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Cladosporol A, a new peroxisome proliferator-activated receptor γ (PPARγ) ligand, inhibits colorectal cancer cells proliferation through β-catenin/TCF pathway inactivation

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ABSTRACT

Background: Cladosporol A, a secondary metabolite from *Cladosporium tenuissimum*, exhibits antiproliferative properties in human colorectal cancer cells by modulating the expression of some cell cycle genes ($p21^{waf1/cip1}$, cyclin D1).
Methods: PPARγ activation by cladosporol A was studied by overexpression and RNA interference assays. The interactions between PPARγ and Sp1 were investigated by co-immunoprecipitation and ChIp assays. β-Catenin subcellular distribution and β-catenin/TCF pathway inactivation were analyzed by western blot and RTqPCR, respectively. Cladosporol A-induced β-catenin proteasomal degradation was examined in the presence of the specific inhibitor MG132.
Results: Cladosporol A inhibits cell growth through upregulation of $p21^{waf1/cip1}$ gene expression mediated by Sp1-PPARγ interaction. Exposure of HT-29 cells to cladosporol A causes β-catenin nuclear export, proteasome degradation and reduced expression of its target genes. Upon treatment, PPARγ also activates E-cadherin gene at the mRNA and protein levels.
Conclusion: In this work we provide evidence that PPARγ mediates the anti-proliferative action of cladosporol A in colorectal cancer cells. Upon ligand activation, PPARγ interacts with Sp1 and stimulates $p21^{waf1/cip1}$ gene transcription. PPARγ activation causes degradation of β-catenin and inactivation of the downstream target pathway and, in addition, upregulates E-cadherin expression reinforcing cell–cell interactions and a differentiated phenotype.
General significance: We elucidated the molecular mechanisms by which PPARγ mediates the anticancer activity of cladosporol A.

1. Introduction

Colorectal cancer (CRC) is one of the most widespread tumors and the third cause of cancer-related death in Western Countries [1,2]. Despite the efforts to ameliorate the survival, only few achievements have been obtained in the care of patients with advanced cancers. In addition to surgical resection, chemotherapy with traditional cytotoxic agents is still the favored approach in fighting cancers. Unfortunately, most of these drugs act as non-specific inhibitors of cell division and proliferation, interfering with DNA or RNA synthesis, not only in fast-dividing malignant cells, but also high proliferating normal cells, like hematopoietic, epithelial intestinal and germ cells. The efficacy of these drugs in the treatment of cancer is shadowed by these secondary undesired effects, not significantly changing the mortality rates of most tumors. These considerations strongly stimulated the search for new therapeutic tools in CRC treatment by targeting specific molecular defects. Great effort has also been made to clarify the pathways underlying normal tissue development and identify the targets of these new drugs. About two hundred gene products, with pivotal functions in many cellular processes, have been found and their targets identified. Interestingly, several of them are structurally and/or functionally modified in cancer cells after exposure to environmental carcinogens, proinflammatory and tumor agents [3–5].

Peroxisome proliferators-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily and were identified as mediators of peroxisome proliferation in rodent liver parenchymal cells in response to the hypolipidemic drug clorofibrate [6]. PPARs regulate gene expression acting as ligand-activated transcription factors in different physiological and pathophysiological processes, including lipid

Abbreviations: CRC, colorectal cancer; PPARs, peroxisome proliferators-activated receptors; PPARγ, peroxisome proliferators-activated receptor γ; PPRE, PPARs response element; TCF, T cell factor; LEF, lymphoid-enhancing factor; RT-PCR, reverse transcriptase-polymerase chain reaction; ChIp, chromatin immunoprecipitation

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metabolism and adipocyte differentiation, glucose metabolism and insulin sensitivity, inhibition of cancer cell proliferation and inflammation [7–9]. In order to stimulate transcription, PPARs are activated by natural (long chain unsaturated fatty acids and eicosanoid derivatives) or synthetic ligands (fibrates and thiazolinediones), form heterodimeric complexes with retinoid X receptors (RXRs) and bind specific response elements (PPRE) in the promoter region of the target genes [10,11]. The PPARγ isoform, besides its metabolic actions, plays a crucial role in adipocyte differentiation and regulates cellular proliferation, differentiation and apoptosis in the gastrointestinal tract [12–14]. Based on these observations, PPARγ has been proposed to be involved in colorectal carcinogenesis. Indeed, PPARγ activation by thiazolinediones induces suppression of sporadic CRC formation in rodent models [15–17]. Although some papers have reported that the treatment with PPARγ ligands induces CRC in APC^{Min} mice, most recent data support the role as tumor suppressor gene [18–21].

The Wnt/β-catenin pathway is crucial in differentiation, development and tissue homeostasis and is abnormally modulated in various human diseases, including cancer [22,23]. β-Catenin stability and, consequently, its overall amount in the cell is finely regulated by a degradation complex that includes the adenomatosis polyposis coli protein (APC), glycogen synthase kinase-3β (GSK-3β), casein kinase 1 (CK1) and the scaffolding protein axin. In the absence of Wnt signaling, β-catenin is phosphorylated by GSK-3β and degraded via proteasome activation [23]. The binding of a Wnt ligand to its receptor, composed by a member of the Frizzled (Fzd) family and low-density lipoprotein-receptor-related proteins (LRP5 or LRP6), recruits the cytoplasmic adapter protein disheveled (Dvl) to the destruction complex through interaction with axin, inhibiting β-catenin phosphorylation by GSK-3β. The unphosphorylated form of β-catenin accumulates in the cytoplasm and translocates to the nucleus where it binds to the T cell (TCF) and lymphoid-enhancing (LEF) factors to activate transcription of Wnt target genes [24,25]. β-Catenin promotes cell growth and CRC initiation through induction of genes controlling cell cycle progression; as PPARγ, in contrast, inhibits CRC, it has been proposed that it might interfere with β-catenin-dependent transcriptional modulation and thus inhibit CRC initiation/progression [26,27].

Several experimental evidences support the existence of a functional link between PPARγ and the β-catenin pathways and a direct interaction between them has been disclosed [28,29]. This interaction seems to involve the TCF/LEF binding domain of β-catenin and the β-catenin binding domain (CBD) of PPARγ [28]. Natural and/or synthetic molecules, recognized as PPARγ ligands, display a potential ability to inhibit β-catenin and could be useful tools for chemoprevention in cancer management.

β-catenin interacts also with E-cadherin and contributes, along with other plasma membrane-associated proteins, to the formation of adherent junctions that are crucial for epithelial cell–cell adhesion. Notably, E-cadherin and APC compete for the binding to the same region of β-catenin, thus determining its accumulation in distinctive cell compartments [30]. Through these interactions, E-cadherin inhibits epithelial to mesenchymal transition and hence metastasis and invasion [31,32]. The E-cadherin and β-catenin complex is pivotal in determining the progression of several human carcinomas [33]. E-cadherin promoter contains a canonical PPRE that has been demonstrated to mediate the PPARγ ligand-activated response in prostate cancer cell lines [34]. This finding suggests a functional and direct link between PPARγ and E-cadherin.

We previously tested the antiproliferative properties of cladosporol A on three CRC derived cell lines (HT-29, SW480 and CaCo-2) and sought to elucidate the underlying molecular mechanisms. Specifically, we showed that exposure of HT-29 cells to the drug caused cell cycle arrest at the G1/S phase, supported by a robust $p21^{waf1/cip1}$ overexpression, a significant CDK2, CDK4, cyclin D1 and cyclin E downregulation and inhibition of the CDK2 and CDK4 kinase activities [35]. We also showed that the cell-cycle block was $p21^{waf1/cip1}$-dependent and p53-independent and provided evidence that growth inhibition and induced redox response might be mediated by ERK and JNK. Finally, we demonstrated that the increase of $p21^{waf1/cip1}$ gene transcription was achieved through an Sp1-mediated mechanism [35].

In this work we provide evidence that PPARγ plays a central role in mediating the antiproliferative action of cladosporol A through the direct interaction with Sp1, binding to the promoter and activation of $p21^{waf1/cip1}$ gene transcription. In addition, cladosporol A induces β-catenin degradation and β-catenin/TCF pathway impairment as shown by reduced c-Myc and cyclin D1 transcription. Finally, cladosporol A induces E-cadherin expression thus antagonizing metastasis and invasion.

2. Materials and methods

2.1. Cells, antibodies and reagents

HT-29, LoVo, HCT116, SW480 and Geo (from human colon cancer) cells were obtained from the American Type Culture Collection (Rockville, MD). p53 −/− HCT116 were a kind gift from Dr. Bevilacqua (University of Naples "Federico II").

These cell lines bear different genetic abnormalities typical of human CRC, because they have truncated or mutant adenomatous polyposis coli (APC) gene. Moreover, SW480 cells express mutated forms of both *TP53* (Arg273 > His and Pro309 > Ser) and *RAS* (Val12 > Glu). HT-29 cells bear a mutated *TP53* (Arg 273 > His), but a wild-type *RAS* allele. HCT116 cells carry a mutated form of *RAS* and a wild-type *TP53*. Finally, CaCo-2 cells express wild-type *RAS*, but carry a mutated *TP53* allele form [36].

Antibodies against $p21^{waf1/cip1}$, cyclin D1, p53, Sp1, PPARγ, PPARα, β-actin and Protein A/G Plus Agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against E-cadherin and β-catenin were purchased from BD Transduction Laboratories (BD, New Jersey, USA). Anti-mouse and anti-rabbit IgG peroxidase-linked secondary antibodies, ECL and ECL Plus Western blotting detection kit were purchased from Amersham Life Science (Little Chalfont, England). D-MEM (Dulbecco's Modified Eagle's Medium), D-luciferin sodium salt, thiazolinedione, GW9662 were from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin–streptomycin, L-glutamine, trypsin-EDTA and OptiMEM I were obtained from Gibco (Carlsbad, CA, USA). Charcoal/dextran-treated FBS was purchased from Hyclone (Logan, Utah, USA). Fetal calf serum (FCS), lipofectamine 2000, TRIZOL, and SuperScript II reverse transcriptase were from Invitrogen (Carlsbad, CA, USA). SYBR Green was purchased from BioRad (Hercules, CA, USA). AmpliTaq Gold was purchased from Applied Biosystems (Foster City, CA, USA).

2.2. Cell culture and cladosporol A treatment

Human colon adenocarcinoma cell lines HT-29, SW480 and Geo were grown as a monolayer in D-MEM containing 10% FBS, 1% penicillin–streptomycin and 1% L-glutamine; LoVo and HCT116 were grown as a monolayer in RPMI 1640 completed with 10% FBS, 1% penicillin–streptomycin and 1% L-glutamine. Cells were cultured in 100 mm plates, up to 70–80% confluence, at 37 °C in a 5% CO_2 humidified atmosphere. Cladosporol A was dissolved in dimethylsulfoxide (DMSO) and mixed with fresh medium to a 20 μM final concentration. Cladosporol A was obtained from cultures of a strain of *Cladosporium tenuissimum* designated ITT21 and grown on sugar-rich malt agar [37]. This fungus is a hyperparasite of rust fungi and its derived compounds possess antifungal activity [38]. Cladosporol A was the main metabolite present in the culture and was isolated as a white powder. The molecule was purified at 98% [37]. Cells at 40% confluence were treated with Cladosporol A in the presence of 10% charcoal/dextran-treated FBS containing 1% penicillin–streptomycin and 1% L-glutamine. In all treatments, the DMSO final concentration in the medium was less than 0.1%.

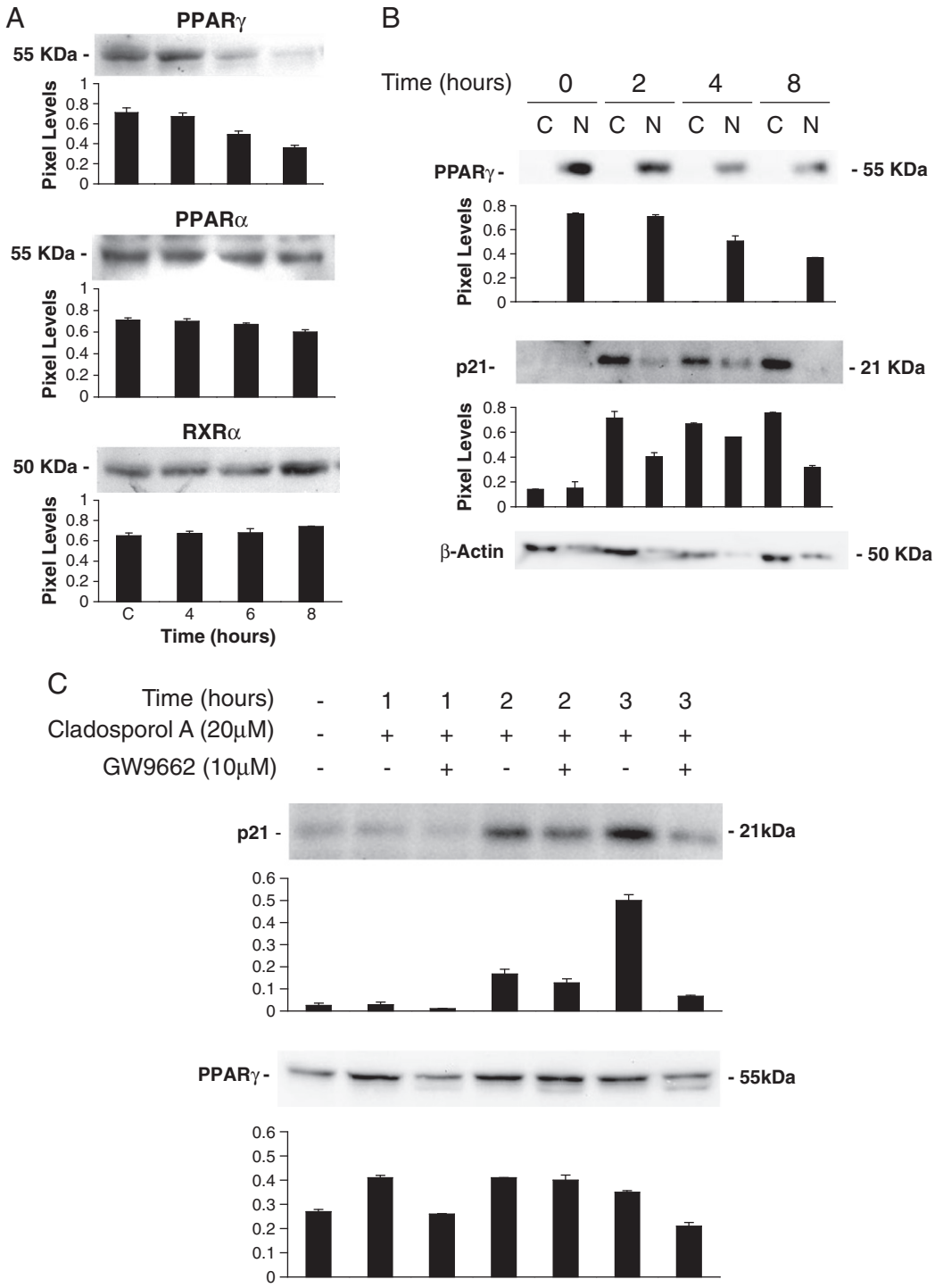

Fig. 1. Cladosporol A modulates PPARγ expression in HT-29 cells. (A) Western blotting analysis on total protein extracts produced from HT-29 cells treated or not with 20 μM cladosporol A for 4, 6 and 8 h. Anti-PPARγ, anti-PPARα and anti-RXRα antibodies were used to probe untreated and cladosporol A-treated HT-29 cells. Total extracts from untreated cells are indicated by C (control). (B) Western blotting analysis on nuclear (N) and cytoplasmic (C) protein extracts produced from HT-29 cells treated with 20 μM cladosporol A for 2, 4 and 8 h. Anti-PPARγ anti-$p21^{waf1/cip1}$ and anti-β-actin antibodies were used to probe cladosporol A-treated HT-29 cells. (C) Western blotting analysis on total protein extracts produced from HT-29 cells treated or not with 20 μM cladosporol A for 1, 2 and 3 h and in presence or absence of GW9662 (10 μM). Anti-PPARγ and anti-$p21^{waf1/cip1}$ antibodies were used to probe untreated and cladosporol A-treated HT-29 cells. To control the samples loaded derived from untreated and cladosporol A-treated HT-29 cells, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of proteins/β-actin of at least 3 independent experiments. The Western blotting assays reported here are representative of a single exemplificative experiment.

2.3. Protein extract preparation and western blotting analysis

Treated and untreated cells were lysed in Ripa buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.6, 10 mM EDTA, 1% NP-40) containing also a protease inhibitors cocktail (AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin hemisulfate salt, Pepstatin A) and then centrifugated at 13,000 rpm for 10 min, at 4 °C. Supernatant containing total proteins was quantified and 80 μg of each sample were used for western blot experiments. To prepare nuclear and cytoplasmatic extracts, cells were collected after cladosporol A treatment and lysed using Lysis

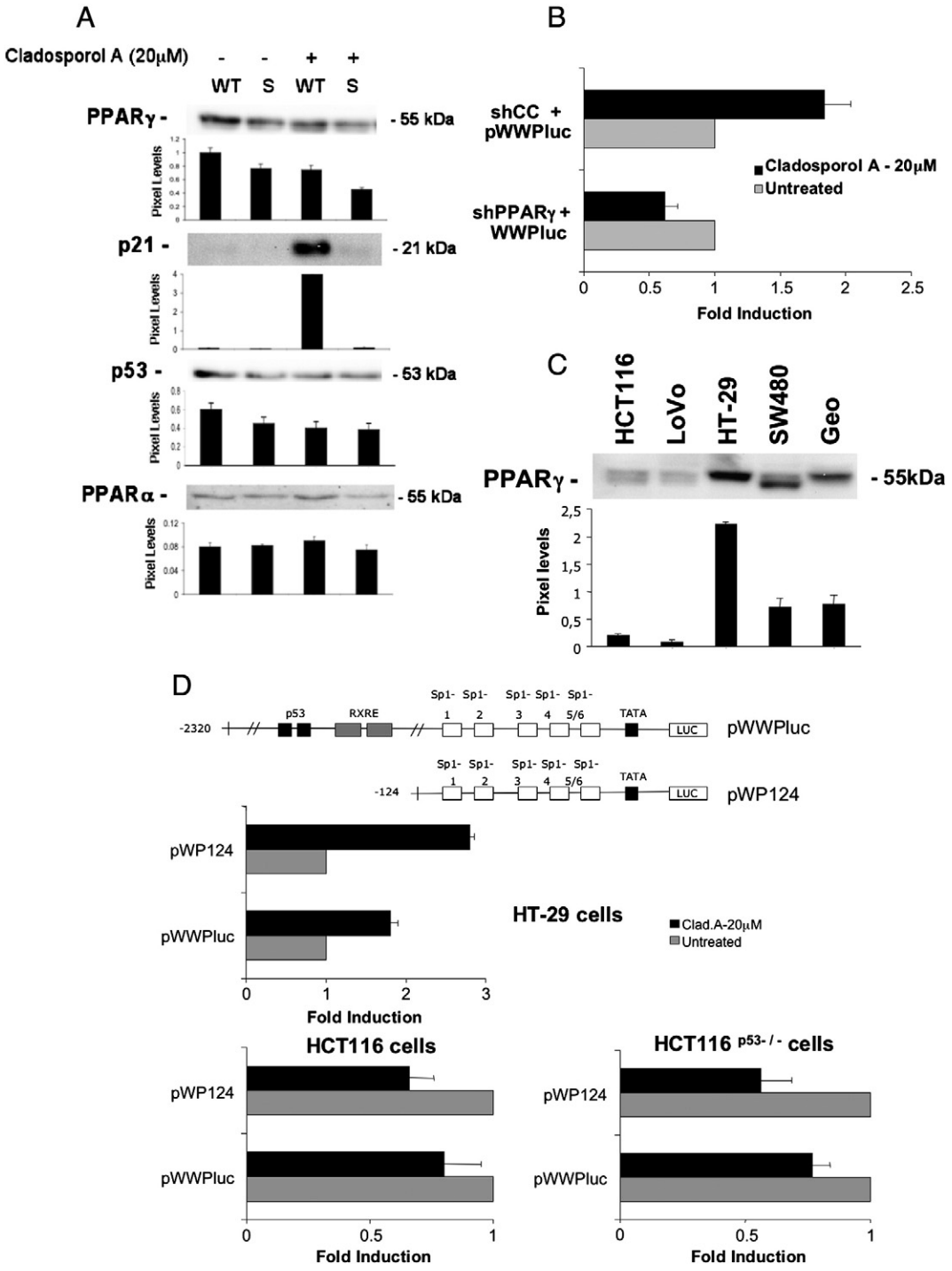

Fig. 2. A functional PPARγ is required for cladosporol A-induced p21$^{waf1/cip1}$ expression. (A) Western blotting analysis on total protein extracts produced from HT-29 cells transiently transfected with a PPARγ siRNA recombinant plasmid and, afterwards, treated or not with 20 μM cladosporol A for 24 h. Anti-PPARγ and anti-p21$^{waf1/cip1}$, anti-p53 and anti-PPARα antibodies were used to probe untreated and cladosporol A-treated HT-29 cells. To control the samples loaded derived from untreated and cladosporol A-treated HT-29 cells, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of proteins/β-actin of at least 3 independent experiments. The Western blotting assays reported here are representative of a single exemplificative experiment. (B) Transient transfection assay in HT-29 cells in the presence of the PPARγ siRNA recombinant plasmid (shPPARγ) together with the pWWPluc reporter construct bearing the p21$^{waf1/cip1}$ promoter. The results of the transiently transfections as fold induction of luciferase activity are reported. The luciferase activities were normalized to the β-galattosidase ones used as control. Data shown are mean ± SD of 3 independent experiments performed in duplicate. (C) Western blotting analysis on total protein extracts produced from HCT116, LoVo, HT-29, SW480 and Geo cells. Anti-PPARγ antibody was used to probe. To control the samples loaded an anti-β-actin antibody was used and the bar graph represents the mean ± SD of PPARγ/β-actin of at least 3 independent experiments. The Western blotting assay reported here is representative of a single exemplificative experiment. (D) Transient transfection assay in HT-29, HCT116 wt, and HCT116 p53 −/− cells with the two reporter plasmid bearing the entire promoter region of p21$^{waf1/cip1}$ gene (pWWPluc) and its 124 bp-long mutated version (pWP124), respectively. The results of the transiently transfections as fold induction of luciferase activity are reported. The luciferase activities were normalized to the β-galattosidase ones used as control. Data shown are mean ± SD of 3 independent experiments performed in duplicate. (E) Transient transfection assay in HCT116 wt, HCT116 p53 −/− and Lovo cells with the reporter plasmid pWWPluc in presence or absence of the expression plasmid pCDNA3-Flag-PPARγ1wt and treated or not with 20 μM cladosporol A for 24 h. In the upper part of figure is reported the western blotting analysis showing the level of the expression of ectopic PPARγ protein, as well as its molecular mass (60 kDa) compared with that of the endogenous protein (55 kDa). The results of the transiently transfections as fold induction of luciferase activity are reported. The luciferase activities were normalized to the β-galattosidase ones used as control. Data shown are mean ± SD of 3 independent experiments performed in duplicate.

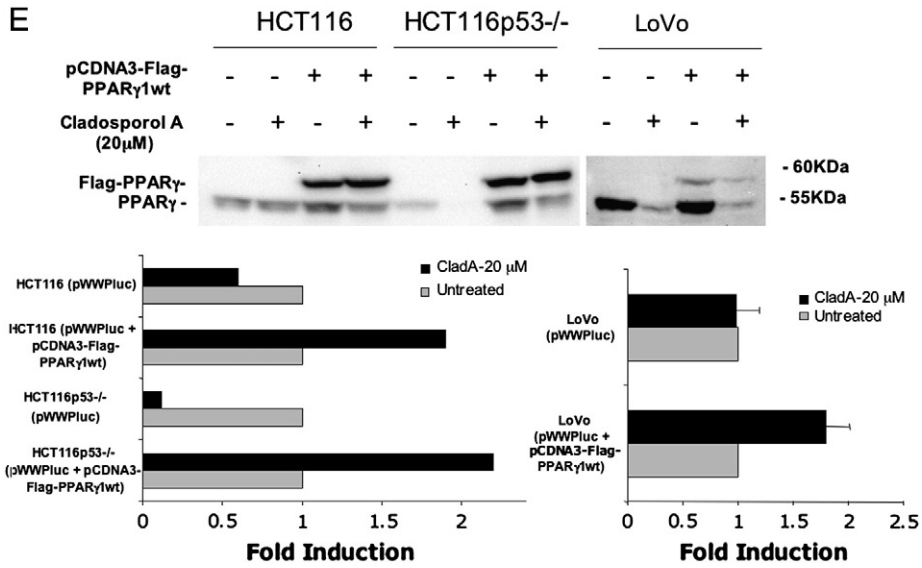

Fig. 2 (*continued*).

Buffer (25 mM Hepes pH 7.8, 0.5% NP40, 50 mM KCl, 1 mM PMSF, 100 μM DTT) completed with protease inhibitor cocktail. Then, nuclear fraction was pelleted by centrifugation and separated from cytoplasmic fraction. After washing (25 mM Hepes pH 7.8, 50 mM KCl, 1 mM PMSF, 100 μM DTT, protease inhibitors cocktail), nuclei were incubated in Extraction buffer (25 mM Hepes pH 7.8, 10% Glycerol, 500 mM KCl, 1 mM PMSF, 100 μM DTT) and completed with protease inhibitor cocktail and nuclear proteins were obtained. Nuclear and cytoplasmic extracts were analyzed by western blotting assay as previously described [35]. The relative intensity of protein bands was measured using the Molecular Imager Chemi-Doc imaging system (Bio-Rad, Hercules CA, USA) and evaluated by the Quantity One software (Bio-Rad, Hercules CA, USA).

2.4. Reverse transcription-PCR (RT-PCR) and real-time quantitative PCR (RT-qPCR) assays

RNA was isolated from treated and untreated HT-29 cells using TRIZOL reagent according to the manufacturer's instructions. The purity,

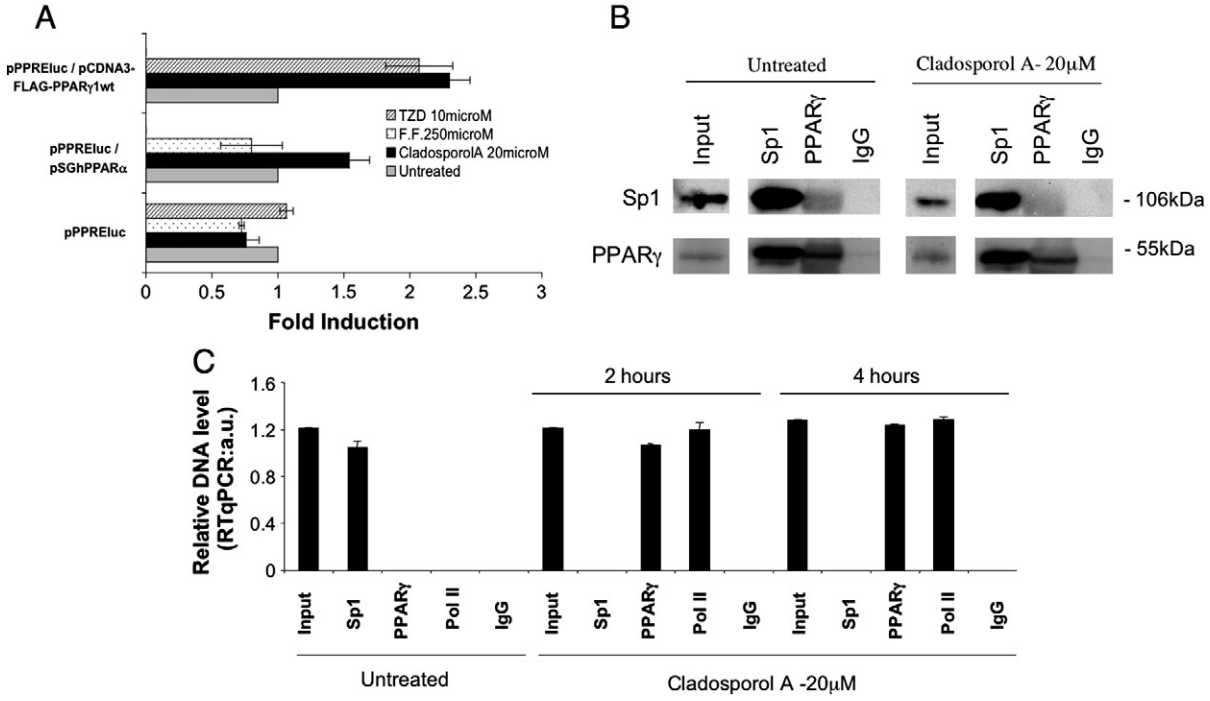

Fig. 3. A PPRE-independent mechanism is required for activation of the $p21^{waf1/cip1}$ gene transcription mediated by cladosporol A. (A) Transient transfection assay in HCT116 wt with the luciferase reporter gene driven by a minimal promoter containing three copies of PPRE motif and in the presence or in the absence of the expression plasmid pCDNA3-Flag-PPARγ1wt and pSG5h-PPARα, alternatively. After transfection cells were treated or not with drugs reported in figure for 24 h. The results of the transiently transfections as fold induction of luciferase activity are reported. The luciferase activities were normalized to the β-galattosidase ones used as control. Data shown are mean ± SD of 3 independent experiments performed in duplicate. (B) Soluble extracts from untreated and cladosporol A-treated HT-29 cells were immunoprecipitated with anti-PPARγ and Sp1 antibodies, respectively. The presence of PPARγ and Sp1 in the immunoprecipitates was determined by western blotting assay with their specific antibodies. (C) Chromatin immunoprecipitation assay on untreated and 20 μM cladosporol A-treated HT-29 for 2 and 4 h. Cross-linked chromatin was immunoprecipitated with the indicated antibodies. The DNA was immunoprecipitated in the presence of the specific anti-Sp1 and anti-PPARγ antibodies and amplified using specific primers for $p21^{waf1/cip1}$. The levels of the amplified DNA fragment, containing the Sp1 binding sites of the minimal $p21^{waf1/cip1}$ promoter, determined by RT-qPCR are reported in the figure. Data shown are mean ± SD of 3 independent experiments performed in duplicate.

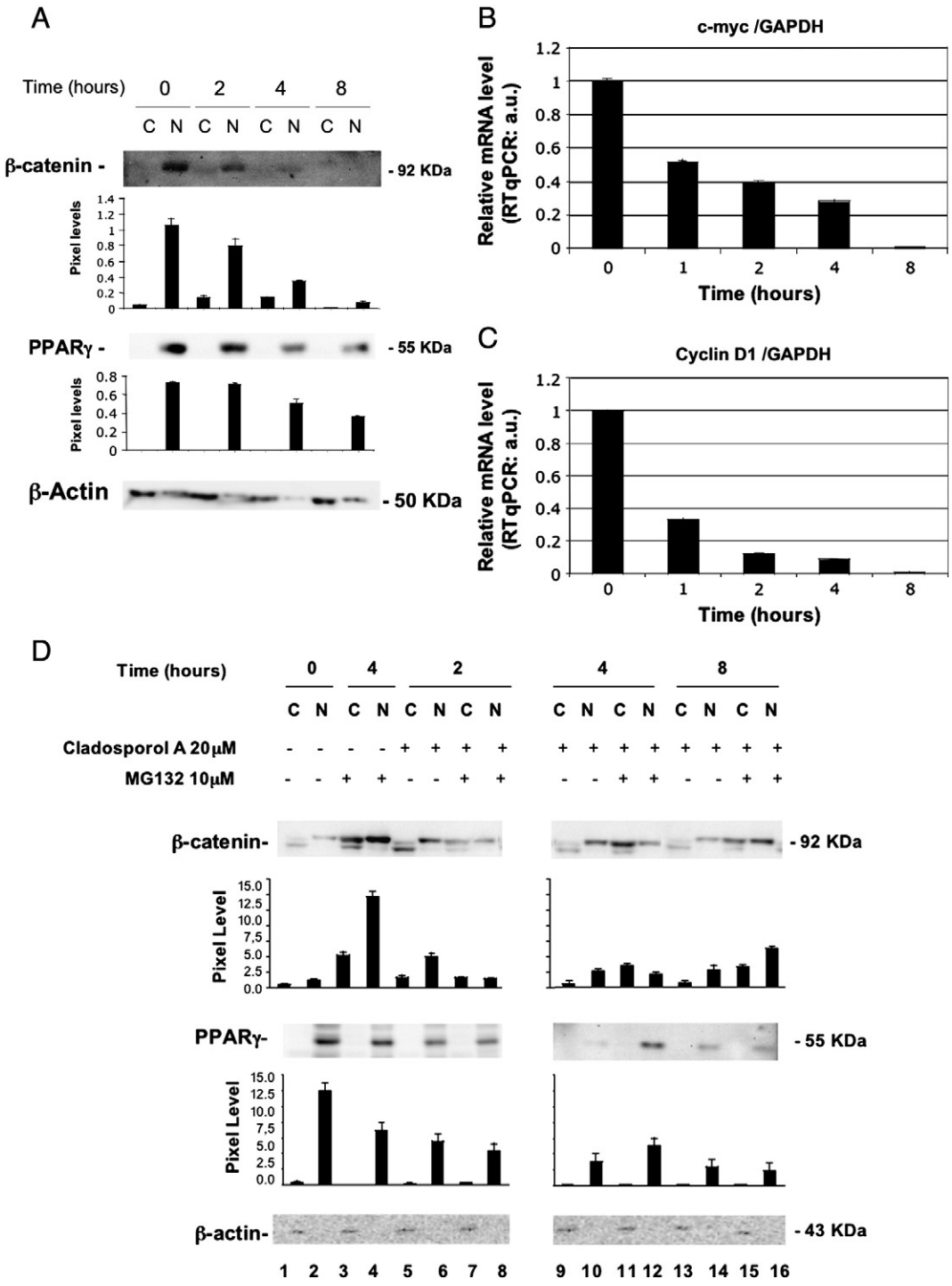

Fig. 4. Cladosporol A interferes in the β-catenin/TCF pathway and determines cell proliferation arrest. (A) Western blotting analysis on nuclear (N) and cytoplasmic (C) protein extracts produced from HT-29 cells treated with 20 μM cladosporol A for 2, 4 and 8 h. Anti-β-catenin, anti-PPARγ and anti-β-actin antibodies were used to probe cladosporol A-treated HT-29 cells. To control the samples loaded derived from untreated and cladosporol A-treated HT-29 cells, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of proteins/β-actin of at least 3 independent experiments. The Western blotting assays reported here are representative of a single exemplificative experiment. (B) Real-time RT-qPCR was performed using total RNA extracted from proliferating HT-29 cells treated or not with 20 μM of Cladosporol A for 1, 2, 4 and 8 h. Amplification of the GAPDH gene was used as internal control. The bar graphs represent the mean ± SD of *c-myc*/GAPDH produced after 3 independent experiments. (C) Real-time RT-qPCR was performed using total RNA extracted from proliferating HT-29 cells treated or not with 20 μM of Cladosporol A for 1, 2, 4 and 8 h. Amplification of the GAPDH gene was used as internal control. The bar graphs represent the mean ± SD of cyclinD1/GAPDH produced after 3 independent experiments. (D) Western blotting analysis on nuclear (N) and cytoplasmic (C) protein extracts produced from HT-29 cells treated with 20 μM cladosporol A for 2, 4 and 8 h in the presence or absence of 10 μM MG132. Anti-β-catenin and anti-PPARγ antibodies were used to probe protein extracts from cladosporol A-treated HT-29 cells. To control the samples loaded derived from untreated and cladosporol A-treated HT-29 cells, an anti-β-actin antibody was used. The Western blotting assays reported here are representative of a single exemplificative experiment. (E) Soluble extracts from untreated and cladosporol A-treated HT-29 cells for 3 and 6 h were immunoprecipitated with anti-β-catenin and anti-PPARγ antibodies, respectively. The presence of β-catenin and PPARγ in the immunoprecipitates was determined by western blotting assay with their specific antibodies. (F) Soluble extracts from HT-29 cells transiently transfected with pCDNA3-Flag-PPARγ1wt and pSG5.HA-β-catenin expression vectors and, afterwards, treated with 20 μM of cladosporol A for 3 and 6 h. Recombinant proteins were immunoprecipitated with anti-HA and anti-FLAG antibodies, respectively. The presence of HA-β-catenin and FLAG-PPARγ in the immunoprecipitates was determined by western blotting assay with the specific antibodies directed against both epitopes.

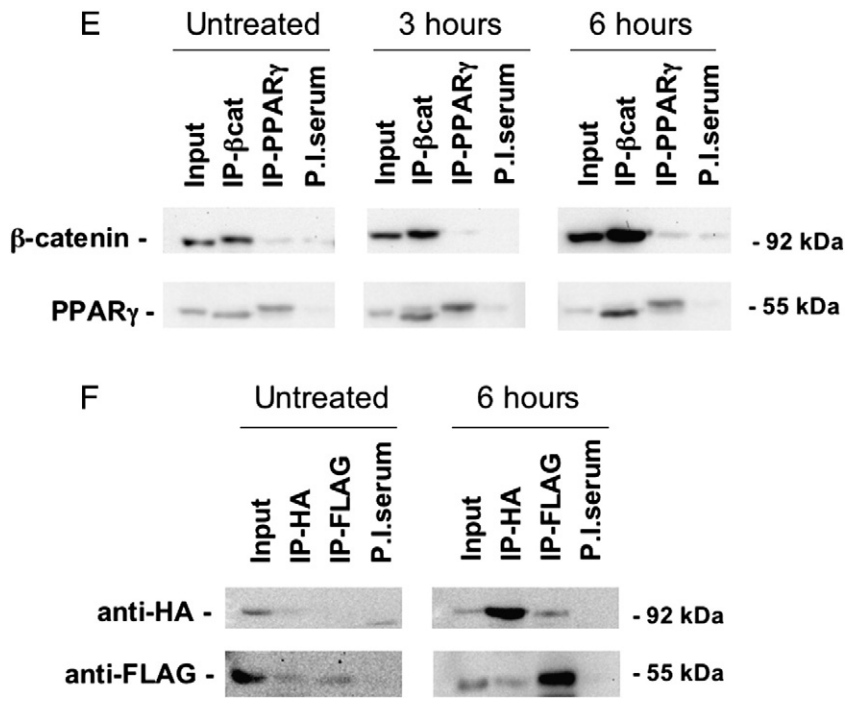

Fig. 4 (*continued*).

integrity and concentration of total RNA were determined by gel electrophoresis and UV spectroscopy. The cDNAs were obtained through reverse transcription as previously described [35]. PCR analysis was performed for E-cadherin gene by using the following primers: hCDH1 Fw 5′-ATGAGTGTCCCCCGGTATCTTC-3′ and hCDH1 Rev 3′-GCGGAATACTAAGAGACGAGCA-5′. As an internal control for the densitometric analysis of the amplified fragments, the housekeeping human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was utilized by using the following primers: 5′-GACCCCTTCATT GACCTCAACTACATG-3′ and 3′-GTGCACCACCCTGTTGCTGTAGCC-5′. PCR reaction was performed in accordance with the protocol previously reported [35]. DNA amplification was carried out allowing 20, 25 and 35 PCR cycles of reactions (94 °C for 1 min, 58 °C for 30 s, 72 °C for 1 min, for each cycle). PCR products were analyzed on 1.2% agarose gels containing ethidium bromide. Gel images were acquired with the Chemi-Doc imaging system (Bio-Rad).

For RT-qPCR, a 20 μl mixture containing 1 μg of cDNA, 10 μl of SYBR Green and 0.4 μM primers of c-Myc (Fw 5′-CACCACCAGCAGCGACTCT-3′, Rev 5′-GCCTGCCTCTTTTCCACAGA-3′) and cyclin D1 (Fw 5′-CCGTCC ATGCGGAAGATC-3′, Rev 5′-ATGGCCAGCGGGAAGAC-3′) or GAPDH was added to each sample. Thirty-five amplification cycles were performed according to the program used in RT-qPCR assay. For each sample, analysis was carried out in triplicate and the results were evaluated by the Gene Expression Relative Quantitation program (Bio-Rad).

2.5. Plasmids and transient transfection experiments

Silencing of PPARγ gene was obtained using pSM2C retroviral plasmid Cat.n. RHS1764-SH2330H6 (Open Biosystem Products, Huntsville, AL, USA), carrying a puromycin resistant gene and directed against the fourth exon of PPARγ gene. As a silencing negative control, we used the SH2330H6 analog (shCC vector) that carries a scrambled RNA. The human wild-type $p21^{waf1/cip1}$ promoter-luciferase fusion construct (pWWP) and its mutant pWP124 were a generous gift from Dr. Y. Sowa (Department of Molecular-Targeting Cancer Prevention, Kyoto Prefectural University of Medicine, Kyoto, Japan) [35]. PPARγ and PPARα overexpression was obtained using, respectively, a PCDNA3-FLAG tagged carrying the complete PPARγ cDNA and pSG5hPPARα containing the human PPARα cDNA blunt-end cloned in BamHI filled site of pSG5 vector (Stratagene, Santa Clara, CA). PPRE-Luc plasmid has a luciferase reporter gene under the transcriptional control of the herpes simplex thymidine kinase (TK) promoter fused to three copies of the PPRE derived from Acyl-CoA oxidase gene. As an internal control for all transient transfection assays, we used the RSV-βGal plasmid, expressing β-galactosidase cDNA driven by the strong Rous Sarcoma Virus (RSV) promoter.

The day before transient transfection, HT-29 or HCT116 cells were plated in 12-well plates to reach a 70% confluence. After 24 h, growth medium was replaced with OPTI-MEM®I, without serum and antibiotics, and cells were transfected using lipofectamine 2000 reagent according to manufacturer's instructions. About 10–12 h after transfection, cells were washed and treated with 20 μM cladosporol A. Transfection samples were carried out in triplicate and the transactivation activities evaluated by luciferase assay. The values were normalized by β-galactosidase assay and the average value for each triplicate was calculated. Total extracts from an aliquot of cells, simultaneously treated with 20 μM cladosporol A, were analyzed by Western Blotting assay to check the expression of PCDNA3-FLAG-PPARγ and pSG5-HA-hPPARα tagged proteins.

2.6. Coimmunoprecipitation and ChIp assay

In protein coimmunoprecipitation assay, HT-29 cells were collected and lysed in JS buffer (HEPES 50 mM pH 7.5, NaCl 150 mM, Glycerol 1%, Triton X-100 1%, MgCl2 1.5 mM, EGTA 5 mM, protease inhibitors). Proteins were extracted and 500 μg incubated o/n at 4 °C with Sp1, PPARγ and β-catenin specific antibodies, previously attached to A/G plus Agarose resin (1 h incubation at 4 °C). After incubation, the obtained pellet was washed 3–4 times in JS buffer, dissolved in Loading Buffer 4× (without DTT and β-mercaptoethanol) and boiled 10 min at 100 °C to denaturate and detach from A/G plus Agarose resin the precipitated proteins. Finally, proteins were examined in a Western Blotting assay. The same procedure was followed to reveal the exogenous proteins

after transfection of wt HCT116 cells with plasmids expressing PCDNA3-FLAG-PPARγ and pSG5-HA-hPPARα tagged proteins.

ChIP assay was carried out according to EpiQuik™ Chromatin Immunoprecipitation Kit (Epigentek Group Inc. Cat. No. P-2002, Farmingdale, NY, USA) protocol as previously described [35]. The amplified DNAs from the precipitated fragments were detected by Real Time quantitative PCR (RT-qPCR).

2.7. Statistical analysis

All experiments were repeated at least three times. Data from Western blotting, RT-PCR, RT-qPCR, transient transfection experiments are expressed as means ± SD.

3. Results

3.1. A functional PPARγ is required for cladosporol A-induced $p21^{waf1/cip1}$ expression

We are interested in identifying new molecules with antiproliferative properties that act as specific PPARγ ligands. To verify whether cladosporol A is a PPARγ ligand, we treated HT-29 cells with 20 μM of cladosporol A for short times and analyzed total protein extracts by western blotting (Fig. 1A). PPARγ was greatly reduced, in particular its nuclear fraction, suggesting that cladosporol A affects its cell content and subcellular distribution (Fig. 1B). It is well known that PPARγ cyclically fluctuates in response to specific ligands [39], an effect that appears to be specific as PPARα and RXRα were not affected (Fig. 1A). As we previously identified $p21^{waf1/cip1}$ as one of the targets of the antiproliferative action of cladosporol A [35], we thought that PPARγ could mediate the increased expression of $p21^{waf1/cip1}$ and its nuclear translocation (already after 2 and 4 h) upon cladosporol A-treatment (Fig. 1B). To this goal, we treated HT-29 cells with 20 μM of cladosporol A, in the presence or absence of GW9662, an irreversible PPARγ antagonist, and analyzed the protein extracts by western blotting. Exposure to GW9662 completely abrogated the $p21^{waf1/cip1}$ increase already 2 h after treatment, reaching the maximum at 3 h (Fig. 1C). Furthermore, we transiently transfected a plasmid carrying a specific sh*PPARG* or a scrambled shRNA in HT-29 cells treated with 20 μM of cladosporol A for 48 h. The specific shRNA reduced PPARγ by about 50% and completely abrogated $p21^{waf1/cip1}$ upregulation; no effects were observed on p53 and PPARα, ruling neither out off-target effects nor with a scrambled shRNA (Fig. 2A). To further verify that the observed effect on $p21^{waf1/cip1}$ gene transcription was dependent upon a functional PPARγ, we cotransfected HT-29 cells with a luciferase reporter construct containing the entire $p21^{waf1/cip1}$ promoter (pWWPluc) with or without the shPPARG plasmid. PPARγ greatly increased luciferase activity whereas its knockdown dramatically inhibited it (Fig. 2B). PPARγ activation by cladosporol A thus well correlates with $p21^{waf1/cip1}$ gene induction, suggesting that the PPARγ-$p21^{waf1/cip1}$ axis controls CRC cell proliferation. Consistently, in HCT116, p53 −/− HCT116 and LoVo CRC derived cells that exhibit low PPARγ, cladosporol A induced $p21^{waf1/cip1}$ gene expression less than in HT-29 cells that instead have higher levels (Fig. 2C). In fact, transfection of the two luciferase reporter plasmids bearing either the entire $p21^{waf1/cip1}$ gene promoter region (pWWPluc) or its 124 bp-long shorter version (pWP124) resulted in a significant increase of luciferase activity only in cladosporol A-treated HT-29 cells (Fig. 2D). The relationship between PPARγ and $p21^{waf1/cip1}$ induction was further confirmed by cotransfecting the reporter plasmid pWWPluc described above and the expression vector bearing the PPARγ cDNA under the control of a strong eukaryotic promoter (pCDNA3-FLAG-PPARγ). Ectopic PPARγ expression significantly stimulated the $p21^{waf1/cip1}$ promoter transcription also in HCT116, p53 −/− HCT116 and LoVo cells exposed to 20 μM cladosporol A for 24 h (Fig. 2E). All together these data demonstrate that $p21^{waf1/cip1}$ gene induction is dependent on the cellular context and on a discrete amount of a functional PPARγ.

3.2. A PPRE-independent mechanism is required for activation of the $p21^{waf1/cip1}$ gene transcription mediated by cladosporol A

We have previously shown that cladosporol A stimulates $p21^{waf1/cip1}$ gene expression via an Sp1-dependent p53-independent mechanism. A 124 bp-long version of the $p21^{waf1/cip1}$ promoter region (pWP124) that includes 5 Sp1 sites, was sufficient to drive transcription from a luciferase reporter gene and site-directed mutagenesis of the Sp1 binding sites abolished such a stimulation [35]. Inspection of the $p21^{waf1/cip1}$ promoter region did not show any canonical PPRE motif, suggesting that a PPRE-independent mechanism was responsible for the induction of the gene. Several examples have reported that transcription stimulation can occur through the specific interaction of Sp1 with PPARγ [40–42]. That cladosporol A stimulates transcription of a target gene, through the binding of PPARγ to its response element, was proved by cotransfection of the PPRE-TK-luc-reporter plasmid and PPARγ expression vector in HCT116 cells. Cladosporol A stimulated luciferase activity at even higher levels than those obtained with TZD, a specific PPARγ-ligand (Fig. 3A). A mild effect was also observed in the same treated cells in the presence of an ectopically expressed PPAR.

To demonstrate that PPARγ activates transcription through a PPRE-independent mechanism also in the case of $p21^{waf1/cip1}$, we performed co-immunoprecipitation assays in cladosporol A-treated and untreated HT-29 cells. A specific interaction between Sp1 and PPARγ was detected in both cases (Fig. 3B). To further prove that Sp1 and PPARγ directly bind and activate $p21^{waf1/cip1}$ gene transcription, we performed chromatin immunoprecipitations on the $p21^{waf1/cip1}$ minimal promoter fragment in the presence or absence of cladosporol A. While Sp1 was steadily recruited on the promoter, PPARγ was engaged only after treatment (Fig. 3C) along with RNA Polymerase II, suggesting that the simultaneous presence of both factors and their interaction is a prerequisite to recruit RNA Polymerase II and trigger transcription initiation. That Sp1 is bound to the promoter also in untreated cells may indicate that it is constitutively present on this region likely to maintain an open chromatin configuration ready to interact with a ligand-activated PPARγ, recruit the basal machinery and drive transcription.

3.3. Cladosporol A interferes with the β-catenin/TCF pathway and determines cell proliferation arrest

To evaluate whether cladosporol A could interfere with the β-catenin/TCF pathway and, in turn, with its target gene transcription, we initially checked β-catenin protein levels in basal conditions and after treatment. A marked and progressive decrease of nuclear β-catenin was shown, after 2 hour exposure to 20 μM cladosporol A, by western blot analysis of HT-29 cell-fractionated extracts (Fig. 4A). An early and dramatic decrease of c-*MYC* and cyclin D1 gene transcription was also detected, indicating that a reduced nuclear β-catenin was associated with a lower transcription of its target genes (Fig. 4B and C). We also assessed whether the effects reported were due to a cladosporol A-bound PPARγ and an increased PPARγ-mediated proteasomal degradation. Treatment of HT-29 cells with 20 μM cladosporol A, for 2, 4 and 8 h, caused a marked reduction of β-catenin in the nucleus (Fig. 4D, lanes 6, 10 and 14); addition of MG132, a proteasome inhibitor, counteracted this effect (Fig. 4D, lanes 8, 12 and 16). Also the cytosolic fraction of β-catenin increased after MG132, indicating that both the nuclear and cytosolic fractions were targeted by PPARγ for proteasome degradation (compare lanes 5, 9 and 13 with lanes 7, 11 and 15 of Fig. 4D). As control, we checked PPARγ levels: the reduction observed upon cladosporol A exposure (Figs. 1B and 4D, lanes 6, 10, 14) was reversed by MG132 (Fig. 4D, lanes 8, 12 and 16).

Finally, we carried out co-immunoprecipitation assays on total extracts from untreated and cladosporol A-treated HT-29 cells for 3

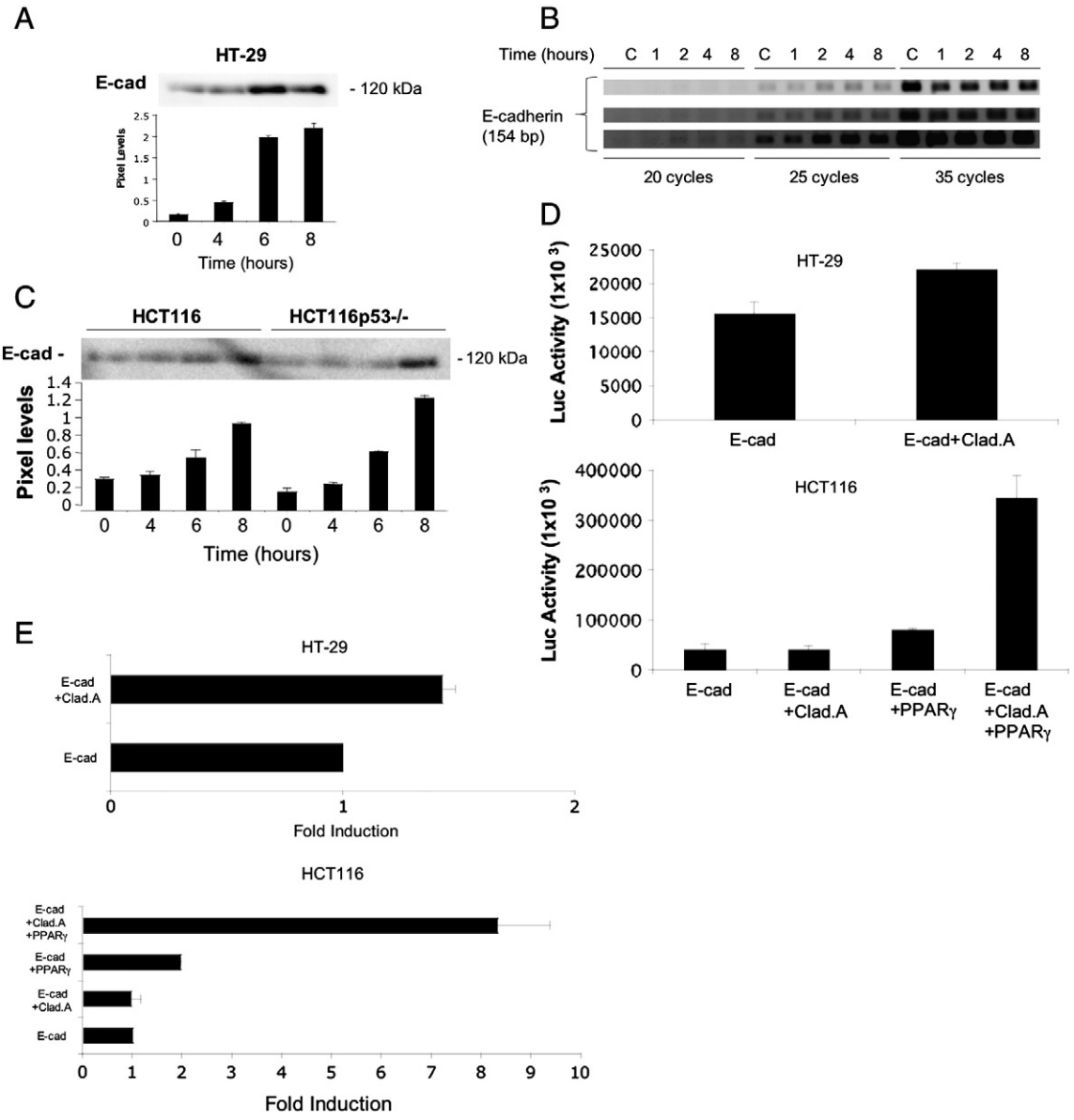

Fig. 5. Increased expression of E-cadherin mRNA and protein in human colon cancer derived cell lines in response to cladosporol A. (A) Western blotting analysis on total protein extracts produced from HT-29 cells treated or not with 20 μM cladosporol A for 4, 6 and 8 h. An anti-E-cadherin antibody was used to probe untreated and cladosporol A-treated HT-29 cells. To control the samples loaded derived from untreated and cladosporol A-treated HT-29 cells, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of E-cadherin/β-actin of at least 3 independent experiments. The Western blotting assay reported here is representative of a single exemplificative experiment. (B) mRNA expression of E-cadherin in HT-29 cells evaluated by semiquantitative RT-PCR. Total RNA extracted from proliferating HT-29 cells treated or not with 20 μM of cladosporol A, for 1, 2, 4 and 8 h, was used to amplify the specific E-cadherin 154 bp-long product for 20, 25, 35 cycles. (C) Western blotting analysis on total protein extracts produced from HCT116 wt, HCT116 p53 −/− cells treated or not with 20 μM cladosporol A for 4, 6 and 8 h. An anti-E-cadherin antibody was used to probe untreated and cladosporol A-treated cells. To control the samples loaded derived from untreated and cladosporol A-treated cells, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of E-cadherin/β-actin of at least 3 independent experiments. The Western blotting assays reported here are representative of a single exemplificative experiment. (D Upper panel) Transient transfection assay in HT-29 cells with the reporter plasmid bearing the entire promoter region of E-cadherin gene driving the expression of luciferase gene. After transfection, cells were treated with 20 μM of cladosporol A; (lower panel) Transient transfection assay in HCT116wt cells with the plasmid above described. After transfection, cells were treated with 20 μM of cladosporol A for 24 h. The results of the transient transfections in terms of luciferase activity are reported. The luciferase activities were normalized to the β-galattosidase ones used as control. Data shown are mean ± SD of 3 independent experiments performed in duplicate. (E) The results as fold induction are reported here.

and 6 h to prove the physical interaction between β-catenin and PPARγ. A complex formed by both proteins was clearly recognized by the specific antibodies, but not by a preimmune serum used as a control (Fig. 4E). We confirmed the interaction between PPARγ and β-catenin by carrying out co-immunoprecipitation assays in p53 −/− HCT116 cells expressing both proteins. After 6 h of treatment the interaction between the two exogeneous proteins was clearly visible (Fig. 4 F). Altogether these results indicate that cladosporol A reduces the amount of nuclear and cytosolic β-catenin by promoting a PPARγ-dependent proteasomal degradation. As result, transcription of c-*MYC* and cyclin D1, two well-recognized target genes, involved in the mitogenic response and cell cycle control are reduced.

3.4. Increased expression of E-cadherin mRNA and protein in human colon cancer derived cell lines in response to cladosporol A

Cytosolic β-catenin is mainly located in the proximity of the plasma membrane where it interacts with E-cadherin, an important membrane-bound protein involved in cell–cell interactions. It is well-established that loss of E-cadherin is the hallmark of the Epithelial–

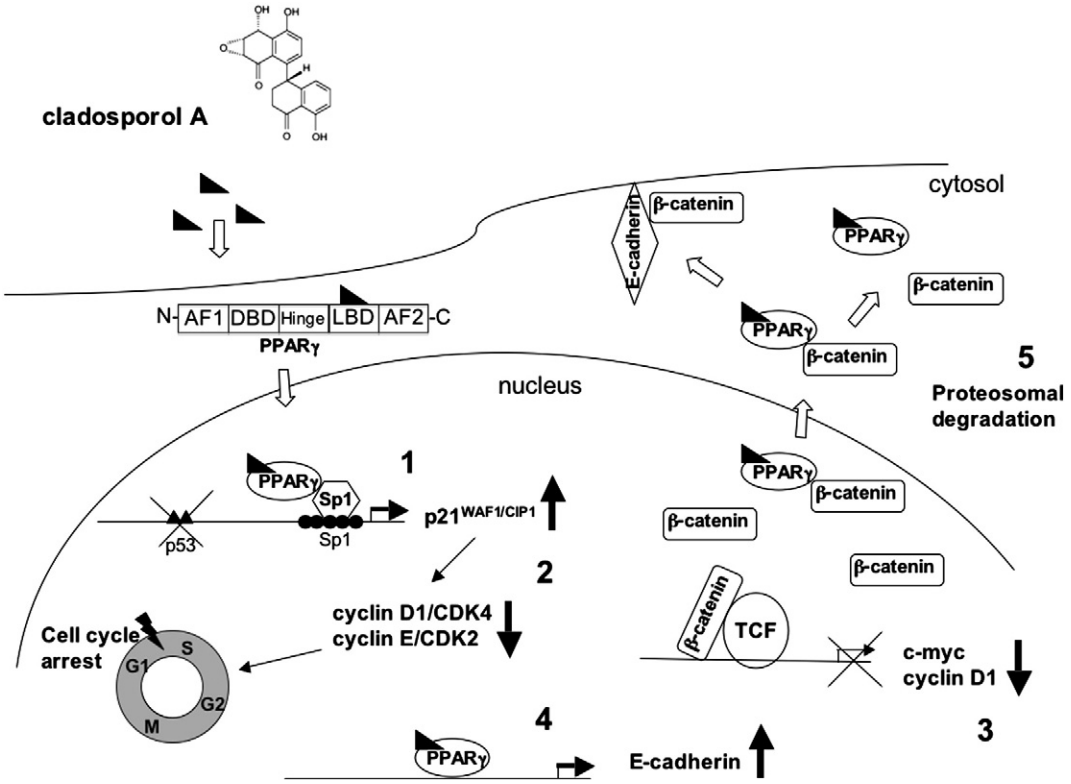

Fig. 6. Cladosporol A actions regulating cell proliferation in colon cancer derived cells. 1) Increase of $p21^{waf1/cip1}$ transcription mediated by PPARγ and Sp1. 2) Down-regulation of cyclin D1, cyclin E, CDK2 and CDK4 expression and their phosphorylation activities. 3) Down-regulation of the β-catenin-dependent target gene transcription, i.e. cyclin D1 and c-myc. 4) PPARγ-dependent increase of E-cadherin mRNA transcription. 5) Proteosomal degradation of PPARγ and β-catenin.

Mesenchymal Transition (EMT), a process that promotes tumor de-differentiation, progression and metastasis [21]. We have shown that E-cadherin is a PPARγ target gene and *PPARG* modulation is directly associated with E-cadherin expression [20,21]. Accordingly, a putative PPRE located at nucleotides −2476 to −2464 from the transcription start site was identified by a bioinformatic analysis of the E-cadherin promoter. To investigate whether cladosporol A influences E-cadherin gene expression, we tested its levels in cladosporol A-treated and untreated HT-29 cells. Both E-cadherin protein and mRNA levels increased after treatment with cladosporol A, as assessed by western blot and semiquantitative RT-PCR assay of the total RNA extracted after 2 and 4 h of treatment, respectively (Fig. 5A and B). The same increase of E-cadherin protein levels was obtained by administering cladosporol A to p53 +/+ HCT116 and p53 −/− HCT116 cells (Fig. 5C).

Moreover, transfection of the full-length E-cadherin promoter, containing the PPRE site, fused to the luciferase reporter gene resulted in luciferase activity that was about 1.5 and 7-fold higher in treated HT-29 and HCT116 cells, respectively, than in untreated cells (Fig. 5D–E), consistent with the increase of the endogenous mRNA and protein levels (Fig. 5A–C). These results indicate that cladosporol A may act as a potential anti-invasive drug by increasing E-cadherin levels, favoring cell–cell adhesion and preserving the epithelial identity.

4. Discussion

The discovery of new molecules to be used in cancer therapy has gained interest and focused a great wealth of research worldwide. Thousands of new drugs, obtained from plants, fungi, microorganisms, are going to be investigated and characterized for their therapeutical properties. America's biopharma research companies are just now testing 981 new drugs to fight different cancers affecting millions of patients all over the world (http://www.phrma.org).

The interest for new molecules in cancer treatment is mainly due to the increased comprehension of the molecular mechanisms underlying cancer biology and to the possibility to avoid or reduce the cytotoxic effects associated with traditional molecules that target DNA, RNA or the cell division machinery. The new (natural and/or synthetic) drugs, in fact, should display the ability to selectively target proteins along the molecular pathways involved in tumor survival, growth and metastases.

We have previously demonstrated that cladosporol A, a secondary metabolite from *C. tenuissimum*, has antiproliferative properties in human colorectal cancer cells [35] by inhibiting HT-29, SW480 and CaCo-2 cell growth through the early and robust up-regulation of $p21^{waf1/cip1}$, down-regulation of cyclin D1, cyclin E, CDK2 and CDK4 expression and their phosphorylation activities [35].

In this work, we sought to investigate whether PPARγ is a mediator of cladosporol A action. PPARγ is expressed in the colonic epithelium, regulates cell differentiation and interferes with colon tumorigenesis by inducing cell cycle arrest, cell differentiation and apoptosis [13–17].

Here we provide evidence that cladosporol A acts as a ligand of PPARγ (Fig. 1A–C). By gain- and loss-of-function experiments, we also definitely prove that cladosporol A affects cell growth via a PPARγ-mediated induction of $p21^{waf1/cip1}$ gene transcription (Fig. 2A–E) that is Sp1-dependent and p53-independent, as previously reported [35]. The induction entails the interaction between Sp1 and PPARγ as shown for other genes and takes place in a PPRE-independent manner, as confirmed by the absence of a canonical PPRE in the promoter region [40–42]. Indeed, Sp1 and PPARγ form a specific complex both in untreated and cladosporol A-treated cells as shown in co-immunoprecipitation and ChIP assays (Fig. 3B, C). Interestingly, these latter experiments clearly indicate that Sp1 is already bound

to the $p21^{waf1/cip1}$ promoter in basal conditions, whereas PPARγ is recruited only after cladosporol A treatment suggesting that Sp1 binding to the promoter holds the chromatin in an open configuration that allows PPARγ recruitment upon activation by specific ligands. Intriguingly, only in this latter condition, RNA Polymerase II is engaged on the promoter to trigger or resume transcription from a poised state. More experiments are needed to fully decipher the $p21^{waf1/cip1}$ gene promoter chromatin status i.e. to verify the presence of coactivators/corepressors, specific histone modifications and modifying enzymes involved in the specific chromatin remodeling after cladosporol A treatment.

The anticancer activities exhibited by several PPARγ ligands are due to increased $p21^{waf1/cip1}$ and $p27^{Kip1}$ expression or reduced levels of cyclin D1, cyclin E, CDK2, CDK4 or inflammatory cytokines and NFkB [9,43,44]. Similar effects have been shown also for Cladosporol A as well. Other ligands, instead, exert their activity by repressing β-catenin, a key molecule involved in the carcinogenesis of various tissues, including colon, as it activates transcription of target genes as c-myc and cyclin D1 [24,25]. This putative dual ability should strengthen their antiproliferative properties. Here we demonstrate that cladosporol A displays such a dual activity as PPARγ directly interacts with β-catenin promoting the nuclear export and its proteasomal degradation (Fig. 4A–F). This effect likely depends on the cellular context, in particular on the relative amount of PPARγ and RXR and activation of the APC/β-catenin pathway usually associated with mutations of the components' genes, as in HT-29 cells [45,46]. Finally, we show that cladosporol A-activated PPARγ mediates also the induction of E-cadherin transcription through the binding to the PPRE in the promoter region (Fig. 5A–C). E-cadherin activation favors cell adhesion and preserves the epithelial phenotype, further supporting its antiproliferative and differentiation-prone behavior in line with published data [33,47,48]. E-cadherin, APC and TCF specifically interact with the β-catenin central repeat domains; by changing the relative intracellular amounts of these factors, it is conceivable that a competition for the same binding domains may result, influencing β-catenin functions and hence a different cell fate.

In conclusion, we identify cladosporol A as a new PPARγ ligand and show that its anti-proliferative properties are due to induction of $p21^{waf1/cip1}$ gene expression in an PPARγ/Sp1-dependent manner. We also demonstrate that cladosporol-A bound PPARγ targets β-catenin to proteasomal degradation, reducing the overall cell content and, in turn, transcription of the target genes. These dual effects enhance the antiproliferative potential of the molecule. As E-cadherin itself is target of PPARγ transcriptional stimulation, the accumulation of the E-cadherin/β-catenin complex at the membrane compartment is also stimulated, generating new cell–cell interactions, preserving the epithelial features and further supports a role as an anti-proliferative and pro-differentiation molecule. The beneficial effects of cladosporol A, here described, are summarized in the scheme of Fig. 6 [35 and this work].

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