



• BASIC RESEARCH •

## Antidiabetic thiazolidinediones induce ductal differentiation but not apoptosis in pancreatic cancer cells

Elisabetta Ceni, Tommaso Mello, Mirko Tarocchi, David W Crabb, Anna Caldini, Pietro Invernizzi, Calogero Surrenti, Stefano Milani, Andrea Galli

Elisabetta Ceni, Stefano Milani, Mirko Tarocchi, Calogero Surrenti, Andrea Galli, Gastroenterology Unit, Department of Clinical Pathophysiology, University of Florence, Florence, Italy  
David W Crabb, Departments of Medicine and of Biochemistry and Molecular Biology, Indiana University School of Medicine, IN, USA

Anna Caldini, Clinical Chemistry Laboratories, Careggi Hospital, Florence, Italy

Pietro Invernizzi, Department of Medicine Ospedale San Paolo, University of Milan, Milan, Italy

Tommaso Mello, Farmacogenomic Foundation FiorGen, Florence, Italy

Supported by Ministero dell'Università, della Ricerca Scientifica e Tecnologica, (MURST)

Correspondence to: Andrea Galli, M.D., Ph.D., Gastroenterology Unit, Department of Clinical Pathophysiology, University of Florence, Viale Morgani 85, 50134 Firenze Italy. [a.galli@dfc.unifi.it](mailto:a.galli@dfc.unifi.it)  
Telephone: +39-554271294 Fax: +39-554271297

Received: 2004-07-09 Accepted: 2004-10-13

### Abstract

**AIM:** Thiazolidinediones (TZD) are a new class of oral antidiabetic drugs that have been shown to inhibit growth of some epithelial cancer cells. Although TZD were found to be ligands for peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), the mechanism by which TZD exert their anticancer effect is presently unclear. In this study, we analyzed the mechanism by which TZD inhibit growth of human pancreatic carcinoma cell lines in order to evaluate the potential therapeutic use of these drugs in pancreatic adenocarcinoma.

**METHODS:** The effects of TZD in pancreatic cancer cells were assessed in anchorage-independent growth assay. Expression of PPAR $\gamma$  was measured by reverse-transcription polymerase chain reaction and confirmed by Western blot analysis. PPAR $\gamma$  activity was evaluated by transient reporter gene assay. Flow cytometry and DNA fragmentation assay were used to determine the effect of TZD on cell cycle progression and apoptosis respectively. The effect of TZD on ductal differentiation markers was performed by Western blot.

**RESULTS:** Exposure to TZD inhibited colony formation in a PPAR $\gamma$ -dependent manner. Growth inhibition was linked to G1 phase cell cycle arrest through induction of the ductal differentiation program without any increase of the apoptotic rate.

**CONCLUSION:** TZD treatment in pancreatic cancer cells

has potent inhibitory effects on growth by a PPAR-dependent induction of pancreatic ductal differentiation.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

**Key words:** Thiazolidinediones; Pancreatic cancer; PPAR $\gamma$ ; Cancer growth; Differentiation

Ceni E, Mello T, Tarocchi M, Crabb DW, Caldini A, Invernizzi P, Surrenti C, Milani S, Galli A. Antidiabetic thiazolidinediones induce ductal differentiation but not apoptosis in pancreatic cancer cells. *World J Gastroenterol* 2005; 11(8): 1122-1130  
<http://www.wjgnet.com/1007-9327/11/1122.asp>

### INTRODUCTION

Pancreatic cancer is a devastating disease characterized by an increased incidence in western industrialized countries, an extremely poor median survival of 4-6 mo after diagnosis<sup>[1]</sup>, and limited therapeutic options<sup>[2]</sup>.

The majority of pancreatic cancers arises from the pancreatic duct cells and is characterized by uncontrolled growth, inability to express the differentiated features of normal duct cells and progressive accumulation of multiple genetic abnormalities<sup>[3]</sup>.

It has been suggested that the pharmacological induction of cellular differentiation might be an alternative to conventional tumor chemotherapy. The activation of the terminal differentiation program in genetically abnormal tumor cells is strictly associated with irreversible growth arrest<sup>[4]</sup>. In pancreatic cancer cells, retinoids, for example, induce differentiation and inhibit growth by activation of their specific nuclear receptors<sup>[5,6]</sup> suggesting that other nuclear receptors involved in the regulation of cellular differentiation might be targets for novel therapeutic strategies of pancreatic cancer.

Thiazolidinediones (TZD) such as pioglitazone (PGZ) and rosiglitazone (RGZ) are a new class of antidiabetic drugs, which attenuate the insulin resistance associated with obesity, hypertension and impaired glucose tolerance in humans as well as in several animal models of non-insulin-dependent diabetes mellitus<sup>[7]</sup>. TZD were found to be ligands for peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), a member of the nuclear receptor superfamily of ligand-dependent transcription factors that is predominantly expressed in adipose tissue although it is also expressed in other tissue at much lower levels<sup>[8-10]</sup>. In adipose tissue PPAR $\gamma$  functions as a master regulator of adipogenesis and

its ligand-mediated activation in fibroblasts induces adipocyte differentiation and lipid storage<sup>[11,12]</sup>. TZD activate PPAR $\gamma$  and promote association with 9-cis retinoic acid receptor (RXR) to form functional heterodimers, which recognize their cognate response element at the level of target genes<sup>[13,14]</sup>.

Several recent studies have indicated that TZD may have anticancer properties in a variety of different epithelial malignancies including breast, prostate and gastrointestinal cancers<sup>[15-17]</sup>. Treatment of cultured breast and colon cancer cells with TZD resulted in a reduction in growth rate and induction of apoptosis<sup>[18,19]</sup>. Furthermore, human colorectal cancer cells implanted in nude mice were shown to grow more slowly in mice treated with TZD, with a 50% reduction of tumor volume<sup>[20]</sup>. In addition loss-of-function mutation of the PPAR $\gamma$  gene has been found in some human colon and thyroid carcinomas<sup>[21,22]</sup>. As a consequence, PPAR $\gamma$  has become a molecular target for anticancer drug development, and TZD have been proposed for therapy of PPAR $\gamma$ -expressing tumors.

Although preliminary evidence has shown that, troglitazone, the first TZD marketed for use in humans, inhibited pancreatic cell proliferation<sup>[23,24]</sup>, the mechanism by which these drugs inhibit cell growth in these cells has not been conclusively established.

In this study we demonstrated that exposure of pancreatic cancer cells to TZD inhibited anchorage-independent growth with a PPAR $\gamma$ -dependent differentiation-inducing mechanism. Surprisingly the ductal pro-differentiation program induced by PPAR $\gamma$  activation was not associated with apoptosis in these cells.

## MATERIALS AND METHODS

### Materials

Most chemicals and supplies were purchased from Sigma Chemical Company (St. Luis, MO). Nitrocellulose and Nytran were from Schleicher and Schuell, Inc., (Keene, NH). Agarose, trypsin, all restriction endonucleases, DNA-modifying enzymes, and tissue culture media were purchased from Gibco BRL (New Brunswick, NJ). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). D-threo-[dichloroacetyl-1,2-<sup>14</sup>C]-chloramphenicol was purchased from New England Nuclear (Boston, MA). Rosiglitazone and pioglitazone were from SmithKline Beecham Pharmaceuticals (Welwyn, UK) and Takeda Chemicals (Tokyo, Japan) respectively.

### Tissue sample and cell cultures

The pancreatic adenocarcinoma cell lines PANC-1, CAPAN-2, and HPAC were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Dulbecco's modified Eagles medium (DMEM; GIBCO Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco). Culture medium was supplemented with 50 U/mL penicillin and 50  $\mu$ g/mL streptomycin. All experiments were performed in phenol red-free medium.

### Tumor clonogenic assay

Pancreatic cancer cells were trypsinized and disaggregated

through a 19-gauge needle into a single-cell suspension, as evaluated by microscopy. Cells  $2 \times 10^4$  were mixed with DMEM/5% dialyzed FBS containing 0.3% agarose and TZD at the concentration indicated. Cells were then layered over a solid base of 0.5% agarose in the same medium, in 60-mm dishes. The cultures were incubated in humidified 50 mL/L CO<sub>2</sub>/95% air at 37 °C. After 14 d the colonies were counted by an Omnicon 3 600 Colony Counter and photographed.

### Cell cycle analysis

Cells ( $4 \times 10^5$ ) were exposed to TDZ for 4 d in medium supplemented with 5% dialyzed FBS. Total cells both in suspension and adherent, were collected, washed, suspended in cold PBS, and stained in trypan blue. Both blue and non-blue cells were counted. The cells were adjusted to  $1 \times 10^6$  viable cells/mL and fixed in 2:1 ratio (v/v) in methanol overnight before staining with propidium iodide. Cell cycle status was analyzed with Becton Dickinson Flow Cytometer and CellFIT Cell-Cycle Analysis software.

### Protein extract and Western blot

Whole-cell proteins were extracted from the different adenocarcinoma cell lines. Cells were cultured in the presence or absence of test agents and were homogenized in Laemmli buffer<sup>[25]</sup>. Nuclear proteins were isolated from treated and untreated cells based on micro preparation methods<sup>[26]</sup>. The nuclear extracts were suspended in 20 mmol/L HEPES (pH 7.9), 40 mmol/L NaCl, 1.5 MgCl<sub>2</sub>, 0.2 mmol/L ethylenediaminetetraacetic acid, 25% glycerol, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride. Lysates were centrifuged at 20 000 g for 10 min at 4 °C and supernatants were frozen in liquid nitrogen and stored at -80 °C until use. Nuclear and whole-cell extracts (40  $\mu$ g protein) were fractionated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro blotted onto nitrocellulose filters. Proteins were detected by incubating the filters with the following primary antibody: mouse anti-human PPAR $\gamma$  (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), sheep anti-human carbonic anhydrase II (1:500)<sup>[27]</sup> cytokeratin 7 (1:1 500) (Novocastra Lab, Newcastle UK), p21 (1:1 000) (Santa Cruz Biotechnology, Santa Cruz, CA) and p27 (1:1 000) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human bcl-2, bax, and bcl-xl (1:1 000) (Transduction, Laboratories, San Diego CA) mouse anti human bax (1:1 000), and mouse anti-human bak (1:1 000) (CalBiochem, San Diego CA). Detection of the protein bands was performed using the Amersham ECL kit (Arlington Heights, IL).

### RNA extraction and RT-PCR

Total RNA was extracted from cultured cells and by guanidinium-phenol-chloroform methods of Chomczynski and Sacchi<sup>[28]</sup> with minor modifications<sup>[29]</sup>. One mg of RNA from tumor cells was reverse transcribed with Molony murine leukemia virus (MMLV) reverse transcriptase (Life Technologies Inc., Paisley, UK) at 42 °C for 60 min in a 20- $\mu$ L mixture in the presence of random hexamers. The nucleotide bases used for human PPAR $\gamma$  were 5'-TCTGGCCACC-AACTTTGGG-3' and 5'-CTTCACAAGCATGA-

ACTCCA-3'. Two  $\mu\text{L}$  of a reverse-transcribed mixture was subjected to PCR in a 20- $\mu\text{L}$  reaction solution [10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.0 mmol/L  $\text{MgCl}_2$ , 0.01% gelatin, 20 mmol/L deoxynucleotide triphosphate, 0.5 units of Taq polymerase (Life Technologies Inc., Paisley, UK), and 0.25 pmol of primers]. Twenty-five cycles of reaction at 94 °C for 50 s, 60 °C for 45 s, and 72 °C for 90 s were carried out by DNA thermal cycler (Perkin-Elmer Cetus Norwalk, CT). Efficiency of RT was controlled in each sample by PCR amplification of human  $\beta_2$ -microglobulin (5'-GCAAAAGATGAGTATGCCTG-3', 5'-TTCACCTCAATCCAAATGCGG-3').

### Transient transfection of culture cells

Cells were transfected at the density of  $5 \times 10^5$  cells/60 mm dish with 2.5  $\mu\text{g}$  of peroxisome proliferator response element (ARE)<sub>3</sub>-tk-luciferase reporter plasmid (containing 3 copies of the PPRE from the adipocyte lipid-binding protein (aP2) gene ligated to a herpes simplex thymidine kinase promoter upstream of a luciferase gene)<sup>[30,31]</sup>, and 5  $\mu\text{g}$  of pSV2CAT (vector containing SV40 early promoter and enhancer sequences that drives a chimeric chloramphenicol acetyl transferase [CAT] gene) as an internal control by calcium phosphate precipitation. Total amount of DNA transfected was normalized with a carrier DNA (pcDNA3.1; Invitrogen Corporation, Carlsbad, CA). Four hours later the cells were exposed to PBS containing 15% glycerol for 3 min. The cells were rinsed twice with PBS and fresh serum supplemented with 5% dialyzed FBS was added. Twenty four hours after transfection cells were treated with TZD. Twenty four hours later, the cells were harvested, washed twice with PBS, and lysed in 150  $\mu\text{L}$  of buffer containing 25 mmol/L Tris, pH 7.8, 2 mmol/L ethylenediaminetetraacetic acid, 20 mmol/L dithiothreitol, 10% glycerol, and 1% Triton X-100. Fifty microliters of cell extract were incubated with luciferase assay reagent based on the original protocol of de Wet *et al*<sup>[32]</sup>. The number of relative light units with a 3-s delay and 30-s incubation were measured by Sirius1 luminometer (Berthold Detection System, Pforzheim/Germany). CAT activity was measured as described previously<sup>[33]</sup>. The conversion of chloramphenicol to its acetylated products was quantified on Ambis beta scanner (Ambis System, San Diego, CA).

### Generation of the PPAR $\gamma$ -expressing cell line

Human PPAR $\gamma$  cDNA was cloned into pLNCX, a retroviral vector driving expression of the cloned cDNA from the cytomegalovirus promoter and conferring resistance to G418. Retrovirus plasmid was transfected into a packaging cell line, PA317, and used to transduce HPAC cells as previously described<sup>[34]</sup>. In brief, PA317 were transfected with 10  $\mu\text{g}$  of the cloned construct (PPAR $\gamma$ -pLNCX) or the empty retroviral vector, using calcium phosphate precipitation followed by glycerol stock as described above. Twenty four hours after transfection the virus-containing supernatant was removed, filtered through a 45- $\mu\text{m}$  filter, and stored at -70 °C. A total of  $5 \times 10^5$  HPAC cells were infected with  $10^6$  cfu/mL virus in the presence of 6  $\mu\text{g}/\text{mL}$  polybrene. Twenty four hours later the cells were replated in duplicate and selected with 600  $\mu\text{g}/\text{mL}$  of the antibiotic

G418. After selection of G418-resistant cells, the clones were expanded and screened for PPAR $\gamma$  expression by Western blotting.

### Apoptosis assay

Following the indicated treatments, apoptosis was measured by a DNA fragmentation assay (Apo-Direct) as recommended by the manufacturer (PharMingen, San Diego, CA). Briefly, cells (adherent and floating) were fixed in 1% formaldehyde in PBS overnight. After washing,  $10^6$  fixed cells were incubated with terminal deoxynucleotidyl transferase enzyme (TdT) and FITC-dUTP for 90 min at 37 °C to label DNA breaks. Cells were rinsed, incubated in RNaseA/propidium iodide in the dark for 30 min at room temperature to stain total DNA, and then analyzed by flow cytometry. Cells doublets and clumps were eliminated from the analysis by gating.

### Statistical analysis

Data was expressed as mean  $\pm$  SD. Statistical correlation of data was checked for significance by ANOVA and paired Student's *t* test. The corresponding probability (*P*) is given.

## RESULTS

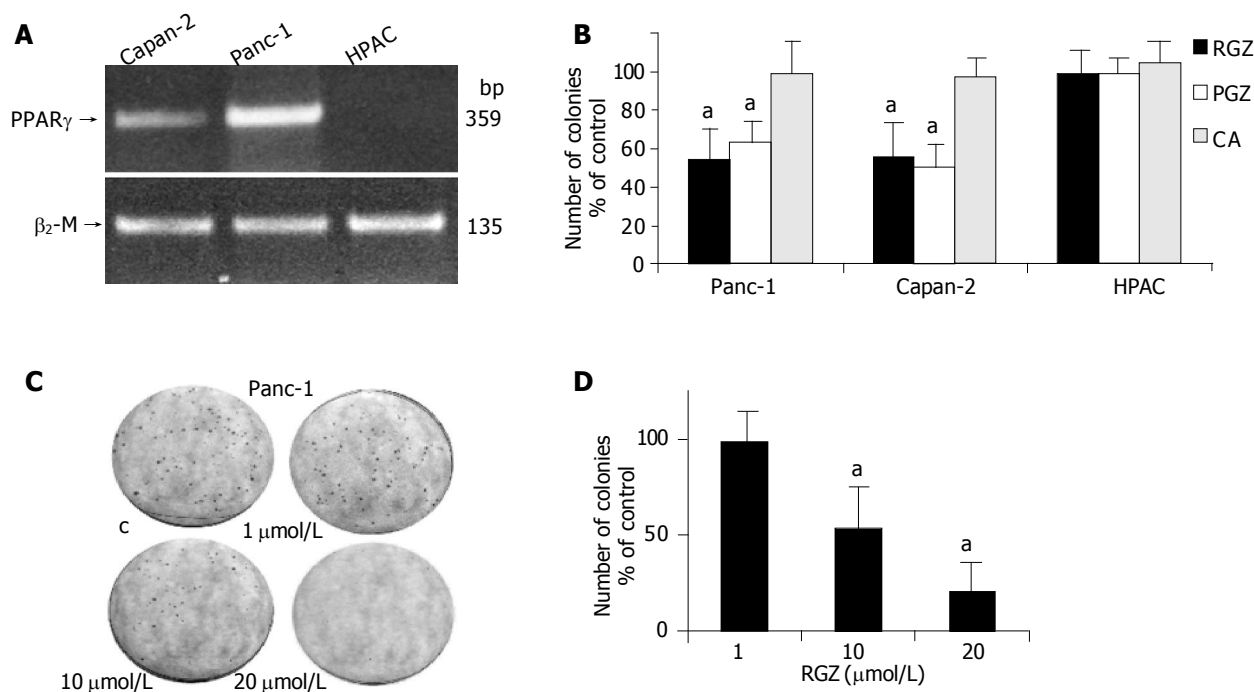
### TZD inhibited anchorage-independent growth of human pancreatic cancer cells with a PPAR $\gamma$ -dependent mechanism

We initially investigated the effect of TZD on pancreatic cancer cell growth. The effect of TZD on anchorage-independent growth was assessed by cloning cancer cells in soft agarose. Treatment with both TZD at the concentration of 10  $\mu\text{mol}/\text{L}$  resulted in a significant inhibition of colony formation by Panc-1 and Capan-2 cell lines, whereas drug treatment was ineffective in the PPAR $\gamma$  non-expressing HPAC cells (Figure 1A, B). Clofibric acid was used as a negative control. The dose dependency for the anchorage-independent growth inhibition was further characterized in Panc-1 cells. PGZ inhibited colony formation in a dose-dependent manner in Panc-1 cell line (Figure 1C). Moreover, the size of the colonies was significantly smaller in the RGZ-treated cells compared to cells treated with vehicle alone.

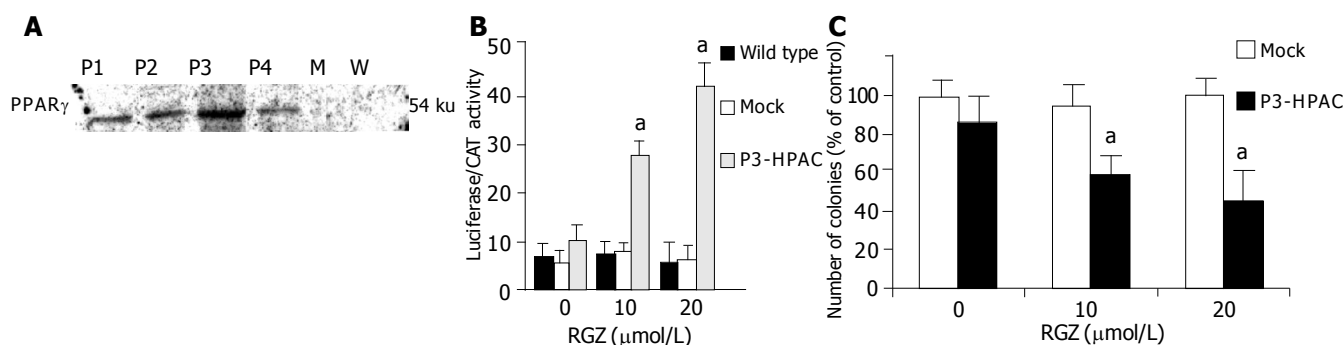
To confirm the role of PPAR $\gamma$  in TZD-induced growth arrest, we generated a PPAR $\gamma$ -expressing HPAC cells using transducing retroviruses. Four isolated HPAC clones showed selective overexpression of PPAR $\gamma$  protein compared to parental wild type and mock-transfected controls (Figure 2A). Of the selected clones, number 3 (P3-HPAC) was used for further studies. In these cells, PGZ induced the activity of the ARE-7<sub>3</sub>-tk-luc reporter in a dose-dependent manner (Figure 2B) and this effect was correlated to a significant inhibition of anchorage-independent growth (Figure 2C).

### PPAR $\gamma$ activation by TZD alters cell cycle progression but does not induce apoptosis in pancreatic cancer cells

Preliminary experiments evaluating trypan blue exclusion and lactate dehydrogenase leakage from pancreatic cancer cells into the culture medium showed that both TZD induced growth inhibition rather than cytotoxicity, because the number of vitally stained cells was higher than 90% in all experiments at any given time point. Based on this observation,



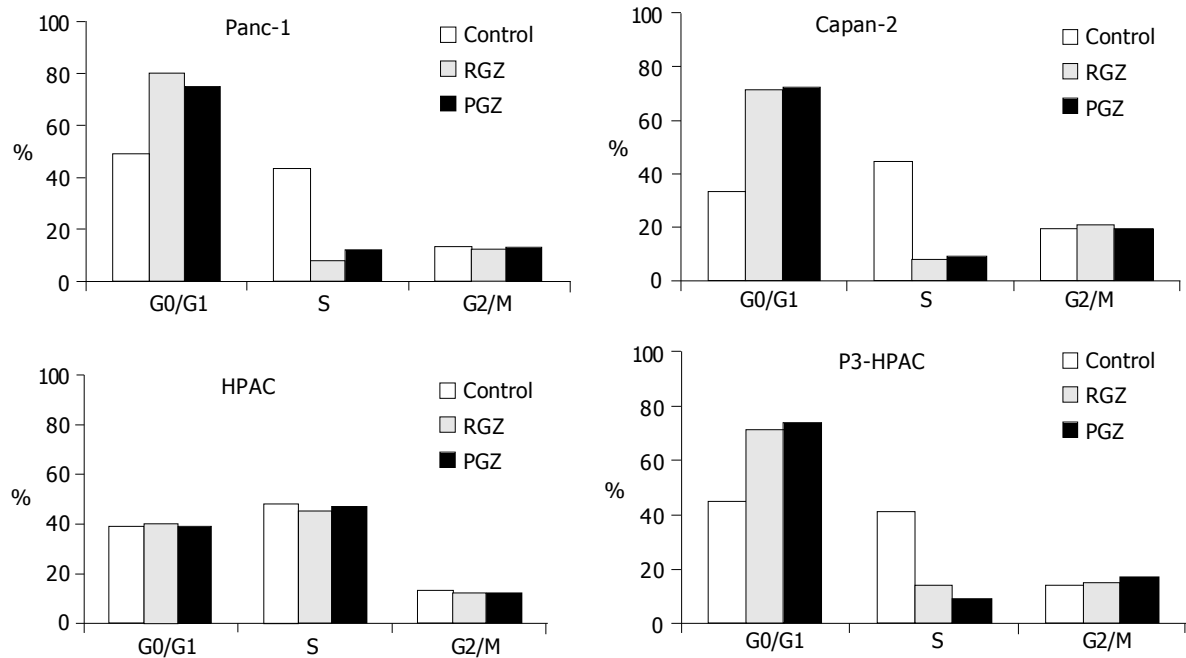
**Figure 1** Effect of TZD on anchorage-independent growth in human pancreatic adenocarcinoma cells. **A:** PPAR $\gamma$  expression in human pancreatic adenocarcinoma. One microgram of total RNA extracted from three pancreatic cancer cell lines (Capan-2, Panc-1, and HPAC) was reverse transcribed using random hexamers and amplified by polymerase chain reaction using specific primers for PPAR $\gamma$  and for  $\beta_2$ -microglobulin ( $\beta_2$ -M) as described in Methods. The reverse-transcription polymerase chain reaction products were electrophoresed on ethidium bromide-containing agarose gel; **B:** TZD inhibit anchorage-independent growth.  $2 \times 10^4$  cells were plated into media containing 0.3% agarose, supplemented with either 10  $\mu\text{mol/L}$  of TZD (RGZ or PGZ), or 1 mmol/L clofibrate (CA). After 14 d the number of colonies was determined and then expressed as the percentage of control cells treated with vehicle (DMSO) alone. The mean  $\pm$  SD of six independent experiments performed for each cell line in triplicate are shown.  $^a P < 0.05$  (or higher degree of significance) vs control; **C:** Dose-dependent inhibition of anchorage-independent growth by TZD in Panc-1 cells. Clonogenic assay of Panc-1 cells treated with the indicated concentrations of RGZ was performed as described in Methods. The number of colonies was then given as the percentage of control cells treated with vehicle alone. The mean  $\pm$  SD of five independent experiments performed for each in triplicate are shown.  $^a P < 0.05$  (or higher degree of significance) vs control.



**Figure 2** PPAR $\gamma$  expression and transcriptional activity in HPAC cells transduced with PPAR $\gamma$ -expressing retrovirus. **A:** HPAC cells were stable transduced by retrovirus driving expression of human PPAR $\gamma$  (hPPAR $\gamma$ -pLNCX) as described in methods. After selection with G418 four resistant clones were expanded and screened for hPPAR $\gamma$  expression. The cell extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted to nitrocellulose. The proteins (40  $\mu\text{g}$ ) were detected with antibody raised against human PPAR $\gamma$ . P1-P4 represent nuclear protein extracted from G418-resistant HPAC cell clones; M represents nuclear proteins from mock (pLNCX) transduced HPAC cells; W (wild type) represents nuclear proteins from untransduced, parental HPAC cells; **B:** After overnight attachment cells were transfected with ARE-7 $_3$ -tk-luciferase reporter plasmid and pSV $_2$ -CAT as internal control for transfection efficiency. Twenty-four hours after transfection cells were treated with RGZ at the indicated concentration. Twenty-four hours after treatment the cells were harvested for luciferase and CAT assay as described in Methods. The data is expressed as mean  $\pm$  SD for 4 replicate experiments performed in triplicate;  $^a P < 0.05$  vs control; **C:** Effect of TDZ treatment on anchorage-independent growth of HPAC cells transduced with PPAR $\gamma$ -expressing retrovirus. Clonogenic assay of P3-HPAC cells treated with the indicated concentration of RGZ was performed as described in materials and methods. The number of colonies was then given as the percentage of control cells treated with vehicle alone. The mean  $\pm$  SD of five independent experiments performed for each in triplicate are shown.  $^a P < 0.05$  vs Mock transduced cells.

we assessed the effect of RGZ and PGZ on cell cycle progression. Little change in cell distribution was observed at 12 h with 20  $\mu\text{mol/L}$  of TZD in all cell lines (not shown). In PPAR $\gamma$ -expressing cells both RGZ and PGZ increased

the proportion of cells in G $_0$ /G $_1$  phase at 24 h and the arrest persisted at later time points (Figure 3). The increased number of cells in G $_0$ /G $_1$  phase was mirrored by a proportional decrease of cells in S phase. No effect was

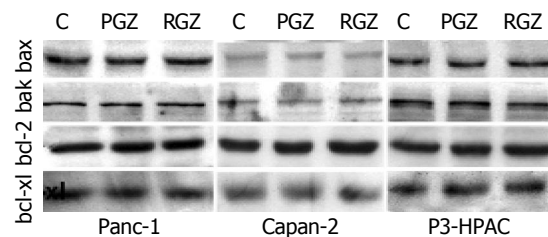


**Figure 3** Cell cycle phase distribution of pancreatic tumor cells treated with TZD. After overnight attachment cells were treated with 20  $\mu\text{mol/L}$  of TZD (RGZ or PGZ) for 72 h, followed by staining with propidium iodide and flow cytometric analysis of DNA content. The percentage of cells in each cell cycle phase was determined by analysis of the DNA content histograms using Modfit software as described in Methods. Data show the percentage of cells in each phase of cell cycle in a representative experiment. Similar results were obtained in at least three independent experiments.

documented in the PPAR $\gamma$ -deficient HPAC line, whereas the inhibition of cell cycle progression by TZD was restored in P3-HPAC cells. To determine whether the inhibitory effect of TZD was in part mediated by inducing apoptosis, cells were treated with RGZ or PGZ for 72 h before the analysis. The extent of apoptosis was measured by incorporation of FITC dUTP in the presence of TdT enzyme to detect DNA fragmentation. Both compounds had negligible effect on the extent of apoptosis at the higher concentration used (Table 1). In addition, the expression of the bcl-2 family members involved in regulating apoptosis was also determined in PANC-1, Capan-2 and P3-HPAC cells following treatment with RGZ or PGZ. After 24-h incubation with TZD, no change in the expression of the inducers of apoptosis, bax or bak, or inhibitors of apoptosis such as bcl-2 and its close homologue bcl-xL was detected (Figure 4). These results suggest that pancreatic tumor cells are relatively resistant to apoptosis induced by TZD.

**Table 1** Effect of TZD on apoptosis in pancreatic tumor cells. Extent of apoptosis following treatment of pancreatic cancer cell lines with 20  $\mu\text{mol/L}$  of TZD (RGZ or PGZ) for three days was measured by the incorporation of FITC-dUTP in the presence of TdT enzyme to detect DNA fragmentation as described in Methods. Cells were stained with RNase A/PI and analyzed by flow cytometry. The mean $\pm$ SD of five independent experiments each performed in triplicate are shown

Treatment	Apoptosis (%)			
	Panc-1	Capan-2	HPAC	P3-HPAC
Control (DMSO)	1.16 $\pm$ 0.15	0.88 $\pm$ 0.14	0.46 $\pm$ 0.19	0.66 $\pm$ 0.17
RGZ	1.20 $\pm$ 0.17	0.90 $\pm$ 0.22	0.38 $\pm$ 0.17	0.58 $\pm$ 0.19
PGZ	1.19 $\pm$ 0.14	0.93 $\pm$ 0.17	0.40 $\pm$ 0.18	0.57 $\pm$ 0.13

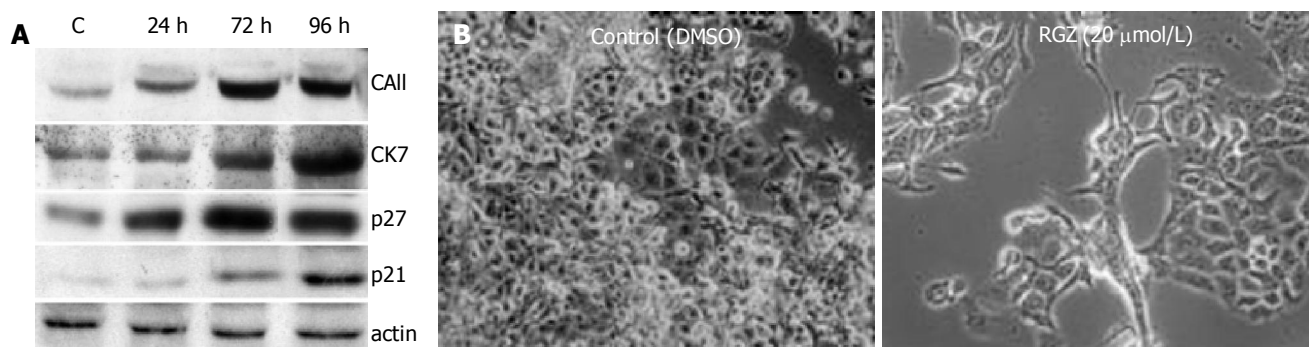


**Figure 4** Expression of apoptotic proteins in cells treated with TZD. Sub-confluent cells were treated with 20  $\mu\text{mol/L}$  of TZD (RGZ or PGZ) for 24 h. Cells were then harvested, and whole-cell protein extracts were fractionated by sodium dodecyl sulfate-polyacrylamide electrophoresis and transferred to nitrocellulose paper as described in Methods. Different proteins were detected by incubating the filter with specific antibodies.

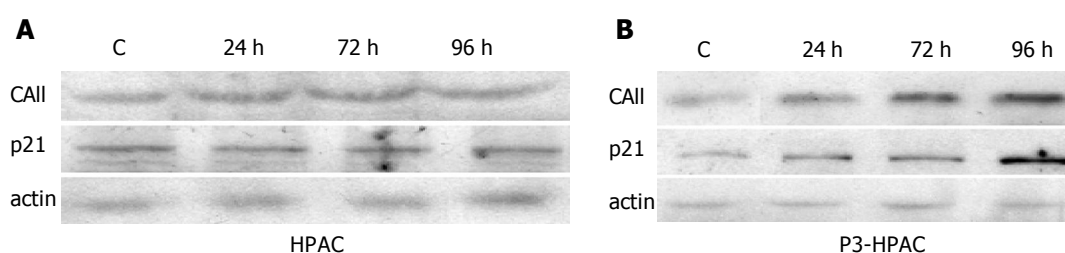
#### TZD promoted differentiation and reversal of the transformed phenotype in PPAR $\gamma$ -expressing cells

In the PPAR $\gamma$ -expressing cell line, PANC-1, 72-h incubation with RGZ resulted in morphological changes with more abundant, flattened cytoplasm, and increased cytoplasmic/nuclear ratio, as is consistent with a more mature phenotype (Figure 5B). To determine whether morphological changes and growth arrest were accompanied by differentiation, analysis of markers of the differentiated state was performed.

Treatment with 20  $\mu\text{mol/L}$  of RGZ resulted in a time-dependent increased expression of ductal specific markers such as carbonic anhydrase II (CA II)<sup>[5]</sup> and cytokeratin 7<sup>[35]</sup> as well as "general" differentiation markers such as the cell cycle inhibitors p21 and p27 (Figure 5A). Expression of



**Figure 5** TZD-induced ductal differentiation in pancreatic adenocarcinoma cells. **A:** Effect of RGZ on the expression of differentiation markers in Panc-1 cells. After 24 h of plating, cells were incubated with 20  $\mu\text{mol/L}$  of RGZ for the indicated time intervals. Protein extracts were then extracted and separated by Western blotting as described in Methods. Immunoreactive proteins were detected by incubating the filters with the specific primary antibodies. A representative of three independent experiments, yielding similar results, is shown; **B:** Effect of RGZ on tumor cells morphology. Panc-1 cells were plated onto sterile culture chambers and then treated with 20  $\mu\text{mol/L}$  of RGZ for 96 h. After the incubation period cells were photographed with Axiovert 200 Image System (Zeiss, Gottingen, Germany). Original magnification  $\times 200$ .



**Figure 6** Effect of TZD on expression of differentiation markers in HPAC cells transduced with PPAR $\gamma$ -expressing retrovirus. Untransduced parental HPAC cells. **A:** or PPAR $\gamma$ -overexpressing P3-HPAC cells; **B:** were incubated with 20  $\mu\text{mol/L}$  of RGZ for the indicated time intervals. Protein extracts were then extracted and separated by Western blotting as described in Methods. Immunoreactive proteins were detected by incubating the filters with the specific primary antibodies. A representative of three independent experiments, yielding similar results, is shown.

$\beta$ -actin, used as an internal control, did not change under any experimental condition. Treatment with PGZ had similar effect (not shown). In contrast, HPAC cells did not show any induction of differentiation markers (Figure 6A). TZD induced p21 and CA II expression in the P3-HPAC clone (Figure 6B), thus supporting the role of PPAR $\gamma$  in the acquisition of a more differentiated state.

Because PPAR $\gamma$  activation can enhance adipocyte differentiation<sup>[11]</sup>, we examined the lipid accumulation in PANC-1 and in Capan-2 cells. Oil Red O staining revealed no lipid accumulation after PGZ or RGZ treatment for 4 d (not shown).

## DISCUSSION

Here we studied the effects of two TZD, pioglitazone (PGZ) and rosiglitazone (RGZ), on pancreatic cancer cell growth, and we characterized the relationship between PPAR $\gamma$  expression and their anticancer properties. The two TZD similarly induced a strong inhibition of anchorage-independent growth on PPAR $\gamma$ -expressing cell lines but they had no effect on colony formation of the PPAR $\gamma$ -deficient cells, HPAC (Figure 1B). The role of PPAR $\gamma$  in the antiproliferative effect of TZD in pancreatic cancer cells was confirmed by overexpression of the receptor in HPAC cells. Expression of PPAR $\gamma$  in HPAC cells, obtained

by transducing retrovirus, restored the growth inhibitory effect of TZD in parallel with a significant induction of PPRE reporter activity (Figure 2B, C). We therefore, conclude that PPAR $\gamma$  expression determines TZD sensitivity in pancreatic carcinoma cells. Confirming our result, the reduction of DNA accumulation by RGZ in glioma cells is strictly dependent on PPAR $\gamma$  expression<sup>[36]</sup>. In addition RGZ exclusively inhibits anchorage-independent growth in human colorectal cancer cells that express a transcriptionally active PPAR $\gamma$ <sup>[18]</sup>. This data is, however, partially in contrast with the observation that the anticancer effects of TZD are independent of PPAR $\gamma$  and mediated by inhibition of translation initiation<sup>[37]</sup>. In fact, in PPAR $\gamma$  ES cells troglitazone induced cell cycle arrest by partial depletion of intracellular Ca<sup>2+</sup> stores, activation of the double-stranded RNA-dependent protein kinase (PKR), and phosphorylation of the eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ). Furthermore, Abe *et al* have indicated that troglitazone suppressed cell growth and histamine secretion by a PPAR $\gamma$ -independent mechanism in the human basophilic leukemia cell line KU812<sup>[38]</sup>. A possible explanation of this controversy may be due to the different chemical structure of the various members of TZD family. Only troglitazone, for instance, has the chroman structure of vitamin E, suggesting that this TZD could regulate signal pathways by mimicking the effects of vitamin E and independently of PPAR $\gamma$  transcriptional

activation<sup>[39]</sup>.

To define the mechanisms by which TZD inhibit the growth of pancreatic carcinoma cells, we analyzed the cell cycle profile of cells treated with RGZ and PGZ. Both TZD increased the population of cells in G<sub>1</sub>/G<sub>0</sub> phase and reduced the population of cells in S phase in PPAR $\gamma$ -expressing cells (Figure 3). These results support recent observations in other growing cells, such as colon cancer cells and myeloid leukemia cells, showing that PPAR $\gamma$  activation induce G<sub>1</sub> cell cycle arrest<sup>[18,40]</sup>. These cycle alterations were achieved at TZD concentrations that repressed pancreatic cell growth, indicating that cell-cycle arrest is one of the primary mechanisms responsible for the anti-proliferative action of TZD in pancreatic cancer cell *in vitro*.

Apoptosis and cell differentiation are tightly linked to cell cycle control mechanisms, particularly those that regulate the transit through G<sub>1</sub> phase<sup>[41,42]</sup>. Thus, the induction of G<sub>1</sub> arrest by TZD-activation of PPAR $\gamma$  may be the precipitating molecular events for subsequent cell differentiation or death. We did not find any pro-apoptotic effects in PPAR $\gamma$ -expressing and non-expressing cells after TZD treatment at the highest concentration used (Table 1 and Figure 4), whereas a significant time-dependent induction of both general and ductal-specific differentiation markers was observed after TZD treatment in PANC-1 cells and in the PPAR $\gamma$  overexpressing clone, P3-HPAC. This suggests that the *in vitro* anti-proliferative effects of TZD in pancreatic tumor cells are not primarily mediated by the induction of apoptotic cell death. Our findings are consistent with the apoptotic-resistant phenotype characteristic of pancreatic tumor cells that are resistant to undergoing apoptosis induced by chemotherapeutic agents, activation of surface receptors such as CD 95 or by serum and growth factor withdrawal<sup>[43,44]</sup>. Similarly to TZD, non-steroidal anti-inflammatory drugs (NSAID) such as indomethacin and sulindac inhibit pancreatic cell growth by cell-cycle arrest without apoptosis via a COX<sub>2</sub>-independent mechanism<sup>[45]</sup>. Interestingly, it has been shown that NSAID are PPAR $\gamma$  activators and their growth-inhibitory effect in pancreatic cells could be mediated, at least in part, by this receptor<sup>[46]</sup>. Furthermore, Wick *et al*, have recently demonstrated that PPAR $\gamma$  is one of the molecular targets of NSAID mediating COX-independent inhibition of lung cancer cell growth<sup>[47]</sup>. In contrast, the natural PPAR $\gamma$  ligand, 15d-PGJ<sub>2</sub>, induced substantial apoptosis in pancreatic cancer cells<sup>[48]</sup>. These discrepancies could be explained assuming that the effect of 15d-PGJ<sub>2</sub> may be partially PPAR $\gamma$ -independent. Indeed, cyclopentenone prostaglandins have been shown to induce apoptotic cell death of human hepatic myofibroblasts, which do not express PPAR $\gamma$ , by a mechanism involving the production of reactive oxygen species<sup>[49]</sup>. Furthermore, specific inhibition of PPAR $\gamma$  does not prevent 15d-PGJ<sub>2</sub>-induced apoptosis in breast cancer cells, suggesting that this eicosanoid requires mechanisms others than activation of PPAR $\gamma$  to induce apoptosis<sup>[50]</sup>.

Although inhibition of cloning efficiency is generally considered the hallmark of differentiation, we investigated additional characteristics that would point towards a more differentiated phenotype of ductal carcinoma cells. Whereas much is known about PPAR $\gamma$  and its role in adipocytic

differentiation<sup>[11]</sup>, in part because of the identification of well-established markers of the terminally differentiated adipocyte<sup>[12]</sup>, the pancreatic ductal epithelium represents a more complex and challenging system. We found that TZD treatment induced the expression of differentiated ductal cells markers such as CK-7<sup>[35]</sup> and CAII<sup>[5]</sup> in parallel with a significant up-regulation of the cell cycle inhibitors p21 and p27 in Panc-1 cells (Figure 5A). By light microscopy, we observed elongation and flattening of cells with extending cellular process after TZD treatment (Figure 5B). These morphological changes are strikingly similar to the ones observed after butyrate and retinoid induced differentiation in the same cell lines<sup>[5,51]</sup> and represented a more differentiated and less malignant state. In agreement with the negligible effect of TZD on growth of PPAR $\gamma$  non-expressing cells, these drugs have no effect on the expression of CAII and p21 in HPAC cells (Figure 6A). Ectopic expression of PPAR $\gamma$  completely restored the ability of TZD to induce ductal differentiation in HPAC cells, suggesting that the expression and activation of PPAR $\gamma$  pathway is a key step in pancreatic-specific differentiation (Figure 6B). Chang *et al*, have recently reported growth inhibition and increased expression of markers of bronchoalveolar progenitor cells in non-small cell lung cancer after treatment with PPAR $\gamma$  agonists<sup>[52]</sup>. Similarly to our results, they did not document either lipid accumulation or adipocyte-specific gene expression, thus excluding adipocytic transdifferentiation of these cell lines. The signaling events evoked by PPAR $\gamma$  activation in epithelial cancer cells remain unclear. The ability of TZD to establish various lineage-specific differentiated states that differ according to the cellular type would suggest that the PPAR $\gamma$  pathway functions early in the induction of differentiation, before lineage-specific events occur.

Overall, our study demonstrates that TZD inhibits growth of pancreatic cancer cells via a PPAR $\gamma$ -dependent induction of ductal differentiation. Given the favorable toxicity profile of these drugs and the limited treatment options that are currently available for patients with pancreatic malignancies, TZD might be a new effective approach to complement conventional chemotherapeutic regimens for pancreatic cancer therapy.

## ACKNOWLEDGEMENTS

We are indebted to Dr. Mizukami for human PPAR $\gamma$  expression plasmid. The authors express their appreciation to Professor M. Serio and M. Mannelli for many helpful comments and suggestion.

## REFERENCES

- 1 National Cancer: Annual cancer statistic review 1973-1988. Bethesda, Department of health and human service 1991, NIH publication No 91-2789
- 2 Washaw AL, Fernandez-Del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992; **326**: 455-465
- 3 Hohne MW, Halatsch ME, Kahl G, Weinel RJ. Frequent loss of expression of the potential tumor suppressor gene DCC in ductal pancreatic adenocarcinoma. *Cancer Res* 1992; **52**: 2616-2619
- 4 Leszczyniecka M, Roberts T, Dent P, Grant S, Fisher PB. Differentiation therapy of human cancer: basic science and

- clinical applications. *Pharmacol Ther* 2001; **90**: 105-156
- 5 **Rosewicz S**, Stier U, Brembeck F, Kaiser A, Papadimitriou CA, Berdel WE, Wiedenmann B, Riecken EO. Retinoids: effects on growth, differentiation, and nuclear receptor expression in human pancreatic carcinoma cell lines. *Gastroenterology* 1995; **109**: 1646-1660
  - 6 **Rosewicz S**, Wollbergs K, Von Lampe B, Matthes H, Kaiser A, Riecken EO. Retinoids inhibit adhesion to laminin in human pancreatic carcinoma cells via the alpha 6 beta 1-integrin receptor. *Gastroenterology* 1997; **112**: 532-542
  - 7 **Olefsky JM**. Treatment of insulin resistance with peroxisome-proliferator-activated receptor gamma agonists. *J Clin Invest* 2000; **106**: 467-472
  - 8 **Braissant O**, Foufelle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* 1996; **137**: 354-366
  - 9 **Kliwer SA**, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, Evans RM. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci USA* 1994; **91**: 7355-7359
  - 10 **Roberts-Thomson SJ**. Peroxisome proliferator-activated receptors in tumorigenesis: targets of tumour promotion and treatment. *Immunol Cell Biol* 2000; **78**: 436-441
  - 11 **Tontonoz P**, Hu E, Spiegelman BM. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator-activated receptor gamma. *Curr Opin Genet Dev* 1995; **5**: 571-576
  - 12 **Tontonoz P**, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid activated transcription factor. *Cell* 1994; **79**: 1147-1156
  - 13 **Reginato MJ**, Bailey ST, Krakow SL, Minami C, Ishii S, Tanaka H, Lazar MA. A potent antidiabetic thiazolidinedione with unique peroxisome proliferator-activated receptor gamma-activating properties. *J Biol Chem* 1998; **273**: 32679-32684
  - 14 **Juge-Aubry C**, Pernin A, Favez T, Burger AG, Wahli W, Meier CA, Desvergne B. DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5'-flanking region. *J Biol Chem* 1997; **272**: 25252-25259
  - 15 **Pignatelli M**, Cortes-Canteli M, Lai C, Santos A, Perez-Castillo A. The peroxisome proliferator-activated receptor gamma is an inhibitor of ErbBs activity in human breast cancer cells. *J Cell Sci* 2001; **114**: 4117-4126
  - 16 **Kubota T**, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I, Koeffler HP. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Cancer Res* 1998; **58**: 3344-3352
  - 17 **Tsujie M**, Nakamori S, Okami J, Hayashi N, Hiraoka N, Nagano H, Dono K, Umeshita K, Sakon M, Monden M. Thiazolidinediones inhibit growth of gastrointestinal, biliary, and pancreatic adenocarcinoma cells through activation of the peroxisome proliferator-activated receptor gamma/retinoid X receptor alpha pathway. *Exp Cell Res* 2003; **289**: 143-151
  - 18 **Brockman JA**, Gupta RA, Dubois RN. Activation of PPARgamma leads to inhibition of anchorage-independent growth of human colorectal cancer cells. *Gastroenterology* 1995; **115**: 1049-1055
  - 19 **Elstner E**, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, Koeffler HP. Ligands for peroxisome proliferator-activated receptor gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. *Proc Natl Acad Sci USA* 1998; **95**: 8806-8811
  - 20 **Sarraf P**, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, Spiegelman BM. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. *Nat Med* 1998; **4**: 1046-1052
  - 21 **Kroll TG**, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM, Fletcher JA. PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected]. *Science* 2000; **289**: 1357-1360
  - 22 **Sarraf P**, Mueller E, Smith WM, Wright HM, Kum JB, Aaltonen LA, de la Chapelle A, Spiegelman BM, Eng C. Loss-of-function mutations in PPAR gamma associated with human colon cancer. *Mol Cell* 1999; **3**: 799-804
  - 23 **Motomura W**, Okumura T, Takahashi N, Obara T, Kohgo Y. Activation of peroxisome proliferator-activated receptor gamma by troglitazone inhibits cell growth through the increase of p27Kip1 in human. Pancreatic carcinoma cells. *Cancer Res* 2000; **60**: 5558-5564
  - 24 **Kawa S**, Nikaido T, Unno H, Usuda N, Nakayama K, Kiyosawa K. Growth inhibition and differentiation of pancreatic cancer cell lines by PPAR gamma ligand troglitazone. *Pancreas* 2002; **24**: 1-7
  - 25 **Laemmli UK**. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680-685
  - 26 **Andrews NC**, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 1991; **19**: 2499
  - 27 **Invernizzi P**, Battezzati PM, Crosignani A, Zermiani P, Bignotto M, Del Papa N, Zuin M, Poddà M. Antibody to carbonic anhydrase II is present in primary biliary cirrhosis (PBC) irrespective of antimitochondrial antibody status. *Clin Exp Immunol* 1998; **114**: 448-454
  - 28 **Chomczynski P**, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156-159
  - 29 **Casini A**, Pinzani M, Milani S, Grappone C, Galli G, Jezequel AM, Schuppan D, Rotella CM, Surrenti C. Regulation of extracellular matrix synthesis by transforming growth factor beta1 in human fat-storing cells. *Gastroenterology* 1993; **105**: 245-253
  - 30 **Galli A**, Stewart M, Dorris R, Crabb D. High-level expression of RXRalpha and the presence of endogenous ligands contribute to expression of a peroxisome proliferator-activated receptor-responsive gene in hepatoma cells. *Arch Biochem Biophys* 1998; **354**: 288-294
  - 31 **Camp HS**, Li O, Wise SC, Hong YH, Frankowski CL, Shen X, Vanbogelen R, Leff T. Differential activation of peroxisome proliferator-activated receptor-gamma by troglitazone and rosiglitazone. *Diabetes* 2000; **49**: 539-547
  - 32 **de Wet JR**, Wood KV, DeLuca M, Helinski DR, Subramani S. Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* 1987; **7**: 725-737
  - 33 **Crabb DW**, Dixon JE. A method for increasing the sensitivity of chloramphenicol acetyltransferase assays in extracts of cultured cells. *Anal Biochem* 1987; **163**: 88-92
  - 34 **Galli A**, Price D, Crabb D. High-level expression of rat class I alcohol dehydrogenase is sufficient for ethanol-induced fat accumulation in transduced HeLa cells. *Hepatology* 1999; **29**: 1164-1170
  - 35 **Schussler MH**, Skoudy A, Ramaekers F, Real FX. Intermediate filaments as differentiation markers of normal pancreas and pancreas cancer. *Am J Pathol* 1992; **140**: 559-568
  - 36 **Berge K**, Tronstad KJ, Flindt EN, Rasmussen TH, Madsen L, Kristiansen K, Berge RK. Tetradecylthioacetic acid inhibits growth of rat glioma cells ex vivo and in vivo via PPARgamma-dependent and PPARgamma-independent pathways. *Carcinogenesis* 2001; **22**: 1747-1755
  - 37 **Palakurthi SS**, Aktas H, Grubisich LM, Mortensen RM, Halperin JA. Anticancer effects of thiazolidinediones are independent of peroxisome proliferator-activated receptor gamma and mediated by inhibition of translation initiation. *Cancer Res* 2001; **61**: 6213-6218
  - 38 **Abe A**, Kiriya Y, Hirano M, Miura T, Kamiya H, Harashima H, Tokumitsu Y. Troglitazone suppresses cell growth of KU812 cells independently of PPARgamma. *Eur J Pharmacol* 2002; **436**: 7-13
  - 39 **Inoue I**, Katayama S, Takahashi K, Negishi K, Miyazaki T, Sonoda M, Komoda T. Troglitazone has a scavenging effect



- on reactive oxygen species. *Biochem Biophys Res Commun* 1997; **235**: 113-116
- 40 **Zhu L**, Gong B, Bisgaier CL, Aviram M, Newton RS. Induction of PPARgamma1 expression in human THP-1 monocytic leukemia cells by 9-cis-retinoic acid is associated with cellular growth suppression. *Biochem Biophys Res Commun* 1998; **251**: 842-848
- 41 **King KL**, Cidlowski JA. Cell cycle regulation and apoptosis. *Annu Rev Physiol* 1998; **60**: 601-617
- 42 **Gao CY**, Zelenka PS. Cyclins, cyclin-dependent kinases and differentiation. *Bioessays* 1997; **19**: 307-315
- 43 **Ungefroren H**, Voss M, Jansen M, Roeder C, Henne-Bruns D, Kremer B, Kalthoff H. Human pancreatic adenocarcinomas express Fas and Fas ligand yet are resistant to Fas-mediated apoptosis. *Cancer Res* 1998; **58**: 1741-1749
- 44 **Raitano AB**, Scuderi P, Korc M. Binding and biological effects of tumor necrosis factor and gamma interferon in human pancreatic carcinoma cells. *Pancreas* 1990; **5**: 267-277
- 45 **Yip-Schneider MT**, Sweeney CJ, Jung SH, Crowell PL, Marshall MS. Cell cycle effects of nonsteroidal anti-inflammatory drugs and enhanced growth inhibition in combination with gemcitabine in pancreatic carcinoma cells. *J Pharmacol Exp Ther* 2001; **298**: 976-985
- 46 **Lehmann JM**, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* 1997; **272**: 3406-3410
- 47 **Wick M**, Hurteau G, Dessev C, Chan D, Geraci MW, Winn RA, Heasley LE, Nemenoff RA. Peroxisome proliferator-activated receptor-gamma is a target of nonsteroidal anti-inflammatory drugs mediating cyclooxygenase-independent inhibition of lung cancer cell growth. *Mol Pharmacol* 2002; **62**: 1207-1214
- 48 **Eibl G**, Wente MN, Reber HA, Hines OJ. Peroxisome proliferator-activated receptor gamma induces pancreatic cancer cell apoptosis. *Biochem Biophys Res Commun* 2001; **287**: 522-529
- 49 **Li L**, Tao J, Davaille J, Feral C, Mallat A, Rieusset J, Vidal H, Lotersztajn S. 15-deoxy-Delta 12,14-prostaglandin J2 induces apoptosis of human hepatic myofibroblasts. A pathway involving oxidative stress independently of peroxisome-proliferator-activated receptors. *J Biol Chem* 2001; **276**: 38152-38158
- 50 **Clay CE**, Monjabez A, Thorburn J, Chilton FH, High KP. 15-Deoxy-delta12,14-prostaglandin J2-induced apoptosis does not require PPARgamma in breast cancer cells. *J Lipid Res* 2002; **43**: 1818-1828
- 51 **el-Deriny SE**, O'Brien MJ, Christensen TG, Kupchik HZ. Ultrastructural differentiation and CEA expression of butyrate-treated human pancreatic carcinoma cells. *Pancreas* 1987; **2**: 25-33
- 52 **Chang TH**, Szabo E. Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor gamma in non-small cell lung cancer. *Cancer Res* 2000; **60**: 1129-1138