Deficiency of caveolin-1 in Apc<sup>min/+</sup> mice promotes colorectal tumorigenesis

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Caveolin-1 (Cav1), a scaffold protein of membrane caveolae and coactivator of peroxisome proliferator-activated receptor gamma (PPARγ), inhibits oncogenic signaling through Ras and wingless. However, the in vivo role of Cav1 in colorectal cancer (CRC) remained unknown. To test whether loss of Cav1 accelerates tumorigenesis, we generated a novel mouse model of CRC by crossing C57BL/6 Apc<sup>min/+</sup> with B6129 Cav1 knockout (Cav1<sup>−/−</sup>) mice. Apc<sup>min/+</sup> Cav1<sup>−/−</sup> mice developed large, microinvasive and vascularized intraepithelial adenocarcinomas in the distal colon and rectum with higher incidence than Apc<sup>min/+</sup> Cav1<sup>−/+</sup> and Apc<sup>min/+</sup> Cav1<sup>+/−</sup> littersmates. Intratumoral gene signatures related to Ras and wingless signaling were elevated, nuclear localization of PPARγ protein and expression of PPARγ-target genes were reduced independently of Cav1. The PPARγ-agonist rosiglitazone prevented tumor formation in mice irrespectively of the Cav1 status and upregulated expression of the Ras-inhibitory protein docking protein-1. Thus, codeficiency of Cav1 and adenomatous polyposis coli facilitated formation of CRC, and activation of PPARγ may offer novel strategies for treatment of CRC.

Introduction

Adenomatous polyposis coli (APC) is an inhibitor of canonical wingless (WNT) signaling and mutated in the majority of human colorectal cancers (CRCs) (1). The Apc<sup>min/+</sup> mouse model has been widely utilized to study WNT-driven carcinogenesis in vivo (1). Caveolin-1 (Cav1), a scaffold protein of plasma membrane caveolae and an inhibitor of oncogenic Ras and WNT signaling (2,3), is also frequently lost in human cancers (4,5). However, the role of Cav1 in CRC remained unknown. We, therefore, raised the hypothesis that Cav1 deficiency accelerates WNT-driven formation of CRC in vivo.

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-regulated nuclear transcription factor that promotes differentiation of intestinal epithelial cells and immune responses in the gastrointestinal tract (6). PPARγ is expressed in adipocytes, macrophages and the colorectum and is activated by antidiabetic drugs (e.g. thiazolidinediones) and dietary/inflammatory lipids (e.g. during colitis). PPARγ transcriptionally regulates a broad spectrum of target genes (such as p21, PTEN and Cyclin D1), including the WNT/β-catenin signaling pathway and genes involved in cell cycle control and apoptosis (7). However, the role of Cav1 in CRC remained unknown. We, therefore, raised the hypothesis that Cav1 deficiency accelerates WNT-driven formation of CRC in vivo.

Abbreviations: APC, adenomatous polyposis coli; Cav1, caveolin-1; cDNA, complementary DNA; CRC, colorectal cancer; Dok1, docking protein-1; EGFR, epidermal growth factor receptor; KO, knockout; I-TU, large tumors; MOM, modifier of min; PPARγ, peroxisome proliferator-activated receptor gamma; RT-qPCR, reverse transcription–quantitative PCR; s-TU, small tumors; WNT, wingless-type MMTV integration site family; WT, wild type.

Ras-inhibitory proteins Cav1 (7,8) and docking protein-1 (Dok1) (9,10) and thereby inhibits proliferation and may prevent cancer (6,11). Cav1 is not only a ‘passive’ target gene (12) of PPARγ but rather an ‘active’ nuclear receptor coactivator (13–15), which directly interacts with PPARγ in the cytosol and amplifies the sensitivity of PPARγ to stimulation by ligand (9). Through this molecular mechanism, Cav1 promotes the translocation of PPARγ to the nucleus and potentiates its ligand-dependent transcriptional activity (9). Thereby, Cav1 actively counteracts the phosphorylation, retention and inactivation of PPARγ in the cytosol by the Ras-Raf-MEK-ERK cascade (16,17). Canonical WNT signaling also leads to inhibition of PPARγ, thus abrogating its protective effects (18,19). Loss of PPARγ correlates with a poor survival prognosis (20) and epigenetic silencing of the PPARγ gene in CRC patients (21). We, therefore, hypothesized that PPARγ is a downstream target of oncogenic WNT/Ras-signaling, which may be exploited for chemoprevention of CRC.

Our data show that absence of Cav1 accelerates colorectal tumorigenesis in Apc<sup>min/+</sup> mice preferentially in the distal colon and rectum, emulating the anatomic localization in humans. CRCs were characterized by a gain of WNT- and Ras-related gene expression, cytosolic accumulation of PPARγ protein and loss of PPARγ’s transcriptional activity in the nucleus, events that were independent of Cav1’s presence. Pharmacological activation of PPARγ reduced expression of Ras/WNT-related target genes and prevented development of CRCs in mice irrespectively of Cav1 as well. These results contrasted with the synergism observed for Cav1 and PPARγ in previous in vitro studies (9,16) in favor of a more dominant antitumoral efficacy of PPARγ over Cav1 in vivo.

In sum, our data point to novel strategies for treatment of human CRC where frequent mutations or alterations in signaling activity of WNT/Ras proteins are an obstacle to successful treatment.

Materials and methods

Animals

Homozygous Cav1 knockout (Cav1-ko) (strain Cav1tm1Mls/J; stock number 004585) and matched control wild-type (WT) (strain B6129SF2/J; stock number 009204) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) (9). Apc<sup>−/+</sup> mice (Charles River Laboratories, Wilmington, MA) were maintained on a pure C57BL/6J background. For generation of the CRC tumor model, male Apc<sup>−/+</sup> mice were interbred with female Cav1-ko mice to obtain double heterozygous F1 progeny. F1 littersmates were intercrossed for at least eight generations to generate Apc<sup>−/+</sup> Cav1<sup>−/+</sup>, Apc<sup>−/+</sup> Cav1<sup>−/−</sup> and Apc<sup>−/+</sup> Cav1<sup>−/+</sup> mice and the remaining three APC WT genotypes (Apc<sup>−/+</sup>, Apc<sup>−/−</sup> Cav1<sup>−/−</sup>, Apc<sup>−/+</sup> Cav1<sup>−/−</sup>). The mice (n = 5-6 per group) received a Chow diet or a chow diet (both from Altromin, Lage, Germany) supplemented with 0.02% (w/w) rosiglitazone maleate (Chemos GmbH, Regensburg, Germany) for 4 months (~25 mg/kg/day) (9). Animal studies were conducted in agreement with the ethical guidelines of the Technische Universität München and were approved by the Government of Bavaria, Munich.

Reagents

Chemicals were from Merck (Darmstadt, Germany) or Sigma (Taufkirchen, Germany). Rosiglitazone maleate was provided by F.Hoffmann La Roche AG (Basel, Switzerland). Rabbit polyclonal antiserum were Cav1 (N-20, sc-894), PPARγ (H-100, sc-7196; both from Santa Cruz Biotech., CA), phospho-c-Raf (Ser259, #9421), phospho-GSK3β (Ser9, #9322), phospho-p38 MAPK (Thr180/Tyr182, #4511), phospho-SAPK/JNK (Thr183/Tyr185, #4686), phospho-MEK1/2 (Ser277/212, #9121) (all from Cell Signaling, Danvers, MA), Hsp90α (H-114, sc-7947), Lamina A/C (H-110, sc-20681; both from Santa Cruz Biotech.). Goat polyclonal antibody was β-catenin (C-18, sc-1496, Santa Cruz Biotech.).

Western blotting, immunohistochemistry and subcellular fractionation

Detection of immunoprecipitated proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16), immunohistochemistry and hematoxylin and eosin (H&E) staining (22) and fractionation of frozen tissues (9,16) were performed as described previously.
DNA microarray, reverse transcription–PCR and quantitative PCR

Total RNA (1 μg) from CRC and normal colon tissue of Apc(min/+)Cav1−/− mice were labeled using the One-Cycle cRNA labeling kit (Affymetrix, Wycombe, UK), and cRNA was hybridized to GeneChip® Mouse Exon 1.0 ST arrays (Affymetrix). Gene signatures were identified using Gene Set Enrichment Analysis (9). PCR primers are listed in Supplementary Table S1, available at Carcinogenesis Online.

Statistics

Results are mean ± standard error from at least three animals per genotype. Matched tumor (TU) and normal (NT) tissues from the same animal were analyzed. The software Graphpad Prism (version 4.0) and SAS (version 9.3) were used to analyze the in vivo data. P-values < 0.05 were considered significant.

Results

Codification of APC and Cav1 promotes tumorigenesis in the mouse distal colon

C57BL/6J Apc(min/+) mice were interbred with B6129 Cav1−/− mice to investigate the influence of Cav1 deficiency on CRC formation. All genotypes were born according to expected Mendelian ratios, except for Apc(min/+)Cav1−/− mice, which exhibited a significantly lower frequency than the other five genotypes, however, upon birth, were viable and fertile. Consistent with a gene-dosage effect, Cav1 mRNA was already diminished (by 80%) within the normal (NT) colon tissue in the heterozygous state (Apc(min/+)Cav1+/−) compared with Apc(min/+)Cav1+/+ mice. Compared with NT tissue of the same genotype, Cav1 mRNA was further decreased to approximately 50% in the TU tissue of Apc(min/+)Cav1−/− mice and to 80% in Apc(min/+)Cav1−/− mice (n = 3 per genotype, t-test: *P = 0.034 NT and *P = 0.038 TU Apc(min/+)Cav1+/− and *P = 0.0003 NT and *P = 0.0002 TU Apc(min/+)Cav1−/− versus NT Apc(min/+)Cav1+/+). Similar results were obtained on the protein level (Figure 1A).

Apc(min/+)Cav1−/− (n = 8) mice had a reduced mean life span (Figure 1B) of approximately 30 days compared with Apc(min/+)Cav1+/− (n = 49) and Apc(min/+)Cav1+/+ (n = 16) (log-rank test: *P = 0.0211 −/− versus +/+; Supplementary Table S2, available at Carcinogenesis Online), presumably due to anemia and increased tumor burden causing stenosis/obstruction in the colon and rectal bleeding. Although the survival of Apc(min/+)Cav1+/− mice appeared slightly inferior to that of Apc(min/+)Cav1+/+ mice (Figure 1B), the difference was not statistically significant (log-rank test: *P = 0.57 n.s.; Supplementary Table S2, available at Carcinogenesis Online).

Macroscopic observations revealed that Apc(min/+)Cav1−/− mice (Figure 1C) preferentially developed tumors in the distal colon (and rectum) with robust vascularization. In contrast, Apc(min/+)Cav1+/− mice developed fewer lesions in the distal colon (and rectum) and mainly exhibited tumors in the proximal colon (cecum). All genotypes had multiple benign polyps in the small intestine and occasional duodenal tumors (data not shown). No significant bias in tumor size, incidence or multiplicity was observed among the genders or generations (F1–F8).

Microscopically (Figure 1D), all tumors showed a tubular growth pattern consisting of hyperchromatic cells with multilayered irregular nuclei and loss of mucin, and a high nucleus-to-cytoplasm ratio and loss of polarity with nuclear stratification. In accordance with the Vienna classification (23) for gastrointestinal epithelial neoplasia in humans, the lesions would correspond to Category 4 (tubular adenoma with high-grade neoplasia/intraductal carcinoma). Although no definitive submucosal growth pattern was observable, a few cases showed invasion into the muscularis mucosa. Neither a serrated growth pattern nor a massive inflammatory infiltrate was demonstrable.

Apc(min/+)Cav1−/− (n = 8) developed colon tumors (Figure 2A) with an incidence of 100% compared with 78% in Apc(min/+)Cav1+/− mice (n = 59) and 74% in Apc(min/+)Cav1+/+ (n = 19) (Supplementary Table S2, available at Carcinogenesis Online). Apc(min/+)Cav1−/− and Cav1+/− mice exhibited tumors at an early age (0–3 months), whereas Apc(min/+)Cav1−/− mice exhibited macroscopic tumors at later time points (7–8 months) where almost all homozygous mice had already succumbed to the disease (Figure 2B). Notably, about 88% of Apc(min/+)Cav1−/− mice had tumors that were larger than 3 mm compared with 29% of Apc(min/+)Cav1+/− and 42% of the Apc(min/+)Cav1+/+ mice (Cochran-Mantel-Häenzel test: *P = 0.0168 Apc(min/+)Cav1+/− versus Cav1+/− and Cav1+/+) (Figure 2C). Reverse transcription–quantitative PCR (RT–qPCR) analyses (n = 3 per genotype, t-test; Figure 2D) showed that Wnt6 mRNA was upregulated 242-fold (*P = 0.03) in Apc(min/+)Cav1−/−, 67-fold (*P = 0.0044) in Apc(min/+)Cav1+/− and 20-fold in Apc(min/+)Cav1−/− TU tissue compared with Apc(min/+)Cav1+/− NT tissue. CyclinD1 mRNA was increased 6-fold (*P = 0.03) in Apc(min/+)Cav1−/− but only 2- to 2.5-fold in TU tissue of Apc(min/+)Cav1+/− and Apc(min/+)Cav1+/− mice compared with Apc(min/+)Cav1+/− NT tissue. In sum, these data indicated that loss of Cav1 accelerated the development of distal colorectal tumors in Apc(min/+) mice.

Consistent with the macroscopic observations, the multiplicity of colon tumors was reduced in Apc(min/+)Cav1−/− mice with an average of 1.2 colon tumors per mouse compared with 1.7 in Apc(min/+)Cav1+/− mice and a maximum of 3–6 single lesions, including small, not vascularized tumors (Supplementary Table S3, available at Carcinogenesis Online). About 46% of Apc(min/+)Cav1−/− mice suffered from cecum tumors in contrast to 25% of Apc(min/+)Cav1+/− and Cav1+/− mice. Conclusively, Apc(min/+)Cav1−/− mice tend to develop large distal CRCs compared with Apc(min/+)Cav1+/− littersmates with more but smaller proximal lesions. In addition, 80% of Apc(min/+)Cav1−/− mice were positive for mucus-filled cystically dilated glands in the tumors compared with 58% of Apc(min/+)Cav1+/− and 43% of Apc(min/+)Cav1+/− littersmates, a phenotype possibly caused by cell cohesion defects in absence of Cav1 (24).

Loss of PPAR gene signature in CRCs of Apc(min/+) mice occurs independently of Cav1

Transcriptome profiles of normal colon (NT) and TU tissue from the most frequent genotype Apc(min/+)Cav1+/− were collected from complementary DNA (cDNA) microarrays (Supplementary material: Excel microarray, available at Carcinogenesis Online). Gene Set Enrichment Analysis revealed reduced expression of genes regulated by PPAR transcription factors in TU compared with NT tissue (Supplementary Table S4, available at Carcinogenesis Online). In contrast, an enrichment of a Ras-related gene signature with increased expression of Egfr (epidermal growth factor receptor), Ras, Nras (neuroblastoma Ras proto-oncogene) and Met (Met proto-oncogene) was observed in TU tissue (Figure 3A).

To validate these signatures in all three Cav1 genotypes, mRNA was isolated from NT and TU tissue of Apc(min/+)Cav1−/−, Apc(min/+)Cav1+/− and Apc(min/+)Cav1+/+ mice, and RT-qPCRs were performed. In CRC samples (Figure 3B), we detected increased mRNA levels of the WNT-pathway gene Myc (3.5-fold, *P = 0.03), the proto-oncogene Met (90-fold) and of Nras and Kras (3-fold, P = 0.054 for Nras) (n = 13 NT, n = 9 TU, t-test, NT versus TU). Sequencing of Kras CDNA isolated from NT and TU tissue revealed mutation neither in the codon 12 nor in the codon 13 (data not shown). Although there was no reduction of Ppary mRNA in the TU tissue, mRNA levels of cognate PPARα-target genes were lowered by about 40–90% including Acly (acyl-CoA oxidase, P = 0.05), Ppck (phosphoethanol pyruvate carboxylase, *P = 0.04) and Hmgcs2 (hydroxymethylglutaryl-CoA synthase 2, *P = 0.008) (n = 13 NT, n = 9 TU, t-test, NT versus TU) (Figure 3C). No significant difference between the three Cav1 genotypes (Apc(min/+)Cav1+, Apc(min/+)Cav1−/−, Apc(min/+)Cav1+/−) was observed. These data suggested that Apc(min/+) CRCs lose transcriptional activity of PPARα independently of Cav1.

Reduced nuclear localization of PPARα protein in CRCs of Apc(min/+) mice

Western blot (WB) analysis (Figure 4A) failed to reveal a change in total PPARα protein levels in whole tissue lysates from NT compared with TU samples either. Instead, protein levels of β-catenin and active (dephosphorylated) GSK3β, active (dephosphorylated) c-Raf and active (phosphorylated) MEK1/2 were increased, confirming functional WNT- and Ras-signaling in the TU tissue. Immunohistochemistry evinced regular nuclear PPARα staining patterns in normal colon crypts and villi (Figure 4B). In contrast, PPARα protein was...
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predominantly distributed in the cytosol of the TU tissue, and the nuclear staining was lost. In small tumors (s-TU, <3 mm), we found a mixture of nuclear and cytosolic staining, indicating that nuclear PPARg is lost during progression of small polyps to large tumors (l-TU, >3 mm) and intraepithelial adenocarcinomas.

Ex vivo subcellular fractionation confirmed the reduction of PPARg protein levels in membrane and nuclear fractions of TU tissue (Figure 4C). In the cytosolic fraction, PPARg protein remained unchanged, indicating that PPARg is not subjected to quantitative degradation, for example by the proteasome. PPARg protein was more robustly reduced in l-TU compared with s-TU. Quantification of WBs revealed an over 70% reduction of PPARg in neoplastic tissue (l-TU and s-TU were summarized, \( n = 3 \) per genotype, \( t \)-test: *\( P = 3.6 \times 10^{-5} \) for membrane and *\( P = 1.4 \times 10^{-9} \) for nucleus TU versus NT).

![Figure 1.](https://academic.oup.com/carcin/article-abstract/34/9/2109/2463121)

**Fig. 1.** Loss of Cav1 promotes formation of CRC in Apc<sup>−/−</sup> mice. (A) Reduction of Cav1 mRNA and protein expression in murine colorectal cancers. Left panel: Cav1 mRNA quantified by RT–qPCR in TU versus NT colon tissue. CT values were normalized to \( \beta \)-2-microglobulin and calculated as mean ± standard error (\( n = 3 \) per genotype); *\( P < 0.05 \) NT Apc<sup>+/+</sup>Cav1<sup>+/+</sup> versus NT and TU of Apc<sup>+/−</sup>Cav1<sup>+/−</sup> and Apc<sup>−/−</sup>Cav1<sup>−/−</sup>. Right panel: Representative western blot of Cav1 protein: TU versus NT in ileum and colon tissue. (B) Cav1 deficiency shortens the lifetime of tumor-bearing mice. Kaplan–Meier survival analysis; log-rank test: *\( P = 0.0211 \) Apc<sup>+/−</sup>Cav1<sup>−/−</sup> (<\( n = 8 \)) versus Apc<sup>−/−</sup>Cav1<sup>−/−</sup> (<\( n = 8 \)); \( P = 0.17 \), n.s. Apc<sup>+/+</sup>Cav1<sup>+/+</sup> versus Apc<sup>−/−</sup>Cav1<sup>−/−</sup> (<\( n = 16 \)); \( P = 0.57 \), n.s. Apc<sup>+/−</sup>Cav1<sup>+/−</sup> (<\( n = 49 \)) versus Apc<sup>−/−</sup>Cav1<sup>−/−</sup> (<\( n = 16 \)). (C) Macroscopic images of representative vascularized distal CRCs from Apc<sup>−/−</sup>Cav1<sup>−/−</sup> (i: top panel, resected tumor; middle panels, tumor in situ) and Apc<sup>+/+</sup>Cav1<sup>+/+</sup> mice (ii: lower panels, overview of resected large intestine). TU = tumor, p = proximal, d = distal, r = rectum, c = cecum. Scale bars = 5 mm. (D) Microscopic images of H&E stainings in paraffin sections. Top and middle panel: TU of the Apc<sup>−/−</sup>Cav1<sup>−/−</sup> mouse shown in (Ci) with high-grade intraepithelial neoplasia. Magnification \( \times 10 \) and \( \times 200 \). Lower panel: TU from an Apc<sup>+/−</sup>Cav1<sup>+/−</sup> mouse with microinvasive adenocarcinoma. Magnification \( \times 16 \). TU = tumor, c = prominent cystically dilated gland, inv = invasion into muscularis mucosae.
Loss of Cav1 accelerates growth of CRC in Apc<sup>min/+</sup> mice. (A) H&E staining of paraffin sections from representative CRCs in Apc<sup>min/+</sup> Cav1<sup>+/-</sup>, Apc<sup>min/+</sup> Cav1<sup>+/+</sup> and Apc<sup>min/+</sup> Cav1<sup>−/−</sup> mice (all females, age 186–214 days). Scale bar = 50 μm. (B) Cav1 deficiency accelerates CRC formation. Tumor size was measured in μm<sup>2</sup> in H&E stained slides and compared with the age (in months) of the mice. Data are mean ± standard error from n ≥ 3 animals per genotype. (C) Cav1 deficiency promotes formation of large CRCs. Macroscopic analysis of tumor incidence in Apc<sup>min/+</sup> (Cav1<sup>−/−</sup> n = 8, Cav1<sup>+/−</sup> n = 59, Cav1<sup>++/+</sup> n = 19) mice. Colons were analyzed for TUs bigger or smaller than 3 mm; *P = 0.0168 Apc<sup>min/+</sup>Cav1<sup>+/−</sup> versus Apc<sup>min/+</sup>Cav1<sup>++/+</sup> for TU > 3 mm. (D) Increased expression of Wnt6 and Cyclind1 in Apc<sup>min/+</sup> CRCs. The mRNAs were quantified by RT–qPCR in TU versus NT colon tissue. CT values were normalized to β2-microglobulin and calculated as mean ± standard error (n = 3 per genotype); *P < 0.05 NT Apc<sup>min/+</sup>Cav1<sup>+/−</sup> versus NT and TU of Apc<sup>min/+</sup>Cav1<sup>++/+</sup> and Apc<sup>min/+</sup>Cav1<sup>−/−</sup>.

Again, there was no significant difference regarding loss of nuclear PPARγ protein between the three Cav1 genotypes. These findings indicated that nuclear PPARγ protein is reduced in Apc<sup>min/+</sup> CRCs independently of Cav1.

The PPARγ-agonist rosiglitazone prevents formation of CRC in Apc<sup>min/+</sup> mice irrespectively of Cav1

To test whether pharmacological activation of PPARγ inhibits colorectal tumorigenesis, Apc<sup>min/+</sup> (n = 12) and Apc<sup>min/+</sup> (Cav1<sup>+/−</sup>, Cav1<sup>++/+</sup> and Cav1<sup>−/−</sup>) mice (n = 11), at an age of 3 months, were fed a chow diet with 0.02% (w/w) rosiglitazone for additional 4 months (~25 mg/kg/day) (9) (Figure 5A). H&E stainings revealed no histopathological abnormalities in ligand-treated Apc<sup>min/+</sup> mice (Figure 5B). Instead, in Apc<sup>min/+</sup> mice, tumor incidence was reduced from 79% to 36% by rosiglitazone (chi-square test: *P = 0.0034; Fisher exact test: *P = 0.0073) (Figure 5A, Supplementary Table S5, available at Carcinogenesis Online). The overall tumor size was decreased to 29% (Wilcoxon two-sample test: *P = 0.0126; Kruskall–Wallis test: *P = 0.0123), and only 27% of rosiglitazone (rosi)-treated mice developed tumors that were larger than 5 mm<sup>2</sup>, compared with 55% in the control group (Figure 5A). Tumor multiplicity was diminished from 1.4 tumors per mouse compared with 0.7 in treated mice (Cochran-Armitage trend test: *P = 0.0482) (Supplementary Table S5, available at Carcinogenesis Online). We defined the antitumor response to rosiglitazone as follows: absence of colon and cecum tumors (full responder), reduction of colon tumor size or presence of cecum tumors (partial responder), no reduction in tumor size and presence of cecum tumors (non-responder) (Figure 5B).

In sum, 8 of 11 Apc<sup>min/+</sup> mice displayed a full or partial response to rosiglitazone, whereas 3 of 11 animals were non-responders. This ratio corresponded to a non-responder rate of 27.3%. There was no significant difference in the response rate between the three Cav1 genotypes. The intratumoral expression of PPARγ-target genes including Aco (2-fold, *P = 0.059) and Pepck (2-fold, *P = 0.0053) together with Cav1 (2.2-fold, *P = 0.054) and a second Ras-inhibitory protein, docking protein 1 (Dock1) (1.6-fold, *P = 0.008), was increased by rosiglitazone (n = 11 rosi versus n = 12 chow, t-test, rosi versus chow). The mRNAs of Cyclind1 and Kras (P = 0.07) were decreased by approximately 40% (Figure 5C). Pparγ itself was upregulated by 20% and did not reach statistical significance. Again, there was no difference between the three Cav1 genotypes regarding the pharmacologic efficacy of rosiglitazone. Thus, activation of PPARγ prevented Apc<sup>min/+</sup>-driven CRC formation in vivo independently of Cav1.

Discussion

In this study, we demonstrate a novel in vivo role for Cav1 in CRC. Cav1-deficient mice per se suffer from a hyperplastic phenotype in the gastrointestinal tract, lung and mammary glands (9,24,25).
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Cav1-KO mice are also hypersensitive to challenging environmental stimuli or pathogens resulting in fibrosis, vascular dysfunction and abnormal angiogenesis, inflammation and an altered immunological response (24,26–29). Nonetheless, a second genetic or chemocarcinogenic “hit” is necessary, in addition to loss of Cav1, to drive cancer (3), for example upon deletion of tumor-suppressor genes (such as INK4a) (30) or transgenic expression of an oncogene as described for the MMTV-driven breast cancer model (31). Likewise, loss of function of the APC tumor-suppressor protein in the WNT pathway is a consequence of one of the most frequent genetic alterations in human CRCs (1). However, the role of Cav1 in WNT-driven CRC remained unknown.

To test whether absence of Cav1 accelerates \( \text{Apc}^{\text{min/+}} \)-driven formation of CRC \text{in vivo}, we crossed \( \text{Apc}^{\text{min/+}} \) mice with Cav1-KO mice, which, preferentially in the homozygous state, formed large and vascularized colorectal tumors. The C57BL/6J \( \text{Apc}^{\text{min/+}} \) mouse is a preclinical model that recapitulates many aspects of the human disease (1). However, malignant carcinomas are rare; most lesions are adenomas in the small intestine. Interbreeding of C57BL/6J \( \text{Apc}^{\text{min/+}} \) mice to tumor-suppressor-deficient strains, for example Tgbr1 (32), Smad3 (33) or Ephrinbr (34), also resulted in the formation of CRCs accompanied by an extended life span of the mouse (>1 year) compared with pure C57BL/6J \( \text{Apc}^{\text{min/+}} \) mice (~4 months). Similarly, a crossing of C57BL/6J \( \text{Apc}^{\text{min/+}} \) mice to mice transgenic for K-RasG12D (35) developed colon tumors, in good accordance with models combining K-RasG12V (36–39) or other Ras mutants (40,41) with loss-of-function APC variants. Advanced intestinal cancers also appeared in long-lived \( \text{Apc}^{\text{min/+}} \) mice upon deviation of the genetic background from pure C57BL/6J (42), presumably through differences in modifier of min (MOM) loci (1).

Likewise, genetic loci that cooperate with the Cav1 gene may influence tumor localization, incidence and multiplicity and the overall survival of the individual. In humans, the \( \text{CAV1} \) locus is located in a fragile chromosomal region \( \text{FRA7G} \) in 7q31.1 (next to the Met proto-oncogene), which is frequently deleted or amplified in tumors (43). The murine \( \text{Cav1/Cav2/Met} \) genes are arranged in tandem series within a DNA-locus 6-A2 that corresponds to the human region (44). Several MOM loci have been identified, which influence tumorigenesis dependently or independently of the gene of interest (1), that is as a result of variations in the genetic background of a given strain. In an elegant study, Lisanti et al. (45) demonstrated that the interbreeding of Cav1-KO to \( \text{Apc}^{\text{min/+}} \) mice on a pure C57BL/6J background resulted in an increased tumor multiplicity in the small and large intestines and a close genetic association with novel MOM loci flanking the \( \text{Cav1} \) gene. Thus, the variations observed in tumor load and survival in our mouse model may be caused by MOM loci as well, which work independently of the primary alterations in the Cav1 and APC genes. Future genetic studies have to elaborate this possibility.

Notably, the absolute life span did not significantly differ between \( \text{Apc}^{\text{min/+}} \) \( \text{Cav1}^{+/−} \) (180 ± 10 days) and \( \text{Cav1}^{+/−} \) mice (186 ± 5 days), whereas \( \text{Cav1}^{-/−} \) littermates lived an average of 153 ± 16 days. Thus, the ‘in-between’ life expectancy of \( \text{Cav1}^{+/−} \) mice in Kaplan–Meier plots (Figure 1B) shall not be considered a true survival advantage but

Fig. 3. Loss of PPARγ’s transcriptional activity in CRC of \( \text{Apc}^{\text{min/+}} \) mice. (A) Gene Set Enrichment Analysis of cDNA array data from \( \text{Apc}^{\text{min/+}} \text{Cav1}^{+/−} \) NT colon tissue compared with colorectal TU tissue. Enrichment plots and heat maps showing (right panel) downregulation of PPAR signature and (left panel) upregulation of Ras signature in TU compared with NT. (B and C) Ras and PPAR-related mRNAs detected in cDNA microarrays were quantified by RT–qPCR comparing TU versus NT of all \( \text{Apc}^{\text{min/+}} \) genotypes. CT values were normalized to \( β_2 \)-microglobulin and calculated as mean ± standard error (\( n = 13 \) NT, \( n = 9 \) TU mice); *\( P < 0.05 \) NT versus TU.
Fig. 4. Loss of nuclear PPARg localization in CRC of Apc<sup>min</sup> mice. (A) Top panel: WB quantification of WNT and Ras/MAPK signaling proteins. Optical density values from bands were normalized to Hsp90 and calculated as-fold ± standard error (n = 3 from all three Cav1 genotypes, *P < 0.05 NT versus TU tissue). Lower panel: Representative WBs from whole tissue lysates of the Apc<sup>min</sup>Cav1<sup>+/-</sup> genotype. (B) Immunohistochemistry detecting PPARg in Apc<sup>min</sup>Cav1<sup>−/−</sup> NT and TU tissues showing nuclear staining of PPARg in NT, reduced nuclear and cytosolic staining in small tumors (s-TU, <3 mm) and exclusive cytosolic staining in large tumors (l-TU, >3 mm). (C) Subcellular fractionation of NT versus s-TU and l-TU in three different Apc<sup>min</sup>Cav1<sup>−/−</sup> mice. Right: WB detecting PPARg in membrane, cytosolic and nuclear fraction. Left: Quantification of WBs. Relative band intensities were normalized to Hsp90 or LaminA/C and are shown as-fold ± standard error (n = 3 per genotype, *P < 0.05 NT versus TU).
Fig. 5. The PPARγ agonist rosiglitazone prevents formation of CRC in Apc<sup>min/+</sup> mice. (A) Left: Microscopic analysis of rosiglitazone-treated (rosi) Apc<sup>min/+</sup> mice showing incidence of colon TU bigger or smaller than 3 mm. Right: Mean TU area comparing chow and rosi-fed Apc<sup>min/+</sup> mice (chow, n = 66; rosi, n = 11,
Fig. 6. Proposed model of Ras/WNT (tumor promoters) and Cav1/PPARg (tumor suppressors) signaling in CRC. Blue = tumor-suppressive proteins, red = tumor-promoting proteins. Black arrows = subcellular translocations. In normal tissue, Cav1 and WT APC inhibit proliferation (and stabilize cell junctions) by excluding β-catenin from the nucleus and by inhibition of Ras. Cav1 interacts with PPARg and promotes ligand-dependent signaling of PPARg (e.g. upon uptake of rosiglitazone from the extracellular medium). In absence of Ras-signaling, active PPARg, thus, inhibits β-catenin and promotes expression of antiproliferative/ proliferation genes in the nucleus, such as Dok1, Cav1, Pten, p21 e.a. and Pparg itself, which enforce inhibition of Ras through a negative feedback loop. Upon loss of Cav1 or mutation of APC (as exist in CRC tissue of humans and mice and in human CRC cells), β-catenin is released to the nucleus, represses PPARg-target genes and activates genes involved in proliferation such as Cyclind1 and Myc. Cav1-mediated inhibition of WNT- and Ras-signaling is alleviated, and Ras activity may be additionally potentiated by mutations (G12D,G12V,G13D). Active Ras-signaling leads to cytosolic sequestration and inactivation of PPARg by MEK1/2 resulting in loss of PPARg’s transcriptional activity in the nucleus. Raf/MEK-inhibitors in combination with PPARg agonists may overcome the unresponsiveness of Ras-mutated human CRC cells to anti-EGFR therapy, exemplified by cetuximab (CTXmab) or erlotinib.

rather reflects the natural variation in this spontaneous carcinogenesis model. In accordance with our results, others have already reported that Cav1−/− mice per se show a dramatic reduction in life span (46), which may be a consequence of pulmonary and cardiac defects in the homozygous state (24). Thus, a second oncogenic hit (30,31), such as the APC mutation used here, may exacerbate this predisposition to a shortened life expectancy in presence of life-threatening bowel obstruction, bleeding and tumor burden.

Our model fits into the series of spontaneous CRC models suitable for preclinical research, because it emulates the genetic changes and the natural (distal) anatomical localization of CRC in humans (47,48). However, the molecular mechanisms and factors that determine the localization of the tumor in the proximal (cecum) versus distal (colon-rectum) regions of the large intestine remain to be elucidated. An interesting study by Thompson et al. (49) evinced that the PPAR- dependent gene expression profiles differed considerably between the proximal and the distal colon in mice due to activation of metabolic genes versus repression of genes involved in adhesion, migration and signaling. Similarly, active Ras was found to be more oncogenic in the small intestine and the proximal colon than in the distal colon (37,50), whereas PPARg and PPAR beta/delta expression was the highest in the distal colon in mice (51,52). Region-specific gradients have been also described for mutations in APC, BRAF e.a. genes in humans (47,48). These gradients may explain the differential susceptibility of distinct anatomic regions of the intestine to alterations in expression or function of oncopgenes and tumor suppressors. Whether such a gradient exists for Cav1 remains to be determined.

Previous studies have provided evidence for a functional antagonism between β-catenin/WNT and PPARg signaling pathways (18,53,54). For example, PPARg-deficient Apc−/− mice develop CRC with higher incidence and multiplicity than PPARg-WTs (53). PPARg ligands modify intestinal tumorigenesis depending on the type, dose and duration of administration and on the status of the Apc gene in mice (6,55–57). Thus, different ligand regimens increased or decreased tumor load in Apc−/− mice. However, whether aberrant subcellular localization of PPARg can explain the paradoxical effects of PPARg ligands on tumor growth needs to be clarified.

We demonstrated before that active MEK1/2 led to nuclear export of PPARg and inhibited transcription of PPARg-target genes involved in differentiation and growth inhibition in vitro (16). The endogenous Ras-inhibitors Cav1 and Dok1 acted as sensitizers/coactivators for PPARg where they counteredacted MEK1-dependent cytosolic sequestration of PPARg, promoted its transport to the nucleus and enhanced its transcriptional activity in human cancer cells (9) (Fig. 6). In clear support of these in vitro data (9,16), we showed here that intratumoral Pparg RNA levels remained unchanged, instead nuclear localization of and transcription driven by the PPARg protein were diminished in murine CRC as well.

$*P < 0.05$ rosi versus chow. (B) Representative H&E images of Apc−/− mice treated with rosi. Non-responder to rosi with colon TU (and cecum TU, not shown); partial responder to rosi with no TU in colon (but TU in cecum, not shown); full responder to rosi without TU in colon (or cecum, not shown). Apc−/− mice treated with rosi (n = 12) showed no colon lesions. Scale bar = 50 μm. (C) Ras and PPAR-related mRNAs were quantified by RT-qPCR. NT tissue of mice fed with chow diet was compared with NT tissue of rosi-fed mice of all Apc−/− genotypes. Rosiglitazone induces expression of PPARg-target genes that inhibit Ras (Dok1, Cav1). CT values were normalized to β2M and calculated as mean ± standard error (n = 11 rosi, n = 12 chow, $*P < 0.05$ rosi versus chow).

<ref>Fig. 6. Proposed model of Ras/WNT (tumor promoters) and Cav1/PPARg (tumor suppressors) signaling in CRC. Blue = tumor-suppressive proteins, red = tumor-promoting proteins. Black arrows = subcellular translocations. In normal tissue, Cav1 and WT APC inhibit proliferation (and stabilize cell junctions) by excluding β-catenin from the nucleus and by inhibition of Ras. Cav1 interacts with PPARg and promotes ligand-dependent signaling of PPARg (e.g. upon uptake of rosiglitazone from the extracellular medium). In absence of Ras-signaling, active PPARg, thus, inhibits β-catenin and promotes expression of antiproliferative/ proliferation genes in the nucleus, such as Dok1, Cav1, Pten, p21 e.a. and Pparg itself, which enforce inhibition of Ras through a negative feedback loop. Upon loss of Cav1 or mutation of APC (as exist in CRC tissue of humans and mice and in human CRC cells), β-catenin is released to the nucleus, represses PPARg-target genes and activates genes involved in proliferation such as Cyclind1 and Myc. Cav1-mediated inhibition of WNT- and Ras-signaling is alleviated, and Ras activity may be additionally potentiated by mutations (G12D,G12V,G13D). Active Ras-signaling leads to cytosolic sequestration and inactivation of PPARg by MEK1/2 resulting in loss of PPARg’s transcriptional activity in the nucleus. Raf/MEK-inhibitors in combination with PPARg agonists may overcome the unresponsiveness of Ras-mutated human CRC cells to anti-EGFR therapy, exemplified by cetuximab (CTXmab) or erlotinib.</ref>
Similar to the localization shift observed in human cell lines, the PPARγ protein was sequestered in the cytoplasm, an event presumably caused by active Ras-MEK-ERK signaling that was detectable in the tumor tissue and that might result in additional posttranslational modifications including inactivation or degradation of PPARγ. However, in contrast to our in vitro findings (9,16), this ‘silencing’ phenomenon occurred irrespectively of Cav1’s presence. Long-term (~4 months) pharmacological activation of PPARγ by its cognate ligand rosiglitazone in Apc<sup>min/+</sup> mice, at an age of 3 months where tumor initiation has already commenced, reduced the incidence and multiplicity of CRC, and this antitumoral efficacy was again independent of Cav1. In human cancer cells, PPARγ-mediated inhibition of cell proliferation was potentiated by Cav1’s presence (9,16), whereas ligand-activated PPARγ did not require Cav1 to prevent WNT-driven development of CRC in mice.

This discrepancy may be explained by the following observation. Altered expression of Cav1 is frequently associated with changes in tumor growth in preclinical models and survival prognosis in patients (4,5). Loss of Cav1, through genetic or epigenetic mechanisms, occurs in many human cancer entities (4,5) including colon and gastric cancer (3,58). In accordance with these findings, Cav1 mRNA/protein levels were already diminished in tumor tissue of Apc<sup>min/+</sup>Cav1<sup>+/−</sup> mice compared with the normal colon. The WT APC protein positively regulates Cav1 gene expression via FOXO transcription factors (59), whereas truncated APC contributes to downregulation of Cav1 via Myc (39,60). Thus, transcriptional repression, methylation or loss of heterozygosity may lead to silencing of Cav1 expression during colorectal carcinogenesis already in Cav1−/− mice. Hence, a generally reduced Cav1 protein/mRNA expression in CRC tissue of all three Apc<sup>min/−</sup> Cav1<sup>+/−</sup> Cav1<sup>+/−</sup> Cav1<sup>+/−</sup> genotypes may explain why there was no difference in response to PPARγ ligand between the three groups (Cav1<sup>+/−</sup> Cav1<sup>+/−</sup> Cav1<sup>+/−</sup>). Further conclusions are also limited due to the small animal numbers per treatment group.

Our pharmacological approach preserved proper nuclear localization of PPARγ, induced expression of PPARγ-target genes encoding for the Ras-inhibitory proteins Cav1 (12) and Dok1 (9) and decreased the expression of Kras and Cyclind1 mRNAs (Figure 6). Similar to alterations in the expression of components in the Cav1 and WNT pathways, mutations in K-Ras occur frequently in human CRC and are an obstacle to successful treatment with antibodies directed against the epidermal growth factor receptor (61). Our data propose that elevated Ras activity (as observed from increased Kras or Kras mRNA levels in Apc<sup>min/+</sup> tumors) may be indirectly targeted by PPARγ ligands that upregulate endogenous Ras-pathway inhibitors (Dok1, Cav1) and additional growth-inhibitory or differentiation-promoting genes (e.g. PTEN, p21, trefoil factors e.a.). The latter are expected to exert negative feedback loops (i) toward inhibition of Ras and WNT pathway activities at the plasma membrane and (ii) through negative crosstalk between β-catenin and PPARγ in the nucleus (Figure 6).

This concept is in accordance with the major conclusion from our work that Cav1 is sufficient but not necessary for ligand-activated PPARγ to exert its antitumoral efficacy in vivo. Because PPARγ ligands regulate a plethora of multiple genes that cooperate in growth inhibition and tissue differentiation, this approach is not limited to Cav1 induction alone. Clinical studies in cancer patients using PPARγ ligands as single treatment regimens have been disappointing (6). Novel combinations with agents (61) that inhibit kinases (B-Raf, MEK1/2) downstream of the mutated K-Ras or receptor tyrosine kinases (EGFR/HER) may open future perspectives for treatment of human CRC, for example by relocalization of nuclear receptors from the cytosol to their proper site of action in the nucleus.

Supplementary material

Supplementary Tables S1-S5 and Excel microarray can be found at http://carcin.oxfordjournals.org/

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References


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