

Inhibition of peroxisome proliferator-activated receptor γ activity suppresses pancreatic cancer cell motility

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(Received March 30, 2008/Revised June 6, 2008/Accepted June 9, 2008/Online publication October 9, 2008)

Peroxisome proliferator-activated receptor γ (PPAR γ) is a ligand-activated transcription factor that has been implicated in the carcinogenesis and progression of various solid tumors, including pancreatic carcinomas. We aimed to clarify the role of this receptor in pancreatic cell motility *in vitro* and in metastasis *in vivo*. Cell motility was examined by assaying transwell migration and wound filling in Capan-1 and Panc-1 pancreatic cancer cells, with or without the PPAR γ -specific inhibitor T0070907. A severe combined immunodeficiency xenograft metastasis model was used to examine the *in vivo* effect of PPAR γ inhibition on pancreatic cancer metastasis. In both transwell-migration and wound-filling assays, inhibition of PPAR γ activity suppressed pancreatic cell motility without affecting *in vitro* cell proliferation. Inhibition of PPAR γ also suppressed liver metastasis *in vivo* in metastatic mice. In PPAR γ -inhibited cells, p120 catenin accumulation was induced predominantly in cell membranes, and the Ras-homologous GTPases Rac1 and Cdc42 were inactive. Inhibition of PPAR γ in pancreatic cancer cells decreased cell motility by altering p120ctn localization and by suppressing the activity of the Ras-homologous GTPases Rac1 and Cdc42. Based on these findings, PPAR γ could function as a novel target for the therapeutic control of cancer cell invasion or metastasis. (*Cancer Sci* 2008; 99: 1892–1900)

Pancreatic ductal adenocarcinoma is associated with one of the highest mortality rates in patients with malignancies.⁽¹⁾ Because of a lack of early symptoms, PDAC is often diagnosed only after a local tumor has disseminated and metastatic disease has already developed in regional lymph nodes or distant organ sites. To overcome this dismal situation, development of novel PDAC therapies involving drugs that target disease-specific molecules is urgently required. PPAR γ , a member of the nuclear receptor family of ligand-activated transcription factors, is one promising target for such therapies.⁽²⁾

Activation of PPAR γ , which is expressed mainly in adipose tissue, is known to play a central role in adipocyte differentiation and insulin sensitivity.⁽³⁾ For this reason, synthetic PPAR γ -activating ligands such as TZD are used commonly as oral antihyperglycemic agents to control non-insulin-dependent diabetes mellitus. More recently, PPAR γ has been investigated as a target for the treatment of a variety of cancers.^(4–6) The fact that PPAR γ is overexpressed in many tumors, including examples in the esophagus, stomach, breast, lung, and colon, suggests that PPAR γ function impacts tumor survival.^(4–8) Initial efforts to alter PPAR γ activity focused on activation with TZD ligands, which have been shown to induce G₁ cell-cycle arrest in a variety of tumor cell lines.^(9,10) However, the reported benefits of

TZD for pancreatic carcinoma patients in clinical trials are modest at best.^(11,12)

Several observations suggest that inhibition of PPAR γ function may be beneficial in treating neoplasms.^(13,14) Although PPAR γ is overexpressed in many cancer cell types, loss-of-function mutations are rare,⁽¹⁵⁾ which suggests that the receptor is a tumor cell survival factor. Evidence that PPAR γ function can contribute to carcinogenesis or cancer cell survival includes reports of a murine colon cancer model in which PPAR γ activation leads to increased tumor formation.^(16,17)

Profiles of PPAR γ expression in a variety of human malignancies, including pancreatic cancer, have been described. One recent report showed a significant association between high levels of PPAR γ expression in pancreatic cancer cells and shorter overall survival time.⁽¹⁸⁾ Prior investigations demonstrating that PPAR γ inhibition induces apoptosis in epithelial tumor lines suggest strongly that PPAR γ inhibition may also be beneficial in PDAC treatment.^(19–21) In hepatocellular carcinoma cell lines, PPAR γ inhibitors have been shown to inhibit cell adhesion and induce morphological changes that normally occur prior to the commitment to apoptosis; in contrast, caspase inhibitors do not prevent these changes.⁽²⁰⁾ We hypothesize that PPAR γ inhibition interferes with adhesion-dependent epithelial cell survival signals, leading to cell death (anoikis). Two additional reports have shown that high doses of PPAR γ inhibitors also interfere with Caco-2 cell survival.^(22,23) The effect of PPAR γ inhibitors (especially at low concentrations) on pancreatic cancer cells has not been investigated.

Ras-homologous GTPases play a pivotal role in the regulation of numerous cellular functions associated with malignant transformation and metastasis. Members of the Rho family of small GTPases are key regulators of actin reorganization and cell motility, as well as cell–cell and cell–extracellular matrix adhesion. These processes all play critical roles during the development and progression of cancer. Because of their pleiotropic functions, Rho proteins appear to be promising targets for the development of novel anticancer drugs,^(24,25) including those for PDAC.⁽²⁵⁾ The ability to modulate pathways regulated by Rho could not only improve the therapeutic efficiency, but also reduce the side effects of conventional antineoplastic therapies.

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Abbreviations: FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; p120ctn, p120 catenin; PDAC, pancreatic ductal adenocarcinoma cells; PPAR γ , peroxisome proliferator-activated receptor γ ; PPRE, PPAR γ -response element; Rho, Ras-homologous; SCID, severe combined immunodeficiency; siRNA, small interfering RNA; TZD, thiazolidinedione.

The protein p120ctn is the prototypic member of a subfamily of armadillo repeat-domain proteins involved in intercellular adhesion. A recent report demonstrated clearly that p120 regulates, at least in part, the activity of Rho GTPases, and that p120 association with classical cadherins regulates their stability.⁽²⁶⁾ Ectopic expression of p120ctn has been shown to promote cell migration and to induce a wide variety of morphological changes.⁽²⁶⁾

In the present study, we investigated the effects of PPAR γ inhibitors on pancreatic cell lines and xenograft metastatic tissues that function as models for PDAC. Our data demonstrate that inhibition of PPAR γ in pancreatic cancer cells decreases cell motility by altering p120ctn localization and suppressing the activity of the Rho GTPases Rac1 and Cdc42. These findings suggest that PPAR γ inhibitors may improve the benefit of current PDAC therapeutics.

Materials and Methods

Cell lines and reagents. The PDAC cell line Panc-1 was purchased from the American Type Culture Collection (Rockville, MD, USA). Other cell lines were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). All cell lines were grown in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO₂. The PPAR γ -specific inhibitor T0070907 and PPAR γ ligand rosiglitazone were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Western blot analysis. Adherent cells were washed in phosphate-buffered saline, and cell extracts were prepared in Laemmli lysis buffer. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA, USA) following the manufacturer's suggested procedure. After electrophoresis of extract aliquots (20 μ g protein) on 10% sodium dodecylsulfate-polyacrylamide gels, proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), blocked at room temperature for 1 h in Tris-buffered saline with 5% bovine serum albumin, and then incubated with primary monoclonal antibody for 1 h. Anti-PPAR γ antibody (E-8) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal antibodies against p120ctn and Rac1 were obtained from BD Transduction Laboratories (Palo Alto, CA, USA). After three washes the membranes were incubated for 1 h at room temperature with secondary antibody, and immune complexes were visualized using the enhanced chemiluminescence detection kit (Amersham, London, UK) following the manufacturer's procedure. Images were captured and analyzed using a LAS-3000 imaging system (Fujifilm, Tokyo, Japan). The ProteoExtract Subcellular Proteome Extraction Kit (EMD Biosciences, Darmstadt, Germany) was used for the preparation of cytosolic protein extracts.

Cell proliferation and apoptosis assays. Cell proliferation was measured using MTT assays.⁽²⁷⁾ Approximately 5×10^3 cells in 100 μ L medium were plated per well in a 96-well plate. After 24 h incubation, the medium was changed and supplemented with various concentrations of T0070907 in dimethylsulfoxide, and the cells were incubated for another 24–72 h. After incubating the plates for an additional 4 h with MTT solution (0.5%), sodium dodecylsulfate was added to a final concentration of 20% and absorbance at 595 nm was determined for each well using a microplate reader (Model 550; Bio-Rad). Control wells were treated with dimethylsulfoxide alone. Three independent experiments were carried out for each cell line. Annexin V staining with the annexin V-FITC apoptosis detection kit (Becton Dickinson, San Jose, CA, USA) followed by FACScan flow cytometry (Becton Dickinson) was used to identify apoptotic cells. Apoptosis measures were carried out in triplicate.

Cell-motility assays. Motility was assessed by migration of cells in porous-membrane culture inserts (8.0- μ m pore size; Becton Dickinson). After 24 h of incubation, cells that did not migrate were removed from the upper surface of the membrane with a cotton swab, and migrating cells on the lower surface of the membrane were fixed and stained with toluidine blue. Migrating cell counts were estimated from counts of three independent microscopic visual fields ($\times 100$). To estimate cell-migration activity during wound healing, cells were grown for 2 days (to confluency), after which a scrape in the form of a cross was made through the confluent monolayers with a plastic pipette tip. To measure migration, several wounded areas within each plate were marked for orientation and then photographed periodically by phase-contact microscopy for 24 h after wounding.

Inhibition of PPAR γ function using siRNA. PPAR γ siRNA was purchased from Santa Cruz Biotechnology. Panc-1 and Capan-1 cells at 70% confluence were transfected with PPAR γ siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The cells were treated with 10 nmol/L PPAR γ siRNA for 24 h. Stealth RNAi Negative Control Medium GC (Invitrogen) was used for control specimens. Using real-time reverse transcription-polymerase chain reaction to measure steady-state mRNA levels in cells, PPAR γ -specific siRNA was found to inhibit PPAR γ expression to levels less than 30% of those in control cells (data not shown).

Measuring the effect of T0070907 on PPAR γ -dependent transcription. Capan-1 cells transfected with plasmid encoding a PPAR γ -response element fused to a luciferase reporter (pHD[$\times 3$]PPRE-Luc) were stimulated as described previously with 1 μ mol/L rosiglitazone and various concentrations of T0070907.⁽²⁰⁾ Luciferase activity was measured 16 h after transfection. Because Renilla luciferase control plasmids are sensitive to steroid/thyroid/retinoid nuclear-receptor stimulation, variability in transfection efficiencies (<20%) were assessed in parallel experiments using the pRL-TK plasmid (Promega, Madison, WI, USA).

Immunofluorescence staining. Cells (5×10^5 per well) were grown on collagen-1-coated glass coverslips in six-well flat-bottom plates for 24 h. After 24 h incubation, T0070907 was added to a final concentration of 0.1 μ mol/L and the cells were grown for an additional 24 h. The cells were then fixed in 4% paraformaldehyde followed by 100% ethanol at -20°C. After permeabilization with 0.1% Triton-X, non-specific binding of antibody to the cells was blocked with 2% normal swine serum. Cells were incubated subsequently with anti-p120 catenin antibody followed by FITC-labeled secondary antibody. Samples were then mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined using confocal laser-scanning microscopy (Carl Zeiss, Oberkochen, Germany). All experiments were repeated in triplicate.

Measurement of Rac-1 and Cdc42 activities. GST pull-down assays using a Rac-1/Cdc42 activation kit were used to evaluate Rac1/Cdc42 activities according to the manufacturer's protocol (Stressgen, Ann Arbor, MI, USA). Briefly, we used a GST fusion polypeptide composed of GST fused to the interactive domain of human p21-activated kinase-1, which interacts specifically with GTP-bound Cdc42 and Rac1 GTPases.⁽²⁵⁾ The GST fusion target was incubated with cell lysates and then applied to GST-specific beads to estimate the relative abundance of active Cdc42 and Rac1. Bound Rac1 and Cdc42 proteins were resolved on 12% denaturing polyacrylamide gels and distinguished by western blotting using antibodies specific to each protein. The amount of active GTP-bound enzyme was quantified relative to the total amount of each GTPase present in whole unprecipitated cell lysates. The experiments were carried out six times.

In vivo metastasis study. Five-week-old male SCID mice were obtained from CLEA Japan (Tokyo, Japan) and maintained in a

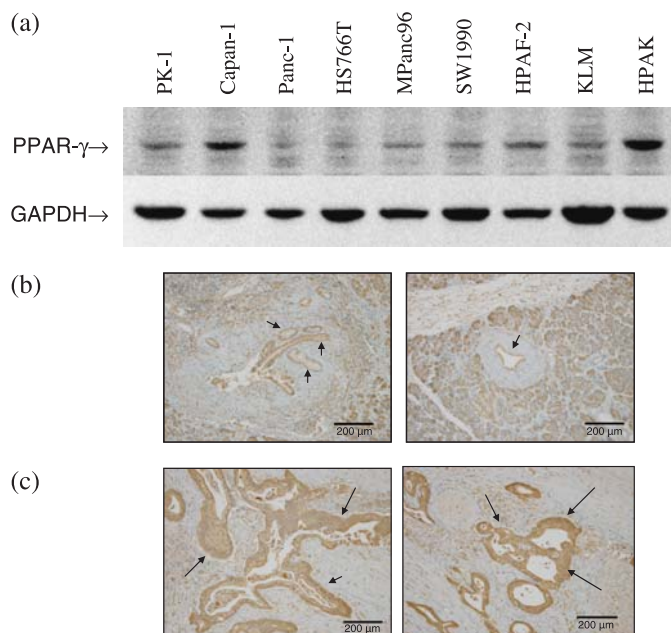


Fig. 1. Expression of peroxisome proliferator-activated receptor γ (PPAR γ) in pancreatic cancer cells. (a) Western blots showing PPAR γ expression in various pancreatic ductal adenocarcinoma cell lines, as well as a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control. Immunohistochemical staining of (b) normal ductal epithelium (left and right panels) and (c) pancreatic ductal adenocarcinoma (left and right panels) with anti-PPAR γ antibody. Arrows mark staining of normal pancreatic ductal epithelium in (b) and pancreatic ductal adenocarcinoma in (c).

specific pathogen-free environment. Experiments were carried out according to the guidelines of Yokohama City University. Six-week-old mice were used in this experiment. To assay metastatic capability, viable cancer cells were suspended in serum-free medium, and 20- μ L aliquots of cell suspension containing 2×10^6 cells were inoculated into the spleens of SCID mice under anesthesia. After inoculation, the mice were randomized into two treatment groups ($n = 6$) and one control group ($n = 6$). Administration of T0070907 (5 mg/kg/day) to each treatment group began 1 day after cell inoculation and continued daily for 4 weeks. Four weeks after inoculation, the mice were killed and autopsied immediately. Liver metastasis was measured by counting macroscopic lesions, and measuring them to calculate tumor volume:

$$\text{length}/2 \times \text{width}/2 \times \text{height}/2 \times 4/3 \times \pi. \text{ (28)}$$

Examination of hematoxylin–eosin-stained sections of each lesion resulted in assessments of histopathological alterations in liver metastases.

Results

Expression of PPAR γ in pancreatic ductal adenocarcinoma cells. Western blotting of PPAR γ with the E8 antibody revealed a specific band between 50 and 60 kDa present in all PDAC cell lines examined (Fig. 1a). Among these lines, steady-state levels of PPAR γ protein were highest in Capan-1 and HPAK cells, and lowest in Panc-1 and HS766T cells. Immunohistochemical staining with a PPAR γ -specific antibody demonstrated that PPAR γ expression in PDAC tissues (Fig. 1c) was similar to normal pancreatic ductal epithelium (Fig. 1b).

Peroxisome proliferator-activated receptor γ inhibitors reduce migration of PDAC cells. The effect of the PPAR γ antagonist

T0070907 on PDAC cell migration was measured using *in vitro* wound-filling assays. The migration of wounded cells treated with T0070907 was inhibited significantly (Fig. 2a,b) relative to untreated, wounded Panc-1 and Capan-1 cells. Among cells transfected with PPAR γ siRNA to reduce PPAR γ expression levels, Capan-1 cell migration was more severely inhibited than Panc-1 (Fig. 2c,d). These results indicate that chemical inhibitors or inhibitory siRNA molecules that reduce PPAR γ activity lead to inhibition of wound filling. Migration of Capan-1 and Panc-1 cells in the absence or presence of several concentrations of T0070907 were also measured in 24-h transwell migration assays (Fig. 3). In both cell lines, the presence of T0070907 reduced cell migration significantly and in a dose-dependent manner (Fig. 3a,c). Reduced migration of cells with PPAR γ siRNA relative to untreated controls (Fig. 3b,d) demonstrates that transwell migration is inhibited specifically by a reduction in PPAR γ activity.

Effect of PPAR γ inhibitor on cell proliferation and apoptosis. To investigate whether chemical inhibition of PPAR γ affects cancer cell proliferation and apoptosis, we used MTT assays to measure cell proliferation and apoptosis in cultured Panc-1 and Capan-1 PDAC cell lines. No significant changes in cell proliferation (Fig. 4a,b) or apoptosis (Fig. 4c,d) were observed in T0070907-treated versus untreated PDAC cells. These results demonstrate that suppression of cell proliferation or apoptosis is not necessarily consequent to T0070907-mediated suppression of PDAC cell motility.

Inhibition of PPAR γ alters the subcellular localization of p120ctn. Association of p120ctn with the intracellular domains of cadherins promotes cell–cell adhesion and cell motility by regulating the activation of Rho GTPases.^(29,30) Because cytoplasmic p120ctn is the only known activator of Rho GTPases that functions in cell motility, the ratio of cadherin-bound p120ctn to p120ctn in the cytoplasmic pool is an important factor regulating motility. To examine the involvement of p120ctn in T0070907-mediated suppression of cell motility, we used immunocytochemical analyses to examine the subcellular distribution of p120ctn in PDAC cells. In T0070907-treated Capan-1 cells, p120ctn was found predominantly on the plasma membranes (relative to more free p120ctn in the cytoplasm of untreated cells) (Fig. 5a). In contrast, there were no significant changes in the distribution of p120ctn in T0070907-treated Panc-1 cells (data not shown). The intracellular distributions of PPAR γ and p120ctn did not overlap (merged) (Fig. 5a).

Although western blots of fractionated cells revealed that cytoplasmic p120ctn levels decreased in T0070907-treated Capan-1 cells (Fig. 5b), no significant change in distribution was observed between untreated and treated Panc-1 cells (data not shown). These results indicate that in Capan-1 cells, PPAR γ inhibition increases the relative amount of cadherin-bound p120ctn. We speculate that relatively low levels of PPAR γ expression in Panc-1 cells may confound our ability to measure any similar change in p120ctn subcellular localization following T0070907 treatment. To investigate whether PPAR γ activity in Capan-1 cells is inhibited by low concentrations of T0070907, the effect of a range of T0070907 concentrations on PPARE-dependent transcription was measured (Fig. 5c). With 0.1 μ mol/L T0070907, PPARE-dependent transcription in Capan-1 cells was inhibited to approximately half maximum.

Peroxisome proliferator-activated receptor γ inhibitor suppresses the activity of Rac-1 and Cdc42. Previous reports suggest that p120ctn affects cell motility in association with Rac1 and Cdc42 Rho GTPases.^(30–33) The activities of Rac1 and Cdc42 GTPases were measured in lysates of T0070907-treated and -untreated Capan-1 cells using a GST pull-down target that interacts specifically with active GTPases. In T0070907-treated cells, we observed a significant decrease in the percent-active fractions of Rac1 and Cdc42 GTPases (Fig. 6).

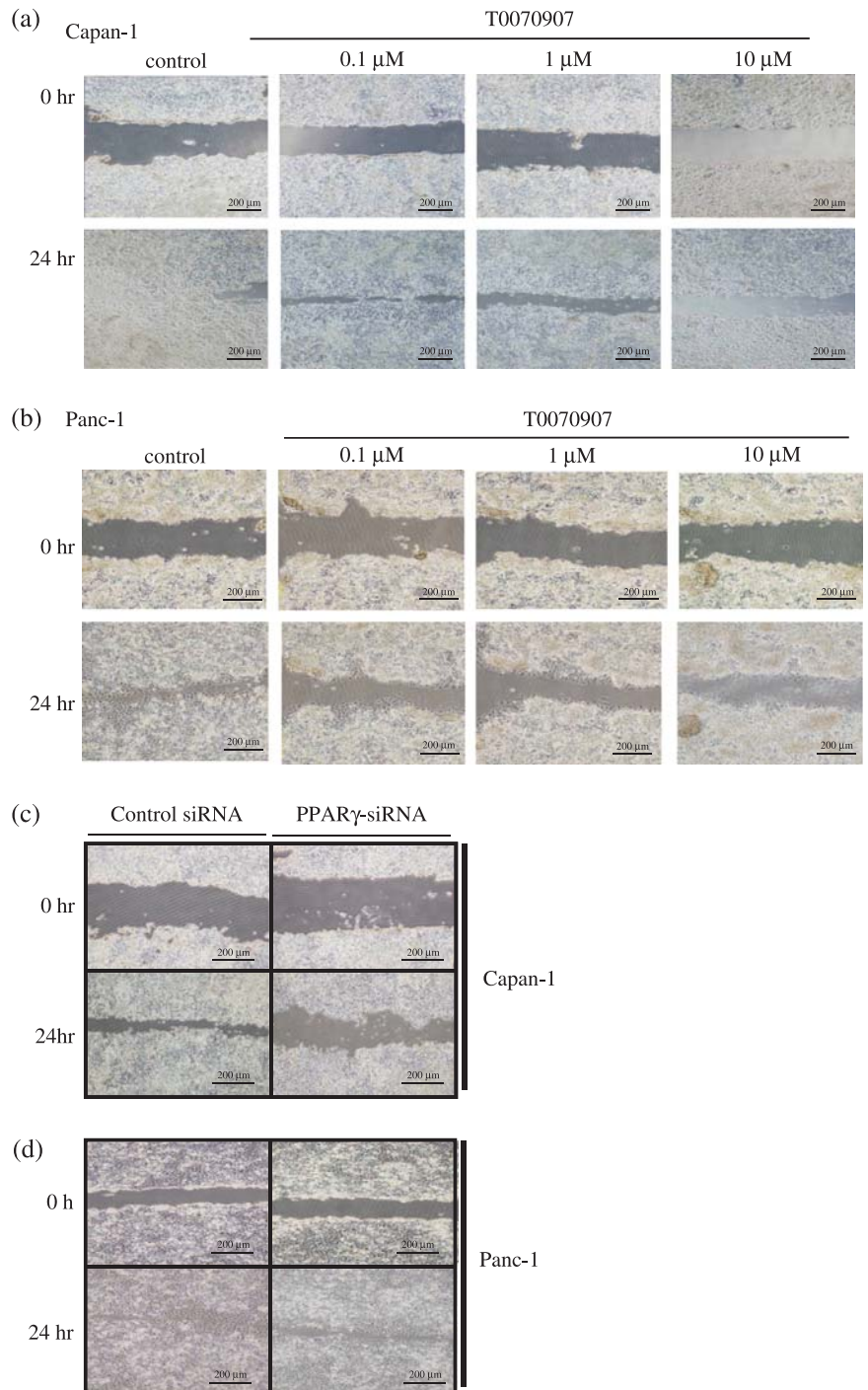


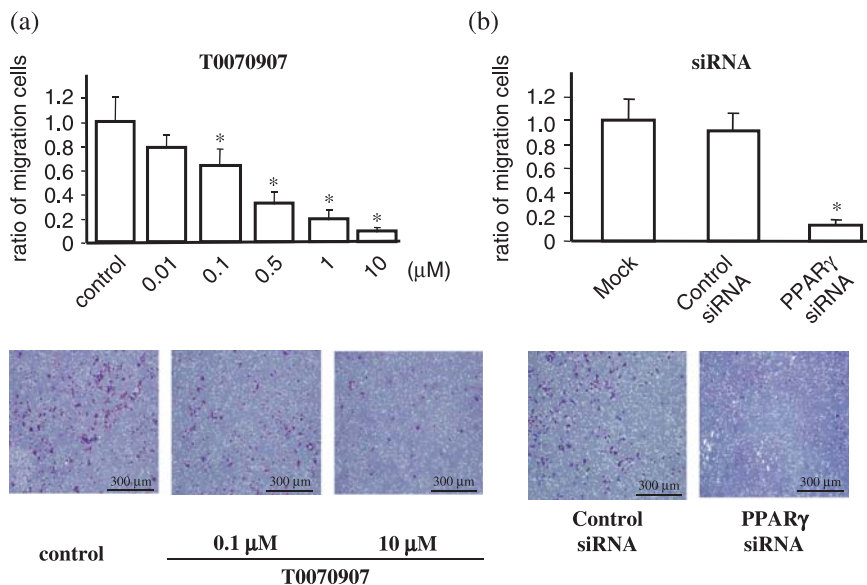
Fig. 2. Peroxisome proliferator-activated receptor γ (PPAR γ) inhibition reduces the wound-filling ability of PDAC cells. Wound-filling assays in (a) Capan-1 and (b) Panc-1 cells treated with various concentrations of T0070907 for 24 h. In both lines, all concentrations of PPAR γ inhibitor result in slower cell migration into wound areas. (c) Capan-1 and (d) Panc-1 cells transfected with PPAR γ small interfering RNA (siRNA) also migrated more slowly into wound areas than cells transfected with control siRNA.

Peroxisome proliferator-activated receptor γ inhibitor reduces liver metastasis in a mouse xenograft model. To investigate whether PPAR γ inhibitors affect metastatic cell spreading, we tested the ability of T0070907 to reduce metastatic tumor formation in a Capan-1/SCID mouse xenograft model. Capan-1 cells were injected into the spleens of SCID mice, and the number and size of metastatic lesions in livers were measured after 4 weeks (Fig. 7). Mice treated orally with 5 mg/kg/day of T0070907 contained two-thirds fewer metastatic foci ($P < 0.05$), with an average tumor volume of only 12% of tumors in control mice ($P < 0.05$) (Table 1). Serum L-alanine aminotransferase (ALT) levels were within the normal range in the all mice (Suppl. Fig. S1).

Discussion

We demonstrated that levels of PPAR γ expression vary among pancreatic adenocarcinoma cell lines (Fig. 1) and tested the effect of the PPAR γ -specific inhibitor T0070907 on PDAC cells. In Capan-1 and Panc-1 cells, both T0070907 and PPAR γ siRNA suppressed cell motility, migration, and invasion, but did not inhibit cell proliferation (Figs 2–4). These results suggest strongly that PPAR γ plays a crucial role in PDAC cell motility, migration, and invasion. Elucidating the mechanism that underlies cell motility is of clinical importance as a means for controlling tumor cell invasion, dissemination, and metastasis in patients with pancreatic cancer.

Capan-1



Panc-1

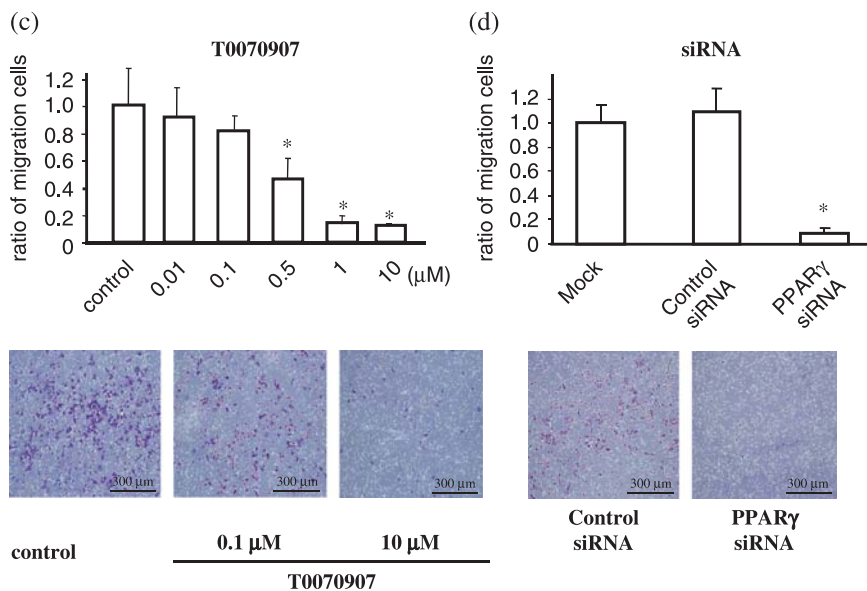


Fig. 3. Peroxisome proliferator-activated receptor γ (PPAR γ) inhibition reduces migration of PDAC cells. Cell migrations were estimated from transwell migration assays, in which migrated cells are stained violet and membrane pores can be seen as white dots. Relative to control-treated cells, (a) Capan-1 and (c) Panc-1 cell migration decreased significantly and in a dose-dependent manner in response to increasing concentrations of PPAR γ inhibitor T0070907 (* $P < 0.05$). (b,d) Significant decreases in cell migration were also observed in PPAR γ small interfering RNA (siRNA)-transfected cells relative to control siRNA-transfected cells.

Table 1. Effects of peroxisome proliferator-activated receptor γ inhibitor (T0070907) on liver metastasis of Capan-1 cells

Incidence	Number of metastatic colonies (mean \pm SD)	Total tumor volume (mm ³) (mean \pm SD)
Vehicle	3.50 \pm 1.05	738.6 \pm 415.7
T0070907 (5 mg/kg/day)	1.00 \pm 1.27	86.8 \pm 173.2
	$P < 0.05$	$P < 0.05$

Capan-1 cells were injected into the spleen of male severe combined immunodeficiency mice. One day after injection, three groups ($n = 6$) were randomized into vehicle or 5 mg/kg/day T0070907. After 4 weeks, livers were harvested, and the number of metastases and total tumor volume of all metastatic lesions was determined.

Following treatment with T0070907 PPAR γ inhibitor, p120ctn was found predominantly in Capan-1 cell membranes. Recent reports demonstrate that p120ctn associates with all classic cadherin subtypes, and is involved in the regulation of cell motility and cell

adhesion.⁽²⁶⁾ p120ctn is known to also regulate actin cytoskeleton configuration. We did not observe colocalization of PPAR γ and p120ctn expression in PPAR γ inhibitor-treated or -untreated cells, indicating that PPAR γ may not interact directly with p120ctn.

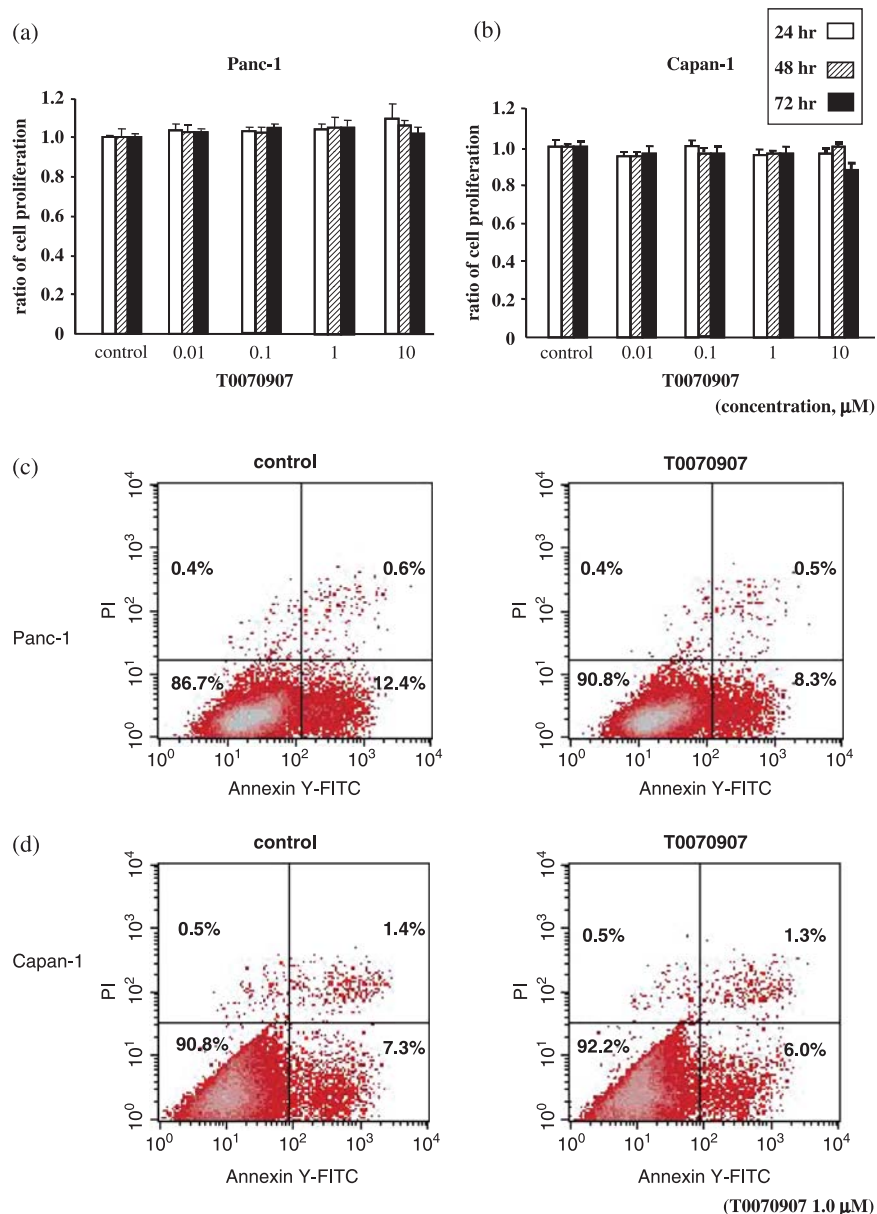


Fig. 4. Effect of a peroxisome proliferator-activated receptor γ (PPAR γ) inhibitor on cell proliferation and apoptosis in PDAC cells. Cell proliferation was calculated from 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays. (a) Panc-1 and (b) Capan-1 cells treated with 1.0 $\mu\text{mol/L}$ T0070907 for 24, 48, and 72 h showed no significant change in MTT values relative to control cells (y -axis values represent the ratio of MTT optical density readings from treated and untreated cells; columns represent ratio mean \pm SD). Apoptotic cell counts were measured by fluorescence activated cell sorting (FACS) after treatment of (c) Panc-1 or (d) Capan-1 cells without or with 1.0 $\mu\text{mol/L}$ T0070907 for 24 h. T0070907 treatments did not result in any significant changes in the percentage of apoptotic cells.

Ras-homologous GTPases, which localize to membranes in a GDP-bound state, are activated to a GTP-bound state upon stimulation of cell-surface receptors. Upon activation, Rho GTPases bind effectors that trigger specific cellular responses. As Rho proteins are known to play essential roles in signaling events that regulate cadherin-dependent motility, specific inhibitors of individual Rho functions (notably RhoA-, RhoB-, Rac1-, or Cdc42-related functions) could provide therapeutic benefits in controlling cancer metastasis. Indeed, compounds developed as specific inhibitors of the RhoA-effector molecule Rho-kinase have been demonstrated to exert antimetastatic activity *in vivo*.⁽²⁴⁾

The inactivation of Rac1 and Cdc42 that we observed in response to PPAR γ inhibition indicates that these molecules are involved in PPAR γ -mediated PDAC cell motility. We also demonstrated liver metastasis inhibition in response to PPAR γ inhibition in an *in vivo* metastatic model. Previous reports have demonstrated an induction in apoptosis in response to PPAR γ inhibition in other epithelial tumor lines.^(19–21) Anoikis, which is a loss of adhesion-induced apoptosis, was also reported in response to PPAR γ inhibition by T0070907; however, concen-

trations greater than 10 $\mu\text{mol/L}$ T0070907 have been shown to be required to induce anoikis in a variety of carcinoma cell lines. In the present study, we observed a significant inhibitory effect of T0070907 on cell migration at much lower T0070907 concentrations (0.01–1 $\mu\text{mol/L}$; Fig. 5c) that had no effect on cell proliferation or cell death as measured in MTT and apoptosis assays (Fig. 4). Our findings at low concentrations of T0070907 suggest that inhibition of cancer cell migration is due to the specificity of T0070907's pharmacological effect on PPAR γ , and not by anoikis, which is induced at higher concentrations of PPAR γ inhibitor. The relatively low concentrations of T0070907 required for inhibition and the dose-dependent effect of the inhibitor on cell migration make it unlikely that inhibition was non-specific.

Transwell migration and wound-filling assays in both Capan-1 and Panc-1 pancreatic cancer cell lines demonstrated the inhibitory effects of PPAR γ on cell motility *in vitro*. In contrast, the effects of PPAR γ inhibitor on p120^{ctn} subcellular localization, and on Rac1 and Cdc42 GTPase activities, were exclusive to Capan-1 cells, and not seen in Panc-1. We speculate that relative

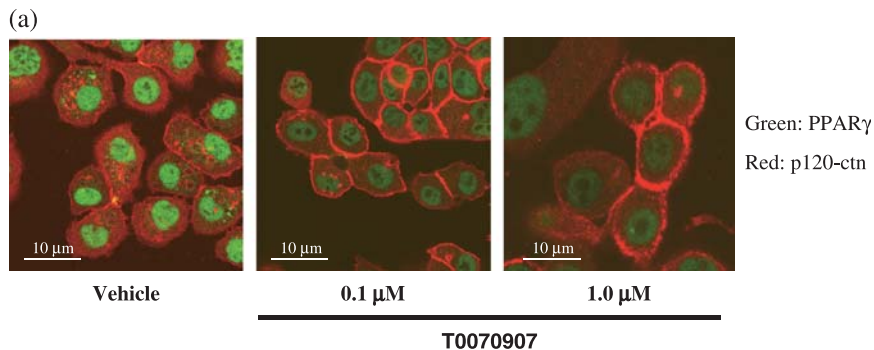


Fig. 5. Peroxisome proliferator-activated receptor γ (PPAR γ) inhibitor T0070907 affects the subcellular localization of p120 catenin (p120ctn) in Capan-1 cells. (a) The subcellular distribution of p120ctn in Capan-1 control cells or those treated with 0.1 or 1.0 μ mol/L T0070907 were compared following immunostaining with anti-p120ctn (red) and anti-PPAR γ (green). Although p120ctn accumulated in the cytoplasm of vehicle-treated cells, it localized predominantly to cell membranes in T0070907-treated Capan-1 cells. Merged images of PPAR γ and p120ctn immunohistochemical staining patterns show no colocalization. (b) Western-blot analysis of cytosolic protein extracts with p120ctn antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control antibody. T0070907 treatment resulted in reduced signal strength from the cytosolic p120ctn band. Changes in the cytosolic p120ctn expression can be seen by the reduction in the ratio of p120ctn : GAPDH signal. Bars represents the mean ratio of p120 : GAPDH signal strength \pm SD. * P < 0.05. (c) Capan-1 cells transfected with a PPAR γ -response element (PPRE)-luciferase reporter plasmid were stimulated with 1 mmol/L rosiglitazone (synthetic ligand of PPAR γ) in the presence of concentrations of T0070907 shown. Luciferase activity was measured after 16 h of treatment. Bars represent relative PPRE activity as measured by the ratio of PPAR γ -dependent luciferase activity in treated cells relative to untreated cells.

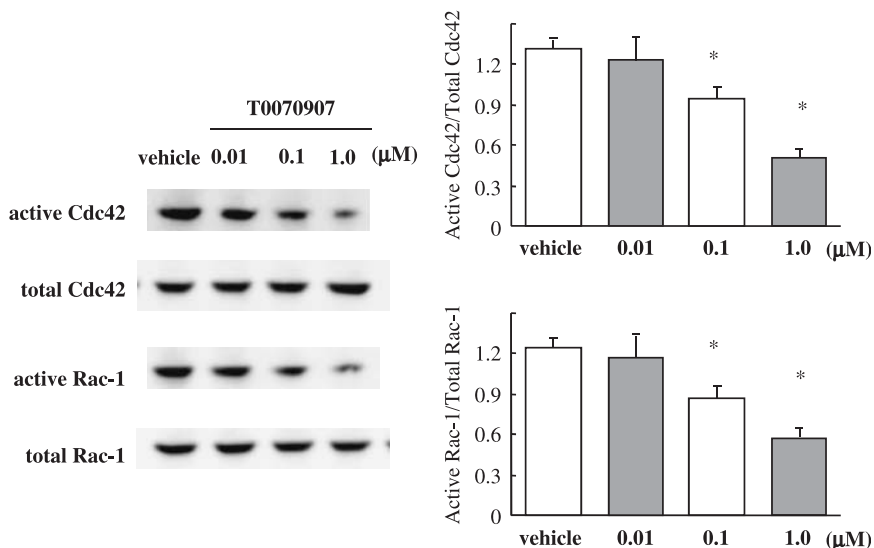
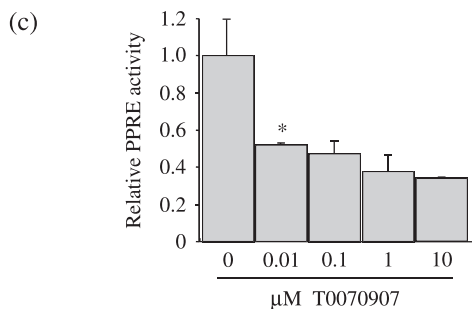
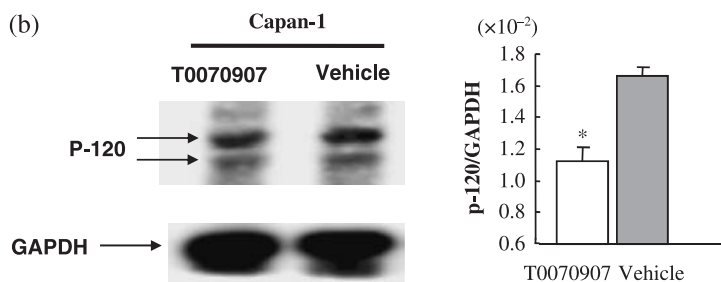
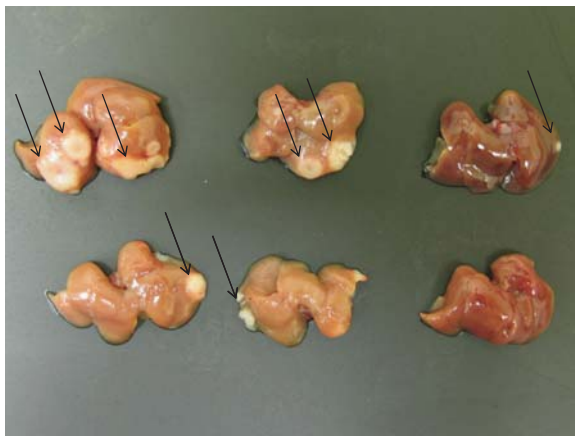


Fig. 6. Effect of the peroxisome proliferator-activated receptor γ (PPAR γ) inhibitor T0070907 on the activity of Rac-1 and Cdc42 GTPases in Capan-1 cells. GTPase activity for Rac-1 and Cdc42 was measured using GST pull-down assays that are capable of distinguishing the percentage of the active fraction (relative to total) of Rac-1 and Cdc42 in lysates of Capan-1 cells. Treatment with T0070907 (0.1 and 1.0 μ mol/L) resulted in a significant decrease in the percentage of active Rac-1 and Cdc42. Each column represents the mean \pm SD. The experiments were carried out six times. * P < 0.05.



Vehicle T0070907
(1 mg/kg/day) T0070907
(5 mg/kg/day)

Fig. 7. Peroxisome proliferator-activated receptor γ (PPAR γ) inhibitor T0070907 reduces the number and volume of metastases in a murine xenograft model. Numbers and areas of metastases (arrows) in the liver decreased markedly in mice treated with T0070907 relative to control mice.

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to Panc-1 cells, steady-state levels of PPAR γ in Capan-1 cells are much higher (Fig. 1a), which may contribute to these discrepancies. This hypothesis is supported by a recent clinical report in which a significant positive association was measured between high levels of PPAR γ expression in pancreatic cancer cells and shorter overall survival time.⁽¹⁸⁾ Further investigation will be required to better understand the mechanism of PPAR γ inhibition of pancreatic cancer cell motility, invasion, and metastasis.

In conclusion, we have demonstrated that inhibition of PPAR γ in pancreatic cancer cells decreases cell motility by altering p120ctn localization and suppressing Rac1 and Cdc42 Rho GTPase activities. PPAR γ could function as a novel therapeutic target for controlling cancer cell dissemination or metastasis.

Acknowledgments

We thank Machiko Hiraga and Yuko Satoh for their technical assistance. The present work was supported in part by a Grant-in-Aid for research on the Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare, Japan to A.N., a grant from the National Institute of Biomedical Innovation to A.N., a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (KIBAN-B) to A.N., a grant from the Princess Takamatsu Cancer Research Foundation to A.N., and a grant from the Japanese Human Science Research Foundation to A.N.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Serum levels of alanine aminotransferase (ALT) in the T0070907-treated metastatic model mice. There was no liver injury at this dose of T0070907.

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