

Peroxisome proliferator-activated receptor γ inhibits follicular and anaplastic thyroid carcinoma cells growth by upregulating p21^{Cip1/WAF1} gene in a Sp1-dependent manner

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Abstract

Peroxisome proliferator-activated receptor γ (PPAR γ) has been demonstrated to be anti-neoplastic against various human tumors. The aim of this study was to delineate the molecular mechanism underlying PPAR γ ligand rosiglitazone (BRL) antiproliferative effects in follicular WRO and anaplastic FRO human thyroid carcinoma cells. BRL upregulated the p21^{Cip1/WAF1} levels in the two thyroid cancer cells, while did not modify the p53 protein content. Different evidences indicate that the p21^{Cip1/WAF1} upregulation by BRL requires a functional PPAR γ , since it was reversed by silencing PPAR γ and pretreatment with GW9662, an irreversible PPAR γ antagonist. Transient transfection assays showed that BRL triggered the transcriptional activity of p21^{Cip1/WAF1} promoter gene in a p53-independent way, being a p21^{Cip1/WAF1} promoter construct deleted in the p53 sites still activated by BRL. The Sp1 inhibitor mithramycin silenced the p21^{Cip1/WAF1} promoter activity suggesting an important role of Sp1 in mediating BRL activation. The electrophoretic mobility shift and chromatin immunoprecipitation (ChIP) assays evidenced a functional interaction between PPAR γ and Sp1 in regulating p21^{Cip1/WAF1}. Intriguingly, ChIP analysis revealed in the p21^{Cip1/WAF1} gene promoter an increased recruitment of the RNA Pol II associated with an increased histone H3 acetylation and a reduced H3 methylation. The biological event, consistent with PPAR γ -induced WRO and FRO cell growth inhibition, was reversed by p21^{Cip1/WAF1} antisense oligonucleotides and was confirmed by increasing the PPAR γ expression, suggesting a crucial role exerted by p21^{Cip1/WAF1} in PPAR γ action. Our results further candidate BRL as a potential agent able to inhibit tumor progression of follicular and anaplastic thyroid carcinoma.

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Introduction

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of the nuclear receptor (NR) family of ligand-induced transcription factors, which is best known for its differentiating effects on adipocytes and insulin-mediated metabolic functions (Desvergne & Wahli 1999). However, PPAR γ is also involved in cell cycle

control, inflammation, atherosclerosis, apoptosis, and carcinogenesis (Auwerx 1999). Consistently, PPAR γ agonists decrease the growth rate of various malignant cell lines and induce differentiation and apoptosis in diverse carcinoma, including thyroid cancer cells (Tontonoz *et al.* 1997, Elstner *et al.* 1998, Kuboto *et al.* 1998, Mueller *et al.* 1998, Chung *et al.* 2002,

Martelli et al. 2002, Klopper et al. 2004, Aiello et al. 2006, Chen et al. 2006, Copland et al. 2006).

Thyroid cancers are the most common malignancy of endocrine organs (Parkin et al. 2005), with an incidence rate that steadily increased over the past few decades (Liu et al. 2001). More than 95% of thyroid carcinomas are derived from follicular epithelial cells, while a minority of tumors (2%) is referred as undifferentiated or anaplastic carcinoma, one of the most aggressive human malignancies (Kondo et al. 2006). A majority of well-differentiated thyroid cancers can be effectively managed by surgical resection with or without radioactive iodine ablation and only a subset of these tumors can behave aggressively. By contrast, there is no effective form of treatment presently available for patients affected by anaplastic neoplasia and hence they have an extremely poor prognosis (Ain 1998).

The growth activity of well-differentiated compared with undifferentiated thyroid carcinoma is low and this difference is governed by altered cell cycle regulators (Kondo et al. 2006 and references therein). Among a family of negative cell cycle regulators, the p21^{Cip1/WAF1} member of the cyclin-dependent-kinase interacting protein/kinase inhibitory protein (CIP/KIP) family plays a crucial role in the growth arrest mediated by either p53-dependent (el-Diery et al. 1993) or p53-independent mechanism (Jiang et al. 1994). The p21^{Cip1/WAF1} was shown to be involved in PPAR γ -mediated growth regulation in different cancer cells, including thyroid (Han et al. 2004, Hong et al. 2004, Bonofiglio et al. 2006, Copland et al. 2006). Particularly, PPAR γ mediates the upregulation of p21^{Cip1/WAF1} (Chung et al. 2002, Han et al. 2004, Hong et al. 2004) and a mechanism through which PPAR γ mediates p21^{Cip1/WAF1} mRNA induction was shown to be via Sp1-enhanced binding to the p21^{Cip1/WAF1}, KDR and hormone-sensitive lipase promoters (Han et al. 2004, Hong et al. 2004, Sassa et al. 2004, Deng et al. 2006).

In this study, we have investigated the way through which the PPAR γ ligand rosiglitazone (BRL49653, BRL) induces growth inhibition in follicular WRO and anaplastic FRO thyroid cancer cells. Independent of the p53 pathway, the underlying molecular mechanism involves a functional interaction between PPAR γ and Sp1 to Sp1 sites of the p21^{Cip1/WAF1} promoter gene.

Materials and methods

Reagents

BRL was purchased from Alexis (San Diego, CA, USA); the irreversible PPAR γ -antagonist GW9662

(GW) and the Sp1-specific inhibitor, mithramycin, were from Sigma. All compounds were solubilized in dimethyl sulfoxide (Sigma) also used as the vehicle.

Cell cultures

Human follicular WRO and anaplastic FRO thyroid cancer cells (a gift from Dr F Arturi, University of Magna Grecia, Catanzaro, Italy) were grown in Dulbecco's Modified Eagle's Medium (DMEM) plus glutamax containing 10% fetal bovine serum (FBS; Invitrogen) and 1 mg/ml penicillin–streptomycin (P/S).

Plasmids

The human wild-type p21^{Cip1/WAF1} promoter-luciferase (luc) reporter (p21^{Cip1/WAF1} wt) and its deletion construct that lacks the two p53-binding sites (p21^{Cip1/WAF1} Δ p53) were kind gifts from Dr T Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). The PPAR γ expression plasmid was a gift from Dr R Evans (The Salk Institute, San Diego, CA, USA). As an internal transfection control we co-transfected the plasmid pRL-CMV (Promega), which expresses *Renilla luciferase* enzymatically distinguishable from firefly *luciferase* by the strong cytomegalovirus enhancer/promoter.

[³H]thymidine incorporation

WRO and FRO cells were seeded in 12-well plates in a regular growth medium. On the second day, the cells were incubated in DMEM supplemented with 1% charcoal-stripped-FBS in the presence of vehicle or with treatments. The medium was renewed every 2 days together with the appropriate treatments. [³H]thymidine (1 μ Ci/ml; New England Nuclear, Newton, MA, USA) was added to the medium for the last 6 h of the sixth day. After rinsing with PBS, the cells were washed once with 10% and thrice with 5% trichloroacetic acid. The cells were lysed by adding 0.1 M NaOH and then incubated for 30 min at 37 °C. Thymidine incorporation was determined by scintillation counting.

Immunoblotting

The cells were grown in 10 cm dishes to 70–80% confluence and exposed to treatments for 24 h in a serum-free medium (SFM), as indicated. They were then harvested in cold PBS and resuspended in a lysis buffer containing 20 mM HEPES (pH 8), 0.1 mM EDTA, 5 mM MgCl₂, 0.5 M NaCl, 20% glycerol, 1% NP-40, and inhibitors (0.1 mM Na₃VO₄, 1% phenylmethylsulphonyl fluoride (PMSF), 20 mg/ml

aprotinin). The protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories). A 50 µg portion of protein lysates was used for western blotting (WB), resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with an antibody directed against the p21^{Cip1/WAF1}, p53, PPAR γ and Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As the internal control, all membranes were subsequently stripped (0.2 M glycine (pH 2.6) for 30 min at room temperature) of the first antibody and reprobed with anti- β -actin antibody (Santa Cruz Biotechnology). The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-coupled goat anti-mouse or anti-rabbit IgG and revealed using the enhanced chemiluminescence system (ECL system, Amersham Pharmacia). The blots were then exposed to Kodak film (Sigma). The intensity of bands representing relevant proteins was measured using the Scion Image laser densitometry scanning program.

Reverse transcription-PCR (RT-PCR) assay

Cells were grown in 10 cm dishes to 60–70% confluence and exposed to treatments for 24 h in SFM. The total cellular RNA was extracted using TRIZOL reagent (Invitrogen) as suggested by the manufacturer. The purity and integrity were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures. The evaluation of gene expression was performed by the semiquantitative RT-PCR method as described previously (Maggiolini *et al.* 1999). For p21^{Cip1/WAF1} and the internal control gene 36B4, the primers were: 5'-GCTTCATGCCAGCTACTTCC-3' (p21^{Cip1/WAF1} forward) and 5'-CTGTGCTCACTTCAGGGTCA-3' (p21^{Cip1/WAF1} reverse), and 5'-CTCAACATCTCCC CTTCTC-3' (36B4 forward) and 5'-CAAATCCCA TATCCTCGTCC-3' (36B4 reverse) to yield respectively the products of 270 bp with 20 cycles and 408 bp with 12 cycles. The results obtained as optical density arbitrary values were transformed to percentage of the control (percent control) taking the samples from untreated cells as 100%.

Transfection assay

The cells were transferred into 24-well plates with 500 µl regular growth medium/well the day before transfection. The medium was replaced with SFM on the day of transfection, which was performed using Fugene 6 reagent as recommended by the manufacturer (Roche) with a mixture containing 0.5 µg promoter-luc

reporter plasmids and 5 ng pRL-CMV. After 24 h transfection, SFM was treated as indicated and the cells were incubated for a further 24 h. Firefly and *R. luciferase* activities were measured using the Dual Luciferase Kit (Promega).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from thyroid cancer cells were prepared as described previously for EMSA (Andrews & Faller 1991). Briefly, both cell lines plated into 10 cm dishes were grown to 70–80% confluence, shifted to SFM for 24 h, and then treated with 1 µM BRL for 6 h. Thereafter, the cells were scraped into 1.5 ml cold PBS. They were pelleted for 10 s and resuspended in 400 µl cold buffer A (10 mM HEPES–KOH (pH 7.9) at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were then allowed to swell on ice for 10 min and then vortexed for 10 s. The samples were then centrifuged for 10 s and the supernatant fraction discarded. The pellet was resuspended in 50 µl cold Buffer B (20 mM HEPES–KOH (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated in ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 °C and the supernatant fraction (containing DNA-binding proteins) was stored at –70 °C. The probe was generated by annealing single-stranded oligonucleotides and labeled with [γ -³²P] ATP (Amersham Pharmacia) and T4 polynucleotide kinase (Promega), and then purified using Sephadex G50 spin columns (Amersham Pharmacia). The DNA sequence of Sp1, present in the native human p21^{Cip1/WAF1} promoter gene, used as the probe or the cold competitor is the following: Sp1, 5'-GGGGGT CCCGCCTCCTTGA-3' (Sigma). The protein-binding reactions were carried out in 20 µl buffer (20 mM HEPES (pH 8), 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glycerol, 1 mg/ml BSA, 50 µg/ml poly dI/dC) with 50 000 c.p.m. labeled probe, 5 µg nuclear protein or 1 µl human Sp1 recombinant protein (Promega), and 5 µg poly(dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. For the experiments involving the anti-PPAR γ and anti-Sp1 antibodies and IgG (Santa Cruz Biotechnology), the reaction mixture was incubated with these antibodies at 4 °C for 30 min before the addition of labeled probe. Mithramycin (100 nM) was incubated with the labeled probe for 30 min at 4 °C before the addition of nuclear extracts. The entire

reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 \times Tris borate–EDTA for 3 h at 150 V. The gel was dried and subjected to autoradiography at -70°C .

Chromatin immunoprecipitation and re-immunoprecipitation (ChIP and reChIP) assay

For ChIP assay, thyroid carcinoma cells were grown in 10 cm dishes to 50–60% confluence, shifted to SFM for 24 h, and then treated with 1 μM BRL for 1 h or pre-incubated with mithramycin for 1 h where required. Thereafter, the cells were washed twice with PBS and cross-linked with 1% formaldehyde at 37°C for 10 min. Next, the cells were washed twice with PBS at 4°C , collected, and resuspended in 200 μl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1)) and left on ice for 10 min. Then, the cells were sonicated four times for 10 s at 30% of maximal power (Sonics and Materials Inc., Vibra Cell 500 W) and collected by centrifugation at 11 000 g for 10 min, at 4°C . The supernatants were diluted in 1.3 ml IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.1), 16.7 mM NaCl) followed by immunoclearing with 80 μl sonicated salmon sperm DNA/protein A agarose (UBI, DBA Srl, Milan, Italy) for 1 h at 4°C . The precleared chromatin was immunoprecipitated with anti-PPAR γ , anti-Sp1, anti-RNA Pol II (Santa Cruz Biotechnology), anti-acetyl histone H3, and anti-trimethyl histone H3 antibodies (Upstate, Milan, Italy). The chromatin immunoprecipitated with anti-PPAR γ was re-immunoprecipitated with the anti-Sp1 antibody. At this point, 60 μl salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 h at 4°C . After pelleting, the precipitates were washed sequentially for 5 min with the following buffers: wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 150 mM NaCl); wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 500 mM NaCl); and wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl (pH 8.1)), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluted with the elution buffer (1% SDS, 0.1 M NaHCO₃). The eluates were reverse cross-linked by heating at 65°C and digested with proteinase K (0.5 mg/ml) at 45°C for overnight. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. To each sample, 2 μl of 10 mg/ml yeast tRNA (Sigma) were added and DNA was precipitated with 70% ethanol for 24 h at -20°C , and then washed with 95% ethanol and resuspended in 20 μl TE buffer.

A 5 μl volume of each sample was used for PCR amplification with the following primers flanking a sequence of p21^{Cip1/WAF1} promoter: 5'-GATTTGT GGCTCACTTCGTGGG-3' (forward) and 5'-GCA GCTGCTCACACCTCAGCT-3' (reverse) (Gene Bank, accession number U24170). The PCR conditions were 1 min at 95°C , 1 min at 60°C , and 1 min at 72°C . The amplification products obtained in 30 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. The negative control was provided by PCR amplification without the DNA sample. The specificity of the reactions was ensured using normal mouse and rabbit IgG (Santa Cruz Biotechnology).

ChIP/immunoblot assay

For the ChIP/immunoblot assay, thyroid carcinoma cells were subjected to the procedure of ChIP assay, as described above, until we obtained the immunocomplexes. At this time, we added the Laemmli buffer to the immunocomplexes and performed the WB analysis, as described by the Upstate protocol.

Antisense oligodeoxynucleotide experiments

The oligonucleotides used were: 5'-GACATCACC AGGATCGGACAT-3' for p21, and 5'-GATCTCAG CACGGCAAAT-3' for the scrambled control (cs). For antisense experiments, a concentration of 200 nM of the indicated oligonucleotides (ODN) was transfected using Fugene 6 reagent as recommended by the manufacturer for 4 h, before treatment with vehicle or BRL. The transfection was renewed every 2 days together with the appropriate treatments.

RNA interference (RNAi)

Cells were plated in six-well dishes in the regular growth medium the day before transfection to 60–70% confluence. On the second day, the medium was changed with SFM without P/S and the cells were transfected with 25 bp RNA duplex of validated RNAi-targeted human PPAR γ mRNA sequence 5'-AGA AUAAUAAGGUGGAGAUGCAGGC-3' sequence (Invitrogen) or with a stealth RNAi-negative control low GC (Invitrogen) to a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. After 5 h, the transfection medium was changed with SFM in order to avoid Lipofectamine 2000 toxicity and the cells were exposed to vehicle or BRL for the next 24 h and then lysed as described for WB analysis.

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls testing to determine the differences in means. $P < 0.05$ was considered statistically significant.

Results

BRL induces growth inhibitory effects in WRO and FRO cells

On the basis of other (Kuboto *et al.* 1998, Clay *et al.* 1999, Ohta *et al.* 2001) and our (Bonfiglio *et al.* 2005, 2006) studies demonstrating the inhibitory effects of the PPAR γ agonists on the proliferation of different cancer cells, we first treated human follicular WRO and anaplastic FRO thyroid cancer cells with increasing BRL concentrations for 5 days. BRL inhibited the growth of both thyroid cancer cells in a dose- and PPAR γ -dependent manner, since this effect was no longer notable in the presence of its specific antagonist GW9662 (GW; Fig. 1A and B).

BRL upregulates p21^{Cip1/WAF1} expression in thyroid cancer cells

The p53 tumor suppressor gene plays an undisputed role as a major mediator of cell cycle arrest and/or apoptosis in the response of mammalian cells to stress stimuli and p21^{Cip1/WAF1}, a known p53-downstream gene, has been suggested to mediate p53-induced growth arrest triggered by DNA damage (Vousden & Lu 2002). Therefore, we aimed to examine the potential ability of the PPAR γ agonist BRL to modulate p53 along with its natural target gene p21^{Cip1/WAF1} in both thyroid cancer cells. Interestingly, BRL upregulated the protein expression of p21^{Cip1/WAF1} in a dose-dependent manner in the WRO and FRO cells, while did not modify the p53 protein content (Fig. 2A and B). Next, we investigated the mRNA expression of p21^{Cip1/WAF1}, which was induced by BRL at an increasing concentration in both thyroid cancer cells (Fig. 3A). Using GW, BRL action was abrogated, suggesting a direct involvement of PPAR γ in mediating this effect (Fig. 3A). To definitively confirm the mechanism by which BRL upregulates p21^{Cip1/WAF1} levels, we inhibited PPAR γ expression by RNAi (Fig. 3B). The effect of BRL on p21^{Cip1/WAF1} expression was prevented in the presence of PPAR γ RNAi, while it was still notable using a control RNAi (Fig. 3C), demonstrating the crucial role of PPAR γ in BRL-induced upregulation of p21^{Cip1/WAF1}.

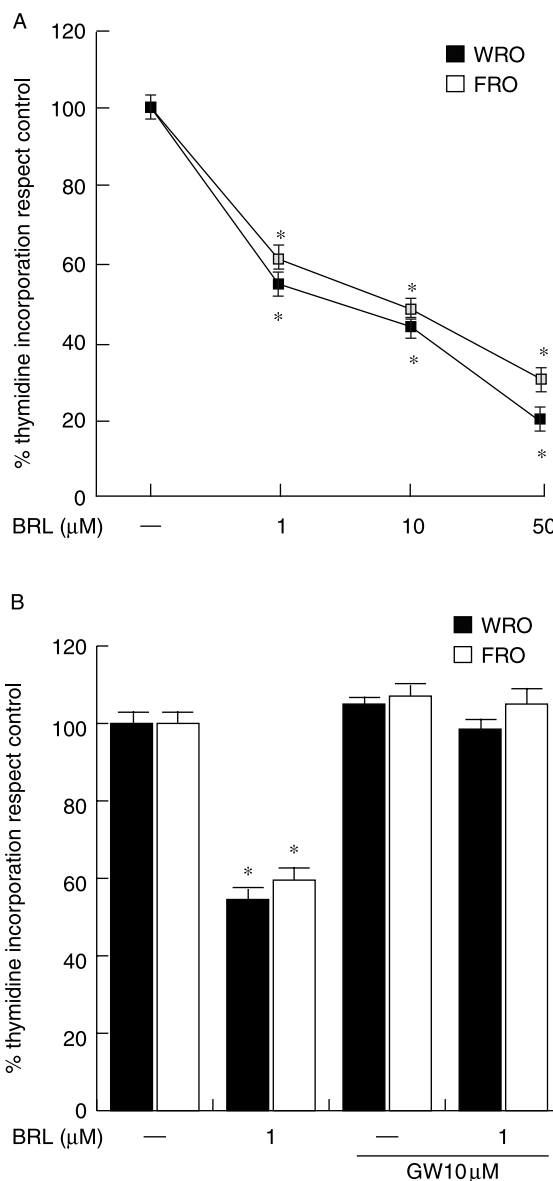


Figure 1 Antiproliferative effects exerted by PPAR γ agonist BRL in follicular WRO and anaplastic FRO thyroid cancer cells. (A) WRO and FRO cells were treated for 5 days with vehicle (—) or with increasing BRL concentrations. (B) Both thyroid cancer cells were treated for 5 days with vehicle (—), 1 μ M BRL, 10 μ M GW alone or in combination with BRL. On day 6, [3 H] thymidine incorporation was determined by scintillation counting. Data are expressed as mean \pm s.d. of three independent experiments performed in triplicate. * $P < 0.05$ BRL-treated versus untreated cells.

BRL transactivates p21^{Cip1/WAF1} gene promoter

The aforementioned observations prompted us to investigate whether the PPAR γ ligand is able to transactivate the p21^{Cip1/WAF1} promoter gene, which contains two p53-response elements (Li *et al.* 1994,

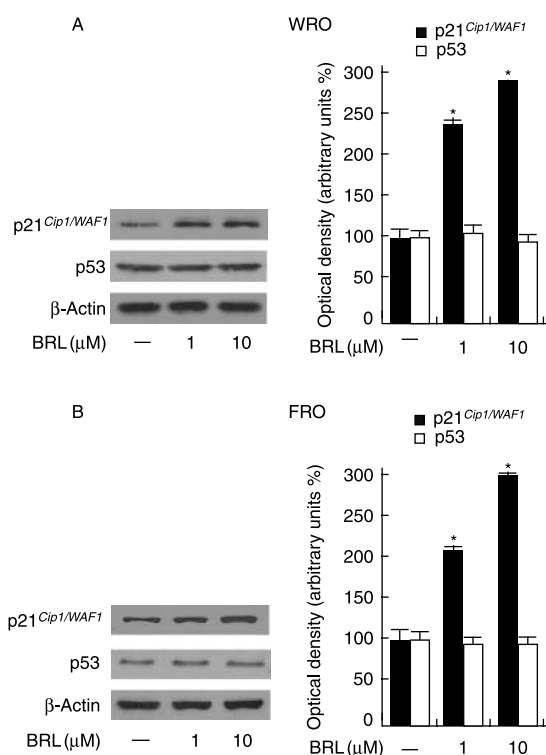


Figure 2 Modulation of p21^{Cip1/WAF1} protein level by BRL. Immunoblots of p21^{Cip1/WAF1} and p53 from (A) WRO and (B) FRO cells treated for 24 h with vehicle (–) and with increasing BRL concentrations. β -Actin was used as the loading control. The side panels show the quantitative representation of data (mean \pm s.d.) of three independent experiments included that presented in A and B respectively. * P < 0.05 BRL-treated versus untreated cells.

Wu & Schonthal 1997). Thus, WRO and FRO cells were transiently transfected with the human wild-type p21^{Cip1/WAF1} promoter–luciferase fusion plasmid (p21^{Cip1/WAF1} wt) or with a promoter construct that lacks the two p53-binding sites (p21^{Cip1/WAF1} Δ p53; Fig. 4A). BRL was able to transactivate both constructs, defining the minimal region of p21^{Cip1/WAF1} promoter responsible for its induction independently of p53 (Fig. 4B). Such effects were reversed by GW, suggesting that the transactivation of p21^{Cip1/WAF1} by BRL occurs in a PPAR γ -dependent manner (Fig. 4B). Since the deleted mutant p21^{Cip1/WAF1} Δ p53 encoding the region from –124 to +11 expresses multiple Sp1 sites, we performed transfection experiments using mithramycin, a specific inhibitor of GC binding (Blume *et al.* 1991). The transactivation of this construct induced by BRL was abrogated by mithramycin, indicating an involvement of Sp1 transcription factor in the PPAR γ action observed in WRO and FRO cells (Fig. 4B).

BRL binds to Sp1 sites of the p21^{Cip1/WAF1} promoter gene in EMSA

In order to further support whether Sp1 sites mediate the BRL-induced upregulation of p21^{Cip1/WAF1}, we performed EMSA using synthetic oligodeoxyribonucleotides corresponding to the Sp1 site located in the human p21^{Cip1/WAF1} gene promoter, as probe. In nuclear extracts from WRO cells (Fig. 5A) we obtained the formation of specific bands (lane 1), which were strongly increased in cells treated with BRL (lane 2). Competition binding studies demonstrated that a 100-fold molar excess of unlabeled probe inhibited the formation of these complexes (lane 3). The addition of mithramycin, which binds to GC boxes and prevents sequential Sp1 binding, decreased the intensity of the bands to Sp1 DNA sequence (lane 4). Of note, the anti-PPAR γ and anti-Sp1 antibodies (lanes 5 and 6 respectively) were able to supershift the specific bands. Different controls were used to assess the specificity of the binding; IgG that did not generate supershifted bands (lane 7), human Sp1 recombinant protein alone (lane 8), and Sp1 in combination with either cold competitor (lane 9) or the anti-Sp1 antibody (lane 10). As shown in Fig. 5B, similar results, such as an increased DNA-binding complex under BRL (lane 2), a reduction of the band intensity by using mithramycin (lane 4), and supershifted bands in the presence of the anti-PPAR γ and anti-Sp1 antibodies (lanes 5 and 6) were obtained in FRO cells.

Functional interaction of PPAR γ with p21^{Cip1/WAF1} by ChIP and reChIP assays

The interaction of PPAR γ with the p21^{Cip1/WAF1} gene promoter was further investigated by ChIP assay. WRO and FRO chromatin were immunoprecipitated with the anti-PPAR γ , the anti-Sp1, the anti-RNA Pol II, the anti-acetyl histone H3, and the anti-trimethyl histone H3 antibodies. The PPAR γ immunoprecipitated chromatin was re-immunoprecipitated with the anti-Sp1 antibody. PCR was used to determine the recruitment of PPAR γ to the p21^{Cip1/WAF1} region containing the Sp1 site. As shown in Fig. 6A, the results indicated that PPAR γ was weakly and constitutively bound to the p21^{Cip1/WAF1} promoter in untreated cells and this recruitment was increased upon BRL treatment, while in the presence of mithramycin the BRL effect was reversed in both thyroid cancer cells. Histone modification plays an important role in controlling gene expression (He & Lehming 2003). For example, acetylation of histone H3 at lysine 9 and methylation of histone H3 at lysine 9 are associated

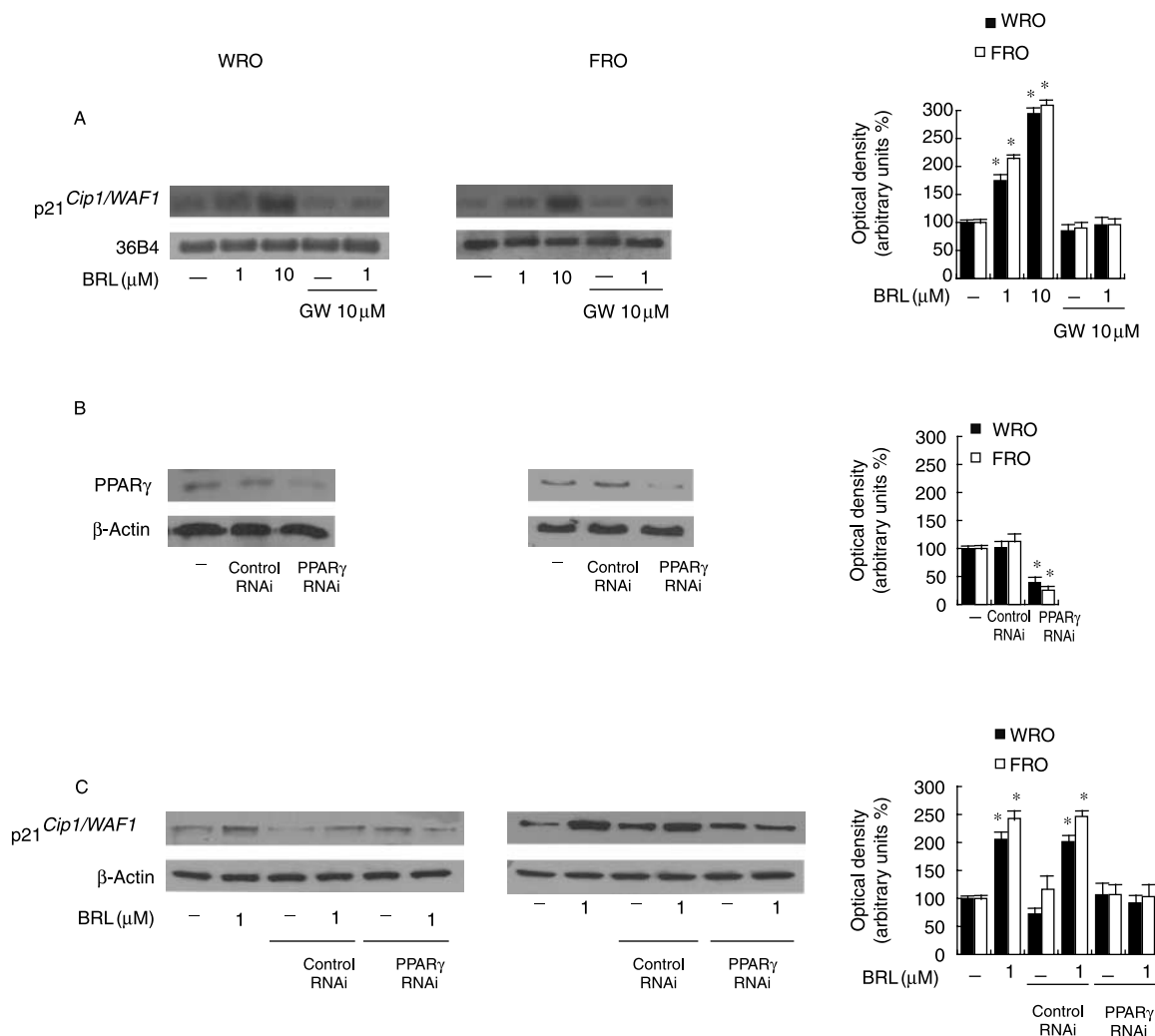


Figure 3 BRL upregulates p21^{Cip1/WAF1} mRNA expression. (A) Semiquantitative RT-PCR evaluation of p21^{Cip1/WAF1} mRNA expression in WRO and FRO cells treated for 24 h in the presence of vehicle (–) or with treatments as indicated. 36B4 mRNA levels were determined as control. In the side panel is shown the quantitative representation of data (mean ± s.d.) of three independent experiments included that presented in A after densitometry and correction for 36B4 expression. **P* < 0.05 BRL-treated versus untreated cells. (B) PPAR γ protein expression (evaluated by WB) in WRO and FRO cells not transfected (–) or transfected with 25 nucleotide of validated RNA interference (RNAi) targeted human PPAR γ mRNA sequence (PPAR γ RNAi) or with stealth RNAi negative control (control RNAi), as reported in Materials and methods. β -Actin was used as loading control. The side panel shows the quantitative representation of data (mean ± s.d.) of three independent experiments included that presented in B. (C) Immunoblots of p21^{Cip1/WAF1} in thyroid cancer cells treated in the presence of vehicle (–) or with BRL 1 μ M, transfected using PPAR γ RNAi or control RNAi. β -Actin was used as loading control. The side panel shows the quantitative representation of data (mean ± s.d.) of three independent experiments included that presented in C.

with transcriptional activation and repression respectively. Besides, since it was speculated that increased degrees of H3 Lys9 methylation are associated with an increased stability of silencing (Rice *et al.* 2003; mono- versus di- versus tri-), we used a specific anti-H3 trimethylated at lysine 9 antibody. Noteworthy, an increased recruitment of H3 acetylated at lysine 9 and a reduction of H3 trimethylated at lysine 9 upon BRL treatment was observed. In addition, the positive

regulation of the p21^{Cip1/WAF1} transcription activity through Sp1 induced by BRL was demonstrated by the increased recruitment of RNA Pol II, which was reversed in the presence of mithramycin. Altogether, the data indicate that PPAR γ binding to the Sp1 transcription factor exerts stimulatory effects on p21^{Cip1/WAF1} gene expression. The physical interaction between the PPAR γ and Sp1 proteins was strengthened by the ChIP/immunoblot assay (Fig. 6B).

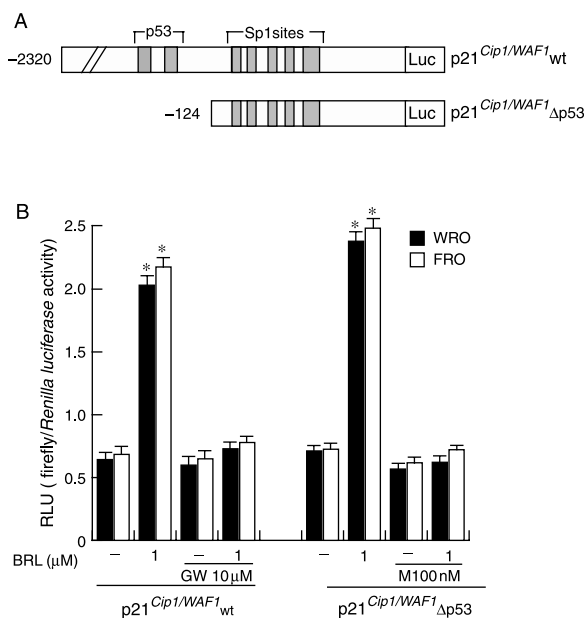


Figure 4 BRL induces the transcriptional activity of p21^{Cip1/WAF1} promoter gene. (A) Schematic map of the p21^{Cip1/WAF1} promoter plasmids used in this study. (B) WRO and FRO cells were transiently transfected with the wild-type p21 promoter-luciferase (p21^{Cip1/WAF1} wt) and then treated with vehicle (-), 1 μM BRL, 10 μM GW alone or in combination with BRL. Both thyroid cancer cells were transiently transfected with deleted construct (p21^{Cip1/WAF1} Δp53) and then treated with vehicle (-), 1 μM BRL, 100 nM M alone or in combination with BRL. The luciferase activities were normalized to the *Renilla luciferase* as internal transfection control and data were reported as RLU values. Columns are mean ± s.d. of three independent experiments performed in triplicate. *P < 0.05 BRL-treated versus untreated cells. RLU, relative light units; M, mithramycin.

p21^{Cip1/WAF1} antisense oligodeoxynucleotides prevent the BRL-induced growth inhibition in thyroid cancer cells

To confirm the BRL-induced growth inhibition of thyroid cancer cells through p21^{Cip1/WAF1}, WRO and FRO cells were transfected with the p21^{Cip1/WAF1} antisense (AS)-ODN or control scrambled (cs)-ODN in the proliferation assay. In previous studies, the utilization of AS-ODN of p21^{Cip1/WAF1} *in vivo* and *in vitro* has been shown to be a sensitive way to examine its role in the inhibition of cell proliferation, angiogenesis, matrix protein production, and induction of apoptosis (Tian *et al.* 2000, Weiss *et al.* 2003). Noteworthy, the inhibitory effect exerted by BRL on cell proliferation was reversed by p21^{Cip1/WAF1} AS-ODN but not by cs-ODN in both thyroid cancer cells (Fig. 7A) in which the p21^{Cip1/WAF1} protein levels under these experimental conditions were assessed by WB (Fig. 7B). To support our data we evaluated a dose-dependence of the growth inhibition (Fig. 7C)

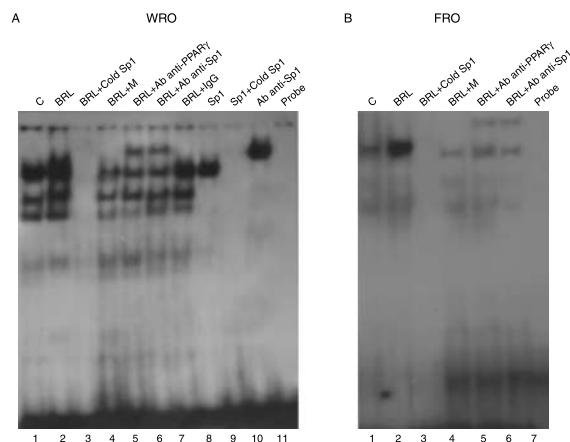


Figure 5 BRL increases the binding to Sp1 sites located within the p21^{Cip1/WAF1} promoter region in EMSA. (A) Nuclear extracts from WRO cells (lane 1) were incubated with a double-stranded Sp1 sequence probe labeled with [γ -³²P] and subjected to electrophoresis in a 6% polyacrylamide gel. In lane 2, nuclear extracts were treated with 1 μM BRL. Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled Sp1 probe (lane 3) or 100 nM mithramycin (M) (lane 4). Anti-PPAR γ and anti-Sp1 antibodies and IgG were incubated with nuclear extracts treated with BRL (lanes 5, 6, and 7). One microliter of human Sp1 recombinant protein (lane 8) was incubated with a double-stranded Sp1 sequence probe. A 100-fold molar excess of unlabeled Sp1 probe (lane 9) or anti-Sp1 antibody (lane 10) was added to Sp1 protein. Lane 11 contains probe alone. (B) Nuclear extracts from FRO cells were incubated with a double-stranded Sp1 sequence probe labeled with [γ -³²P] and subjected to electrophoresis in a 6% polyacrylamide gel. In lane 2, nuclear extracts were treated with 1 μM BRL. Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled Sp1 probe (lane 3) or 100 nM mithramycin (M) (lane 4). Anti-PPAR γ and anti-Sp1 antibodies were incubated with nuclear extracts treated with BRL (lanes 5 and 6 respectively). Lane 7 contains probe alone.

and the p21^{Cip1/WAF1} induction (Fig. 7D) by PPAR γ , transfecting in both thyroid cancer cell lines increasing doses of PPAR γ -expressing vector (0.5, 1, and 2 μg). Taken together, these results indicate that the anti-proliferative effect exerted by BRL in thyroid cancer cells involves a positive crosstalk between PPAR γ - and p21^{Cip1/WAF1}-signaling pathway.

Discussion

An inhibitory action of PPAR γ in the thyroid tumors growth and progression was documented (Ohta *et al.* 2001, Martelli *et al.* 2002, Klopper *et al.* 2004, Aiello *et al.* 2006). Particularly, BRL and other thiazolidinediones inhibit thyroid carcinoma cell proliferation in a PPAR γ -dependent fashion (Chung *et al.* 2002, Martelli *et al.* 2002, Klopper *et al.* 2004, Aiello *et al.* 2006, Chen *et al.* 2006, Copland *et al.* 2006), PPAR γ mediates the upregulation of p21^{Cip1/WAF1}

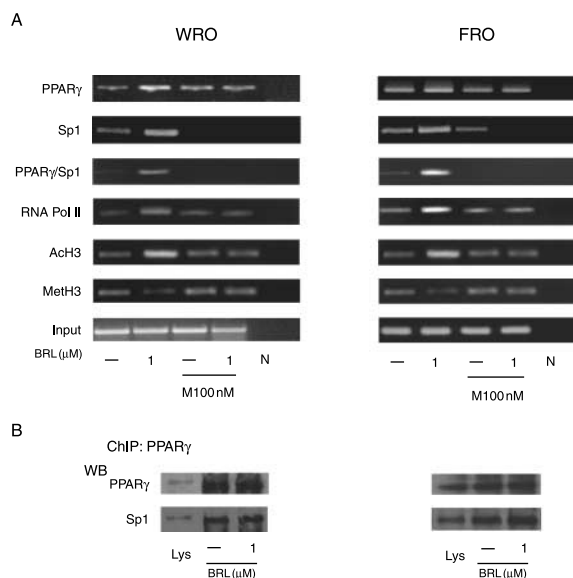


Figure 6 Functional interaction between PPAR γ and p21^{Cip1/WAF1} promoter in ChIP and ReChIP assays. (A) WRO and FRO cells were treated as indicated, then cross-linked with formaldehyde, and lysed. The soluble chromatin was immunoprecipitated with the anti-PPAR γ , anti-Sp1, anti-RNA Pol II, anti-acetyl histone H3 (AcH3), and anti-trimethyl histone H3 (MethH3) antibodies. Chromatin immunoprecipitated with the anti-PPAR γ antibody was re-immunoprecipitated with the anti-Sp1 antibody. The p21^{Cip1/WAF1} promoter sequence containing Sp1 was detected by PCR with specific primers, as described in Materials and methods. To control input DNA, the p21^{Cip1/WAF1} promoter was amplified from 30 μ l initial preparations of soluble chromatin (before immunoprecipitations). N, negative control provided by PCR amplification without DNA sample; M, mithramycin. (B) Immunoblots of PPAR γ and Sp1 from WRO and FRO cells treated with BRL 1 μ M in which chromatin was immunoprecipitated with the anti-PPAR γ antibody. Lys, cell lysates.

(Chung *et al.* 2002, Han *et al.* 2004, Hong *et al.* 2004), p21^{Cip1/WAF1} mediates PPAR γ -induced growth arrest in cancer cells including thyroid (Han *et al.* 2004, Copland *et al.* 2006), and PPAR γ mediates p21^{Cip1/WAF1} mRNA induction via Sp1-enhanced binding to the p21^{Cip1/WAF1}, KDR, and hormone-sensitive lipase promoters (Han *et al.* 2004, Hong *et al.* 2004, Sassa *et al.* 2004, Deng *et al.* 2006). In this report, we demonstrated that PPAR γ acts as a tumor suppressor gene against two different human thyroid carcinoma cell lines. In both WRO, a well-differentiated thyroid follicular carcinoma and FRO, an undifferentiated/anaplastic thyroid carcinoma, PPAR γ inhibits cell growth by stimulating the expression of the cyclin-dependent kinase inhibitor p21^{Cip1/WAF1}.

From our study, the specific role of PPAR γ in upregulating p21^{Cip1/WAF1} raised by the evidence that this effect was completely abrogated either in the presence of specific siRNA to PPAR γ or by the

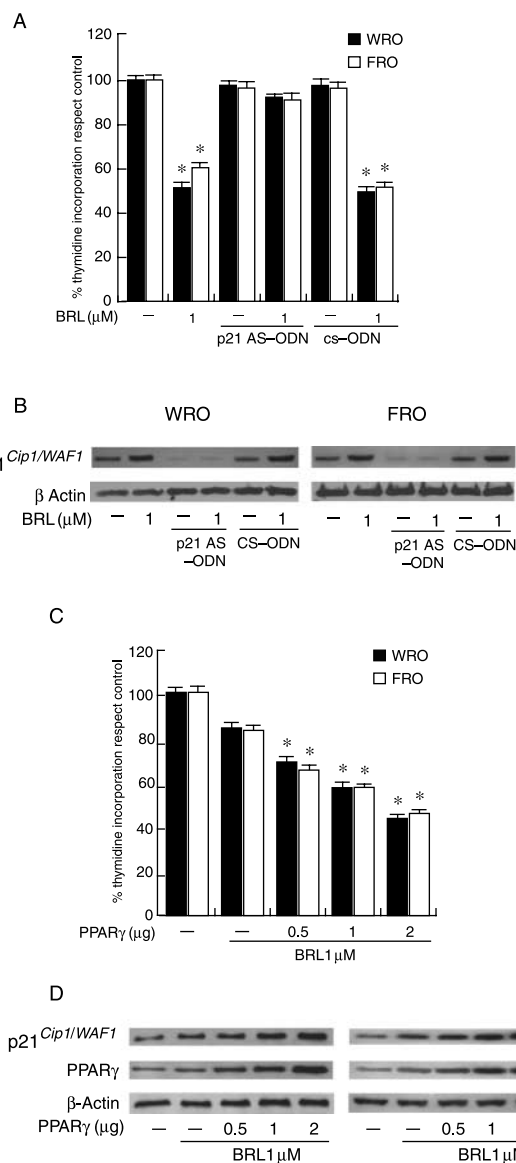


Figure 7 The antiproliferative effects exerted by BRL in thyroid cancer cells are p21^{Cip1/WAF1}-mediated. (A) WRO and FRO cells were treated in the presence of vehicle (-) or with BRL 1 μ M, transfected using p21^{Cip1/WAF1} antisense (p21 AS) or control scrambled (cs) oligonucleotides (ODN) as indicated. On day 6, [³H]thymidine incorporation was determined by scintillation counting. Data are expressed as mean \pm s.d. of three independent experiments performed in triplicate. * P < 0.05 BRL-treated versus untreated cells. (B) Immunoblots of p21^{Cip1/WAF1} from thyroid carcinoma cells treated as in A. β -Actin was used as the loading control. (C) WRO and FRO cells were treated in the presence of vehicle (-) or with BRL 1 μ M and transfected with increasing doses of PPAR γ expression plasmid. After 48 h, [³H]thymidine incorporation was determined by scintillation counting. Data are expressed as mean \pm s.d. of three independent experiments performed in triplicate. * P < 0.05 BRL-treated versus untreated cells. (D) Immunoblots of p21^{Cip1/WAF1} and PPAR γ from thyroid carcinoma cells treated as in C. β -Actin was used as loading control.

pretreatment with GW, a potent and selective antagonist of PPAR γ that causes specific and irreversible loss of binding. The molecular events responsible for the induction of p21^{Cip1/WAF1} by PPAR γ ligands are consistent with the enhanced transcriptional activation of this gene as it raised by the capability of the ligand to activate the promoter of p21^{Cip1/WAF1}. Then, we asked whether the above reported activation was direct or mediated by p53, being p21^{Cip1/WAF1} a classic p53 responsive gene. Although, the p21^{Cip1/WAF1} gene was first identified as a p53-inducible gene, more recently its induction was shown to occur via p53-independent mechanisms in various cell lines stimulated for differentiation and growth arrest, including thyroid carcinoma cells (Park et al. 2001). The p53 tumor suppressor gene is mutated in about half of most types of cancer arising from a wide spectrum of tissues (Bourdon 2007). As it is concerned with thyroid carcinoma, p53 mutations have rarely been detected in follicular carcinomas, while are more frequent (25–85%) in the anaplastic type (Ito et al. 1992, Nakamura et al. 1992, Donghi et al. 1993). In both thyroid cancer cell lines, p21^{Cip1/WAF1} BRL transactivation occurred independent of p53 since the deletion of the two p53 response elements of the promoter maintains promoter activity. Moreover, since p53 levels do not respond to BRL treatment it is reasonable that the mechanism is p53-independent because our thyroid cancer cell lines have either mutated nonfunctional p53 (WRO cells) or scantily expressed p53 wild-type form (FRO cells), as reported previously (Fagin et al. 1993).

Multiple transcription factor binding sites within the human p21^{Cip1/WAF1} promoter have been described to be able to interact with Sp1 including regulatory elements for Sp1 that play important roles in cell cycle arrest and apoptosis in carcinoma cells (Moon et al. 2006, Tvrdík et al. 2006, Fang et al. 2007). Sp1 has also been shown to interact directly with proteins of the basal transcription machinery such as TFIID components (Hilton & Wang 2003). On the other hand, Sp1 interacts physically and cooperates functionally with several sequence-specific activators including NF- κ B, GATA, YY1, E2F1, Rb, and SREBP-1 (Noé et al. 1998, Rotheneder et al. 1999, Flück & Miller 2004, Zhang et al. 2005, Teferedegne et al. 2006). Sp1 has been considered traditionally as a ubiquitous factor associated closely with core promoter activities; it has recently been observed that it participates in several cases of regulated gene transcription triggered by multiple signaling pathways and metabolic or differentiation conditions. Different members of the nuclear hormone receptors, preferentially activate Sp1 and

other Sp family proteins binding to GC-rich sites that in turn regulate a large number of constitutive and induced mammalian genes (Scarpulla 2002).

Physical interaction of Sp1 with the progesterone receptor (Tseng et al. 2003), estrogen receptor (Panno et al. 2006, Li et al. 2007), and with the orphan receptor chicken ovalbumin upstream promoter transcription factor (Pipaón et al. 1999) has already been demonstrated. Recently, it has been showed that NRs can form ternary complexes with Sp1 and GC-rich DNA and in this complex NRs could also serve as auxiliary factors for Sp1 bound to GC-rich DNA (Husmann et al. 2000). Our data sustain this functional model of a tripartite complex of Sp1, PPAR γ , and GC-rich DNA. For instance, in our study, EMSA and ChIP assays demonstrated the coexistence of the two proteins in the DNA-binding complexes, addressing that the functional interaction between PPAR γ and Sp1 could modulate the activity of the human p21^{Cip1/WAF1} promoter under basal other than BRL-inducible conditions in WRO and FRO cell lines. Our data show a discrepancy with those of previous publication in which Sp1 interacts with the consensus GC-rich sequence in p21^{Cip1/WAF1} promoter gene and this band was not directly associated with PPAR γ under the conditions used for the gel mobility shift assay (Hong et al. 2004). Besides, it has also been reported in another cell system that PPAR γ ligands enhanced nuclear protein-binding activities of Sp1 and C/EBP sites in p21^{Cip1/WAF1} promoter (Han et al. 2004). Our data show that treatment with mithramycin, a specific inhibitor of Sp1, completely reversed the activation of p21^{Cip1/WAF1} promoter by BRL, suggesting furthermore that the interaction between PPAR γ and Sp1 is essential for such activation.

Noteworthy, ChIP analysis also evidenced how the functional interaction between PPAR γ and Sp1 in human p21^{Cip1/WAF1} promoter is concomitant with an increase in the RNA Pol II recruitment, associated with an enhanced histone H3 acetylation and a reduced H3 methylation. All these events feature an 'open' chromatin conformation and address the coordinated action of the general transcription machinery with chromatin modifying and remodeling enzymes.

The crucial role of p21^{Cip1/WAF1} in mediating the inhibitory effect of PPAR γ in cell proliferation has been definitively demonstrated in thyroid cells transfected with p21 AS-ODN and with increasing PPAR γ plasmid amounts in the cell growth assay. Finally, our findings evidenced how PPAR γ inhibits thyroid cancer cell growth circumventing p53 mutation. This supports PPAR γ agonists as single agents or as part of combination regimens in the

treatment of patients affected by follicular and particularly anaplastic thyroid cancer.

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