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**Quantitative real-time detection PCR**

Total RNA was isolated from frozen liver tissue samples using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's protocol. cDNA was synthesized from 100 ng total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) then mixed with the TaqMan Universal Master Mix (Applied Biosystems) and each TaqMan probe. TaqMan probes used were PDGFR-α/β, VEGFR1/2, α-SMA, collagen 1/4, β-catenin, CyclinD1, and Myc (Applied Biosystems). Relative expression levels were calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Western blotting**

Western blotting was conducted as described previously (20). Whole-cell lysates from mouse liver were prepared and lysed by CelLytic MT cell lysis reagent (Sigma-Aldrich) containing Complete Mini EDTA-free Protease Inhibitor cocktail tablets (Roche). Cytoplasmic and nuclear protein extracts were prepared using the NE-PER nuclear extraction reagent kit (Pierce Biotechnology). Primary antibodies used were PDGFR-α (1:1,000 dilution), PDGFR-β (1:1,000 dilution), VEGFR2 (1:1,000 dilution), p44/42 MAPK (1:1,000 dilution), total AKT (1:1,000 dilution), p-p44/42 MAPK (1:1,000 dilution), p-AKT (Ser473; 1:1,000 dilution), p-AKT (Thr308; 1:1,000 dilution), β-catenin (1:2,000 dilution), cyclin D1 (1:400 dilution), and lamin A/C (1:1,000 dilution; all Cell Signaling Technology); α-SMA (1:200 dilution; DAKO); 4-HNE (1:200 dilution; NOF); and GAPDH (1:1,000 dilution) and Myc (1:1,000 dilution; both Santa Cruz).

**Statistical analysis**

Results are expressed as mean ± SD. Significance was tested by 1-way analysis of variance with Bonferroni's method, and differences were considered statistically significant at $P < 0.05$.

## Results

**Peretinoin prevented the development of hepatic fibrosis in *Pdgf-c Tg***

To evaluate the HCC chemopreventive effects of peretinoin, we used a mouse model of *Pdgf-c Tg* in which PDGF-C is expressed under the control of the albumin promoter (7). Experimental mice were male mice expressing the PDGF-C transgene (*Pdgf-c Tg*); whereas male mice not expressing the transgene were considered WT. After weaning at week 4, *Pdgf-c Tg* or nontransgenic WT mice were fed a basal diet or a diet containing 0.03% or 0.06% peretinoin. At week 20, mice were sacrificed to analyze the progression of hepatic fibrosis. At week 48, mice were sacrificed to analyze the development of hepatic tumors (Fig. 1A). At week 20, Azan staining showed that predominant pericellular fibrosis had developed in *Pdgf-c Tg* mice (Fig. 1B). Densitometric analysis showed a significant dose-dependent reduction in the size of the fibrotic area in mice that received a diet containing peretinoin at both weeks 20 and 48 (Fig. 1C). Peretinoin therefore efficiently repressed the development of hepatic fibrosis in *Pdgf-c Tg* mice.

The expression of fibrosis-related genes in *Pdgf-c Tg* mice was evaluated by IHC staining, quantitative real-time detection PCR (RTD-PCR), and Western blotting. The expression of PDGFR-α and PDGFR-β, essential receptors for intracellular PDGF-C signaling, was upregulated mainly in the intracellular or portal area in *Pdgf-c Tg* mice livers (Fig. 2), but was significantly repressed by peretinoin after weaning at week 4. Similarly, the expression of collagen 1, collagen 4, and desmin was significantly upregulated in *Pdgf-c Tg* mice, but repressed by peretinoin (Fig. 2 and Supplementary Fig. S1A).

RTD-PCR results confirmed that these genes were substantially upregulated in *Pdgf-c Tg* mice and significantly repressed by both 0.03% and 0.06% peretinoin (Fig. 3A). Western blotting showed that the expression of phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2 and cyclin D1, representative markers of the cell proliferation signaling pathway, was upregulated in *Pdgf-c Tg* mice, and repressed by peretinoin (Fig. 3B). Thus, peretinoin could partially but significantly prevent the development of hepatic fibrosis in *Pdgf-c Tg* mice during the study observation period of 48 weeks.

**Peretinoin prevented the development of HCC in *Pdgf-c Tg* mice**

At week 48, *Pdgf-c Tg* mice developed hepatic tumors with an incidence of 90% (Fig. 4A). Histologic assessment of these tumors verified that 54% (15/28) were adenomas and 46% (13/28) were HCC (Fig. 4A and C and Supplementary Fig. S2; ref. 21). Peretinoin (0.03%) dose-dependently repressed the incidence of hepatic tumors to 53% (19/36) and to 29% (5/17) at 0.06%. Correlating with tumor incidence, maximum tumor size and liver weight were also significantly repressed by peretinoin (Fig. 4B). Thus, peretinoin repressed the development of hepatic tumors in *Pdgf-c Tg* mice.

**Serial gene expression profiling in the liver of *Pdgf-c Tg* mice that developed hepatic fibrosis and tumors**

To examine which signaling pathways were altered during the progression of hepatic fibrosis and tumor development, we analyzed gene expression profiling in the liver of *Pdgf-c Tg* mice using Affymetrix gene chips. By filtering criteria for $P < 0.001$ and more than 2-fold differences, 538 genes were selected as differentially expressed. One-way hierarchical clustering analysis of differentially expressed genes is shown in Supplementary Fig. S3.

Of the 3 main clusters, 2 were upregulated (clusters A and B) and 1 was downregulated (cluster C). Cluster A consisted of immune-related [chemokine (C-C motif) receptor (CCR)4, CCR2, toll-like receptor (TLR)3 and TLR4], apoptosis-related [caspase (CASP)1 and CASP9], angiogenesis- and/or growth factor–related (PDGF-C, VEGF-C, osteopontin, HGF), oncogene-related [v-ets erythroblastosis virus E26 oncogene homologue (Ets)1, Ets2, CD44, N-myc downstream-regulated (NDRG)1], and fibrosis-related (tubulin) genes. The expression of cluster A genes was further upregulated in tumors at week

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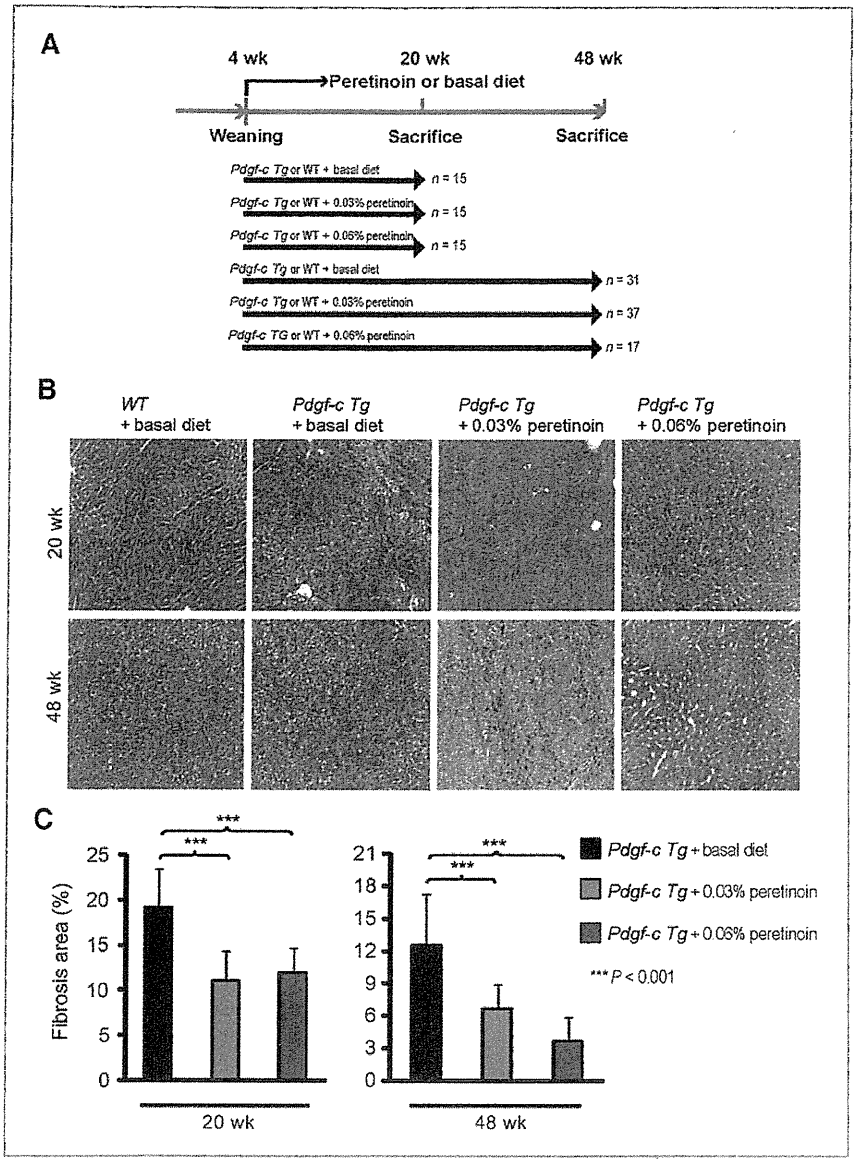


Figure 1. A, feeding schedule of *Pdgf-c Tg* and WT mice. After weaning, male mice were randomly divided into 3 groups: (i) *Pdgf-c Tg* or WT mice receiving basal diet, (ii) *Pdgf-c Tg* or WT mice receiving 0.03% peretinoin-containing diet, and (iii) *Pdgf-c Tg* or WT mice receiving 0.06% peretinoin-containing diet. B, Azan staining of WT or *Pdgf-c Tg* mouse livers fed with different diets at 20 weeks and 48 weeks. C, densitometric analysis of *Pdgf-c Tg* mouse liver fibrotic areas at 20 weeks ($n = 15$) and 48 weeks ($n = 15$).

48. Cluster B consisted mainly of connective tissue- and/or fibrosis-related [vascular cell adhesion molecule (VCAM)1, collagen I, III, IV, V, VI, integrin, decorin, TGF-βRII, PDGFR-α, and PDGFR-β] genes, the expression of which declined slightly at week 48. In contrast, cluster C, containing differentiation and liver function related genes [cytochrome P450, family 2, subfamily c (CYP2C)], were downregulated during the course of hepatic fibrosis and tumor development (Supplementary Fig. S4). Cluster C included xenobiotic- and metabolic process–related genes, which are potential targets of peretinoin. Peretinoin treatment prevented hepatic fibrosis and it preserved liver function. In addition, peretinoin might induce its target genes. Thus, peretinoin reduced the expression of upregulated genes (clusters A and B) and restored the expression of downregulated genes (cluster C) at both weeks 20 and 48 (Supplementary Figs. S3 and S4).

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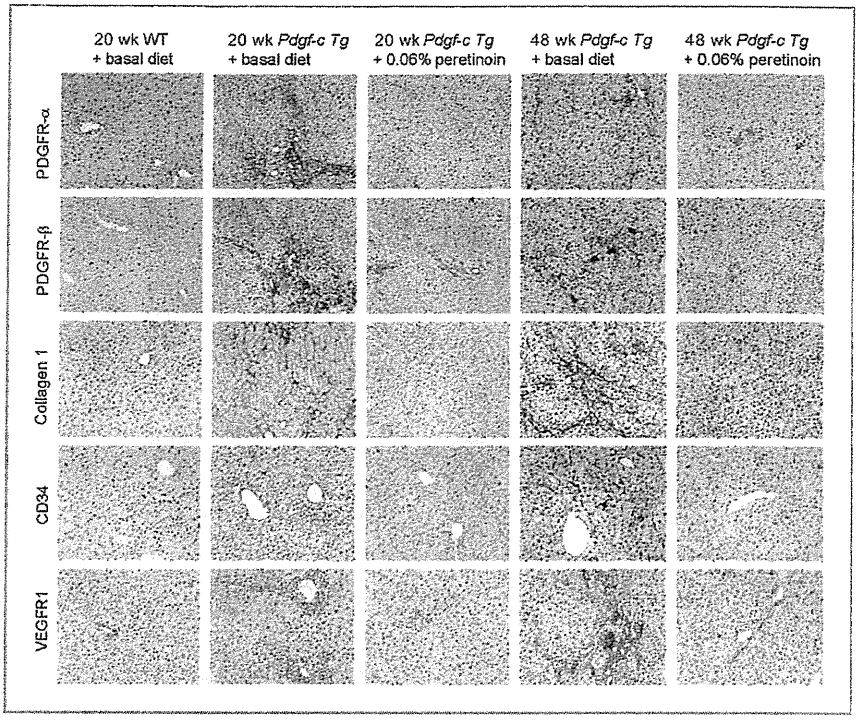


Figure 2. IHC staining of PDGFR-α, PDGFR-β, collagen 1, CD34, and VEGFR1 expression in *Pdgf-c Tg* or WT mouse livers fed a basal diet or 0.06% peretinoin.

To examine the molecular network consisting of differentially expressed genes in *Pdgf-c Tg* mice with or without peretinoin administration, the direct interactions of 513 genes were analyzed by MetaCore (i.e., 413 genes were downregulated and 100 genes were upregulated in *Pdgf-c Tg* mice treated with peretinoin compared with untreated mice; $P < 0.002$). A core gene network consisting of 41 genes was obtained (Supplementary Fig. S5) including interactions between representative growth factors, receptors (PDGFR and TGFβR), and transcriptional factors. Of these genes, the transcriptional factors Sp1 and Ap1 seem to be key regulators in the network (Supplementary Fig. S5).

**Peretinoin inhibits PDGFR *in vitro***

Gene expression profiling landscaped the dynamic changes of signaling pathways in *Pdgf-c Tg* mice. To determine the effects of peretinoin *in vitro*, primary HSCs from normal C57BL/6J mice were stimulated by PDGF-C (Fig. 5) to induce the expression of PDGFR-α, PDGFR-β, alpha smooth muscle actin (α-SMA), and collagen 1a2; activated HSCs thus transformed into myofibroblasts (Fig. 5A and B). Peretinoin significantly reduced the expression of these genes and inhibited HSC activation.

We next evaluated the effects of peretinoin on human hepatoma cell lines (Huh-7, HepG2, and HLE), mouse embryonic fibroblast cells (NIH3T3), HUVECs, and Lx-2 (ref. 22; Supplementary Fig. S6A). Experimental conditions were optimized so that more than 90% of cells were variable at 20 μmol/L peretinoin, as determined by an MTS cell proliferation assay (data not shown). Peretinoin dose-dependently inhibited the expression of PDGFR-α and PDGFR-β in Huh-7, HepG2, HLE, NIH3T3, HUVEC, and Lx-2 cells, whereas no obvious expression of PDGFR-α was observed in HepG2 cells and HUVECs (Supplementary Fig. S6A). Peretinoin also inhibited VEGFR2 expression in HUVEC. These results were confirmed by RTD-PCR (data not shown). Correlating with these results, the expression of phosphorylated serine/threonine kinase AKT (p-AKT) and p-ERK1/2, downstream signaling molecules of PDGFR-α, PDGFR-β, and VEGFR2, was also dose-dependently repressed. The expression of collagen 1a2 was significantly repressed by peretinoin in Lx-2, HLE, and Huh-7 cells (Supplementary Fig. S6B). These results suggest that peretinoin may inhibit hepatic fibrosis, angiogenesis, and tumor growth through reduction of the PDGF and VEGF signaling pathway.

We examined the expression of 2 key regulators in peretinoin signaling, Sp1 and Ap1, in Huh-7 cells. Interestingly, the expression of Sp1 was decreased, which correlates with that of PDGFR-α, whereas expression of phosphorylated c-Jun (p-c-Jun) was increased in Huh-7 cells (Supplementary Fig. S6C). Therefore, peretinoin seems to repress the expression of PDGFR, partially through the inhibition of Sp1.

**Peretinoin inhibits hepatic angiogenesis in *Pdgf-c Tg* mice**

The effect of peretinoin on liver angiogenesis in *Pdgf-c Tg* mice was further analyzed. IHC staining of *Pdgf-c Tg* mouse

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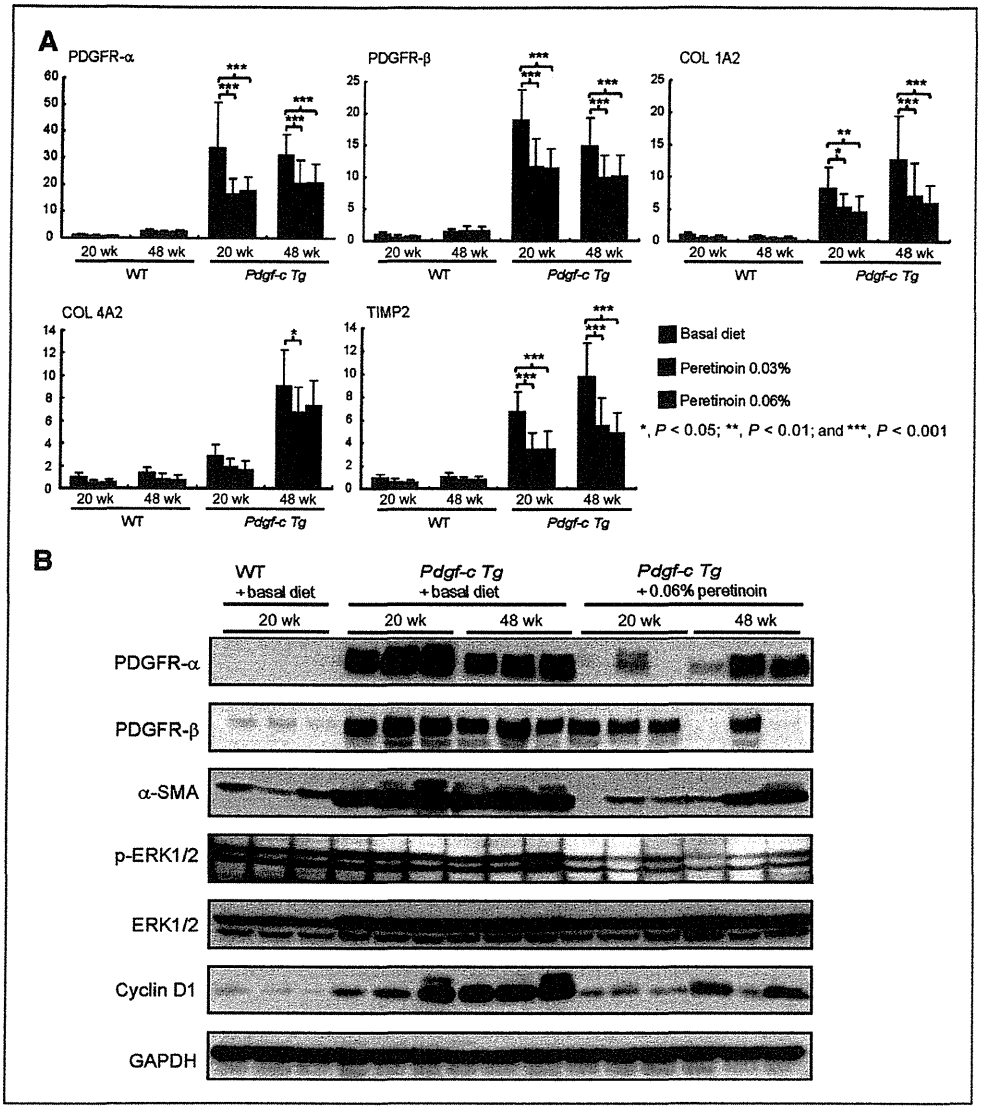


Figure 3. A, RTD-PCR analysis of PDGFR-α, PDGFR-β, collagen (COL) 1a2, collagen 4 a2, and TIMP2 expression in *Pdgf-c Tg* ($n = 5$) or WT mouse livers ($n = 15$). B, Western blotting of PDGFR-α, PDGFR-β, α-SMA, p-ERK, ERK, cyclin D1, and GAPDH expression in PDGF-C Tg or WT mouse livers fed a basal diet or 0.06% peretinoin at 20 or 48 weeks ($n = 3$).

livers at weeks 20 and 48 revealed overexpression of the endothelial markers CD31 and CD34 and the endothelial growth factors VEGFR1 and endothelium-specific receptor tyrosine kinase 2 (Tie2) in the mesenchymal region (Fig. 6 and Supplementary Fig. S1A). This expression was significantly repressed by peretinoin as determined by the densitometric area (Supplemental Fig. S1B). RTD-PCR results revealed significant upregulation of VEGFR1 (Flt-1) in *Pdgf-c Tg* mice compared with WT mice at both weeks 20 and 48, whereas the expression of VEGFR2 (Flk-1) and Tie2 was only upregulated at week 48. The expression of these genes was significantly repressed by peretinoin (Fig. 6A). Western blotting confirmed the upregulation of CD31 and VEGFR1 (Flk-1) at week 48 (Fig. 6B). In addition, p-AKT (Thr 308 and Ser 473) and 4-hydroxy-2-nonenal (4-HNE), an oxidative stress marker, were upregulated in *Pdgf-c Tg* mice and repressed by peretinoin (Fig. 6B).

We also assessed circulating endothelial cells (CEC), a useful biomarker for angiogenesis in the blood, and found that the $CD31^+/CD34^+$ CEC population was significantly upregulated in *Pdgf-c Tg* mice at week 48 but significantly repressed by peretinoin (Fig. 6C and D). Thus, peretinoin

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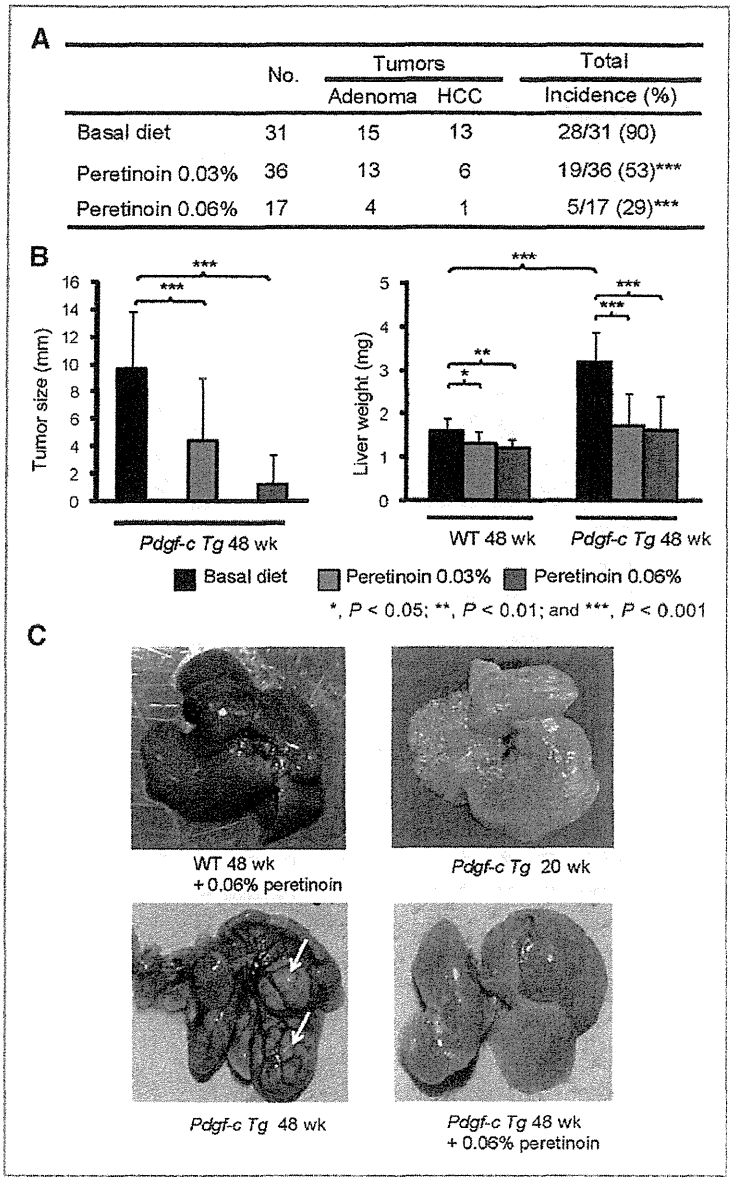


Figure 4. A, incidence of hepatic tumors (adenoma or HCC) in *Pdgf-c Tg* mouse livers fed with different diets. B, tumor sizes and liver weights of *Pdgf-c Tg* and WT mice fed with basal diet ($n = 31$ for *Pdgf-c* Tg, $n = 15$ for WT mice) or 0.03% ($n = 36$ for *Pdgf-c* Tg, $n = 15$ for WT mice) and 0.06% ($n = 17$ for *Pdgf-c* Tg, $n = 15$ for WT mice) peretinoin at 48 weeks. C, macroscopic findings of *Pdgf-c Tg* or WT mouse livers. No obvious change was observed in the liver of WT mice fed with 0.06% peretinoin for 48 weeks (top left). Fibrosis and steatosis were observed in the liver of *Pdgf-c Tg* mice fed a basal diet for 20 weeks (top right). Multiple tumors developed (white arrows) in the liver of *Pdgf-c Tg* mice fed a basal diet for 48 weeks (bottom left). Suppression of tumor development in the liver of *Pdgf-c Tg* mice fed a 0.06% peretinoin diet for 48 weeks (bottom right).

seems to inhibit angiogenesis in the liver of *Pdgf-c Tg* mice, which might prevent the development of hepatic tumors.

### Peretinoin inhibits canonical Wnt/β-catenin signaling in *Pdgf-c Tg* mice

The activation of the Wnt/β-catenin signaling pathway is seen in 17% to 40% of patients with primary HCC (23, 24). Moreover, recent reports suggested an interaction between PDGF signaling and Wnt/β-catenin signaling (25–27). We evaluated Wnt/β-catenin signaling in *Pdgf-c Tg* mice and showed by IHC staining that β-catenin was overexpressed in the submembrane at week 48 (Fig. 7A). Peretinoin significantly reduced this expression (Fig. 7A and B), and Western blotting revealed that accumulation of β-catenin in the nuclear fraction of liver tumor tissues was more preferentially repressed by peretinoin than in the cytoplasmic fraction, although expression was repressed in both fractions (Fig. 7C). Wnt ligand (Wnt5a) and frizzled receptor (Fzd1) expression was significantly upregulated in hepatic tumors compared with normal liver (Fig. 7D). These results together suggest that canonical Wnt/β-catenin

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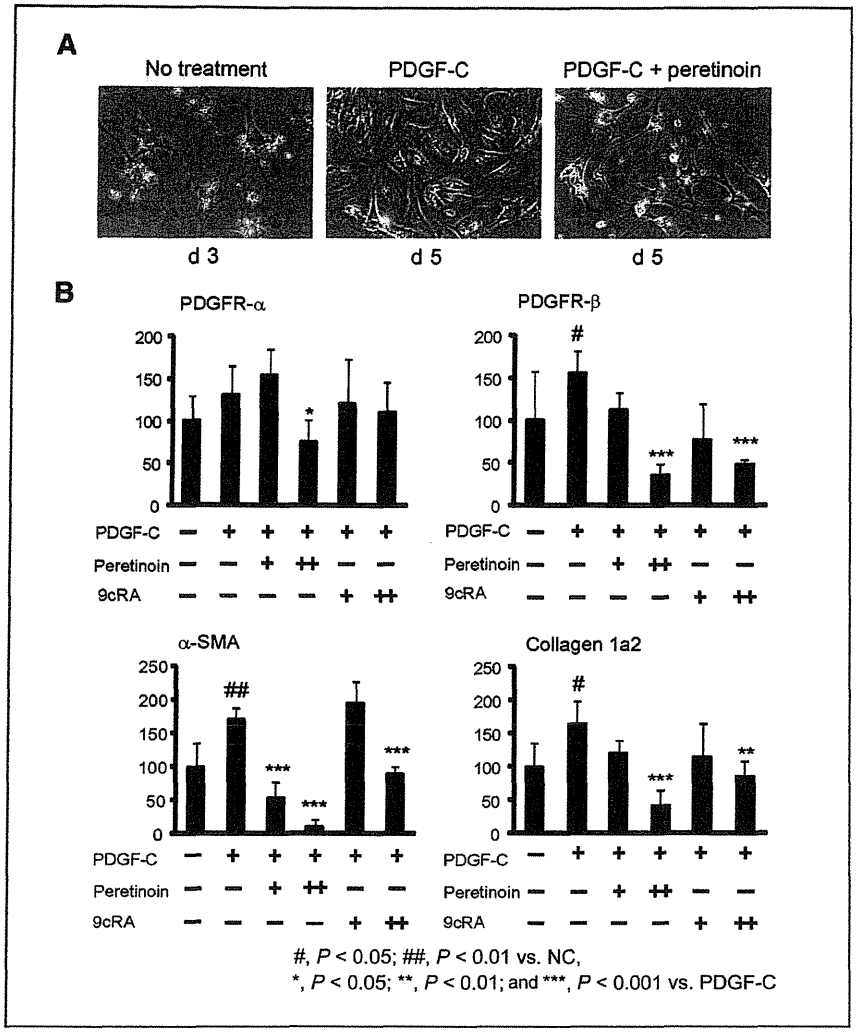


Figure 5. A, microscopic view of freshly isolated primary mouse HSCs after PDGF-C transformation into myofibroblasts (left). Peretinoin inhibited the transformation of HSCs by PDGF-C. B, RTD-PCR analysis of PDGFR-α, PDGFR-β, α-SMA, and collagen 1a2 expression in HSCs treated with or without PDGF-C, peretinoin, and 9cRA ($n = 4$). PDGF-C (+), 80 ng/mL; peretinoin (+), 5 μmol/L; (++), 10 μmol/L; 9cRA (+), 5 μmol/L; (++), 10 μmol/L. NC, no control.

signaling is activated in hepatic tumors and repressed by peretinoin.

Growth factors such as PDGF or HGF potentially activate Wnt/β-catenin signaling (26, 28), which promotes cancer progression and metastasis. We evaluated whether such growth factor signaling could be repressed by peretinoin in hepatic tumors. The expression of c-myc, β-catenin, Tie2, Fit-1, and Flk-1 were significantly upregulated from 1.5- to 4-fold in hepatic tumors compared with normal liver, and this expression was significantly repressed by peretinoin. Similarly, the expression of PDGFR-α, PDGFR-β, collagen 1a2, collagen 4a2, tissue inhibitor of metalloproteinase 2 (TIMP2), and cyclin D1 was substantially upregulated from 5- to 15-fold in hepatic tumors, and significantly repressed by peretinoin (Fig. 7D). Thus, growth factor signaling as well as canonical Wnt/β-catenin signaling in hepatic tumors seems to be repressed by peretinoin. These results explain the inhibitory effect of peretinoin in the development of HCC in *Pdgf-c Tg* mice.

## Discussion

HCC often develops in association with liver cirrhosis and its high recurrence rate leads to poor patient prognosis. Indeed, the 10-year recurrence-free survival rate after liver resection for HCC with curative intent was shown to be only 20% (29). Therefore, there is a pressing need to develop effective preventive therapy for HCC recurrence to improve its prognosis.

Peretinoin, a member of the acyclic retinoid family, is expected to be an effective chemopreventive drug for HCC (11, 12, 30) as shown by a previous phase II/III trial in which 600 mg peretinoin per day in the Child-Pugh A subgroup reduced the risk of HCC recurrence or death by 40% [HR = 0.60 (95% CI, 0.40–0.89); ref. 31]. However, further clinical

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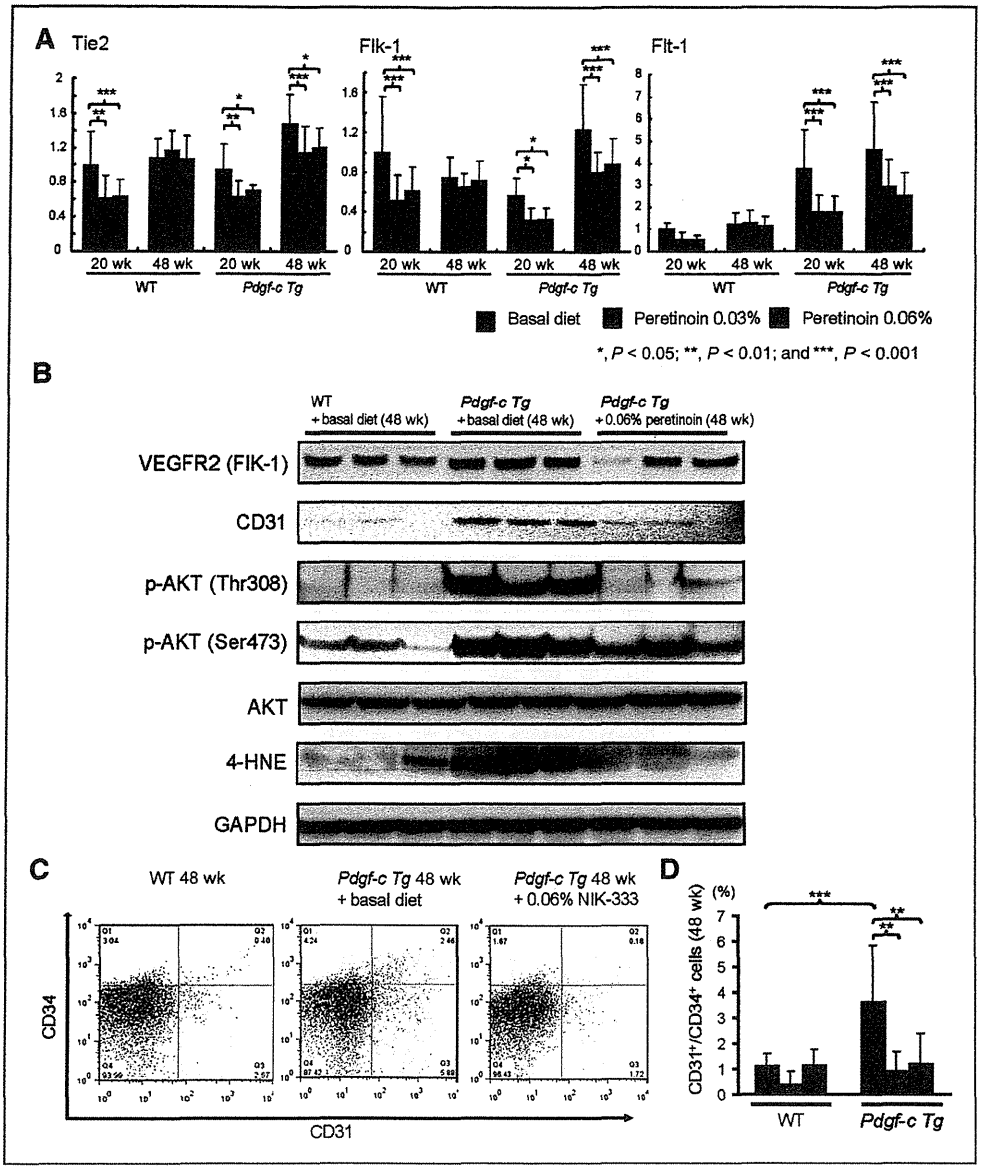


Figure 6. A, RTD-PCR analysis of Tie2, Flk-1, and Flt-1 expression in the liver of *Pdgf-c Tg* and WT mice fed with different diets ($n = 15$). B, Western blotting of Flk-1, CD31, p-AKT (Thr 308, Ser473), AKT, 4-HNE, and GAPDH expression in the liver of *Pdgf-c Tg* or WT mice fed a basal diet or 0.06% peretinoin at 48 weeks ($n = 3$). C, fluorescence-activated cell-sorting analysis of CD31- and CD34-positive CEC in blood of *Pdgf-c Tg* or WT mice fed a basal diet or 0.06% peretinoin at 48 weeks. D, frequency of CD31- and CD34-positive CEC in blood of *Pdgf-c Tg* or WT mice fed a basal diet or 0.06% peretinoin at 48 weeks ($n = 10$).

studies are needed to confirm the clinical efficacy of peretinoin, and a large scale study involving several countries is currently being planned.

During the course of chronic hepatitis, nonparenchymal cells including Kupffer, endothelial and activated stellate cells release a variety of cytokines and growth factors that might accelerate hepatocarcinogenesis. Although peretinoin has been shown to suppress the growth of HCC-derived cells by inducing apoptosis and differentiation (32–35), increasing p21 and reducing cyclin D1 (13), limited data have been published about its effects on hepatic mesenchymal cells such as stellate cells and endothelial cells (14).

In parallel with a phase II/III trial, we conducted a pharmacokinetics study of peretinoin focusing on 12

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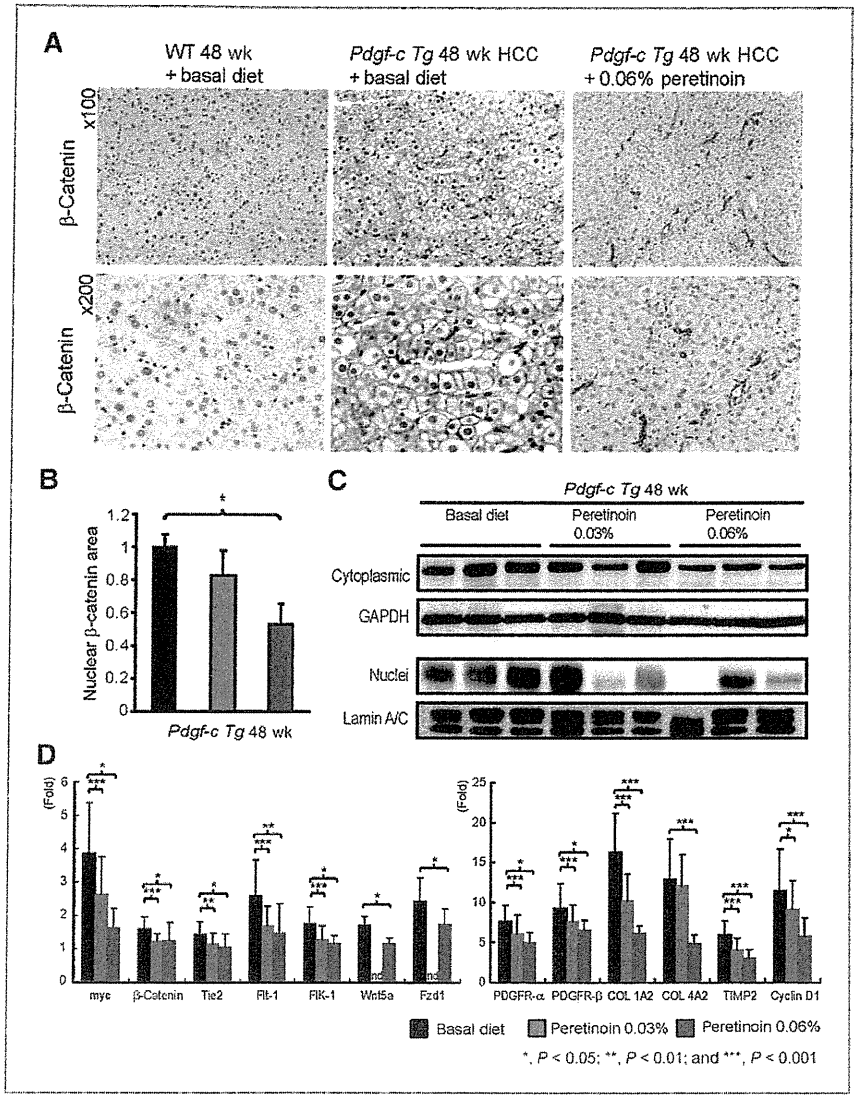


Figure 7. A, IHC staining of β-catenin expression in HCC tissues of *Pdgf-c Tg* mice fed a basal diet or 0.06% peretinoin at 48 weeks. B, densitometric analysis of β-catenin expression in the liver of *Pdgf-c Tg* mice fed with different diets ($n = 15$ for basal diet, $n = 15$ for 0.03% peretinoin, $n = 5$ for 0.06% peretinoin). C, Western blotting of β-catenin expression in cytoplasmic and nuclear fractions of *Pdgf-c Tg* mouse livers fed with different diets. GAPDH was used to standardize cytoplasmic protein and lamin A/C to standardize nuclear protein ($n = 3$). D, RTD-PCR analysis of myc, β-catenin, Tie2, Flt-1, Flk-1, Wnt5a, Fzd1, PDGFR-α, PDGFR-β, collagen (COL) 1a2, collagen 4a2, TIMP2, and cyclin D1 expression in HCC tissues of *Pdgf-c Tg* mice fed with different diets ($n = 15$ for basal diet, $n = 15$ for 0.03% peretinoin, $n = 5$ for 0.06% peretinoin). Relative fold expressions compared with WT mice are shown.

patients with CH-C and HCC to monitor the biological behavior of peretinoin in the liver. Gene expression profiling during peretinoin administration revealed that HCC recurrence within 2 years could be predicted and that PDGF-C expression was one of the strongest predictors. In addition, other genes related to angiogenesis, cancer stem cell and tumor progression were downregulated, whereas expression of genes related to hepatocyte differentiation and tumor suppression was upregulated by peretinoin (data not shown). Moreover, a recent report revealed the emerging significance of PDGF-C–mediated angiogenic and tumorigenic properties (7, 8, 36). In this study, we therefore used the mouse model of *Pdgf-c Tg*, which displays the phenotypes of hepatic fibrosis, steatosis, and HCC development that resemble human HCC arising from chronic hepatitis usually associated with advanced hepatic fibrosis.

We showed that peretinoin effectively inhibits the progression of hepatic fibrosis and tumors in *Pdgf-c Tg* mice (Figs. 1 and 4). Affymetrix gene chips analysis revealed dynamic changes in hepatic gene expression (Supplementary Fig. S3), which were confirmed by IHC staining, RTD-PCR and Western blotting. Pathway analysis of differentially expressed genes suggested that the transcriptional regulators Sp1 and Ap1 are key regulators in the peretinoin inhibition of hepatic fibrosis and tumor development in *Pdgf-c Tg* mice (Supplementary Fig. S5).

We clearly showed that peretinoin inhibited PDGF signaling through the inhibition of PDGFRs (Figs. 2 and 3). In

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addition, we showed that PDGFR repression by peretinoin inhibited primary stellate cell activation (Fig. 5). Interestingly, this inhibitory effect was more pronounced than the effects of 9cRA (Fig. 5B). Normal mouse and human hepatocytes neither express PDGF receptors (J.S. Campbell and N. Fausto, unpublished data), nor proliferate in response to treatment with PDGF ligands (7). However, peretinoin inhibited the expression of PDGFRs, collagens, and their downstream signaling molecules in cell lines of hepatoma (Huh-7, HepG2, and HLE), fibroblast (NIH3T3), endothelial cells (HUVEC), and stellate cells (Lx-2; Supplementary Fig. S6). Furthermore, Sp1 but not Ap1, might be involved in the repression of PDGFR-α in Huh-7 cells (Supplementary Fig. 6C). The overexpression of Sp1-activated PDGFR-α promoter activity, whereas siRNA knockdown of Sp1 repressed PDGFR-α promoter activity in Huh-7 cells (data not shown). Therefore, this seems to confirm that Sp1 is involved in the regulation of PDGFR, as reported previously (37, 38), although these findings should be further investigated in different cell lines. A recent report showed the involvement of transglutaminase 2, caspase3, and Sp1 in peretinoin signaling (35).

Peretinoin was shown to inhibit angiogenesis in the liver of *Pdgf-c Tg* mice in this study, as shown by the decreased expression of VEGFR1/2 and Tie 2 (Figs. 2 and 6 and Supplementary Fig. S1). Moreover, peretinoin inhibited the number of $CD31^+$ and $CD34^+$ endothelial cells (CEC) in the blood and liver (Fig. 6C and D), while also inhibiting the expression of EGFR, c-kit, PDGFRs, and VEGFR1/2 in *Pdgf-c Tg* mice (data not shown). We also showed that peretinoin inhibited the expression of multiple growth factors such as HGF, IGF, VEGF, PDGF, and HDGF, which were upregulated from 3- to 10-fold in *Pdgf-c Tg* mice (Supplementary Fig. S3). These activities collectively might contribute to the antitumor effect of peretinoin in *Pdgf-c Tg* mice. The inhibition of both PDGFRs and VEGFR signaling by peretinoin was previously shown to have a significant effect on tumor growth (36), and we confirmed herein that peretinoin inhibited the expression of VEGFR2 in HUVECs (Supplementary Fig. S6; ref. 39). Finally, we showed that peretinoin inhibited canonical Wnt/β-catenin signaling by showing the decreased nuclear accumulation of β-catenin (Fig. 7). These data confirm the previous hypothesis of transrepression of the β-catenin promoter by 9cRA *in vitro* (40).

Although we showed that the PDGF signaling pathway is a target of peretinoin for preventing the development of hepatic fibrosis and tumors in mice, retinoid-inducing genes such as G0S2 (41), TGM2 (35), CEBPA (42), ATF, TP53BP, metallothionein 1H (MT1H), MT2A, and hemopexin (HPX) were upregulated in peretinoin-treated mice (data not shown). These canonical retinoid pathways are likely to participate in preventing disease progression in conjunction with anti-PDGF effects.

The precise mechanism of peretinoin toxicity, in which 5% of mice treated with 0.06% peretinoin died after 24 weeks of treatment, is currently under investigation. These mice showed severe osteopenia and we speculate that the toxicity might be caused by retinoid-induced osteopenia, as observed in a hypervitaminosis A rat model (43). However, the toxicity of prolonged treatment with oral retinoids in humans remains controversial (44) and severe osteopenia has so far only been seen in a rodent model.

In summary, we show that peretinoin effectively inhibits hepatic fibrosis and HCC development in *Pdgf-c Tg* mice. Further studies are needed to elucidate the detailed molecular mechanisms of peretinoin action and the effect of peretinoin on PDGF-C in human HCC. The recently developed multikinase inhibitor Sorafenib (BAY 43-9006, Nexavar) was shown to improve the prognosis of patients with advanced HCC (45). Promisingly, a phase II/III trial of peretinoin showed it to be safe and well tolerated (46). Therefore, combinatorial therapy that incorporates the use of small molecule inhibitors with peretinoin may be beneficial to some patients. The application of peretinoin during pre- or early-fibrosis stage could be beneficial in preventing the progression of fibrosis and subsequent development of HCC in patients with chronic liver disease.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** M. Honda, J.S. Campbell, S. Kaneko
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** H. Okada, M. Honda, J.S. Campbell, Y. Sakai, T. Yamashita, Y. Takebuchi, K. Hada, T. Shirasaki, R. Takabatake, M. Nakamura, H. Sunagozaka, N. Fausto
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.S. Campbell, T. Yamashita, H. Sunagozaka, S Kaneko
**Writing, review, and/or revision of the manuscript:** H. OkadaM. Honda, J.S. Campbell, N. Fausto, S. Kaneko
**Study supervision:** J.S. Campbell, S. Kaneko
**Pathologic examination and evaluation:** T. Tanaka

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## References

1. Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. Gastroenterology 2002;122:1609–19.

2. Mohamed AE, Kew MC, Groeneveld HT. Alcohol consumption as a risk factor for hepatocellular carcinoma in urban southern African blacks. Int J Cancer 1992;51:537–41.

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3. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993; 328:1797–801.
4. Deugnier YM, Charalambous P, Le Quilleuc D, Turlin B, Searle J, Brissot P, et al. Preneoplastic significance of hepatic iron-free foci in genetic hemochromatosis: a study of 185 patients. Hepatology 1993;18:1363–9.
5. Yeoman AD, Al-Chalabi T, Karani JB, Quaglia A, Devlin J, Mieli-Vergani G, et al. Evaluation of risk factors in the development of hepatocellular carcinoma in autoimmune hepatitis: implications for follow-up and screening. Hepatology 2008;48:863–70.
6. Smedile A, Bugianesi E. Steatosis and hepatocellular carcinoma risk. Eur Rev Med Pharmacol Sci 2005;9:291–3.
7. Campbell JS, Hughes SD, Gilbertson DG, Palmer TE, Holdren MS, Haran AC, et al. Platelet-derived growth factor C induces liver fibrosis, steatosis, and hepatocellular carcinoma. Proc Natl Acad Sci U S A 2005;102:3389–94.
8. Crawford Y, Kasman I, Yu L, Zhong C, Wu X, Modrusan Z, et al. PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. Cancer Cell 2009;15:21–34.
9. Lau DT, Luxon BA, Xiao SY, Beard MR, Lemon SM. Intrahepatic gene expression profiles and alpha-smooth muscle actin patterns in hepatitis C virus induced fibrosis. Hepatology 2005;42: 273–81.
10. Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Kaneko S. Different signaling pathways in the livers of patients with chronic hepatitis B or chronic hepatitis C. Hepatology 2006;44: 1122–38.
11. Muto Y, Moriwaki H, Ninomiya M, Adachi S, Saito A, Takasaki KT, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. N Engl J Med 1996;334:1561–7.
12. Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. N Engl J Med 1999;340:1046–7.
13. Suzui M, Masuda M, Lim JT, Albanese C, Pestell RG, Weinstein IB. Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. Cancer Res 2002;62:3997–4006.
14. Sano T, Kagawa M, Okuno M, Ishibashi N, Hashimoto M, Yamamoto M, et al. Prevention of rat hepatocarcinogenesis by acyclic retinoid is accompanied by reduction in emergence of both TGF-alpha-expressing oval-like cells and activated hepatic stellate cells. Nutr Cancer 2005;51:197–206.
15. Muto Y, Moriwaki H, Omori M. *In vitro* binding affinity of novel synthetic polyprenoids (polyprenoic acids) to cellular retinoid-binding proteins. Gann 1981;72:974–7.
16. Yamada Y, Shidoji Y, Fukutomi Y, Ishikawa T, Kaneko T, Nakagama H, et al. Positive and negative regulations of albumin gene expression by retinoids in human hepatoma cell lines. Mol Carcinog 1994;10:151–8.
17. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 2010;139:499–509.
18. Frith C, Ward J, Turusov V. Pathology of tumors in laboratory animals. Vol. 2. Lyon, France: IARC Scientific Publications; 1994. p. 223–70.
19. Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, et al. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. Toxicol Pathol 2010;38: 5S–81S.
20. Honda M, Takehana K, Sakai A, Tagata Y, Shirasaki T, Nishitani S, et al. Malnutrition impairs interferon signaling through mTOR and FoxO pathways in patients with chronic hepatitis C. Gastroenterology 2011;141:128–40, 140.e1–2.
21. Frith CH, Ward JM, Turusov VS. Tumours of the liver. IARC Sci Publ 1994;111:223–69.
22. Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. Gut 2005;54:142–51.
23. Nhieu JT, Renard CA, Wei Y, Cherqui D, Zafrani ES, Buendia MA. Nuclear accumulation of mutated beta-catenin in hepatocellular carcinoma is associated with increased cell proliferation. Am J Pathol 1999;155:703–10.
24. Wong CM, Fan ST, Ng IO. beta-Catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. Cancer 2001;92:136–45.
25. van Zijl F, Mair M, Csiszar A, Schneller D, Zulehner G, Huber H, et al. Hepatic tumor-stroma crosstalk guides epithelial to mesenchymal transition at the tumor edge. Oncogene 2009;28: 4022–33.
26. Fischer AN, Fuchs E, Mikula M, Huber H, Beug H, Mikulits W. PDGF essentially links TGF-beta signaling to nuclear beta-catenin accumulation in hepatocellular carcinoma progression. Oncogene 2007;26: 3395–405.
27. Hou X, Kumar A, Lee C, Wang B, Arjunan P, Dong L, et al. PDGF-CC blockade inhibits pathological angiogenesis by acting on multiple cellular and molecular targets. Proc Natl Acad Sci U S A 2010;107: 12216–21.
28. Apte U, Zeng G, Muller P, Tan X, Micsenyi A, Cieply B, et al. Activation of Wnt/beta-catenin pathway during hepatocyte growth factor-induced hepatomegaly in mice. Hepatology 2006;44:992–1002.
29. Eguchi S, Kanematsu T, Arii S, Omata M, Kudo M, Sakamoto M, et al. Recurrence-free survival more than 10 years after liver resection for hepatocellular carcinoma. Br J Surg 2011;98:552–7.
30. Okusaka T, Ueno H, Ikeda M, Morizane C. Phase I and pharmacokinetic clinical trial of oral administration of the acyclic retinoid NIK-333. Hepatol Res 2011;41:542–52.
31. Okusaka T, Makuuchi M, Matsui O, Kumada H, Tanaka K, Kaneko S, et al. Clinical benefit of peretinoin for the suppression of hepatocellular carcinoma (HCC) recurrence in patients with Child-Pugh grade A (CP-A) and small tumor: a subgroup analysis in a phase II/III randomized, placebo-controlled trial. J Clin Oncol 2011;29 Suppl 4s:165.
32. Araki H, Shidoji Y, Yamada Y, Moriwaki H, Muto Y. Retinoid agonist activities of synthetic geranyl geranoic acid derivatives. Biochem Biophys Res Commun 1995;209:66–72.
33. Nakamura N, Shidoji Y, Yamada Y, Hatakeyama H, Moriwaki H, Muto Y. Induction of apoptosis by acyclic retinoid in the human hepatoma-derived cell line, HuH-7. Biochem Biophys Res Commun 1995;207: 382–8.
34. Yasuda I, Shiratori Y, Adachi S, Obora A, Takemura M, Okuno M, et al. Acyclic retinoid induces partial differentiation, down-regulates telomerase reverse transcriptase mRNA expression and telomerase activity, and induces apoptosis in human hepatoma-derived cell lines. J Hepatol 2002;36:660–71.
35. Tatsukawa H, Sano T, Fukaya Y, Ishibashi N, Watanabe M, Okuno M, et al. Dual induction of caspase 3- and transglutaminase-dependent apoptosis by acyclic retinoid in hepatocellular carcinoma cells. Mol Cancer 2011;10:4.
36. Timke C, Zieher H, Roth A, Hauser K, Lipson KE, Weber KJ, et al. Combination of vascular endothelial growth factor receptor/platelet-derived growth factor receptor inhibition markedly improves radiation tumor therapy. Clin Cancer Res 2008;14: 2210–9.
37. Molander C, Hackzell A, Ohta M, Izumi H, Funa K. Sp1 is a key regulator of the PDGF beta-receptor transcription. Mol Biol Rep 2001;28: 223–33.
38. Bonello MR, Khachigian LM. Fibroblast growth factor-2 represses platelet-derived growth factor receptor-alpha (PDGFR-alpha) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical cis-acting element in the proximal PDGFR-alpha promoter. J Biol Chem 2004;279:2377–82.
39. Komi Y, Sogabe Y, Ishibashi N, Sato Y, Moriwaki H, Shimokado K, et al. Acyclic retinoid inhibits angiogenesis by suppressing the MAPK pathway. Lab Invest 2010;90:52–60.

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40. Shah S, Hecht A, Pestell R, Byers SW. Trans-repression of beta-catenin activity by nuclear receptors. J Biol Chem 2003;278:48137–45.
41. Kitareewan S, Blumen S, Sekula D, Bissonnette RP, Lamph WW, Cui Q, et al. G0S2 is an all-trans-retinoic acid target gene. Int J Oncol 2008;33:397–404.
42. Uray IP, Shen Q, Seo HS, Kim H, Lamph WW, Bissonnette RP, et al. Rexinoid-induced expression of IGFBP-6 requires RARbeta-dependent permissive cooperation of retinoid receptors and AP-1. J Biol Chem 2009;284:345–53.
43. Hough S, Avioli LV, Muir H, Gelderblom D, Jenkins G, Kurasi H, et al. Effects of hypervitaminosis A on the bone and mineral metabolism of the rat. Endocrinology 1988;122:2933–9.
44. Ribaya-Mercado JD, Blumberg JB. Vitamin A: is it a risk factor for osteoporosis and bone fracture? Nutr Rev 2007;65:425–38.
45. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–90.
46. Okita K, Matsui O, Kumada H, Tanaka K, Kaneko S, Moriwaki H, et al. Effect of peretinoin on recurrence of hepatocellular carcinoma (HCC): results of a phase II/III randomized placebo-controlled trial. J Clin Oncol 2010;28 Suppl 15s:4024.

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*Article*

# Preclinical Cancer Chemoprevention Studies Using Animal Model of Inflammation-Associated Colorectal Carcinogenesis

**Takuji Tanaka** [1,2]

[1] Cytopatholgy Division, Tohkai Cytopathology Institute, Cancer Research and Prevention (TCI-CaRP), 5-1-2 Minami-uzura, Gifu 500-8285, Japan; E-Mail: takutt@toukaisaibou.co.jp; Tel.: +81-58-273-4399; Fax: +81-58-273-4392

[2] Department of Tumor Pathology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

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**Abstract:** Inflammation is involved in all stages of carcinogenesis. Inflammatory bowel disease, such as ulcerative colitis and Crohn's disease is a longstanding inflammatory disease of intestine with increased risk for colorectal cancer (CRC). Several molecular events involved in chronic inflammatory process are reported to contribute to multi-step carcinogenesis of CRC in the inflamed colon. They include over-production of free radicals, reactive oxygen and nitrogen species, up-regulation of inflammatory enzymes in arachidonic acid biosynthesis pathway, up-regulation of certain cytokines, and intestinal immune system dysfunction. In this article, firstly I briefly introduce our experimental animal models where colorectal neoplasms rapidly develop in the inflamed colorectum. Secondary, data on preclinical cancer chemoprevention studies of inflammation-associated colon carcinogenesis by morin, bezafibrate, and valproic acid, using this novel inflammation-related colorectal carcinogenesis model is described.

**Keywords:** inflammatory bowel disease; colorectal cancer; chemoprevention; animal model; AOM; DSS; morin; bezafibrate; valproic acid

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## Abbreviations

ACF, aberrant crypt foci; AD, adenoma; ADC, adenocarcinoma; AOM, azoxymetheane; 5-ASA, 5-aminosalicylic acid; CD, Crohn's disease; COX, cyclooxygenase; CRC, colorectal cancer; DMH,

1,2-dimethylhydrazine; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; HATs, histone acetylases; HCC, hepatocellular carcinoma; HDACs, histone decarboxylases; HDACIs, histone decarboxylase inhibitors; HIF, hypoxia-inducible factor; HMG-CoA, 3-hydroxy-3-methylglutary coenzyme A; IBD, inflammatory bowel disease; IL, interleukin; iNOS, inducible nitric oxide synthase; KAD, Kyoto *Apc* Delta; MAM, methylazoxymethanol; NF-κB, nuclear factor-kappaB; NSAIDs, non-steroidal anti-inflammatory drugs; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PPARs, peroxisome proliferator-activated receptors; PSC, primary sclerosing cholangitis; RXR, retinoid X receptor; TNF, tumor necrosis factor; Stat3, signal transducer and activator of transcription; UC, ulcerative colitis; UDCA, ursodeoxycholic acid; VPA, valproic acid.

## 1. Introduction

An association between inflammation and cancer has been suggested for a long time [1] and it is now well-recognized that inflammation is involved in carcinogenesis in several tissues [2,3]. Patients with inflammatory bowel disease (IBD), especially major types of IBD ulcerative colitis (UC) and Crohn's disease (CD) have a significantly increased risk of developing premalignancy (dysplastic lesions) and malignancy (adenocarcinoma, ADC) in the colorectum [4–6]. Although UC-associated colorectal cancer (CRC) accounts for only less than 2% of all CRCs in the general population, it is responsible for 10–15% of deaths in the UC patients [7]. The risk of CRC increases in relation to the degrees of inflammation and the disease duration (duration/risk = 10 years/1.6%, 20 years/8.3%, and 30 years/18.4%) in UC patients [8]. Even younger patients with UC have high risk of CRC [9]. CD is also associated with an increased risk of large and small bowel ADC [10]. Patients with CD have an increased cumulative risk for CRC, from 2.9% at 10 years to 8.3% after 30 years of disease [10]. Patients with UC as well as those with CRC have been increasing in Asian countries including Japan, similarly to Western countries [11]. Therefore, it is necessary to investigate the mechanisms of CRC development with the background of inflammation for establishing the countermeasure strategy such as chemoprevention [12–14]. Also, a novel animal model is required [15–19], as until now there have been few useful ones.

Along with the development of surveillance colonoscopy or prophylactic colonoscopy, recently the concept of chemoprevention has gained increasing importance [20]. Many natural or synthetic pharmacological agents have been evaluated for their chemopreventive efficacy for UC-associated CRC using animal models. The ideal chemopreventive agents would be effective for preventing neoplastic progression, safe (without or low side-effects), and inexpensive [20,21]. The most frequently used chemopreventive agents in UC patients are 5-aminosalicylic acid (5-ASA) compounds (mesalazine and sulfasalazine) as well as ursodeoxycholic acid (UDCA), which is applied in patients with primary sclerosing cholangitis (PSC) [22] being one of the extra-intestinal manifestations. Although the chemopreventive role for UDCA in PSC patients is generally accepted [23,24], there is still debate regarding the chemopreventive capability of 5-ASA derivatives in the patients without PSC [21]. While there are numerous studies supporting the chemopreventive efficacy of 5-ASA [25,26], several studies [27,28] could not find significant reduction in UC-associated dysplasia and CRC. We have reported that chemopreventive efficacy of UDCA is superior to that of 5-ASA in the mouse azoxymethane (AOM)/dextran sodium sulfate (DSS) model [15,17,18] of colitis-related colorectal

carcinogenesis [29]. Therefore, further pharmacological candidates and potential targets should be evaluated for chemoprevention in UC-associated CRC [12–14,17,30].

This article describes our short-term mouse and rat CRC models with the background of colitis mimicking human UC and our exploration of chemopreventive agents [12–14], and finally summarizes our recent data on chemopreventive abilities of morin, bezafibrate, and valproic acid (VPA) against inflammation-related mouse or rat colorectal carcinogenesis.

## 2. Development of an Inflammation-Associated CRC Model

### *2.1. AOM/DSS Mouse Model*

Rats have mostly been employed for animal colorectal carcinogenesis models, and AOM, methylazoxymethanol (MAM) acetate, and 1,2-dimethylhydrazine (DMH) have been widely used as colorectal carcinogens [15]. About 30 weeks are required for development of CRC in about half of rats that are initiated with these colonic carcinogens. On the other hand, in experiments and studies using mice, multiple administrations of the colorectal carcinogens are required and it takes a long-term of 40 weeks or longer to develop CRC [31]. Therefore, I have developed a novel mouse model that would produce CRC in a short-term in the inflamed colorectum [18]. AOM-DSS, here called the TANAKA model [15,17,18], is a well-characterized experimental model for UC-associated colorectal carcinogenesis (Figure 1a–e) [18,32–36]. Mice that received a single injection of a low dose of the classic colon carcinogen AOM prior to administration of DSS in drinking water develop inflammation and mucosal ulcer (Figure 1b) [18,37,38] as well as dysplasia (Figure 1c), adenoma (AD) (Figure 1d) and ADC (Figure 1e) with pathologic features that resemble those of human UC-associated neoplasia [18,33,34,36]. The extent of neoplastic lesions depends on several factors like strain susceptibility as well as duration, dosage, and schedule of cyclic DSS application [39–41].

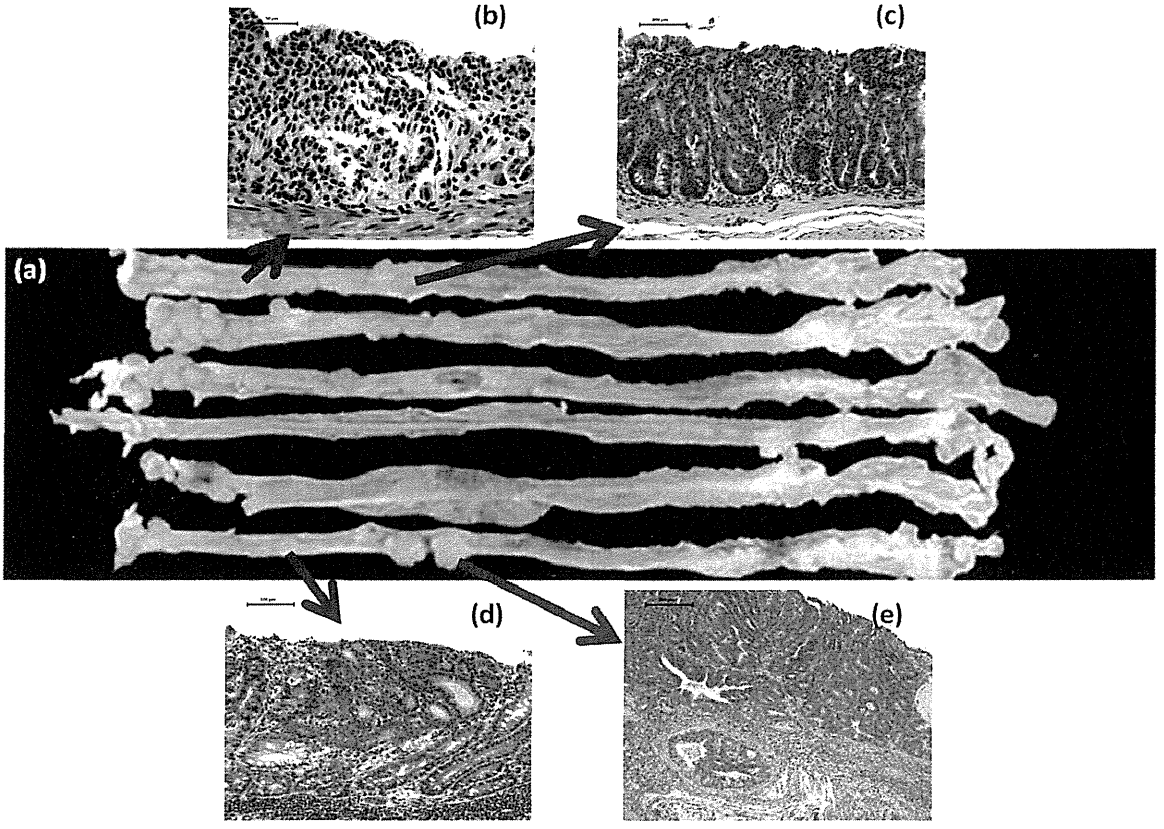
To settle the issue of the influence of peroxisome proliferator-activated receptor (PPAR) agonists on colorectal carcinogenesis, which has been a topic since 1998 [42–44], we confirmed that colitis induced by DSS, which is a non-genotoxic carcinogen [45,46], using aberrant crypt foci (ACF) as a biomarker [16,47–49], had tumor promoter activity to enhance development of ACF in rats and hypothesized that a combination of DSS and AOM would induce CRC in a short-term period in mice as well [50].

### *2.2. DMH/DSS and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)/DSS Mouse Models*

Instead of AOM, experiments with DMH [51] or a heterocyclic amine, PhIP [52] as an initiator (colon carcinogen) and followed by DSS treatment resulted in rapid development of colorectal neoplasms. Histopathologically, ADC induced by DMH/DSS showed severer atypia and more aggressive biological nature than that induced by AOM/DSS. As noticed in the cancers induced by AOM/DSS, the ADC cells developed in the inflamed colon of mice that received DMH and DSS were positive for cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and β-catenin. Mutation patterns of the *β-catenin* gene slightly differ among the ADCs that were induced by the different treatment regimens: AOM/DSS, codon 32–34, 37, and 41; DMH/DSS, codon 32, 34, 37, and 41; and PhIP/DSS, codon 32 and 34.

However, these mutations was limited to the codon 32–34, 37, 41, and 45 that played an important role in degradation of β-catenin protein.

**Figure 1.** (**a**) Macroscopic view of colorectal tumors developed in mice that received AOM and DSS. Histopathology of (**b**) severe mucosal ulcer; (**c**) dysplastic crypts, (**d**) colonic adenoma (AD), and (**e**) adenocarcinoma (ADC) in a mouse that received AOM and DSS.



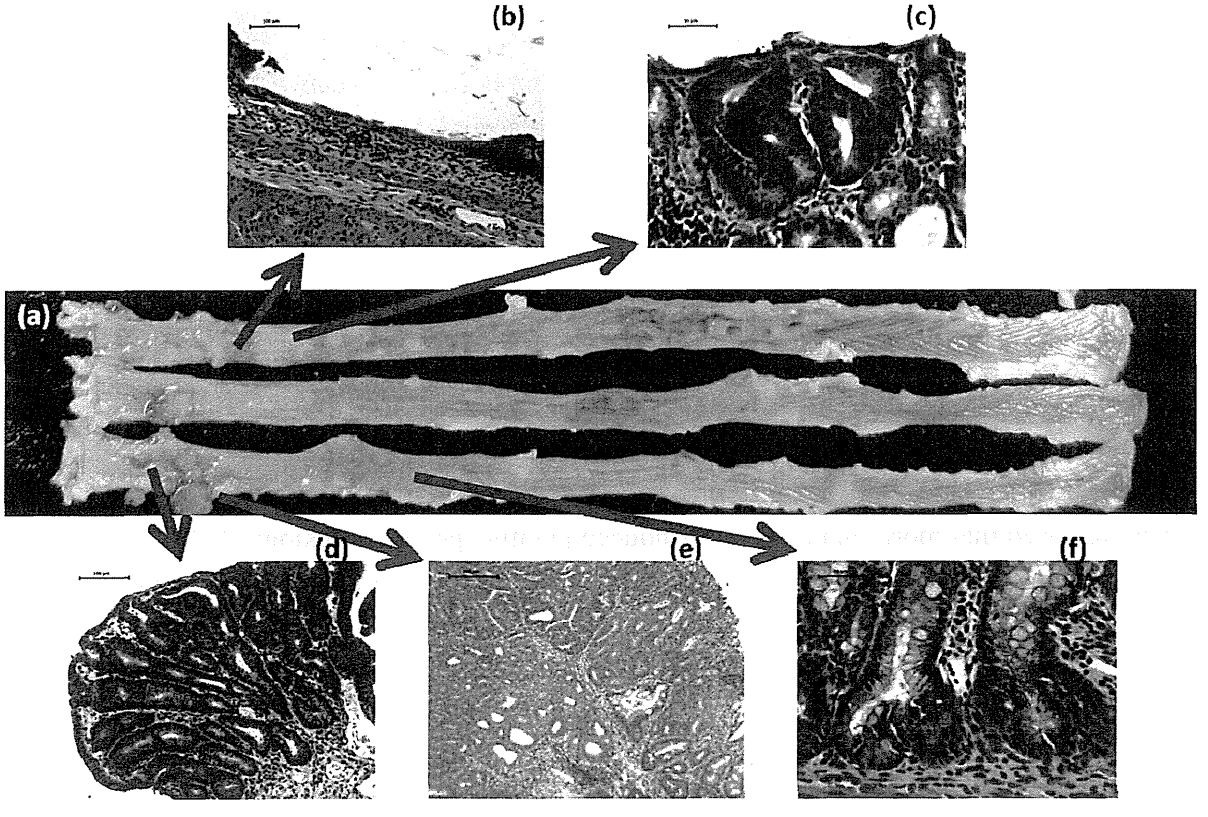

### *2.3. DSS Promotes CRC Development in $Apc^{Min/+}$ Mice*

In $Apc^{Min/+}$ mice, known as an animal model for familial adenomatous polyposis (FAP), multiple tumors (tubular ADs) develop in the small intestine, instead of the large intestine in human FAP, and markedly few tumors develop in the large bowel. However, dysplastic crypts are observed in the colonic mucosa of $Apc^{Min/+}$ mice [53,54]. Therefore, DSS possibly enhances the growth of dysplastic crypts, and finally the lesions progress to ADCs. To investigate whether DSS-induced inflammation in the colonic mucosa would accelerate the growth of dysplastic crypts, $Apc^{Min/+}$ mice were given drinking water containing 2% DSS for one week without the initiation (carcinogen) treatment [53]. Surprisingly, multiple colorectal tumors, which were histopathologically tubular ADs and ADCs, developed four weeks after the end of DSS treatment. Immunohistochemistry showed that the developed colorectal ADCs were positive against β-catenin, COX-2, iNOS, and p53 antibodies, suggesting that these factors were involved in the development of colorectal neoplasms in the $Apc^{Min/+}$ mice by the DSS treatment, in addition to oxidative stress and nitrosative stress. The findings suggested that DSS-induced inflammation in the large bowel of $Apc^{Min/+}$ mice exert powerful tumor-promotion and/or progression effects on the growth of dysplastic crypts, which had already existed after the birth [53,54].

### *2.4. AOM/DSS and DMH/DSS Rat Models*

The mouse inflammation-associated colorectal carcinogenesis model was named the TANAKA model. With this model it was possible to induce colorectal tumors in a short-term period in rats as well as by similar treatment regimens (AOM/DSS and DMH/DSS) [55,56]. The administration dose of colon carcinogens for initiation is too low to induce colon tumors, but it can initiate or induce DNA modification [18]. Treatment with DSS followed by AOM did not produce colonic neoplasms [18]. The TANAKA model will help advance the research on elucidation of the mechanisms of inflammation-associated colorectal carcinogenesis, inhibition of carcinogenesis, and clarification of the mechanisms of the tumor-promotion ability of DSS. In particular, development of challenging research using the Kyoto *Apc* Delta (KAD) rats (Figure 2a–e) [57] and *gpt* delta rats [58] will give new insight in the pathogenesis of CRC development in the inflamed colon [57]. Interestingly, atypical and neoplastic cells of dysplastic crypts, AD, and ADC in the KAD rats that received AOM and DSS contain Paneth's granules (Figure 2f), which consist of several anti-microbial compounds and other compounds that are known to be important in immunity and host-defense, in their cytoplasm. We have thus confirmed that in rats with or without genetically alterations colonic tumors are rapidly produced as observed in mice and the rat AOM/DSS and DMH/DSS models can be applied to determine the chemopreventive ability of target compounds.

**Figure 2.** (**a**) Macroscopic view of colorectal tumors developed in KAD rats that received AOM and DSS. Histopathology of (**b**) severe mucosal ulcer; (**c**) dysplastic crypts; (**d**) colonic adenoma (AD); (**e**) adenocarcinoma (ADC), and (**f**) cryptal cells containing Paneth's granules in a mouse that received AOM and DSS.



### *2.5. Detection of Initiators and Promoters in Colorectal Carcinogenesis*

We could identify initiators and promoters of colorectal carcinogenesis by modifying the regimens of AOM/DSS and DMH/DSS. When applied test compounds to the initiation treatment instead of AOM or DMH and followed by DSS treatment, we could evaluate initiation activity of test compounds [59]. When test chemicals are applied after initiation treatment with AOM or DMH, we could determine tumor promotion activity of test compounds (unpublished data and [60]). Thus, modification of two regimens in the TANAKA model will determine environmental carcinogens and tumor promoters in the colorectum.

## 3. Exploration of Chemopreventive Agents Using an Inflammation-Associated Colorectal Carcinogenic Model and Elucidation of the Mechanisms

Studies on chemoprevention of inflammation-associated colorectal carcinogenesis by several natural and synthetic compounds against have been reported using the AOM/DSS-induced mouse and rat colorectal carcinogenesis models. Several are promising compounds and their clinical application is expected. Representative compounds are: auraptene and nobiletin from citrus fruits [61], collinin [61], β-cyclodextrin inclusion compounds of auraptene and 4'-geranyloxyferulic acid [62], tricin [35], melatonin [55], urosodeoxycholic acid [29], COX-2 selective inhibitor nimesulide [63], iNOS selective inhibitors [64], PPAR ligands (troglitazone and bezafibrate) [63], and the lipophilic statin pitavastatin [65]. All these compounds have anti-inflammatory activity and are able to suppress the expression of COX-2, iNOS, and inflammatory cytokines.

## 4. Preclinical *in Vivo* Chemoprevention Studies

Using our animal models of inflammation-associated colorectal carcinogenesis, cancer chemopreventive abilities of candidate compounds, morin, bezafibrate, and VPA in mice or rats were investigated. All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Institute, TCI-CaRP.

### *4.1. Morin Study*

A flavonol, morin (3,5,7,2',4'-pentahydroxyflavone) found in almonds, mill, fig, mulberry, and other Moraceae, acts as a potent antioxidant, inhibitor of xanthine oxidase, protein kinase C and proliferation, apoptosis inducer and modulator of lipoxygenase and cyclooxygenase activities. This flavone has been reported to inhibit the growth of COLO205 cells in nude mice [66], exhibit intestinal anti-inflammatory activity in the acute phase of rat colitis induced by trinitrobenzenesulfonic acid [67,68]. We have previously reported that morin inhibits AOM-induced putative precursor lesions, ACF, in rats [69] and inhibit chemically-induced rat tongue carcinogenesis [70]. Morin exerts anti-inflammatory effects on septic shock induced by lipopolysaccharide [71]. This study aimed to determine possible inhibitory potential of morin in colitis-associated colon carcinogenesis initiated with AOM and promoted by DSS in male F344 rats.

Materials and methods: A total of 66 male rats (5-week-old) were initiated with a single s.c. injection of AOM (20 mg/kg bw), and then they were given promotion stimuli by the treatment with 1.5% DSS in

their drinking water for seven days. They were then given a basal diet containing 50, 250 and 1,000 ppm of morin for 17 weeks. Experimental groups included the AOM/1.5% DSS (n = 14), AOM/1.5% DSS/50 ppm morin (n = 10), AOM/1.5% DSS/250 ppm morin (n = 11), AOM/1.5% DSS/1,000 ppm morin (n = 11), AOM alone (n = 5), 1.5% DSS alone (n = 5), 500 ppm morin alone (n = 5), and untreated (n = 5) groups. At the end (week 20) of the study histopathological analysis of colorectum was performed on hematoxylin and eosin (H&E)-stained histologic sections (3 μm thickness). Proliferation activity of colonic ADCs was determined by immunofluorescence technique using anti-Mcm2 antibody (BD Biosciences PharMingen, Tokyo, Japan). Apoptotic cells were detected by fluorescein in situ tunnel method, TACS TdT kit (R&D Systems, Inc., Minneapolis, MN, USA). Polyamine levels [72] and mRNA expression of nuclear factor-kappaB (NF-κB), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, Stat3, and hypoxia-inducible factor (HIF)-1α [73] in colonic mucosa were determined in mice randomly selected from each group. All measurements were statistically analyzed using either the Tukey multiple comparison post test or Fisher's extract probability test. Differences were considered to be statistically significant at $p < 0.05$.

Results: At week 20, the treatment with morin inhibited colonic mucosal ulcer (Figure 3) and dysplastic crypts ($p < 0.05$ at 1,000 ppm, Figure 3). The incidence ($p < 0.005$) and multiplicity ($p < 0.01$) of colonic AD were significantly reduced by feeding with 1000 ppm morin (Figure 4). Also, dietary administration with 1,000 ppm morin significantly inhibited the incidence ($p < 0.02$) and multiplicity ($p < 0.05$) of colonic ADC (Figure 4), when compared to the AOM/DSS group (93% incidence and 2.36 ± 1.95 multiplicity). Feeding with 50 ppm and 250 ppm morin lowered the incidence and multiplicity of ADC, but the inhibition rates did not reach statistically significance. The treatments also modulated proliferation and apoptosis in ADCs. Mcm2 positive rates (%) of ADCs in rats fed the diets containing 50 ppm morin (n = 6, 78.7 ± 6.8), 250 ppm morin (n = 7, 63.4 ± 6.3, $p < 0.001$), and 1,000 ppm morin (n = 5, 55.6 ± 12.5, $p < 0.001$) were lower than that of rats given AOM and DSS (n = 13, 83.4 ± 8.8). When compared with the AOM and DSS group (n = 13, 8.23 ± 1.24), apoptotic index (%) of ADCs was increased by feeding with morin: 50 ppm morin (n = 6, 10.83 ± 3.66), 250 ppm morin (n = 7, 11.29 ± 3.95), and 1,000 ppm morin (n = 5, 13.00 ± 2.74, $p < 0.05$).

Growth inhibition and apoptosis induction by morin in CRC might be caused by activation of caspase 3 and increase of p21 protein [66]. Suppression of NF-κB-regulated gene products and enhancement of apoptosis induced by TNF [74] also contribute to inhibition of colitis-related colorectal carcinogenesis by morin. Our findings suggest that dietary morin is able to inhibit colitis-related colon carcinogenesis in rats and a flavonol morin is one of the candidates for clinical application of chemoprevention against CRC development in patients with ulcerative colitis.

Because morin can inhibit inflammation, inhibit tumor promotion, suppress tumor growth, and down-regulate the expression of certain genes regulated by NF-κB, it may be possible that morin modulates the activation of NF-κB and NF-κB-regulated gene expression induced by carcinogens, inflammatory agents, and immune modulators. In fact, Manna *et al.* [74] have recently reported morin suppresses the activation of NF-κB and NF-κB-regulated gene expression that leads to enhancement of apoptosis.

**Figure 3.** Incidences and multiplicities of mucosal ulcer and dysplastic crypts in the morin study.

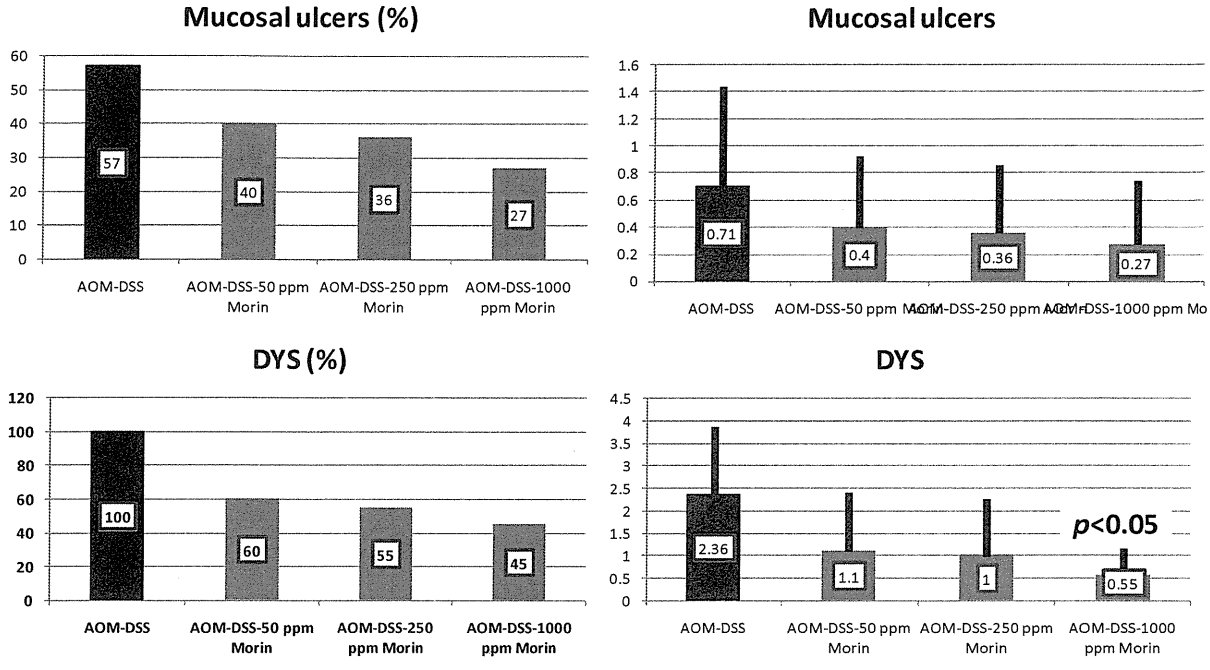
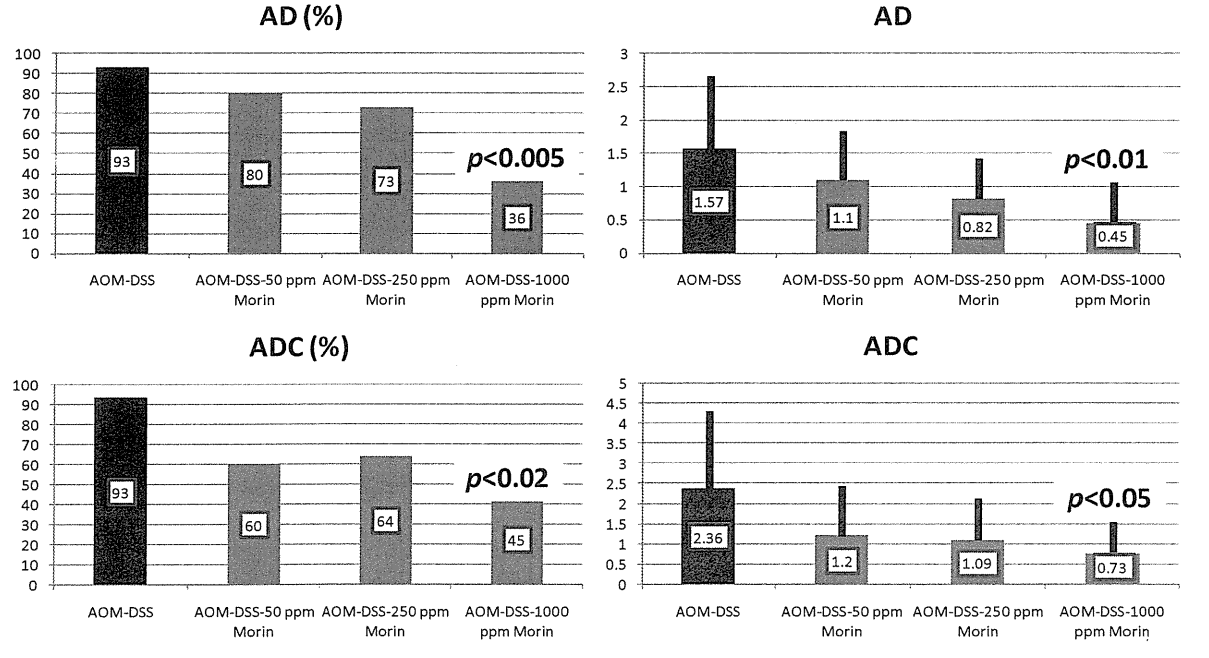



**Figure 4.** Incidences and multiplicities of adenoma (AD) and adenocarcinoma (ADC) in the morin study.




*4.2. Bezafibrate Study*

CRC is one of the leading forms of malignancy in the developed countries. Epidemiologic and animal studies have suggested that risk factors for coronary artery disease like insulin resistance and dyslipidemia are probably related to the development of colon cancer [75–77]. In particular, nuclear peroxisome proliferator-activated receptors (PPARs), mainly PPARα and PPARγ, which play a central role in lipid

and glucose metabolism, had been hypothesized as being involved in colon carcinogenesis [50,78,79]. Furthermore, synthetic PPAR ligands (glitazones and bezafibrate) with proven beneficial effects on insulin resistance and triglyceride levels had been proposed to be candidates as tumor preventive agents [50,63,79]. This study aimed to determine inhibitory potential of bezafibrate in colitis-associated colon carcinogenesis initiated with AOM and promoted by DSS in mice.

Materials and methods: A total of 100 male ICR mice (5-week old) initiated with a single s.c. injection of AOM (10 mg/kg bw) were promoted by 1.5% DSS in their drinking water for seven days. They were then given a basal diet containing 50, 100 and 500 ppm of bezafibrate for 17 weeks. Mice were divided into 8 groups: AOM/1.5% DSS (n = 20), AOM/1.5% DSS/50 ppm bezafibrate (n = 20), AOM/1.5% DSS/100 ppm bezafibrate (n = 20), AOM/1.5% DSS/500 ppm bezafibrate (n = 20), AOM alone (n = 5), 1.5% DSS alone (n = 5), 500 ppm bezafibrate alone (n = 5), and untreated (n = 5) groups. At the end (week 20) of the study histopathological analysis of colorectum was performed on H&E-stained histological sections of 3 μm in thickness. Immunofluorescence technique using anti-Mcm2 antibody (BD Biosciences PharMingen) and fluorescein in situ tunnel method, TACS TdT kit (R&D Systems, Inc.) were used for determination of proliferation activity and apoptosis index of colonic ADCs, respectively. Polyamine levels [72] and mRNA expression of NF-κB, TNF-α, IL-1β, Stat3, and HIF-1α [73] in colonic mucosa were assayed in some mice of each group. Measurements were statistically analyzed using either the Tukey multiple comparison post-test or Fisher's extract probability test. Differences were considered to be statistically significant at $p < 0.05$.

Results: At week 20, the bezafibrate feeding inhibited the occurrence of mucosal ulcer (the incidence at 500 ppm, $p < 0.02$; and the multiplicity at 50, 100 and 500 ppm, $p < 0.01$ or $p < 0.001$, Figure 5) and dysplastic crypts (the multiplicity at 500 ppm, $p < 0.05$, Figure 5). As illustrated in Figure 6, he development of colonic ADC was significantly inhibited by feeding with 500 ppm bezafibrate (incidence: 73% reduction, $p < 0.01$; and multiplicity: 92%, $p < 0.05$), when compared to the AOM/DSS group (73% incidence and 2.53 ± 3.14 multiplicity). Feeding with 50 ppm (47% incidence with a multiplicity of 1.40 ± 1.92) and 100 ppm bezafibrate (67% incidence with a multiplicity of 1.13 ± 1.19) also lowered the incidence and multiplicity of ADC, but the inhibition was not statistically significant when compared to the AOM/DSS group (Figure 6). Although statistically insignificant, feeding with 50 and 100 ppm bezafibrate increased the incidence and multiplicity of ADs, but 500 ppm bezafibrate decreased the development of ADs (Figure 6). These findings may suggest the threshold of chemopreventive ability of bezafibrate. Feeding with bezafibrate lowered Mcm2 positive rates (%) of ADCs, when compared with the AOM and DSS group (n = 10, 72.9 ± 10.1): 50 ppm bezafibrate (n = 7, 54.3 ± 9.5), 100 ppm bezafibrate (n = 4, 38.8 ± 7.4, $p < 0.01$), and 500 ppm bezafibrate (n = 3, 22.7 ± 3.2, $p < 0.001$) were lower than that of rats given AOM and DSS (n = 13, 83.4 ± 8.8). When compared with the AOM and DSS group (n = 10, 8.18 ± 1.17), apoptotic index (%) of ADCs was increased by feeding with bezafibrate: 50 ppm bezafibrate (n = 7, 10.71 ± 3.35), 100 ppm bezafibrate (n = 4, 13.20 ± 2.49, $p < 0.05$), and 500 ppm bezafibrate (n = 3, 13.25 ± 3.10, $p < 0.05$).

Anti-angiogenic effects and anti-inflammatory activity of PPARα agonist [80] are considered to contribute to inhibition of CRC growth. Down-regulation of the anti-apoptotic gene *Mcl-1* by PPARα agonist [81] causes apoptosis. The findings suggest that dietary bezafibrate is able to inhibit colitis-related colon carcinogenesis in mice and a hypolipidemic drug bezafibrate is one of the