Published in final edited form as:

Cancer Res. 2019 September 01; 79(17): 4480–4490. doi:10.1158/0008-5472.CAN-19-0384.

PPAR δ mediates the effect of dietary fat in promoting colorectal cancer metastasis

Dingzhi Wang^{1, #}, Lingchen Fu^{2, #}, Jie Wei^{1, #}, Ying Xiong¹, Raymond N. DuBois^{1, 3, *}

¹Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425

²Laboratory for Inflammation and Cancer, Biodesign Institute of Arizona State University, Tempe, AZ 85287

³Department of Research and Division of Gastroenterology, Mayo Clinic, Scottsdale, AZ 85259

Abstract

The nuclear hormone receptor peroxisome proliferator-activated receptor delta (PPARdelta) is a ligand-dependent transcription factor involved in fatty acid metabolism, obesity, wound healing, inflammation, and cancer. Although PPARdelta has been shown to promote intestinal adenoma formation and growth, the molecular mechanisms underlying the contribution of PPARdelta to colorectal cancer (CRC) remain unclear. Here we demonstrate that activation of PPARdelta induces expansion of colonic cancer stem cell (CSC) and promotes CRC liver metastasis by binding to the Nanog promoter and enhancing Nanog expression. Moreover, PPARdelta mediated the effect of a high-fat diet in promoting liver metastasis and induction of colonic CSC expansion. Our findings uncover a novel role of dietary fats in CRC metastasis and reveal novel mechanisms underlying PPARdelta-mediated induction of CSCs and those responsible for the contribution of dietary fats to CRC progression. These findings may provide a rationale for developing PPARdelta antagonists to therapeutically target CSCs in CRC.

Keywords

PPAR δ ; dietary fat; Nanog; cancer stem cells; metastasis

Introduction

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related deaths in the USA. Although colonoscopy screening is an effective way to detect and prevent CRC by removing precancerous adenomas in many patients, a high percentage of patients continue to present to their physician with advanced cancer. Liver and lung metastases occur in about 20–70% and 10–20% of patients, respectively. Unfortunately, distant metastases are the major cause of death for patients with advanced CRC. The

*Correspondence to: Raymond N. DuBois, MD, Ph.D., 601 Clinical Science Building, 96 Jonathan Lucas Street, Suite 601, Charleston, SC 29425, Tel: 843-792-2842 and Fax: 843-792-2967, duboisrn@muscc.edu.

[#]Equal contribution to the manuscript

Conflict of interest disclosure statement: All authors have no any conflict interests

standard therapies for metastatic CRC have improved considerably but we continue to face a dismal 5-year survival rate when patients present with advanced disease. Clearly, cancer prevention and interception is being considered a plausible approach for the population and individuals at high risk for developing a number of different cancers. Newly developed cancer immunotherapies and targeted therapy are being carefully evaluated on several fronts to achieve better outcomes. However, the development of novel strategies for CRC prevention and interception relies on understanding the molecular mechanisms responsible for CRC initiation, progression, and metastatic spread.

Two primary hypotheses have been proposed to explain cancer initiation and progression. The first postulates is that the initiation and progression of these malignancies depend on a series of somatic mutations and/or epigenetic alterations that occur in different stages of cancer. The second hypothesis considers that heterogeneous solid tumors originate from a rare population of undifferentiated cancer cells (1–3), which could have also acquired driver mutations. Undifferentiated cancer cells that possess the capacity of self-renewal, differentiate into a heterogeneous lineage, and develop innate resistance to cytotoxic agents have been defined as cancer stem cells (CSCs) or tumor-initiating cells (4,5). CSCs are thought to be responsible for tumor initiation, growth, metastatic spread, relapse, and recurrence. Several studies have shown that CSCs are present in human CRC and are capable of initiating tumor development (5,6). However, very little is known about their biology and how they are regulated.

Exposure to diets high in fat content is associated with some human diseases such as obesity, diabetes, dyslipidemias, and cancer (7,8). However, the molecular mechanism by which high-fat diets contribute to cancer remain poorly understood. Nuclear hormone receptors, such as peroxisome proliferator-activated receptors (PPARs), play a central role in regulating the storage and catabolism of dietary fats via complex metabolic pathways (9). These nuclear receptors are also ligand-dependent transcription factors that bind to peroxisome proliferator responsive elements (PPREs) located in promoter regions of responsive genes and initiate the gene transcription. PPARs-regulates genes involved in both physiologic and pathophysiologic processes. PPARs are activated by natural ligands, including certain fatty acids and fatty acid derivatives, and synthetic agonists. To date, three mammalian PPARs have been identified and are referred to as PPAR α , PPAR δ/β and PPAR γ . Each PPAR isotype displays a tissue-selective expression pattern. PPAR α and PPAR γ are predominantly present in the liver and adipose tissue, respectively, while PPAR δ is more broadly expressed in diverse tissues (10) and its expression level is very high in the gastrointestinal tract compared with other tissues (11). Emerging evidence that PPAR δ is required for the maintenance of hematopoietic stem cells (12) prompted us to postulate that it might also promote CRC initiation, growth, and liver metastasis by induction of CSC expansion.

Nanog is one of the key transcription factors governing self-renewal and pluripotency of stem cells (13). For example, forced expression of Nanog is sufficient to maintain pluripotency of embryonic stem (ES) cells, whereas loss of Nanog results in differentiation of ES cells (14). Nanog is expressed in stem cells, yet it is absent in healthy differentiated cells of adult organisms. Interestingly, Nanog expression is upregulated in a variety of human malignancies. For example, high levels of Nanog are associated with poor overall and

recurrence-free survival in CRC (15). Moreover, Nanog expression correlates with advanced cancer stages and liver metastasis in CRC patients, suggesting that Nanog is a potential biomarker for CRC liver metastasis (16). Although cancer cells that express Nanog exhibit stem cell properties (17), how Nanog is regulated in cancers is poorly understood.

Materials and Methods

Cell culture

HCT-116 and LS-174T cell lines were obtained from ATCC (Manassas, VA) in 2014. Parental LS-174T (ATCC), LS-174T/vector, LS-174T/shPPAR δ , LS-174T/shNanog, parental HCT-116 (ATCC), HCT-116/WT, HCT-116/PPAR $\delta^{-/-}$, HCT-116/vector, and HCT-116/shNanog cells were maintained in McCoy's 5A medium (Life Technologies) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). All CRC cells are used between passages 2 to 5. All cell lines have been tested by MycoProbe Mycoplasma Detection Kit (R&D) and also authenticated before the experiment according ATCC STR database. For GW501516 treatment, the cells were cultured with serum-free medium for 24 or 48 h and then treated with vehicle or indicated concentration of GW501516 for 24 h. After treatment, the cells were subjected to *in vitro* sphere-forming assay, subcutaneous (sub-Q) injection of NSG mice, q-PCR, Western blotting, luciferase assay, and ChIP assay.

Animal experiments

All animal experiments conform to our animal protocols that were approved by the Institutional Animal Care and Use Committee at MUSC. NSG and *Apc*^{Min/+} mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Male *Apc*^{Min/+} mice at age of 7 weeks old were fed with a control diet or a diet containing GW501516 (10 mg/kg body weight) for 7 weeks. For the sub-Q injection, the cell numbers indicated in Tables 1–2 and Supplementary Tables S1–S2 were injected into the flanks of male NSG mice at age of 7 weeks old. For the orthotopic mouse model, 2.5×10^4 of cells were injected into the cecal wall of male NSG mice at age of 7 weeks old. After injection, the mice bearing HCT-116-derived cells or LS-174T-derived cells were fed with a control diet, a diet containing GW501516 (10 mg/kg body weight), or a high-fat diet (D12492, Research Diets INC) for 6 or 10 weeks.

Immunohistochemical staining

Paraffin-embedded tissue sections (5 μ m thick; n=5 per animal) were stained with anti-Nanog rabbit antibody (1:100, abcam, Cambridge, MA) and anti-CD44v6 mouse monoclonal antibody (1:50, R&D System) in 4°C overnight. The immunohistochemical staining was completed by using a Zymed-Histostain-SP Kit (Zymed, South San Francisco, CA) according to the manufacturer's instructions.

Isolation of tumor epithelial cells

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. Normal intestinal tissues and intestinal adenomas isolated from *Apc*^{Min/+} mice were minced and digested with PBS containing with 0.1% BSA and 12

mg/ml collagenase I (Gibco). Single-cell suspensions were subjected to flow cytometry analysis. Single-cell suspensions were subjected to flow cytometry analysis. In addition, tumor epithelial cells were purified by Flow Cytometry sorting (see below). Isolated epithelial cells were subjected to *in vitro* sphere-forming assays, sub-Q injection of NSG mice, and q-PCR.

Flow cytometry analysis and sorting

Single-cell suspensions in staining buffer (Biolegend) were incubated with anti-hEpCAM (CD326)-APC-Vio770 (1:100, Miltenyi Biotec), anti-mCD45-PE-Cy7 (1:300, Biolegend), anti-hCD44v6-PE (1:20, R&D System), and/or anti-mEpCAM-APC-Vio770 antibodies for 30 min on ice. After the cells were washed twice with 1 ml of the labeling buffer, they were analyzed on a FortessaX20 Flow Cytometer (BD Biosciences) or were sorted by MoFlo Astrios EQ Cell Sorter (Beckman Coulter). For analysis of Nanog expression in tumor epithelial cells, single-cell suspensions were stained with cell surface markers as described above. Then, the cells were fixed and permeabilized by using a Cytofix/Cytoperm kit (BD Biosciences) followed by intracellular cellular staining with primary anti-human Nanog rabbit antibody (1:100, abcam) in permeabilization buffer for 30 min on ice. After washing with permeabilization buffer, the cells were incubated with second anti-rabbit-IgG-PE (1:200, Biolegend) in permeabilization buffer for 30 min on ice. After the cells were washed twice with 1 ml of the permeabilization buffer, they were analyzed on a FortessaX20 Flow Cytometer (BD Biosciences). The flow cytometric profiles were analyzed by counting 20,000 events using FLOWJO software program (FLOWJO, LLC).

In vitro sphere-forming assay

30,000 cells from human CRC cell lines or purified cecal tumor epithelial 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 for three weeks. The sphere numbers in each well were quantified.

Lentivirus production and stable transfection

PPAR δ and Nanog shRNAs were purchased from Open Biosystems. Lentivirus production and stable transfection were performed according to the manufacturer's instructions.

Western blot analysis

Whole cell extracts were prepared from indicated cells. Transfer membranes were blocked with 5% dry milk in TBS-T buffer for 1 h and then incubated with anti-hPPAR δ (Santa Cruz Biotechnology, 1:500) or anti-hNanog (Cell Signaling, 1:1000) antibody for overnight at 4 °C. The blots were stripped and re-probed with β -actin antibody (Sigma-Aldrich, 1:6000).

Quantitative PCR

Total RNA was isolated from human cell lines and purified cecal tumor epithelial cells 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 CD44 and Nanog were obtained from Life Technologies. The relative expression of each target gene represents an average of triplicates that are normalized against the transcription levels of β -actin.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology). Briefly, cells were treated with 1% formaldehyde for 10 min at room temperature to crosslink proteins to DNA, which was then quenched by adding glycine to 0.125 M for 5 min at room temperature. Crosslinked chromatin was digested with 250 units of Micrococcal Nuclease per IP to reduce the DNA length to 150–900 base pairs. One μ g of anti-PPAR δ antibody (Santa Cruz) was used to immunoprecipitate the crosslinked DNA per IP. After being reverse crosslinked, the DNA was purified and eluted into 50 μ l of elution buffer. The amount of immunoprecipitated DNA residing in the *Nanog* promoter region was detected by PCR with primers targeting *Nanog* promoter. The primers used for PCR were forward 5'-TCCCAGGTTCAAGGGATTCT-3' and reverse 5'-TGGCCAACATGGCGAAA-3'.

Luciferase assay

Human pNanog-Luc plasmid (–1942 to +24) is a gift from Dr. Ren-He Xu. A PPRE site (–524 to –514) of pNanog-Luc was deleted as a mutant pNanog-Luc. For dual luciferase reporter assays, cells were transfected with the firefly luciferase reporter constructs and the control renilla luciferase reporter pRL-CMV using Lipofectamine™ (Invitrogen). After treatment, cells were lysed with cell lysis buffer provided by the dual-luciferase reporter assay kit (Promega, Madison, WI). Luciferase activity was then measured according to the manufacture's instruction.

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

Activation of PPAR δ induces CSC expansion by induction of Nanog expression *in vitro*

To determine whether activation of PPAR δ increases the number of colorectal carcinoma cells with intrinsic self-renewal properties, LS-174T cells were first treated with a PPAR δ agonist (GW501516) at the indicated dosages (Fig. 1A, left panel). After treatment, these cells were subjected to *in vitro* sphere-forming assays in the absence of GW501516 treatment. We found that GW501516-treated cells formed many more spheres than vehicle-treated cells (Fig. 1A, left panel). In contrast, depletion of PPAR δ in LS-174T cells partially attenuated the effect of GW501516 at 0.1 μ M on induction of cells with self-renewal

properties (Fig. 1A, right panel), demonstrating that PPAR δ mediates the effect of GW501516 on the cells with self-renewal properties. Similarly, pretreatment of GW501516 resulted in more sphere formation in wild-type HCT-116 cells, but not in PPAR δ null HCT-116 cells (Fig. 1B). These results demonstrate that activation of PPAR δ induces the expansion of cancer stem-like cells. Limiting dilution transplantation assays in xenograft models are considered to be the gold standard to assess CSCs. Following pre-treatment of LS-174T and HCT-116 cells with GW501516 or vehicle, the cells were then injected into the flanks of NSG (NOD-SCID-*IL-2R $\gamma^{-/-}$*) mice. Seven of 8 mice implanted with as few as 50 GW501516-treated LS-174T cells developed tumors, whereas 2 of 8 mice with vehicle-treated cells formed any measurable tumor (Supplementary Table S1). Similar results were obtained for HCT-116 cells (Supplementary Table S1). Collectively, these results demonstrate that activation of PPAR δ results in an increase of the number of colonic CSCs *in vitro*.

To further identify which targets of PPAR δ are responsible for regulation of CSCs, we first examined whether PPAR δ binding sites were present within the promoters of self-renewal regulatory factors such as Oct4, Nanog, Sox2, and KLF4. One potential PPRE site (–524 to –514) was found within the Nanog promoter, but not within Oct4, Sox2, and KLF4. We next examined whether activation of PPAR δ with GW501516 induced Nanog expression. As shown in Figure 1C–D, LS-174T cells treated with GW501516 exhibited an increase in both Nanog protein and mRNA levels. In contrast, knockdown of PPAR δ inhibited the effect of GW501516 on induction of Nanog expression (Fig. 1C–D). Similar results were observed in HCT-116 cells (Supplementary Fig. S1). These results establish that activation of PPAR δ induces Nanog expression. To further examine whether PPAR δ binds to the Nanog promoter, ChIP assays were performed. After activation by GW501516, PPAR δ bound to the PPRE site (–524 to –514) within the Nanog promoter as compared to vehicle treatment (Fig. 1E). Moreover, GW501516 treatment induced transcription from a wild type Nanog promoter, but not from a mutant Nanog promoter in which the PPRE site was deleted (Fig. 1F). These results reveal that following activation, PPAR δ binds to the Nanog promoter resulting in increased expression of *Nanog* mRNA. Importantly, knockdown of Nanog completely inhibited the ability of PPAR δ to induce CSC expansion (Fig. 1G), demonstrating that Nanog mediates the effect of PPAR δ on induction of CSC expansion. Taken together, these results reveal that activation of PPAR δ induces colonic CSC expansion by direct induction of Nanog expression via binding to its promoter.

Activation of PPAR δ induces CSC expansion and accelerates liver metastasis *in vivo*

Apc^{Min} mice represent a pre-malignant model for human CRC. In *Apc^{Min}* mice, activation of PPAR δ resulted in increased Nanog expression in both normal intestinal and tumor epithelial cells (Supplementary Fig. S2A). Moreover, intestinal tumor cells isolated from GW501516-treated mice formed more spheres in culture than those taken from control mice (Supplementary Fig. S2B), indicating that adenomas from GW501516-treated mice contain more tumor stem cells than those from vehicle-treated mice. As mentioned above, CSCs are thought by many investigators to be responsible for metastatic spread from the primary site. We therefore examined whether activation of PPAR δ induced CSC expansion in primary tumors and promoted liver metastasis. We utilized an orthotopic model of metastatic CRC to

evaluate factors involved in tumor spread. LS-174T/vector, LS-174T/shPPAR δ , HCT-116/WT, or HCT-116/PPAR $\delta^{-/-}$ cells were injected into the cecal wall of NSG mice. After injection, the mice were treated with vehicle or GW501516. Analyses of IHC and flow cytometry revealed that GW501516 increased the numbers of Nanog-positive and CD44v6-positive tumor epithelial cells found in cecal tumors (Fig. 2A–D). Nanog is expressed in the nucleus of epithelial cells, whereas CD44v6 is expressed on the cell surface (Fig. 2A and 2C). CD44 is one of the informative markers for CSCs in CRC. Moreover, mRNA levels of Nanog and CD44 were also higher in cecal tumors from GW501516-treated mice compared to those taken from control mice (Supplementary Fig. S2C). In addition, cecal tumor cells isolated from GW501516-treated mice injected with vector or WT cells formed more spheres than those from vehicle-treated mice (Fig. 2E). In contrast, knockdown of PPAR δ in LS-174T cells or deletion of PPAR δ in HCT-116 cells completely inhibited the effects of GW501516 on induction of Nanog and CD44 expression and sphere formation (Fig. 2A–E), demonstrating that PPAR δ mediates the effects of GW501516 on induction of Nanog and CD44 expression and expansion of cells with self-renewal properties in cecal tumors. We further evaluated the tumor-initiating ability of these carcinoma cells after being placed in the cecum. After completion of limiting dilution transplantation experiments, 7 of 8 NSG mice implanted with 200 LS-174T-derived cecal tumor cells from GW501516-treated mice developed tumors, whereas only 2 of 8 mice implanted with 200 LS-174T-derived cecal tumor cells from vehicle-treated mice formed measurable tumors (Table 1). Similar results were obtained when HCT-116 cells were evaluated (Table 1). Importantly, GW501516 treatment resulted in an increased number of metastatic lesions in the liver and the overall liver tumor burden (Fig. 3A–C). Similarly, knockdown or deletion of PPAR δ completely inhibited the effects of GW501516 on promotion of liver metastasis (Fig. 3A–C). Interestingly, GW501516 treatment did not affect cecal tumor weight (Supplementary Fig. S2D). Collectively, these data establish that activation of PPAR δ induces colonic CSC expansion and promotes liver metastasis. Furthermore, the data imply that Nanog mediates the effects of PPAR δ on induction of colonic CSC expansion and promotion of liver metastasis.

Nanog is required for PPAR δ induction of CSC expansion and liver metastasis

We next addressed whether Nanog is required for PPAR δ induction of CSC expansion, metastatic spread to the liver and growth *in vivo*. LS-174T/vector, LS-174T/shNanog, HCT-116/vector, or HCT-116/shNanog cells were injected into the cecal wall of NSG mice. Following injection, the mice were treated with vehicle or GW501516. Cecal tumor cells isolated from GW501516-treated mice injected with vector cells were found to form more spheres than those from vehicle-treated mice (Fig. 4A–B). In contrast, Nanog knockdown in cecal tumor cells isolated from GW501516-treated mice developed similar spheroid numbers to those from Nanog knockdown in cecal tumor cells isolated from vehicle-treated mice (Fig. 4A–B). These data demonstrate that Nanog mediates PPAR δ induction of CSC expansion in cecal tumors. Moreover, knockdown of Nanog also completely inhibited PPAR δ promotion of liver metastasis (Fig. 4C–D), but did not impact the effect of GW501516 on cecal tumor weight (Supplementary Fig. S3A–B). These results demonstrate that Nanog is required for PPAR δ promotion of liver metastasis and that cecal CSCs contribute to liver metastasis. Taken together, our results demonstrate that activation of

PPAR δ promotes liver metastasis by induction of CSC expansion via directly binding to the Nanog promoter and inducing its expression. To assess human relevance of this pathway, the correlation between PPAR δ levels and Nanog levels in human colorectal carcinoma specimens with matched normal tissues was evaluated. PPAR δ mRNA levels positively correlated with the mRNA levels of Nanog in human colorectal carcinoma specimens (Fig. 4E). Interestingly, there is also a positive correlation between PPAR δ and CD44 in these specimens (Fig. 4F).

The PPAR δ -Nanog pathway mediates the effect of a high-fat diet on induction of CSC expansion and liver metastasis

Although diets high in fat have been shown to increase intestinal polyp burden in mouse models of CRC (18–20), the role of a high-fat diet in CRC metastasis has not really been carefully evaluated. Similar to treatment with a PPAR δ agonist, exposure to a high-fat diet induced the expression of Nanog (Fig. 5A–B) and CD44v6 (Fig. 5C–D) in epithelial cells taken from cecal tumors. In addition, exposure to a high-fat diet also increased the number of CSCs found in cecal tumors (Fig. 5E). In contrast, deletion of PPAR δ completely inhibited the effect of a high-fat diet on induction of Nanog and CD44 expression as well as expansion of CSCs (Fig. 5A–E), demonstrating that PPAR δ mediates the predominant effect of a high-fat diet on CSC expansion. Moreover, 3 of 8 NSG mice implanted with 500 HCT-116-derived cecal tumor cells from a high-fat diet-treated mice developed tumors, whereas 0 of 8 mice with 500 HCT-116-derived cecal tumor cells from control mice formed measurable tumors (Supplementary Table S2). Most importantly, a high-fat diet accelerated metastatic liver tumor formation and growth (Fig. 5F) and loss of PPAR δ completely inhibited the effect of a high-fat diet on promotion of liver metastasis (Fig. 5F). Moreover, exposure to a high-fat diet did not affect cecal tumor weight (Supplementary Fig. S3C). Interestingly, the high-fat diet significantly increased the body weight of NSG mice after two-weeks treatment, but the increase of the body weight is not significant after four- and six-weeks treatment (Supplementary Fig. S3D). Similarly, knockdown of Nanog completely attenuated the effect of a high-fat diet on induction of CD44 expression and CSC expansion (Fig. 6A–B) as well as promotion of liver metastasis (Fig. 6C). Moreover, 5 of 8 NSG mice implanted with 200 LS-174T-derived cecal tumor cells from a high-fat diet-treated mice developed tumors, whereas 1 of 8 mice with 200 LS-174T-derived cecal tumor cells from control mice formed measurable tumors (Supplementary Table S3). Cumulatively, these results demonstrate that the PPAR δ -Nanog pathway mediates the effect of a high-fat diet on induction of CSC expansion and liver metastasis.

Discussion

The role of PPAR δ in metabolism has been well established (21) and PPAR δ agonists have been examined for their potential use for treatment of dyslipidemia, metabolic syndrome, and Type-2 diabetes. In addition, new results from a phase 2 study showed that daily treatment with seladelpar, a PPAR δ agonist, for primary biliary cholangitis was safe and had a potent anti-cholestatic effects (<https://www.healio.com/hepatology/autoimmune-cholestatic-biliary-diseases/news/online/%7B6516e193-4b47-4b3f-9e40-e8e5095ba408%7D/seladelpar-provides-safe-potent-anti-cholestatic-effect-in-pbc>).

However, the biological function of PPAR δ in tumorigenesis remains unclear and somewhat controversial. PPAR δ polymorphisms have been shown to be associated with CRC (22,23). Although investigation of PPAR δ expression in human CRC specimens generated disparate results (24,25), one report showed that the PPAR δ protein accumulated only in human CRC cells with a highly malignant morphology (25). Whether PPAR δ possesses pro-tumor or anti-tumor effects may depend on the context being studied, the tissue type being examined, and the specific genetic strategy utilized to disrupt its functions. In the mammary gland, most *in vivo* studies suggest that PPAR δ has a pro-tumor effect (26). However, the function of PPAR δ in CRC remains disputed. The first evidence linking PPAR δ to carcinogenesis actually emerged from studies on CRC. PPAR δ was initially identified as a direct transcriptional target of APC/ β -catenin/Tcf pathway and as a repressive target of NSAIDs (27,28). A case-control study in a large cohort showed that the protective effect of NSAIDs against colorectal adenomas was reported to be modulated by a polymorphism in the *PPARD* gene (29). PPAR δ expression and activity are also induced by oncogenic K-Ras (30). Moreover, COX-2-derived PGI $_2$, an endogenous fatty-acid derivative, directly transactivates PPAR δ (31) and COX-2-derived PGE $_2$, another endogenous fatty-acid derivative, indirectly induces PPAR δ activation in CRC (32) and other cancer cells (24). Importantly, the elevation of both PPAR δ and COX-2 in tumor tissues was correlated with a poor prognosis in patients with CRC (33). These studies indicate that PPAR δ is a focal point of cross-talk between oncogenic signaling pathways. Our group reported the first *in vivo* evidence showing that activation of PPAR δ by its agonist (GW501516) or a PPAR δ endogenous activator (PGE $_2$) accelerated intestinal tumor formation and growth in *Apc*^{Min/+} mice by promoting tumor cell survival (32,34). In loss-of-function studies, *Apc*^{Min/+} mice in which PPAR δ exons 4–5 were deleted and azoxymethane (AOM)-treated mice in which PPAR δ exon 4 was deleted in only colonic epithelial cells showed that PPAR δ had a pro-tumor effect (32,35,36). In addition, deletion of PPAR δ exons 4–5 also attenuated chronic inflammation-induced colonic tumor formation and growth (37). In contrast, results from mice in which PPAR δ exon 8 was deleted showed that PPAR δ exerts an anti-tumorigenic function in *Apc*^{Min/+} and AOM-treated mice (38,39). Deletion of PPAR δ exon 4 and/or 5, which encodes an essential portion of the DNA binding domain, is thought to completely disrupt PPAR δ function as a nuclear transcriptional factor and to inhibit tumorigenesis. Interestingly, four recurrent variants of PPAR δ polymorphisms were detected in or adjacent to exon 4 in patients with CRC (22). The deletion of exon 8, the last PPAR δ exon, may produce a hypomorphic protein, which retains some apo-receptor function. In a gain-of-function of study, targeted PPAR δ overexpression in intestinal epithelium promoted colonic tumorigenesis in AOM-treated mice (40). Collectively, these results strongly support the notion that activation of PPAR δ accelerates intestinal polyp formation and growth. However, the mechanisms by which PPAR δ promotes CRC formation and growth have remained elusive. A recent *in vivo* study revealed that a PPAR δ agonist enhanced intestinal stemness and tumor-initiating capacity of adenoma cells (41). However, that study did not provide evidence demonstrating that PPAR δ mediates the effect of this agonist on induction of stemness and tumor-initiating capacity since PPAR δ agonists have off-target effects. Our results here provide direct genetic evidence demonstrating that activation of PPAR δ induces colonic CSC expansion *in vitro* (Fig. 1 and Supplementary Table S1) and *in vivo* (Fig. 2 and Table 1). Although a recent report showed that deletion of PPAR δ in HCT-116 cells attenuated tumor cell spread to lung

or liver when cells were into the tail vein or the spleen of immunodeficient mice (42), we provide the first evidence showing that activation of PPAR δ accelerates liver metastasis in a orthotopic mouse model of metastatic CRC (Fig. 3).

Several studies have shown that Nanog enhances cancer cell migration and invasion. For example, *in vitro* studies demonstrated that knockdown of Nanog resulted in reduction of cell migration and invasion, whereas overexpression of Nanog caused cell migration and invasion in ovarian and liver cancers (17,43). An *in vivo* study revealed that Nanog alone was not sufficient to cause tumor formation, but enhanced migration and invasion of breast cancer cells (44). As mentioned earlier, Nanog is one of the self-renewal transcriptional factors which have been used to generate induced pluripotent stem cells (13). It has also been reported that Nanog regulates self-renewal of CSCs via induction of IGF1R in liver cancer (17). Further studies are warranted to investigate how Nanog enhances CSC expansion. In addition, it has been reported that Nanog is regulated by several factors in cancer cells. For example, snail1 was found to up-regulate Nanog expression via the Smad1/Akt/Gsk3b cascade in lung cancer (45). Hedgehog has previously been shown to activate Nanog gene transcription via GLI1 in glioblastoma multiforme (46). β -catenin and c-Jun have been shown to up-regulate Nanog expression in CRC cell lines (47). In our current study, we reveal that activation of PPAR δ induces Nanog expression by directly binding to the Nanog promoter in colorectal carcinoma cells (Fig. 1). Most importantly, we found that Nanog is required for PPAR δ induction of colonic CSC expansion and liver metastasis (Figs. 1G and 4).

In addition to genetic mutations, epigenetic changes, and chronic inflammation, lifestyle such as the “Western” diet is also a risk factor for cancer (8), including CRC (48). A higher intake of a Western-like diet after the diagnosis of CRC at stage III is associated with a significantly worse disease-free survival (49). A Western-style diet often includes high saturated fat content, red and processed meat, with excess total calories. Saturated fat is mainly derived from animal sources of food, such as red meat, poultry and full-fat dairy products. Although several studies have found that high-fat diets accelerated intestinal polyp burden in mouse models of CRC (18–20), it was unclear whether intake of a diet high in fat promotes CRC metastasis. Our results demonstrate that PPAR δ -Nanog pathway mediates the effect of a high-fat diet on acceleration of CRC liver metastasis (Figs. 5F and 6C). Moreover, it has been reported that a high-fat diet correlates with elevation of Nanog expression in mouse gastric mucosa (50). Indeed, our results reveal that a high-fat diet induces Nanog expression through activation of PPAR δ in primary tumors (Fig. 5A–B). A recent *in vivo* study revealed that a PPAR δ agonist mimicked the effect of a high-fat diet on enhancement of intestinal stemness and the tumor-initiating capacity of tumor cells (41). Since most drugs have off-target effects, this study did not provide evidence demonstrating that PPAR δ mediates the effect of the high-fat diet on promotion of stemness and tumor-initiating capacity. Our study revealed that PPAR δ mediates the effect of the high-fat diet on promotion of colon CSC expansion in primary tumors (Fig. 5A–E). Since fatty acids and their derivatives are natural ligands for PPAR δ , we postulate that they can directly activate PPAR δ . Further investigation is needed to identify which endogenous fatty acids or their derivatives are PPAR δ ligands and which are required for PPAR δ induction of CSC expansion and liver metastasis.

In conclusion, our findings uncover a critical role of dietary fat in promotion of CRC metastasis, and reveal a potential mechanism responsible for the contribution of dietary fat to CRC initiation and metastasis. Moreover, our results further reveal a novel mechanism by which PPAR δ induces CSC expansion by binding to the Nanog promoter and inducing Nanog expression. Finally, our findings not only highlight concerns about the use of PPAR δ agonists for treatment of metabolic disorders in patients who are at high risk for CRC, but also support a rationale for development of PPAR δ antagonists as new agents targeting CSCs in CRC treatment. Targeting CSCs may present novel therapeutic approaches for CRC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank the National Colorectal Cancer Research Alliance (NCCRA) for its generous past support (R.N.D.).

Additional Information

Financial support: DW, LF, and RND (NIH R01 DK047297 to R.N. DuBois, NCI R01 CA184820 to R.N. DuBois, NCI P01 CA077839 to R.N. DuBois), JW (NIH R01 DK047297 to R.N. DuBois, NCI R01 CA184820 to R.N. DuBois), YX (NIH R01 DK047297 to R.N. DuBois)

REFERENCES

1. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004;10:789–99. [PubMed: 15286780]
2. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 2003;100:3983–8. [PubMed: 12629218]
3. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432:396–401. [PubMed: 15549107]
4. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;63:5821–8. [PubMed: 14522905]
5. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007;445:111–5. [PubMed: 17122771]
6. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A* 2007;104:10158–63. [PubMed: 17548814]
7. Schmid A The role of meat fat in the human diet. *Critical reviews in food science and nutrition* 2011;51:50–66. [PubMed: 21229418]
8. Woutersen RA, Appel MJ, van Garderen-Hoetmer A, Wijnands MV. Dietary fat and carcinogenesis. *Mutat Res* 1999;443:111–27. [PubMed: 10415435]
9. Berger JP, Akiyama TE, Meinke PT. PPARs: therapeutic targets for metabolic disease. *Trends Pharmacol Sci* 2005;26:244–51. [PubMed: 15860371]
10. Michalik L, Desvergne B, Wahli W. Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat Rev Cancer* 2004;4:61–70. [PubMed: 14708026]
11. Higashiyama H, Billin AN, Okamoto Y, Kinoshita M, Asano S. Expression profiling of peroxisome proliferator-activated receptor-delta (PPAR-delta) in mouse tissues using tissue microarray. *Histochem Cell Biol* 2007;127:485–94. [PubMed: 17333240]
12. Ito K, Carracedo A, Weiss D, Arai F, Ala U, Avigan DE, et al. A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat Med* 2012;18:1350–8. [PubMed: 22902876]

13. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;318:1917–20. [PubMed: 18029452]
14. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 2003;113:631–42. [PubMed: 12787504]
15. Meng HM, Zheng P, Wang XY, Liu C, Sui HM, Wu SJ, et al. Over-expression of Nanog predicts tumor progression and poor prognosis in colorectal cancer. *Cancer Biol Ther* 2010;9(4):295–302. [PubMed: 20026903]
16. Xu F, Dai C, Zhang R, Zhao Y, Peng S, Jia C. Nanog: a potential biomarker for liver metastasis of colorectal cancer. *Dig Dis Sci* 2012;57:2340–6. [PubMed: 22562535]
17. Shan J, Shen J, Liu L, Xia F, Xu C, Duan G, et al. Nanog regulates self-renewal of cancer stem cells through the insulin-like growth factor pathway in human hepatocellular carcinoma. *Hepatology* 2012;56:1004–14. [PubMed: 22473773]
18. Baltgalvis KA, Berger FG, Pena MM, Davis JM, Carson JA. The interaction of a high-fat diet and regular moderate intensity exercise on intestinal polyp development in Apc Min/+ mice. *Cancer Prev Res (Phila)* 2009;2:641–9. [PubMed: 19549797]
19. Park MY, Kim MY, Seo YR, Kim JS, Sung MK. High-fat Diet Accelerates Intestinal Tumorigenesis Through Disrupting Intestinal Cell Membrane Integrity. *J Cancer Prev* 2016;21:95–103. [PubMed: 27390738]
20. Ju J, Liu Y, Hong J, Huang MT, Conney AH, Yang CS. Effects of green tea and high-fat diet on arachidonic acid metabolism and aberrant crypt foci formation in an azoxymethane-induced colon carcinogenesis mouse model. *Nutr Cancer* 2003;46:172–8. [PubMed: 14690793]
21. Botta M, Audano M, Sahebkar A, Sirtori CR, Mitro N, Ruscica M. PPAR Agonists and Metabolic Syndrome: An Established Role? *Int J Mol Sci* 2018;19:e1197. [PubMed: 29662003]
22. Ticha I, Gnosa S, Lindblom A, Liu T, Sun XF. Variants of the PPARD gene and their clinicopathological significance in colorectal cancer. *PloS one* 2013;8:e83952. [PubMed: 24391853]
23. Rosales-Reynoso MA, Wence-Chavez LI, Arredondo-Valdez AR, Dumois-Petersen S, Barros-Nunez P, Gallegos-Arreola MP, et al. Protective role of +294 T/C (rs2016520) polymorphism of PPARD in Mexican patients with colorectal cancer. *Genet Mol Res* 2017;16: doi 10.4238/gmr16019324.
24. Wang D, DuBois RN. Therapeutic potential of peroxisome proliferator-activated receptors in chronic inflammation and colorectal cancer. *Gastroenterol Clin North Am* 2010;39:697–707. [PubMed: 20951925]
25. Takayama O, Yamamoto H, Damdinsuren B, Sugita Y, Ngan CY, Xu X, et al. Expression of PPARdelta in multistage carcinogenesis of the colorectum: implications of malignant cancer morphology. *Br J Cancer* 2006;95(7):889–95. [PubMed: 16969348]
26. Glazer RI. PPARdelta as a Metabolic Initiator of Mammary Neoplasia and Immune Tolerance. *PPAR Res* 2016;2016:3082340. [PubMed: 28077942]
27. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 1999;99:335–45. [PubMed: 10555149]
28. Ouyang N, Williams JL, Rigas B. NO-donating aspirin isomers downregulate peroxisome proliferator-activated receptor (PPAR)delta expression in APC(min/+) mice proportionally to their tumor inhibitory effect: Implications for the role of PPARdelta in carcinogenesis. *Carcinogenesis* 2006;27:232–9. [PubMed: 16141240]
29. Siezen CL, Tijhuis MJ, Kram NR, van Soest EM, de Jong DJ, Fodde R, et al. Protective effect of nonsteroidal anti-inflammatory drugs on colorectal adenomas is modified by a polymorphism in peroxisome proliferator-activated receptor delta. *Pharmacogenet Genomics* 2006;16:43–50. [PubMed: 16344721]
30. Shao J, Sheng H, DuBois RN. Peroxisome proliferator-activated receptors modulate K-Ras-mediated transformation of intestinal epithelial cells. *Cancer Res* 2002;62:3282–8. [PubMed: 12036946]

31. Gupta RA, Tan J, Krause WF, Geraci MW, Willson TM, Dey SK, et al. Prostacyclin-mediated activation of peroxisome proliferator-activated receptor delta in colorectal cancer. *Proc Natl Acad Sci U S A* 2000;97:13275–80. [PubMed: 11087869]
32. Wang D, Wang H, Shi Q, Katkuri S, Walhi W, Desvergne B, et al. Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. *Cancer Cell* 2004;6:285–95. [PubMed: 15380519]
33. Yoshinaga M, Taki K, Somada S, Sakiyama Y, Kubo N, Kaku T, et al. The expression of both peroxisome proliferator-activated receptor delta and cyclooxygenase-2 in tissues is associated with poor prognosis in colorectal cancer patients. *Dig Dis Sci* 2011;56:1194–200. [PubMed: 20824502]
34. Gupta RA, Wang D, Katkuri S, Wang H, Dey SK, DuBois RN. Activation of nuclear hormone receptor peroxisome proliferator-activated receptor-delta accelerates intestinal adenoma growth. *Nat Med* 2004;10:245–7. [PubMed: 14758356]
35. Reddy BS, Wang CX, Kong AN, Khor TO, Zheng X, Steele VE, et al. Prevention of azoxymethane-induced colon cancer by combination of low doses of atorvastatin, aspirin, and celecoxib in F 344 rats. *Cancer Res* 2006;66:4542–6. [PubMed: 16618783]
36. Zuo X, Peng Z, Moussalli MJ, Morris JS, Broaddus RR, Fischer SM, et al. Targeted genetic disruption of peroxisome proliferator-activated receptor-delta and colonic tumorigenesis. *J Natl Cancer Inst* 2009;101:762–7. [PubMed: 19436036]
37. Wang D, Fu L, Ning W, Guo L, Sun X, Dey SK, et al. Peroxisome proliferator-activated receptor delta promotes colonic inflammation and tumor growth. *Proc Natl Acad Sci U S A* 2014;111:7084–9. [PubMed: 24763687]
38. Reed KR, Sansom OJ, Hayes AJ, Gescher AJ, Winton DJ, Peters JM, et al. PPARdelta status and Apc-mediated tumourigenesis in the mouse intestine. *Oncogene* 2004;23:8992–6. [PubMed: 15480419]
39. Harman FS, Nicol CJ, Marin HE, Ward JM, Gonzalez FJ, Peters JM. Peroxisome proliferator-activated receptor-delta attenuates colon carcinogenesis. *Nat Med* 2004;10:481–3. [PubMed: 15048110]
40. Zuo X, Xu M, Yu J, Wu Y, Moussalli MJ, Manyam GC, et al. Potentiation of colon cancer susceptibility in mice by colonic epithelial PPAR-delta/beta overexpression. *J Natl Cancer Inst* 2014;106:dju052. [PubMed: 24681603]
41. Beyaz S, Mana MD, Roper J, Kedrin D, Saadatpour A, Hong SJ, et al. High-fat diet enhances stemness and tumorigenicity of intestinal progenitors. *Nature* 2016;531(7592):53–8. [PubMed: 26935695]
42. Zuo X, Xu W, Xu M, Tian R, Moussalli MJ, Mao F, et al. Metastasis regulation by PPAR expression in cancer cells. *JCI Insight* 2017;2:e91419. [PubMed: 28097239]
43. Siu MK, Wong ES, Kong DS, Chan HY, Jiang L, Wong OG, et al. Stem cell transcription factor NANOG controls cell migration and invasion via dysregulation of E-cadherin and FoxJ1 and contributes to adverse clinical outcome in ovarian cancers. *Oncogene* 2013;32:3500–9. [PubMed: 22945654]
44. Lu X, Mazur SJ, Lin T, Appella E, Xu Y. The pluripotency factor nanog promotes breast cancer tumorigenesis and metastasis. *Oncogene* 2014;33:2655–64. [PubMed: 23770853]
45. Liu CW, Li CH, Peng YJ, Cheng YW, Chen HW, Liao PL, et al. Snail regulates Nanog status during the epithelial-mesenchymal transition via the Smad1/Akt/GSK3beta signaling pathway in non-small-cell lung cancer. *Oncotarget* 2014;5:3880–94. [PubMed: 25003810]
46. Zbinden M, Duquet A, Lorente-Trigos A, Ngwabyt SN, Borges I, Ruiz i Altaba A. NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. *EMBO J* 2010;29:2659–74. [PubMed: 20581802]
47. Ibrahim EE, Babaei-Jadidi R, Saadeddin A, Spencer-Dene B, Hossaini S, Abuzinadah M, et al. Embryonic NANOG activity defines colorectal cancer stem cells and modulates through AP1- and TCF-dependent mechanisms. *Stem Cells* 2012;30:2076–87. [PubMed: 22851508]
48. Wu K, Hu FB, Fuchs C, Rimm EB, Willett WC, Giovannucci E. Dietary patterns and risk of colon cancer and adenoma in a cohort of men (United States). *Cancer Causes Control* 2004;15:853–62. [PubMed: 15577287]

49. Meyerhardt JA, Niedzwiecki D, Hollis D, Saltz LB, Hu FB, Mayer RJ, et al. Association of dietary patterns with cancer recurrence and survival in patients with stage III colon cancer. *JAMA* 2007;298:754–64. [PubMed: 17699009]
50. Arita S, Kinoshita Y, Ushida K, Enomoto A, Inagaki-Ohara K. High-fat diet feeding promotes stemness and precancerous changes in murine gastric mucosa mediated by leptin receptor signaling pathway. *Arch Biochem Biophys* 2016;610:16–24. [PubMed: 27693038]

Statement of Significance

Findings show that PPAR δ contributes to CRC metastasis by expanding the cancer stem cell population, indicating that antagonists that target PPAR δ may be beneficial in treating CRC.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Activation of PPAR δ induces colonic CSC expansion by inducing Nanog expression via binding to Nanog promoter

LS-174T, LS-174T/vector, LS-174T/shPPAR δ , HCT-116/WT, and HCT-116/PPAR $\delta^{-/-}$ cells were treated with vehicle or GW501516. (A-B) After treatment, the indicated cells were subject to *in vitro* sphere-forming assays. LS-174T/vector and LS-174T/shPPAR δ cells were treated with vehicle or 0.1 μM of GW501516. The protein levels of PPAR δ in indicated cells were measured by Western blotting. (C-D) The Nanog expression at protein (C) and mRNA levels (D) in LS-174T, LS-174T/vector, LS-174T/shPPAR δ cells after treatment with GW501516. (E) A representative image of three independent ChIP assays for PPAR δ

binding to the promoter of Nanog in LS-174T cells after treatment. (F) The luciferase activity of WT and mutant Nanog promoters in LS-174T cells was measured after treatment. (G) After treatment, the indicated cells were subject to *in vitro* sphere-forming assays. The protein levels of Nanog in indicated cells were measured by Western blotting. The error bar indicates $\pm$ SEM. * $p < 0.05$.

Figure 2. Activation of PPAR δ induces colonic CSC expansion

The NSG mice were treated with vehicle or GW501516 after HCT-116/WT, HCT-116/*PPAR $\delta^{-/-}$* , LS-174T/vector, or LS-174T/shPPAR δ cells were injected into cecum. (A and C) The representative immunostaining of Nanog (A) and CD44v6 (C) in cecal tumors taken from indicated group of mice (scale bar, 50 μ m). (B and D) Cells isolated from cecal tumors of indicated group of mice were subjected into flow cytometry analysis. Data represents the percentage of EpCAM⁺CD45⁻Nanog⁺ (B) and EpCAM⁺CD45⁻CD44v6⁺ (D) cells in total

cecal tumor epithelial cells. (E) Sphere formation of tumor epithelial cells isolated from cecal tumor cells of indicated group of mice. The error bar indicates $\pm$ SEM. * $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Activation of PPAR δ accelerates liver metastasis

The NSG mice were treated with vehicle or GW501516 after LS-174T/vector, LS-174T/shPPAR δ , HCT-116/WT, or HCT-116/PPAR $\delta^{-/-}$ cells were injected into cecum. (A) Gross view of liver metastasis. (B-C) Average numbers of liver metastatic tumors at different size and total that includes all sizes in indicated group of mice. The error bar indicates $\pm$ SEM.

* $p < 0.05$.

Figure 4. Nanog is required for PPAR δ induction of CSC expansion and liver metastasis
 The NSG mice were treated with vehicle or GW501516 after LS-174T/vector, LS-174T/shNanog, HCT-116/vector, or HCT-116/shNanog cells were injected into cecum. (A-B) Sphere formation of cecal tumor epithelial cells isolated from indicated group of mice. (C-D) Average numbers of liver metastatic tumors at different size and total that includes all sizes in indicated group of mice. (E-F) The *PPAR δ* levels are correlated with the expression of *Nanog* or *CD44* at mRNA levels in 20 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. The error bar indicates $\pm$ SEM. * $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. A high-fat diet induces CSCs and promotes liver metastasis via PPAR δ

The NSG mice were treated with a control diet or a high-fat diet after HCT-116/WT or HCT-116/PPAR $\delta^{-/-}$ cells were injected into cecum. (A and C) The representative immunostaining of Nanog (A) and CD44v6 (C) in cecal tumors taken from indicated group of mice (scale bar, 50 μ m). (B and D) Cells isolated from cecal tumors taken from indicated group of mice were subjected into flow cytometry analysis. Data represents the percentage of EpCAM $^{+}$ CD45 $^{-}$ Nanog $^{+}$ (B) and EpCAM $^{+}$ CD45 $^{-}$ CD44v6 $^{+}$ (D) cells in total cecal tumor epithelial cells. (E) Sphere formation of tumor epithelial cells isolated from cecal tumor cells

of indicated group of mice. (F) Average numbers of liver metastatic tumors at different size and total that includes all sizes in indicated group of mice. The error bar indicates $\pm$ SEM. * $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. A high-fat diet induces CSCs and promotes liver metastasis via Nanog

The NSG mice were treated with a control diet or a high-fat diet after LS-174T/vector or LS-174T/shNanog cells were injected into cecum. (A) Cells isolated from cecal tumors taken from indicated group of mice were subjected into flow cytometry analysis. Data represents the percentage of EpCAM⁺CD45⁻CD44v6⁺ cells in total cecal tumor epithelial cells. (B) Sphere formation of tumor epithelial cells isolated from cecal tumor cells of indicated group of mice. (C) Average numbers of liver metastatic tumors at different size and total that includes all sizes in indicated group of mice. The error bar indicates $\pm$ SEM.

* $p < 0.05$.

Table 1.
Tumorigenicity of cecal tumor cells from mice treated with vehicle or GW501516

The NSG mice were treated with vehicle or GW501516 after LS-174T/vector or HCT-116/WT cells were injected into cecum. Tumor epithelial cells isolated from cecal tumor cells of indicated group of mice were subjected to limiting dilution transplantation assays.

		Number of mice with tumors/total injected mice (% of mice with tumors)			
No. of injected cells					
LS-174T		24 days	27 days	34 days	44 days
Vehicle	5,000	1/6 (17)	2/6 (33)	5/6 (83)	6/6 (100)
	1,000	1/8 (12.5)	2/8 (25)	4/8 (50)	8/8 (100)
	200	0/8 (0)	0/8 (0)	1/8 (12.5)	2/8 (25)
GW501516	5,000	3/ 6 (50)	5/6 (83)	6/6 (100)	6/6 (100)
	1,000	2/8 (25)	5/8 (63)	7/8 (88)	8/8 (100)
	200	0/8 (0)	1/8 (13)	4/8 (50)	7/8 (88)
HCT-116		26 days	33 days	37 days	44 days
Vehicle	1×10 ⁴	1/8 (12.5)	2/8 (25)	4/8 (50)	8/8 (100)
	2,500	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)
	500	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)
GW501516	1×10 ⁴	4/8 (50)	8/8 (100)	8/8 (100)	8/8 (100)
	2,500	2/8 (25)	5/8 (63)	7/8 (88)	7/8 (88)
	500	0/8 (0)	1/8 (13)	2/8 (25)	4/8 (50)