



Interplay between SOX9, β -catenin and PPAR γ activation in colorectal cancer

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ABSTRACT

Colorectal carcinogenesis relies on loss of homeostatic mechanisms regulating cell proliferation, differentiation and survival. These cell processes have been reported to be influenced independently by transcription factors activated downstream of the Wnt pathway, such as SOX9 and β -catenin, and by the nuclear receptor PPAR γ . The purpose of this study was to explore the expression levels and functional link between SOX9, β -catenin and PPAR γ in the pathogenesis of colorectal cancer (CRC). We evaluated SOX9, β -catenin and PPAR γ expression levels on human CRC specimens by qPCR and immunoblot detection. We tested the hypothesis that PPAR γ activation might affect SOX9 and β -catenin expression using four colon cancer cell lines (CaCo2, SW480, HCT116, and HT29 cells). In CRC tissues SOX9 resulted up-regulated at both mRNA and protein levels when compared to matched normal mucosa, β -catenin resulted up-regulated at protein levels, while PPAR γ mRNA and PPAR γ protein levels were down-regulated. A significant relationship was observed between high PPAR γ and SOX9 expression levels in the tumor tissue and female gender ($p = 0.005$ and $p = 0.04$, respectively), and between high SOX9 expression in the tumor tissue and age ($p = 0.04$) and microsatellite instability (MSI), in particular with MSI-H ($p = 0.0002$). Moreover, treatment with the synthetic PPAR γ ligand rosiglitazone induced different changes of SOX9 and β -catenin expression and subcellular localization in the colon cancer cell lines examined. In conclusion, SOX9, β -catenin and PPAR γ expression levels are deregulated in the CRC tissue, and in colon cancer cell lines ligand-dependent PPAR γ activation unevenly influences SOX9 and β -catenin expression and subcellular localization, suggesting a variable mechanistic role in colon carcinogenesis.

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1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies in developed countries and a major cause of cancer-related death worldwide [1]. Presently, the first-choice treatment is surgical excision with or without adjuvant chemotherapy [2], but the identification of novel biomarkers for prognosis and molecular targets for therapeutic intervention is firmly needed. CRC onset and progression are linked to mutational pathways, such as chromosomal instability (CIN) and microsatellite instability (MSI) [3], and colorectal carcinogenesis is related to the progressive loss of homeostatic control of cell proliferation, differentiation and apoptosis [4]. The transcription factors play a key role in the regulation of these cell processes. An

emerging role is attributed to SOX [sex-determining region Y (Sry) box-containing], member of a transcription factor family with homology in their 79-amino acid DNA-binding domain known as the HMG box. SOX family is involved in the control of stem cell maintenance and cell phenotype choice during embryonic development and committed differentiation in multiple organ systems [4]. In particular, SOX9 is considered an important regulator of cell fate and differentiation and is implicated in the maintenance of a non differentiated population of putative stem/progenitor cells for adult tissue renewal in intestinal epithelium, critical for maintaining constant integrity of epithelial barriers [5]. In the colon, SOX9 is localized to the proliferative compartment of the intestinal epithelium and is involved downstream in the Wnt/ β -catenin signaling for the management of epithelium homeostasis [4,6,7]. Aberrant proliferation of tissue-specific stem cells is implicated in gastrointestinal tumorigenesis, and SOX9 has been found deregulated in many types of cancer [8–11] including those of the digestive system and in particular in colorectal cancer [4].

The key effector protein in Wnt pathway is the transcription activator β -catenin, whose coding gene (*CTNNB1*) can function as an oncogene involved in tumor cell phenotype onset [12,13]. Cytoplasmic β -catenin forms a complex with APC (adenomatous polyposis coli), the associated protein Axin/Conductin, protein kinases such as casein kinase (CK) and glycogen synthase kinase-3 β (GSK-3 β), which phosphorylate β -catenin and other elements of the molecular proteolytic complex. Phosphorylated (p) β -catenin is an ephemeral, transcriptionally inactive mediator, whose half life is settled on by the closely controlled equilibrium between phosphorylation, dephosphorylation, ubiquitination, proteasomal degradation and subcellular localization. β -catenin translocates from the cytoplasm to the nucleus and interacts with the TCF/LEF1 family transcription factors, inducing the expression of target-genes such as *MYC* and *CCND1* [13,14]. β -catenin is also involved in cell adhesion, bridging between cadherins and the actin cytoskeleton at the cell–cell adherens junctions. Deregulation of β -catenin levels, partly caused by mutations of the *APC* gene, is thought to play a role in the development of colorectal cancer [15]. In over 80% of human colon cancers occur mutations in *APC*, which determine large deletions in the C-terminus of the protein [16].

Another important cell fate regulator is represented by PPAR γ (Peroxisome Proliferator-Activated Receptor γ), a ligand-activated transcription factor belonging to the large superfamily of nuclear receptors. PPAR γ is known to regulate cellular proliferation/differentiation, and could be involved in gastrointestinal cancerogenesis [17–20]. High levels of PPAR γ expression are measured in the normal mucosa of colon and rectum [21], and enhanced tumorigenicity in mouse small intestine and colon has been found associated with decreased intestinal PPAR γ levels [22]. Accordingly, in a number of colorectal cancer patients decreased PPAR γ expression levels have been found compared with adjacent normal colonic mucosa [23]. On the other hand, in a large series of primary colorectal cancers more than half of tumors were characterized by PPAR γ overexpression when compared to the matched normal mucosa [24], but with a significant association between PPAR γ downregulation, occurrence of distant metastases and poorer overall patients' survival [25,26]. The biomolecular events involved are not yet clear, but PPAR γ deregulation probably plays different and context dependent roles in CRC onset and progression. Recently the effects of PPAR γ activity on gastrointestinal tract tumor biology have been pinpointed by studies exploring genetic mechanisms to assess the role of PPAR γ as a tumor suppressor and inducer of differentiation of cancer stem cells, and evaluating the clinical applications and potential therapeutic employ of PPAR γ agonists in colorectal cancer treatment [24,27–29].

PPAR γ is able to maintain lower steady-state levels of β -catenin in the presence of a normal APC pathway, and ligand related PPAR γ activation decreases β -catenin levels [30].

Based on these premises, considering the effects of PPAR γ ligands on cell fate and its direct interaction on β -catenin [31,32], and the role

of Wnt/ β -catenin target-gene SOX9 in colorectal cancer onset and progression [4,7,8], we hypothesized that the interaction between PPAR γ activation and SOX9 expression may play a role *via* β -catenin signaling in colorectal cancerogenesis. To address this hypothesis, we have investigated PPAR γ , SOX9 and β -catenin expression levels in human colorectal cancer specimens, evaluating their relationships to clinical and pathological features, and assessed the effects of PPAR γ activation on SOX9 and β -catenin expression *in vitro* in colon cancer cell lines.

2. Materials and methods

2.1. Patients and tissue specimens

We analyzed primary tumors and matched normal specimens from a cohort of 31 CRC patients undergoing curative surgery at our Institution (21 men and 10 women, mean age \pm SD 66 \pm 13 years). Clinical and pathological features of colorectal cancer patients are shown in Table 1. Tissue specimens, collected according to the guidelines of the local Ethical Committee, were immediately frozen in liquid nitrogen and stored at -80 °C until nucleic acid and protein extraction. Written informed consent was obtained from each patient in accordance with the Institutional guidelines.

Our investigation has been conducted in accordance with the ethical standards and the Declaration of Helsinki, and according to the national and international guidelines, after approval of our Institutional Review Board.

Table 1
Clinical and pathological features of colorectal cancer patients.

	n = 31	%
Age (years)		
Mean \pm SD	66 \pm 13	
Gender		
Male/Female	21/10	
Tumor location		
Proximal ^a	15	48.4
Distal ^b	16	51.6
Histologic type		
Adenocarcinoma	26	84
Carcinoma in situ	5	16
Grading		
G1–G2	28	90.3
G3–G4	3	9.7
Depth of tumor invasion		
T1–T2	1	3.2
T3	28	90.3
T4	2	6.5
Lymph node metastasis		
N0	12	38.7
N1–N2	19	61.3
Metastasis		
Not	20	64.5
Yes	11	35.5
Modified Dukes staging system		
A	1	3.2
B	11	35.5
C	19	61.3
D	0	/
Vascular invasion		
Not	15	48.4
Yes	16	51.6
MSI frequency		
High	6	20.7
Low	6	20.7
Stable	17	58.6
Missing	2	/

^a Caecum, ascending colon, proximal transverse.

^b Distal transverse, descending colon, sigmoid colon and rectum.

2.2. Cell culture and rosiglitazone treatment

CaCo2, SW480, HCT116, and HT29 cells were cultured at 37 °C in 5% CO₂ atmosphere in DMEM and MEM Alpha media, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 ng/ml streptomycin (Invitrogen Life Technologies, Milan Italy).

The synthetic rosiglitazone (Ros) ligand was used to determine PPAR γ activation. The cells were treated with vehicle (ethanol) or 20 μ M Ros for 24 h.

2.3. Antibodies and chemicals

Rabbit polyclonal antibodies directed against β -catenin and PPAR γ were purchased from Cell Signaling Technology (Euroclone, Milan, Italy). Mouse antibodies directed against β -actin and rabbit polyclonal antibodies directed against SOX9 were purchased from Santa Cruz (D.B.A., Milan, Italy). Rosiglitazone was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA).

2.4. Quantitative real time PCR (qPCR)

Total RNA was extracted from tissue samples and from CaCo2, SW480, HCT116, and HT29 cells upon treatment with rosiglitazone using the RNeasy[®] Mini Kit (QIAGEN S.r.l., Milan, Italy), and subsequently digested by DNase I. cDNA was synthesized from 500 ng total RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and random hexanucleotides. For qPCR, we used the following SYBR Green Quantitect Primers purchased from Qiagen: human SOX9 (QT00001498), human CTNNB1 (QT00077882), human PPAR γ (QT00029841). Reactions were set up in 96-well plates using a 7700 RT-PCR System (Applied Biosystems, Foster City, CA, USA) and all samples were assayed in triplicate. The data were analyzed using the default and variable parameters available in the SDS software package (version 1.9.1; Applied Biosystems, Foster City, CA, USA). Target gene expression levels were normalized using the GAPDH housekeeping control and the mRNA amount of each target gene relative to GAPDH was calculated through the comparative Ct method, also called the $2^{-\Delta\Delta Ct}$ method.

2.5. Immunoblot detection

Total proteins were extracted from 5 snap frozen CRC specimens homogenized through mechanical and detergent based lysis, Ripa buffer (150 mM NaCl, 50 mM TRIS-HCl pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 1% Triton, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1% cholic acid sodium salt), supplemented with protease inhibitor cocktail (COMPLETE; Roche Diagnostics, Mannheim, Germany), 1 mM phenylmethanesulphonyl fluoride and 1 mM sodium orthovanadate, being used as detergent. After boiling at 100 °C for 3 min, equal amounts of proteins were loaded on 10% polyacrylamide gels and separated by electrophoresis. Protein transfer was performed on PVDF membrane (Millipore S.p.A., Milan, Italy). The membranes were blocked with 5% skim milk in wash buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) and incubated with the specific primary antibodies diluted in blocking solution, at appropriate dilutions. Following three washes, membranes were incubated with a secondary goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated antibody (BioRad, Hercules, CA, USA) diluted 1:3000 in wash buffer. After three further washes, proteins were revealed by chemiluminescence (ECL, Amersham Biosciences AB, Uppsala Sweden) and the signal detected on an X-ray film (Amersham Biosciences AB, Uppsala Sweden). For quantitative measurement, the film was scanned and the spots corresponding to the proteins of interest were analyzed using the image processing program ImageJ (NIH Image, Bethesda, MD).

2.6. Indirect immunofluorescence (IFL)

CaCo2, SW480, HCT116, and HT29 cells grown and transfected on coverslips, were fixed for 30 min in 4% paraformaldehyde at 4 °C. Coverslips were then washed three times in PBS and incubated with the β -catenin or SOX9 antibody diluted 1:100 in PBS, 2% bovine serum albumin (BSA), 0.3% Triton X-100 for 2 h at room temperature (RT). After three washes in PBS, cells were incubated 2 h at RT with Alexa 488-labeled anti-rabbit antibodies diluted at 1:50. After rinsing three times in PBS, coverslips were mounted on microscope slides. For fluorescence microscopy, slides mounted for immunofluorescence were observed using a Nikon Eclipse E600 microscope.

2.7. Immunoprecipitation (IP)

Cells treated with 20 μ M of rosiglitazone for 24 h were washed with PBS and lysed in 0.5 ml of Radiolmmuno Precipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, pH 8) for 60 min at 4 °C, centrifuged and the supernatant was collected. For immunoprecipitation (IP) assays a protein G immunoprecipitation kit (Sigma-Aldrich, St. Louis, MO, USA) was used and a pre-clearing step (Protein G Agarose suspension and cell extract sample were incubated for 2 h at 4 °C) was carried out to reduce background caused by non-specific adsorption of irrelevant cellular proteins to the Protein G Agarose. To 200 μ g of the lysate 2.5 μ g of mouse anti- β -Catenin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and left 3 h at 4 °C, followed by addition of 50 μ l of beads suspension overnight at 4 °C. Beads were washed two times with IP 1 \times buffer and five times with IP 0.1 \times buffer following the specific protocol, and immunoprecipitated proteins were eluted by boiling in 40 μ l of 1 \times Laemmli buffer before being loaded onto a SDS gel (10% acrylamide) and transferred to a Nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) followed by immunoblot analysis. The primary antibodies used were mouse anti- β -Catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-PPAR γ (Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit anti-SOX9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. The secondary antibodies used were goat anti-mouse IgG HRP-linked or goat anti-rabbit IgG HRP-linked antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Chromogenic development of the western blots was obtained by using the Immun-Star Western C kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. MTT assays for cell viability and cell proliferation rate

Cell viability assays were carried out in CaCo2, SW480, HCT116, and HT29 cell lines. Cells were plated (1 \times 10⁵ cells/well) in quadruplicate onto a 12-well cultured plate, and treated with 20 μ M of rosiglitazone for 24 h. On 24, 48, 72 and 96 h the cells were harvested by trypsinization and the reduction in cell viability was determined using the MTT assay. Cell viability was expressed as optical density (OD), which was detected using a NANODrop reader at 570 nm. The results are expressed as the mean of absorbances from quadruplicate samples. Two independent experiments were carried out and the results were expressed based on the following formula: cell viability% = number of cells in drug treatment group / number of cells in control group \times 100%.

To evaluate the anti-proliferative effect of rosiglitazone the four cell lines were plated onto 96-well plates at approximately 2 \times 10⁴ cells/well and treated with increasing concentrations (10, 20, 40 and 60 μ M) of rosiglitazone for 48 h. Then MTT reagent was added and the absorbance at 570 nm was recorded using an enzyme-linked immunosorbent assay (ELISA) reader. The proliferation inhibition rate (IR) was calculated according to the following formula: IR% = [1 - absorbance of drug treatment group / absorbance of vehicle control group] \times 100%.

2.9. Cell cycle assay by flow cytometry

To evaluate the effect of PPAR γ activation on the cell cycle, the CaCo2, SW480, HCT116, and HT29 cell lines were treated with 20 μ M of rosiglitazone for 24 h or 48 h, collected by centrifugation permeabilized with ice-cold 70% ethanol for at least 1 h and washed with PBS. The cells were then treated with RNase A at 37 $^{\circ}$ C for 30 min and resuspended in PBS containing 20 μ g/ml propidium iodide and stained at 4 $^{\circ}$ C for 30 min. The DNA contents were then analyzed by EPICS ALTRA (Beckman Coulter, Inc.).

2.10. Microsatellite instability

The microsatellite instability (MSI) analysis was performed using the Bethesda panel of microsatellite (BAT25, BAT26, D5S346, D17S250 and D2S123) evaluated by means of a multiplex-PCR and PAGE analysis. Tumors showing instability in four or more markers were classified as high MSI (MSI-H), those showing it in two markers as low MSI (MSI-L), and those showing no instability as microsatellite-stable (MSS).

2.11. Statistical analysis

SOX9, PPARG and CTNNB1 mRNA expression levels of CRC tissue were compared with those of adjacent normal mucosa, calculated using the formula $2^{-\Delta\Delta Ct}$ and values were reported as median, 25th percentile (first quartile, Q1) and 75th percentile (third quartile, Q3). For continuous variables, normal distribution was verified by the Shapiro–Wilk test and the one-sample Kolmogorov–Smirnov test. For normally distributed variables, hypotheses regarding differences among the groups were compared by means of the paired t-test, Student's t-test or by means of the analysis of variance (ANOVA) as indicated. For non-normally distributed variables, hypotheses regarding differences among the groups were compared by means of a non-parametric Wilcoxon signed rank sum test, the Mann–Whitney rank sum test or the Kruskal–Wallis rank sum test as indicated. Statistical analyses were also performed on the subjects divided into quartile groups and by using Pearson's chi-square test to evaluate interactions between variables. P-values <0.05 were considered statistically significant. Survival rates were calculated by the Kaplan–Meier method for analysis of censored data. All analyses were performed using SPSS Statistical Package (SPSS v17, Chicago, IL, US). For protein expression levels and *in vitro* experiments, the results are expressed as means \pm SE.

3. Results

3.1. SOX9, β -Catenin, PPARG expression levels in tumoral tissue specimens matched to normal mucosa

We previously reported deregulated SOX9, CTNNB1 and PPARG mRNA levels in CRC tissue, evaluated by microarray analysis (ArrayExpress accession n. E-MTAB-57) [33]. As an initial step, we sought to corroborate these data, on 31 pairs of tumor tissue/normal mucosa from 28 well–moderately differentiated (G1–G2) colorectal tumors and from 3 poorly differentiated–undifferentiated (G3–G4) colorectal tumors, resected from 21 male and 10 female patients (mean age \pm SD 66 \pm 13 years). Clinical and pathological features of colorectal cancer patients are shown in Table 1. Using qPCR, in tumor tissue compared to matched normal tissue was observed SOX9 up-regulation (median = 1.73, Q1–Q3 = 0.62–2.86, p = 0.004) and PPARG down-regulation (median = 0.41, Q1–Q3 = 0.23–0.70, p = 0.0005). No statistically significant difference was observed in CTNNB1 expression levels (median = 1.01, Q1–Q3 = 0.58–1.42, p = 0.984). Median values, 25th and 75th percentile, and extremes of GAPDH-Ct value/target gene-Ct value are shown in Fig. 1.

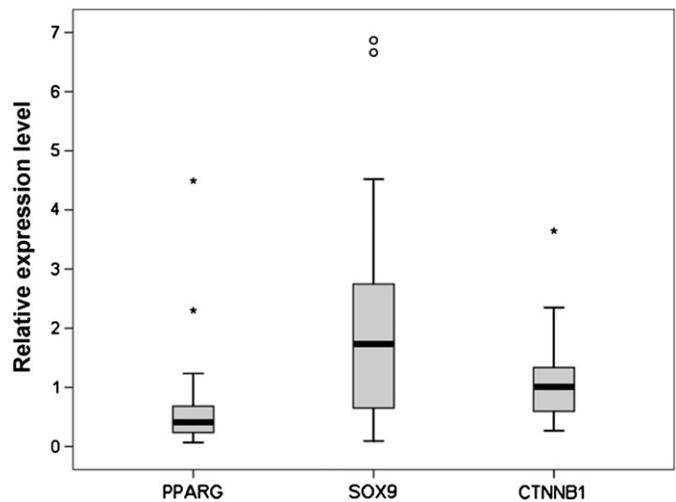


Fig. 1. SOX9, PPARG and CTNNB1 mRNA expression evaluated by qPCR. SOX9, PPARG and CTNNB1 mRNA expression in 31 CRC specimens (T) paired to normal tissues (N) evaluated by qPCR. Outliers are indicated by asterisk (*) or circle (°) where appropriate.

When detected by western blot SOX9 and PPAR γ protein expression showed the same trend of RNA expression in tumors as compared to normal tissue (Fig. 2, Panels A and B) while β -catenin expression resulted in an increase only at the protein level, suggesting that a post transcriptional mechanism may be involved.

3.2. Relationship of gene expression levels to clinical–pathological parameters in CRC patients

Associations between gene expression levels and clinical–pathological features (age, gender, tumor site and stage, and MSI status) of CRC patients are shown in Table 2. A statistically significant relationship was observed between higher PPARG and SOX9 expression levels in the tumor tissue and female gender (p = 0.005 and p = 0.04, respectively), and between high SOX9 expression in the tumor tissue and age (p = 0.04) and MSI status, in particular with MSI-H (p = 0.0002) (Table 2).

Kaplan–Meier method for analysis of censored data showed no statistically significant decrease in survival for the patients with high PPARG expression levels (P = 0.414), high SOX9 expression levels (P = 0.437), or high CTNNB1 expression levels (P = 0.371) (Fig. 3).

3.3. Effect of rosiglitazone treatment on SOX9 and CTNNB1 expression levels in colon cancer cell lines

Considering the role of Wnt/ β -catenin target-gene SOX9 in colorectal cancer onset and progression and the direct interaction of PPAR γ ligands on β -catenin, we tested the effect of treatment with 20 μ M rosiglitazone, a PPAR γ activator, on four colorectal cancer cell lines. As shown in Fig. 4, after treatment a statistically significant decrease was observed in SOX9 and CTNNB1 mRNA expression in CaCo2 (FC = -1.47 , p < 0.05 and FC = -1.50 , p < 0.01, respectively) and in HT-29 (FC = -2.88 , p < 0.001 and FC = -2.55 , p < 0.001, respectively). In HCT116 we observed a statistically significant increase of SOX9 expression (FC = 1.95; p < 0.01). In SW480 rosiglitazone treatment did not cause statistically significant changes of SOX9 and CTNNB1 expression. As a control for the modulation of PPAR γ activity by rosiglitazone, we examined whether this ligand increased the expression levels of fatty acid binding protein 1 (FABP1) or fatty acid synthase (FAS), a well-established target of PPAR γ activity (Fig. 4).

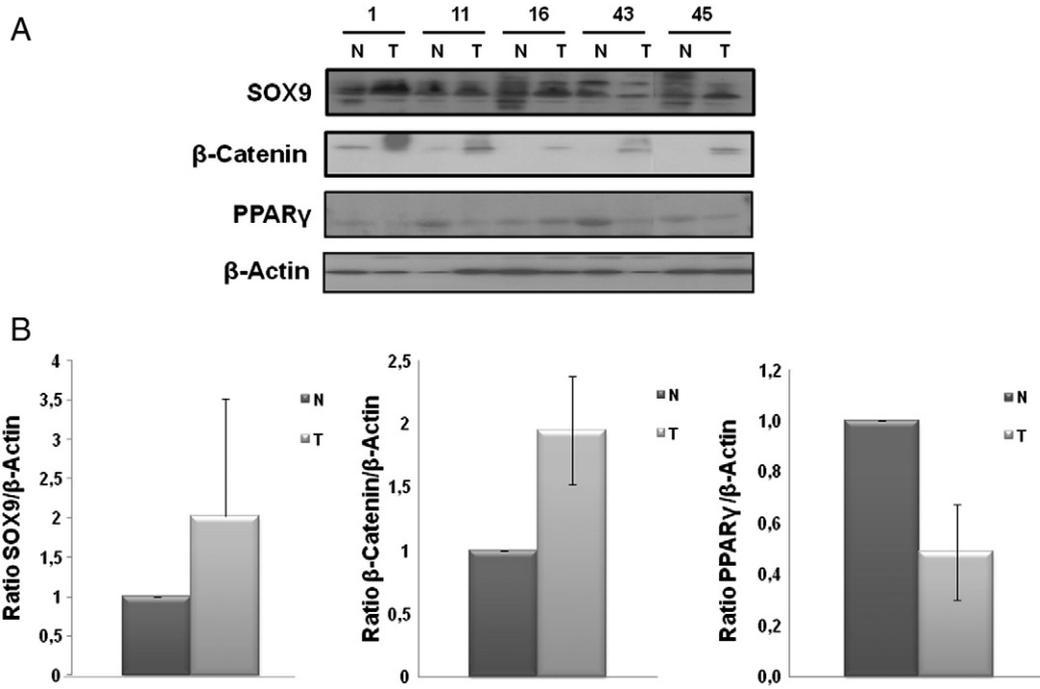


Fig. 2. Immunoblot detection of SOX9, β-catenin and PPARγ in proteins extracted from selected paired normal tissues and CRC specimens. Panel A: Western blot analysis reveals up-regulated SOX9 and β-catenin expression in CRC tissue (T) compared to normal mucosa (N) in 5 different specimens. On the contrary an opposite trend was depicted for PPARγ protein. Patient identification numbers are displayed at the top of figure. Panel B: The plots represent the signal quantification of the western blot experiments by densitometry of SOX9, β-catenin and PPARγ normalized to β-actin expression as reference protein.

3.4. Immunofluorescence detection of SOX9, β-catenin and PPARγ in colon cancer cells upon rosiglitazone treatment

We then performed immunofluorescence staining for SOX9, β-catenin, and PPARγ. Treated and untreated CaCo2 cells displayed nuclear localization for SOX9 staining, and the main difference was represented by decreased levels of SOX9 within nuclei in CaCo2 cells treated with rosiglitazone at a concentration of 20 μM (Fig. 5, Panel e) as compared to vehicle treated cells (Fig. 5, Panel a). As shown in Fig. 6, vehicle treated CaCo2 cells displayed classical β-catenin localization around the cellular membrane and within the nucleus (Fig. 6, Panel a). On the other hand, β-catenin protein was not present within nuclei upon 24-hour treatment with rosiglitazone

at a concentration of 20 μM (Fig. 6, Panel d). SW480 cells displayed nuclear localization of SOX9, which was unaffected by rosiglitazone (Fig. 5, Panels a and e). As for β-catenin, SW480 untreated cells displayed plasma membrane but not nuclear localization (Fig. 6, Panel a). Conversely, β-catenin protein was present within SW480 nuclei upon 24-hour treatment with rosiglitazone at a concentration of 20 μM (Fig. 6, Panel d). No change was observed in the HCT116 cell line for both proteins upon treatment with 20 μM rosiglitazone (Fig. 5, Panels a and e; Fig. 6, Panels a and d). In HT-29 cell line, SOX9 staining displayed nuclear localization in rosiglitazone treated and vehicle treated cells (Fig. 5, Panels a and e). Canonical β-catenin localization around the cellular membrane, but not within nuclei, was observed in vehicle treated cells (Fig. 6, Panel a) with an increase

Table 2
Clinicopathological parameters in CRC patients and relationship with gene expression levels.

	CRC		PPARγ				SOX9				CTNNB1			
	n	n = 31	Median	Q1	Q3	p values	Median	Q1	Q3	p values	Median	Q1	Q3	p values
Age	<62	11	0.50	0.23	1.02		2.21	1.76	3.76		0.70	0.58	1.51	
	62–74	12	0.44	0.26	0.61	0.84	1.21	0.53	1.76	0.04	1.06	0.62	1.61	0.70
	>74	8	0.34	0.15	0.65		0.80	0.48	3.19		1.06	0.63	1.20	
Gender	M	21	0.28	0.14	0.53	0.005	1.07	0.55	1.87		0.70	0.51	1.25	
	F	10	0.72	0.41	1.02		2.37	1.69	4.52	0.04	1.13	0.90	1.51	0.29
Cancer location	Proximal	16	0.53	0.77	0.30		1.86	1.17	3.33		1.10	0.64	1.61	
	Distal	15	0.25	0.13	0.59	0.07	1.07	0.44	1.87	0.11	0.86	0.49	1.22	0.11
Grading	G1/G2	28	0.45	0.20	0.72		1.70	0.58	2.88		1.06	0.58	1.47	
	G3/G4	3	0.24	0.23	0.66	0.63	2.21	1.69	2.64	0.46	0.70	0.63	0.86	0.42
	A	1	0.14	0.14	0.14		0.80	0.80	0.80		0.32	0.32	0.32	
Dukes staging system	B	11	0.32	0.14	0.56	0.16	1.36	0.27	3.76	0.50	0.86	0.37	1.18	0.12
	C	19	0.53	0.25	0.80		1.84	0.98	2.64		1.11	0.63	1.51	
	D	0												
	Carcinoma in situ	5	0.53	0.49	0.74		1.07	0.55	1.67		1.22	1.08	1.42	
Histologic type	Adenocarcinoma	26	0.39	0.23	0.66	0.58	1.81	0.68	2.89	0.24	0.95	0.58	1.25	0.48
	MSI-H	6	0.38	0.16	0.70		5.59	2.89	6.87		0.61	0.49	0.90	
MSI status	MS-L	6	0.44	0.28	0.59	0.94	2.25	1.87	2.86	0.0002	1.10	0.70	1.25	0.27
	MSS	17	0.41	0.25	0.74		0.80	0.53	1.73		1.11	0.65	1.51	
Vascular invasion	Yes	15	0.53	0.14	1.02		1.73	0.68	2.86		1.08	0.49	1.59	
	Not	16	0.40	0.25	0.54	0.65	1.61	0.58	2.76	1.00	0.95	0.62	1.20	0.57

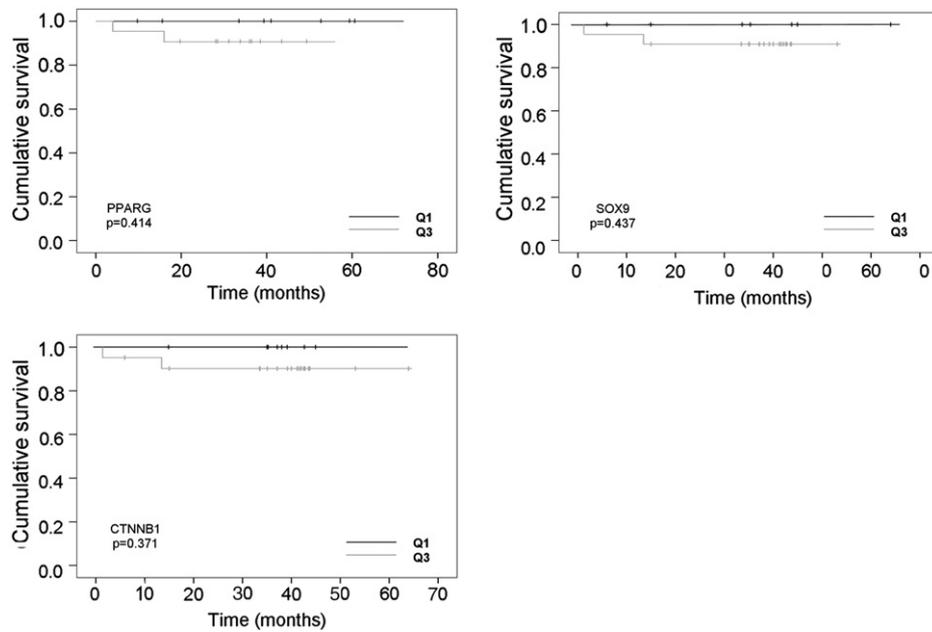


Fig. 3. Cumulative survival of patient subdivided into quartile groups according to PPAR γ expression, SOX9 expression and CTNNB1 expression. No statistically significant difference was found in cumulative survival rates of the patients divided into quartile groups. Q1 = lower quartile and Q3 = upper quartile.

in cell-cell junction localization upon 24-hour treatment with rosiglitazone at a concentration of 20 μ M (Fig. 6, Panel d). No change was observed in all cell lines for PPAR γ protein upon treatment with 20 μ M rosiglitazone (Fig. 7, Panels a–d).

3.5. Immunoprecipitation (IP) of SOX9, β -Catenin and PPAR γ in colon cancer cells upon rosiglitazone treatment

In order to assess whether the interaction among SOX9, β -Catenin and PPAR γ was direct or indirect, an immunoprecipitation experiment was performed upon treatment with rosiglitazone at a concentration

of 20 μ M for 24 h. We observed that β -catenin and PPAR γ co-immunoprecipitated, whereas no band was observed when Sox9 IP was performed (Fig. 8). Immunoprecipitation experiments evidenced a direct interaction between β -catenin and PPAR γ but not for SOX9 protein.

3.6. Effect of SOX9 changes on viability and proliferation of colon cancer cell lines

To assess the effect of SOX9 changes induced by PPAR γ activation on viability and proliferation of human colon cancer cell lines, we

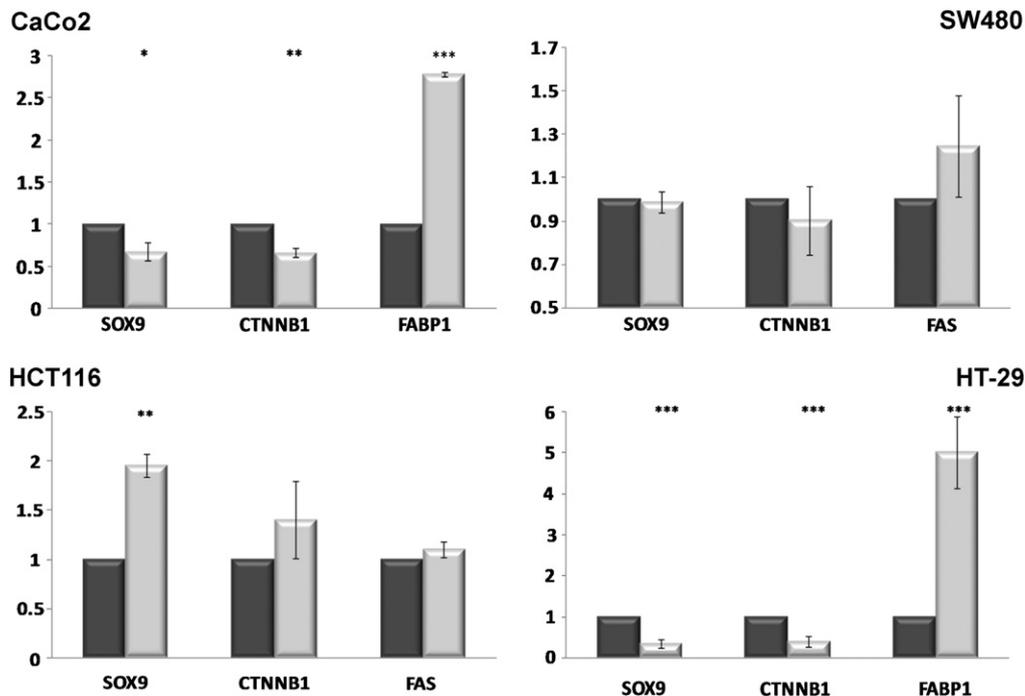


Fig. 4. SOX9 and CTNNB1 mRNAs expression evaluated by qPCR in colon cancer cell lines upon rosiglitazone treatment. mRNA expression analysis by qPCR of SOX9, CTNNB1 and FABP1/FAS in CaCo2, SW480, HCT116 and HT-29 cells upon rosiglitazone treatment at concentration of 20 μ M for 24 h. *, P < 0.05 value; **, P < 0.01; ***, P < 0.001. Dark gray bars correspond to vehicle treated cells, clear gray bars correspond to rosiglitazone treated cells.

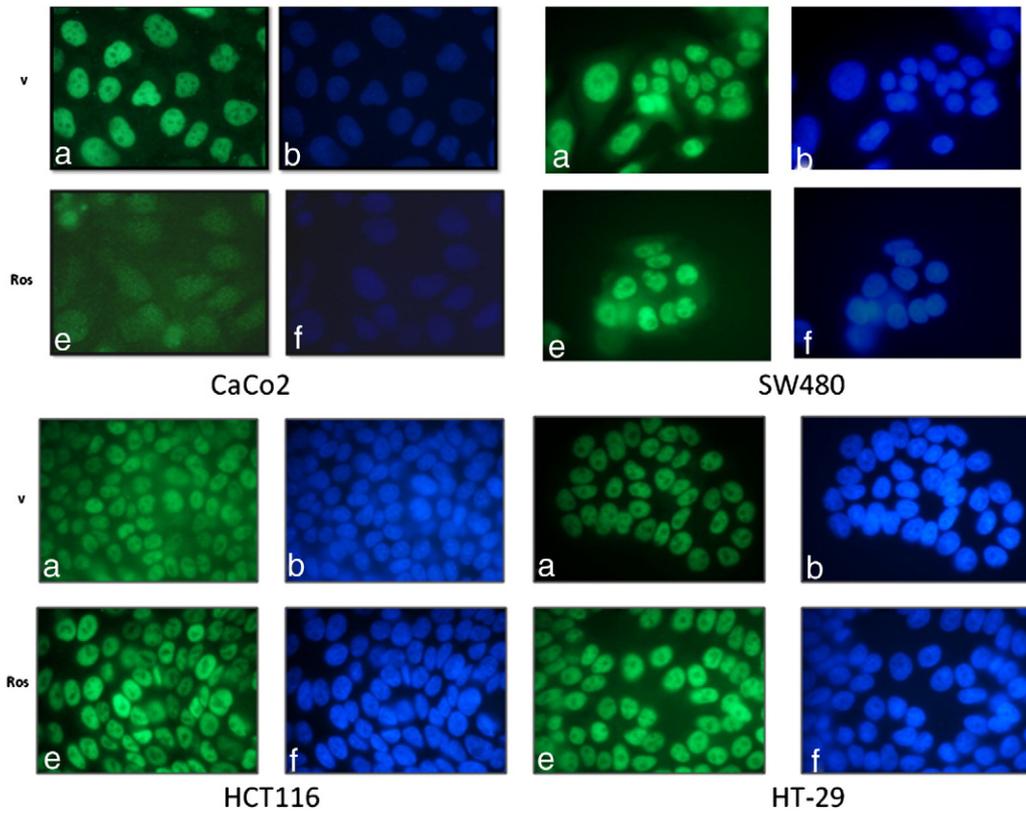


Fig. 5. Immunofluorescence of SOX9 in colon cancer cell lines treated with rosiglitazone. Panels (a) show colon cancer cell lines before treatment stained with anti SOX9 antibody. Panels (e) display SOX9 staining in colon cancer cell lines upon treatment with rosiglitazone. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (blue) (Panels b and f).

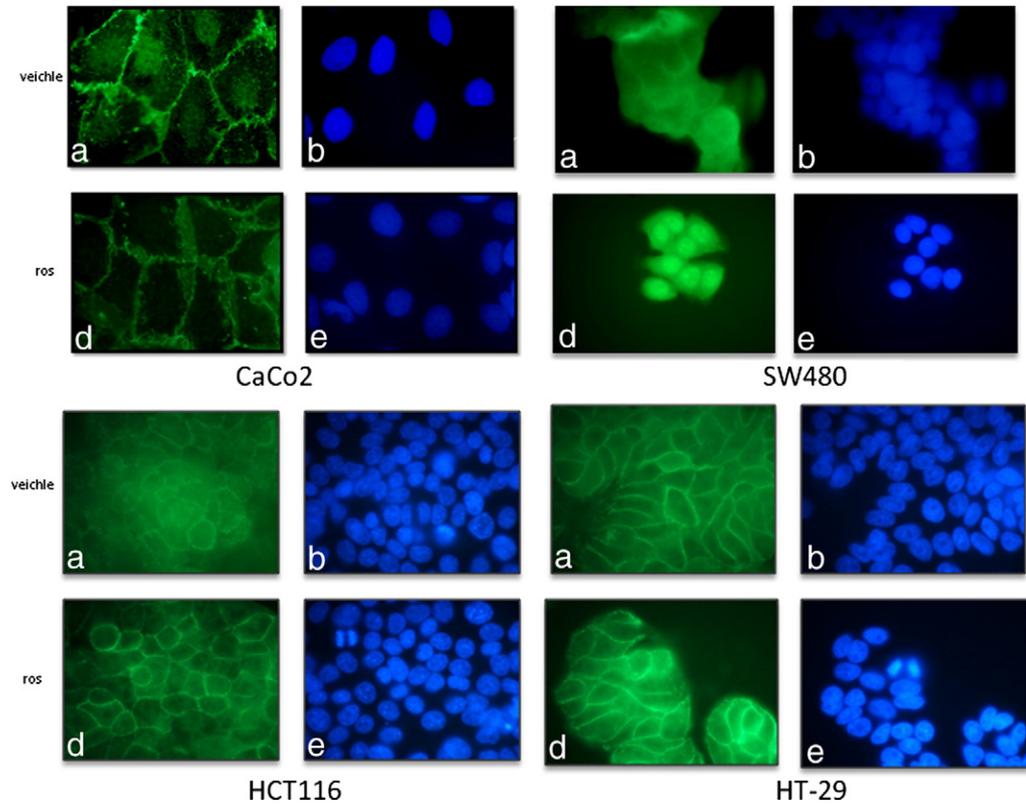


Fig. 6. Immunofluorescence of β -catenin in colon cancer cell lines treated with rosiglitazone. Panels (a) show colon cancer cell lines before treatment stained with anti β -Catenin antibody. Panels (d) display β -catenin staining in colon cancer cell lines upon treatment with rosiglitazone. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (blue) (Panels b and e).

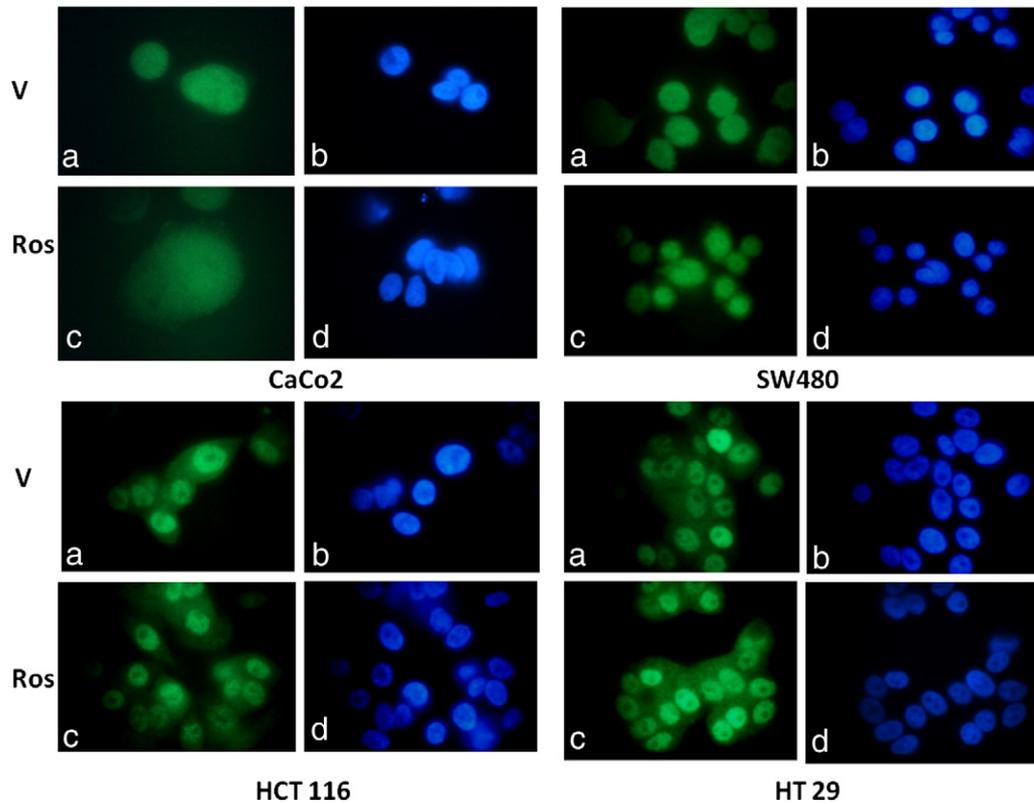


Fig. 7. Immunofluorescence of PPAR γ in colon cancer cell lines treated with rosiglitazone. Panels (a) show colon cancer cell lines before treatment stained with anti PPAR γ antibody. Panels (c) display PPAR γ staining in colon cancer cell lines upon treatment with rosiglitazone. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (blue) (Panels b and d).

performed MTT assay after rosiglitazone treatment. CaCo2, SW480, HCT116, and HT29 cell lines were assessed after incubation with 20 μ M of rosiglitazone at 24, 48, 72, and 96 h. Rosiglitazone treatment reduced HT-29 cells viability up to 20% at 72 h ($p = 0.225$) and up to 80% at 96 h ($p = 0.027$), respectively. Viability of the other three cell lines showed a downward trend at 96 h (Fig. 9, Panel A).

The inhibitory effect on proliferation was evaluated on the cell lines treated with increasing concentrations (10, 20, 40 and 60 μ M) of rosiglitazone for 48 h. After 48 hour incubation statistically significant dose dependent changes were observed in CaCo2 and HT-29 cell lines (Fig. 9, Panel B). The optical density (OD) values of rosiglitazone treated (20 μ M, 40 μ M, and 60 μ M) groups were decreased in a

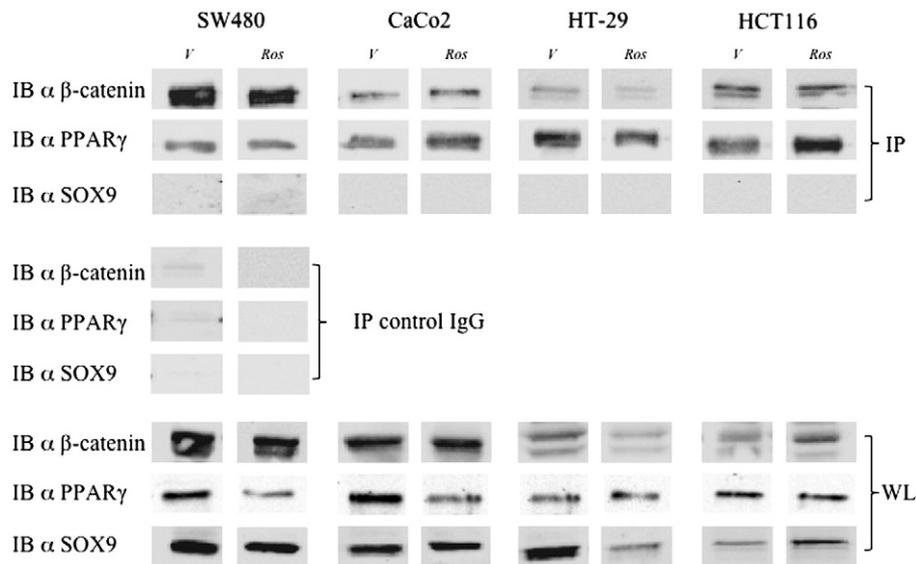


Fig. 8. Immunoprecipitation of β -catenin, PPAR γ and SOX9 in colon cancer cell lines upon rosiglitazone treatment. Sw480, CaCo2, HT-29 and HCT116 cells were treated with ethanol ($v =$ vehicle) or with 20 μ M of rosiglitazone (Ros) for 24 h and total cellular proteins were immunoprecipitated (IP) with an anti- β -catenin antibody. The presence of β -catenin, PPAR γ and SOX9 in the immunoprecipitated fractions was analyzed by immunoblotting (IB). All experiments were repeated at least two times. The negative control was performed using control IgG on cell extract. WL: whole lysate.

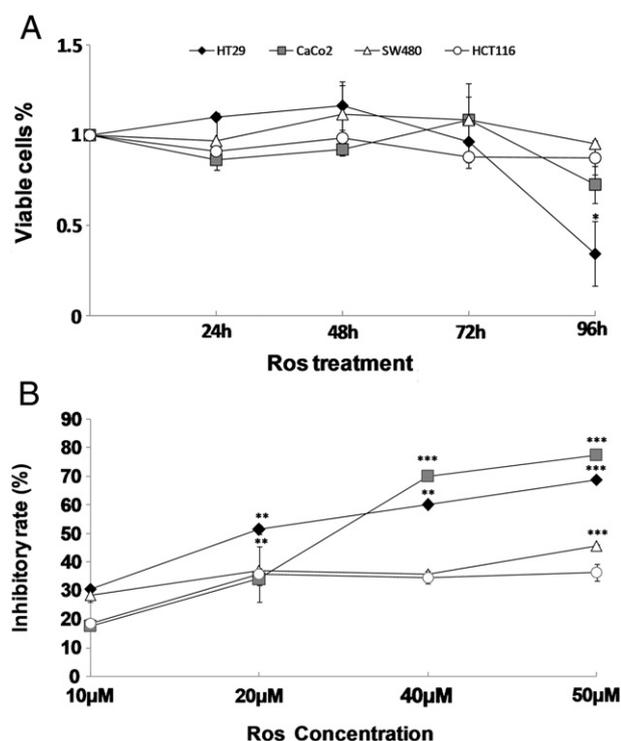


Fig. 9. MTT proliferation assay. Panel A: MTT viability assay of CaCo2, SW480, HCT116 and HT-29 control cells or cells treated with rosiglitazone at concentration of 20 μ M at 24, 48, 72 and 96 h. Panel B: Inhibitory effect on proliferation was evaluated on the cell lines treated with increasing concentrations (10, 20, 40 and 60 μ M) of rosiglitazone for 48 h. *, $P < 0.05$ value; **, $P < 0.01$; ***, $P < 0.001$.

statistically significant way when compared to the untreated groups ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 9, Panel B). The inhibitory effect evidenced on proliferation of SW480 and HCT116 cells was significantly lower with respect to CaCo2 and HT-29 cells.

3.7. Cell cycle analysis

We examined the effect of PPAR γ activation on cell-cycle progression by means of rosiglitazone treatment in the four colon cancer cell lines. As shown in Fig. 10, CaCo2 cells treated with 20 μ M of rosiglitazone for 24 h revealed an increase in the number of cells in the G0/G1 phase and reduction in the number of cells in the S phase when compared with the untreated cells. Furthermore, HT-29 cells treated with 20 μ M of rosiglitazone revealed a slight increase in the number of cells in the G0/G1 phase and a reduction in the number of cells in the S phase at 24 h when compared with the untreated cells, although these effects were more evident at 48 h. No change was observed for SW480 and HCT116 cell lines.

4. Discussion

SOX9 is a target gene of WNT/ β -catenin pathway and PPAR γ activation causes a decrease in β -catenin expression in normal intestinal mucosa in mice, probably controlling the levels of known regulators of β -catenin, such as APC, axin and GSK-3 β [34]. In this study we have evaluated SOX9, CTNNB1 and PPARG expression levels in CRC specimens, and we have put in evidence SOX9 and CTNNB1 up-regulation and PPARG down-regulation, an association with MSI and female gender, but no differences in the survival curves were observed.

Our results are in agreement with previous finding of SOX9 overexpression reported in different papers [11,12], but not with the clinical-pathological correlations and survival rates referred by other authors [35,36]. Anyway, data reported in CRC patients by these authors seem contrasting, considering that high SOX9 expression levels in the

tumor tissue were found associated on the one hand with better survival rates [35] and on the other hand with advanced tumor stage, typically causing poorer survival [36]. Besides, higher tumor tissue levels of SOX9 were found in patients 62 years of age or younger, and higher tumor tissue levels of either PPARG and SOX9 expression were found in female CRC patients. These associations might be correlated to the age and gender related differences in CRC incidence and prognosis reported in epidemiological studies [40–42], and should be interpreted in relationship to the molecular pathways modulated by female hormones. In particular, estrogens have been found to influence PPAR γ expression in a tissue specific-manner [43,44] and to determine SOX9 subcellular localization [45].

Few studies report gender-related differences of PPAR γ expression. No significant gender-related differences were detected in the tissue distribution of PPAR γ subtypes in the zebrafish [46], whereas a gender-related difference in PPAR γ expression with up-regulation in female animals was evidenced in rat adipose tissues depending on the levels of the sex hormones [47], and in bladder smooth muscle of female Ossabaw swines, a porcine model of the metabolic syndrome [48]. In humans, gender-related differences of PPAR γ expression have been evidenced in relationship to particular single nucleotide polymorphisms [49], in para-physiological processes, such as aging [50], and in frank pathological conditions, such as coronary artery disease [51] or diabetes mellitus [52]. Besides, SOX9 is an important transcription factor required for development and male sex determination, which regulates the expression of target genes in the associated pathways and is involved in the expansion and differentiation of multiple cell lineages. Regarding the relationship between SOX9 expression levels and gender in neoplastic disease, it has been evaluated in different types of human cancer, such as hepatocellular carcinoma, gastric cancer, malignant glioma, lung cancer, but no significant association between SOX9 mRNA levels and gender was reported [53–57].

As reported above, high levels of SOX9 expression in tumor tissue are strongly correlated with MSI in our CRC patients. Aggressiveness of colorectal tumors is negatively associated with the phenomenon of hypermutation related to altered DNA repair mechanisms and the consequent exceeding rate of genetic mutation, and approximately 15–20% of CRC are hypermutated with 75% of them presenting MSI. Whole-exome sequencing and integrative analysis of genomic data have put in evidence that 93% of non-hypermutated and 97% of hypermutated CRCs have a deregulated WNT signalling pathway and SOX9 is one of the genes most frequently mutated [58].

MSI and CIN mutational pathways are involved in CRC progression, and MSI influences response to irinotecan versus 5-FU chemotherapy [37,3] and conditions cancer specific survival [38,39].

SOX9 expression was evaluated in MSS and MSI CRC by comparing transcript profiles of normal mucosa and CRC tissue in two independent sample sets, and different expression of this transcription factor was found. It is important to consider that analysis was performed through gene expression profile by microarray to evaluate transcription factors putatively associated with recurrence of stage II MSS and MSI CRC [59]. On the other hand, we did not find a significant correlation for CTNNB1. Anyway, our data show a trend (CTNNB1 expression decreased in MSI-H in respect to MSI-L and MSS) that is in agreement with data evidenced in articles that investigated the relationship between CTNNB1 mutations and underlying pathways of genomic instability: the frequency of β -catenin mutations was significantly higher, and presumably CTNNB1 expression levels lower, in MSI-H than in MSS/MSI-L CRC [60,61].

Besides, Kaplan and Meyer analysis in our study showed no correlation of PPARG, SOX9 and CTNNB1 expression levels with patient survival. As reported by Ogino et al. [26], two studies, one considering 86 CRC patients and one considering 99 CRC patients, did not show a prognostic value of tumoral PPARG expression, and he found statistically significant differences only in a very large cohort (470) of CRC

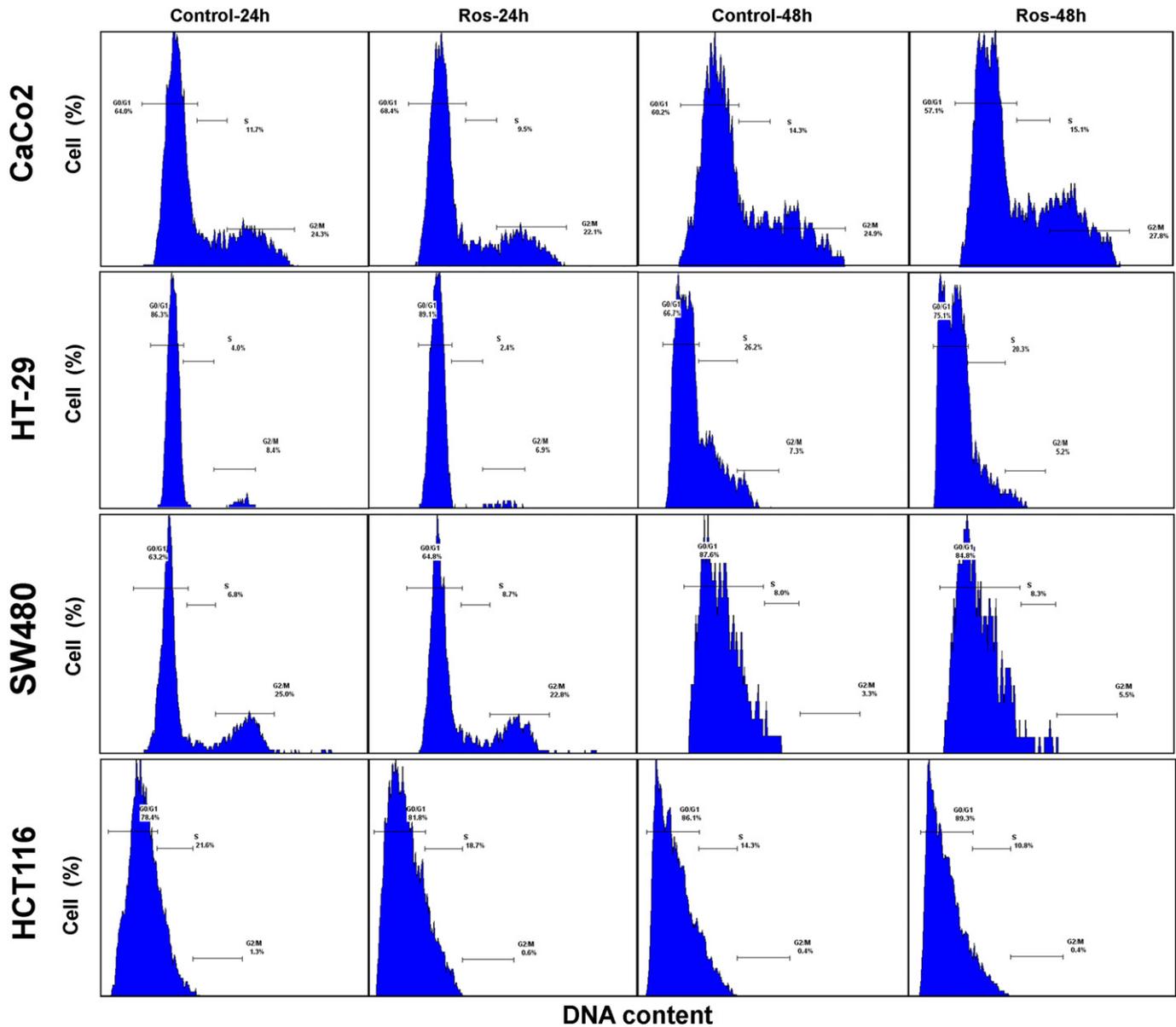


Fig. 10. Effect of SOX9 decrease following PPAR γ activation on cell-cycle. Cell-cycle profile was examined by flow cytometry and percentages of cells in G0/G1, S, and G2/M phase in CaCo2, HT-29, HCT116 and SW480 cell lines are indicated in the graphs.

patients. In this huge number of patients was evidenced a statistically significant difference in overall survival ($p = 0.0047$), and a not statistically significant difference in colorectal cancer specific survival ($p = 0.089$) after 10 years of follow-up. These results suggest that a very long follow-up and a very large sample size are necessary to put in evidence an effect of these transcription factors on survival, maybe in relationship to pleiotropic effects and influence of genetic background.

In this instance, our study was meant to focus on biomolecular interactions and subcellular localizations of SOX9, β -catenin and PPAR γ in different colon cancer cell lines.

To assess if SOX9 and β -catenin expression was influenced by PPAR γ activity we used CaCo2, SW480, HCT116, and HT29 colon cancer cell lines. We found that in some but not all the types of colon cancer cell line examined PPAR γ activation reduced SOX9 and *CTNNB1* gene expression levels. In particular, the modulation with the PPAR γ -agonist rosiglitazone induced a statistically significant decrease of SOX9 and *CTNNB1* expression in CaCo2 and in HT-29 cell

lines, a paradoxical significant increase of SOX9 expression in HCT116 cell line, and no significant expression change in SW480 cells.

In a similar way, immunofluorescence detection of SOX9 and β -catenin in colon cancer cell lines evidenced contrasting patterns of subcellular protein localization upon rosiglitazone treatment. The subcellular localizations of β -catenin at the level of the membrane, cytoplasm and nucleus affect its functions as a tumor suppressor at the membrane or as an oncogene in the nucleus.

The different patterns of SOX9 and *CTNNB1* gene expression and subcellular protein localization evidenced in the colon cancer cell lines examined in our study might be related to different mutational statuses for APC and/or β -catenin. Moreover, the shuttling between the cytosol and the nucleus may occur through the direct interaction of PPAR γ with β -catenin, as evidenced in our IP experiment and already demonstrated [31,32]. On the contrary, our IP experiments did not evidence a direct interaction between PPAR γ / β -catenin and SOX9 proteins, so that an indirect effect of PPAR γ on SOX9 transcription factor could be supposed.

The CaCo2 cell line has both somatic *CTNNB1* mutation (missense variant at codon 245) and *APC* mutation (truncation at position 1367). The *CTNNB1* mutation is located in an amino acid that is directly N-terminal to a serine residue that lies in a consensus site for CK, and might cause a potential change in the accessibility of that site for phosphorylation [62]. Furthermore, PPAR γ activation controls the levels of β -catenin regulators, such as GSK-3 β (crucial for phosphorylation), and has been found able to decrease β -catenin expression in normal intestinal mucosa in mice [30]. Another possible mechanism may be represented by the direct interaction between PPAR γ and β -catenin with subsequent inhibition of β -catenin translocation into the nucleus [32]. The mutation in *APC* stabilizes β -catenin constitutively activating Wnt-target genes [63]. Accordingly, in our study untreated CaCo2 cells displayed β -catenin localization within nuclei, whereas upon 24-hour treatment with rosiglitazone β -catenin protein was not present within the nuclei. Regarding to SOX9 staining, untreated CaCo2 cells displayed nuclear localization, which was slightly decreased in cells treated with rosiglitazone, probably in relationship to the absence of β -catenin nuclear localization.

SW480 are characterized only by a biallelic somatic *APC* mutation (truncation at position 1338) that does not hamper β -catenin phosphorylation, but is not able to promote degradation, determining high p β -catenin levels [7,64]. We evidenced that untreated SW480 cells displayed membrane-bound β -catenin localization, and upon 24-hour treatment with rosiglitazone β -catenin was present within the nuclei, suggesting delocalization from the plasma membrane to the cytoplasm and transport into the nucleus. Regarding to a possible mechanism involved in β -catenin nuclear translocation upon rosiglitazone treatment, we can speculate on the molecular basis relying on previous data reported in the scientific literature. SW480 cells build up high amounts of β -catenin (only 20% phosphorylated) that might saturate the degradation machinery [64], and PPAR γ activation upon rosiglitazone treatment has been found capable of blocking the phosphorylation of β -catenin suggesting that dephosphorylation might be an alternative pathway that can protect β -catenin and induce its nuclear accumulation [31]. In basal condition SW480 cells are characterized by low PPAR γ levels, so that rosiglitazone treatment, by increasing the levels of the nuclear receptor, might subsequently facilitate β -catenin nuclear translocation. Concerning to SOX9 staining, both untreated and rosiglitazone treated SW480 cells displayed nuclear localization.

In HCT 116 cell line *CTNNB1* heterozygous mutation (deletion of codon 45) results in loss of highly conserved serine residues in a region of the protein that may be a target for the enzyme GSK-3 β ; the phosphorylated (p) β -catenin may be derived from the wild-type molecule generated from the intact allele and the proteolytic complex formation is not disrupted. Indeed, no change was observed in the HCT116 cell line for both proteins before and after rosiglitazone treatment, with membrane-bound localization of β -catenin and nuclear localization of SOX9.

HT-29 cells have a mutant form of the *APC* gene (truncation at position 1555) that can support some β -catenin phosphorylation, and is at least partially capable of supporting its degradation [64]. Recently a PPAR γ region has been identified as having high homology to the β -catenin binding domain (CBD) within TCF/LEF, which facilitates its interaction with β -catenin [65]. In HT-29 cells PPAR γ activation has been found able to suppress β -catenin/TCF dependent transcription *via* direct binding, which prevents its degradation and inhibits β -catenin movement into the nucleus [32]. The subcellular distribution of the β -catenin destruction complex is mostly cytoplasmic and the transient localization of β -catenin in junctions may reflect a compartmentalized shelter from degradation [64]. In untreated HT-29 cells we observed membrane-bound β -catenin localization, with an increase in cell-cell junction localization upon 24-hour rosiglitazone treatment. SOX9 staining displayed nuclear localization in untreated and treated cells.

The different patterns of response to rosiglitazone treatment that we observed in the colon cancer cell lines examined mirror the conflicting role played by PPAR γ in gastrointestinal cancerogenesis, as this nuclear receptor inhibits tumor growth only in the presence of functional *APC* [30]. Accordingly, a tumor-promoting effect of PPAR γ agonists has been evidenced in the *Apc*^{Min/+} mice [66], implying that the normal response of intestinal epithelial tumor cells to PPAR γ agonists may be modified by *APC* loss or dysfunction. The expression and subcellular localization data for PPAR γ , SOX9 and β -catenin that we obtained by IF and IP suggest a reciprocal interactions among these transcription factors, considering also that PPAR γ lessens β -catenin levels upon ligand related activation in the presence of a normal *APC* pathway [30], and SOX9 is transcriptionally repressed by WNT signaling, while in turn the SOX9 protein enhances β -catenin degradation [7,8,36]. Our data suggest that the effect of rosiglitazone treatment on expression and trafficking of these transcription factors, and ultimately on cell viability and proliferation rate, might be influenced by SOX9 and β -catenin levels, that are decreased in CaCo2 and HT29 cells, and nuclear localization of SOX9, decreased in CaCo2 cells, and β -catenin, absent in CaCo2 and HT29, but increased in SW480 cells. In HCT116 cells β -catenin is not visible in the nucleus, but the paradoxical increase of SOX9 upon rosiglitazone treatment might boost cell proliferation. SOX9 exhibits several pro-oncogenic properties directly binding and activating the promoter of the polycomb *Bmi1*, an oncogene playing an essential role in proliferation, senescence and carcinogenesis, whose upregulation represses the tumor suppressor *Ink4a/Arf* locus [36]. Besides, expression of SOX9 matches cytoplasmic and nuclear β -catenin, corroborating the link between SOX9 expression and the β -catenin/TCF complex activity [7]. Otherwise, PPAR γ could hinder SOX9 nuclear translocation and maintain it in the cytosol, acting the same way as on β -catenin [32], holding back the transcription processes influenced by SOX9.

Furthermore, in HCT116 cells we observed a statistically significant increase of SOX9 expression upon rosiglitazone treatment.

In these cells the M3 promoter region of *PPARG* is hypermethylated, indicating that extensive promoter methylation influences PPAR γ activity [25]. Besides, HCT116 cells are characterized by elevated PPAR δ expression and are resistant to PPAR γ agonists, because PPAR δ activity antagonizes the ability of PPAR γ to induce its effects [67]. PPAR δ is involved in the Wnt/ β -catenin signaling pathway interacting with β -catenin binding to TCF/LEF transcription factors and influences also SOX9 expression [68,69]. These evidences have important drawbacks, considering that SOX9 overexpression is associated with an adverse prognosis in colorectal cancer [35].

Furthermore, using MTT assay we have explored the effects on cell proliferation of the decrease of SOX9 and *CTNNB1* expression induced by PPAR γ activation after rosiglitazone treatment of the colon cancer cell lines examined. Time and dose dependent significant changes after rosiglitazone treatment were evidenced only for CaCo2 and HT-29 cell lines. This evidence is corroborated by cell-cycle progression study performed by fluorescence-activated cell-sorting analysis. In CaCo2, and HT-29 cells treated with rosiglitazone an increase in the number of cells in the G0/G1 phase and a reduction in the number of cells in the S phase were evidenced when compared with the untreated cells. Our results are in agreement with the regulation of proliferation by SOX9 previously reported in primary cells through control of the progression of the cell cycle from the G0/G1 to the S phase [35]. Moreover, we evaluated the basal expression levels of *PPARG* in the different colon cancer cell lines, and we found very high levels in HT29 cells, high levels in HCT116 cells, and very low levels in SW480 cells, normalizing to *PPARG* expression level in CaCo2 cells (see Supplementary Fig. 1S). The level of *PPARG* expression evidenced in the colon cancer cell lines examined is in agreement with the PPAR γ protein level reported in a seminal study [70]. The effects of PPAR γ activation depend on cell context and set in motion downstream gene activation events directing cell fate, and including decreases in proliferation through either cyclins or cyclin-dependent kinases, cell differentiation through up-regulation of

tissue lineage-specific differentiation proteins, cell death through apoptosis-associated proteins, and growth arrest through overcoming the effects of RAS/MEK/MAP kinase pathway derangement [70]. Furthermore, the role of PPAR γ might depend on a direct interaction with β -catenin, considering that upon ligand-dependent activation PPAR γ keeps β -catenin in the cytosol and decreases β -catenin/TCF complex, restraining β -catenin-mediated transcriptional pathways that uphold cell proliferation [32].

Considering that in HCT116 cells promoter hypermethylation modulates PPAR γ activity and that an effect of rosiglitazone treatment on proliferation and cell cycle is evident only in HT29 and CaCo2 cells, changes in these cell processes seem to be dependent on high basal levels of this transcription factor. In agreement with the literature, the results of our study suggest that basal levels of PPAR γ expression influence the percentages of cells distributed in the different stages of the cell cycle. Higher percentages of cells in G0/G1 and lower percentages of cells in the G2/M phase were found in HT29 and HCT116 cells, which express higher levels of PPAR γ , whereas the lowest percentage of cells in G0/G1 and highest percentage of cells in the G2/M phase were found in the SW480 cell line, which is characterized by very low levels of PPAR γ expression. The CaCo2 cell line showed intermediate values of PPAR γ expression when compared to the other cell lines examined, and basal percentages of distribution of cells in different stages of the cell cycle were similar to those found in SW480 cells. However, upon rosiglitazone treatment the former cell line showed percentage changes with increase of cells in the G0/G1 phase, corresponding to the disappearance of β -catenin nuclear localization, differently from the latter cell line that did not display modifications of distribution of cells in different stages of the cell cycle, but showed *de novo* β -catenin nuclear localization after PPAR γ activation.

In conclusion the expression of SOX9, β -catenin and PPAR γ which is deregulated in colorectal cancer tissue and the modified levels of SOX9 and β -catenin evidenced in some colon cancer cell line after PPAR γ activation suggest a novel gene regulatory interaction in colorectal carcinogenesis. Our results shed light on a possible mechanism through which PPAR γ ligand-dependent activation may affect tumor cell phenotype and behavior, as well as cell viability and proliferation. SOX9 upregulation and β -catenin delocalization are predictive markers of aggressive biological behavior and our study suggests that PPAR γ activation may influence SOX9 and β -catenin expression and subcellular localization, at least in some types of colon cancer cell. A better understanding of these molecular mechanisms may further disclose the role played by SOX9 and β -catenin and clarify the contrasting effects of PPAR γ transcriptional activation on tumorigenesis, testing the feasibility of drug modulation of their gene expression and translating these discoveries into clinical use of new therapeutic targets.

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Conflict of interest statement

The authors declare that there are no conflicts of interest with respect to the authorship and/or publication of this article.

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