

# Anticancer Effects of Thiazolidinediones Are Independent of Peroxisome Proliferator-activated Receptor $\gamma$ and Mediated by Inhibition of Translation Initiation<sup>1</sup>

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## ABSTRACT

The thiazolidinedione (TZD) class of peroxisome proliferator-activated receptor (PPAR)  $\gamma$  ligands, known for their ability to induce adipocyte differentiation and increase insulin sensitivity, also exhibits anticancer properties. Currently, TZDs are being tested in clinical trials for treatment of human cancers expressing high levels of PPAR $\gamma$  because it is assumed that activation of PPAR $\gamma$  mediates their anticancer activity. Using PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  mouse embryonic stem cells, we report here that inhibition of cell proliferation and tumor growth by TZDs is independent of PPAR $\gamma$ . Our studies demonstrate that these compounds block G<sub>1</sub>-S transition by inhibiting translation initiation. Inhibition of translation initiation is the consequence of partial depletion of intracellular calcium stores and the resulting activation of protein kinase R that phosphorylates the  $\alpha$  subunit of eukaryotic initiation factor 2 (eIF2), thus rendering eIF2 inactive. PPAR $\gamma$ -independent inhibition of translation initiation most likely accounts for the anticancer properties of thiazolidinediones.

## INTRODUCTION

The PPAR $\gamma^3$  plays a crucial role in adipocyte differentiation (1, 2), and the TZD class of PPAR $\gamma$  ligands induces differentiation of PPAR $\gamma$  expressing preadipocytes (3) and primary human liposarcoma cells (4). TZDs also inhibit the growth of several cancer cell lines including lung (5), breast (6), colon (7), prostate (8), and hematopoietic (9) cells *in vitro* and in animal models of cancer (6, 7). In addition, loss-of-function mutations of PPAR $\gamma$  have been found in some human colon and thyroid carcinomas (10, 11). As a consequence, PPAR $\gamma$  has become a molecular target for anticancer drug development, and TZDs have been proposed for differentiation-mediated therapy of human cancers that express high levels of PPAR $\gamma$  such as liposarcoma (12), breast (13), and colon (14) cancer.

Cell cycle withdrawal induced by TZDs is assumed to be mediated by PPAR $\gamma$  activation (15, 16) as a necessary step toward terminal differentiation (17). Although the ability of TZDs to induce PPAR $\gamma$ -mediated cell differentiation has been demonstrated clearly, neither a role for PPAR $\gamma$  in cell cycle regulation nor the mechanism by which TZDs inhibit cell growth has been established conclusively. Indeed, the sensitivity of cancer cell lines to the growth-inhibitory effect of TZDs does not seem to correlate with the levels of PPAR $\gamma$  as

exemplified by TZD-resistant but high PPAR $\gamma$ -expressing 21 MT human breast cancer cells (13). We have analyzed the molecular mechanism underlying TZD-induced cell cycle arrest using PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  mouse ES cells as well as cell lines expressing different levels of PPAR $\gamma$ . We report here that TZDs inhibit proliferation of PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells to the same extent. We also show that TZDs induce cell cycle arrest in G<sub>1</sub> by a PPAR $\gamma$ -independent mechanism that involves partial depletion of intracellular Ca<sup>2+</sup> stores, activation of PKR, and phosphorylation of the  $\alpha$  subunit of eIF2 $\alpha$ , resulting in inhibition of translation initiation. Because TZDs have already shown anticancer efficacy in humans, our findings have important implications for human disease because they validate inhibition of translation initiation as a target for cancer therapy and also place TZDs among inhibitors of translation initiation, which are an emerging class of mechanism-specific anticancer drugs.

## MATERIALS AND METHODS

**Cell Culture and Transfection.** PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  mouse ES cells used in this study were derived from cells reported by Milstone *et al.* (18). PPAR $\gamma^{-/-}$  cells are insensitive and PPAR $\gamma^{+/+}$  are sensitive to induction of differentiation under appropriate conditions (2). These cells were routinely cultured in DMEM supplemented with 15% heat inactivated FBS (Hyclone, Logan, UT), 0.1 mM  $\beta$ -mercaptoethanol, 2.0 mM L-glutamine, 20 mM glucose, 25 mM HEPES, and 1000 units/ml of ESGRO (Chemicon International, Temecula, CA; Ref. 19). For the experiments performed within 1–2 h of TZD treatment, FBS concentration was reduced to 1%, and for longer duration experiments FBS concentration was reduced to 5%. NIH 3T3 and 3T3 L1 cells were cultured in DMEM/10% heat-inactivated calf serum (Life Technologies, Inc., Gaithersburg, MD). For all of the experiments performed within 1–2 h of TZD treatment, the medium was replaced by DMEM supplemented with bFGF (5 ng/ml) and 0.1% calf serum. Human cancer cell lines were grown in RPMI 1640 with 5% FBS (Gemini Bio Products, Calabasas, CA). NIH 3T3 cells were transfected with 10  $\mu$ g of the plasmids carrying the mouse pBabe-PPAR $\gamma$ 2. Dominant-negative PKR (PKR-K296) and eIF-2 $\alpha$ 51A expressing cells are described elsewhere (20, 21).

**Cell Growth Assay.** Adherent human solid tumor cells were plated in 96-well plates and maintained for 5 days in the presence of 6.25–100  $\mu$ M TRO (gift from Dr. Allison Goldfine, Joslin Diabetes Center, MA) or CGT (Biomol, Plymouth Meeting, PA), and cell proliferation was measured by the SRB assay as described (22). Briefly, cells were fixed in 10% cold trichloroacetic acid at 4°C for 1 h, extensively washed with double-distilled H<sub>2</sub>O and air-dried. Plates were then incubated with 0.4% SRB in 1% acetic acid for 1 h, washed with 1% acetic acid to remove the unbound dye, and air-dried. The bound dye was solubilized by addition of 10 mM Tris (pH 10), and the absorbance was determined in a Titertek Multiscan plate reader at 490 nm. The data calculations were carried out as described (22).

**DNA Synthesis.** DNA synthesis was determined in 3T3 cells either transfected or not, by measuring incorporation of [<sup>3</sup>H]thymidine as described (23).

**Cell Cycle Analysis.** Exponentially growing PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells were treated with TZDs in ES medium with 5% FBS for 3 days. The cells were fixed with ethanol and stained with propidium iodide for cell cycle analysis by flow cytometry. Nocodazole-treated cells were used to verify the G<sub>2</sub>-M peak.

**Expression of Cell Cycle Regulatory Proteins.** PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells were treated with TZDs for 24 h in ES medium with 2% FBS.

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<sup>3</sup> The abbreviations used are: PPAR $\gamma$ , peroxisome proliferator activated receptor; TZD, thiazolidinedione; eIF2 $\alpha$ , eukaryotic initiation factor 2 $\alpha$ ; ES, embryonic stem; PKR, double-stranded RNA-dependent protein kinase; FBS, fetal bovine serum; bFGF, basic fibroblast growth factor; SRB, sulforhodamine B; TRO, troglitazone; CGT, ciglitazone; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; TG, thapsigargin; SOC, store-operated Ca<sup>2+</sup> channel; cdk, cyclin-dependent kinase.

Expression of cell cycle regulatory proteins was determined by Western blotting with specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

**Polysome Profile Analysis** Exponentially growing PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells were exposed to either TRO or CGT (25  $\mu\text{M}$ ) for 2 h, followed by treatment with cycloheximide (25  $\mu\text{g}/\text{ml}$ ) for 5 min. The cells were washed, collected in ice-cold PBS/cycloheximide, and lysed. Samples of equal absorbance at 260 nm were subjected to sucrose (13–60%) density gradient centrifugation (24). The gradients were eluted from the bottom while monitoring absorbance at 254 nm.

**Phosphorylation of eIF2 $\alpha$**  eIF2 $\alpha$  phosphorylation in exponentially growing ES cells was determined by Western blot analysis using a phospho-specific eIF2 $\alpha$  antibody [Rabbit Pan Anti-eIF2 $\alpha$  (pS51); Biosource International, Hopkinton, MA]. For ES cells, TZDs were used at 25  $\mu\text{M}$  and for all other cells at 12.5  $\mu\text{M}$  concentration.

**Ca $^{2+}$  Measurements.** Exponentially growing cells were loaded with 5  $\mu\text{M}$  Fura-2 AM (Molecular Probes, Eugene, OR) in Krebs-Ringer medium buffered with 25 mM HEPES (pH 7.4 at 37°C) for 25 min. Cells were then transferred to a stirred, thermostated cuvette in a dual-wavelength spectrofluorometer system (Photon Technology International, Inc., South Brunswick, NJ). Fluorescence emission was analyzed at 505 nm, with simultaneous excitation at 340 and 380 nm, as described (25).

**PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  Tumors.** DB2-J male mice, 4 weeks of age, were obtained from The Jackson Laboratory. Twenty-four mice received injections s.c. of  $4 \times 10^6$  PPAR $\gamma^{-/-}$ , and 24 mice received injections of PPAR $\gamma^{+/+}$  mouse ES cells in 0.1 ml PBS/animal. After 2 weeks, the mice bearing distinctly visible tumors were randomly distributed into treatment and vehicle groups. The animals were given either 500 mg/kg/day TRO (Sanyko Parke Davis, Parsippany, NJ) by gavage in gum-arabicum or gum-arabicum (vehicle group) alone 5 days/week. The tumor dimensions were measured weekly using calipers, and tumor volume was calculated using the following formula: tumor volume =  $4/3 \times 3.14 \times (L/2 \times W/2 \times W/2)$ , where  $L$  is the length and  $W$  is the width of the tumor. One mouse in the vehicle group died of bleeding from the tumor site. The data were analyzed by Student's  $t$  test. All animals were sacrificed after 4 weeks of treatment.

## RESULTS AND DISCUSSION

**Effect of TZDs on Cell Growth and DNA Synthesis in Cells Expressing Different PPAR $\gamma$  Levels.** To investigate the potential role of PPAR $\gamma$  in inhibition of cell proliferation by TZDs, PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells (2, 18) were treated with different doses of TRO or CGT for 5 days, and cell growth was monitored by the SRB assay. In both cell lines, TRO and CGT similarly inhibited cell proliferation in a concentration-dependent manner, with IC $_{50}$ s of  $\sim 20$   $\mu\text{M}$  (Fig. 1, *a* and *b*). The IC $_{50}$ s are consistent with the growth-inhibitory (6, 7, 14, 26) and anti-inflammatory (27) effects of these drugs reported by others. Consistently, we have also observed that in human colon cancer cell lines expressing different levels of PPAR $\gamma$ , there is no correlation between the sensitivity of cells toward the growth-inhibitory action of TZDs and their PPAR $\gamma$  expression level (data not shown). These data are consistent with the report indicating that 21 MT human breast cancer cells are relatively resistant to TZDs, although they express a high level of PPAR $\gamma$  (13).

It is known that 3T3 L1 cells, which express high levels of PPAR $\gamma$ , undergo TZD-induced adipocyte differentiation, whereas NIH 3T3 cells, which do not express detectable PPAR $\gamma$ , are resistant to the adipogenic effect of TZDs (1). To further determine whether the cell growth-inhibitory effect of TZDs is attributable to their ability to activate PPAR $\gamma$ , we challenged NIH 3T3 and 3T3 L1 cells with TZDs and measured DNA synthesis by [ $^3\text{H}$ ]thymidine incorporation. The results show that both TZDs inhibit DNA synthesis in NIH 3T3 and 3T3 L1 cells in a dose-dependent manner. The potency of CGT (IC $_{50}$ , 7.5  $\mu\text{M}$ ) is comparable in both cell lines. TRO seems to be more potent in NIH 3T3 cells (IC $_{50}$ , 3.5  $\mu\text{M}$ ) that express no detectable PPAR $\gamma$  compared with 3T3 L1 cells (IC $_{50}$ , 7.5  $\mu\text{M}$ ), which express a high level of PPAR $\gamma$  and show ligand-mediated stimulation of PPAR $\gamma$

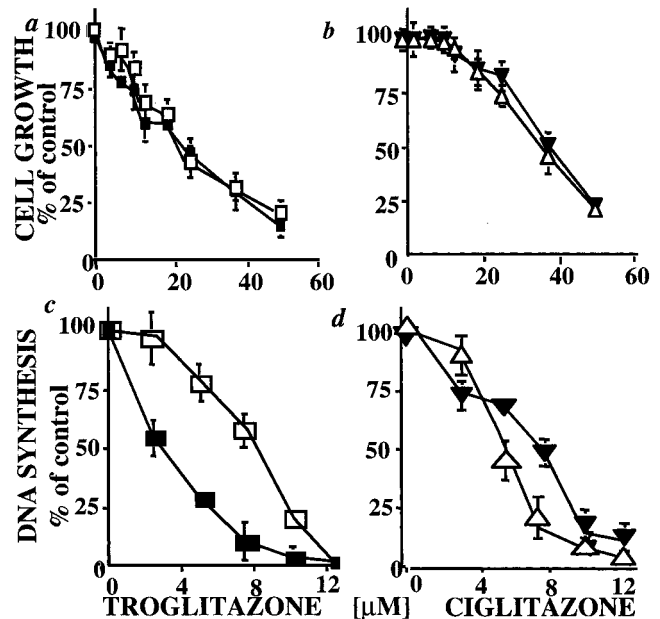


Fig. 1. TZDs inhibit cell growth in PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells and DNA synthesis in 3T3 cells. *a* and *b*, PPAR $\gamma^{-/-}$  (■, ▼) and PPAR $\gamma^{+/+}$  (□, △) ES cells were treated with the indicated concentration of either TRO (*a*) or CGT (*b*) for 5 days, and percentage of growth of the cells was measured. *c* and *d*, DNA synthesis in the absence or presence of TRO (*c*) or CGT (*d*) was measured in quiescent NIH 3T3 (■, ▼) and 3T3 L1 (□, △) cells 15 h after stimulation with bFGF. Data are expressed as means of three different experiments; bars, SEM.

activity (Fig. 1, *c* and *d*). These results indicate that inhibition of DNA synthesis by TZDs bears no correlation with the levels of PPAR $\gamma$  expression. Taken together, these results indicate that inhibition of cell growth by TZDs is not the consequence of PPAR $\gamma$ -mediated differentiation signaling.

To explore the stage of the cell cycle that is blocked by TZDs, we added 25  $\mu\text{M}$  TRO to quiescent NIH 3T3 cells at different times after bFGF stimulation and monitored DNA synthesis by pulse labeling the cells with [ $^3\text{H}$ ]thymidine 12 h after stimulation with bFGF. To determine the time of the G $_1$ -S transition, quiescent cells were pulsed with [ $^3\text{H}$ ]thymidine for 2 h at different times after bFGF stimulation and harvested immediately to measure incorporation of [ $^3\text{H}$ ]thymidine into the DNA. Quiescent NIH 3T3 cells stimulated with bFGF entered S-phase 12 h after mitogenic stimulation (Fig. 2*a*, top). TRO inhibited DNA synthesis when it was added until late G $_1$  but not at later times (Fig. 2*a*, bottom). These data suggest that TZDs inhibit cell growth by blocking cell cycle progression before G $_1$ -S transition but not at S-phase. To further confirm these results, we performed fluorescence-activated cell sorter analysis of exponentially growing PPAR $\gamma^{+/+}$  and PPAR $\gamma^{-/-}$  ES cells exposed to TZDs. ES cells display a cell cycle profile that is very similar to early embryonic cycles, *i.e.*, a short G $_1$  phase and shortened overall duplication time. As a result, in exponentially growing cultures most cells are in S-phase because this is the longest phase of cell cycle (28). Consistently, treatment of ES cells with nocodazole, an inhibitor of G $_2$ -M transition, for as little as 4 h causes accumulation of cells in G $_2$ -M (Fig. 2*b*). Both TRO and CGT blocked ES cells in G $_1$ , with similar potency in both PPAR $\gamma^{+/+}$  and PPAR $\gamma^{-/-}$  cells (Fig. 2*b*), as well as in NIH 3T3 and 3T3 L1 cells (data not shown). These results indicate that TZDs cause G $_1$  arrest independently of PPAR $\gamma$ .

**TZDs Deplete Intracellular Ca $^{2+}$  Stores.** We have demonstrated previously that partial depletion of intracellular calcium stores by compounds such as clotrimazole or EPA inhibits translation initiation, resulting in cell cycle arrest in G $_1$  (20, 21). Clotrimazole and EPA

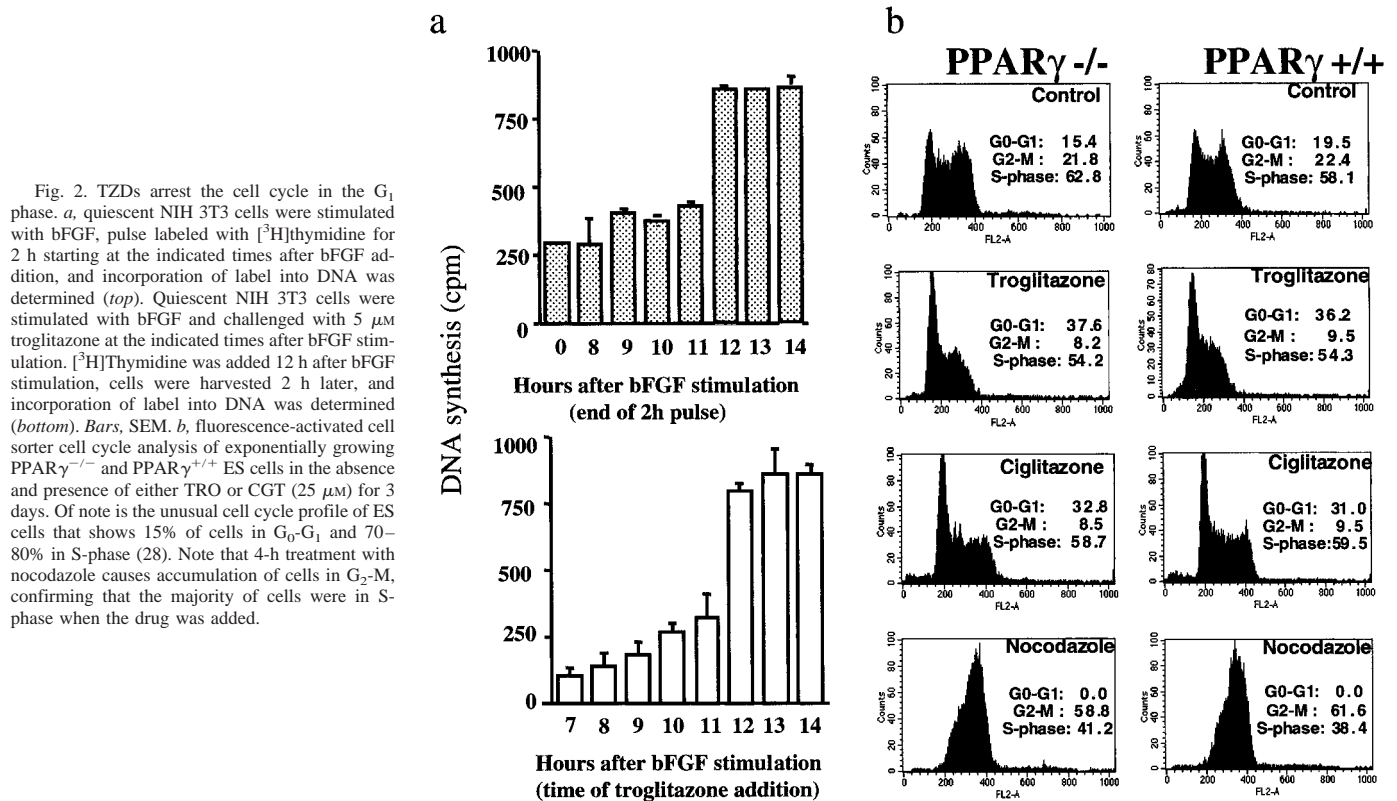


Fig. 2. TZDs arrest the cell cycle in the  $G_1$  phase. *a*, quiescent NIH 3T3 cells were stimulated with bFGF, pulse labeled with [ $^3$ H]thymidine for 2 h starting at the indicated times after bFGF addition, and incorporation of label into DNA was determined (*top*). Quiescent NIH 3T3 cells were stimulated with bFGF and challenged with 5  $\mu$ M troglitazone at the indicated times after bFGF stimulation. [ $^3$ H]Thymidine was added 12 h after bFGF stimulation, cells were harvested 2 h later, and incorporation of label into DNA was determined (*bottom*). Bars, SEM. *b*, fluorescence-activated cell sorter cell cycle analysis of exponentially growing PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells in the absence and presence of either TRO or CGT (25  $\mu$ M) for 3 days. Of note is the unusual cell cycle profile of ES cells that shows 15% of cells in  $G_0$ - $G_1$  and 70–80% in S-phase (28). Note that 4-h treatment with nocodazole causes accumulation of cells in  $G_2$ -M, confirming that the majority of cells were in S-phase when the drug was added.

partially deplete intracellular  $Ca^{2+}$  stores because they induce  $Ca^{2+}$  release from the ER stores and at the same time block capacitative  $Ca^{2+}$  influx across the plasma membrane (21, 23). Depletion of internal  $Ca^{2+}$  stores causes phosphorylation and inactivation of eIF2 $\alpha$  and thus inhibits translation initiation (20, 29). A recent report that TRO inhibits the capacitative influx of  $Ca^{2+}$  in porcine endothelial cells (30) prompted us to speculate that an effect on intracellular  $Ca^{2+}$  homeostasis could mediate TZD-induced cell cycle arrest in  $G_1$ .

To investigate the effect of TZDs on the filling state of internal  $Ca^{2+}$  stores, PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells were loaded with Fura-2 AM and then challenged with TZDs (25). TZDs rapidly

increased cytosolic  $Ca^{2+}$  by release from intracellular stores in a dose-dependent manner (Fig. 3, *a* and *b*). Subsequent addition of TG, a specific inhibitor of the SER- $Ca^{2+}$ ATPase, did not cause further  $Ca^{2+}$  release (data not shown), indicating that TZDs cause depletion of TG-sensitive calcium stores.  $Ca^{2+}$  store depletion activates SOCs that increase capacitative  $Ca^{2+}$  influx from the external medium and refill the  $Ca^{2+}$  stores.

To analyze the effect of TZDs on SOC-mediated capacitative  $Ca^{2+}$  influx, we sequentially added TG and a TZD to Fura-2-loaded PPAR $\gamma^{-/-}$  or PPAR $\gamma^{+/+}$  ES cells. The plateau phase of increased cytosolic  $Ca^{2+}$  seen after the addition of TG in  $Ca^{2+}$ -containing

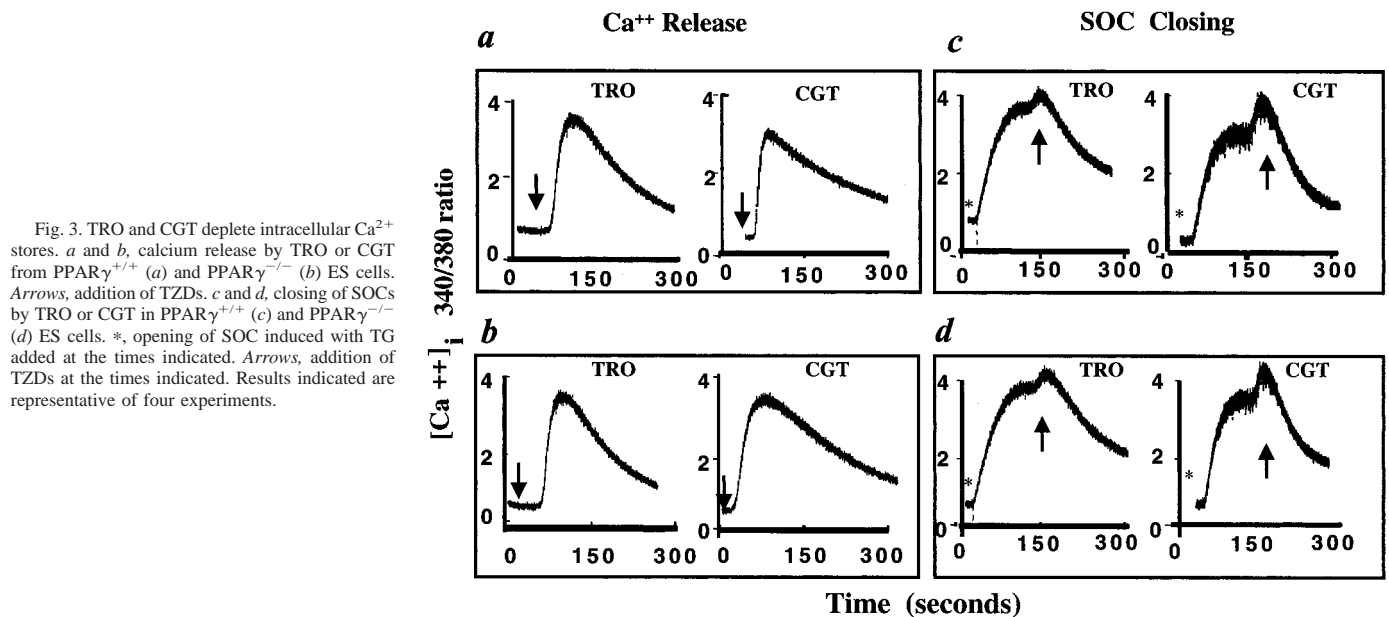


Fig. 3. TRO and CGT deplete intracellular  $Ca^{2+}$  stores. *a* and *b*, calcium release by TRO or CGT from PPAR $\gamma^{+/+}$  (*a*) and PPAR $\gamma^{-/-}$  (*b*) ES cells. Arrows, addition of TZDs. *c* and *d*, closing of SOCs by TRO or CGT in PPAR $\gamma^{+/+}$  (*c*) and PPAR $\gamma^{-/-}$  (*d*) ES cells. \*, opening of SOC induced with TG added at the times indicated. Arrows, addition of TZDs at the times indicated. Results indicated are representative of four experiments.

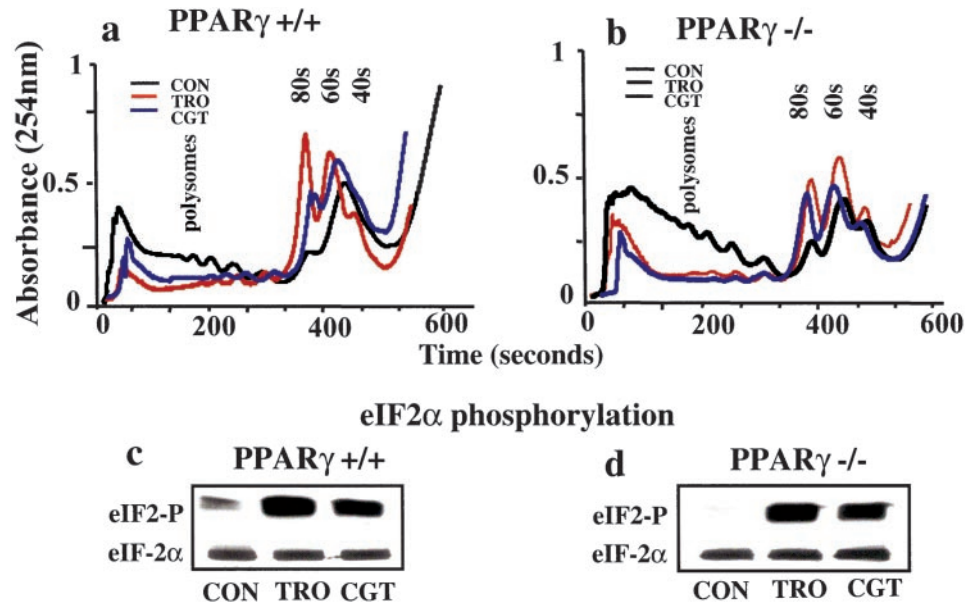


Fig. 4. TZDs inhibit translation initiation by phosphorylation of eIF2 $\alpha$ , similarly in PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells. *a* and *b*, polysome profiles of vehicle control (black), TRO (red), or CGT (blue) treated PPAR $\gamma^{+/+}$  (*a*) and PPAR $\gamma^{-/-}$  (*b*) ES cells. Profiles are representative of three experiments. *c* and *d*, Western blot analysis for total eIF2 $\alpha$  (lower bands) and for phosphorylated eIF2 $\alpha$  (upper bands) in PPAR $\gamma^{+/+}$  (*c*) and PPAR $\gamma^{-/-}$  (*d*) ES cells after 30 min treatment with vehicle control or with either TRO or CGT.

medium represents capacitative Ca<sup>2+</sup> influx (Fig. 3, *c* and *d*). Addition of either TRO or CGT during this plateau phase initially triggered an additional spike of cytosolic Ca<sup>2+</sup>, most likely from a TG-independent pool, and then returned cytosolic Ca<sup>2+</sup> toward its basal levels (Fig. 3, *c* and *d*). The similar effect seen in both cell types indicates that TZDs inhibit capacitative Ca<sup>2+</sup> influx independently of PPAR $\gamma$ . Consistently, TZDs induced Ca<sup>2+</sup> release from intracellular stores and inhibited SOC-mediated Ca<sup>2+</sup> influx in a similar fashion in cells with varying levels of PPAR $\gamma$  expression. TZDs caused depletion of internal Ca<sup>2+</sup> stores in: (*a*) NIH 3T3 cells and 3T3 L1 cells; (*b*) 3T3 L1 cells before differentiation when PPAR $\gamma$  levels are low and after differentiation when PPAR $\gamma$  levels are induced (31); (*c*) human colon cancer cell lines that express different levels of PPAR $\gamma$ ; and (*d*) in murine PPAR $\gamma$  or empty vector-transfected NIH 3T3 cells (data not shown). We have reported previously that both release of intracellular Ca<sup>2+</sup> and inhibition of SOCs are required to induce sustained partial depletion of intracellular Ca<sup>2+</sup> stores, sustained inhibition of protein synthesis, and down-regulation of cell cycle regulatory proteins (20). These results demonstrate that TZDs have a Ca<sup>2+</sup> store-depleting effect that is totally independent of PPAR $\gamma$ , which may account for the antiproliferative effects of these compounds.

#### TZDs Inhibit Translation Initiation by eIF2 Phosphorylation.

To determine whether depletion of internal Ca<sup>2+</sup> stores by TZDs also inhibits translation initiation, we analyzed the ribosomal profile of TZD or vehicle-treated cells by sucrose density gradient centrifugation. Exponentially growing PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells as well as NIH 3T3 and 3T3 L1 cells were challenged with TZDs for 2 h, and cell lysates were subjected to sucrose density gradient centrifugation. In both PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells, TZDs identically shifted the ribosomal profile from heavy to lighter polysomes (Fig. 4, *a* and *b*), as is characteristic of inhibition of translation initiation (32). Identical results were obtained when we studied the effects of TZDs on polysome profiles of NIH 3T3 and 3T3 L1 cells and of human colon cancer cells that express different levels of PPAR $\gamma$  (data not shown). These results conclusively demonstrate that TZDs inhibit translation initiation, regardless of the PPAR $\gamma$  status of the cells.

Most proto-oncogenes and cell cycle regulatory proteins are encoded by mRNAs that contain a highly structured GC-rich 5' untranslated region, a major barrier to translation initiation (33, 34). For this reason, translation of cell growth-promoting proteins is highly dependent on the activity of translation initiation factors such as eIF2 or

eIF4, which play a critical role in cell growth and oncogenesis (35, 36). eIF2 forms a ternary complex with met-tRNA<sup>i</sup> and GTP and recruits the 40S ribosomal subunit to form the 43S preinitiation complex. The preinitiation complex recruits mRNA with the participation of other translation initiation factors and scans mRNA for the initiation codon. The 60S ribosomal subunit is then joined to start the protein synthesis. The ternary complex is dissociated, and GTP is hydrolyzed to GDP in this process. Regeneration of the ternary complex requires GDP to GTP exchange on eIF2, a reaction catalyzed by eIF2B. Phosphorylation of eIF2 $\alpha$  inhibits this exchange reaction by increasing the affinity of eIF2 for eIF2B, locking these two translation initiation factors into stable but unproductive complexes, and thus inhibiting translation initiation.

To test whether TZDs inhibit translation initiation by phosphorylating eIF2 $\alpha$ , we measured phosphorylation of eIF2 $\alpha$  by Western blot analysis using an antibody that specifically recognizes eIF2 $\alpha$  when its serine 51 residue is phosphorylated (37). Treatment of PPAR $\gamma^{-/-}$  or PPAR $\gamma^{+/+}$  ES cells with TZDs induced a comparable phosphorylation of eIF2 $\alpha$  that is evident within 30 min after drug addition (Fig. 4, *c* and *d*). Similar results were obtained by measuring the direct incorporation of <sup>32</sup>P into eIF2 $\alpha$  in NIH 3T3 and 3T3 L1 cells and in the human colon cancer cells (data not shown).

Mutation of serine 51 residue of eIF2 $\alpha$  to alanine (eIF2 $\alpha$ -51A) renders the initiation factor nonphosphorylatable and therefore constitutively active (20, 38). Consistently, NIH 3T3 cells stably transfected with eIF2 $\alpha$ -51A were totally resistant to the phosphorylation of eIF2 $\alpha$  (Fig. 5) and to the DNA synthesis inhibitory effects of both TZDs (TRO<sub>IC50</sub>, 10 ± 0.2 μM; CGT<sub>IC50</sub>, 15 ± 0.4 μM) as compared with vector control cells (TRO<sub>IC50</sub>, 3.8 ± 0.2 μM; CGT<sub>IC50</sub>, 5 ± 0.4 μM). These data indicate that phosphorylation of eIF2 $\alpha$  and inhibition of eIF2 activity mediate inhibition of translation initiation by TZDs.

The eIF2 $\alpha$  is phosphorylated on serine 51 residue by PKR (37). NIH 3T3 cells expressing a dominant-negative mutant of PKR (PKR-K296; Refs. 20, 39) were also significantly resistant to the phosphorylation of eIF2 $\alpha$  (Fig. 5) and to the DNA synthesis inhibitory effects of both TZDs (TRO<sub>IC50</sub>, 10 ± 0.5 μM; CGT<sub>IC50</sub>, 17 ± 0.4 μM) as compared with vector control cells (TRO<sub>IC50</sub>, 3.8 ± 0.2 μM; CGT<sub>IC50</sub>, 5 ± 0.4 μM). Taken together, these results demonstrate that TZDs inhibit translation initiation and cell proliferation by PKR (or related kinase)-mediated phosphorylation of eIF2 $\alpha$ .

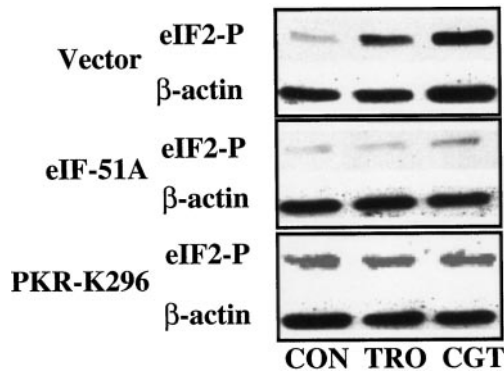


Fig. 5. Phosphorylation of eIF2 $\alpha$  on serine 51 is mediated by PKR. Western blot analysis for phosphorylated eIF2 $\alpha$  (upper bands) and  $\beta$ -actin (lower bands) in vector control and eIF2-51A- and PKR-K296-expressing cells treated with vehicle, TRO, or CGT.

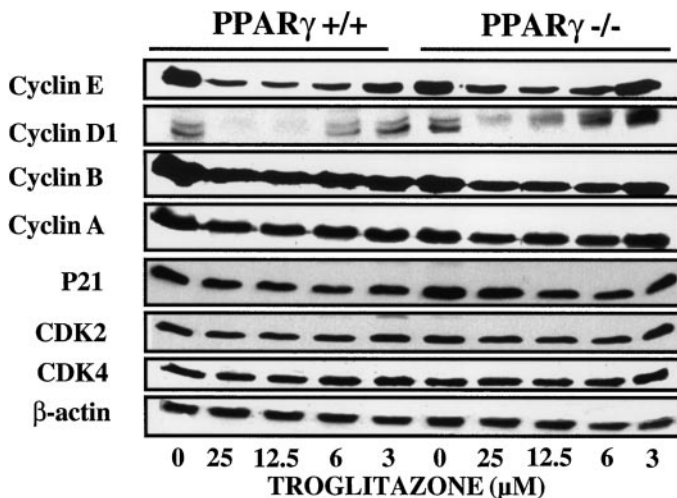


Fig. 6. TZDs preferentially inhibit expression of G1 cyclins in both PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells. Exponentially growing ES cells were treated with indicated doses of TRO for 24 h, and cell lysates were immunoblotted with specific antibodies.

**TZDs Abrogate Expression of G1 Cyclins.** G1 cyclins bind to and activate cdk that drive the cell cycle through the G1 phase and govern G<sub>1</sub>-S transition (40–42). To understand the mechanism of G1 arrest induced by TZDs, we analyzed their effect on the expression of G1 regulatory proteins including G1 cyclins, cdk, and cdk inhibitors in exponentially growing PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells. The results show that in both PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  ES cells, TRO significantly down-regulates cyclin D1 and cyclin E in a dose-dependent manner and has a minimal effect on p21<sup>cip1</sup>, cyclin B, and cyclin A. In contrast, the expression of other cell cycle regulatory proteins, such as cdk4 and cdk2, and of housekeeping proteins, such as  $\beta$ -actin, was not affected (Fig. 6). Identical results were obtained with CGT (data not shown). Consistent with their inhibitory effect on translation initiation, TZDs inhibit synthesis of G1 cyclins without affecting the level of their respective mRNAs (data not shown). These data indicate that TZDs do not interfere with mitogenic signaling upstream from cyclin D1 transcription. Whether down-regulation of cyclin D1 is necessary and/or sufficient for cell cycle-inhibitory effects of TZDs and whether other cell cycle regulatory proteins are also abrogated by these compounds remain to be determined.

**The Antitumor Effect of TZDs *In Vivo* Is Independent of PPAR $\gamma$ .** In an effort to determine whether the antitumor effects of TZDs are also independent of PPAR $\gamma$ , we injected DB2-J male mice with PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  mouse ES cells and treated tumor-bearing mice with either TRO or vehicle alone. Treatment with TRO

almost totally suppressed the growth of tumors established by injection of both PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  mouse ES cells (Fig. 7). These results conclusively demonstrate that the antitumor activity of TZDs is independent of PPAR $\gamma$  and may be mediated through its effects on inhibition of translation initiation.

Extensive experimental evidence indicates that TZDs have intriguing anticancer properties. Because TZDs are high-affinity ligands of the PPAR $\gamma$  receptor, it is widely believed that all anticancer properties of these drugs are part of the PPAR $\gamma$ -mediated differentiation response and involve activation of the PPAR $\gamma$  nuclear receptor. In this report, we identify a novel PPAR $\gamma$ -independent mechanism for the antiproliferative activity of the TZDs. Indeed, Ca<sup>2+</sup> release from ER stores and closing of SOCs in the plasma membrane, PKR-mediated phosphorylation of eIF2 $\alpha$  and inhibition of translation initiation, abrogation of synthesis and expression of cell cycle regulatory proteins, and the consequent cell cycle arrest in G<sub>1</sub> occur identically in both PPAR $\gamma^{+/+}$  and PPAR $\gamma^{-/-}$  ES cells. Furthermore, all these phenomena were observed in cells expressing different levels of PPAR $\gamma$ , either naturally or after transfection with PPAR $\gamma$ . Most importantly, TZDs inhibit the growth of tumors formed by injection of both PPAR $\gamma^{+/+}$  and PPAR $\gamma^{-/-}$  ES cells to the same extent. These data demonstrate clearly that the antitumor effects of TZDs are independent of PPAR $\gamma$ .

Most differentiation-inducing agents, such as sodium butyrate and retinoids (43), induce cell cycle arrest in G<sub>1</sub>, suggesting that G<sub>1</sub> arrest is frequently a prerequisite for cell differentiation. In view of our new findings, it is tempting to postulate that the TZDs have a dual pharmacological effect on the target cells. On one hand they inhibit translation initiation via partial depletion of intracellular Ca<sup>2+</sup> stores, activation of PKR, and phosphorylation of eIF2 $\alpha$ , thus inhibiting cell proliferation. These are rapid epigenetic effects that occur within the first 30 min of drug addition and are totally PPAR $\gamma$  independent. On the other hand, in preadipocytes, liposarcoma, and perhaps some other susceptible cells, TZDs activate PPAR $\gamma$  and the transcription of an array of PPAR $\gamma$ -responsive genes that lead to their differentiation. Interestingly, EPA, another inhibitor of translation initiation via partial ER calcium depletion (21), is also a PPAR $\gamma$  ligand (44). It is conceivable that the ligand-binding pocket of the PPAR $\gamma$  molecule may share some common features with one of the receptors responsible for Ca<sup>2+</sup> release and SOC closing. The other compounds such as clotrimazole that also inhibit translation initiation by ER calcium depletion do not induce differentiation of 3T3 L1 cells (data not

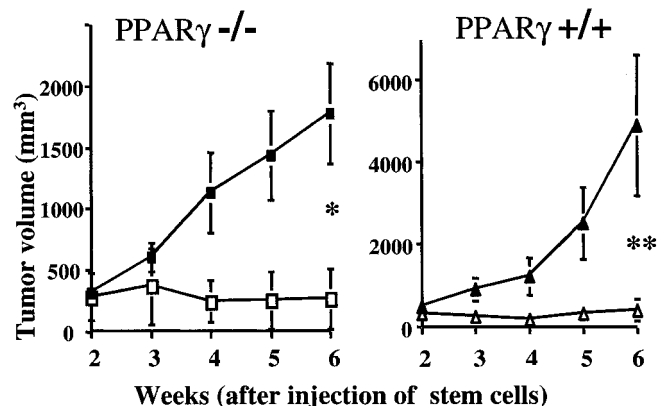


Fig. 7. TZDs inhibit growth of PPAR $\gamma^{-/-}$  and PPAR $\gamma^{+/+}$  tumors. Mice received injections s.c. with either PPAR $\gamma^{-/-}$  (left) or PPAR $\gamma^{+/+}$  (right) mouse ES cells and formed rapidly growing tumors (■, ▲) that were almost totally suppressed by the administration of TRO (p.o., 500 mg/kg/day; □, △). TZD administration started 2 weeks after injection of the stem cells (note that the growth rate of the PPAR $\gamma^{+/+}$  tumors was faster than that of the PPAR $\gamma^{-/-}$  tumors). Bars, SEM;  $n = 12$  (\*, \*\*;  $P < 0.05$  at week 6).

shown), indicating that  $Ca^{2+}$ -mediated inhibition of translation initiation and  $G_1$  arrest are not sufficient to cause cell differentiation. Whether  $Ca^{2+}$  release-mediated cell cycle arrest in  $G_1$  and inhibition of translation initiation are required for the PPAR $\gamma$ -mediated induction of differentiation by TZDs and EPA is not known.

This work defines the TZDs as novel inhibitors of translation initiation. The crucial role of translation initiation in cell growth regulation and oncogenesis makes this cellular process an attractive target for cancer treatment (20, 21). The anticancer activity of the TZDs therefore should be explored in clinical trials independently of the levels and/or genetic status of PPAR $\gamma$  in cancers.

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## Anticancer Effects of Thiazolidinediones Are Independent of Peroxisome Proliferator-activated Receptor $\gamma$ and Mediated by Inhibition of Translation Initiation

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