expression of the adiponectin receptors in colon cancer tissue. Our results suggest the possibility that adiponectin might directly exert action on the promotion of colon cancer through the AdipoRs, which is different from the indirect action mediated by modulating the metabolic environment, such as improving insulin resistance. Further studies are required in order to elucidate the function of AdipoRs activated by adiponectin and the downstream mechanisms of the AdipoRs in colon cancer cells.

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Adiponectin suppresses colorectal carcinogenesis under the high-fat diet condition

T Fujisawa,[1] H Endo,[1] A Tomimoto,[1] M Sugiyama,[1] H Takahashi,[1] S Saito,[1] M Inamori,[1] N Nakajima,[2] M Watanabe,[3] N Kubota,[4] T Yamauchi,[4] T Kadowaki,[4] K Wada,[5] H Nakagama,[6] A Nakajima[1]

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[1] Division of Gastroenterology, Yokohama City University School of Medicine, Yokohama, Japan; [2] Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan; [3] Laboratory for Medical Engineering, Graduate School of Engineering, Yokohama National University, Yokohama, Japan; [4] Department of Internal Medicine, Graduate school of Medicine, University of Tokyo, Tokyo, Japan; [5] Department of Pharmacology, Graduate School of Dentistry, Osaka University, Osaka, Japan; [6] Biochemistry Division, National Cancer Center Research Institute, Tokyo, Japan

Correspondence to: Dr A Nakajima, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan; Nakajima-tky@umin.ac.jp

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ABSTRACT

Background and aims: The effect of adiponectin on colorectal carcinogenesis has been proposed but not fully investigated. We investigated the effect of adiponectin deficiency on the development of colorectal cancer.

Methods: We generated three types of gene-deficient mice (adiponectin-deficient, adiponectin receptor 1-deficient, and adiponectin receptor 2-deficient) and investigated chemical-induced colon polyp formation and cell proliferation in colon epithelium. Western blot analysis was performed to elucidate the mechanism which affected colorectal carcinogenesis by adiponectin deficiency.

Results: The numbers of colon polyps were significantly increased in adiponectin-deficient mice compared with wild-type mice fed a high-fat diet. However, no difference was observed between wild-type and adiponectin-deficient mice fed a basal diet. A significant increase in cell proliferative activity was also observed in the colonic epithelium of the adiponectin-deficient mice when compared with wild-type mice fed a high-fat diet; however, no difference was observed between wild-type and adiponectin-deficient mice fed a basal diet. Similarly, an increase in epithelial cell proliferation was observed in adiponectin receptor 1-deficient mice, but not in adiponectin receptor 2-deficient mice. Western blot analysis revealed activation of mammalian target of rapamycin, p70 S6 kinase, S6 protein and inactivation of AMP-activated protein kinase in the colon epithelium of adiponectin-deficient mice fed with high-fat diet.

Conclusions: Adiponectin suppresses colonic epithelial proliferation via inhibition of the mammalian target of the rapamycin pathway under a high-fat diet, but not under a basal diet. These studies indicate a novel mechanism of suppression of colorectal carcinogenesis induced by a Western-style high-fat diet.

Adipose tissue produces and secretes several bioactive substances[1 2] known as adipocytokines,[3] and obesity is an important risk factor for many human diseases, including colorectal cancer and diabetes mellitus.[4 5] Several case–control studies have shown that high-fat diets may promote the development of colorectal cancer,[6] and the results of animal experiments suggest the existence of a link between fat intake and colorectal cancer[7]. Adiponectin is mainly secreted by adipocytes[8] and is a key hormone responsible for insulin sensitisation.[9 10] While adiponectin protein is abundantly found in the plasma of healthy human subjects,[11] adiponectin mRNA levels in the adipose tissue and plasma are dramatically decreased in patients with obesity and/or type 2 diabetes mellitus.[12–15] Because both obesity and type 2 diabetes have been reported to be associated with an elevated risk of colorectal cancer,[4–16] we hypothesised that the plasma level of adiponectin may be related to the risk of colorectal cancer.

Several contradictory results have been reported from human clinical studies on the relationship between the plasma levels of adiponectin and the risk of colorectal cancer.[17 18] While some clinical studies have been conducted in humans, no studies investigating the relationship between the plasma levels of adiponectin and the risk of colorectal cancer have been reported in animal models. Therefore, the mechanism underlying the promotion of colorectal carcinogenesis by adiponectin deficiency still remains unclear.

It is now well known that the adiponectin receptor exists in two isoforms: adiponectin receptor 1 (AdipoR1), which is abundantly expressed in the skeletal muscle; and adiponectin receptor 2 (AdipoR2), which is predominantly expressed in the liver.[19] These receptors mediate the enhanced activation of AMP-activated protein kinase (AMPK) and the peroxisome proliferator-activated receptor α (PPARα), as well as the increase in fatty-acid oxidation and glucose uptake induced by adiponectin.[20 21]

Recently, involvement of the AMPK/mammalian target of rapamycin (mTOR) pathway in the development of various types of cancer has attracted attention.[22–24] The important role of mTOR in mammalian cells is related to its control of mRNA translation. The targets for mTOR signalling are proteins involved in controlling the translational machinery, including the ribosomal protein S6 kinases and S6 proteins that regulate the initiation and elongation phases of translation.[25 26] With regard to the upstream control, mTOR is regulated by signalling pathways linked to several oncoproteins or tumour suppressors, including AMP-activated protein kinase (AMPK).[25 27] mTOR is located at the intersection of major signalling pathways and is believed to be capable of integrating a large panel of stress signals, including nutrient deprivation, energy depletion, and oxidative or hypoxic stresses. In particular, AMPK activation has been reported to directly inhibit mTOR[25] and suppress cell proliferation.

Using adiponectin-deficient mice (KO) we therefore investigated whether adiponectin deficiency might promote the development of colorectal cancer, and examined the involvement of the AMPK/mTOR pathway in the effect of adiponectin on colon carcinogenesis.

Table 1 Histological findings of azoxymethane-induced colon polyps in adiponectin-deficient (KO) mice and wild-type (WT) mice receiving a high-fat diet

Experimental group	Adenocarcinoma	Adenoma	Total
WT (n = 10)	15 (42%)	21 (58%)	36 (100%)
KO (n = 10)	51 (60%)	34 (40%)	85 (100%)

MATERIALS AND METHODS

Animal models

All mice were treated humanely in accordance with the National Institutes of Health and AERI-BBRI Animal Care and Use Committee guidelines. Adiponectin (ACRP30 or AdipoQ)-deficient (*ACRP30−/−*) mice (KO mice) and adiponectin receptor 1 or 2-deficient mice (*AdipoR1−/−* or *AdipoR2−/−*) were generated by our group as described previously.[21] We performed the experiments in this study using littermate mice backcrossed to C57Bl/6 for 10 generations.

The animals were fed a basal diet or a high-fat diet until the end of the study. The composition of both diets is listed in supplementary table 1. Three to five mice were housed per metallic cage with sterilised softwood chips as bedding, in a barrier-sustained animal room air-conditioned at 24 (SD 2)°C and 55% humidity, under a 12 h light–dark cycle.

Induction of colon polyps

Azoxymethane (AOM) was purchased from Sigma (St. Louis, Missouri, USA). Mice (6 weeks old) were divided into four groups: (1) WT mice fed the basal diet (n = 10), (2) KO mice fed the basal diet (n = 10), (3) WT mice fed the high-fat diet (n = 10), and (4) KO mice fed the high-fat diet (n = 10). Mice were injected intraperitoneally with 10 mg/kg of AOM once a week for 6 weeks and sacrificed at 20 weeks following initiation of AOM injection to evaluate the difference in the extent of polyp formation between the KO and WT mice (supplementary fig 1A).

Induction of aberrant crypt foci

Mice (6 weeks old) were divided into four groups: (1) WT mice fed the basal diet (n = 12 mice), (2) KO mice fed the basal diet (n = 11), (3) WT mice fed the high-fat diet (n = 11), and (4) KO mice fed the high-fat diet (n = 11). Mice were given two weekly intraperitoneal injections of 10 mg/kg of AOM and sacrificed at 6 weeks following initiation of AOM injection (supplementary fig 1B). The protocol of the 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP)-induced aberrant crypt foci (ACF) model is shown in supplementary fig 2A.[29]

Effect of the AMP kinase activator and mTOR inhibitor on colon carcinogenesis

The AMP kinase activator 5-aminoimidazole-4-carboxamide-l-β-D-ribofuranoside (AICAR) and the mTOR inhibitor rapamycin were purchased from BIOMOL (Plymouth Meeting, PA, USA). WT and KO mice (6 weeks old) were intraperitoneally injected with AICAR (0.1 mg/kg/day), rapamycin (0.2, 0.4, 0.8 mg/kg) or vehicle (saline) until the end of the experiment. The mice in each group were fed the high-fat diet and received AOM injections according to the ACF protocol.

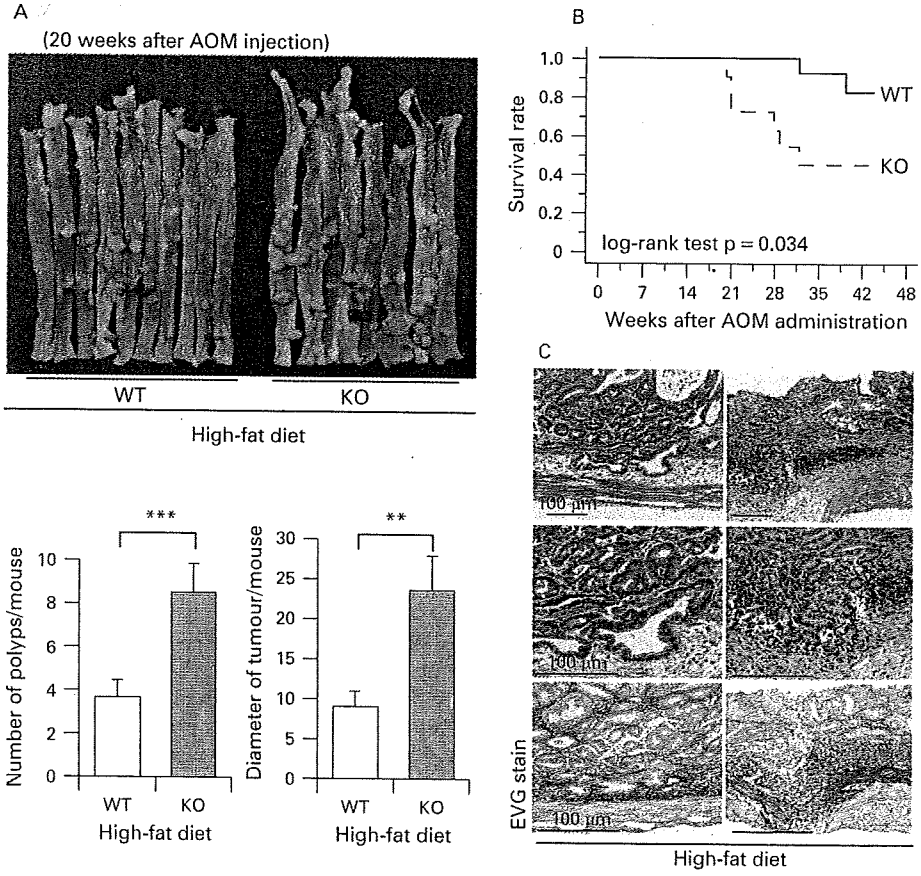

Figure 1 Promotion of colon polyp formation in adiponectin-deficient (KO) mice under the high-fat diet condition. (A) Upper panel: Macroscopic findings of colon polyp in wild-type (WT) and KO littermate mice under the high-fat diet condition at 20 weeks following initiation of azoxymethane (AOM) injection. Lower panel: Number and diameter of polyps per mouse in the high-fat diet groups. Each column represents the mean (with the SEM), *p<0.05. (B) Survival rate of the WT and KO littermates under the high-fat diet condition. The survival rate of the WT mice (solid line) was significantly higher than that of the KO mice (broken line) under the high-fat diet condition. More than half of the KO mice died by the end of the study, while only two of the WT mice died. (C) Invasive polyps in the colons obtained from the KO mice under the high-fat diet condition. Haematoxylin and eosin staining (upper and middle panels) and Elastica van Gieson staining (EVG stain, lower panel) were performed using samples isolated from three individual animals.

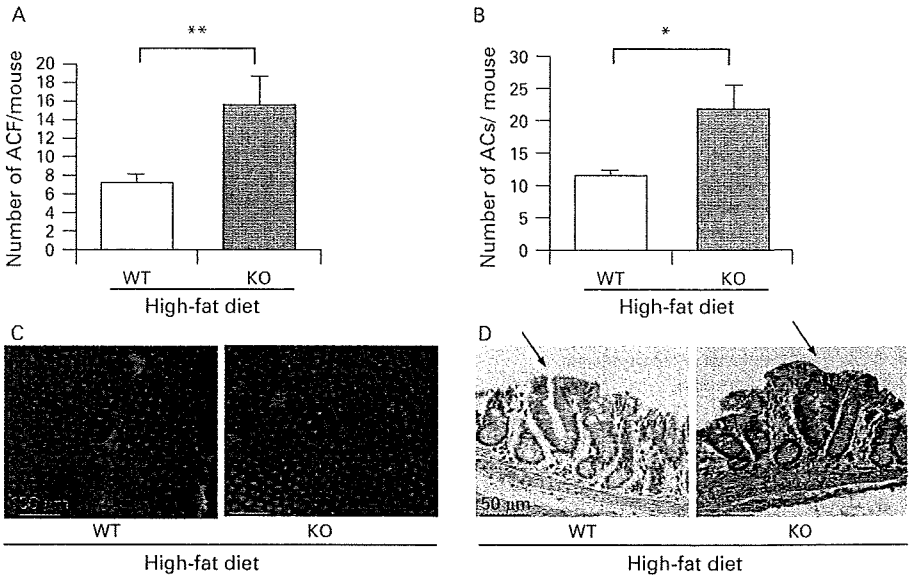

Figure 2 Enhancement of the formation of aberrant crypt foci (ACF) in adiponectin-deficient (KO) mice under the high-fat diet condition. (A,B) Average number of ACF (A) and aberrant crypts (ACs) (B) in the two groups, wild-type (WT) and KO littermate mice, under the high-fat condition, respectively. Each column represents the mean (with the SEM), *$p<0.05$, **$p<0.01$. (C) Stereomicroscopic observations of ACF in colon tissue from each group. The samples were stained with 0.2% methylene blue. (D) Representative haematoxylin and eosin staining of ACF in WT and KO mice under the high-fat diet condition.

Histological analysis of the aberrant crypt foci and colon polyps

The entire colon was removed and fixed in 10% neutralised formalin and the numbers of polyps, ACF and aberrant crypts (ACs) were counted as described previously.[30] To facilitate counting, the colons were stained with 0.2% methylene blue solution and observed by stereomicroscopy. After being counted, they were removed and embedded in paraffin blocks according to standard procedures. Paraffin sections were then prepared at 3.0 μm thickness, stained with hematoxylin & eosin and Elastica van Gieson staining for a detection of submucosal invasion, and subjected to histological analysis.

Analysis of the survival rate

In the polyp induction experiment, both the KO ($n = 11$) and WT ($n = 12$) mouse groups were continuously observed for 45 weeks. Survival curves were drawn using the Kaplan–Meier method and analysed using the log-rank test.

Assay for assessment of the proliferative activity of the colon epithelial cells

We evaluated the bromodeoxyuridine (BrdU) and the proliferating cell nuclear antigen (PCNA) labelling indices to determine the proliferative activity of the colon epithelial cells. BrdU (BD Biosciences, New Jersey, USA) was diluted in phosphate-buffered saline at 1 mg/ml and administered intraperitoneally at a dose of 50 mg/kg, 1 h prior to the sacrifice of the mice. Immunohistochemical detection of BrdU was performed using a commercial kit (BD Biosciences) and a PCNA detection kit (Zymed Laboratories, South San Francisco, California, USA) was used for PCNA detection. The BrdU and PCNA labelling indices were expressed as the ratio of the number of positively stained nuclei to the total number of nuclei counted in the crypts of the colon. The criteria for selecting the crypts included the presence of a clearly visible and continuous cell column on each side of the crypt. Twenty crypts were evaluated each mouse.

Immunoblotting

The extracted protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, London, UK). The membranes were probed with primary antibodies specific for adiponectin receptor 1, adiponectin receptor 2 (Santa Cruz Biotech, California, USA), phospho-AMPK, AMPK, phospho-mTOR, mTOR, phospho-S6K, S6K, phospho-S6 protein, S6 protein (Cell Signaling Technology, Danvers, Massachusetts, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, Maryland, USA). Horseradish-peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection kit (Amersham) were used for the detection of specific proteins.

Statistical analysis

Statistical analyses for the number of ACF, number of colon polyps, BrdU labelling index and PCNA labelling index were conducted using the Mann–Whitney test. The results for western blot analysis were obtained using the Student t test. Values of $p<0.05$ were regarded as denoting statistical significance.

RESULTS

Promotion of colon polyp formation and lower survival rate in adiponectin-deficient mice under the high-fat diet

The number of polyps in the KO mice was significantly higher than that in the WT mice under the high-fat diet (fig 1A), while there was no difference in the total number of polyps between the WT and KO mice under the basal diet (supplementary fig 3). The sum of the diameter of the polyps per mouse was also measured and similar results were obtained. Table 1 shows histological findings of polyps in mice under high-fat diet. We also observed the survival rate of the WT and KO mice under the high-fat diet condition in the AOM model, and there was a significantly higher survival rate in the WT mice than in the KO mice. While more than half of the KO mice were dead by the end of the study, only two WT mice died (fig 1B).

Interestingly, invasion by malignant cells was observed in parts of the polyps exclusively in the KO mice under the high-fat diet, and the malignant cells were found to have destroyed the muscularis mucosae and invaded the submucosal layer in

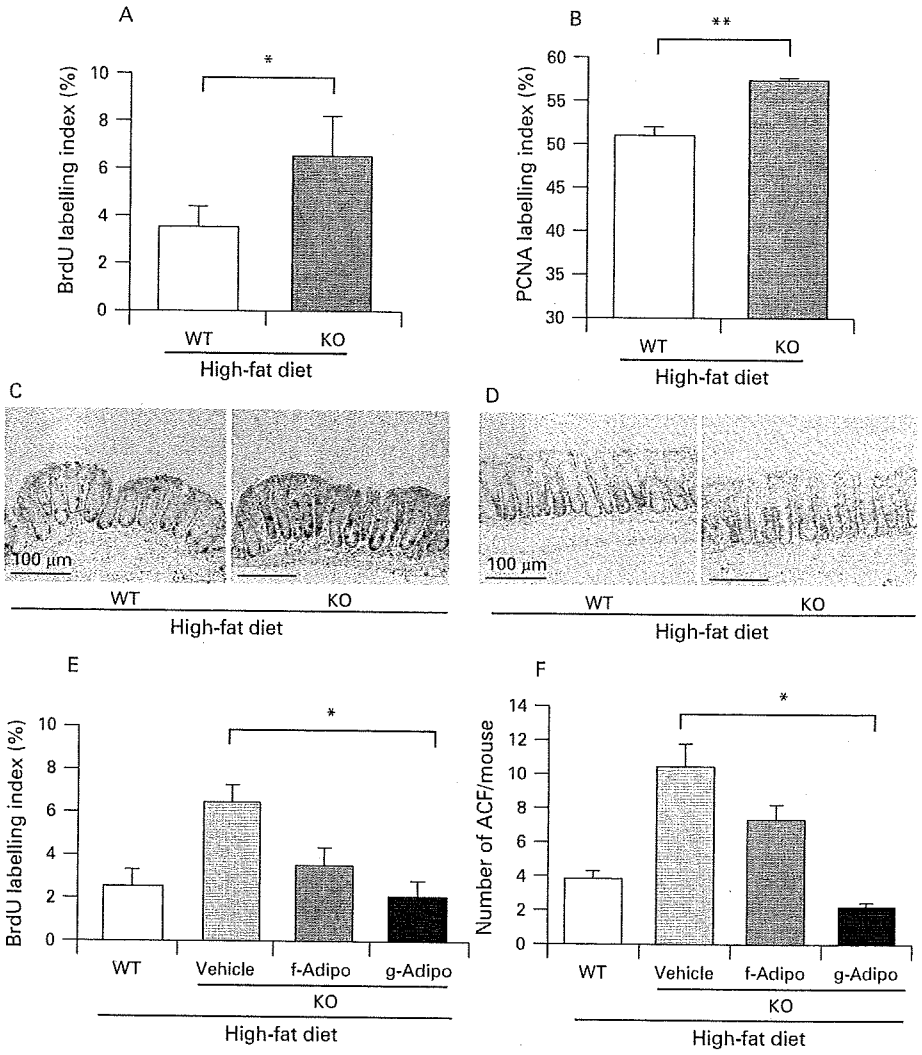

Figure 3 Increase in the colonic epithelial cell proliferative activity in adiponectin-deficient (KO) mice under the high-fat diet condition. (A,B) Average bromodeoxyuridine (BrdU) labelling index (A) and proliferating cell nuclear antigen (PCNA) labelling index (B) in each group in the aberrant crypt foci (ACF) formation experiment. BrdU was administered intraperitoneally 1 h prior to the sacrifice of the animals. Both indices were expressed as the percentage of positively stained nuclei out of the total number of nuclei counted in the crypts of the colon. Each bar represents the mean (with the SEM), *p<0.05, **p<0.01. (C,D) Representative immunohistochemical staining for BrdU (C) and PCNA (D) in each group. (E,F) Wild-type (WT) mice and KO littermate mice fed the high-fat diet were injected intraperitoneally with 50 μg/body recombinant full-length adiponectin (f-Adipo) or 5 μg/body recombinant globular adiponectin domain (g-Adipo) or only vehicle every other day for 6 weeks in an ACF experiment. Adiponectin ameliorates the epithelial cell hyperproliferation in the KO mice under the high-fat diet condition (E). The same effect was observed on the suppression of ACF formation (F).

the tissue specimens (fig 1C), whereas no such invasion was observed in the WT mice. At the end of experiment, the body weight in KO mice was decreased compared to WT mice under the high-fat diet (supplementary fig 4A,B).

Enhanced formation of aberrant crypt foci in adiponectin-deficient mice under the high-fat diet

To investigate the effect of adiponectin in suppressing colon carcinogenesis under the high-fat diet, we analysed colon specimens for the formation of ACF, defined as clusters of aberrant crypts, as a marker of the early stage of colorectal carcinogenesis.[51 52] Although there were no significant differences in the total number of ACF and ACs between the WT and KO littermates mice under the basal diet (data not shown), the numbers of ACF and ACs in the KO mice were significantly higher than those in the WT littermates under the high-fat diet (fig 2A,B). The macroscopic and microscopic characteristics of the ACF in the WT and KO mice under the high-fat diet condition are shown in fig 2C,D; no morphological differences of the ACF were observed between the WT and the KO mice. The differences in the body weight (supplementary fig 4C,D) and the serum levels of adiponectin, glucose, insulin, lipids, and tumour necrosis factor α between the WT and the KO mice are shown in supplementary table 2. In agreement with previous metabolic studies of adiponectin-deficient mice,[33] there were no differences between the two groups under the high-fat diet. To confirm the protective role of adiponectin in colorectal carcinogenesis, the food-borne carcinogen PhIP was used as a second model of colon carcinogenesis in mice fed a high-fat diet. Similar results to those obtained using the AOM-induced carcinogenesis model was obtained (supplementary fig 2).

Increase in cell proliferative activity in adiponectin-deficient mice under the high-fat diet condition

We investigated the proliferative activity of the colon epithelium by determining the BrdU and PCNA labelling indices. Both indices were increased in the KO mice as compared with their WT littermates under the high-fat diet (fig 3A–D). On the other hand, there was no difference under the basal diet (data not shown). Moreover, we examined both indices in colon polyps under the high-fat diet but there was no difference between WT and KO mice (supplementary fig 5).

Adiponectin ameliorates epithelial cell hyper-proliferation in adiponectin-deficient mice under the high-fat diet condition

We administered recombinant adiponectin via an intraperitoneal injection to KO mice in comparison to their WT littermates under the high-fat diet condition. Globular domain adiponectin

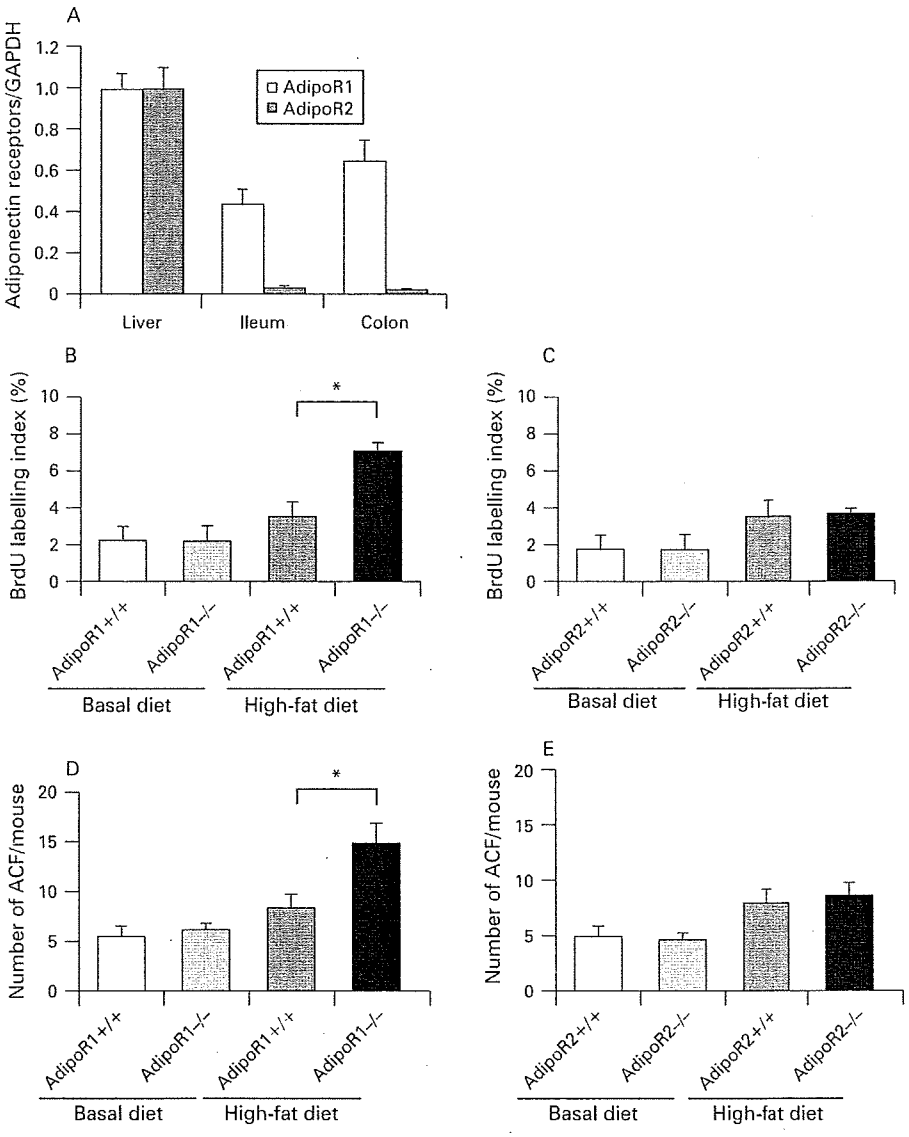

Figure 4 Expression of the adiponectin receptors AdipoR1 and AdipoR2 in the liver, ileum and colon, and promotion of epithelial cell proliferation in AdipoR1−/− mice under high-fat diet condition. (A) Western blot analysis to investigate the expression of AdipoR1 and AdipoR2 was performed on protein obtained from the liver, ileum and colon. The ratio in the liver was defined as 1.0. (B,C): Average bromodeoxyuridine (BrdU) labelling index on colon epithelium from AdipoR1−/− and AdipoR2−/− mice in comparison to the wild-type (WT) littermate mice under basal and high-fat diet condition. The increase in the epithelial cell proliferation was observed in AdipoR1−/− mice, but not in AdipoR2−/− mice under the high-fat diet condition. (D,E) Average number of aberrant crypt foci (ACF) in AdipoR1−/− (D) and AdipoR2−/− mice (E). GADPH, glyceraldehyde-3-phosphate dehydrogenase.

exerted a more potent effect on the suppression of proliferative activity of colon epithelial cells than full-length adiponectin under the high-fat diet (fig 3E). The same effect was observed on the suppression of ACF formation (fig 3F).

The increase in cell proliferative activity in adiponectin receptor 1-deficient mice under the high-fat diet

By using western blot analyses we investigated whether adiponectin receptors are expressed in the colon epithelium, and observed that AdipoR1 was predominantly expressed in the colon in comparison to AdipoR2 (fig 4A).

The BrdU index in the *AdipoR1*−/− mice was significantly higher than in their WT littermates under the high-fat diet (fig 4B). The numbers of ACF and ACs in the *AdipoR1*−/− mice were also significantly higher than their WT littermates (fig 4D). However, no difference in BrdU index and the number of ACF was observed in *AdipoR2*−/− mice and their WT littermates (fig 4C,E). These results suggest that the AdipoR1-mediated, but not the AdipoR2-mediated, pathway may play an important role in the suppressive effect of adiponectin on colorectal carcinogenesis under the high-fat diet, not under the basal diet.

The mTOR pathway is relatively activated in the colon epithelium of adiponectin-deficient mice in comparison to wild-type mice under the high-fat diet

In order to clarify the mechanisms underlying the enhanced proliferative activity of the colon epithelial cells in the presence of adiponectin deficiency, we investigated the expression levels of various potential target proteins in colonic specimens prepared from the WT mice and KO mice under the high-fat diet. The results of western blot analysis revealed that the amounts of phosphorylated mTOR, S6 kinase and S6 protein were significantly higher in the KO mice compared with the WT mice under the high-fat diet (fig 5B–D). It has been reported that adiponectin activates AMPK via AdipoR1, and AMPK is known to suppress the mTOR pathway.[30 34] A significant decrease in the level of phosphorylated AMPK was observed in the KO mice compared with that in the WT mice under the high-fat diet (fig 5A). Moreover, adiponectin administration ameliorated activation of the AMPK/mTOR pathway in KO mice under the high-fat diet condition (supplementary fig 6). These results indicate that, under the high-fat diet, deficiency of adiponectin suppresses AMPK activation, which results in

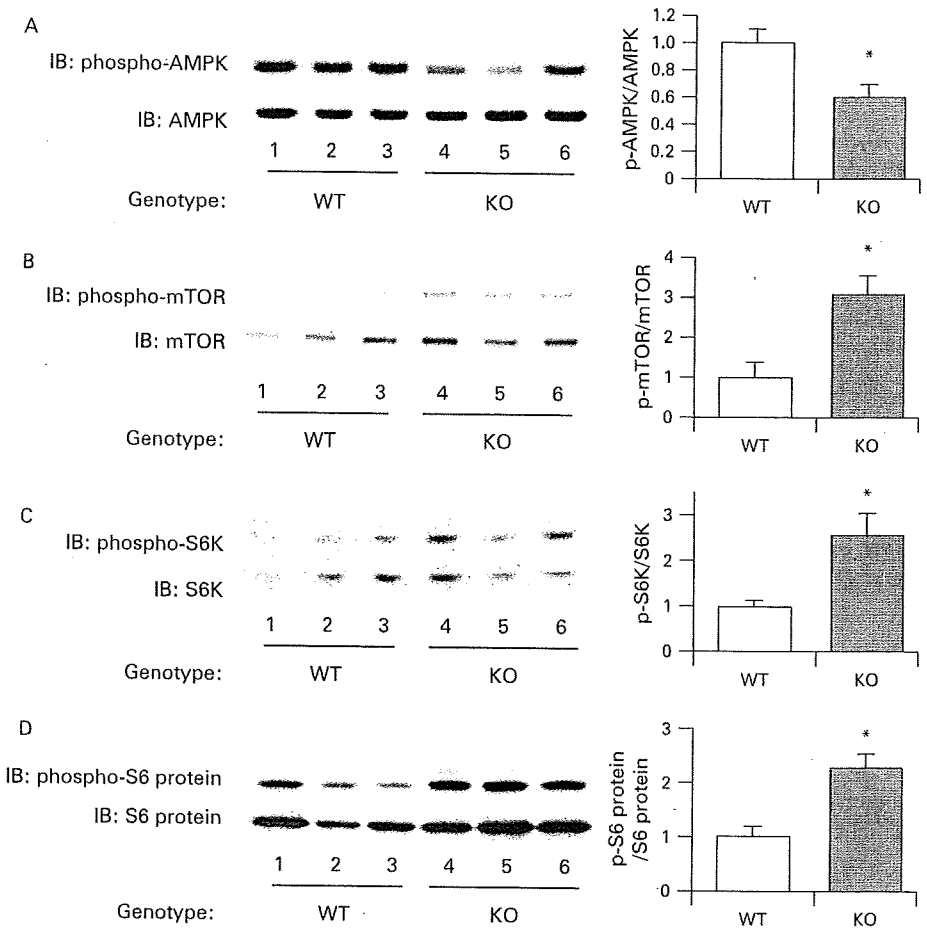

Figure 5 Activation of the mammalian target of rapamycin (mTOR) pathway in adiponectin-deficient (KO) mice compared with that in wild-type (WT) mice under the high-fat diet condition. Western blot analysis for phosphorylated and total AMP-activated protein kinase (AMPK) (A), mTOR (B), p70 S6 kinase (C) and S6 protein (D) in the colon from the WT and KO mice under the high-fat diet condition. Left panels: Pictures of the western blotting. Right panels: Graphs showing the ratios of the phosphorylated protein to the total protein. Each column represents the mean (with the SEM), *$p<0.05$.

activation of the mTOR pathway directly involved in cell proliferation. To confirm whether adiponectin actually suppresses the AMPK/mTOR pathway, we treated mice with the specific AMPK activator AICAR, or the mTOR inhibitor rapamycin. The increase of cell proliferation in the colon epithelium was significantly suppressed by AICAR in the KO mice under the high-fat diet, but no effect in WT mice under high-fat diet (fig 6A,B). Similarly, ACF formation was significantly suppressed by AICAR in the KO mice under the high-fat diet, but not in the WT mice (fig 6C). In the KO mice under the high-fat diet, treatment with rapamycin significantly reduced the BrdU index in a dose dependent manner and ACF formation (fig 6D,E). These results indicate that the activation of the mTOR pathway may play important roles in the increase in epithelial cell proliferation in KO mice under the high-fat diet, and may play an important role in the promotion of colon carcinogenesis in KO mice under the high-fat diet condition.

DISCUSSION

The existence of a relationship between high-fat diets and colorectal cancer has been speculated for a long time, but no definitive conclusions have been arrived at yet.[35] It has been reported that the secretion of adiponectin from adipocytes is suppressed in obese humans.[11] Considered together with the knowledge that obesity is also an important risk factor for colorectal cancer,[4] we speculated that adiponectin might suppress the development of colorectal cancer.

We demonstrated significantly enhanced formation of polyps and ACF in the KO mice compared with that in the WT mice under the high-fat diet. Furthermore, an increase in proliferative activity of colonic epithelial cells was also observed in the KO mice under the high-fat diet, but not under the basal diet. These results suggest that under the high-fat diet, but not under basal diet, a deficiency of adiponectin significantly promotes the proliferative activity of the colonic epithelial cells, and thereby may be promoting colorectal carcinogenesis. We demonstrated that the AdipoR1 is predominantly expressed in colon epithelium. The increase in the proliferative activity of the colonic epithelial cells and the number of ACF were observed in AdipoR1−/− mice, not in the AdipoR2−/− mice, under high-fat diet condition. These results suggest that the AdipoR1-mediated, but not the AdipoR2-mediated, pathway may play an important role in the suppressive effect of adiponectin on the increased in epithelial cell proliferation under the high-fat diet.

We demonstrated the activation of the mTOR pathway and inactivation of AMPK in colon epithelial cells in the KO mice under the high-fat diet, but there was no difference under the basal diet (data not shown). Moreover, the replacement of adiponectin ameliorated the activated mTOR pathway by adiponectin deficiency. AICAR, the AMPK specific activator, suppressed the increase in epithelial cell proliferation in KO mice, but not in WT littermates, under high-fat diet. Furthermore, rapamycin, an mTOR inhibitor, also significantly suppressed the increase in epithelial cell proliferation only in KO mice under high-fat diet in a dose dependent manner,

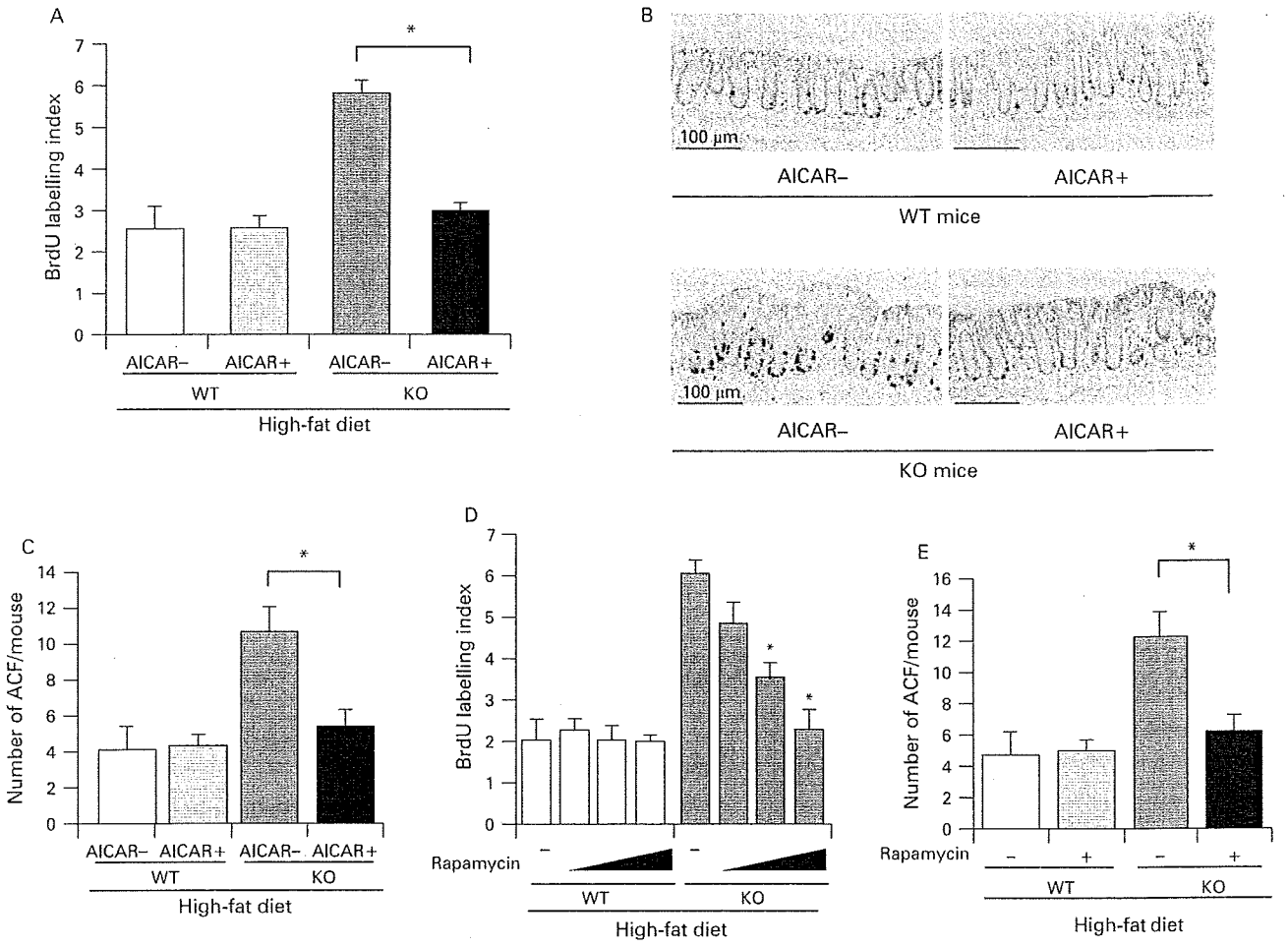

Figure 6 Suppression of epithelial cell hyper-proliferation by activation of AMP-activated protein kinase (AMPK) or by inhibition of the mammalian target of rapamycin (mTOR) in adiponectin-deficient (KO) mice under the high-fat diet condition. Wild-type (WT) mice (6 weeks old) were injected intraperitoneally with the AMPK activator 5-aminoimidazole-4-carboxamide-l-β-D-ribofuranoside (AICAR) (0.1 mg/kg/day), or vehicle until the end of the experiment. Mice in the high-fat diet group were also given two weekly intraperitoneal injections of 10 mg/kg of azoxymethane (AOM). (A) Average bromodeoxyuridine (BrdU) labelling index in the WT and KO mice treated (+) or not treated (−) with AICAR under the high fat diet condition. BrdU was administered intraperitoneally 1 h prior to the sacrifice of the animals. Each column represents the mean (with the SEM), *$p<0.05$. (B) Representative immunohistochemical staining patterns for BrdU in each group. (C) Average number of aberrant crypt foci (ACF) in the WT and KO mice treated (+) or not treated (−) with AICAR under the high fat diet condition. (D) WT and KO mice fed the high-fat (HF) diet were injected intraperitoneally with various doses of rapamycin (0.2, 0.4, 0.8 mg/kg) or only with vehicle every other day for 6 weeks. Average values of the BrdU index were decreased in the KO mice in a dose dependent manner, but not in the WT mice. *$p<0.05$ compared to non-treated KO mice. (E) Average number of ACF in the WT and KO mice treated (+) or not treated (−) with rapamycin (0.8 mg/kg) under the high fat diet condition. Each bar represents the mean (with the SEM), *$p<0.05$.

suggesting that mTOR plays an important role in promoting epithelial cell proliferation where there is a lack of adiponectin under a high-fat diet. It has been reported that AMPK directly inhibits mTOR.[36] Therefore we speculate that the AMPK/mTOR pathway is a possible mechanism closely involved in the protective effect of adiponectin in colon carcinogenesis under the high-fat diet (supplementary fig 7). Concerning other major pathways in the carcinogenesis, it was reported that adiponectin attenuated the adenomatous polyposis coli (APC)/β-catenin pathway,[56] and increased p53 expression[57] in different kinds of cancer cells. p53 also suppresses the mTOR pathway through activation of phosphatase and tensin homologue deleted on chromosome ten (PTEN), AMPK, insulin-like growth factor-1-binding protein 3 (IGF1-BP3) and tuberous sclerosis complex-2 (TSC-2).[58] Although the mechanism underlying the promotion of colon carcinogenesis by a high-fat diet is still unknown, our present data strongly suggest that plasma adiponectin derived from adipocytes suppresses the mTOR pathway through the activation of AMPK, resulting in suppression of the cell proliferative activity and, thereby, suppression of colon carcinogenesis, under the high-fat diet. However, in the event of a decrease in plasma adiponectin level, AMPK activity is suppressed, resulting in the activation of mTOR and the members downstream in the pathway, such as the p70 S6 kinase and S6 protein. We speculate that activation of the mTOR pathway directly promotes colonic epithelial cell proliferation and, thereby, colorectal carcinogenesis.

It has been reported that the plasma adiponectin levels are decreased in humans under the conditions of obesity and/or diabetes mellitus.[21] However, it was reported that plasma levels of adiponectin in the mice are not decreased in response to high-fat feeding for several weeks.[59] Therefore, we used

adiponectin-deficient mice to elucidate the role of adiponectin on colonic epithelial proliferation and carcinogenesis under the high-fat diet. Our experimental condition in which we used adiponectin-deficient mice fed a high-fat diet may well have reflected these pathophysiological conditions in humans.

The purpose of our study was to elucidate the role of adiponectin on colon carcinogenesis, not to elucidate the mechanism whereby a high-fat diet promotes carcinogenesis. This mechanism remains unknown. However, we could provide a possible mechanism underlying the protective roles of adiponectin in colorectal carcinogenesis promoted by a high-fat diet We consider that AMPK and mTOR may be novel therapeutic targets for the prevention of colorectal cancer under the low levels of plasma adiponectin in an obese population where the obesity is a result of a Western-style diet with a high fat content. Our results shed light on a novel mechanism by which adiponectin might suppress carcinogenesis mediated by a high-fat diet. Continued investigation to elucidate the precise mechanisms involved is necessary because of the major clinical implications.

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Competing interests: None.

Ethics approval: All animal experiments were approved by the institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

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Full Paper

Peroxisome Proliferator-Activated Receptor γ (PPARγ) Suppresses Colonic Epithelial Cell Turnover and Colon Carcinogenesis Through Inhibition of the β-Catenin / T Cell Factor (TCF) Pathway

Toshio Fujisawa[1,†], Atsushi Nakajima[1,2,†], Nobutaka Fujisawa[1], Hirokazu Takahashi[1], Ikuko Ikeda[1], Ayako Tomimoto[1], Kyoko Yonemitsu[1], Noriko Nakajima[3], Chiho Kudo[4], Koichiro Wada[4,*], Naoto Kubota[5], Yasuo Terauchi[6], Takashi Kadowaki[5], Hitoshi Nakagama[7], and Richard S. Blumberg[2]

[1]*Division of Gastroenterology, and* [6]*Division of Endocrinology and Metabolism, Yokohama City University School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan*
[2]*Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA*
[3]*Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
[4]*Department of Pharmacology, Graduate School of Dentistry, Osaka University, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan*
[5]*Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[7]*Biochemistry Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

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Abstract. Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor superfamily member, plays a major role in lipid metabolism and insulin sensitivity. We investigated the role of PPARγ in colonic epithelial cell turnover and carcinogenesis in colon because PPARγ is strongly expressed in colonic epithelium. Administration of PPARγ agonists suppressed epithelial cell turnover in mice. Expression level of β-catenin protein, a key molecule in carcinogenesis, was increased in mouse colon treated with PPARγ ligands. A direct interaction between β-catenin and PPARγ in cultured cell lines and colonic epithelium in mice was observed. Ligand-activated PPARγ ligand directly interacts with β-catenin, retaining it in the cytosol and reducing β-catenin / T cell factor (TCF) transcriptional activity that is functionally important on aberrant crypt foci (ACF) formation. PPARγ hetero-deficiency promoted the induction of ACF, but had no effect on the incidence of colonic polyps. These results indicate that PPARγ regulates colonic epithelial cell turnover via direct interactions with β-catenin, resulting in inhibition of β-catenin–mediated transcriptional pathways that are involved in promoting cell proliferation. Our findings suggest that PPARγ plays a role as a physiological regulator of colonic epithelial cell turnover and consequently predisposition to the development of colon cancer in early stage.

Keywords: peroxisome proliferator-activated receptor γ (PPARγ), epithelial cell turnover, β-catenin, colon cancer

Introduction

Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor superfamily member, is strongly expressed in adipocytes (1, 2) and plays a major role in lipid metabolism (3 – 5) and insulin sensitivity (6, 7). It has been demonstrated that 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ_2) is a potential endogenous ligand (8) and that thiazolidinediones (TZD), such as pioglitazone and rosiglitazone, widely used as oral hypoglycemic agents, are specific exogenous ligands for PPARγ. PPARγ has also been reported to be expressed in the intestinal epithelium (9). In intestinal epithelium,

†These authors contributed equally to this work.
*Corresponding author. kwada@dent.osaka-u.ac.jp
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PPARγ has been suggested to play an important role as an endogenous inhibitor of NF-κB–mediated inflammation (10). PPARγ has also been detected in colon cancer cells where it has been indirectly linked to colon cancer pathogenesis (11). However, the relationship between PPARγ and colon cancer have been contradictory. The groups of Lefebvre et al. and Saez et al. have shown that treatment with PPARγ ligands enhances colon carcinogenesis in APC^{Min} mice (12, 13). In contrast, Sarraf et al. have demonstrated that troglitazone and rosiglitazone suppress the growth of human colonic cancer cells (14). We previously conducted a study to clarify these controversial results and demonstrated that PPARγ ligands significantly suppress colon carcinogenesis in the azoxymethane (AOM)-induced colon cancer model (15). PPARγ ligands also significantly reduced the incidence of aberrant crypt foci (ACF), a putative precancerous lesion, and reduced the incidence of tumors developing in the colon in this model. In addition, Tanaka et al. also reported on the suppressive effect of PPARγ ligands on the formation of ACF in the AOM-induced colon cancer model in rats (16). Niho et al. demonstrated that PPARγ ligands suppress tumor formation in APC^{Min} mice (17, 18). These results, including our own report, indicate that activation of PPARγ by various ligands suppresses colon carcinogenesis at the initiation, promotion, and progression stages of the disease. However, the molecular mechanisms underlying the suppression of colonic carcinogenesis by PPARγ activation have not yet been clarified.

It has been reported that the β-catenin–Wnt signaling pathway controls cell proliferation and body patterning throughout development (19). In addition, β-catenin plays an important role as a transcriptional activator in colonic carcinogenesis (20 – 22). Specifically, accumulation of β-catenin in the cytosol results in its nuclear translocation in the presence of active Wnt signaling, leading to induction of a genetic program that is involved in the promotion of cell growth at the initiation of colonic carcinogenesis (23, 24). Thus, given the effects of PPARγ ligands on inhibiting the development of ACF and the direct role of Wnt-β–catenin pathways in mediating ACF formation (25), we hypothesized that PPARγ might affect β-catenin–mediated transcriptional regulation as a mechanism to inhibit colon carcinogenesis. Consistent with this, Girnun et al. showed an increase in the levels of β-catenin in the colonic epithelium of PPARγ hetero–deficient mice (26), and concluded that PPARγ suppresses colonic carcinogenesis by regulating the expression levels of β-catenin. However these studies do not show a direct relationship between PPARγ and β-catenin. Namely, the relationship between PPARγ and β-catenin in colon carcinogenesis remains unclear. The aim of this study was, therefore, to clarify the direct interaction between PPARγ and β-catenin in colon carcinogenesis. To clarify the direct interaction between PPARγ and β-catenin may reveal the exact role of PPARγ in influencing epithelial cell turnover and the initial stages of colonic carcinogenesis because β-catenin is the key molecule in the carcinogenesis. Here, we observed that PPARγ directly interacts with β-catenin and diminishes the translocation of β-catenin into the nucleus, which suppresses the activity of β-catenin in mediating its transcriptional program on culture cells and the AOM-induced colon cancer model.

Materials and Methods

Cell lines and culture conditions

HT-29, Lovo, Caco2, and DLD1 cells were purchased from the Japanese Collection of Research Bioresources (Osaka). HT-29 cells were maintained in D-MEM medium supplemented with 10% fetal bovine serum, 200 U/ml of penicillin, and 200 μg of streptomycin. Lovo cells were maintained in McCoy's 5A medium supplemented with 20% fetal bovine serum, 200 U/ml of penicillin, and 200 μg of streptomycin.

Reagents

Pioglitazone and rosiglitazone were kindly provided by Takeda Chemical (Osaka) and by SmithKline Beecham (West Sussex, UK), respectively. PGJ_2 was purchased from ALEXIS Biochemicals (Lausen, Switzerland).

BrdU and PCNA assay

Bromodeoxyuridine (BrdU; BD Biosciences, Franklin Lakes, NJ, USA) was used to label the colonic epithelial cells undergoing DNA replication. BrdU was diluted in phosphate-buffered saline at 1 mg/ml and used at 50 mg /kg body weight. It was given intraperitoneally 1 h prior to the sacrifice of the animals. The immunohistochemical detection of BrdU was conducted using a commercial kit (BD Biosciences). Proliferating cell nuclear antigen (PCNA) was also used to label the colonic epithelial cells undergoing DNA replication and the immunohistochemical detection of PCNA was conducted using a commercial kit (Zymed Laboratories Inc., South San Francisco, CA, USA).

Reporter assay

Lovo cells were seeded at a density of 1×10^4 cells/well in 24-well plates and cultured for 24 h, prior to transfection. Transfections were performed in accordance with the manufacturer's procedure using 3 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA),

2 μg of Firefly Luciferase Reporter DNA (TOPflash or control FOP flash; Upstate Biotechnology, Inc., New York, NY, USA), and 0.5 μg of phRL-TK-Renilla Luciferase (Promega, Madison, WI, USA) as a control for transfection efficiency in 200 μl of Opti-mem (Invitrogen). At 24 h after the transfection, the medium was removed and the cells were treated with medium containing or not containing 10 μM of PGJ_2 for 8 h. The medium was removed and the Firefly and Renilla luciferase activities were sequentially measured using the Dual-Glo buffer system (Promega). Transfections were performed in triplicate and repeated five times for each set. To normalize the data, the Firefly luciferase activity in each well was divided by the Renilla luciferase value. Each experiment was repeated at least three times. Data represent the TOP/FOP relative luciferase activity and the mean ± S.D. from three independent experiments.

Knockdown of PPARγ using siRNA

PPARγ siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). This siRNA was transfected into 70%-confluent Lovo cells using Lipofectamine 2000. The cells were treated with 12 nM of PPARγ siRNA for 24 h prior to the reporter assay. The Stealth RNAi Negative Control Medium GC (Invitrogen) was used as the negative control.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells using the RNAeasy Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was prepared and RT-PCR was performed using the RNA PCR kit (Takara, Otsu). The PCR primers were designed according to the reported sequences as follows: c-myc (ref. 27) (forward 5'-GGTCTTCCCCTACCCT CTCAACGA-3', reverse 5'-GGCAGCAGGATAGTCC TTCCGAGT-3'), cyclin D1 (ref. 28) (forward 5'-TGT GCTGCGAAGTGGAAACC-3', reverse 5'-AAATCG TGCGGGGTCATTGC-3'), β-actin (ref. 27) (forward 5'-AAGAGAGGCATCCTCACCCT-3', reverse 5'-TAC ATGGCTGGGGTGTTGA-3'). After initial denaturation for 10 min at 9°C, the reaction was conducted over 20 PCR cycles of 95°C for 60 s and 56°C for 60 s. β-Actin mRNA quantification was also performed for standardization.

Immunoprecipitation

Cell extracts were prepared in lysis buffer (20 mM HEPES, 2 mM EGTA, 50 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 1 mM vanadate, and 0.04 mM phenylmethylsulfonyl fluoride) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA). To determine the interaction between PPARγ and β-catenin in various colon cancer cell lines in the presence or absence of PPARγ ligands, immunoprecipitation was performed using a specific anti-PPARγ antibody (Santa Cruz Biotechnology) or anti-β-catenin antibody (BD Transduction Laboratories, Lexington, KY, USA). The antibodies were pre-adsorbed on protein G-Sepharose beads for 1 h at 4°C. Equal amounts of cell extracts (400 μg protein each) were incubated with the respective antibody-crosslinked beads overnight at 4°C, with purification of the antibody-protein complex beads by centrifugation. The samples were then resolved by SDS-PAGE, followed by Western blotting. The signals were detected with an ECL-plus kit (Amersham Pharmacia Biotech, London, UK) in accordance with the manufacturer's protocol.

GST-PPARγ fusion protein and GST pull-down assay

The full-length human PPARγ1 cDNA was subcloned into the pGEX-4T vector (Amersham Pharmacia Biotech). The GST-PPARγ fusion protein was synthesized and purified using the B-PER GST Fusion Protein Purification Kit (PIERCE, Rockford, IL, USA). A clone of β-catenin (GeneStorm clones: RG001399) was purchased from Invitrogen. In vitro transcription and translation were performed using the TNT kit (Promega) in the presence of [^{35}S]-methionine (Amersham Pharmacia Biotech). Lysates were prepared by solubilizing the cells in immunoprecipitation buffer. The β-catenin synthesized in vitro was pulled down with either the GST-PPARγ fusion protein or the GST protein alone and the immunoprecipitates resolved by SDS-PAGE under reducing conditions, followed by autoradiography.

Animal models

All mice were treated humanely in accordance with the National Institutes of Health and AERI-BBRI Animal Care and Use Committee guidelines. All animal experiments were approved by the institutional Animal Care and Use Committee of Yokohama City University School of Medicine. AOM was purchased from Sigma. Heterozygous PPARγ-deficient [PPARγ (+/−)] mice and wild-type [(PPARγ (+/+)] mice were generated as described previously (29). They were fed a standard diet (Oriental MF; Oriental Co., Tokyo) until sacrifice. Female BALB/c mice were purchased from CLEA Japan (Tokyo). All the animals were housed in a ventilated, temperature-controlled room (23 ± 1°C) under a 12-h light/dark cycle. To prepare the experimental diet, pioglitazone was mixed with the powdered standard diet (Oriental MF) and stored at 4°C until use; the concentration of pioglitazone in the experimental diet was 200 ppm. After acclimatization for 3 days to the housing

environment and the standard diet, the BALB/c mice were divided into groups that were fed either the standard diet or the experimental diet until sacrifice.

Induction of ACF

Eight-week-old PPARγ (+/+) (n = 14) and PPARγ (+/−) (n = 14) mice were injected intraperitoneally with 10 mg/kg of AOM once a week for 2 weeks. Another set of 8-week-old PPARγ (+/+) (n = 14) and PPARγ (+/−) (n = 14) mice were treated with saline as a control. After the first administration of AOM or saline for four weeks, the mice were sacrificed at experimental week 5. The entire colon was immediately removed and fixed, and the numbers of ACF and aberrant crypts (ACs) were counted as described previously (15).

Induction of colonic tumors

Eight-week old PPARγ (+/+) (n = 20) and PPARγ (+/−) (n = 20) mice were injected intraperitoneally with 10 mg/kg of AOM once a week for 6 weeks. All the surviving mice were sacrificed 37 weeks after the last injection of AOM. The colons were removed immediately and fixed in 10% neutralized formalin as described previously (15).

Histological analysis of the ACF and colonic tumors

All the colons were removed and fixed in neutralized 10% formalin overnight at 4°C. To facilitate counting of the ACF and colonic tumors, the colons were stained with 0.2% methylene blue solution and examined under a dissecting microscope. The ACF and colonic tumors were removed and embedded in paraffin blocks in accordance with standard procedures.

Immunohistochemical analysis

Paraffin-embedded sections were deparaffinized and subjected to immunohistochemical staining with an anti-mouse β-catenin monoclonal antibody (BD Transduction Laboratories) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) in accordance with the manufacturer's instructions. The primary β-catenin antibody was used at a dilution of 1:800 and the PPARγ antibody at a dilution of 1:500, with nuclear counterstaining performed using hematoxylin.

Immunoblotting and immunofluorescence

Colonic epithelium was isolated and protein extraction performed. Western blotting was performed using anti-PPARγ (Santa Cruz Biotechnology), anti–β-catenin (BD Transduction Laboratories), or anti-G3PDH (Trevigen, Gaithersburg, MD, USA) antibodies. Segments of colonic epithelium from mice fed the experimental or standard diet were embedded in the same block and frozen sections cut. For the immunofluorescence analyses, the frozen tissue sections were incubated in a working solution of M.O.M. Mouse IgG Blocking Reagent (Vector Laboratories) at 37°C for 1 h. The sections were then washed with PBS and incubated in a working solution of M.O.M. Dilute (Vector Laboratories) at room temperature for 30 min. The sections were then exposed to an anti–β-catenin mouse monoclonal antibody (BD Transduction Laboratories) (1:100 diluted with M.O.M. Dilute) or anti-rabbit PPARγ polyclonal antibody (Santa Cruz Biotechnology) (1:10 diluted with M.O.M. Dilute) overnight at 4°C. After washing with PBS, the sections were incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) or Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probe) at 37°C for 1 h. Imaging was performed using a confocal microscope equipped with an argon-krypton laser (LSM-Micro-System; Carl Zeiss, Oberkochen, Germany). Alexa Fluor 488 dye exhibited green fluorescence and Alexa Fluor 555 dye exhibited red fluorescence. The emission patterns of the two fluorescence labels were collected separately and the data overlaid to generate two-color images.

Statistical analyses

Statistical analyses for ACF and colonic tumor multiplicity were conducted by using the Mann-Whitney *U* test. Other statistical analyses of the differences in the incidence and histologic characteristics of the colonic tumors were performed by Student's *t*-test. Differences were considered significant when P values were <0.05.

Results

PPARγ modulates the levels of β-catenin

To determine the effect of PPARγ on β-catenin levels, we examined the quantities of PPARγ and β-catenin proteins in the colonic epithelium from PPARγ (+/−) and PPARγ (+/+) mice. The results of Western-blot analysis indicated that the PPARγ protein levels were reduced in the colonic epithelium of the PPARγ (+/−) mice as compared to that observed in the colonic epithelium of wild-type mice (Fig. 1A). Interestingly, the β-catenin protein levels in the colonic epithelium of the PPARγ (+/−) mice were also reduced in comparison to that observed in the colonic epithelium of the wild-type mice (Fig. 1B). These results indicate that the expression of PPARγ and β-catenin in the colonic epithelium are coordinately regulated. We, therefore, examined whether PPARγ might upregulate β-catenin levels in the colonic epithelium of PPARγ-ligand–treated mice. As expected, Western-blot analysis showed significant upregulation of β-catenin protein levels in the colonic epithelium of

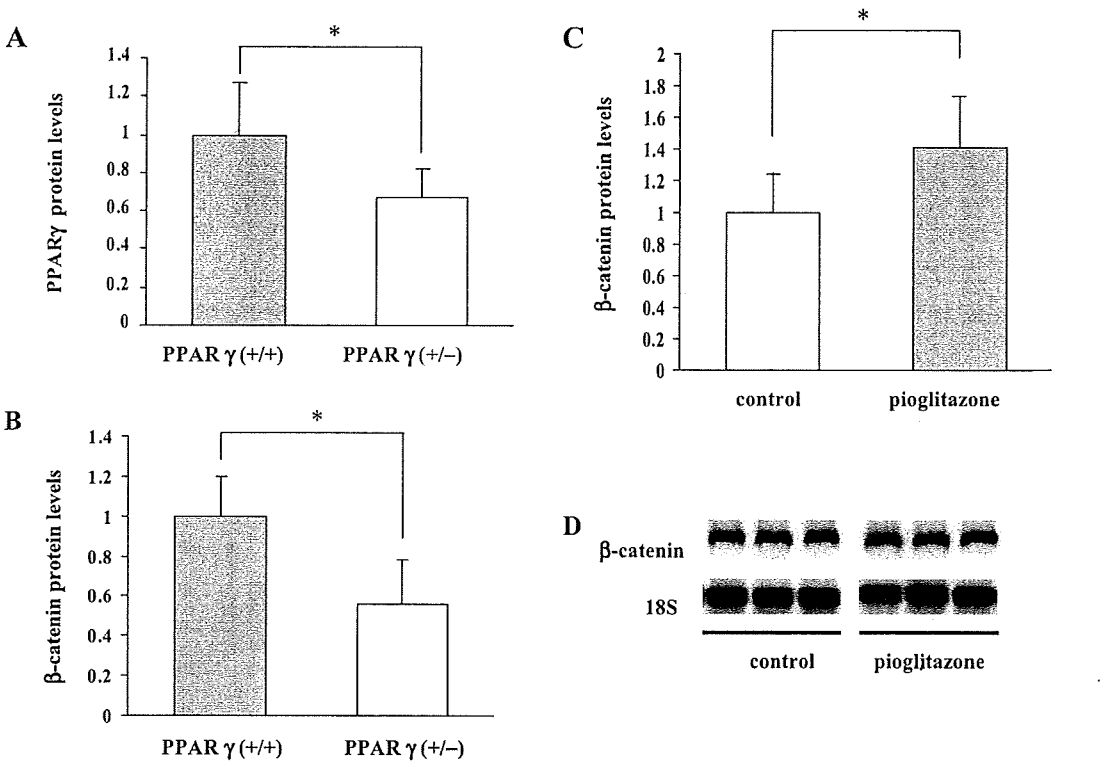

Fig. 1. β-Catenin expression in the colonic epithelium of PPARγ (+/–)/(+/+) and TZD-treated mice. A and B: Expression of PPARγ (A) or β-catenin (B) in the colonic epithelium of PPARγ (+/–) and PPARγ (+/+) mice. Protein lysates were prepared from the colonic epithelium of five mice per group, and the levels of PPARγ and β-catenin were analyzed by Western-blot analysis. The averaged expression of each protein in wild type (PPARγ (+/+)) mice was defined as 1.0. The relative expression level of each protein in the PPARγ (+/–) mice was expressed as "protein level" compared to that in wild type (PPARγ (+/+)) mice. *$P<0.05$, as compared with the result in the PPARγ (+/–) mice. C: β-Catenin protein levels in the colonic epithelium of BALB/c mice treated with pioglitazone. Protein lysates were prepared from the colonic epithelium of five mice per group and the levels of β-catenin were analyzed by Western-blot analysis. The *Y* axis indicates the relative expression levels of β-catenin in the control mice. *$P<0.05$, as compared with the results in the control mice. D: β-Catenin mRNA levels in the colonic epithelium of BALB/c mice treated with pioglitazone. Total RNA was isolated from the colonic epithelium of five mice per group, and the levels of β-catenin mRNA were determined by northern blot analysis. Levels of 18S were determined as an internal control. The results of Northern blot analysis indicated no differences in the β-catenin mRNA levels between the pioglitazone-treated mice and control mice.

the pioglitazone-treated mice as compared to that detected in the colonic epithelium of the vehicle-treated control mice (Fig. 1C).

To clarify whether the PPARγ-mediated increase in β-catenin protein levels was due to a transcriptional-related mechanism, we performed northern blot analysis on RNA from the colonic epithelium of the pioglitazone-treated and control mice. The results of northern blot analysis showed no differences in the β-catenin mRNA levels in the colonic epithelium from the pioglitazone-treated and control mice (Fig. 1D). These results indicate that the PPARγ-mediated increase in β-catenin protein levels is not due to transcriptional regulation, but rather due to a post-transcriptional event.

Activation of PPARγ by ligand treatment suppresses β-catenin/TCF–mediated transcription in colonic carcinogenesis

It is well known that β-catenin which accumulates in the cytosol is translocated into the nucleus whereupon it binds with members of the T cell factor (TCF) / lymphoid enhancing factor (LEF) family and induces target gene transcription, such as c-myc and cyclin D1. To determine whether ligation of PPARγ regulates β-catenin /TCF activity, Lovo, a colonic cancer cell line, was transfected with a TCF reporter vector, TOPflash, which contains a combination of TCF-binding elements. Incubation with the PPARγ ligand PGJ_2 significantly suppressed the transcriptional activity of TOPflash (Fig. 2A). In contrast, knockdown of PPARγ protein expression by treatment of Lovo cells with siRNA

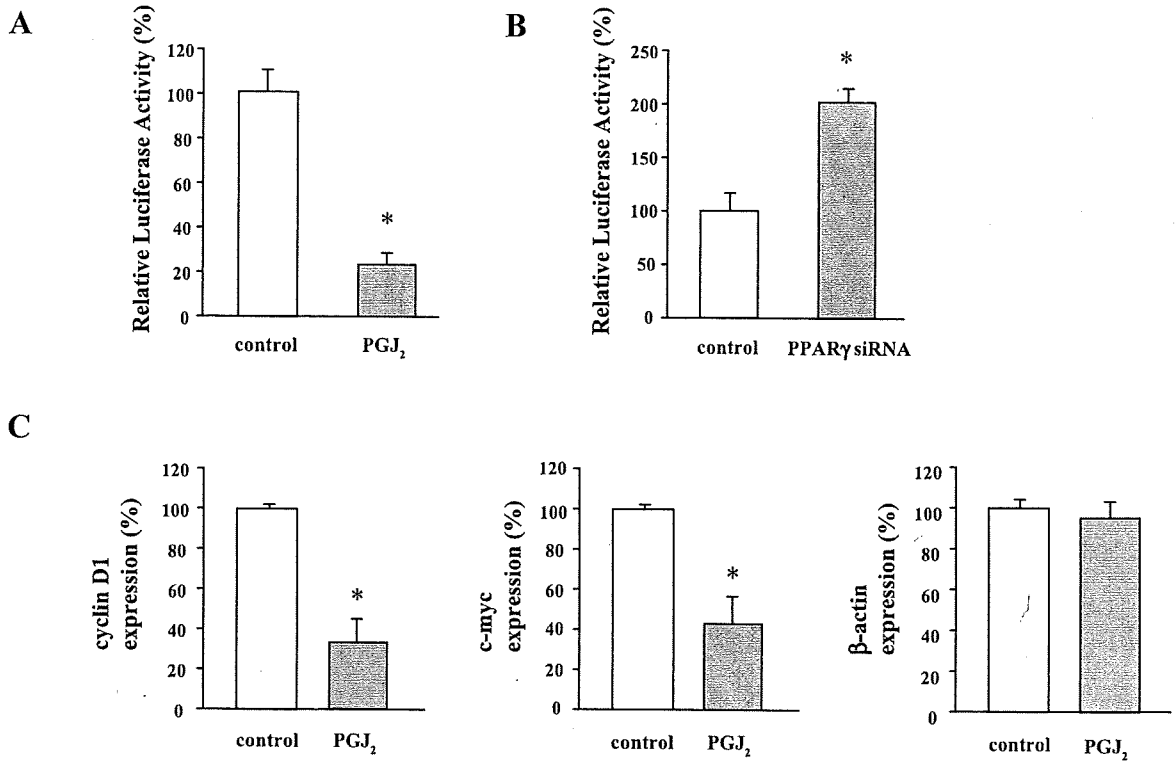

Fig. 2. PPARγ ligand suppresses the β-catenin/TCF–mediated transcription activity in colonic carcinogenesis. A: Ligand activation of PPARγ reduces β-catenin/TCF activity. Lovo cells were transiently transfected with the TCF-responsive promoter TOPflash or FOP flash. After incubation with 10 μM PGJ_2 for 8 h, dual-luciferase assays were performed. B: siRNA inhibition of PPARγ induces β-catenin/TCF activity. Lovo cells were treated with 12 nM PPARγ siRNA for 24 h before performing the reporter assay. Each experiment was repeated at least three times. Data represent the TOPflash/FOP flash relative luciferase activity, and each reported value is the mean ± S.D. from three independent experiments. C: Ligand activation of PPARγ suppresses expression of the β-catenin/TCF target genes c-myc and cyclin D1. Lovo cells were treated with 10 μM PGJ_2 for 5 h. The levels of c-myc and cyclin D1 mRNA were determined by RT-PCR analysis. The level of β-actin as an internal control was also determined. *$P<0.05$, as compared with the result in the control mice.

promoted TOPflash transcriptional activity (Fig. 2B). These results indicate that PPARγ negatively regulates the transcriptional activity of β-catenin/TCF. We then performed RT-PCR to determine whether the PPARγ ligand PGJ_2 regulates expression of β-catenin and TCF target genes. The PPARγ ligand significantly inhibited the expression of c-myc and cyclin D1 (Fig. 2C). Taken together, these results indicate that activation of PPARγ significantly suppresses β-catenin/TCF activity in vitro despite increased levels of β-catenin.

PPARγ directly interacts with β-catenin

To elucidate the mechanism by which activation of PPARγ suppresses the activation of β-catenin and TCF, we investigated the interaction between PPARγ and β-catenin both in vivo and in vitro. Immunoprecipitation of cell extracts from various colonic cancer cell lines with a PPARγ-specific antibody revealed a direct interaction between PPARγ and β-catenin which was increased by treatment with the PPARγ ligand pioglitazone (Fig. 3: A and B). Similar results were observed in the colonic epithelium of mice treated with the PPARγ ligand in vivo (Fig. 3C). In addition, a GST pull-down assay confirmed the direct interaction between PPARγ and β-catenin (Fig. 3D). These data indicate that activation of PPARγ causes it to interact with β-catenin which, in turn, is associated with inhibition of β-catenin translocation into the nucleus. This inhibition of β-catenin translocation into the nucleus is further associated with suppression of the ability of β-catenin and TCF to regulate its normal transcriptional program of activity.

To confirm the direct interaction of PPARγ with β-catenin in vivo, we investigated the localization of PPARγ and β-catenin in the colonic epithelium of mice. As shown in Fig. 4 (A and B), β-catenin was mostly localized to the cell membrane with PPARγ localizing to the nucleus in the crypt of the colon. However, in the surface epithelium, PPARγ and β-catenin co-localized

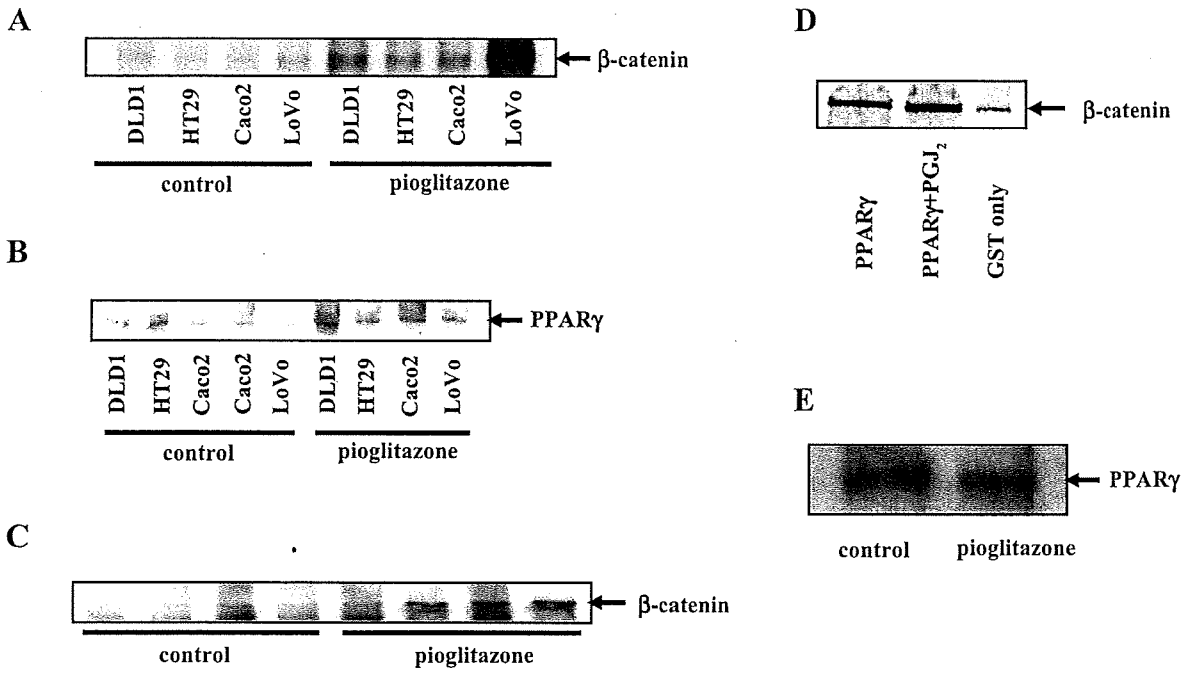

Fig. 3. PPARγ directly associates with β-catenin both in vivo and in vitro. A: Soluble extracts from several colon cancer cells treated/not treated with pioglitazone were immunoprecipitated with anti-PPARγ antibody, as indicated. The presence of β-catenin in the immunoprecipitates was determined by Western blotting with anti–β-catenin antibody. B: Soluble extracts from several colon cancer cells treated/not treated with pioglitazone were immunoprecipitated with anti–β-catenin antibody, as indicated. The presence of PPARγ in the immunoprecipitates was determined by Western blotting with anti-PPARγ antibody. C: Soluble extracts of colonic epithelial cells of BALB/c mice treated/not treated with pioglitazone were immunoprecipitated with anti-PPARγ antibody, as indicated. The presence of β-catenin proteins in the immunoprecipitates was determined by Western blotting with anti–β-catenin antibody. D: β-Catenin–containing plasmids were transcript and translated in vitro in the presence of ^{35}S, and radiolabel proteins were incubated with either GST alone or GST-PPARγ fusion protein. The bindings were assayed in the presence or absence of the specific ligand PGJ_2. The protein complexes formed were pulled down with glutathione-Sepharose beads and resolved by SDS-PAGE, followed by autoradiography. E: Confirmation of PPARγ protein levels treated with or without pioglitazone in Lovo cells. No marked difference was observed.

with each other in the cytosol (Fig. 4C). These data indicate that PPARγ directly interacts with β-catenin, retaining the latter in the cytosol and preventing its transcriptional activity in the nucleus. Taken together, activation of PPARγ by its ligand upregulates the direct interaction between PPARγ and β-catenin in the colonic epithelial cells, suppressing β-catenin translocation into the nucleus. Furthermore, our data suggest that natural PPARγ ligands are more highly expressed in surface epithelium at steady-state.

PPARγ ligand suppresses colonic epithelial cell proliferation

To clarify the mechanism underlying the inhibitory effect of PPARγ on β-catenin transcriptional activity, we investigated the effect of a PPARγ ligand on colonic epithelial cell proliferation in vivo. To do so, we used both BrdU and PCNA labeling for the quantitation of cell proliferation. BrdU is a modified pyrimidine analogue that is readily incorporated into nuclei during the DNA synthetic phase of the cell cycle (S-phase). PCNA is a nuclear antigen whose expression increases during the G_1-phase, peaks at the S-phase, and declines during the G_2/M-phases of the cell cycle. The BrdU-labeled tissue sections and the number of BrdU-labeled cells are shown in Fig. 5 (A – D). The number of BrdU-labeled cells per crypt in the distal colon of pioglitazone-treated mice was significantly reduced as compared with that observed in the colonic epithelium of the control, untreated mice (Fig. 5D). BrdU-stained cells in the colonic epithelium of the pioglitazone- or rosiglitazone-treated mice were decreased and mainly detectable near the crypt base. In contrast, in control mice, BrdU-positive cells extended up from the base towards the surface epithelium. Furthermore, treatment of mice with pioglitazone and rosiglitazone resulted in significantly shorter lengths of the crypts in comparison to control mice (Fig. 5: A – C).

Similarly, the PCNA-labeling index in the colonic epithelium of mice treated with pioglitazone and

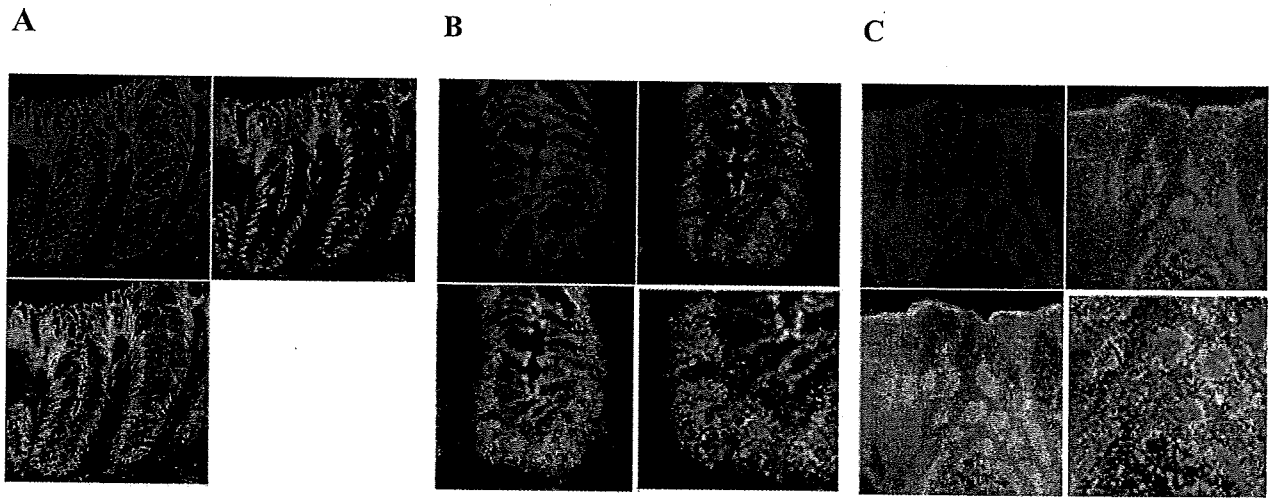

Fig. 4. Immunofluorescence microscopy of PPARγ and β-catenin in the colonic epithelium of BALB/c mice. Immunostaining for PPARγ (Alexa Fluor 488, green) and β-catenin (Alexa Fluor 555, red). A: An entire crypt (magnification = 40). B: The bottom of a crypt (magnification = 62). C: The top of a crypt (magnification = 62). Right bottom corner in (B) and (C) represents a high magnification picture.

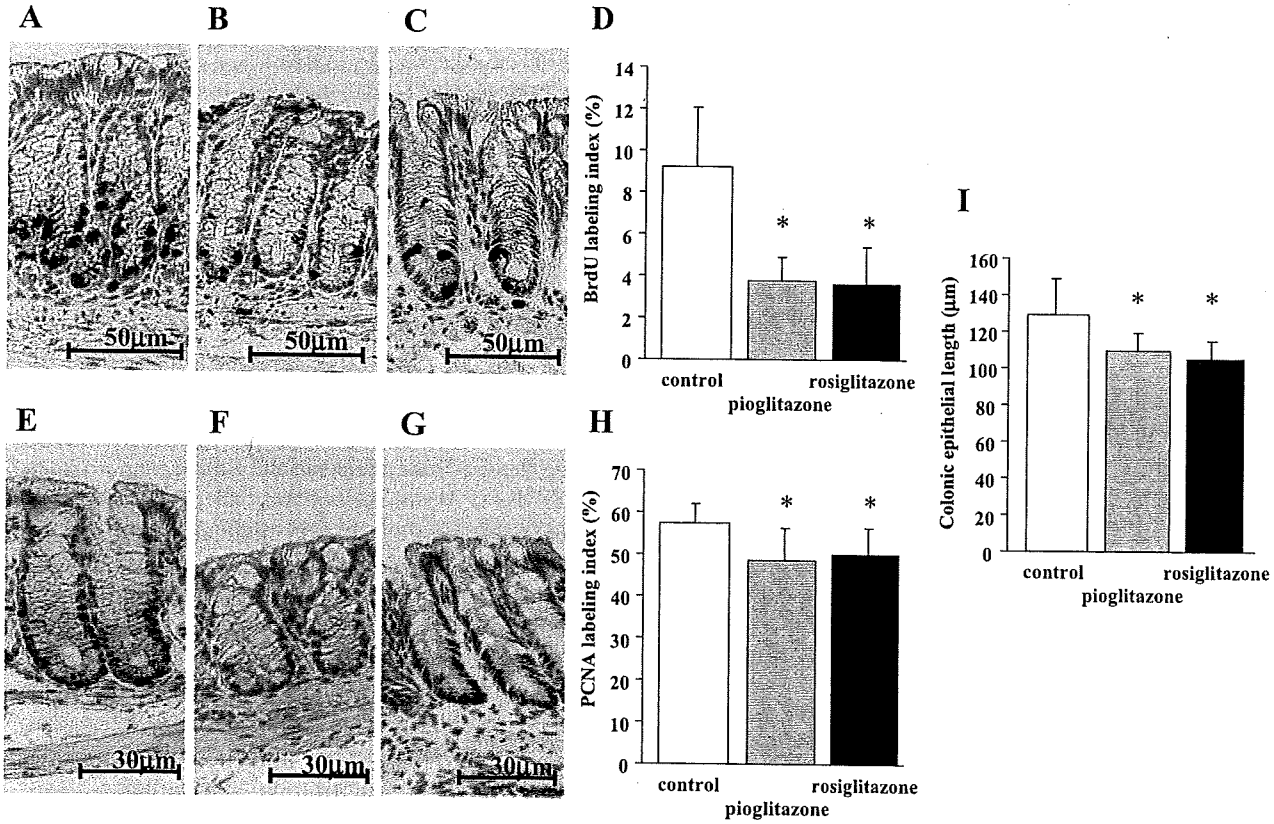

Fig. 5. PPARγ ligand suppresses colonic epithelial cell proliferation. Immunohistochemical staining of BrdU in the colonic epithelium of control mice (A), pioglitazone-treated mice (B), and rosiglitazone-treated mice (C). D: BrdU-labeling index in pioglitazone-treated, rosiglitazone-treated, and control mice. Immunohistochemical staining of PCNA in the colonic epithelium of control mice (E), pioglitazone-treated mice (F), and rosiglitazone-treated mice (G). H: PCNA-labeling index in pioglitazone-treated, rosiglitazone-treated, and control mice. I: Comparison of the colonic epithelial length in pioglitazone-treated, rosiglitazone-treated, and control mice. The colonic epithelial length in the TZD-treated mice was significantly shorter than that in the control mice. *$P<0.05$ as compared with the results in control mice.

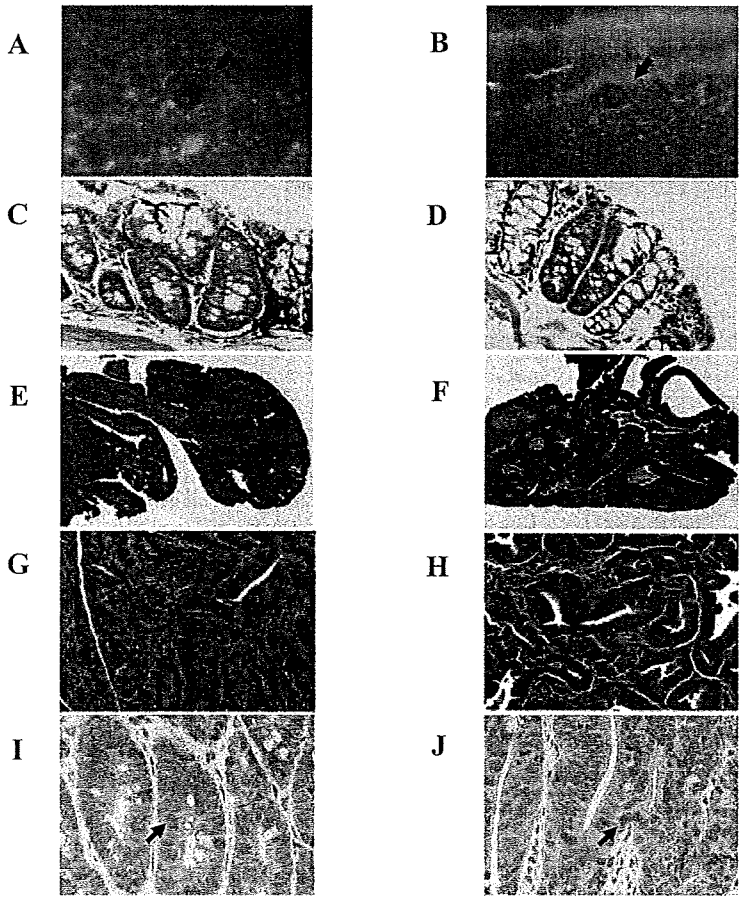

Fig. 6. Histology of ACF and colonic tumors arising in the mice after AOM treatment. ACF arising in the colons of PPARγ (+/–) (A, C) and PPARγ (+/+) (B, D) mice. A and B: 0.2% metylene blue staining: ACF is indicated by the solid arrow; C and D: H & E staining (magnification = ×20). Colonic tumors arising in the colons of PPARγ (+/–) (E, G) and PPARγ (+/+) (F, H) mice. E and F: H & E staining (magnification = ×10). G and H: H & E staining (magnification = ×40). Immunohistochemical staining of β-catenin in the adenomas of PPARγ (+/–) (I) and PPARγ (+/+) (J) mice. β-Catenin was detectable in the cytosol and nuclei (arrows) in the adenomas of both groups of animals

rosiglitazone was also significantly reduced in comparison to that observed in the control mice (Fig. 5: E – H). Importantly, the apoptotic index of the colonic epithelium as determined by TUNEL staining was unchanged in the pioglitazone-treated and control mice (data not shown). These results indicate that PPARγ ligands suppress colonic epithelial cell proliferation and turnover.

Heterozygous PPARγ-deficiency promotes ACF formation in AOM-induced colon carcinogenesis

To investigate the effect of PPARγ-mediated inhibition of β-catenin transcriptional activity in colon carcinogenesis in vivo, we examined the formation of ACF in the AOM-induced colon cancer model in PPARγ (+/–) mice (Fig. 6: A and B). AOM treatment of PPARγ (+/–) mice resulted in a significant increase in the formation of ACF in the colon in comparison to that observed in the colons of wild-type mice similarly treated with AOM (Table 1). However, PPARγ heterodeficiency did not result in an increased incidence or multiplicity of adenocarcinomas (Table 1). Histological analysis of the tumors from both genotypes of mice identified them as either adenomas or adenocarcinomas. Moreover, there were no significant differences in the histologic characteristics of the ACF, adenomas, or adenocarcinomas observed in PPARγ (+/–) mice and PPARγ (+/+) mice (Fig. 6: C – H). As expected, β-catenin was detectable in the cytosol and nuclei in the adenomas of both groups of animals (Fig. 6: I and J). Figure 7 shows that the survival of PPARγ (+/–) mice and PPARγ (+/+) mice was identical and observed to be 85% at 37 weeks.

Discussion

The development of colon cancer is the consequence of a multi-step process (30). This genetically driven program causes cellular hyper-proliferation of normal colonic epithelium, resulting in formation of adenomas and, consequently, carcinomas (31 – 34). In addition, this process is enhanced by chronic inflammation (35). Since PPARγ is regulated by and, in turn, regulated cellular proliferation and inflammation (36, 37), it is of significant importance to the pathogenesis of colon cancer. However, the physiological role of this receptor in the colonic epithelium remains incompletely charac-

Table 1. Effect of PPARγ heterodeficiency on the formation of (a) ACF and (b) colonic tumors in the AOM-induced colon cancer model

a

Genotype	AOM	n	No. of ACF/mouse	No. of ACs/mouse
PPARγ (+/+)	+	14	11.71 ± 5.44	18.21 ± 8.62
PPARγ (+/–)	+	14	16.21 ± 4.28*	26.00 ± 6.00*
PPARγ (+/+)	–	14	0	0
PPARγ (+/–)	–	14	0	0

b

Experimental group	Incidence (%)	Histology			Multiplicity
		Ad	AdCa	total	
PPARγ (+/+)	10/19 (53)	7	17	24	1.26 ± 1.81
PPARγ (+/–)	11/17 (65)¶	6	13	19	1.12 ± 1.10¶

*$P<0.05$, as compared with the results in the PPARγ (+/+) mice treated with AOM. ¶No difference in the number of colon tumors arising in the colons of PPARγ (+/+) and PPARγ (+/–). ACs, aberrant crypts; Ad, adenoma; AdCa, adenocarcinoma.

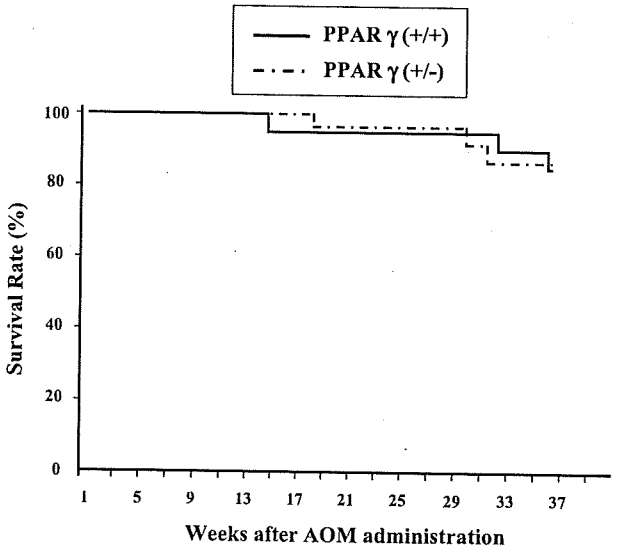

Fig. 7. Survival of PPARγ (+/–) and PPARγ (+/+) mice after AOM treatment. PPARγ (+/–) and PPARγ (+/+) mice were treated with 10 mg/kg of AOM and observed until 37 weeks after AOM administration. There was no significant difference in the survival rates between the two groups.

terized. In addition, it is still unknown how PPARγ affects the cell turnover in colon.

In the present study, we observed that PPARγ ligand markedly suppressed colonic epithelial cell proliferation, independently of apoptosis, resulting in decreased colonic crypt length. These results may indicate that activation of PPARγ induces a cellular program that decreases epithelial cell proliferation. Namely, PPARγ negatively regulates colonic epithelial cell turnover by affecting cell cycle kinetics rather than inducing apoptosis under physiological conditions in vivo. In fact, PPARγ is expressed abundantly in the colonic epithelium, and it is suggested that PPARγ is a key molecule in the regulation of colonic epithelial cell turnover.

To clarify how PPARγ affects the epithelial cell turnover, we investigated the interaction between PPARγ and β-catenin because β-catenin is a key molecule in cell proliferation and ACF formation in the colon (25). We showed that PPARγ activation leads to increased β-catenin protein levels in the colonic epithelium. However, this PPARγ mediated increase in β-catenin protein expression in the colonic epithelium was not associated with the nuclear translocation of β-catenin. Furthermore, it was observed that PPARγ directly interacted with β-catenin in both colonic cancer cell lines and native colonic epithelial cells in vivo and that this interaction was enhanced by treatment with PPARγ ligands, suggesting that binding of β-catenin by PPARγ may prevent its degradation. Moreover, the increase in the direct binding of PPARγ to β-catenin observed here was associated with the decreased β-catenin translocation into the nucleus, despite increased protein levels, and inhibition of β-catenin and TCF dependent transcription.

It has been reported that ligation and activation of PPARγ in HT-29 and Lovo colon cancer cells significantly suppresses cellular proliferation (38). Consistent with this, we observed that PPARγ activation suppressed β-catenin/TCF transcriptional activity. These results indicate that PPARγ negatively regulates epithelial cell turnover via inhibition of β-catenin movement into the nucleus, resulting in the inhibition of β-catenin/TCF–

dependent transcription.

In the present study, we observed that heterodeficiency of PPARγ promoted the formation of ACF, a precancerous pathologic marker in the very early stage of carcinogenesis. These results indicate that PPARγ suppresses tumorigenesis at early stages of initiation. ACF are the formative lesions that lead to adenoma and, consequently, carcinoma. However, PPARγ heterodeficiency did not result in an increased incidence of tumors. These results may indicate that strong activation with non-endogenous ligands is required to suppress tumor progression, although activation of PPARγ by endogenous ligands is sufficient to suppress colonic carcinogenesis at early stages of colon cancer development such as ACF formation (15).

In conclusion, PPARγ regulates colonic epithelial cell turnover via direct interactions with β-catenin, resulting in inhibition of β-catenin translocation into the nucleus. The inhibition of β-catenin translocation prevents β-catenin-mediated transcriptional pathways that are involved in promoting cell proliferation. Our findings indicate that PPARγ plays a role as a physiological regulator of colonic epithelial cell turnover and consequently predisposition to the development of colon cancer.

Acknowledgments

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