carcinoma cells. Collectively, our findings demonstrate that PGE₂ induces CSC expansion and promotes liver metastasis. Our results also suggest that PGE₂ promotes CRC initiation, growth, and metastasis via CSCs.

Multiple lines of evidence show that EP1, EP2, and EP4 contribute to intestinal tumorigenesis³⁷. In contrast, EP3 has been shown to inhibit intestinal tumorigenesis^{37, 38}. Previous work showed that treatment of C3L5 breast cancer cells with an EP4 antagonist resulted in reduction of sphere formation *in vitro* and decreased levels of CSC markers *in* C3L5 tumor-bearing mice³⁹, indicating that EP4 may mediate PGE₂ induction of CSCs in breast cancer. Our *in vitro* and *in vivo* results provide direct evidence demonstrating that EP4 mediates the effect of PGE₂ on induction of colonic CSC expansion and promotion of liver metastasis. Activation of PI3K-AKT signaling has been shown to enhance intestinal stem cell formation and expansion by enhancing β -catenin nuclear localization and transcriptional activation in mice⁴⁰. Moreover, a Ras-MAPK pathway is required for maintenance and proliferation of intestinal stem cells in *Drosophila*^{41, 42}. It is well established that PGE₂ activates PI3K and MAPK pathways via EP4 and both pathways can induce NF- κ B activation. Here we demonstrate that PGE₂ induces CSCs by activating NF- κ B via PI3K and MAPK pathways. Although both PI3K and MAPK pathways downstream of EP4 are independent, our results showed that both pathways are required for PGE₂ activation of NF- κ B. We further demonstrate that EP4, but not EP1 and EP2, mediates the effect of PGE₂ on induction of NF- κ B activation. More importantly, our *in vivo* results demonstrate that NF- κ B is required for PGE₂ induction of CSCs and liver metastasis. These results indicate that an inflammatory pathway, PGE₂-NF- κ B, mediates the effects of chronic inflammation on CSC expansion. Although PGE₂ has been shown to play a role in the regulation of embryonic and adult stem cell homeostasis^{43–45} as well as in mouse colonic stem cells⁴⁶, we extended the scope of our research to reveal that PGE₂ can also induce CSC expansion.

In conclusion, our findings not only uncover a critical role of PGE₂ in regulation of CSCs, but also reveal a novel mechanism for the contribution of chronic inflammation to CSC

expansion. Moreover, our *in vitro* and *in vivo* results are likely to be clinical relevant because the concentration of PGE₂ is positively correlated with the expression of CSS markers in human CRC specimens. Finally, our findings may provide a rationale for development of EP4 antagonists as new therapeutic agents in treatment of CRC patients via eliminating CSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CSC	cancer stem cells
NSAIDs	nonsteroidal anti-inflammatory drugs
PGE₂	Prostaglandin E ₂
PTGS (COX)	prostaglandin-endoperoxide synthases (cyclooxygenase)

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Figure 1. PGE₂ levels correlate with the expression of CSC markers in human CRC
 The levels of PGE₂ are correlated with the expression of *CD133* (panel A), *CD44* (panel B), *LRG5* (panel C), and *SOX2* (panel D) at mRNA levels in 25 pairs of human colorectal carcinomas (T) with matched normal tissues (N). Data were presented as fold changes in cancer specimens as compared to matched normal tissues. Nonparametric Spearman correlation analysis (R value) was performed.

Figure 2. PGE₂ increases CSCs and promotes liver metastasis

(A) Left panel, the representative immunostaining of CD133 and CD44 (brown) in colonic adenomas taken from *Apc^{Min/+}* mice treated with PGE₂ or vehicle (scale bar, 50 μ m). Right panel, relative expression of CD133 and CD44 at mRNA levels in polyps. (B) Formation of spheres from colonic epithelial adenoma cells isolated from *Apc^{Min/+}* mice treated with PGE₂ or vehicle. (C) The representative immunostaining of CD44v6 and CD133 (brown) in cecal tumors taken from indicated group of mice treated with PGE₂ or vehicle (scale bar, 50 μ m). (D) Cells isolated from indicated cecal tumors were subjected into flow cytometry analysis. Data represents the percentage of EpCAM⁺CD44v6⁺ (top panel) and EpCAM⁺CD133⁺ (bottom panel) cells in total cells of cecal tumor taken from above groups. (E) Formation of spheres from cecal tumor cells isolated from mice treated with PGE₂ or vehicle in above groups. (F) Incidence (left panel) of liver metastatic tumors and average numbers of liver metastatic tumors (middle and right panels) in mice treated with PGE₂ or vehicle in above groups. The error bar indicates $\pm$ SEM. * $p < 0.05$.

Figure 3. Reduction of PGE₂ suppresses CSCs and inhibits liver metastasis

(A) Formation of spheres from intestinal adenoma cells isolated from *Apc^{Min/+}* mice treated with vehicle, celecoxib, or Ono-AE3-208. (B) Average numbers of polyps at different size and total that includes all sizes in above treatment groups. (C) Intestinal PGE₂ levels in above treatment groups. (D) Formation of spheres from cecal tumor cells isolated from NSG mice treated with vehicle, celecoxib, PGE₂, or celecoxib plus PGE₂ after primary carcinoma cells from human CRC specimen were injected into cecum. (E) Average numbers of liver metastatic tumors at different size and total that includes all sizes in mice as described in panel D. (F) Cecal PGE₂ levels in mice as described in panel D. The error bar indicates $\pm$ SEM. *p<0.05.

Figure 4. PGE₂ directly induces CSCs in tumor epithelial cells

(A–D) LS-174T cells (A), mouse colonic epithelial tumor cells (B), primary carcinoma cells isolated from three CRC patients (C), and both CD133^{-/-}CD44^{-/-} and CD133^{+/+}CD44^{+/+} LS-174T cells (D) were treated with vehicle or PGE₂ during *in vitro* sphere-forming assays. Left panels are representative phase contrast photos in panels A–C (scale bar, 50μm). Data in right panels represent the average of sphere numbers from three independent experiments. (E) The LS-174T cells and primary carcinoma cells from human CRC specimens were pretreated with vehicle or PGE₂ in regular tissue culture plates for 48 h. After treatment, the cells were subject to *in vitro* sphere-forming assays without PGE₂ treatment. The error bar for panels C indicates ± SD. The error bar for other panels indicates ± SEM. * p<0.05.

Figure 5. PGE₂ induces CSCs by activating NF-κB via EP4-PI3K and EP4-MAPK-pathways
 (A) Formation of spheres in LS-174T cells (left panel) and human primary colon carcinoma cells (right panel) treated with vehicle, PGE₂, ONO-AE-208 (Ono), PD98059 (PD), and/or LY294002 (Ly). (B) Formation of spheres in LS-174T/con-sh (non-silencing control shRNA), LS-174T/shERK1, and LS-174T/shp85α cells treated with PGE₂ or vehicle (left panel). The protein levels of ERK1 and p85α in indicated cells were measured by Western blotting (right panels). (C) The nuclear NF-κB p65 levels in LS-174T/con-sh, LS-174T/shp85α, and LS-174T/shERK1 cells treated with PGE₂ or vehicle for 24 hr. The band density was quantitated by densitometry scanning. The band density of p65 was normalized by the band density of Lamin A. The results were reported as a mean of fold-induction from three independent experiments (low panel). (D) Formation of spheres in LS-174T/con-sh and LS-174T/shp65 cells treated with PGE₂ or vehicle (left panel). The protein levels of p65 in these cells were measured as described above (right panel).

Fig. 6. EP4 and NF- κ B are required for PGE₂ induction of CSCs and liver metastasis
 (A–B) LS-174T/con-sh or LS-174T/shp65 cells were injected into the cecal walls of NSG mice. After injection, these mice were treated with vehicle or PGE₂. (A) The representative immunostaining of CD44v6 and CD133 (brown) in cecal tumors taken from mice treated with PGE₂ or vehicle in vector group (left panel) and shp65 group (right panel), scale bar, 50 μm. (B) Data represents the percentage of EpCAM⁺CD44v6⁺ (top panel) and EpCAM⁺CD133⁺ (bottom panel) cells in total cells of cecal tumor in indicated group of mice treated with PGE₂ or vehicle. (C) Formation of spheres from cecal tumor cells isolated from mice treated with PGE₂ or vehicle in above groups. (D) Average numbers of liver metastatic tumors in above groups. (E) Formation of spheres from cecal tumor cells isolated from mice treated with vehicle, Ono-AE3-208, PGE₂, or Ono-AE3-208 plus PGE₂ after LS-174T cells were injected into mouse cecum. (F) Average numbers of liver metastatic tumors at different size and total that includes all sizes in groups as described in panel E. The error bar indicates $\pm$ SEM. *p<0.05.

Table 1

Tumorigenicity of cecal tumor cells from mice treated with PGE₂ or vehicle

No. of injected cells	Number of mice with tumors/total injected mice (% of mice with tumors)				
	27 days	35 days	45 days	56 days	
Vehicle	10,000	0/6 (0)	3/6 (50)	4/6 (67)	4/6 (67)
	2,000	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)
	500	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)
PGE ₂	10,000	3/6 (50)	5/6 (83)	6/6 (100)	6/6 (100)
	2,000	0/6 (0)	2/6 (33)	3/6 (50)	3/6 (50)
	500	0/6 (0)	0/6 (0)	1/6 (17)	1/6 (17)