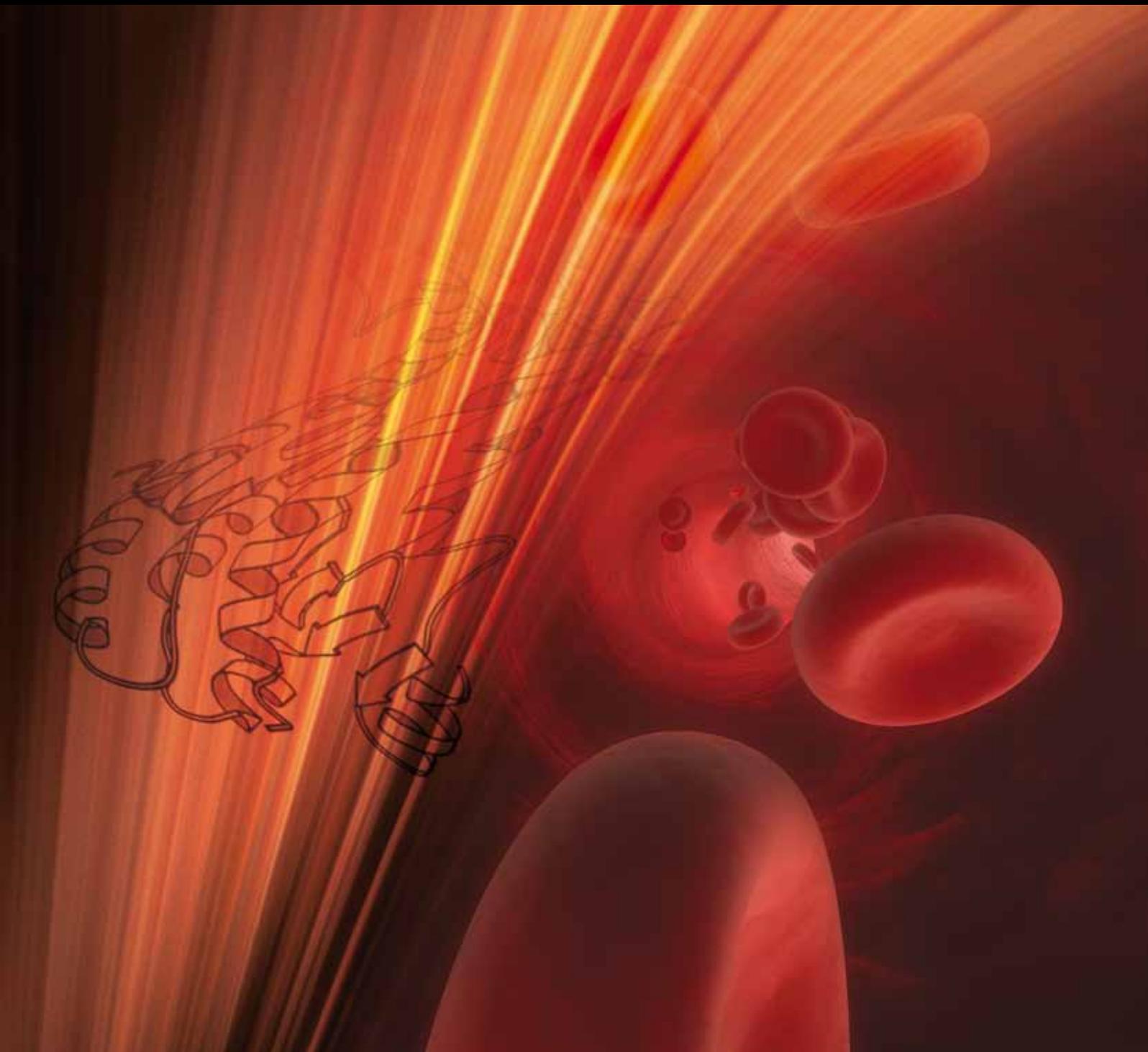


# PPARs and Anticancer Therapies

Guest Editors: Michael E. C. Robbins, Christine Linard,  
and Dipak Panigrahy





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# **PPARs and Anticancer Therapies**

PPAR Research

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## Editorial

# PPARs and Anticancer Therapies

**Michael E. C. Robbins,<sup>1</sup> Christine Linard,<sup>2</sup> and Dipak Panigrahy<sup>3,4</sup>**

<sup>1</sup> *Department of Radiation Oncology, Brain Tumor Center of Excellence, Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*

<sup>2</sup> *Institute for Radioprotection and Nuclear Safety, 92262 Fontenay aux Roses Cedex, France*

<sup>3</sup> *Vascular Biology Program, Children's Hospital, Harvard Medical Center, Boston, MA 02115, USA*

<sup>4</sup> *Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA*

Correspondence should be addressed to Michael E. C. Robbins, [mrobbins@wfubmc.edu](mailto:mrobbins@wfubmc.edu)

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Welcome to this special issue of PPAR research, PPARs, and anticancer therapies. Peroxisomal proliferator-activated receptor (PPAR)  $\alpha$ ,  $\delta$ , and  $\gamma$  are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors first identified some 20 years ago. Although initial studies focused on their role in regulating cellular metabolism, there has been an increasing appreciation for PPARs' role in regulating a wide variety of biological processes, particularly inflammation and cancer. Modulation of these processes is of particular importance with regard to increasing the therapeutic outcome for cancer patients. This special issue brings together reviews and research articles that focus on the role of PPARs with respect to prognostic outcome, anticancer activity, and normal tissue morbidity, thus demonstrating the exciting therapeutic opportunities that are within our grasp.

The ability of PPARs to promote or inhibit cancer has been discussed for some time. In this special issue of PPAR research, the first section contains two research articles that focus on the role of PPAR $\gamma$  in malignant melanoma. A mechanistic study described by Klopfer and colleagues illustrates how the response of poorly differentiated melanoma cells to PPAR $\gamma$  and retinoid X receptor agonists is mediated through the calcium binding protein S100A2. The necessary presence of S100A2 for the maximum antiproliferative effect of PPARs indicates a potential mechanism of tumor response or resistance to PPAR $\gamma$ -directed anticancer therapy. Additional support for a therapeutic role for PPAR $\gamma$  comes from tissue microarray analyses of normal and tumor tissue from patients with malignant melanoma described by Meyer and

colleagues. They report that PPAR $\gamma$  and cyclooxygenase 2 (Cox2) are stage-dependent prognostic markers of malignant melanoma. Moreover, in metastatic malignant melanoma, PPAR $\gamma$  expression may predict for response to bimodulatory stroma-targeted therapy combining Cox/PPAR targeting with metronomic, low-dose chemotherapy. In addition, this article opens the concept of the stroma-tumor interaction complexity.

These research articles are followed by five reviews that demonstrate the potential ability of PPARs to increase the therapeutic window for cancer patients. All of these items strengthen the growing evidence that PPARs' ligands serve as adjuvants in protection of healthy tissue and in enhancing anticancer therapies. With ongoing improvements in cancer therapy and health care, the population of long-term cancer survivors continues to grow. In the US approximately 62% of adult and over 75% of pediatric cancer patients survive beyond 5 years. For this growing population, late effects of anticancer therapies pose a significant risk and can adversely affect the quality of life of these individuals. Ramanan et al. discuss the growing problem of radiation-induced brain injury, particularly cognitive impairment, observed in patients who survive 6 months or more after partial or whole-brain irradiation (WBI) for primary or metastatic brain cancer. Recent studies suggest that the pathogenesis of radiation-induced brain injury involves WBI-mediated increases in oxidative stress and/or inflammatory responses in the brain. PPAR agonists can cross the blood brain barrier, have well-described anti-inflammatory and neuroprotective properties, and as reported in this review do appear to

modulate late radiation-induced brain injury, including cognitive impairment, in rodent models.

PPAR-mediated modulation of radiation-induced gastrointestinal toxicity is similarly reviewed by Linard and Souidi. Radiation-induced gastrointestinal morbidity for the treatment of pelvic and abdominal tumors is more common than generally recognized and can negatively impact the patient's quality of life. Although the role of PPAR $\gamma$  in colon cancer has been investigated for several years, relatively little is known regarding its potential to protect the gut against radiation-induced injury. This review discusses the effects of abdominal radiation on PPARs, their role and function in radiation-induced toxicity, and the possibility of using PPAR agonists as radioprotectors.

Translating these findings to the clinic is predicated by demonstrating that PPAR agonists will not similarly protect cancer cells. The therapeutic implications of using PPAR agonists in the treatment of human neuroepithelial tumors, including astrocytomas, the highly aggressive glioblastoma multiforme, and pediatric neuroblastomas, are reviewed by Benedetti and colleagues. The majority of malignant neuroepithelial tumors have poor prognosis, and despite multimodality therapy consisting of surgery, radiation therapy, and chemotherapy, long-term survival rates remain poor. These tumor cells express PPARs; thus natural and synthetic PPAR ligands may represent promising adjuvant therapeutic agents when used with existing anticancer therapies. Simpson-Haidaris and colleagues highlight the potential uses for PPAR $\gamma$  agonists in anticancer therapy, with special emphasis on their role as an adjuvant or in combined therapy in the treatment of hematological malignancies found in the vasculature, marrow, and eyes. In addition, they review that the potential roles of PPAR $\gamma$  and its ligands may play in modulating cancer-associated angiogenesis and tumor-stromal microenvironment crosstalk in the bone marrow. Finally, Wagner and colleagues review how PPAR agonists and retinoids can inhibit osteosarcoma proliferation, induce apoptosis, and inhibit tumor growth by promoting osteoblastic terminal differentiation. PPAR agonists have the potential to be used not only as adjuvant therapeutic drugs for osteosarcoma but also as chemopreventive agents for those patients who undergo resection of primary bone tumors in order to prevent local recurrence and/or pulmonary metastasis.

In conclusion, we hope that you will find these recent advances in the potential role that PPARs and their agonists play in anticancer therapies both informative and exciting. We thank many authors who have contributed to the articles in this special issue and encourage you to further stimulate your research into the role of PPARs and cancer. The millions of individuals who are impacted either directly or indirectly by cancer deserve no less.

*Michael E. C. Robbins  
Christine Linard  
Dipak Panigrahy*

## Research Article

# Combination PPAR $\gamma$ and RXR Agonist Treatment in Melanoma Cells: Functional Importance of S100A2

Joshua P. Klopper,<sup>1,2</sup> Vibha Sharma,<sup>1</sup> Reid Bissonnette,<sup>3</sup> and Bryan R. Haugen<sup>1,2</sup>

<sup>1</sup> Division of Endocrinology, Metabolism and Diabetes, Department of Medicine, University of Colorado Denver, Aurora, CO 80045, USA

<sup>2</sup> University of Colorado Cancer Center, University of Colorado Denver, Aurora, CO 80045, USA

<sup>3</sup> Department of Molecular Oncology, Ligand Pharmaceuticals, San Diego, CA 92121, USA

Correspondence should be addressed to Joshua P. Klopper, joshua.klopper@ucdenver.edu

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Nuclear hormone receptors, including RXR and PPAR $\gamma$ , represent novel therapeutic targets in melanoma. We have previously shown that the DRO subline of the amelanotic melanoma A375 responds to rexinoid and thiazolidinedione (TZD) treatment *in vitro* and *in vivo*. We performed microarray analysis of A375(DRO) after TZD and combination rexinoid/TZD treatment in which the calcium binding protein S100A2 had increased expression after rexinoid or TZD treatment and a synergistic increase to combination treatment. Increased S100A2 expression is dependent on an intact PPAR $\gamma$  receptor, but it is not sufficient to mediate the antiproliferative effects of rexinoid/TZD treatment. Over expression of S100A2 enhanced the effect of rexinoid and TZD treatment while inhibition of S100A2 expression attenuated the response to rexinoid/TZD treatment, suggesting that S100A2 is necessary for optimal response to RXR and PPAR $\gamma$  activation by respective ligands. In summary, we have identified potential downstream mediators of rexinoid and TZD treatment in a poorly differentiated melanoma and found that alterations in S100A2 expression affect RXR and PPAR $\gamma$  signaling in A375(DRO) cells. These studies provide insight into potential mechanisms of tumor response or resistance to these novel therapies.

## 1. Introduction

Melanoma represents a significant public health problem with a rising incidence over the last 3 decades [1]. More than 7700 patients will die of this disease annually, almost all with metastases [2]. The median survival in patients with metastatic disease is 7–9 months [3]. While some prognostic factors correlate with a more favorable prognosis, such as lack of visceral metastases, younger age, and treatment with biochemotherapy, the 5–10-year survival rates still remain less than 20% [4]. Thus, a search for novel therapies is warranted in this aggressive disease given the suboptimal choices available.

We have reported the efficacy of rexinoid, thiazolidinedione, and combination therapy in the melanoma cell line A375(DRO) (DRO was originally thought to be an anaplastic thyroid cancer cell line) [5–7]. Additionally, we have shown that RXR and PPAR $\gamma$  receptors are necessary for optimal

response to rexinoid or TZD therapies, as knock down of either receptor attenuates the antiproliferative response to its own ligand alone or the ligand of its heterodimer partner [8].

In this report, we explore potential downstream mediators of the rexinoid and TZD treatment effect in the A375(DRO) melanoma cancer cell line using comparative gene expression microarray analysis. We have identified the calcium binding protein S100A2 as a potential mediator of rexinoid and TZD signaling in melanoma. S100A2 is one of 24 members of S100 proteins that regulate cellular processes including neoplasia and has significantly increased gene expression in A375(DRO) with rexinoid and TZD, while a synergistic effect is seen with combination therapy [9].

## 2. Materials and Methods

**2.1. Cell Line and Chemicals.** A375(DRO) was provided by Dr. G.J. Juillard (University of California at Los Angeles, Los

Angeles, CA). DRO was previously thought to be derived from an anaplastic thyroid cancer. We have shown that it is genetically identical to the melanoma cell line A375 and is therefore designated as a subline of A375, A375(DRO) [7, 8]. A375(DRO) was grown in RPMI 1640 (Invitrogen Corporation) supplemented with 2% fetal bovine serum (Hyclone) and 0.5% penicillin/streptomycin. LGD1069 was provided by Ligand Pharmaceuticals (San Diego, CA), and Rosiglitazone (ROSI) was provided by GlaxoSmithKline.

**2.2. Microarray Analysis.** Four million A375(DRO) cells were plated in triplicate into 100 mm plates and incubated overnight. The next day, the medium was changed, and medium with volume equivalent vehicle (DMSO) or 1  $\mu\text{mol/L}$  of LGD1069, ROSI, or the combination (500 nM of each) was added in the set of cells to incubate for 24 hours. RNA was extracted from treated cells using the QIAGEN RNeasy Mini Kit and was quantified by standard spectrophotometry. RNA integrity was verified by gel electrophoresis using an Agilent 2100 Bioanalyzer. Total RNA (5  $\mu\text{g}$ ) was converted to ds-cDNA using the Superscript Choice System (Life Technologies). A high-pressure liquid chromatography-purified T7-(dT)<sub>24</sub> primer was used to initiate the cDNA reverse transcription. After the synthesis of both strands of DNA, the ds-cDNA was extracted with phenol-chloroform-isoamyl alcohol and recovered by ethanol precipitation. Next, in vitro transcription of cRNA was done and the transcript underwent biotin labeling using an RNA Transcript Labeling Kit (Enzo). Biotin-labeled cRNA was purified using the QIAGEN RNeasy Mini Kit. The RNA was then fragmented by incubating the cRNA at 94°C for 35 minutes to allow optimal hybridization to the cDNA oligonucleotide array. We used the Affymetrix GeneChip Human Genome U133A platform, and all gene chip processing and analyses occurred in the UCHSC Affymetrix microarray core facility. Each condition was run in triplicate from three independent experiments. Data analysis, including background adjustment and normalization, was done using Affymetrix GeneSpring software. For microarray analysis, those genes that had at least a >2-fold change in more than three of six gene chips between ligand and vehicle treatment of DRO cells were selected as significant. Significance was determined by a one-way ANOVA ( $P < .05$ ).

**2.3. Quantitative Reverse Transcription-PCR (qRT-PCR).** Total RNA was isolated from A375(DRO) in single samples under the same conditions used for the microarray experiment using the RNeasy Mini Kit (Quiagen, Valencia, CA) as per the manufacturer's protocol. The mRNA for S100A2 was measured by real-time Quantitative RT-PCR using ABI PRISM 7700. The sequences of forward and reverse primers as designed by Primer Express (PE ABI) were 5'-TTCCTGGGTCTGTCTCTGCC-3' and 5'-AGC-GCCTGCTCCAGAGAAC-3'.

The TaqMan fluorogenic probe used was 6FAM-TGG-TCTGCCACAGATCCATGATGTGC-TAMRA.

Amplification reactions, thermal cycling conditions, and generation of a standard curve have been described previously [6].

**2.4. S100A2 Overexpression.** Human S100A2 in pcDNA3 vector was the generous gift from Professor C. Heizmann (University of Zurich). A375(DRO) cells were stably transfected with S100A2 in pcDNA3 vector and empty vector using lipofectamine method in 6-well cell culture plates (4  $\mu\text{g/well}$ ) as previously described [10]. Thereafter stable clones were selected and continuously cultured in 150  $\mu\text{g/mL}$  G418(Gibco/BRL).

**2.5. shRNA.** We used a lentiviral mediated shRNA system from Sigma (St. Louis, MO) and followed the manufacturer's protocol. Lentiviral particles contain shRNA toward S100A2 or PPAR $\gamma$  or RXR $\gamma$ -specific sequences as well as a scrambled (SCR) sequence that consists of 5 nucleotides that do not match any known gene transcript in both the murine and human genome. The infected cells are selected by a puromycin resistance and then assessed for correct insertion/RNA inhibition by qRT-PCR or western blot for S100A2, PPAR $\gamma$ , or RXR $\gamma$ . The concentration of puromycin used to select for DNA construct incorporation cells was 0.4  $\mu\text{g/mL}$ .

**2.6. Western Blot Analysis.** Whole cell protein extracts were obtained from A375(DRO) under conditions of volume equivalent vehicle, LGD1069/ROSI combination treatment, and with overexpressed S100A2 or shRNA directed at S100A2. The protein content of lysates was measured using a commercial protein assay kit (DC from Bio-Rad). Diluted samples containing equal amounts of protein (60  $\mu\text{g}$ ) were mixed with 2x Laemmli sample buffer (Bio-Rad Laboratories). Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 1x TBST (20 mmol/L Tris-HCl (pH 7.6), 8.5% NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at room temperature for 2 hours and incubated in the appropriate primary antibody in 1x TBST containing 5% nonfat dry milk at 4°C overnight. S100A2 protein antibodies (Sigma S6797), RXR $\alpha$  (sc 553 and D-20), RXR $\beta$  (sc 831 and C-20), and RXR $\gamma$  (sc Y-20 and JC-555) receptor antibodies were used at a concentration of 1 : 1000, and PPAR $\gamma$  (sc 7196 and H-100) rabbit polyclonal antibody was used at 1 : 500. After washing, membranes were incubated for 1 hour at room temperature with antirabbit IgG conjugated to horseradish peroxidase at a 1 : 5000 dilution for RXRs and 1 : 1000 for PPAR $\gamma$  (GE Healthcare UK).  $\beta$ -Actin was probed for loading control. The enhanced chemiluminescence detection reagent from Amersham Biosciences was used for immunodetection.

**2.7. Cell Growth and Proliferation.** A375(DRO) cells at baseline, with S100A2 overexpressed, with infected SCR shRNA, and with shS100A2 cells were grown to approximately 80% confluence in 100 mm tissue culture plates. Cells were then harvested using Trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA) and counted using a hemocytometer. Cells were then transferred to a 96-well plate at a concentration of 500 cells/200  $\mu\text{L}$  of media. Each row of eight wells received the same cell type and subsequently the same drug. After

cells were plated, media with the appropriate concentration of ligand or equivalent volume of vehicle was added to each well. Cells were treated with volume equivalent vehicle, 1  $\mu$ M LGD1069, 1  $\mu$ M rosiglitazone, or the 1  $\mu$ M combination (500 nM of each). Fresh media with vehicle or ligand was added every 72 hours. At the completion of 6 days, cell proliferation was assessed following the manufacturers instructions using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Following a two-hour incubation at 37°C, each plate was analyzed by a MRX Micro plate Reader (Dynatech Laboratories, Chantilly, VA) using Revelation software.

**2.8. Statistics.** Cell growth between control and treatment conditions quantified using the group mean  $\pm$  SE and significance was compared between control and treatment conditions with a Student's *t*-test between conditions (SISA online statistical tool).

### 3. Results

**3.1. Microarray Analysis of LGD1069/ROSI-Treated A375 (DRO) Cells.** The antiproliferative effects of rexinoid and TZD treatment on A375(DRO) occur at or beyond six days of treatment [5]. However, we chose to analyze gene expression changes in A375(DRO) at 24 hours since RXR $\gamma$  and PPAR $\gamma$  are nuclear hormone receptors, and we would predict direct gene expression effects of these liganded transcription factors to occur early. The results of the LGD1069 1  $\mu$ M treatment arm have been previously published [6]. Microarray analysis revealed that the combination rexinoid/TZD treatment resulted in 212 genes with increased expression and 1050 genes with decreased expression (one-way ANOVA  $P < .05$ ). These genes broadly fell into the categories of cell growth, nucleic acid binding, and cell signal transduction. The 20 genes with the largest change in expression after treatment are listed in Tables 1, 2, 3, and 4 (excluding affymetrix specific cDNA sequences without related searchable genes). The complete data set is in the supplementary materials (see Supplementary Material available online at doi:10.1155/2010/729876). Four genes were upregulated by rexinoid/TZD combination therapy greater than 20-fold: TIE1 (121.5-fold), S100A2 (69.1-fold), ILB-1 (40.1-fold), and ANGPTL4 (32.2-fold) (Table 5). Of these genes, S100A2 was increased by both the rexinoid (3.4-fold) and TZD (4.9-fold) but also demonstrated a synergistic stimulation (69.1-fold) with the combination treatment. In addition, 171 genes had increased expression and 1006 genes had decreased expression by at least 2-fold with ROSI alone (Tables 1, 2, 3, and 4 —one-way ANOVA  $P < .05$ ). Based upon the significant increase of S100A2 mRNA levels with each ligand alone and the synergistic increase with combination therapy, we performed additional experiments with S100A2 to define its role in mediating the effects of combination rexinoid/TZD treatment in melanoma cells. We have previously published confirmation of ANGPTL4 regulation by rexinoids [6]. The effects of TZD and rexinoids on TIE-1 and ILB-1 mRNA and protein expression have not yet been confirmed by other methods.

TABLE 1: Microarray Analysis of A375(DRO) cells after TZD/rexinoid combination treatment—2-fold up regulated genes. A375(DRO) cells were treated in vitro with 1  $\mu$ mol/L of PIO or combination LGD1069/PIO for 24 hours compared with volume-equivalent vehicle in triplicate with a cutoff of a 2-fold change ( $P < .5$ , one-way ANOVA). Gene symbols were derived from the Affymetrix web site.

Gene Symbol	fold change	GenBank ID
TIE1	121.5	NM_005424
S100A2	69.1	NM_005978
IL1B	40.1	NM_000576
ANGPTL4	32.2	NM_001039667
AREG	18.2	NM_001657
RPL37A	12.6	NM_000998
CORO2B	10.9	NM_006091
RPL27A	8.7	NM_000990
INHBA	7.6	NM_002192
ST3GAL1	5.4	NM_003033
TXN	5.0	NM_003329
ITGA3	5.0	NM_002204
FGFR2	5.0	NM_000141
GEM	4.9	NM_005261
COL5A2	4.9	NM_000393
RPL38	4.8	NM_000999
PMEPA1	4.7	NM_020182
MCL1	4.5	NM_021960
RPL38	4.2	NM_000999
SMARCA2	3.8	NM_003070

**3.2. An Intact RXR $\gamma$  and PPAR $\gamma$  Receptor Is Required for Optimal S100A2 Expression.** S100A2 levels were measured after treatment of A375(DRO) with 1  $\mu$ M combination of LGD1069 and ROSI (500 nM each). To determine the relative contribution of PPAR $\gamma$  and RXR $\gamma$ , we compared control cells stably infected with scrambled shRNA with sublines stably infected with shRNA against PPAR $\gamma$  and RXR $\gamma$  which greatly reduced the levels of each receptor [8]. Figure 1 shows that the rexinoid/TZD-induced expression of S100A2 was attenuated by lack of either receptor. PPAR $\gamma$  appears to have the greatest effect on this response.

**3.3. S100A2 Overexpression Enhances the Antiproliferative Response to LGD1069 and ROSI Treatment.** S100A2 protein was overexpressed in A375(DRO) cells, and the empty vector (EV) was used as a control. Figure 2 shows that the levels of S100A2 protein in the overexpressing subline (S100A2) are similar to levels seen after treating A375(DRO) cells with rexinoid/TZD combination. After plating equivalent numbers of control (A375(DRO) + EV) and S100A2 overexpressing cells, we observed no difference in growth rate at 3 and 6 days (Figure 3(a)). However, with 6 days of 1  $\mu$ M LGD1069, 1  $\mu$ M ROSI, or 1  $\mu$ M combination therapy the S100A2 overexpressing cells had a significant decrease in proliferation compared to the EV cells relative to vehicle treatment (64% versus 46% for LGD1069, 86% versus 72%

TABLE 2: Microarray Analysis of A375(DRO) cells after TZD/rexinoid combination treatment—2-fold down regulated genes.

Gene Symbol	fold change	GenBank ID
ABAT	-17.5	NM_000663
TSEFM	-10.4	NM_005726
XRCC4	-9.0	NM_003401
NUDT1	-8.9	NM_002452
ZIC1	-8.3	NM_003412
BARD1	-8.2	NM_000465
CDC25A	-8.2	NM_001789
MAP9	-8.1	NM_001039580
QPCT	-7.9	NM_012413
C12orf52	-7.8	NM_032848
PTCD2	-7.3	NM_024754
TLE1	-6.8	NM_005077
MYO6	-6.4	NM_004999
WDR59	-6.0	NM_030581
RARS	-5.6	NM_002887
BIN1	-5.2	NM_004305
WBP1	-5.2	NM_012477
CSGALNACT1	-5.1	NM_018371
CAMTA1	-5.1	NM_015215
TRIM23	-5.1	NM_001656

TABLE 3: Microarray Analysis of A375(DRO) cells after TZD/rexinoid combination treatment—2-fold up regulated with ROSI alone.

Gene Symbol	fold change	GenBank ID
ANGPTL4	29.4	NM_001039667
TIE1	13.1	NM_005424
RPL27A	10.9	NM_000990
IL1B	9.3	NM_000576
RPL37A	8.4	NM_000998
AREG	6.1	NM_001657
RPL38	6.1	NM_000999
TACR1	5.6	NM_001058
TXN	5.5	NM_003329
MCL1	5.5	NM_021960
PGK1	5.5	NM_000291
RPL38	5.4	NM_000999
FGFR2	5.2	NM_000141
LOC653505	5.0	NM_001123068
RAB2A	5.0	NM_002865
S100A2	4.9	NM_005978
LOC100131637	4.8	XM_001716033
GBA3	4.7	NM_020973
JUN	4.4	NM_002228
SMARCA2	4.2	NM_003070

TABLE 4: Microarray Analysis of A375(DRO) cells after TZD/rexinoid combination treatment—2-fold down regulated with ROSI alone.

Gene Symbol	fold change	GenBank ID
ORC4L	-15.9	NM_002552
TAF12	-8.9	NM_005644
ZNF443	-8.1	NM_005815
HIGD1A	-8.0	NM_032775
CYB5R2	-7.9	NM_001039580
DAZAP2	-7.5	NM_005726
STK32B	-7.1	NM_024754
ZBTB43	-6.7	NM_014007
CAMTA1	-6.6	NM_015215
HCG4	-6.4	NR_002139
BSC12 /// HNRNPUL2	-6.3	NM_001079559
SLC39A4	-6.0	NM_017414
DZIP3	-6.0	NM_014648
MSRB2	-6.0	NM_018009
KLHL22	-5.8	NM_032848
MPP5	-5.7	NM_024899
RNF41	-5.6	NM_005785
VRK3	-5.5	NM_030581
ABAT	-5.4	NM_000663
BRCA2	-5.3	NM_000059
RARS	-5.3	NM_002887

TABLE 5: Four genes with the highest mRNA stimulation after combination TZD/rexinoid treatment.

	TIE 1	S100A2	IL1B	ANGPTL4
LGD1069/ROSI	121.5	69.1	40.1	32.2
ROSI	13.1	4.9	9.3	29.4
LGD1069*	<2	3.4	<2	6.5

\*data published in [6].

for ROSI, and 94% versus 88% for the combination;  $P \leq .008$  for all conditions; Figure 3(b)).

*3.4. Knock Down of S100A2 Attenuates the Growth Inhibition of A375(DRO) Cells by Rexinoid, TZD, and Combination Treatment.* Knock down of S100A2 with specific shRNA did not affect growth of A375(DRO) cells (data not shown). S100A2 shRNA stably expressed in A375(DRO) cells resulted in a decrease in S100A2 mRNA and protein expression after treatment with LGD1069/ROSI (Figure 4). Stable expression of scrambled shRNA (SCR) had no effect. Figure 5 shows that melanoma cells stably transfected with S100A2 shRNA had significantly blunted growth suppression by treatment with rexinoid, TZD, or combination ( $P < .002$  for all shS100A2 versus SCR treatment conditions). These data indicate that S100A2 is required to mediate the growth suppressive effects of TZD and rexinoids in melanoma cells. Western blot analysis of the cells expressing S100A2 shRNA revealed decreased levels of RXR $\gamma$  but RXR $\alpha$  and PPAR $\gamma$  protein levels were unaffected (Figure 6). RXR $\beta$  protein levels

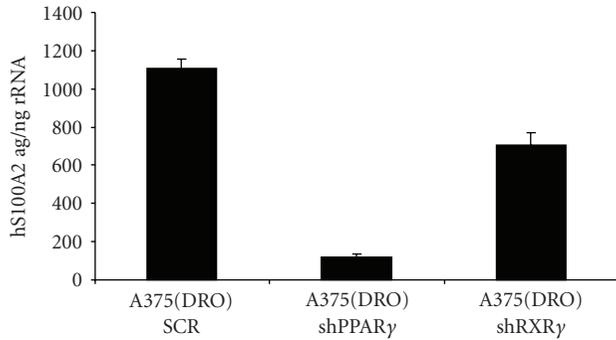


FIGURE 1: S100A2 mRNA stimulation by TZD/rexinoid treatment is dependent on intact PPAR $\gamma$  and RXR $\gamma$ . One microgram of total RNA was used for the S100A2 quantitative reverse transcription-PCR analysis (ABIPRISM7700; Perkin-Elmer), and absolute values were derived from a standard curve using a known amount of sense strand RNA (ag, attograms of sense strand RNA). Isoform RNA was normalized to total input RNA (18s rRNA measured from 1 ng of total RNA). A375(DRO) cells were infected with either shPPAR $\gamma$  or shRXR $\gamma$  lentiviral particles and then treated with LGD1069/ROSI 1  $\mu$ M for 24 hours. S100A2 mRNA levels were compared to levels from A375(DRO) cells infected with the shSCR control under the same treatment conditions.

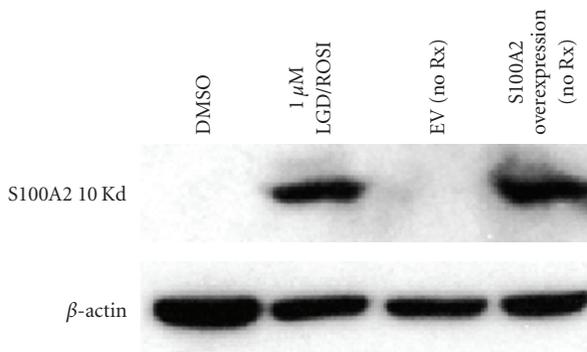


FIGURE 2: Protein expression of S100A2 in A375(DRO) cells. 60  $\mu$ g of nuclear protein extract from A375(DRO) before (DMSO—lane 1) and after combination treatment (lane 2) or transfected with empty vector (EV—lane 3) or S100A2 in pcDNA3 vector with no treatment (lane 4) was size-separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was blocked with 10% nonfat milk and incubated with S100A2 receptor antibodies (sc Y-20).  $\beta$ -Actin was measured as a loading control.

are very low in these cells [11]. Analysis of shS100A2 and RXR $\gamma$  sequences revealed no sequence homology.

#### 4. Discussion

In this report, we have examined global gene expression in a poorly differentiated cancer model, the amelanotic melanoma cell line A375(DRO), after treatment with PPAR $\gamma$  and RXR ligands. S100A2 was shown to be a potentially important target based on increased levels with rexinoid or TZD treatment and synergistically increased levels with combination therapy. Furthermore, S100A2 appears to be

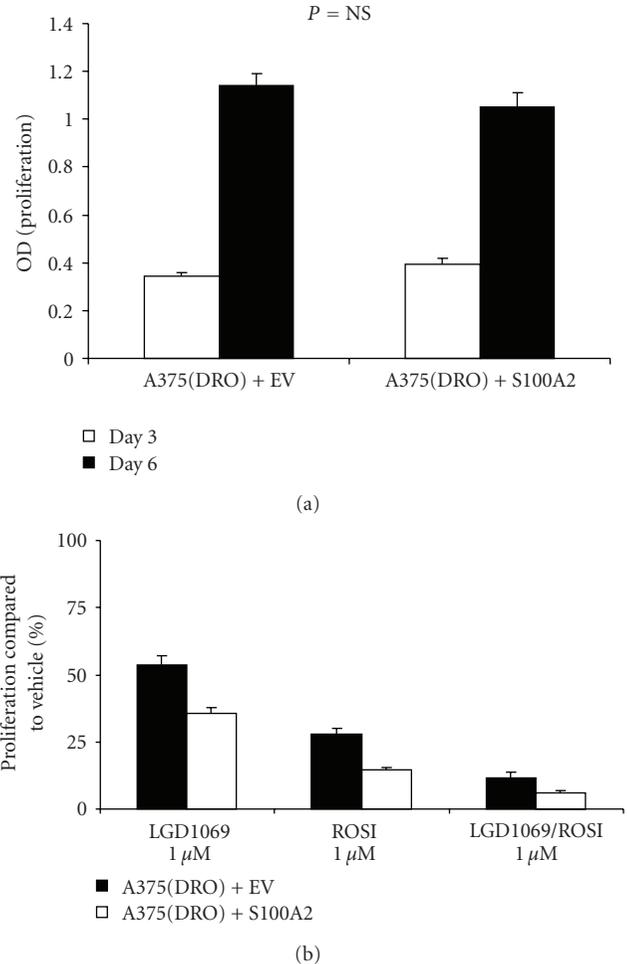


FIGURE 3: Proliferation of A375(DRO) cells overexpressing S100A2. Cells were grown in 2% fetal bovine serum RPMI in the presence of 1  $\mu$ mol/L of LGD1069, TZD, or the combination for 6 days. Cell growth was analyzed using a nonradioactive cell proliferation assay. Proliferation was compared directly between EV and S100A2 (a) and then to that of cells grown in volume equivalent vehicle (set at 100% which represents cell growth in control conditions) (b). Proliferation was significantly decreased in cells with S100A2 overexpression compared to EV for all treatment conditions compared to control. Columns mean; bars, SEM.

required for the maximal antiproliferative effects of rexinoids and TZD in these melanoma cells.

The S100 proteins have a broad range of intracellular functions including the regulation of protein phosphorylation and enzyme activity, calcium homeostasis, regulation of cytoskeletal proteins, and transcriptional factors [12]. S100 proteins appear to regulate tumorigenesis. For example, S100A2 proteins enhance p53 transcriptional activity whereas S100A4 increases p53 apoptosis in models of adenocarcinoma, osteosarcoma, and oral carcinoma [12–14]. Thus, a relative imbalance of S100 proteins may promote or inhibit neoplastic transformation or progression. S100A2 seems to have a variable pattern of expression with some evidence pointing to higher expression in normal tissues and

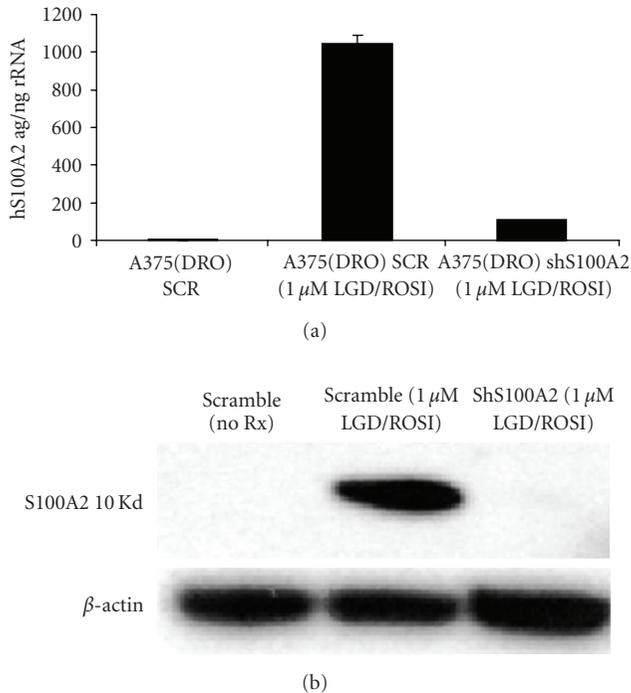


FIGURE 4: shS100A2 decreases S100A2 mRNA and protein in A375(DRO) cells. qRT-PCR and Western blot of S100A2. (a) One microgram of total RNA was used for the S100A2 quantitative reverse transcription-PCR analysis (ABIPRISM7700; Perkin-Elmer), and absolute values were derived from a standard curve using a known amount of sense strand RNA (ag, attograms of sense strand RNA). Isoform RNA was normalized to total input RNA (18s rRNA measured from 1 ng of total RNA). (b) 60 μg of nuclear protein extract from A375(DRO), the SCR shRNA infected control cell, and shS100A2 infected cells. Proteins were size-separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was blocked with 10% nonfat milk and incubated with S100A2 primary antibody (sc Y20) and then secondary antibody with antimouse IgG conjugated to horse-radish peroxidase as previously described. β-actin was measured as a loading control.

early or premalignant issues, but other types of cancer, such as lung cancer, have a higher expression in advanced lesions that correlate with poorer clinical prognosis. However, even within the lung cancer literature, there is disagreement regarding the prognostic significance of S100A2 expression [13, 15, 16].

In melanoma, S100 proteins may play a critical role in regulating the transformation of nevi to melanoma. S100A4 levels are lower in metastatic melanoma compared with primary tumors, while S100A7, S100A8, and S100A9 levels appear to be higher in malignant melanoma compared with normal melanocytes [17]. In a study of 105 patients with stage IV melanoma, elevated serum levels of S100B were associated with a significantly shorter survival [18].

S100A2 expression is higher in premalignant nevi than in cells from primary melanoma tumors or metastases suggesting that loss of S100A2 may be important for neoplastic transformation [19]. In an in vitro model of uveal melanoma, S100A2 gene expression was significantly

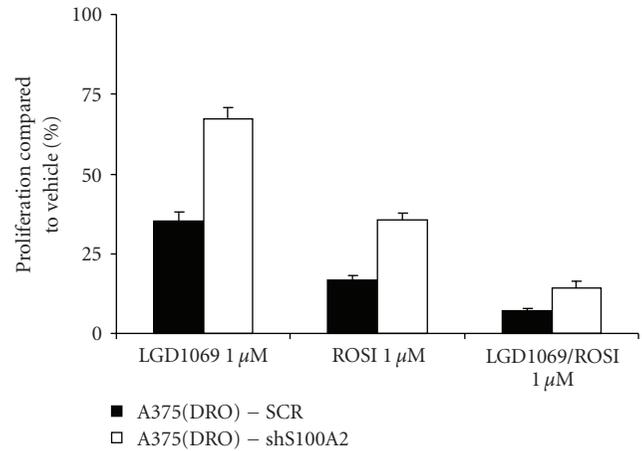


FIGURE 5: Knock down of S100A2 blunts the antiproliferative effect of TZD and rexinoids in A375(DR) cells. A375(DRO), the SCR infected, and an shS100A2 infected subline were grown in 2% fetal bovine serum RPMI in the presence of 1 μmol/L of LGD1069, TZD, or the combination for 6 days. Cell growth was analyzed using a nonradioactive cell proliferation assay. Proliferation was compared to that of cells grown in volume equivalent vehicle (DMSO—set at 100%). Proliferation was statistically significantly attenuated compared to the A375(DRO) SCR subline in all treatment conditions. Columns, mean; bars, SEM.

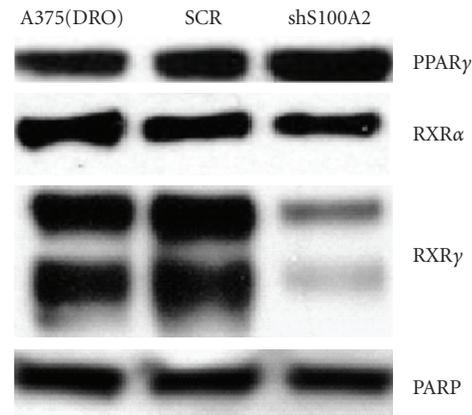


FIGURE 6: Western blot of rexinoid receptors in A375(DRO) cells after knock down of S100A2. 60 μg of nuclear protein extract from A375(DRO), the SCR shRNA-infected control cell, and a clone of shS100A2 infected cells were size-separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was blocked with 10% nonfat milk and incubated with PPARγ, RXRγ, and RXRα primary antibodies and then secondary antibody with antirabbit IgG conjugated to horse-radish peroxidase as previously described. PARP was measured as a loading control.

up-regulated by the methyltransferase inhibitor decitabine, which was correlated with cell death [20]. We have demonstrated that S100A2 levels are low in the A375(DRO) melanoma cell line, and these levels are increased by treatment with PPARγ and RXR agonists, which is associated with a significant reduction in growth and increase in apoptosis [8].

Though other S100 proteins have been shown to increase with retinoid therapy in models of cancer including teratocarcinoma, breast cancer, and gastric carcinoma [21–23], this is the first report of increased S100A2 expression by either rexinoids or TZDs. We have previously demonstrated that, in A375(DRO), the combination of LGD1069 and ROSI synergistically decreases *in vitro* cell proliferation and *in vivo* tumor growth [5, 8]. Our data indicates that S100A2 is necessary to mediate the antiproliferative effects of rexinoid and TZD treatment but is not sufficient to mediate this effect. However, with overexpression of S100A2, we observe an enhanced effect of rexinoid and TZD treatment on the melanoma cells.

This observed relative resistance to rexinoid and TZD treatment with shS100A2 was found in conjunction with decreased RXR $\gamma$  protein levels (though the shS100A2 sequence does not overlap with RXR $\gamma$ ). We have previously shown that decreasing RXR $\gamma$  by shRXR $\gamma$  in A375(DRO) decreases response to rexinoid, TZD, and the combination [8]. Modulators of retinoid receptors have been described in melanoma and include HSP 90 and Cyclophilin B [24], but we were unable to find any direct link between S100A2 expression and RXR regulation. It appears as if the presence of S100A2 is important for optimal RXR $\gamma$  expression, but this is most likely not a direct interaction as measurable and at least partially functional RXR $\gamma$  (as evidenced by response to LGD1069) was seen after shS100A2 infection. Further studies will be needed to elucidate the exact interaction of S100A2 and retinoid receptors.

In summary, we have performed a microarray analysis of a poorly differentiated melanoma after rexinoid and TZD treatment. S100A2 gene expression is significantly increased by both rexinoid and TZD treatment alone and is synergistically increased by combination therapy. S100A2 is necessary for the maximal antiproliferative effect of rexinoid and TZD in this model, but it is not sufficient to mediate this effect.

## Acknowledgments

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## Research Article

# Cyclooxygenase 2 (COX2) and Peroxisome Proliferator-Activated Receptor Gamma (PPARG) Are Stage-Dependent Prognostic Markers of Malignant Melanoma

Stefanie Meyer,<sup>1</sup> Thomas Vogt,<sup>1</sup> Michael Landthaler,<sup>1</sup> Anna Berand,<sup>2</sup> Albrecht Reichle,<sup>2</sup> Frauke Bataille,<sup>3</sup> Andreas H. Marx,<sup>4</sup> Anne Menz,<sup>4</sup> Arndt Hartmann,<sup>5</sup> Leoni A. Kunz-Schughart,<sup>6</sup> and Peter J. Wild<sup>7</sup>

<sup>1</sup>Department of Dermatology, University of Regensburg, 93042 Regensburg, Germany

<sup>2</sup>Department of Hematology and Oncology, University of Regensburg, 93042 Regensburg, Germany

<sup>3</sup>Institute of Pathology, University of Regensburg, 93042 Regensburg, Germany

<sup>4</sup>Institute of Pathology, University Medical Center Hamburg-Eppendorf, 20251 Hamburg, Germany

<sup>5</sup>Institute of Clinical Pathology, University of Erlangen, 91054 Erlangen, Germany

<sup>6</sup>OncoRay - Center for Radiation Research in Oncology, TU Dresden, 01307 Dresden, Germany

<sup>7</sup>Institute of Surgical Pathology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland

Correspondence should be addressed to Peter J. Wild, peter.wild@usz.ch

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Using tissue microarrays (TMAs) we studied COX2/PPARG immunoreactivity in a broad spectrum of tumors focussing on clinicopathological correlations and the outcome of patients with malignant melanoma (MM). TMA-1 contained normal and tumor tissues ( $n = 3448$ ) from 47 organs including skin neoplasms ( $n = 323$ ); TMA-2 88 primary MM, 101 metastases, and 161 benign nevi. Based on a biomodulatory approach combining COX/PPAR-targeting with metronomic low-dose chemotherapy metastases of 36 patients participating in a randomized trial with metastatic (stage IV) melanoma were investigated using TMA-3. COX2/PPARG immunoreactivity significantly increased from nevi to primary MM and metastases; COX2 positivity was associated with advanced Clark levels and shorter recurrence-free survival. Patients with PPARG-positive metastases and biomodulatory metronomic chemotherapy alone or combined with COX2/PPARG-targeting showed a significantly prolonged progression-free survival. Regarding primary MM, COX2 expression indicates an increased risk of tumor recurrence. In metastatic MM, PPARG expression may be a predictive marker for response to biomodulatory stroma-targeted therapy.

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## 1. Introduction

Cyclooxygenases (COXs) catalyze the first rate-limiting step in the conversion of arachidonic acid to prostaglandins. Two COX isoenzymes have been identified: COX1 is constitutively expressed in most tissues and mediates the synthesis of prostaglandins in normal physiological processes, whereas COX2 is not detectable in most normal tissues but is rapidly induced by various stimuli such as inflammatory reactions [1]. COX2 is also expressed in various tumor types [2], and levels of expression have been shown to correlate with invasiveness and prognosis in some tumor entities, suggesting an important role of COX2 in tumor

development and progression. Epidemiological studies show that prolonged COX2 inhibition through acetylsalicylic acid or other nonsteroidal anti-inflammatory drugs (NSAIDs) might offer some protection against colon cancer and some other malignancies [3, 4]. Accordingly, in animal experiments COX2 inhibitors can reduce the incidence of colon carcinoma in APC knockout mice treated with chemical carcinogens [5]. The mechanism by which COX2 expression accelerates tumorigenesis is poorly understood. However, a potential role of COX2 in epithelial and melanocytic skin cancer development is also not unlikely, since COX2 is frequently expressed in malignant melanomas (MMs) [6, 7] and squamous cell carcinomas of the skin [8, 9].

The peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear hormone receptor subfamily of ligand-activated transcription factors. There are three known subtypes of peroxisome proliferator-activated receptors; PPARA, PPARD, and PPARG. The latter is involved in physiological adipocyte differentiation and differentially expressed in several types of human cancers [10], for example, in prostate cancer [11, 12], breast adenocarcinomas [13], ovarian cancer [14, 15], lung cancer [16], and colon cancer [17]. Accordingly, PPAR ligands were shown to inhibit the growth of cells from different cancer lineages in vitro [18]. In human melanoma cell lines the antiproliferative and apoptosis-inducing effect of PPARG ligands was demonstrated, too [19, 20].

Current research data and clinical experience suggest that PPARA/G can mediate both direct antitumoral and immunomodulatory effects and a broad spectrum of stroma modulating activity including antiangiogenic, anti-inflammatory, and immunoenhancing effects [21, 22]. Examples of superadditive complementation of PPARG agonists by COX2 inhibitors and metronomic chemotherapy are well-documented experimentally and in clinical trials, respectively [10, 16, 23].

We had studied such combined tumor-stroma-targeted cancer therapy using PPARG agonists and COX2 inhibitors in the second-line treatment of advanced metastatic melanoma disease [22, 23]. In a randomized multi-institutional phase II trial including 76 mostly chemorefractory patients with progression of metastatic melanoma (stage IV melanoma according to AJCC criteria), we had observed a significantly prolonged progression-free survival in the group of patients that received angiostatically scheduled low-dose metronomic chemotherapy (trofosfamide) in combination with a PPARG agonist (pioglitazone) and a COX2 inhibitor (rofecoxib) compared to the group of patients who received metronomic chemotherapy alone [22]. Accordingly, tumor-associated inflammatory and angiogenic processes mediated by COX2 overexpression or PPARG deficiency were suggested to play a pivotal role in the biology of melanoma progression [22]. However, there is insufficient data on the expression of both target molecules; therefore, their prognostic and therapeutic relevance in MM is still unclear.

The study presented herein is based on a high-throughput tissue microarray (TMA) analysis, a highly efficient technology for investigating large numbers of tumors. To the best of our knowledge this is the largest study of this topic which can link expression data with extensive follow-up data of melanoma patients, respectively. In addition, as we gather extensive data on various other cancers and normal tissues (47 organs and tissue entities) we can put the specificities of the melanoma data into a broader oncologic context.

## 2. Materials and Methods

**2.1. Tissue Microarrays (TMAs).** TMA construction was performed as described previously [24]. The local Institutional

Review Boards of the Universities of Regensburg and Basel granted approval for this project.

The first TMA (*TMA-1*) contained formalin-fixed, paraffin-embedded tissue punches from the archives of the Institute of Pathology, University of Basel, Switzerland. A comprehensive TMA was created by transferring representative tissue cylinders with a diameter of 0.6 mm to seven new paraffin blocks as described by Bubendorf et al. [25]. Representative areas of different subtypes for the most frequent tumor entities and their corresponding nontumorous tissue were selected for analysis. Four  $\mu\text{m}$  sections of the resulting TMA block were cut and mounted to an adhesive-coated slide system (Instrumedics Inc. Hackensack, NJ, USA). The constructed multitumor TMA-1 consisted of 3448 primary tumors from 132 different tumor subtypes and 26 different normal tissues and allowed us to determine the prevalence of COX2 and PPARG expression in nontumorous tissues and corresponding malignant tumors. Samples from skin ( $n = 330$ ), lung ( $n = 217$ ), brain ( $n = 228$ ), breast ( $n = 218$ ), colon ( $n = 204$ ), soft tissue ( $n = 150$ ), salivary gland ( $n = 152$ ), testis ( $n = 126$ ), ovary ( $n = 140$ ), and kidney ( $n = 144$ ) were the major tissues assembled on this TMA. The evaluation of tissue and clinical data was performed on the basis of anonymized patient data according to the regulations of the University of Basel Institutional Review Board. Detailed tumor and tissue characteristics can be found in supplementary Tables 1 and 2 in Supplementary Material available online at doi:10.1155/2010/848645. The skin-related data sets were extracted and are summarized in Table 1.

The second TMA (*TMA-2*) was constructed as described by Wild et al. [26] and contained a total of 350 formalin-fixed, paraffin-embedded human tissues: 88 (25.1%) primary malignant melanomas, 101 (28.9%) metastases, and 161 (46.0%) benign nevi. H&E-stained slides of all tumors were evaluated by two surgical pathologists (T.V., P.J.W.). Clinical follow-up data, provided by the Central Tumor Registry of the University of Regensburg, were available for all patients with primary malignant melanomas ( $n = 88$ ). The median follow-up for all patients was 54 months (range 0 to 135 months), whereas the median follow-up for censored patients ( $n = 74$ ) was 63.5 months. Characteristic parameters of TMA-2 are summarized in Table 2.

The third TMA (*TMA-3*) was constructed on the basis of a randomized multi-institutional phase II trial using an angiostatic biomodulatory approach to assess the impact of COX2- and PPAR-targeted therapy in combination with metronomic low-dose chemotherapy in patients with advanced metastatic stage IV melanoma [22]. The clinical trial was designed to select metronomic chemotherapy alone (arm A: trofosfamide 50 mg orally three times daily, day 1+) or combined anti-inflammatory/angiostatic treatment (arm B: trofosfamide as mentioned above plus rofecoxib 25 mg orally, day 1+, and pioglitazone 60 mg orally, day 1+) for further evaluation. A total of 76 patients, mostly (>60%) refractory to at least one previous chemotherapy with maximum tolerated doses, and progression of metastatic melanoma were included; from the Institute of Pathology and the Department of Dermatology (University of Regensburg,

TABLE 1: COX2 and PPARG expression analysis of skin tumors using TMA-1.

Tumor entity	Cytoplasmic COX2 immunoreactivity					<i>P</i> *	Nuclear PPARG immunoreactivity					<i>P</i> †	
	<i>n</i> analyzable	0 ( <i>n</i> )	1+ ( <i>n</i> )	2+ ( <i>n</i> )	3+ ( <i>n</i> )		<i>n</i> analyzable	0 ( <i>n</i> )	1+ ( <i>n</i> )	2+ ( <i>n</i> )	3+ ( <i>n</i> )		<i>P</i> *
<i>TMA-1</i> : total ( <i>n</i> = 323)	186	34	86	54	12		212	143	50	19	0	<b>.0003</b>	
Melanocytic lesions													
Malignant melanoma	38	0	16	17	5	<b>.02</b>	41	21	8	12	0	<b>.001</b>	<b>.01</b>
Benign nevus	19	4	7	8	0		24	22	2	0	0		
Epithelial tumors													
Squamous cell carcinoma	30	3	10	11	6	<b>.07</b>	33	23	7	3	0	<b>.001</b>	<b>.62</b>
Basal cell carcinoma	31	7	16	7	1		33	11	21	1	0		
Connective tissue tumors													
Kaposi sarcoma	15	6	8	1	0		18	13	5	0	0		1.00
Benign histiocytoma	16	8	7	1	0	<b>.61</b>	22	19	1	2	0	<b>.13</b>	<b>.47</b>
Capillary hemangioma	14	3	9	2	0		18	16	2	0	0		
Appendix tumors													
Benign sebaceous adenoma	23	3	13	7	0		23	18	4	1	0		1.00

\* Fisher's exact test (2-sided); bold face representing significant data.

† Fisher's exact test (2-sided); association of COX2 and PPARG IHC within single tumor entities.

TABLE 2: Clinicopathologic parameters in relation to COX2 immunohistochemistry using TMA-2.

Variable	Categorization	Cytoplasmic COX2 immunoreactivity					<i>P</i> †	Nuclear PPARG immunoreactivity					<i>P</i> †
		<i>n</i> analyzable	0 ( <i>n</i> )	1+ ( <i>n</i> )	2+ ( <i>n</i> )	3+ ( <i>n</i> )		<i>n</i> analyzable	0 ( <i>n</i> )	1+ ( <i>n</i> )	2+ ( <i>n</i> )	3+ ( <i>n</i> )	
Primary malignant melanomas													
Clark level													
	II	4	3	1	0	0	<b>.004</b>	2	2	0	0	0	<b>.793</b>
	III	14	3	6	3	2		12	11	1	0	0	
	IV	52	2	27	15	8		52	39	10	2	1	
	V	13	4	2	6	1		11	9	1	0	1	
Tumor thickness													
	≤2.0 mm	35	8	17	6	4	<b>.104</b>	31	24	6	1	0	<b>.762</b>
	>2.0 mm	49	4	20	18	7		47	37	7	1	2	
Growth pattern*													
	SSM	37	6	15	11	5	<b>.748</b>	8	5	3	0	0	<b>.685</b>
	LMM	3	2	0	1	0		36	29	6	1	0	
	NM	29	2	14	9	4		5	4	1	0	0	
	ALM	6	1	3	1	1		27	21	3	1	2	
	ONA	9	1	5	2	1		2	2	0	0	0	
TP53 immunoreactivity													
	<5%	67	11	28	20	8	<b>.308</b>	63	49	10	2	2	<b>.883</b>
	≥5%	15	0	8	4	3		15	12	3	0	0	
Ki-67 labeling index													
	<5%	68	11	29	18	10	<b>.295</b>	64	53	9	1	1	<b>.101</b>
	≥5%	14	0	7	6	1		14	8	4	1	1	
Melanoma metastases													
	Lymph node	42	3	9	4	26	<b>.013</b>	42	32	6	2	2	<b>.136</b>
	Skin	56	6	27	6	17		53	32	18	1	2	
Benign nevi													
	Compound & junctional	47	39	7	1	0	<b>&lt;.001</b>	53	53	0	0	0	—
	Dermal	21	15	6	0	0		45	45	0	0	0	
	Congenital	51	4	45	2	0		50	50	0	0	0	

\*SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; ALM, akro-lentiginous melanoma; NOS, not otherwise specified.

† Fisher's exact test (two-sided), bold face representing significant data.

Germany) 194 formalin-fixed paraffin-embedded metastatic tissues of 36 patients (47%) were available for further immunohistochemical analysis. The local ethic committee had approved the study.

Prior to TMA-construction, H&E-stained slides of all specimens were evaluated by two dermatopathologists (T.V., S.M.) to identify representative metastatic areas. Clinical follow-up data with a median follow-up period of 9 months (range 1–43 months) were available for 35 melanoma patients (97%), that is, 12 patients (33%) who received metronomic chemotherapy alone (arm A) and 23 patients (64%) with combined anti-inflammatory/angiostatic treatment (arm B). Median follow-up of censored patients was 7 months (range 2–43 months). Characteristic parameters of TMA-3 are given in Table 4.

**2.2. Immunohistochemistry (IHC).** Immunohistochemical studies utilized an avidin-biotin peroxidase method with a 3-amino-9-ethylcarbazole (AEC) chromogen. After antigen retrieval (steam boiler with citrate-buffer, pH 6.0 for 20 minutes) immunohistochemistry was carried out applying the ZytoChemPlus HRP Broad Spectrum Kit (Zytomed Systems, Berlin, Germany) according to the manufacturer's instructions. The following primary antibodies were used: anti-COX2 (mouse monoclonal, Cayman Chemical, Ann Arbor, Mich, USA; dilution 1 : 200, final concentration 2.5 µg/mL), anti-PPARG (rabbit monoclonal, Cell Signalling, New England Biolabs GmbH, Frankfurt am Main, Germany; dilution 1 : 400), anti-TP53 (mouse monoclonal IgG, clone Bp53-12 (sc-263), Santa Cruz Biotechnology Santa Cruz, Calif, USA; dilution 1 : 1000), and anti-Ki-67 (rabbit monoclonal, clone MIB1; DakoCytomation GmbH, Hamburg, Germany; dilution 1 : 10, final concentration 5 µg/mL). As a positive control for COX2 and PPARG IHC, a colon carcinoma with known COX2 and PPARG expression was chosen. Normal tissue samples of 10 different organs were considered as negative controls. Two pathologists performed a blinded evaluation of the stained slides. Cytoplasmic COX2 and nuclear PPARG immunoreactivity were estimated using an arbitrary semiquantitative four-step scoring system (0–3+), based on the intensity of cytoplasmic COX2 staining [6] and the percentage of PPARG positive cell nuclei [7]: 0 (negative): no cytoplasmic COX2 staining/PPARG staining 0% of cell nuclei; 1+: weak COX2 staining/PPARG staining 1 to 9%; 2+: moderate COX2 staining/PPARG staining 10 to 50%; 3+: strong COX2 staining/ PPARG staining greater than 50%. Causes of noninterpretable results included lack of tumor tissue and presence of necrosis or crush artifact. The percentage of tumor cells with nuclear Ki-67 and TP53 staining was determined as described previously [27]. Ki-67/TP53 labeling was considered high if at least 5% of the tumor cells were positive.

**2.3. Statistical Analysis.** Specimens on TMA-1 and TMA-2 were considered independently. Concerning TMA-3, COX2 and PPARG immunoreactivity were examined for a mean of 5 metastatic samples per patient (range 1–15); the median level of COX2 and PPARG immunoreactivity was chosen for further analyses using the SPSS version 16.0 (SPSS,

Chicago, Ill, USA). *P*-values <.05 were considered significant. Contingency table analysis and two-sided Fisher's exact tests or  $X^2$ -tests were used to study statistical associations between clinicopathological and immunohistochemical data. Retrospective overall and progression-free survival curves comparing patients with and without any of the variables were calculated using the Kaplan-Meier method, with significance evaluated by two-sided log rank statistics. For the analysis of progression-free survival, patients were censored at the time of their last progression-free clinical follow-up appointment. For the analysis of overall survival, patients were censored at the time of their last clinical follow-up appointment or at their date of death not related to the tumor. For multiple testing, the closed test principle was used.

### 3. Results

**3.1. TMA-1.** Investigation of COX2 and PPARG protein expression in 323 benign and malignant skin tumors using a comprehensive multitumor TMA (TMA-1) was informative in 57.6% (186/323) and 65.6% (212/323) of cases. COX2 and PPARG expression of any intensity (score 1+–3+) was detected in 81.7% (152/186) and 32.5% (69/212) of informative cases, respectively. Table 1 summarizes the expression data and statistical analysis of COX2 and PPARG immunoreactivity of each skin tumor entity on TMA-1. For connective tissue tumors (Kaposi sarcoma, capillary hemangioma, benign histiocytoma) no significant differences could be found in benign versus malignant tumors ( $P = .61$  and  $P = .13$ ). Regarding epithelial tumors (squamous cell carcinomas, basal cell carcinomas) positive PPARG staining was detected significantly more often in basal cell carcinomas than in squamous cell carcinomas ( $P = .001$ ). Surprisingly, 86.9% of benign skin adnexal tumors (sebaceous adenomas) were positive for COX2; 21.7% positive for PPARG. Regarding melanocytic lesions, 100% (38/38) of primary melanomas and 78.9% (15/19) of benign nevi revealed at least weak COX2 immunoreactivity (score 1+–3+); 48.7% (20/41) of primary melanomas and 8.3% (2/24) of benign nevi demonstrated PPARG positivity (1+–2+). Accordingly, compared to benign nevi, expression of both COX2 and PPARG was significantly increased in primary melanomas ( $P = .02$  and  $P = .001$ ).

Besides skin tumors, COX2 and PPARG expression was analyzed in many other benign and malignant tissue types from 46 different organs using a comprehensive multitumor TMA-1. As shown in supplementary Tables 1 and 2, differential COX2 and PPARG expression between normal and neoplastic tissue could be observed for almost every tissue type investigated. In prostate cancer, for example, COX2 expression continuously increased from prostatic hyperplasia to prostatic intraepithelial neoplasia (PIN) to organ-confined prostate cancer to hormone-refractory prostate cancer to metastatic disease (supplementary Figures 1A).

**3.2. TMA-2.** Based on the results of TMA-1, a second TMA (TMA-2) with clinical follow-up data sampling primary

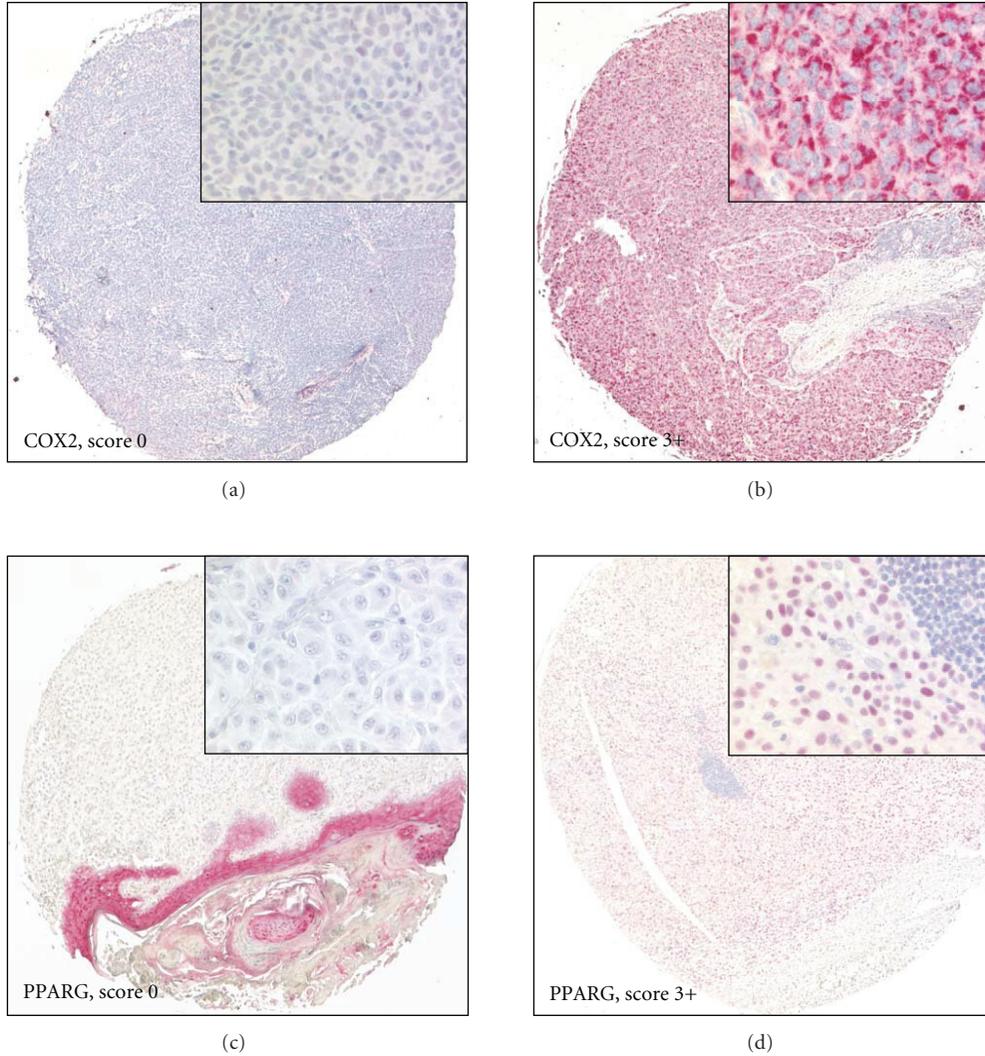


FIGURE 1: Immunohistochemical COX2 and PPARG staining of malignant melanomas on TMA-2. Original magnification 10x (insets 200x). Representative examples of a primary malignant melanoma with negative (a) and strong (b) immunoreactivity for COX2. Representative examples of a primary malignant melanoma with negative (c) and strong (d) immunoreactivity for PPARG.

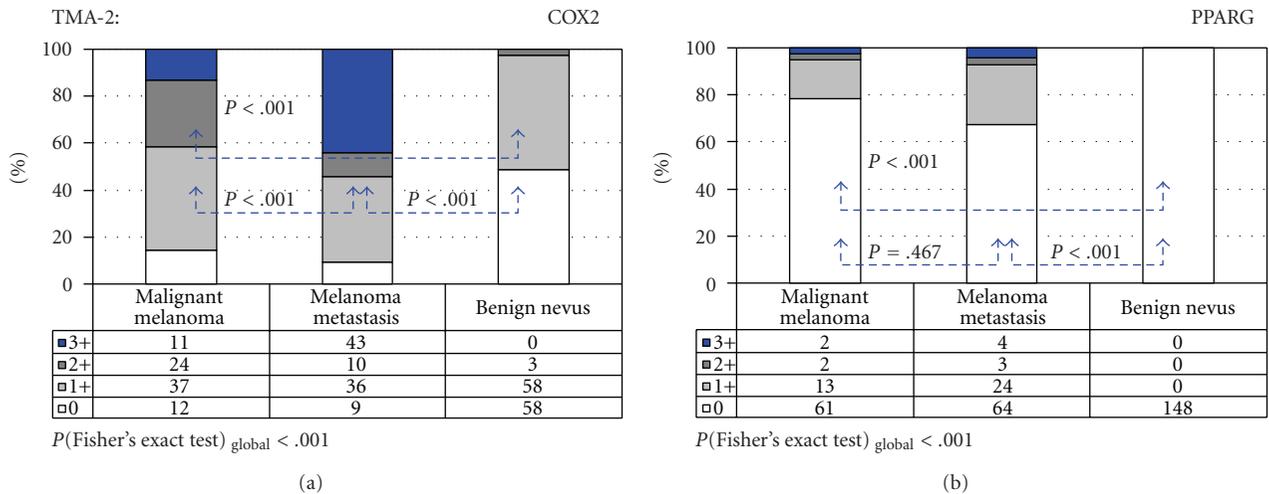


FIGURE 2: Cumulative bar charts of COX2 (a) and PPARG (b) immunoreactivity in melanocytic skin tumors using TMA-2.

malignant melanomas and melanoma metastases as well as benign nevi was constructed. COX2 and PPARG immunoreactivity was informative in 86.0% (301/350) and 91.7% (321/350) of cases, respectively. Expression of COX2 and PPARG of any intensity was detected in 73.8% (222/301) and in 15.0% (48/321) of informative cases. Representative negative and positive COX2 and PPARG immunostaining patterns in malignant melanoma are shown in Figures 1(a)–1(d). Figures 2(a) and 2(b) summarize the results of COX2 and PPARG IHC for primary melanomas, metastases, and nevi on TMA-2. The percentage of COX2 positive cases significantly increased from benign nevi (51%) to primary melanomas (86%) and melanoma metastases (91%;  $P < .001$ ; Figure 2(a)). Likewise, PPARG immunoreactivity significantly increased from benign nevi (0%) to malignant melanomas (22%) and melanoma metastases (33%;  $P < .001$ ; Figure 2(b)). Clinicopathologic variables of melanoma patients were correlated with COX2 and PPARG expression (Table 2). In primary melanomas, positive COX2 immunoreactivity was significantly related to advanced Clark levels ( $P = .004$ ), but no other clinicopathologic variables such as tumor growth pattern, p53 immunoreactivity, and Ki-67 labeling index. Skin metastases demonstrated a gradually weaker COX2 immunoreactivity compared with lymph node metastases ( $P = .013$ ). Among the various types of benign nevi on TMA-2, COX2 expression was significantly increased in congenital nevi compared to compound, junctional, and dermal melanocytic nevi ( $P < .001$ ).

According to a univariate analysis, tumor progression was significantly related to both melanoma thickness and COX2 immunoreactivity, respectively ( $P = .03$ ; Table 3); that is, expression of COX2 was associated with shorter progression-free survival ( $P = .03$ ; Figure 3). In contrast, PPARG expression of primary melanomas was not associated with any of the variables neither the clinicopathologic ones nor progression-free and overall survival (Tables 2 and 3).

**3.3. TMA-3.** Using TMA-3, the prognostic and therapeutic meaning of COX2 and PPARG expression was analyzed in patients with advanced metastatic melanoma disease ( $n = 36$ ). All patients received angiostatic biomodulatory treatment with trofosfamide alone (arm A,  $n = 12$ ) or in combination with rofecoxib and pioglitazone (arm B,  $n = 24$ ). COX2 and PPARG protein expression of metastatic tissues was informative in all 36 cases. Expression of COX2 and PPARG of any intensity was detected in 97.2% (35/36) and in 38.9% (14/36) of patients, respectively. Clinicopathologic variables of this cohort of patients with advanced metastatic melanoma disease were compared relative to COX2 and PPARG expression (Table 4).

Considering all 36 patients receiving biomodulatory therapy expression of PPARG (score 1+–3+) in the metastases was significantly associated with longer progression-free survival ( $P = .044$ ) but not with overall survival ( $P = .179$ ; Figures 4(a) and 4(b)). Expression of COX2 (score 2+–3+) in the metastases, however, was not associated with overall and progression-free survival, respectively (Figures 4(c) and 4(d)). Besides PPARG immunoreactivity, stage of

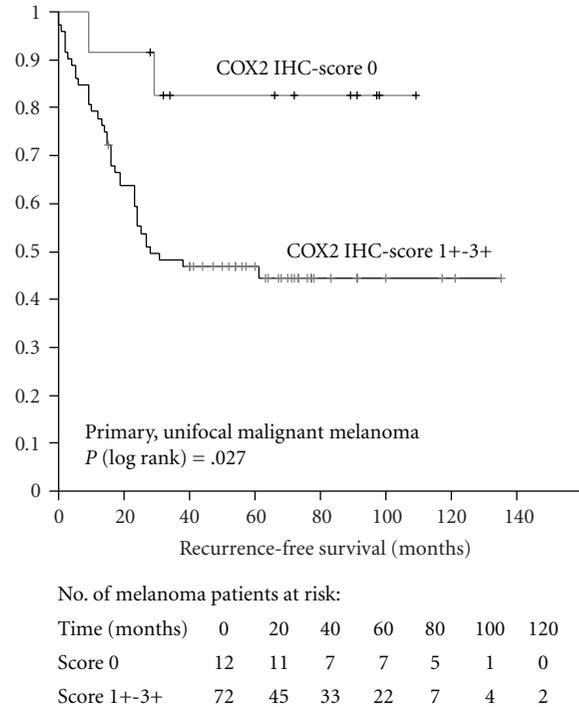


FIGURE 3: Distribution of time (months) to tumor-related death among patients with primary malignant melanomas showing negative (0) or positive (1+ to 3+) COX2 immunoreactivity as estimated by the Kaplan Meier method.

the primary melanoma was also a significant prognostic factor for progression-free survival ( $P = .016$ ; Table 4). In a multivariate Cox regression model, using primary tumor stage (pTis–pT3 versus pT4) and PPARG expression (negative versus positive) as covariates, neither PPARG immunoreactivity nor primary tumor stage remained significant (data not shown).

#### 4. Discussion

In this study, we demonstrate by a comprehensive multitumor TMA that COX2 and PPARG are differentially expressed in a broad spectrum of normal and malignant tissues. Focussing on tumors of the skin we can further confirm that COX2 immunoreactivity of primary MM is significantly associated with advanced Clark levels ( $P = .004$ ) and shorter recurrence-free survival ( $P = .03$ ). PPARG expression of primary MM, however, does not provide significant prognostic information. Yet, by analysis of COX2 and PPARG expression in MM metastases of patients who had received biomodulatory therapy, we can show that only the expression of PPARG is significantly associated with longer progression-free survival ( $P = .044$ ). These findings suggest that COX2 may mainly contribute to early steps in melanoma progression, that is, growth and invasion of primary MM, and becomes less essential in the advanced metastatic setting of melanoma disease. Our study confirms the prognostic meaning of COX2 in patients with primary

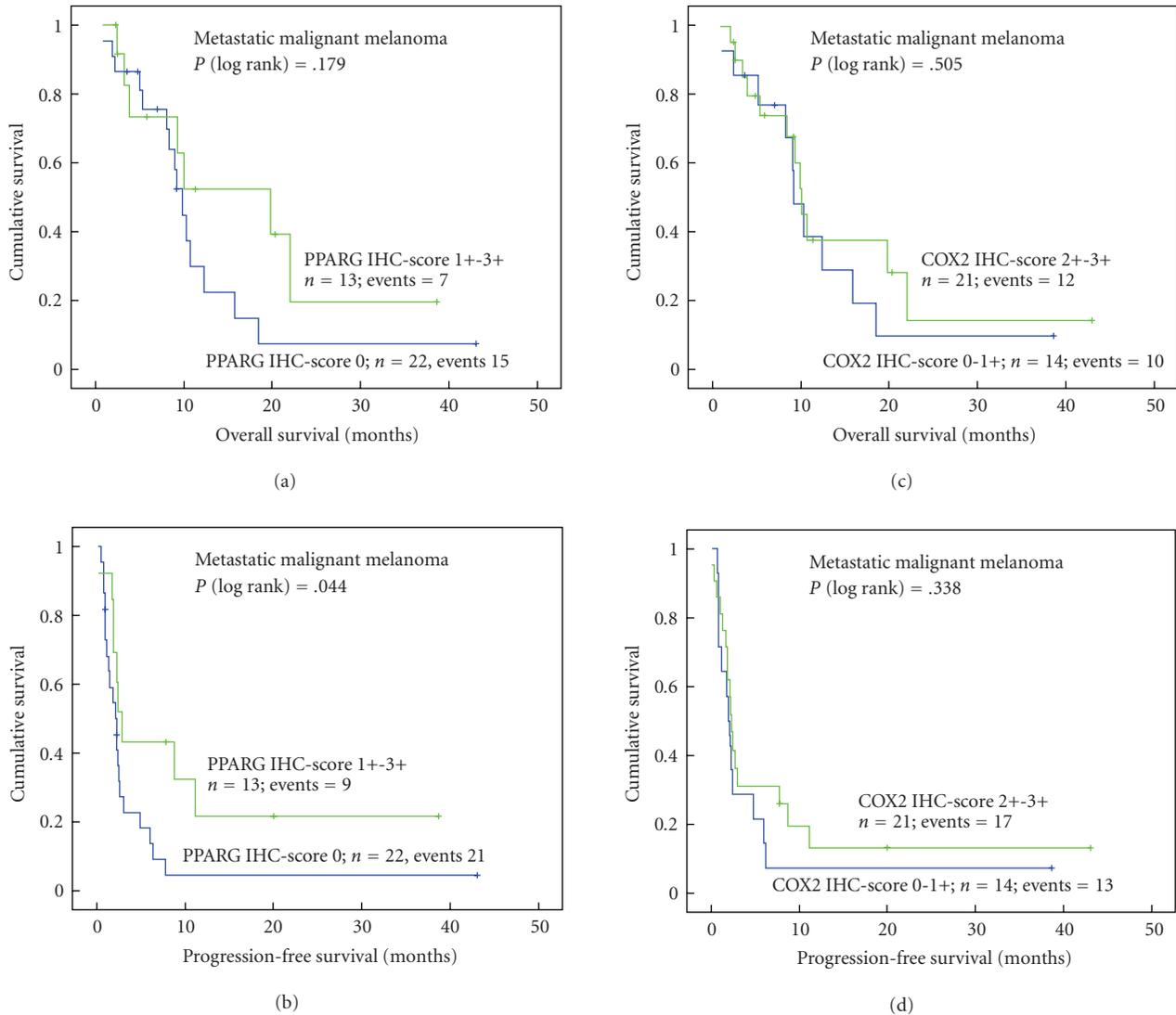


FIGURE 4: Distribution of time (months) to death and tumor progression among patients with advanced metastatic melanomas in correlation with immunoreactivity of PPARG (a), (b) or COX2 (c), (d). All patients received biomodulatory treatment. The calculation was performed according to the method of Kaplan and Meier.

MM and adds a new late-stage histopathological marker, PPARG, which may be predictive for responsiveness to biomodulatory therapy in advanced metastatic MM. To our knowledge this is the first TMA study demonstrating that PPARG protein expression may be a positive prognostic marker indicating responsiveness to stroma-targeted therapy in the late metastatic stage (IV) of MM disease, that is, in patients refractory to conventional first-line chemotherapy, mostly with dacarbazine.

Consistent with previously published data on melanocytic skin lesions [6, 7] our immunohistochemical analysis of benign nevi, primary MM and MM metastases show that COX2 and PPARG immunoreactivity significantly increases from benign nevi to primary MM and MM metastases. In other organs, however, for example, in primary cancers of the lung versus normal lung tissues, decreased expression levels of PPARG were found and associated with poor prognosis

[16]. At first sight, these findings are in contrast to the upregulation of PPARG in primary MM and MM metastases versus benign nevi observed with TMA-2. But, as our data also show, this upregulation does not correlate with the outcome of MM patients indicating a distinct role of PPARG in primary MM and MM metastases. Notably, in the advanced metastatic stages of MM enclosed in this study, patients with PPARG-positive metastases versus PPARG-negative metastases show a significant survival benefit concerning progression-free survival ( $P = .044$ ) not dependent on whether angiostatically scheduled metronomic chemotherapy (trofosfamide) was administered alone or in combination with pioglitazone (PPARG agonist) and rofecoxib (COX2 inhibitor) as additional biomodulatory therapy. Considering PPARG or COX2 as candidate substrates for targeted cancer therapy, it could be assumed that only patients with PPARG- or COX2-positive metastases and additional

TABLE 3: Univariate analysis of factors regarding tumor progression and death.

Variable	Categorization	Tumor progression			Death		
		<i>n</i> <sup>a</sup>	events	<i>p</i> <sup>b</sup>	<i>n</i> <sup>a</sup>	events	<i>p</i> <sup>b</sup>
Age at diagnosis							
	≤60 years	48	25	0.7	48	7	0.6
	>60 years	40	18		40	7	
Gender							
	female	39	15	0.06	39	5	0.4
	male	49	28		49	9	
Clark level <sup>(c)</sup>							
	II	5	0	0.4	5	0	0.3
	III	15	8		15	2	
	IV	54	28		54	8	
	V	13	7		13	4	
Tumor thickness							
	≤2.0 mm	38	14	<b>0.03</b>	38	4	0.2
	>2.0 mm	50	29		50	10	
Ki67 labeling index							
	<5%	33	17	0.7	33	7	0.9
	≥5%	36	16		36	7	
Cytoplasmic COX2 IHC							
	score 0	12	2	<b>0.03</b>	12	0	0.1
	score 1+-3+	72	39		72	14	
Nuclear PPARG IHC							
	score 0	61	28	0.2	61	11	0.6
	score 1+-3+	17	10		17	2	

<sup>a</sup>Only initial and unifocal malignant melanomas were included;

<sup>b</sup>Log rank test (two-sided), bold face representing significant data;

<sup>c</sup>According to UICC: TNM Classification of Malignant Tumours. 6th edn (2002) Sobin LH, Wittekind CH (eds.) Wiley, New York.

PPARG-agonistic or COX2-inhibitory therapy would show a survival benefit compared with patients treated with metronomic chemotherapy alone. Yet, subgroup analysis with TMA-3 did not show a significant survival benefit for these patients. Thus, our study supports current concepts that targeting COX2 and PPAR is more a tumor-stroma effective approach than an approach depending on the status of target expression of the tumor itself [21, 22]. Possible explanations of this paradoxon are multifaceted and complex. There may be numerous “off-target” effects of the involved drugs, for example, modulation of COX2/PPARG-independent pathways [16, 18, 21]. According to the paradigm of biomodulatory stroma targeting approaches [21, 28] the effects may be indirect due to modifying the tumor stroma; that is, the therapy mainly exploits the dependence of cancer tissues on functions of the stroma providing a permissive and supportive environment for tumor cell survival, growth, invasion, and formation of metastases. A variety of soluble agents such as chemokines, growth factors, lipids, angiogenic factors, proteinases, and proteinase inhibitors are involved in a complex crosstalk between tumor and stroma. Stroma targeted approaches aim to inhibit tumor growth and invasion by disruption of this tumor-stroma interaction. Interestingly, stromal cells in the tumoral microenvironment

can also differ from their normal counterparts in the expression of biologically meaningful molecules [29] including also COX2 and PPARG expression. For instance, upregulation of these effectors could be detected in stromal myofibroblasts surrounding colon adenocarcinomas [30].

Therefore, to fully evaluate and understand the potential of COX2 and PPAR modulation in MM further studies using TMAs punching the surrounding stroma may be interesting future work. Based on the large comprehensive amount of data gained in this study it seems to be promising to further develop experimental protocols that employ COX2/PPAR biomodulation. The combination of both drugs is a logical consequence of experimental studies indicating that COX2 and PPARG signalling pathways are multiply intertwined: PPARG ligands suppress COX2 expression induced by lipopolysaccharide and phorbol myristate acetate in macrophages, astrocytes, and epithelial cells [16]. Moreover, expression of COX2 was suggested to be regulated by a negative feedback loop involving PPARG and NF- $\kappa$ B [31, 32]. PPARG agonists were shown to downregulate COX2, potentiate the apoptotic effects of chemotherapeutic agents, and inhibit the growth of human melanoma cell lines in vitro [19, 20]. Consistently, the randomized phase II trial by Reichle et al. [22] including chemorefractory patients with

TABLE 4: Univariate analysis of factors regarding tumor progression and death using TMA-3.

Variable	Categorization	Death			Tumor progression		
		<i>n</i>	events	<i>P</i> *	<i>n</i>	events	<i>P</i> *
Advance melanoma patients							
Age							
	<60 years	12	7	0.152	12	11	0.163
	≥60 years	22	14		22	18	
Initial tumor stage							
	pT1	2	1	0.690	2	2	<b>0.016</b>
	pT2	1	1		1	1	
	pT3	13	9		13	11	
	pT4	9	6		9	6	
	Melanoma in situ	1	0		1	1	
Initial regional lymph node status							
	pN0	11	6	0.980	11	9	0.894
	pN1	9	8		9	8	
	pN2	6	4		6	5	
	pN3	2	1		2	1	
Study therapy							
	A: trofosfamide	12	10	0.570	12	10	0.898
	B: trofosfamide + rofecoxib + pioglitazone	23	12		23	20	
CRP							
	normal or < 30% decrease or increase	14	9	0.115	14	11	0.128
	> 30% decrease	10	10		10	10	
Cytoplasmic COX2 IHC							
	score 0 to 1+	14	10	0.505	14	13	0.338
	score 2+ to 3+	21	12		21	17	
Nuclear PPARG IHC							
	score 0	22	15	0.179	22	21	<b>0.044</b>
	score 1+ to 3+	13	7		13	9	

\*Log-rank test (two-sided).

progressive metastatic stage IV melanoma disease demonstrated a significantly prolonged progression-free survival if metronomic low-dose chemotherapy (trofosfamide) was combined with pioglitazone (PPARA and G agonist) and rofecoxib (COX2 inhibitor). In summary, COX inhibitors and PPAR agonists are a beneficial adjunct in biomodulatory therapy of MM rather independent of the presence of the targeted substrates in the cancer cells themselves. The expression of PPARG in the cancer, however, can indicate a higher probability to respond to stroma-targeted approaches also without drugs aiming on PPAR.

In conclusion, our study provides a late-stage prognostic marker, PPARG expression, which correlates with responsiveness to biomodulatory stroma-targeted therapy. But it should be kept in mind that the indication for such approaches cannot be solely based on selected features of the cancer cell itself but must consider the complexity of the stroma-tumor interaction, that is, the microenvironment, including angiogenesis, immunoeffects, and functions of the connective tissue as well. Therefore, further prospective clinical trials are needed to validate the meaning of PPARG

and COX2 targeting as a part of biomodulatory therapeutic approaches.

## Abbreviations

MM: Malignant melanoma  
TMA: Tissue microarray  
IHC: Immunohistochemistry  
COX2: Cyclooxygenase 2  
PPARG: Peroxisome proliferator-activated receptor gamma.

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## Review Article

# Role of PPARs in Radiation-Induced Brain Injury

**Sriram Ramanan,<sup>1,2</sup> Weiling Zhao,<sup>2,3</sup> David R. Riddle,<sup>2,4</sup> and Mike E. Robbins<sup>2,3</sup>**

<sup>1</sup> Department of Cancer Biology, Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA

<sup>2</sup> Brain Tumor Center of Excellence, Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA

<sup>3</sup> Department of Radiation Oncology, Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA

<sup>4</sup> Department of Neurobiology and Anatomy, Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA

Correspondence should be addressed to Mike E. Robbins, mrobbins@wfubmc.edu

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Whole-brain irradiation (WBI) represents the primary mode of treatment for brain metastases; about 200 000 patients receive WBI each year in the USA. Up to 50% of adult and 100% of pediatric brain cancer patients who survive >6 months post-WBI will suffer from a progressive, cognitive impairment. At present, there are no proven long-term treatments or preventive strategies for this significant radiation-induced late effect. Recent studies suggest that the pathogenesis of radiation-induced brain injury involves WBI-mediated increases in oxidative stress and/or inflammatory responses in the brain. Therefore, anti-inflammatory strategies can be employed to modulate radiation-induced brain injury. Peroxisomal proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the steroid/thyroid hormone nuclear receptor superfamily. Although traditionally known to play a role in metabolism, increasing evidence suggests a role for PPARs in regulating the response to inflammation and oxidative injury. PPAR agonists have been shown to cross the blood-brain barrier and confer neuroprotection in animal models of CNS disorders such as stroke, multiple sclerosis and Parkinson's disease. However, the role of PPARs in radiation-induced brain injury is unclear. In this manuscript, we review the current knowledge and the emerging insights about the role of PPARs in modulating radiation-induced brain injury.

## 1. Introduction

PPARs are ligand-activated transcription factors that belong to the steroid/thyroid hormone superfamily of nuclear receptors [1, 2]. To date, three PPAR isotypes have been identified—PPAR $\alpha$  (NR1C1), PPAR $\beta$  (NR1C2 or PPAR $\delta$ ), and PPAR $\gamma$  (NR1C3) [3]. Each is encoded by a separate gene, and each has a unique tissue distribution pattern [4, 5]. PPARs regulate gene transcription by heterodimerizing with the retinoid X receptor (RXR) and binding to specific consensus sequences (termed PPAR response elements, PPREs) in the enhancer regions of genes [6]. PPREs consist of a direct repeat (DR) of the nuclear receptor hexameric recognition sequence AGGTCA separated by one or two

nucleotides (DR-1 or DR-2) [6]. The protein structure of the PPAR isotypes reveals two well-characterized domains—a highly conserved DNA binding domain and a ligand-binding domain (LBD) that is less well conserved across the subtypes. Variation in the sequence of amino acids that line the ligand-binding pocket is a major determinant of ligand isotype specificity [7, 8]. In the absence of ligand binding, PPAR-RXR heterodimers are bound to corepressor proteins such as HDACs and N-CoRs that maintain the chromatin in the condensed state and inhibit the transcriptional apparatus from assembling. Upon ligand binding, PPARs undergo a conformational change that leads to dissociation of the corepressor proteins. Subsequently, the ligand-bound PPARs complex with coactivator proteins such as p300 leading

to nucleosome remodeling and transcriptional preinitiation complex assembly on target gene promoters [7]. The transcriptional response is strongly influenced by the structure of the promoter and the expression levels of coactivators and corepressors in a given cell-type [9].

## 2. Tissue Distribution and Physiological Role of PPARs

PPAR $\alpha$  is predominantly expressed in tissues that catabolize high amounts of fatty acids such as the liver, skeletal muscle, and heart and regulates many metabolic pathways, including activation of fatty acid  $\beta$ -oxidation and apolipoprotein expression [10]. Natural ligands such as eicosanoids, mono- and polyunsaturated fatty acids and long-chain fatty acyl-CoenzymeA can bind and activate PPAR $\alpha$  [7]. Hypolipidemic fibrate drugs that are routinely prescribed to patients for lowering triglyceride and cholesterol levels have been demonstrated to be synthetic ligands of PPAR $\alpha$  [11, 12].

PPAR $\gamma$  is most abundantly expressed in fat cells, the large intestine, and cells of the monocyte lineage. It is primarily involved in the general transcriptional control of adipocyte differentiation, immune responses, and glucose homeostasis [13, 14]. PPAR $\gamma$  exists as two distinct forms,  $\gamma$ 1 and  $\gamma$ 2, which arise by differential transcription start sites and alternative splicing [15]. Whereas PPAR $\gamma$ 1 is low in most tissues, PPAR $\gamma$ 2 is fat-selective and is expressed at very high levels in adipose tissue [14]. PPAR $\gamma$  is bound and activated by several naturally occurring compounds, such as the eicosanoids 9- and 13-hydroxyoctadecadienoic acids [8]. More recently, a type of nitrated lipids known as nitroalkenes has been demonstrated to be potent, endogenous ligands of PPAR $\gamma$  [16]. In addition, several high-affinity synthetic PPAR $\gamma$  agonists have been synthesized, including the thiazolidinedione (TZD) class of compounds [17], which are used clinically as insulin sensitizers in patients with type 2 diabetes [18], and certain nonsteroidal antiinflammatory drugs [19].

Unlike the PPAR $\alpha$  and PPAR $\gamma$  isotypes, the expression of PPAR $\delta$  appears to be ubiquitous. Ligands of PPAR $\delta$  include fatty acids such as bromopalmitate [20] and the prostanoid prostacyclin PGI<sub>2</sub> [21]. Studies suggest key roles for PPAR $\delta$  in proliferation [22], differentiation, and survival as well as in embryonic development and fatty acid  $\beta$ -oxidation in skeletal muscles and adipose tissues [22]. More recently, PPAR $\delta$  agonists have been shown to enhance oligodendrocyte maturation and differentiation [23]. Mice that are knocked-out for PPAR $\delta$  have altered myelination in the corpus callosum suggesting a role for PPAR $\delta$  in myelination [24].

## 3. PPARs in the Central Nervous System

All PPAR isotypes have been identified in the rodent brain, and their expression has been shown to peak between day 13.5 and 18.5 of gestation. The degree of expression and specific localization varies among the receptors. PPAR $\delta$  appears to be expressed ubiquitously in all regions of the brain [4], primarily in oligodendrocytes and neurons [25] and to a lesser extent in astrocytes [4]. In the spinal cord

white matter, expression is localized to oligodendrocytes [26]. PPAR $\alpha$  expression in the brain appears limited to the olfactory bulbs, hippocampus, and cerebellum, primarily in cerebellar granule neurons (CGNs) [5] and astrocytes. In the spinal cord white matter, expression of PPAR $\alpha$  is localized to astrocytes [26]. PPAR $\gamma$  has been observed, albeit at relatively low levels, in the hippocampus, cerebellum as well as in cortical astrocytes and CGN [4]. All three PPAR isoforms are expressed in the microglia [27].

All PPAR isoforms have been proposed to play an important role in the developing and adult brain [28]. PPAR $\alpha$  has been shown to play a major role in acetylcholine biosynthesis, excitatory amino acid neurotransmission, and defense against oxidative stress [29]. PPAR $\gamma$  is coexpressed along with dopaminergic receptors in several regions of the brain suggesting that it could regulate the action of dopamine on gene transcription [29]. PPAR $\delta$ -mediated transcriptional upregulation of Acyl-CoA synthetase 2 mediates fatty acid utilization and plays an important role in brain lipid metabolism. Experiments using neurosphere cultures derived from mouse neural stem cells isolated from the subventricular zone (SVZ) have demonstrated that PPARs regulate the proliferation and fate of these cells [30, 31].

## 4. Anti-Inflammatory/Neuroprotective Role of PPARs in Neurodegenerative Disorders

In addition to their well-known functions on cellular metabolism, PPARs have been shown to play a major role in inflammation. The anti-inflammatory functions of PPARs in several peripheral tissues have been reviewed elsewhere [2, 32] and beyond the scope of this review. With reference to the CNS, several studies have documented the anti-inflammatory and neuroprotective effects of PPAR ligands in a number of neuropathological conditions [27, 33].

In vitro models of Alzheimer's disease (AD), PPAR $\gamma$  agonists inhibited the neuronal death induced by the amyloid- $\beta$  ( $A\beta$ ) peptide by inhibiting the microglial and monocytic proinflammatory response and astrocytic proliferation [34]. In vivo, oral administration of the PPAR $\gamma$  agonist pioglitazone reduced glial activation and the accumulation of  $A\beta$ -positive plaques in the hippocampus and cortex [35, 36]. In a clinical trial involving 500 AD patients, a significant improvement in cognitive function was observed following treatment with the PPAR $\gamma$  agonist, rosiglitazone for 6 months [37].

In a mouse model of Parkinson's disease, oral administration of pioglitazone inhibited the glial activation induced by the neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and prevented the loss of dopaminergic neurons in the substantia nigra pars compacta. Mechanistically, the neuronal death was prevented by (i) inhibiting the nuclear translocation of the redox-regulated proinflammatory transcription factor NF- $\kappa$ B subunit p65 and (ii) preventing the subsequent induction of inducible nitric oxide synthase (iNOS) gene [38]. Similar protective effects on dopaminergic neurons were demonstrated following administration of the PPAR $\alpha$  agonist fenofibrate [39].

PPAR agonists have also been shown to reduce the severity of cerebral ischemic injury in rodents. Oral administration of PPAR $\alpha$  agonists decreased the incidence of stroke in apolipoprotein-E deficient mice and reduced the cerebral infarct volume in wild-type mice following transient middle cerebral artery occlusion (MCAO) [40, 41]. These effects were associated with decreased oxidative stress and adhesion molecule expression in the brain [40]. Other studies have reported that administration of PPAR $\alpha$  agonists either prior to cerebral ischemia or during the reperfusion period can also have a neuroprotective effect [42, 43]. Likewise, administration of the PPAR $\gamma$  agonists troglitazone or pioglitazone, or the PPAR $\delta$  agonists L-165041 or GW501516, prior to or during transient MCAO reduces the infarct volume [44–46].

In the mouse model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), PPAR agonists have been shown to delay the onset and reduce the severity of the disease. PPAR $\alpha$  agonists inhibited the proliferation of CD4<sup>+</sup> T-cells and shifted their differentiation pattern from the proinflammatory Th1-type to the anti-inflammatory Th2-type cells [47]. In addition, oral administration of the PPAR $\alpha$  agonists, gemfibrozil and fenofibrate, alleviated the clinical symptoms of EAE [47, 48]. Administering the PPAR $\delta$  agonist GW0742 in the mouse diet during the peak of EAE can improve clinical recovery, partly by reducing lymphocyte infiltration into the CNS and by decreasing resident glial activation [49]. Numerous research studies have demonstrated the anti-inflammatory and neuroprotective role of PPAR $\gamma$  agonists in reducing the neurological symptoms of chronic progressive and relapsing forms of EAE [50–52].

Since resident glial cell inflammation and immune cell infiltration into the brain are considered hallmarks of several neuroinflammatory disorders, numerous research groups have hypothesized that the neuroprotective effects of PPAR agonists might result, in part, from inhibition of proinflammatory responses during the CNS pathology. Consistent with this hypothesis, PPAR agonists have been shown to inhibit myelin oligodendrocyte glycoprotein-, cytokine-, and lipopolysaccharide-induced increases in proinflammatory mediators such as tumor necrosis factor alpha (TNF $\alpha$ ), members of the interleukin (IL) family such as interleukin 1 beta (IL-1 $\beta$ ) and IL-12, cyclooxygenase-2 (Cox-2), iNOS, and interferon gamma (IFN- $\gamma$ ) as well as the expression of adhesion molecules such as monocyte chemoattractant protein-1 (MCP-1) in the astrocytes, microglia, and T-cells *in vitro* [53–57].

Taken together, these data suggest that PPAR agonists show promise as efficacious anti-inflammatory agents in ameliorating the clinical symptoms and disease severity of a variety of CNS pathologies.

## 5. Whole-Brain Irradiation and Radiation-Induced Brain Injury

Ongoing advancements in cancer treatment and healthcare have led to an increase in the long-term survivors of cancer; >67% of adult and >75% of pediatric cancer patients will

survive longer than 5 years after initial diagnosis. As a result, late effects remain a significant risk for these ~11 million cancer survivors. Given the increasing population of long-term survivors, the need to mitigate or treat late effects has emerged as a primary area of research in radiation biology [58, 59].

The total dose of radiation therapy that can be administered safely to the brains of patients presenting with primary or metastatic brain tumors is limited by the risk of normal brain morbidity. The need to both understand and minimize the side effects of brain irradiation is intensified by the ever-increasing number of patients with secondary brain metastases (mets) that require treatment with partial or whole-brain irradiation (WBI). Of the ~1 500 000 new cancer patients diagnosed in 2008 [60], up to 30% will develop brain mets [61, 62], making this the 2nd most common site of metastatic cancer, the most common neurological manifestation of cancer, and a cancer problem more common than newly diagnosed lung, breast, and prostate cancer combined. The annual incidence in the US appears to be increasing, as a result of an aging population, better treatment of systemic cancer, and the application of superior imaging techniques such as magnetic resonance imaging (MRI) to detect smaller and micrometastatic lesions in asymptomatic patients [63]. WBI is the primary mode of treatment for brain mets; up to 170 000 individuals will ultimately be treated with large field or WBI each year in the USA. Over half of these patients will survive long enough to develop radiation-induced brain injury, including cognitive impairment. Presently, there are no successful long-term treatments or effective preventive strategies for radiation-induced brain injury [64].

Classically, based on the time of expression, radiation-induced brain injury has been subdivided into acute, subacute (early delayed), and late delayed responses [65]. Acute injury is expressed days to weeks after irradiation and is often characterized by drowsiness, vomiting, headache, and nausea. This type of injury can be treated with corticosteroids and is fairly uncommon under current radiotherapy regimens [65, 66]. Early delayed injury typically occurs from 1- to 6- months postradiation therapy and can involve transient demyelination, short-term memory loss, fatigability, and somnolence. While both these early injuries can result in severe reactions, they are normally reversible and resolve spontaneously. In sharp contrast, late delayed effects, distinguished by demyelination, vascular abnormalities and ultimate radionecrosis of the white matter are observed >6 months postirradiation and are usually irreversible and progressive [67]. Intellectual deterioration is also seen in patients receiving brain irradiation [68]. Data suggest that 20%–50% of brain tumor patients who are long-term survivors suffer from progressive cognitive dysfunction, ranging from mild lassitude to significant memory loss and severe dementia [69–72]. More importantly, in both clinical and preclinical models, the cognitive impairment has been shown to occur in the absence of gross histopathological and radiographic alterations [73–75].

## 6. Mechanisms of Radiation-Induced Brain Injury: Role of Oxidative Stress, Neuroinflammation, and Impaired Neurogenesis

Conventionally, late effects were thought to be the consequence of a reduction in the number of surviving clonogens of either the parenchymal or the vascular target cell populations [65, 76]. Radiation-induced late normal tissue injury was considered to be inevitable, progressive, and untreatable. However, recent data suggest that this view is over-simplistic and that radiation-injury involves complex, intercellular, and intracellular interactions between various cell types [59, 65, 77–79] (in the brain these include astrocytes, microglia, neurons, etc.) within an organ and can be modulated [59]. In general, irradiating late responding normal tissues is hypothesized to activate autocrine and paracrine signal transduction events that initiate downstream reactive processes marked by a persistent oxidative stress and cytokine production ultimately contributing to tissue injury. Although the cellular, molecular, and biochemical mechanisms of radiation-induced brain injury are ill-defined, several studies lend support to the hypothesis that such an injury is driven, in part, via increased oxidative stress and/or inflammation [73, 80–83].

Irradiating one hemisphere of postnatal day 8 rats or of postnatal day 10 mice with a single dose of 4–12 Gy of 4 MV X-rays led to time-dependent increases in nitrotyrosine, a marker for protein nitrosylation, in the SVZ and the granule cell layer (GCL) of the hippocampus 2–12 hours postirradiation [84]. WBI of the mouse brain with a single dose of 6 Gy led to a significant increase in markers of lipid peroxidation and DNA oxidation such as 4-hydroxynonenal and 8-hydroxy-2'-deoxyguanosine, respectively, 1-month postirradiation in the dentate gyrus (DG), and hilus of the hippocampus [85]. In addition to their direct damaging effects on the DNA, lipids, and proteins, reactive oxygen species can act as second messengers to initiate neuroinflammation [86].

Although the brain traditionally has been considered to be immune-privileged, it is widely accepted now that the brain does exhibit inflammation [87]. An acute molecular response characterized by increased expression of inflammatory cytokines/mediators such as TNF $\alpha$ , IL-1 $\beta$ , intracellular adhesion molecule-1 (ICAM-1), Cox-2, and activation of transcription factors such as NF- $\kappa$ B and activator protein-1 (AP-1) is observed within hours of irradiating the rodent brain [88–90]. In addition, a chronic elevation of TNF $\alpha$  has been observed in the mouse brain up to 6-month postirradiation [91].

More recently, the detrimental effect of WBI on ongoing hippocampal neurogenesis and the associated neuroinflammatory response characterized by activated microglia have been proposed as a key mechanism of radiation-induced cognitive impairment [73, 92]. The hippocampus is situated in the medial-temporal lobe and is one of two regions in the mammalian brain where active neurogenesis occurs throughout adulthood. Neurogenesis is a complex multistep

process which starts with the proliferation of the neural precursor cells residing in a specialized region called the subgranular zone (SGZ) of the hippocampus, followed by commitment to a neuronal phenotype, physiological, and morphological maturation with the development of synaptic and electrophysiological properties and ending with the integration of a functional neuron into the GCL [93]. Adult neurogenesis has been shown to play an important role in certain types of hippocampal-dependent cognitive function [94].

One of the earliest observations that led to the proposed involvement of the hippocampus in radiation-induced brain injury was that the extent of cognitive impairment experienced by patients receiving radiotherapy correlated with the dose delivered to the medial-temporal lobe [95]. Subsequently, experimental studies have demonstrated that the neural precursor cells in the SGZ are extremely sensitive to radiation [80]. In vitro, irradiation reduces the proliferative capacity of cultured neural precursor cells [96]. In vivo, the ability of these precursor cells to give rise to new neurons in the GCL is significantly ablated by WBI [80, 96]. More importantly, the WBI-induced decrease in neurogenesis is associated with deficits in hippocampal-dependent spatial learning and memory tasks in mice [97–99]. Furthermore, the deleterious effect of WBI on hippocampal neurogenesis was associated with an increase in the number of activated microglia, suggesting an inflammatory response in the brain following irradiation [80, 96, 98]. A role for the brain microenvironment in the ablation of neurogenesis was further supported by the demonstration that neural precursor cells isolated from nonirradiated brains failed to give rise to new neurons when transplanted into irradiated brains [96]. A negative correlation between activated microglia and hippocampal neurogenesis has been demonstrated, suggesting that the WBI-induced neuroinflammatory response could lead to the impaired neurogenesis [80, 96, 100]. Moreover, administration of the anti-inflammatory drug, indomethacin, decreased radiation-induced microglial activation and partially restored neurogenesis [81]. Together, these data suggest that altered neurogenesis as a result of oxidative stress and/or neuroinflammation is one of the mechanisms of radiation-induced brain injury. Thus, anti-inflammatory strategies might be useful in preventing radiation-induced late effects in the brain.

An additional and intriguing aspect of inflammation is the putative link between inflammation and impaired PPAR expression. Analysis of gene expression in postmortem brain tissue obtained from AD patients revealed significant decreases in PPAR $\alpha$  and PPAR $\delta$  gene expression, determined using real time quantitative PCR [101]. Preliminary studies from our own laboratory indicate that treating rat brain microvascular endothelial cells with ionizing radiation or hydrogen peroxide leads to a rapid and significant reduction in PPAR $\gamma$  mRNA and protein (*unpublished data*). Similar changes have been observed in vivo; one year after a fractionated dose of 40 Gy  $\gamma$ -rays, PPAR $\gamma$  gene expression was markedly lower than that observed in age-matched sham-irradiated rat brains. Although preliminary, these data confirm previous studies in which radiation has been shown

to reduce PPAR expression within hours to several days of treatment in the kidney [102] and the colon [103].

Given that several PPAR agonists are potent neuroprotective/anti-inflammatory agents in several neuroinflammatory disorders, we hypothesized that activation of PPARs will ameliorate the WBI-induced brain injury.

## 7. Effect of PPAR Agonists on Radiation-Induced Brain Injury

**7.1. In Vitro Studies.** A growing body of evidence suggests that the microglial proinflammatory response following radiation contributes to the observed radiation-induced late effects. In vitro studies suggest that irradiating microglia leads to a marked increase in expression of proinflammatory genes including TNF $\alpha$ , IL-1 $\beta$ , IL-6, and Cox-2 [104–106]. Radiation-induced expression of microglial TNF $\alpha$  and IL-1 $\beta$  has been shown to enhance leukocyte adhesion in the brain, partly via increased expression of ICAM-1 in astrocytes [104]. Cox-2-mediated production of prostaglandin E<sub>2</sub>, TNF $\alpha$ , and IL-1 $\beta$  from the conditioned media of irradiated BV-2 cells has been shown to induce astrogliosis [106]. These studies are supported by in vivo experiments in rodents which indicate that brain irradiation leads to a marked increase in microglial activation associated with both a concomitant decrease in neurogenesis in the hippocampus and spatial memory retention deficits as mentioned previously [97–99]. Thus, modulating the microglial proinflammatory response presents a promising approach to ameliorate radiation-induced brain injury.

Extending previous findings, we observed that irradiating BV-2 microglial cells led to a significant increase in TNF $\alpha$  and IL-1 $\beta$  gene expression and Cox-2 protein levels [107]. The promoter regions of TNF $\alpha$ , IL-1 $\beta$ , and Cox-2 contain numerous transcription factor binding sites including AP-1 and NF- $\kappa$ B and numerous reports suggest that their expression in the microglia is regulated by these transcription factors [108–112]. Consistent with this, marked increases in the DNA binding activity of AP-1 and NF- $\kappa$ B as early as 30-minutes postirradiation were observed in the microglial cells. Moreover, using specific inhibitors of AP-1 and NF- $\kappa$ B, it was demonstrated that the radiation-induced increase in TNF $\alpha$  and Cox-2 expression was AP-1 mediated while that of IL-1 $\beta$  was mediated by both NF- $\kappa$ B and AP-1.

Given the potent anti-inflammatory properties of PPAR $\alpha$  ligands in a variety of cell types including microglia [54, 55, 113], we hypothesized that activation of PPAR $\alpha$  in the microglia would inhibit the radiation-induced proinflammatory response. Indeed, the radiation-induced increases in TNF $\alpha$ , IL-1 $\beta$  gene expression, and Cox-2 protein were significantly inhibited by the PPAR $\alpha$  agonists, GW7647, and fenofibrate. Mechanistically, PPAR $\alpha$  agonists prevented the activation of AP-1 (by inhibiting nuclear c-jun phosphorylation) and NF- $\kappa$ B (by preventing p65 nuclear translocation) following irradiation thereby inhibiting the microglial proinflammatory response [107]. These findings emphasize the pleiotropic effects of PPAR $\alpha$  agonists in response to inflammation as they target multiple proinflammatory microglial

cytokines that might be involved in the development and progression of radiation-induced brain injury.

**7.2. In Vivo Studies.** The potent in vitro efficacy of PPAR $\alpha$  ligands in modulating the radiation-induced microglial proinflammatory response, along with the negative correlation between microglial activation and hippocampal neurogenesis, led to the hypothesis that activation of PPAR $\alpha$  in vivo would prevent the detrimental effect of WBI on neurogenesis and inhibit microglial activation (Ramanan et al. *unpublished data*). In this study, wild-type (WT) mice were divided into 4 groups: (1) Sham-irradiation and control diet, (2) Sham-irradiation and fenofibrate (Fen; 0.2% w/w), (3) WBI (delivered as a single dose of 10 Gy  $\gamma$ -rays with half the dose (5 Gy) delivered to each side of the head) and control diet, (4) WBI and fenofibrate. For measuring neurogenesis in the DG, all groups of mice received I.P injections of bromodeoxyuridine (BrdU; 50 mg/Kg body weight) to label the surviving neural precursor cells in the SGZ at 1 month post-WBI (as previously reported in [80, 96, 98]). The number of newborn neurons arising out of these surviving cells was assessed 2-month post-WBI by using double immunofluorescence to detect BrdU and NeuN (a neuronal marker). Consistent with previous findings [80, 96], WBI led to a significant decrease in the number of newborn neurons in the DG that was prevented in the irradiated mice that received fenofibrate in their diet. Furthermore, fenofibrate increased the total number of BrdU<sup>+</sup> cells in the DG of irradiated animals, suggesting that the PPAR $\alpha$  agonist promoted the survival of newborn cells following irradiation.

For the assessment of neuroinflammation, brains isolated either 1-week or 2-month post-WBI were subjected to staining with anti-CD68 antibody to label activated microglia. Consistent with our hypothesis, fenofibrate inhibited the WBI-induced increase in number of activated microglia at 1-week post-WBI. Therefore, the preservation of hippocampal neurogenesis by fenofibrate is associated with decreased microglial activation following WBI. Moreover, the number of activated microglia returned to control levels by 2-month post-WBI, the time point at which we observed a significant decrease in the number of newborn neurons. Thus, the radiation-induced neuroinflammatory response characterized by increased microglial activation might be an early event and could be one of the key components driving the detrimental effects of radiation on ongoing hippocampal neurogenesis.

Some studies have documented that fenofibrate can act independently of PPAR $\alpha$  [48, 114, 115]. To address this issue, the studies above were replicated in PPAR $\alpha$  knock-out (KO) mice. The genetic ablation of PPAR $\alpha$  prevented the protective effect of fenofibrate following WBI. These findings highlight the critical role played by PPAR $\alpha$  in modulating radiation-induced brain injury as well as providing mechanistic insight into the neuroprotective and anti-inflammatory properties of fenofibrate.

A striking difference was observed in the response of the microglial cells to WBI between the WT and PPAR $\alpha$  KO mice. Whereas the number of activated microglia returned

to control levels by 2 -month post-WBI in the WT mice, activated microglia remained significantly elevated in the KO mice. This suggests that the KO mice show a sustained neuroinflammatory response following WBI. Consistent with these data, preliminary findings from our laboratory suggest that the KO mice brains have a sustained increase in NF- $\kappa$ B DNA binding activity up to 24-hour post-WBI. In addition, the SGZs of these mice have a significantly lower level of basal proliferation compared to age-matched WT mice (*unpublished observations*). These findings are not surprising; PPAR $\alpha$  KO mice exhibit a prolonged response to inflammatory stimuli such as lipopolysaccharide and leukotriene B4 [116, 117]. In addition, they develop a physiologically aged phenotype earlier in life compared to the WT mice indicating a role for PPAR $\alpha$  in maintaining the cellular redox balance [118]. Thus, it is possible that the lack of PPAR $\alpha$  enhances the basal level of inflammation and thereby leads to a protracted response to radiation injury. Nevertheless, the experiments using the KO mice served as a reliable experimental control for the off-target effects of fenofibrate and underlined the importance of PPAR $\alpha$  in radiation-induced brain injury (Ramanan et al. *unpublished data*). Whether PPAR $\gamma$  and  $\delta$  ligands mediate similar protective effects on hippocampal neurogenesis following WBI is not yet known and is being actively investigated in our laboratory.

## 8. Effect of PPAR Agonists on WBI-Induced Cognitive Impairment

Functionally, radiation-induced brain injury is characterized by a progressive, cognitive impairment that severely compromises the quality of life (QOL) of cancer patients receiving radiotherapy. Given the increasing evidence for a role of oxidative stress/inflammation in radiation-induced brain injury, Zhao et al. tested the hypothesis that the PPAR $\gamma$  agonist pioglitazone (Pio) would ameliorate the severity of radiation-induced cognitive impairment in a well-characterized rat model of fractionated WBI. Young adult male F344 rats were divided into five experimental groups: (1) fractionated WBI; 40 or 45 Gy  $\gamma$  rays delivered as eight or nine 5 Gy fractions over 4 or 4.5 weeks, respectively and normal diet; (2) sham irradiation and normal diet; (3) WBI plus Pio (120 ppm) prior, during and for 4 or 54 weeks postirradiation; (4) sham irradiation and Pio diet; (5) WBI plus Pio starting 24 hours after completion of WBI. This study found that administering Pio prior to, during, and up to 4- or 54- weeks post-WBI significantly mitigated the WBI-induced cognitive impairment as measured by the object recognition test. However, the mechanism(s) involved in the radiation protection by PPAR $\gamma$  is not known at present.

With reference to PPAR $\alpha$ , although we demonstrated that fenofibrate prevented the detrimental effect of WBI on hippocampal neurogenesis and inhibited microglial activation, we were unable to use the mouse model to test whether it can inhibit radiation-induced cognitive impairment. These mice have a 129/sv background and perform poorly in cognitive function tasks due to defects in their corpus callosum [119]. Currently we are using the existing rat model to investigate whether fenofibrate mitigates radiation-induced cognitive

impairment. At present, the role of PPAR $\delta$  in radiation-induced brain injury is not known and is actively being investigated in our laboratory.

## 9. Putative Mechanism(s) of PPARs in Radiation-Induced Brain Injury

Both clinical and experimental evidence point out that the radiation-induced cognitive impairment can occur in the absence of gross histopathological and radiographical changes alterations [73–75]. These data suggest that more subtle cellular/molecular and functional changes (glial activation, neural precursor, endothelial and neuronal dysfunction) as result of increased inflammation/oxidative stress might play a role in the pathogenesis of radiation-induced late effects.

Based on our findings, we propose a model for the role of PPARs in the regulation of radiation-induced brain injury. Irradiating the brain leads to increased proinflammatory response as evidenced by (1) increased activity of NF- $\kappa$ B and AP-1 and (2) increased levels of TNF $\alpha$ , IL-1 $\beta$  and Cox-2. Microglia probably are the primary source of these mediators, although other cells likely contribute to the proinflammatory response [107, 120]. These cytokines might diffuse into the extracellular space and act on astrocytes, endothelial cells, neurons, and neighboring microglia, initiating a cytokine signaling cascade that alters the brain microenvironment (enhanced neuroinflammation, decreased hippocampal neurogenesis) and ultimately contributes to radiation-induced cognitive impairment (Figure 1).

While the exact role of these proinflammatory mediators in the pathogenesis of radiation-induced brain injury is currently under investigation, a clue to their function is suggested by studies with other brain injury models. Although required for the normal brain development, NF- $\kappa$ B and AP-1 have been shown to be dysregulated in a number of CNS disorders. An up-regulation of c-jun activity has been observed following neuronal injury [121]. In addition, mutating the activating phosphorylation sites on c-jun protects against neuronal apoptosis in the hippocampus [122]. Similarly, in an experimental spinal cord injury model, the p65 subunit of NF- $\kappa$ B was chronically activated in the microglia, endothelial cells and neurons adjacent to the lesion [123]. Increased levels of proinflammatory cytokines have been associated with a number of neuroinflammatory conditions such as Alzheimer's disease [124], Parkinson's disease [125], and multiple sclerosis [126]. Experimental augmentation of TNF $\alpha$ , IL-1 $\beta$ , and Cox-2 levels has been shown to induce behavioral and memory impairments in rodents [127–129]. TNF $\alpha$  and IL-1 $\beta$  have been shown to be potent inducers of apoptosis in oligodendrocytes and neural progenitor cells [130–132]. These data suggest that the elevated levels of proinflammatory mediators in the brain following irradiation could contribute to the pathogenesis of radiation-induced brain injury.

In a number of model systems, activation of PPARs has been shown to downregulate the expression of proinflammatory mediators, such as TNF $\alpha$ , IL-1 $\beta$ , Cox-2, and iNOS by

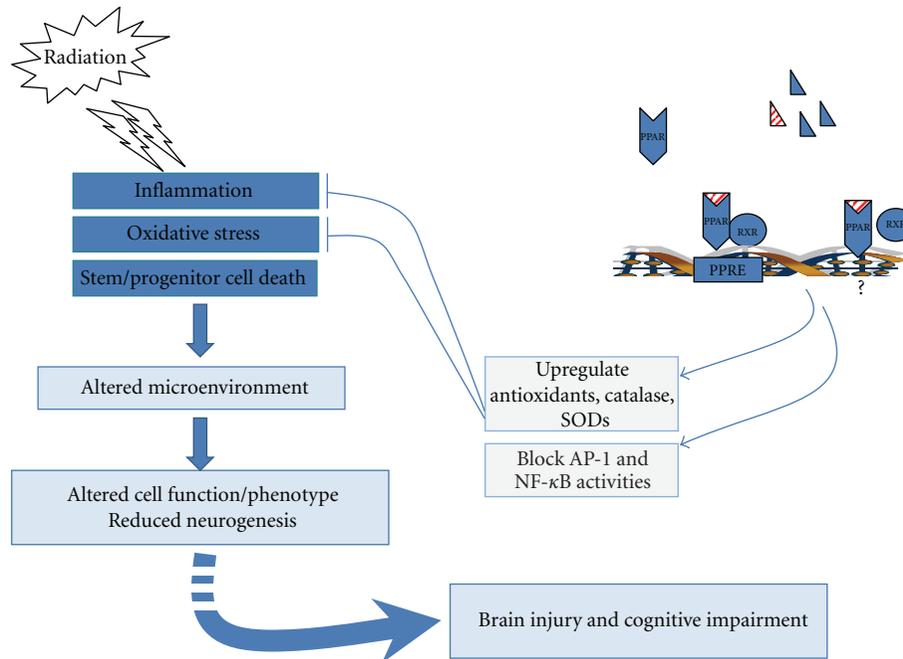


FIGURE 1: Model for the role of PPARs in radiation-induced brain injury. Irradiation is hypothesized to modify the brain microenvironment via the generation of an inflammatory and/or oxidative stress response which is also characterized by increased cell death of the neural precursor cells residing in the neurogenic regions of the brain. This alteration in the microenvironment is proposed to play a role in the dysfunction of the various cell-types in the brain (e.g., astrocytes, endothelial cells, microglia, neurons, and oligodendrocytes) and the reduction in ongoing adult neurogenesis ultimately contributing to radiation-induced brain injury including cognitive impairment. Activation of PPARs using specific ligands is hypothesized to play a role in normalizing the brain microenvironment and preserving cellular function following irradiation in part via inhibition of proinflammatory signaling pathways and by upregulation of antioxidant enzyme activities thus ameliorating the detrimental effects of radiation on the brain.

interfering with the activity of transcription factors NF- $\kappa$ B, AP-1, and STAT-1 (extensively reviewed in [2, 32]). Moreover, PPAR ligands have been shown to maintain the redox balance by upregulating the expression and activity of several antioxidant enzymes. A putative PPRE has been identified in the rat catalase promoter, the activity and expression of which was induced upon PPAR $\gamma$  ligand treatment [133]. PPAR $\alpha$  and  $\gamma$  ligands downregulated phorbol ester-induced expression of NAPDH oxidase subunits p22<sup>phox</sup> (message level) and p47<sup>phox</sup> (protein levels), which was accompanied by increased superoxide dismutase (SOD) activity [134, 135]. PPAR $\delta$  agonists have been shown to inhibit TNF $\alpha$ -induced ROS generation by upregulating expression of antioxidant enzymes catalase, SOD-1, and thioredoxin in human umbilical vein endothelial cells [136]. Moreover, as described previously, PPAR ligands can modulate the severity of several CNS disorders. These data, along with our findings, support the hypothesis that activation of PPARs, via anti-inflammatory and/or antioxidant mechanisms, normalizes the brain microenvironment (characterized by reduced glial activation and preserved hippocampal neurogenesis) following WBI contributing to the amelioration of cognitive impairment following irradiation.

From our model, it is important to appreciate that altered hippocampal neurogenesis and microglial activation following WBI account for only one aspect of the pathogenesis of radiation-induced brain injury. The radiation response of the

brain is complex and involves multiple pathways turned on in multiple cell types via autocrine, paracrine, and juxtacrine signaling mechanisms. Therefore, the relative contribution of other brain cells—astrocytes, endothelial cells, neurons and oligodendrocytes—to radiation injury cannot be excluded. In the future, investigating the radiation response of these cells and whether PPAR ligands can prevent their cellular dysfunction following irradiation will help shed more light on the mechanism(s) of radiation-induced brain injury and how it can be modulated.

## 10. Conclusions

Of the ~200 000 patients who receive WBI each year in the USA, up to 50% of them will develop progressive, cognitive impairment. There are no long-term treatment or prevention strategies for this debilitating side effect. Although the exact mechanisms remain ill-defined, increasing experimental evidence suggests a role for inflammation and/or oxidative stress during the pathogenesis of radiation-induced brain injury. PPAR ligands, given their propensity to target and modulate multiple proinflammatory pathways and their ability to upregulate antioxidant enzymes, appear to be an effective therapeutic strategy to modulate late effects following WBI. PPAR $\alpha$  ligands inhibit the radiation-induced proinflammatory responses in the microglia in vitro and prevent the detrimental effect of WBI on hippocampal

neurogenesis in vivo. Moreover, the PPAR $\gamma$  ligand Pio mitigated the WBI-induced cognitive impairment. Most importantly, PPAR $\alpha$  and  $\gamma$  ligands are FDA-approved and are routinely prescribed for the treatment of several chronic disorders such as hypertriglyceridemia, dyslipidemia, and Type 2 diabetes [17, 137, 138]. PPAR $\delta$  agonists are currently in Phase II clinical trials for dyslipidemia [139]. Therefore, these compounds offer the promise of enhancing the quality of life and long-term survival of cancer patients receiving brain irradiation.

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## Review Article

# PPARs in Irradiation-Induced Gastrointestinal Toxicity

**Christine Linard and Maâmar Souidi**

*Institute for Radioprotection and Nuclear Safety, B.P. no.17, 92262 Fontenay aux Roses Cedex, France*

Correspondence should be addressed to Christine Linard, christine.linard@irsn.fr

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The use of radiation therapy to treat cancer inevitably involves exposure of normal tissues. Although the benefits of this treatment are well established, many patients experience distressing complications due to injury to normal tissue. These side effects are related to inflammatory processes, and they decrease therapeutic benefit by increasing the overall treatment time. Emerging evidence indicates that PPARs and their ligands are important in the modulation of immune and inflammatory reactions. This paper discusses the effects of abdominal irradiation on PPARs, their role and functions in irradiation toxicity, and the possibility of using their ligands for radioprotection.

## 1. Introduction

Recent advances in radiotherapy delivery, such as the development of dose-sculpting techniques, have led to an overall reduction in normal tissue exposure during radiation therapy. Nevertheless, radiation toxicity to normal tissue remains the most important dose-limiting factor in radiotherapy and is a major obstacle to uncomplicated cancer cure. Gastrointestinal symptoms after pelvic radiotherapy affect the patient's quality of life, are substantially more common than generally recognized, and are frequently inadequately managed [1, 2]. They develop because radiation induces changes in one or more specific physiological functions in widely separated parts of the gastrointestinal tract that lie in the path of the radiotherapy beam.

Among the potential molecular targets for the treatment or even prevention of these disturbances are peroxisome proliferator-activated receptors (PPARs), a subclass of the nuclear hormone receptor superfamily. The two PPAR isotypes covered in this article have been identified in vertebrates: PPAR- $\alpha$ , and PPAR- $\gamma$ . The PPAR- $\alpha$  isoform is abundantly expressed in the liver and is expressed as well in the kidney, testes, heart, small intestines, pancreas and smooth muscle. It is also detectable in the lungs, placenta, and adipose tissue. PPAR- $\gamma$  is specifically expressed at high levels in the adipose tissue but is also detected in the

intestines, particularly the colon [3]. Relatively low PPAR mRNA levels are observed in the skeletal muscle, liver, and bone marrow stromal cells. In view of its expression and involvement in immune response and cell proliferation, PPAR- $\gamma$  has become an especially important research topic in gastroenterology, particularly in two important disorders— inflammatory bowel diseases (IBDs) and colon cancer. At this time, relatively little is known about the potential protective and healing characteristics of PPAR- $\gamma$  in radiotherapy-induced bowel damage.

## 2. Molecular Mechanisms of PPAR Activity

*2.1. Mechanism of Transcriptional Transactivation.* The variety of their functions makes it difficult to define the molecular mechanisms of PPAR activity. PPAR- $\gamma$  acts by blocking gene transcription by transrepression, a feature of transcriptional crosstalk between nuclear receptors (NRs) and signaling cascades that modulates inflammation and immunity in a variety of cells, including the epithelial cells of the gut. Several models have been suggested to describe transrepression by PPAR- $\gamma$  interacting with the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B). NF- $\kappa$ B plays an important role in the regulation of immune and inflammatory responses, for it induces the expression of diverse target genes that promote cell proliferation, regulate apoptosis, facilitate

angiogenesis and, in carcinogenesis, stimulate invasion and metastasis [4].

In one coactivator competition model, NF- $\kappa$ B and PPARs use an overlapping set of coactivator proteins [5] and compete with each other to bind to these coactivators. For example, under steady-state conditions, some genes (e.g., iNOS) are occupied and actively repressed by the nuclear receptor corepressor (NCoR) that serves simultaneously to repress PPAR-mediated transcription. The switch from repression to activation requires a reduction in affinity for the corepressor. In addition to the conformational change in the ligand binding domain, ligand binding removes NCoR complexes from promoters of nuclear receptor target genes and thus increases affinity for coactivators [6]. The subsequent finding that transrepression still occurs in the presence of excess coactivators [7] raises serious questions about this model, however.

A second proposed model involves direct interactions between nuclear receptors and negatively regulated transcription factors, interactions that result in the inhibition of the DNA-binding or transactivating activity of one or both factors [5, 8]. In endothelial cell lines, for example, PPAR- $\alpha$  activation inhibits the inflammatory response by direct protein-protein interaction with NF- $\kappa$ Bp65 [9]. Similarly, PPAR- $\gamma$  inhibits the production of cytokines in LPS-stimulated macrophages by direct interaction with NF- $\kappa$ Bp65/p50 [10]. In smooth muscle cells and hepatocytes, PPAR ligands induce the expression of I $\kappa$ B $\alpha$ , which leads to retention of the NF- $\kappa$ B subunits in the cytoplasm and the consequent suppression of their DNA binding activity [11]. It is worth pointing out that PPAR- $\gamma$  ligands, besides promoting PPAR- $\gamma$  interaction with NF- $\kappa$ B subunits, may also act independently of PPAR- $\gamma$ . For example, the PPAR- $\gamma$  ligand 15d-PGJ<sub>2</sub> inhibits the secretion of TNF- $\alpha$  and IL-6 in macrophages stimulated by LPS and directly blocks activity of the I $\kappa$ B kinase complex, completely independently of PPAR- $\gamma$  [12].

More recent studies have produced another model involving a corepressor-dependent mechanism. Ligand-binding of PPAR- $\gamma$  induces the SUMOylation of a fraction of some PPAR- $\gamma$  molecules, which bind to NCoR and prevent its clearance from the promoter. This leads to a sustained repressed state. Using yeast two-hybrid screen assays, Pascual et al. [13] showed that PPAR- $\gamma$  interacts with the protein inhibitor of the activated transcription factor, STAT-1 (PIAS1). The physiological role of PIAS1 is to facilitate the localization of PPAR- $\gamma$  to the NCoR complexes on the promoter of inflammatory genes, including iNOS, in the presence of PPAR- $\gamma$  ligands. Consequently, NF- $\kappa$ B-mediated inflammatory gene expression is downregulated [14].

**2.2. Natural and Synthetic Ligands.** PPAR- $\gamma$  receptors are activated by several natural lipophilic ligands, including long-chain polyunsaturated fatty acids, arachidonic acid metabolites derived from the cyclooxygenase, and lipoxygenase pathways (such as 15-deoxy-d-12,14-prostaglandin J<sub>2</sub> (15PG-J<sub>2</sub>) and 15-HETE) and fatty acid-derived components of oxidized low density lipoproteins (OxLDLs)

(such as 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) [15]. The antidiabetic class of thiazolidinedione drugs includes troglitazone, rosiglitazone, pioglitazone and ciglitazone, all synthetic ligands of PPAR- $\gamma$ . Other synthetic compounds that can function as ligands include certain nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, ibuprofen, flufenamic acid, and fenoprofen [16]. Saturated and unsaturated fatty acids are the primary natural PPAR- $\alpha$  ligands, and eicosanoids and leukotriene  $\beta$ 4 are among the higher affinity endogenous PPAR- $\alpha$  ligands. The best-studied synthetic PPAR- $\alpha$  ligands are the fibrate class of hypolipidemic drugs (bezafibrate, clofibrate, and fenofibrate), which have been used therapeutically in humans for many years [16].

### 3. Pathophysiology of Normal Intestinal Tissue after Pelvic Irradiation

Any clinician involved in the treatment of pelvic malignancies with radiation is aware of the danger of colorectal injury. Although the severe effects were described long ago, consideration of lesser degrees of colorectal injury is much more recent [1, 2]. It remains difficult to define exactly when symptoms start to affect quality of life and why some people seek help for specific gastrointestinal symptoms while others do not. The pathological processes of radiation injury begin immediately after radiation exposure, but its clinical and histological features may not become apparent for weeks, months, or even years afterwards. Radiation injury is commonly classified as acute, consequential, or late effects, according to the time of appearance of symptoms. Acute (early) effects are observed during the second week of treatment (when histological changes probably reach their zenith) and peak by the fourth to fifth weeks (when the histological changes have stabilized or are improving). Some randomized studies show that the prevalence of mild acute intestinal effects ranges from 6 to 37% and severe effects (resulting in treatment interruption) from 0 to 10% [17]. Consequential effects appear later and are caused by persistent acute damage [18]. They tend to peak at 18 months [19]. These symptoms concern fewer patients (half of those with late effects) but are clinically important because of their chronic progressive nature and their significant long-term morbidity. Late effects emerge months to years after radiation exposure. Prevalence estimates of moderate and severe late bowel effects vary from 5% to 30% [20, 21] and affect patients' quality of life. Indeed 2.5% of all pelvic radiotherapy patients require surgery as a result of its complications.

Among the most common side effects of pelvic radiotherapy are acute radiation-induced proctosigmoiditis, enteritis or colitis, depending on the symptoms and localization. They result from delivering radiation to the distal colon and rectum during radiotherapy that touches other pelvic structures. The acute symptoms are pain and diarrhea or constipation due to the loss of epithelial integrity and increased mucus secretion. While the specific causes of

constipation have not been investigated, the many causes of diarrhea include accelerated small and large bowel transit, bacterial overgrowth, and malabsorption of bile salts. Consequently, the tissue develops edema, and hyperemia.

The most common late effects include increased stool frequency and urgency, spotting of blood, and partial incontinence. Less common are ulceration, severe bleeding, pain, stricture, severe incontinence, and fistula [17]. Ulceration results from the tissue necrosis induced by the release of radical species, persistence of the inflammatory process, and the local ischemia associated with the vascular damage. Severe complications, such as bleeding, fistulas, and obstruction, may require surgical intervention. Ischemia and fibrosis in the submucosa and muscularis layer are mainly responsible for these effects, which are accompanied by vascular sclerosis, collagen deposition, and abnormal fibroblast activity. Compromised vascularization of fibrotic tissues [22] contributes to an adverse postoperative outcome, and symptoms of this radiation enteropathy frequently persist after surgery. Proctitis alone is estimated to account for more than 75% of all gastrointestinal radiation injuries [23] and usually begins in the second or third week of radiotherapy. Acute radiation-induced proctosigmoiditis is quite similar to distal ulcerative colitis, both in its symptoms and its acute histopathologic effects. Studies of patients receiving bladder and prostate radiotherapy demonstrate continued active inflammation and repair processes at 3 months after treatment [24]. The acute and chronic effects of radiation to the rectum appear to be correlated [25–27].

#### 4. PPARs and Control of Gastrointestinal Inflammation

**4.1. Inflammatory Response.** Inflammation is a major component of early healing, and its control is essential for efficient repair. It plays a causative role in radiation-induced toxicity. The first observation of this inflammatory process showed that levels of eicosanoids, leukotriene B<sub>4</sub>, thromboxane B<sub>2</sub>, and prostaglandin E<sub>2</sub> all rise markedly in the rectum in response to pelvic irradiation and fall after radiotherapy is complete [28]. Irradiation activates various cell signaling pathways that lead to expression and activation of proinflammatory and profibrotic cytokines [29–32], to vascular injury [33] and to activation of the coagulation cascade [34, 35].

Intestinal homeostasis is generally maintained through balanced immune and inflammatory responses, epithelial integrity, and adequate lymphoepithelial interactions. Su et al. [36] did essential work on the role of PPAR- $\gamma$  in intestinal inflammation, developing the first model of colitis induced by oral administration of dextran sodium sulphate [36, 37] and showing the response of inflammation to treatment by PPAR- $\gamma$  ligands. Another model of colitis, induced by intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS), showed that treatment with troglitazone attenuates colitis [38]. Because it has been reproduced by activation of RXR with specific rexinoids, this beneficial effect has been directly attributed to the RXR/PPAR- $\gamma$  heterodimer

[38]. Further evidence in support of the involvement of this heterodimer comes from the enhanced susceptibility of PPAR- $\gamma^{+/-}$  and RXR $^{+/-}$  mice to TNBS-induced colon inflammation [38]. The high expression of PPAR- $\gamma$  in epithelial cells suggests that these cells are the main target of the RXR/PPAR- $\gamma$  activators, and this hypothesis is reinforced by the persistence of inflammation in deeper colonic layers in animals treated with PPAR- $\gamma$  or RXR agonists [38].

The use of exogenously administered PPAR- $\gamma$  ligands alone in this study left several questions open, especially since only disease-activity measurements were considered, rather than other objective colitis indicators. Can the anti-inflammatory action of PPAR- $\gamma$  activation be proved by other objective indicators of colitis-associated inflammation? Does endogenous PPAR- $\gamma$  play an anti-inflammatory role in physiological conditions? Are the levels of PPAR- $\gamma$  protein lower in IBD patients than those in healthy controls and does that make these patients more susceptible to chronic inflammation? Nakajima et al. answered these questions, showing not only that PPAR- $\gamma$  ligands inhibit inflammation, but also that a PPAR- $\gamma$  deficiency is associated with aggravated injury [39]. This report showed clearly that PPAR- $\gamma$  functions as an endogenous anti-inflammatory substance in the intestines and thus that a decrease in PPAR- $\gamma$  levels may exacerbate inflammation. IBD patients may have low levels of PPAR- $\gamma$  in their intestinal mucosa, potentially predisposing them to unrestrained inflammation, as observed in PPAR- $\gamma$ -deficient mice. Human studies are rare but appear to indicate that patients with ulcerative colitis, but not those with Crohn's disease, have reduced levels of PPAR- $\gamma$  protein in their colonic epithelial lining [40]. This discrepancy between the two diseases has not yet been explained.

Study of the role of PPARs in irradiation-induced inflammatory process began only recently. Experiments in our laboratory have measured the level of colonic PPARs after abdominal irradiation. As in patients with ulcerative colitis, PPAR- $\alpha$ , - $\gamma$ , and heterodimer RXR $\alpha$  expression (of both genes and proteins) fell drastically three days after a single 10-Gy abdominal irradiation, and acute intestinal injury was observed [41]. Similarly Zhao et al. showed that whole-body irradiation downregulated PPAR- $\alpha$  and - $\gamma$  protein levels in the mouse kidney [42]. The PPARs, notably PPAR- $\alpha$  and RXR- $\alpha$ , have been shown to be highly radiosensitive, with PPAR repression observed from the first doses during a fractionated colorectal irradiation (4 Gy/fraction, 3 fractions/week and total dose 52 Gy) that mimicked a radiotherapy protocol in a rat model (unpublished results).

In vivo, irradiation produces an acute and a chronic increase in ROS generation that leads to persistent chronic oxidative stress [43]. PPAR expression is sensitive to oxidative stress and to inflammatory processes, as shown by the decreased PPAR- $\alpha$  expression induced by LPS or proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [44]. In the acute phase of irradiation, the downregulation of PPARs may be a mechanism that contributes to the development or exacerbation of inflammation. Because PPAR- $\gamma$ /RXR $\alpha$  is highly expressed in epithelial cells and given that irradiation may erode the epithelial layer, it was thought that PPAR downregulation might well be correlated

with direct irradiation of the epithelium. But at the doses delivered and during the experimental period, no epithelial erosion was observed [41]. Currently, we have no explanation for the impairment of PPAR expression that is induced by irradiation. The direct impact of this impairment on the severity of radiation enteritis remains to be determined. Zhao et al. showed that the reduction of PPAR levels aggravates radiation nephropathy [42]. Their study shows that radiation-induced apoptosis is inhibited in PPAR- $\alpha$  knockout mice by the enhanced activation of NF- $\kappa$ B and the increased expression of pro-survival factors. The activation of apoptosis signaling pathways prevents the DNA damage that increases the risk of genomic instability, mutations, and long-term carcinogenesis. The absence of this response in PPAR knockout mice might thus increase carcinogenesis in these mice.

Many molecular mechanisms and cellular targets play a role in controlling colonic inflammation by PPAR expression and activation in epithelial and various types of immune cells, especially macrophages. Activation of PPAR- $\gamma$  in the colon inhibits mucosal production of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) by downregulating the NF- $\kappa$ B and MAP kinase signaling pathways [38]. Studies with cell culture models have established that PPAR- $\gamma$  agonists such as thiazolidinedione can reduce NF- $\kappa$ B activation and inflammatory gene expression in colonic epithelial cells [45], macrophages [46–48], dendritic cells [49], and T cells [50, 51]. Although the efficacy of thiazolidinedione depends on numerous factors including the cell model, concentration, duration, type of thiazolidinedione (rosiglitazone, pioglitazone) used, and inflammatory model, it is nonetheless generally accepted that these drugs can reduce inflammatory gene expression.

In our laboratory we tested the strength of the reduction of the inflammatory process induced by GW1929, a nonthiazolidinedione that has been identified as a high-affinity ligand for human PPAR- $\gamma$  and is reported to be more potent than troglitazone [52]. Surprisingly, the treatment exacerbated the irradiation-induced inflammatory process, causing drastic weight loss and more severe mucosal inflammation (unpublished results). These findings suggest that it is absolutely necessary to analyze inflammatory and immune status before treatment. No treatment is universal for every inflammatory disease of the colon. Questions remain about the role of inflammatory (macrophages, T cells) and epithelial cells in PPAR response in other models of acute and chronic enteritis, such as radiotherapy-induced inflammatory intestinal response. The diversity of colitis models may be helpful in demonstrating the importance of targeting the specific ligand-activated PPAR.

**4.2. PPARs and Macrophages.** It is well established that each PPAR isoform is expressed in monocyte/macrophage lineages and influences their phenotypes [46]. In monocytes/macrophages, PPAR- $\gamma$  activation inhibits the expression of inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Similarly, in macrophages, treatment by PPAR- $\gamma$  ligands induces a resting phenotype and suppresses iNOS [48].

These observations indicate that PPAR- $\gamma$  might be a target for anti-inflammatory therapy. More recently, studies with mutations that disrupt one of the PPAR genes confirm the *in vivo* anti-inflammatory effects of PPARs. Thus, Chawla et al. [53] reported that both 15d-PGJ<sub>2</sub> and troglitazone exert anti-inflammatory effects in macrophages derived from mice homozygous for a null mutation in the PPAR- $\gamma$  gene and therefore appear to work by a PPAR- $\gamma$ -independent mechanism. In their study of PPAR- $\gamma$ -deficient macrophages, Crosby et al. [54] showed that PPAR- $\gamma$  is not necessary for the successful inhibition of inflammatory mediators, in particular, iNOS, by synthetic agonists.

Recently, we showed that 5-aminosalicylic acid (5-ASA) reduces the overexpression of TNF- $\alpha$ , MCP1, and iNOS that is induced during the acute phase of irradiation. These effects could be seen by the reduction of macrophage infiltration in the mucosa [41]. It thus appears to us that the role of PPAR- $\gamma$  may be overestimated in these irradiation protocols, since the ligands are used at concentration exceeding those required to bind PPAR- $\gamma$  [53]. 5-ASA acts on inflammatory processes even at the time when irradiation substantially impairs PPAR/RXR $\alpha$  expression. These observations show that the anti-inflammatory effects of PPAR- $\gamma$  ligands in inflammatory cells may be independent of their ability to activate PPAR- $\gamma$  and may involve instead mechanisms such as interference with early signals of the transduction cascade that block NF- $\kappa$ B transcription (see Section 4.4).

In the past several years, studies of macrophages have focused on their role as key cells of innate immune system and their fundamental activities in killing and removing foreign organisms and cell debris. Response to macrophage stimulation is usually seen as proinflammatory and proimmunogenic. More recently, different forms of macrophage activation have been proposed with noninflammatory and even anti-inflammatory consequences. A state of macrophage activation, in response to infectious agents, particularly Mycobacteria, involves IFN $\gamma$  and other cytokines, in conjunction with stimulation by LPS or other Toll-like receptor (TLR) ligands. These so-called classically activated or M1 macrophages have upregulated iNOS and COX2. These cells play an important role in protection against intracellular pathogens and cancer cells.

More recently, research has focused on the activation of macrophages that produce inflammatory mediators. General suppression is achieved after stimulation with IL-10, but exposure to IL-4 or related cytokines initiates a so-called “alternatively activated” or M2 macrophage [55]. Its capacity for an oxidative burst, that is, to produce inflammatory mediators, such as iNOS, in response to LPS, is reduced. These cells have distinct functional properties, and are integrated in polarized Th2 responses, tissue remodeling, and repair [55].

Although sufficient data are not yet available, macrophage polarization is an interesting vantage point for studying the effects of irradiation and their long-term consequences. In a Danish clinical study during radiotherapy of the pelvic-abdominal area, immunohistochemical staining of the CD68 antigen showed increased density of macrophages at 2 weeks (during the radiotherapy), but

no further change at 6 weeks (the end of the protocol). The stained macrophages were localized mainly beneath the epithelium in close proximity and intermingled with other inflammatory cells in the lamina propria [56]. In our laboratory fractionated irradiation of an animal colorectal model confirmed and extended these observations; macrophage immunostaining increased over the period of the protocol, while 6 months after the protocol intended, staining was diminished compared with the control animals [57].

In vivo, irradiation modulates the functional abilities of some cells, in particular, NO production by activated macrophages in an arthritis model [58]. Activation of macrophages to produce iNOS depends on the dose delivered; a dose-dependent modulation of the NO pathway was observed with radiation doses used in anti-inflammatory radiotherapy and with superstimulation by the high radiation doses used for cancer treatment. PPAR- $\gamma$  expression is not necessary for some macrophage functions, including activation and differentiation of this cell lineage. PPAR- $\alpha$  is thought to be involved in NO pathways, including in the inhibition of iNOS expression by murine macrophages [59]. Inversely, PPAR downregulation is associated with the overexpression of macrophage-related inflammatory mediators, including iNOS, TNF- $\alpha$ , and MCP1, and with macrophage infiltration [41] during the acute phase of irradiation. Interestingly, 6 months after the end of the radiotherapy protocol in the rodent, the PPAR level returned to normal but iNOS expression was cut in half. A potential explanation of this finding is that the modification of macrophage polarization is a long-term effect induced by irradiation. One study shows that peritoneal resident macrophages induced by the typical M2 inducers, IL-4, strong inducers PPAR- $\gamma$  (gene and protein) and thus suggests that high PPAR- $\gamma$  activity is preferentially associated with M2 [60]. Overall, it is clear that the regulation of PPARs by pro- or anti-inflammatory signals is an important factor that triggers macrophage polarization. The functional activity of irradiation-induced macrophages and their involvement in long-term effects (fibrosis, lack of response to infection) must be considered in this light.

#### 4.3. Immune Response to Irradiation: PPAR Involvement.

Cytokines are especially important for regulating immune and inflammatory responses with pro- and anti-inflammatory functions, and perform crucial functions in controlling both the innate and adaptive immune response. Not only do cytokines govern the development and homeostasis of lymphocytes, but they also direct the differentiation of helper T cells and promote the generation of memory cells [61]. Immune-mediated damage to intestinal mucosal surfaces can be triggered by both polarized Th1 and Th2 effector CD4<sup>+</sup> T cells, and it can be improved or prevented. Three functional categories of terminally differentiated immune cells such as T helper (Th) cells have been characterized on the basis of their cytokine production and homing capacity: Th1 (IFN- $\gamma$ , TNF- $\alpha$ , IL-12), Th2 (IL-4, IL13), and regulatory Th cells (Treg) that produce IL-10 able to inactivate Th1 and Th2. Th1 cells are mainly involved in cell-mediated immune responses; whereas Th2 cells participate in

humoral responses and promote growth and differentiation of mast cells and eosinophils. Because the Th1 and Th2 subpopulations tend to function antagonistically towards one another, the persistence of disease susceptibility and resistance depends on the cytokine profile secreted by each type. One molecular mechanism for cell polarization may be the regulation of cytokine genes by transcription factors, although the expression of these genes, such as that for IFN- $\gamma$ , can also be regulated at posttranscriptional stages. Induction of T-bet transcription factor expression in primary T cells is strongly correlated with Th1 lineage differentiation and IFN- $\gamma$  expression. In Th2 lineage differentiation and expression of Th2 cytokines (IL-4, IL-5, and IL-13), on the other hand, the GATA3 transcription factor plays a role analogous to that of T-bet in Type 1 lineage differentiation and IFN- $\gamma$  expression. GATA3 expression polarizes T cells towards a Th2 lineage and represses IFN- $\gamma$  expression. It has been suggested that the balance between T-bet and GATA3 expression is crucial for Th1/Th2 T-cell differentiation [62]. Recent reports show that ionizing radiation induces the preferential differentiation of Th cells into Th2 cells in the spleen [63, 64] and more recently, in the intestines [65] where this Th2 dominance is characterized by repression of Th1-specific sets (IFN- $\gamma$ , T-Bet). Indeed, irradiation initiates and maintains a Th2-like immune response over the long term [57]. Th2 cells play a critical role in the pathogenesis of radiation-induced pneumonitis, which precedes lung fibrosis [66]. PPAR- $\gamma$  involvement has previously been described in the regulation of innate immune response [48], and more recently its role has been investigated in adaptive immunity [67, 68]. Although PPAR- $\gamma$  is expressed on dendritic, T and B cells, little is known about their specific expression of PPARs, notably in colitis. PPAR- $\gamma$  ligands have a negative effect on the APC functions of dendritic cells [69]. Remarkably, adoptive transfer of antigen-presenting dendritic cells pretreated with PPAR- $\gamma$  resulted in CD4<sup>+</sup> T cell anergy [70], characterized by impaired differentiation that led to the absence of both Th1 and Th2 cytokine production.

PPAR- $\alpha$  and PPAR- $\gamma$  are differentially expressed after lymphocyte activation. PPAR- $\alpha$  expression was downregulated after T cell activation while PPAR- $\gamma$  expression increased under the same activating conditions. PPAR ligands inhibit the proliferation of T lymphocytes as well as of B cells [71] and repress the production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 [67]. Saubermann et al. [72] showed that the reduction in inflammation induced by dextran sodium sulphate, which has been noted with PPAR- $\gamma$  ligand treatment, is associated with decreased IFN- $\gamma$  and TNF- $\alpha$  and increased IL-4 and IL-10 expression. Consistent with this shift towards Th2 cytokine dominance, treatment by PPAR- $\gamma$  ligands (troglitazone, pioglitazone, and rosiglitazone) increases the expression of the specific Th2 transcription factor, GATA-3. On the other hand, Jones et al. showed that CD4<sup>+</sup> T cells lacking PPAR- $\alpha$  produce high levels of IFN- $\gamma$ . These results indicate that the protective effects exhibited by PPAR- $\gamma$  ligands in intestinal inflammation may be due to immune deviation away from Th1 and towards Th2 cytokine production. Surprisingly, by using another PPAR- $\gamma$  ligand, 5-aminosalicylate, we restored the PPAR/RXR- $\alpha$  level and

normalized the downregulation of the irradiation-induced IFN- $\gamma$ /STAT1 pathway [57]. These results are inconsistent with reports that PPARs negatively regulate IFN- $\gamma$  and presents an essential question: how may PPAR ligands modulate the overexpression of IFN- $\gamma$  in a Th1 model of colitis and upregulate IFN- $\gamma$  in Th2 colitis?

The twofold immunological effect of PPAR ligands has been observed in two different acute lung inflammatory models—carrageenan-induced pleurisy [73] and bleomycin-induced lung injury [74]. In both, PPAR- $\gamma$  ligands reduced inflammatory cell infiltration (by neutrophils and possibly other leukocytes). Others have hypothesized that PPAR- $\gamma$  ligands act by a direct effect on the migration of inflammatory cells in response to endogenous chemoattractant [75]. Other studies show that PPAR- $\gamma$  ligands may modulate leukocyte-endothelial interactions during inflammation through the regulation of endothelial adhesion molecules [74]. Considerable evidence suggests that PPAR activation may limit the endothelial responses that promote T cell adhesion and entry into the vessel wall.

PPAR ligands may act during several different stages of acute irradiation effects. Firstly, PPARs may inhibit Th2 trafficking into the intestinal mucosa by interfering with the endothelial adhesion molecules. Secondly, in vivo irradiation leads to acute and chronic increases in ROS generation and thus to persistent oxidative stress [43]. The free radicals produced by irradiation cause vital cell damage that results in the apoptosis of cells in the first division or within the first few divisions. Some infiltrating cells, especially macrophages, serve as the major source of inflammatory molecules and can increase cell damage. Because Th1 cells are highly sensitive to apoptosis [76], modification of their oxidative status by PPARs may help to normalize the Th1/Th2 balance. Thirdly, we can rule out any direct action by PPAR ligands on transcription factors. Although no synthetic inhibitors of GATA-3 currently exist, PPAR- $\gamma$  agonists have been shown to inhibit GATA-3 expression and Th2-driven inflammatory responses in murine models [77]. The yin-yang cytokine balance of the Th1 and Th2 cytokines is a common feature of inflammatory response and is thought to reflect the operation of a feedback control mechanism. The effects of PPARs on T lymphocytes are thus complex and require further study, especially in the field of radiopathology. Possible explanations for these observed differences include PPAR-independent effects of specific ligands or differences in the model system used.

*4.4. Possible Mechanisms: Crosstalk with Signal Transduction Pathways.* The mechanisms that perpetuate responses after irradiation remain a mystery. No evidence of the expression of immune cytokines (e.g., IL-2, IL-4, and IL-12) has been found in irradiated mouse brains [78], but immune mechanisms may have more importance in other tissues, such as the lung and the intestine [65, 79]. Mitotic death of specific cell populations responding to cell loss may result in lethal damage at different times after irradiation and “waves” of responses. Other possible mechanisms include dysregulated intercellular signaling due to the loss of specific cell

populations, the disruption of negative regulatory cytokine pathways [80], or the development of hypoxia [81].

It is thought that PPAR- $\alpha$  and PPAR- $\gamma$  repress inflammation by physical interaction and form inactive complexes with the proinflammatory transcription factors NF- $\kappa$ B and Activating Protein1 (AP1) [82]. These transcription factors normally induce the transcription of proinflammatory genes, such as cytokines (IL-6, TNF- $\alpha$ ), iNOS [83] and COX-2 [84], chemokines (IL-8, MCP-1), and cellular adhesion molecules (VCAM1, ICAM1). It has also been suggested that PPAR- $\gamma$  can interact with two transcription factors, signal transducer and activator of transcription (STAT) and nuclear factor of activated T-cells (NFAT), and thereby reduce the transcription of proinflammatory genes [82]. Another way in which PPAR- $\alpha$  and PPAR- $\gamma$  control inflammation is more indirect, by influencing the transcription of genes that inhibit NF $\kappa$ B signaling, such as I $\kappa$ B $\alpha$  and IKK. For example, ligand-activated PPAR- $\alpha$  can induce expression of I $\kappa$ B $\alpha$  [9]. By linking to NF $\kappa$ B subunits, it prevents the translocation of NF $\kappa$ B to the cell nucleus and the subsequent transcription of proinflammatory genes. The induction of I $\kappa$ B $\alpha$  via PPAR- $\alpha$  is therefore positively correlated with decreased inflammation and thus helpful in preventing or countering low-level chronic inflammation. In the acute irradiation intestinal model, the PPAR ligand 5-ASA also acted by overexpressing I $\kappa$ B $\alpha$  and thus reducing nuclear translocation and activation [41].

Several reports indicate that activated PPAR- $\gamma$  crosstalks with cytokine-mediated signal transduction pathways in the modulation of immune response. Several lines of evidence indicate that STAT signaling may be involved in the anti-inflammatory action of PPAR ligands [85]. Wang et al. explored the interactions between PPAR- $\gamma$  and STAT3 in detail [86]. Two distinct PPAR- $\gamma$  ligands suppress IL-6 activated STAT3 through various types of crosstalk including direct or mediated by the corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptor). The exact mechanism through which PPAR- $\gamma$  represses STAT3 is not yet fully elucidated. Most studies of multiple myeloma cells show that the inhibitory effects of the PPAR- $\gamma$  ligand on STAT3 activity depend directly on the expression and activation of PPAR- $\gamma$  [86]. A direct physical protein-protein interaction occurs between nuclear receptor PPAR- $\gamma$  and activated transcription factor STAT3. Moreover, the enhancement of this interaction by 15-d-PGJ2, but not troglitazone, suggests that these two classes of PPAR- $\gamma$  ligands inactivate STAT3 through different mechanisms.

Interestingly in the intestinal irradiation model with PPAR- $\alpha$  and PPAR- $\gamma$  repressed (gene and protein levels), STAT3 repression was also observed, again of both gene and protein levels [41]. By normalizing PPAR expression, 5-ASA elevated STAT3 protein levels (nucleus and cytosol). In addition the direct effect of PPAR- $\gamma$  on STAT3 repression, an alternative mechanism for PPAR ligand-mediated STAT3 expression, has been suggested, in which the PPAR ligand acts indirectly by stabilizing inflammatory transcription factor suppressor molecules [87].

Overwhelming evidence shows that STAT3 is the only obligate factor required for IL-10-mediated anti-inflammatory signaling [88]. In the mouse, thiazolidinedione treatment of acute colitis is accompanied by a marked increase in IL-10 expression [71], especially in mature dendritic cells and in activated CD4<sup>+</sup> T cells; this increase depends on both dose and PPAR- $\gamma$  [49] when the presence of a functional PPAR response element (PPRE) in the IL-10 promoter region has been demonstrated. As in colitis, intestinal irradiation represses IL-10 [89] and all strategies that interfere with irradiation-induced NF- $\kappa$ B activation restore the IL-10 level, including 5-ASA treatment (unpublished results).

The obligate role of STAT3 in IL-10 signaling raises the issue of pathway redundancy and specificity, for many receptors use STAT3. For example, IL-6 signaling also activates the STAT3 pathway but is incapable of activating the anti-inflammatory response. One explanation involves the effects of suppressor of cytokine signaling 3 (SOCS3) on the IL-6 receptor. SOCS3 plays an essential role as a negative inhibitor of IL-6 by interfering with STAT3 [90]. Overexpression of SOCS3 is observed immediately (3 hours) after intestinal irradiation [89] and the SOCS3 level remains high for a long time after irradiation ends (6 months) [57]. In our laboratory, we showed that interference with the NF- $\kappa$ B pathway—either direct, by a specific inhibitor (CAPE), or indirect, by PPAR activation (5-ASA)—normalizes the SOCS3 level and in a roundabout way re-establishes the STAT3 level [41, 65]. STAT3 phosphorylation continues for a prolonged period in the absence of SOCS3 [90]. PPAR ligands can activate anti-inflammatory pathways both dependently and independently of IL-10, under the control of the negative regulatory influence of SOCS3. The multiplicity of crosstalk between nuclear receptors and transcriptional factors is an important factor that contributes to signal diversification and specification.

## 5. Radiotoxicity Prevention and Treatment

The improvement in survival after the development of new schedules and techniques for delivering radiotherapy underlines the importance of addressing the problems faced by long-term survivors. Priority must be given to assessing simple methods of preventing bowel toxicity in the first place, without compromising either tumor control or prevention of a secondary cancer. An earlier review of prophylactic treatments for radiation toxicity failed to find that any current regimen was effective [91].

In the late 1970s, studies showed that 5-aminosalicylate is the active moiety of sulfasalazine in patients with ulcerative colitis and Crohn's disease. Since then, this agent, its efficacy, drug profile, precise indications, and adverse events have remained topics of unceasing discussion [92]. At the same time, controversy arose about the prevention of radiation-induced bowel toxicity. A small double-blinded, balanced and randomised trial study showed that acetylsalicylate was effective against the side effects of uterine radiotherapy; it reduced the number of bowel motions and relieved abdominal pain [93].

More recently, Jahraus et al. conducted a double-blinded, randomized, placebo-controlled trial of balsalazide and observed a marked reduction in the classic symptoms of acute radiation-induced proctosigmoiditis [94]. Balsalazide is one of a class of functional drugs whose active metabolite is 5-ASA. It inhibits the synthesis and release of proinflammatory mediators (NO, leukotrienes) and the function of natural killer cells, mast cells, neutrophils, mucosal lymphocytes and macrophages [95]. These results were somewhat surprising, since several other large randomized studies show that 5-ASA either does not improve or worsens the symptoms of acute radiation enteritis. Early randomized trials of olsalazine [96] and mesalamine [97] were disappointing, with mesalamine showing no benefit and olsalazine showing an increased incidence of diarrhea. The negative result for olsalazine was not surprising, because a similar increase in diarrhea prevalence was seen in IBD patients [98]. A study of balsalazide found that it did not significantly alleviate clinical symptoms [99]. In contrast, the randomized, double-blind controlled trial of sulfasalazine conducted by Kiliç et al. in patients with a variety of pelvic radiotherapy malignancies reached the opposite conclusion. This larger, controlled study with a detailed grading of acute radiation enteritis reported that none of the patients in the sulfasalazine-treated group experienced Grade 4 diarrhea compared with the placebo group [100].

The use of 5-ASA in radiation-induced gastrointestinal complications has long been the topic of debate. It would be more effective if given before the radiotherapy insult initiated the cascade of oxidative enzymes rather than after this cascade has already begun [96]. At this time, these inconsistent early clinical observations warrant further investigation into the immunobiology of PPARs and their potential role as a therapeutic target in the protection of patients undergoing radiotherapy. Overall, the benefit of 5-ASA appears to depend on its dose, formula, and mode of administration. The new-generation 5-ASA (i.e., Balsalazide), which produces a high concentration of active drug in the colon, seems off to a promising start in making pelvic radiotherapy more tolerable.

## 6. PPAR Ligand-Associated Toxicities

Wound healing after injury is a high priority for survival. In this situation, epithelial cells change their intracellular contacts, modify their matrix, proliferate, and migrate over the wound. Interestingly, each of these healing behavior is similarly involved in tumorigenesis and metastasis. Epithelia are highly susceptible to injury and also heal injuries effectively. At the same time, 95% of all cancer deaths are from epithelial tumors. Together these facts suggest that the repair mechanisms activated in response to injury may, if not controlled, promote cancer.

Although PPARs may be involved in tumor-associated pathways, their regulation of wound-healing genes within specific tumors remains largely unexplored [101]. PPARs, especially PPAR- $\gamma$ , are expressed or overexpressed in several abdominal malignancies, including colorectal carcinoma [102, 103], prostate carcinoma [104], and pancreatic

carcinoma [105]. Another study reports that 8% of primary colorectal tumors harbor a loss of function mutation in one allele of the PPAR- $\gamma$  gene and emphasizes the potential role of this receptor as a tumor suppressor in humans [106].

In vivo evidence to support an antitumorigenic role of PPAR- $\gamma$  is also conflicting. Some experiments show that PPAR- $\gamma$  can behave as a ligand-activated tumor suppressor. The ligands that activate PPAR- $\gamma$  can inhibit proliferation and induce differentiation and apoptosis of a wide range of neoplastic cell types in vitro and in murine xenograft tumor models. PPAR $\gamma^{-/-}$  mice are more susceptible than wild-type mice to mammary, colon, ovarian, and skin tumors after exposure to carcinogens. PPAR- $\gamma$  also enhances tumor formation in some genetic models of cancer. Significantly, troglitazone reduced the tumor incidence in wild-type but not heterozygote mice [107]. Chen et al. showed that the PPAR- $\gamma$  ligand 15-Deoxy- $\Delta$ 12,14-prostaglandin J2 (15dPGJ2), or ciglitazone, induces apoptosis in HT-29 by inhibiting NF- $\kappa$ B activity, which upregulates various anti-apoptotic genes, and by suppressing the expression of BCL-2, which protects cells against apoptosis [108]. In contrast, however, both troglitazone and rosiglitazone treatments increased the frequency and size of colon tumors in APC<sup>min</sup> mice, a clinically relevant model for both human familial adenomatous polyposis and sporadic colon cancer [109, 110]. These mice have a germ-line mutation of the APC gene resulting in deregulated  $\beta$ -catenin signaling and a very significantly increased frequency of small and large intestinal adenocarcinomas. Subsequently, however, generation of the APC<sup>min</sup> bigenic mouse with an intestinal-specific PPAR- $\gamma$  deficiency demonstrated unequivocally that PPAR- $\gamma$  suppresses tumor formation and suggested that thiazolidinedione has significant off-target effects in mice, especially in the APC<sup>min</sup> mouse colon cancer model [111]. These off-target effects of thiazolidinedione generally appear to have broad anticancerous properties; therefore, the findings in this model appear quite unusual.

Clinical studies have not yet provided a conclusive answer to the question of whether PPAR- $\gamma$  activity favors or inhibits cancer formation and progression, but their outcome has been largely disappointing and the clinical benefits were rather limited. The best results were obtained in three patients with liposarcoma, in which lineage-appropriate differentiation was induced [112]. At present, not enough evidence is available to establish with certainty whether PPAR- $\gamma$  has pro- or antitumorigenic activities, and the field remains confusing. Some of this confusion, however, results from differences in experimental design. First, the differentiation state of the cells and tumors may affect the outcome. PPAR- $\gamma$  activity is influenced by numerous other factors (cofactors, mutations in genes such as APC, and differentiation status). A final point is that the concentration of PPAR- $\gamma$  agonist used is important. In breast cancer cell lines, for instance, low concentrations of PPAR- $\gamma$  agonists induce cell proliferation; whereas higher concentrations of the same agonists correlate with cell cycle arrest and apoptosis [113]. A further point worth stressing is that troglitazone, a compound with significant antioxidant properties, is reported to be associated with the largest number of antitumorigenic effects.

In contrast to normal cells and tissue, irradiation induces a progressive but significant tendency to overexpress PPAR- $\gamma$  in HT-29 at one day after irradiation. Surprisingly, troglitazone treatment significantly reduced irradiation-induced PPAR- $\gamma$  expression (personal communication) and may thus contribute to the lack of efficacy of its anticancer properties. At this time no information is available about any hypothetical deregulation of  $\beta$ -catenin signaling directly due to irradiation. Nonetheless, indirectly, irradiation is a nitric oxide (NO) producer, and NO induces  $\beta$ -catenin degradation and downregulates its transcriptional activity in colon cancer cells, thus revealing a so-far-unidentified mechanism of  $\beta$ -catenin regulation [114]. The actions of these receptors may be attenuated in malignancies by genetic, cytogenetic, and environmental molecular mechanisms, which may compromise ligand generation. This understanding may have important implications for the necessary molecular diagnosis required to target PPAR $\gamma$  therapies most effectively.

## 7. Conclusion

The development of gastrointestinal dysfunction after pelvic radiotherapy depends on a complex pathological process. These gastrointestinal symptoms affect the quality of life, are substantially more common than generally recognized and are frequently inadequately managed. They develop because radiation can induce changes in one or more specific physiological functions in widely separated parts of the gastrointestinal tract that lie in the path of the radiotherapy beam. The increasing weight of evidence suggesting a relation between acute and late effects may encourage clinicians to look at methods for decreasing tissue toxicity and thus improving the patient's quality of life. But decisions about the treatment options for reducing radiotherapy toxicity must be weighed after a careful benefit-risk analysis that depends on the target organ. Short-term preconditioning strategies with PPAR agonists can be protective in several animal models. Thus PPARs may provide a new strategy for intestinal injury prevention in radiation toxicity as in IBD. The tissue level of PPARs appears to be very radiosensitive. Accordingly some additional experiments may be needed to establish the mode of action of PPAR agonists in radiation protection and the stage at which PPARs can influence normal tissue self-renewal. A patient's ability to respond to appropriate therapy has a great impact on the actual toxicity of the treatment. However, as mentioned previously, it remains unclear whether PPARs act as oncogenes or as tumor suppressors. Further studies are needed to confirm this effect, especially in the absence of any effect on tumor control.

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## Review Article

# PPARs in Human Neuroepithelial Tumors: PPAR Ligands as Anticancer Therapies for the Most Common Human Neuroepithelial Tumors

Elisabetta Benedetti,<sup>1</sup> Renato Galzio,<sup>2</sup> Barbara D'Angelo,<sup>1</sup> Maria Paola Cerù,<sup>1</sup> and Annamaria Cimini<sup>1</sup>

<sup>1</sup> Department of Basic and Applied Biology, University of L'Aquila, 67100 L'Aquila, Italy

<sup>2</sup> Department of Health Sciences (Neurosurgery), University of L'Aquila, 67100 L'Aquila, Italy

Correspondence should be addressed to Annamaria Cimini, cimini@univaq.it

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Neuroepithelial tumors represent a heterogeneous class of human tumors including benign and malignant tumors. The incidence of central nervous system neoplasms ranges from 3.8 to 5.1 cases per 100,000 in the population. Among malignant neuroepithelial tumors, with regard to PPAR ligands, the most extensively studied were tumors of astrocytic origin and neuroblastoma. PPARs are expressed in developing and adult neuroepithelial cells, even if with different localization and relative abundance. The majority of malignant neuroepithelial tumors have poor prognosis and do not respond to conventional therapeutic protocols, therefore, new therapeutic approaches are needed. Natural and synthetic PPAR ligands may represent a starting point for the formulation of new therapeutic approaches to be used as adjuvants to the standard therapeutic protocols. This review will focus on the major studies dealing with PPAR expression in gliomas and neuroblastoma and the therapeutic implications of using PPAR agonists for the treatment of these neoplasms.

## 1. Neuroepithelial Tumors

Human neuroepithelial tumors are classified according to the World Health Organization (WHO). The incidence of central nervous system (CNS) neoplasms ranges from 3.8 to 5.1 cases per 100,000 in the population. Among neuroepithelial tumors, with regard to PPAR ligands, the most extensively studied are tumors of astrocytic origin and neuroblastoma.

Astrocytic tumors are classified as: (1) Astrocytoma (WHO grade II), (2) Anaplastic (malignant) astrocytoma (WHO grade III), (3) Glioblastoma multiforme (WHO grade IV); (4) Pilocytic astrocytoma noninvasive, (WHO grade I), (5) Subependymal giant cell astrocytoma (noninvasive, WHO grade I), (6) Pleomorphic xanthoastrocytoma (noninvasive, WHO grade I) [1–4].

Malignant astrocytic tumors are the most common primary brain tumors. High-grade gliomas show high cellular proliferation rate and infiltrate the adjacent brain

tissue [5]. They initially respond to radiation and, to a lesser degree, to chemotherapy; however, they invariably recur. The malignant gliomas with poor prognosis and fatal outcome are mainly represented by anaplastic astrocytoma and glioblastoma.

*1.1. Anaplastic Astrocytoma (WHO Grade III).* Also known as malignant astrocytoma and high-grade astrocytoma, it may arise from a diffuse astrocytoma or may arise *de novo* without indication of a less malignant precursor [6]. Histologically, these tumors show increased cellularity, distinct nuclear atypia, and marked mitotic activity when compared with low-grade astrocytomas. Anaplastic astrocytomas possess an intrinsic tendency to progress to glioblastoma. The mean age at diagnosis is approximately 41 years. This tumor primarily affects the cerebral hemispheres. It has a high frequency of *TP53* mutations, which

is similar to that of low-grade astrocytomas; chromosomal abnormalities are nonspecific. Many of the genetic alterations seen in anaplastic astrocytomas involve genes that regulate cell cycle progression [4]. The mean time to progression is 2 years. Positive predictive factors include young age, high performance status, and gross total tumor resection.

**1.2. Glioblastoma (WHO Grade IV).** Also known as glioblastoma multiforme (GBM), it may develop from low-grade astrocytomas or anaplastic astrocytomas but more commonly it arises *de novo* without evidence of a less malignant precursor [7]. GBM, the most common malignant brain tumor (34%) in adults, is among the most lethal of all cancers [8]. Histologically, GBMs are anaplastic, cellular gliomas composed of poorly differentiated, often pleomorphic astrocytic tumor cells with marked nuclear atypia and brisk mitotic activity. Typically, they affect adults and are preferentially located in cerebral hemispheres. Most patients with GBM survive less than 1 year, thus new therapeutic strategies are urgently needed [9, 10]. Genetic analyses suggest that there are two different types of glioblastoma: *de novo* glioblastoma, which arises from mutated neural stem cells or progenitor cells, and secondary glioblastoma, which arises from lower grade tumors. The secondary GBMs occur in younger patients [11–13]. The peak incidence occurs between the ages of 45 and 70 years. GBMs have been associated with more specific genetic abnormalities than any other astrocytic neoplasm, but none are specific. Amplification of the epidermal growth factor receptor locus is found in approximately 40% of primary GBMs but is rarely found in secondary glioblastomas; mutations of the *PTEN* gene are observed in 45% of primary GBMs and to a lesser extent in secondary glioblastomas [4]. Loss of heterozygosity (LOH) of chromosome 10 and loss of an entire copy of chromosome 10 are the most frequently observed chromosomal alterations.

**1.3. Neuroblastoma.** Neuroblastomas are paediatric tumors originating from neuroblasts in the developing peripheral nervous system. Most primary tumors (65%) occur within the abdomen, with at least half of these arising in the adrenal medulla. Other common sites of disease include the neck, chest, and pelvis. It is the most common extracranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy [14]. Neuroblastoma accounts for more than 7% of malignancies in patients younger than 15 years and around 15% of paediatric deaths [15]. The mortality is high due to rapid tumor progression to advanced stages. The genetic aberration most consistently associated with poor outcome in neuroblastoma is genomic amplification of *MYCN*, which occurs in roughly 20% of primary tumors and is strongly correlated with advanced stage of disease and treatment failure [14, 16, 17]. Deletions of the short arm of chromosome 1 (1p) can be identified in 25–35% of neuroblastomas. These deletions correlate not only with *MYCN* amplification, but also with advanced disease stage [18, 19]. However, the gene or genes within chromosome

1p involved in the pathogenesis of neuroblastoma have not been identified despite intensive investigation. It has been suggested that a strategy to halt the malignancy of these cells could be to induce them to differentiate towards mature neurons. Accordingly, several neuroblastoma differentiation protocols have been proposed, for instance treatment with phenyl acetate and retinoic acid [20]. The SH-SY5Y cell line was established from a high malignant tumor with no N-myc amplification [21]. Treatment of this cell line with phorbol esters leads to sympathetic neuronal differentiation with neurite outgrowth and increased synthesis of noradrenaline and expression of neuropeptide Y and growth-associated protein 43 (GAP-43) [22]. These effects are mediated by and dependent on PKC [20, 23, 24].

**1.4. PPARs.** The peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors are ligand-activated transcription factors which have been implicated in different human pathologies. PPAR $\gamma$  ligands are currently used for treatment of type II diabetes, PPAR $\alpha$  ligands are used to treat cardiovascular diseases [25–27]. After the isolation of PPAR $\alpha$  (NR1C1), in 1990 by Issemann and Green [28] as the nuclear receptor mediating peroxisome proliferation by peroxisome proliferators (PP) in rodent hepatocytes, two related isotypes, PPAR $\beta/\delta$  (NR1C2; referred to as PPAR $\beta$ ) and PPAR $\gamma$  (NR1C3) have been characterized [29]. Since then, these receptors have been linked to many systemic and cellular functions ranging far beyond the process after which they were initially named. Like the other members of the superfamily, PPARs have a canonical nuclear receptor organization [30]. The DNA-binding domain, named the C domain, is highly conserved and its zinc finger domain is a common attribute of all members of the nuclear receptor (NR) superfamily. The C domain is linked to the C-terminal ligand-binding domain (LBD), named the E domain, by the hinge region, named the D domain. The LBD contains a ligand-dependent transactivation function referred to as AF-2 and comprises 12 $\alpha$  helices and 4 $\beta$  sheets that fold to create a large hydrophobic cavity where ligands are buried [31]. In addition, the E domain offers the main surface for dimerization with the 9-cis retinoic acid receptor (RXR) as well as for interaction with regulatory proteins called cofactors. The N-terminal domain, named the A/B domain, is involved in ligand-independent regulation of receptor activity [32]; this domain harbors a weak-ligand-independent transactivation function, called AF-1. PPARs form heterodimers with the RXR and exhibit ligand-induced transcriptional regulatory activity through sequence-specific PPAR-responsive elements (PPRE) in their target genes [33]. Free PPARs may be associated with corepressors that inactivate the transcription function of the nuclear receptor. When the nuclear receptor is activated by a particular ligand binding to the ligand binding domain, this results in conformational changes to PPAR, and the receptor is released from binding with the corepressor. PPAR forms a heterodimeric complex with RXR and then recruits coactivator proteins. This complex binds to a PPRE on DNA and regulates transcription. Peroxisome proliferators, like fatty acids, modulate

tissue-specific responses; for example, they stimulate the expression of enzymes involved in lipid catabolism, namely, the peroxisomal  $\beta$ -oxidation system [34, 35].

PPARs exhibit a broad but isotype-specific tissue expression pattern which can account for the variety of cellular functions they regulate. PPAR $\alpha$  and  $\gamma$  transcripts appear late during fetal development of rat and mouse (day 13.5 of gestation), with a pattern of expression similar to their adult distribution, with the exception of the placenta tissue, where PPAR $\gamma$  is abundantly expressed as early as E8.5 [36, 37]. It has been demonstrated that PPAR $\gamma$  functions in the placenta are crucial for trophoblast terminal differentiation and consequently for placental vascularization and integrity [37]. In the adipose tissue, the two PPAR $\gamma$  isoforms,  $\gamma 1$  and  $\gamma 2$ , act in the brown and white tissues, respectively, to promote adipocyte differentiation and lipid storage, while the expression of the PPAR $\gamma 1$  is preferentially shown in other tissues such as the gut or the immune cells [38]. PPAR $\alpha$  is expressed in tissues with high fatty acid catabolism such as the liver, heart, skeletal muscle, adrenal gland and pancreas, kidney, and intestine. In comparison with the two other isotypes, PPAR $\beta/\delta$  is expressed more ubiquitously and earlier during fetal development [39]. Its transcript is present in all organ tested, and it is often more abundant than the PPAR $\alpha$  and  $\gamma$  transcripts [40]. Shi et al. and Bastie have suggested the involvement of unliganded PPAR $\beta/\delta$  in modulating the expression and transcriptional activity of the other two PPARs [41, 42]. In addition, it has been shown that it is required for placenta development, in the control of cell proliferation and survival, especially in keratinocytes and enterocytes, and in the control of lipid metabolism, even though the underlying mechanisms still need investigations [43].

For more than a decade, work on PPARs was driven by their important role in the regulation of cellular metabolism, PPAR $\alpha$  in tissues known for high  $\beta$ -oxidation rates such as liver, heart, muscle, and kidney [44], while PPAR $\gamma$  was mainly studied for its adipogenic activity. At present, they are receiving growing attention for their involvement in the regulation of cell proliferation, death, and differentiation of both normal and malignant cells.

**1.5. PPAR Ligands.** PPARs are activated by a wide range of naturally occurring or metabolically produced lipids derived from the diet or from endogenous lipid molecules functioning in intracellular signalling pathways, which include saturated and unsaturated fatty acids and fatty acid derivatives such as prostaglandins and leukotrienes [45]. Whereas most natural agonists bind with a relatively weak affinity (in the order of  $\mu$ molar concentration), some high-affinity endogenous ligands have been characterized [46, 47]. Interestingly, some ligands, including 15-deoxy-prostaglandin J2 (15d-PGJ2), associate irreversibly to the receptor through covalent binding [48]. The delivery of PPAR ligands to the nucleus, where the receptors reside, is achieved by different cellular fatty acid binding proteins (FABPs), which are thought to specifically interact with the three PPAR isotypes [49]. PPAR $\alpha$  agonists include both fibrates commonly used

for the treatment of hypertriglyceridemia and the synthetic agonists WY 14,643 and GW7647. The best-characterized PPAR $\gamma$  agonists are thiazolidinediones (TZDs), including pioglitazone and rosiglitazone, which have insulin sensitizing activity and are currently used for the treatment of type 2 diabetes (Actos and Avandia, respectively) [44]. There are a number of nonTZD based PPAR $\gamma$  agonists, such as GW347845 and others that have been synthesized.

Apart from well-defined metabolic actions, PPAR $\gamma$  agonists exhibit several antineoplastic effects [50] and induce apoptotic cell death in various malignant cell lineages, including liposarcoma [51], breast adenocarcinoma [52, 53], prostate carcinoma [54], colorectal carcinoma [55, 56], nonsmall-cell lung carcinoma [57], pancreatic carcinoma [58], bladder cancer [59], and gastric carcinoma [60].

PPAR $\beta/\delta$  agonists include the prostacyclin PGI $_2$ , oleic acid, and the agents, GW0742, GW501516 and GW7842.

## 2. PPARs in the Brain

All three PPAR isotypes are coexpressed in the rat CNS during late embryogenesis, with PPAR $\beta/\delta$  being the more abundantly and precociously expressed. The expression of the three PPAR isotypes peaks in the rat CNS between day 13.5 and 18.5 of gestation. Whereas PPAR $\beta/\delta$  remains highly expressed, the expression of PPAR $\alpha$  and PPAR $\gamma$  decreases postnatally in the brain [61]. Both *in vitro* and *in vivo* observations show that PPAR $\beta/\delta$  is the prevalent isoform in the brain, found in all nervous cell types, whereas PPAR $\alpha$  is expressed at very low levels predominantly in astrocytes [30]. Acyl-CoA synthetase 2 (ACS2), an enzyme crucial for fatty acid activation and utilization, is regulated by PPAR $\beta/\delta$  at the transcriptional level, providing a simple measure of PPAR $\beta/\delta$  action [62]. ACS2 has a role in maturation of neurons (i.e., their cytodifferentiation and formation of neuronal connectivity); in addition, its over-expression in PC12 cells enhances internalization of fatty acids, namely oleic acid (OA), arachidonic acid (AA), and docosahexaenoic acid (DHA) and promotes neurite outgrowth [63]. These observations strongly suggest that PPAR $\beta/\delta$  participates in the regulation of lipid metabolism in the brain, a hypothesis further supported by the observation that PPAR $\beta/\delta$  null mice exhibit an altered myelination of the corpus callosum [64]. The fact that no cytoarchitectural alterations of cerebral cortex was described is in contrast with the results obtained by Michalik et al. and Cimini et al., demonstrating the existence of a close correlation between PPAR $\beta/\delta$  and ACS2 in PPAR $\beta/\delta$  null mice brain and in the rat cortical neurons, respectively [65, 66]. However, the study from Peters et al. is a mainly *in situ* study and may be only in apparent contrast with other authors because it does not exclude that neuronal function and competence may be impaired in PPAR $\beta/\delta$  null mice [64].

All PPARs have been described in the adult and developing brain and spinal cord [67–69]. While PPAR $\beta/\delta$  has been found in neurons of numerous brain areas, PPAR $\alpha$  and PPAR $\gamma$  have been localized to more restricted brain areas [67, 68]. The localization of PPARs has been also

investigated in purified cultures of neural cells. Previous studies have reported that PPAR $\beta/\delta$  is strongly expressed in immature oligodendrocytes (OL) where its activation promotes differentiation; PPAR $\gamma$  is mainly present in microglia, while astrocytes possess all three PPAR isotypes, although to different degrees depending on the brain area and animal age [70–74]. The role of PPARs in the CNS has mainly been related to lipid metabolism but these receptors have been recently implicated in neural cell differentiation and death as well as in inflammation and neurodegeneration [36]. PPAR $\alpha$  has been suggested to be involved in astrocyte maturation and differentiation both in primary adult mouse astroglial cells and in adult neural stem cells (NSC) [75–77]. In addition, this isotype has been suggested to be involved in acetylcholine metabolism and oxidative stress defense [68, 78].

PPAR $\gamma$ , besides playing a role in early phases of oligodendrocyte differentiation, has been mainly studied in relation to inflammation, cancer, and neurodegeneration [79]. Concerning PPAR $\beta/\delta$ , its involvement in neuronal differentiation and in CNS development has been suggested by Basu-Modak et al., Michalik et al., Braissant and Whali, on the basis of its high levels in rat neural tube, in reaggregated neural cell cultures and in adult CNS [62, 65, 80]. Roles for PPAR $\beta/\delta$  in the regulation of pain sensation and transmission in adult spinal cord [69] and in learning and memory in mouse hippocampus and in entorhinal cortex have been proposed [67, 69]. In a previous work we showed that PPAR $\beta/\delta$  is the main isotype present in primary cultures of rat cortical neurons, where it seems necessary for neuronal maturation together with the reduction of PPAR $\gamma$  expression and the activation of PPAR $\alpha$ ; PPAR $\beta/\delta$ , in fact, is gradually increased and activated during neuronal maturation, and this increase correlates with the expression of its target gene ACS2 [81].

### 3. PPARs in Neuroepithelial Tumors

Among neuroepithelial tumors, gliomas, especially glioblastoma and neuroblastoma, have been the most extensively studied as to regard PPARs, and in this context, the PPAR $\gamma$  isotype was the most extensively studied.

**3.1. PPARs and GBM.** Glioblastoma expresses all three PPAR isotypes both *in vitro* and *in situ*. The majority of the studies performed on this neoplasm, both on rat and human gliomas, reported on the antiproliferative activity of different PPAR $\gamma$  ligands, both natural and synthetic, by promoting apoptotic cell death or by increasing reactive oxygen species production [82–86]. PPAR $\gamma$  has been identified in transformed neural cells of human origin, and PPAR $\gamma$  agonists have been shown to decrease cell proliferation, stimulate apoptosis, and induce morphological changes as well as expression of markers typical of a more differentiated phenotype in glioblastoma and astrocytoma cell lines [87–89]. These findings have more recently been confirmed in glioblastoma primary cultures; treating pri-

mary cultures of glioblastoma cells with natural or synthetic PPAR $\gamma$  ligands decreases the expression of markers of undifferentiated stages, such as CD133, nestin and fibronectin, while increasing the expression of differentiation markers such as A2B5, GFAP,  $\beta$ -catenin, and N-cadherin. Conjugated linoleic acid (CLA) and the PPAR $\gamma$  synthetic agonist GW347845 also suppress proliferation and induce apoptosis in primary cultures of glioblastoma cells [90]. Consistent with growth inhibition, both ligands downregulate cyclinD1 and CDk4 protein levels, while inducing the transcription of the tumor suppressor gene PTEN. Both CLA and PPAR $\gamma$  agonist lead to a significant decrease of the VEGF isoforms and NOSII, thus indicating that even in glioblastoma PPAR $\gamma$  is able to inhibit the angiogenic pathways [90]. It has also been reported that ciglitazone induces apoptosis in four human glioblastoma cell lines by decreasing cyclin D1 and Bcl-2 proteins and increasing p27 and p21 proteins [91, 92]. Finally, there is some evidence to suggest that TZDs are potent inhibitors of glioma cell migration and brain invasion largely by transcriptional repression of TGF- $\beta$  [93]. This is particularly important because TGF- $\beta$  is an immunosuppressive cytokine that has been shown to play a major role in the malignant phenotype of gliomas [94]. Furthermore, inhibition of TGF- $\beta$  signaling restores immune surveillance and is associated with improved survival in a glioma model [95].

Apoptosis-based therapies gained interest as promising experimental treatment strategies since direct induction of apoptotic cell death can overcome many of the classical resistance mechanisms such as activated DNA repair or detoxification. The death ligand TRAIL/Apo2L might be a useful tool to trigger apoptosis in cancer, since TRAIL kills tumor cells of diverse cellular origin without severe toxic side effects [96, 97]. However, despite the common expression of death receptors, not all glioblastoma cells are susceptible to TRAIL, due to intracellular blockage of apoptotic signalling cascades. A group of PPAR $\gamma$ -modulating agents sensitize tumor cells to TRAIL-induced apoptosis [98]. It has been reported that glioblastoma cells are sensitized to TRAIL-induced apoptosis by troglitazone via various mechanisms. Troglitazone lead to a marked down-regulation of the antiapoptotic proteins FLIP and Survivin. Moreover, in some cell lines, the cell surface expression of agonistic and antagonistic TRAIL receptors was altered towards a higher susceptibility to death receptor-induced apoptosis. Troglitazone might counteract the capability of tumor cells to become resistant to apoptosis by modulating the apoptotic machinery at different levels [98].

It should be noted that only PPAR $\gamma$  ligands have been described as antiproliferative compounds in gliomas; no effects have been reported for other PPAR ligands. However, some evidences, obtained by us [99] in human gliomas at different grades of malignancy, strongly indicate an upregulation of PPAR $\alpha$  and its direct relationship with malignancy grade, suggesting that PPAR $\alpha$  antagonists can be used to halt malignancy, and suggesting that in some cases dual PPAR agonists should be carefully used.

Taken together, these data reported in glioma cells indicate that PPAR $\gamma$  activation by both synthetic and natural

ligands, results in cell cycle arrest, apoptosis promotion, inhibition of cell migration and invasion as well as suppression of antiapoptotic proteins, induction of differentiation markers, thus suggesting their potential use in the formulation of new therapeutic strategies against this neoplasm and recurrences.

**3.2. PPARs and Neuroblastoma.** Neuroblastoma cells express all three isotypes of PPARs. PPAR $\gamma$  is present in neuroblastoma cell lines [100], as well as in primary neuroblastoma cell culture [50]. Few studies report the expression of PPAR $\alpha$  at mRNA or protein level in human neuroblastoma cell lines [101] and data on the expression of PPAR $\beta/\delta$  in neuroblastomas are scarce [102]. To assess the roles of PPARs on neuroblastoma, most studies evaluate the impact of their natural or synthetic ligands on cell proliferation, death and differentiation. The putative natural PPAR $\gamma$  agonist, 15d-PGJ2, inhibits cellular growth, decreases cellular viability and induces apoptosis in human neuroblastoma cells *in vitro* [100, 103, 104], although some effects have been demonstrated to be PPAR $\gamma$ -independent [105]. Rodway et al. [103] showed that the PPAR $\alpha$  agonist WY-14643 has no effect on the growth of the IMR32 neuroblastoma cell line, whereas PGJ2 induces growth inhibition in the same neuroblastoma cells. This occurs through programmed cell death type II or autophagy, and the serum lysolipid, the lysophosphatidic acid (LPA), is responsible for modulating this cellular response. In the neuroblastoma cell line ND-7, the same group showed that the degree of PPAR $\gamma$  activation induced by PGJ2 is modulated through an interaction with the retinoblastoma protein (Rb) and histone deacetylase [106]. A combination therapy consisting of PGJ2 and the histone deacetylase inhibitor trichostatin A enhances the growth inhibition effects and is therefore proposed as a promising new strategy in the treatment of neuroblastoma. It should be noted that the effects of 15d-PGJ2 can also depend on its action on the NF $\kappa$ B pathway [107]. Valentiner et al. [108] tested four synthetic PPAR $\gamma$  TZD agonists (ciglitazone, pioglitazone, troglitazone, rosiglitazone) and reported their *in vitro* effects on cell growth of seven human neuroblastoma cell lines (Kelly, LAN-1, LAN-5, LS, IMR-32, SK-N-SH, and SH-SY5Y). All TZDs inhibited cell growth and viability of the cells in a dose-dependent manner, whereas the effectiveness of the single drugs was strongly different among cell lines. Similar results for ciglitazone and rosiglitazone have been reported [100, 109]. Cellai and colleagues [109] showed that high concentrations of rosiglitazone *in vitro* significantly inhibit cell adhesion, invasiveness, and apoptosis in SK-N-AS, but not in SH-SY5Y human neuroblastoma cells. The authors argued that this effect may be related to cellular differences in PPAR $\gamma$  activation. We have recently demonstrated [110] that PPAR $\beta/\delta$  agonists, both natural and synthetic (oleic acid and GW0742, respectively), are able to induce cell cycle arrest in G1 phase and neuronal differentiation in human neuroblastoma cell line SH-NH-5YSY by increasing p16 levels and decreasing cyclin D1 levels as well as by inducing the expression of neuronal differentiation markers and downregulating TrkB full length expression. In the case of neuroblastoma, both PPAR $\gamma$  and PPAR $\beta/\delta$

ligands showed antiproliferative effects but it should be noted that PPAR $\gamma$  activation results in apoptosis promotion, while PPAR $\beta/\delta$  activation results in cell cycle arrest and neuronal differentiation, thus suggesting the possibility to use dual agonists to counteract tumor progression and recurrences.

#### 4. Future Perspectives

The majority of malignant neuroepithelial tumors have poor prognosis and do not respond to conventional therapeutic protocols. Studying and validating both natural and synthetic PPAR ligands may represent a starting point for the formulation of new therapeutic approaches to be used as adjuvants to the standard therapeutic protocols. Another point to be considered is the targeting efficiency of these new drugs. The progress in the understanding of the biology and genetic of neuroepithelial tumors together with the use of truly manipulable experimental models, now offer real opportunities for the development of effective targeted therapy. Despite significant gaps in our understanding, a wealth of information now exists about the clinical and biological behaviours of these tumors, the genetic pathways involved in tumorigenesis, and the nature and role of signature alterations in these pathways. The challenge now is to integrate this knowledge in an interdisciplinary way to fully understand these diseases, particularly how their signature heterogeneity contributes to their intractability in order to design efficient drugs delivered exclusively to malignant cells.

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## Review Article

# Therapeutic Implications of PPAR $\gamma$ in Human Osteosarcoma

**Eric R. Wagner,<sup>1</sup> Bai-Cheng He,<sup>1,2</sup> Liang Chen,<sup>1,2</sup> Guo-Wei Zuo,<sup>1,2</sup> Wenli Zhang,<sup>1,3</sup> Qiong Shi,<sup>1,2</sup> Qing Luo,<sup>1,2</sup> Xiaoji Luo,<sup>1,2</sup> Bo Liu,<sup>1,2</sup> Jinyong Luo,<sup>1,2</sup> Farbod Rastegar,<sup>1</sup> Connie J. He,<sup>1</sup> Yawen Hu,<sup>1</sup> Barrett Boody,<sup>1</sup> Hue H. Luu,<sup>1</sup> Tong-Chuan He,<sup>1,2</sup> Zhong-Liang Deng,<sup>1,2</sup> and Rex C. Haydon<sup>1</sup>**

<sup>1</sup> *Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, Chicago, IL 60637, USA*

<sup>2</sup> *Key Laboratory of Diagnostic Medicine, Chongqing Medical University, Chinese Ministry of Education, Chongqing 400016, China*

<sup>3</sup> *Department of Orthopaedics, West China Hospital, Sichuan University, Sichuan 610041, China*

Correspondence should be addressed to Tong-Chuan He, tche@surgery.bsd.uchicago.edu

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Osteosarcoma (OS) is the most common nonhematologic malignancy of bone in children and adults. Although dysregulation of tumor suppressor genes and oncogenes, such as Rb, p53, and the genes critical to cell cycle control, genetic stability, and apoptosis have been identified in OS, consensus genetic changes that lead to OS development are poorly understood. Disruption of the osteogenic differentiation pathway may be at least in part responsible for OS tumorigenesis. Current OS management involves chemotherapy and surgery. Peroxisome proliferator-activated receptor (PPAR) agonists and/or retinoids can inhibit OS proliferation and induce apoptosis and may inhibit OS growth by promoting osteoblastic terminal differentiation. Thus, safe and effective PPAR agonists and/or retinoid derivatives can be then used as adjuvant therapeutic drugs for OS therapy. Furthermore, these agents have the potential to be used as chemopreventive agents for the OS patients who undergo the resection of the primary bone tumors in order to prevent local recurrence and/or distal pulmonary metastasis.

## 1. Introduction

Osteosarcoma (OS) is the most common nonhematologic malignant tumor of bone in adults and children, with the peak incidence in early childhood [1, 2]. It is associated with a poor prognosis due to its high grade at presentation, resistance to chemotherapy, and propensity to metastasize to the lungs [3, 4]. Furthermore, while 80% of OS patients are believed to have micrometastatic disease, only 10%–15% present as radiographically detectable lesions [5, 6]. Herein lies the challenge in identifying the 20% OS patients without micrometastases and modifying medical and surgical management accordingly. Genetic markers associated with metastatic disease could potentially spare those patients that need for chemotherapeutic agents, such as adriamycin, cisplatin, or methotrexate, and experience its severe toxicities ranging from cardiotoxicity to renal dysfunction.

It has been shown that OS cells are similar to undifferentiated osteoblasts, and increasing evidence suggests that

osteogenic differentiation defects may be responsible for OS tumorigenesis [2, 7–10]. Osteoblasts are derived from mesenchymal stem cells (MSCs), and osteoblastic differentiation is a tightly regulated process by numerous growth and differentiation factors, such as bone morphogenetic proteins (BMPs) and Wnts [2] (Figure 1). It is conceivable that any disruption of osteogenic terminal differentiation may result in the development of OS. The aggressiveness of OS may depend on the stage of disruption; that is, more aggressive OS phenotypes may be developed from mutant early osteoblast progenitors, whereas benign tumors may arise from disruptions of late stage osteoblasts [2, 7] (Figure 2).

Current cancer therapies primarily target the proliferative compartment of tumor cells. While effective in initial treatment, these strategies are often nullified by subsequent drug resistance. An attractive alternative is to overcome the uncontrolled cell proliferation through promoting terminal differentiation [8, 9, 11–13]. One possibility deals with

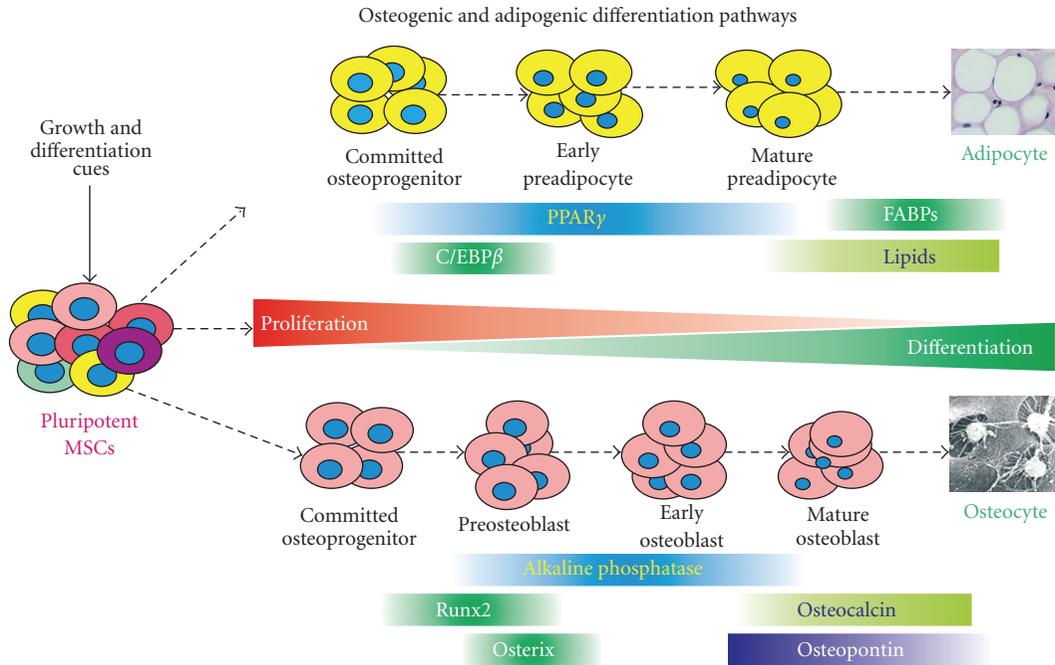


FIGURE 1: Osteogenic and adipogenic differentiation pathways in mesenchymal stem cells (MSCs). MSCs are pluripotent progenitor cells that are able to differentiate into several lineages, including osteogenic and adipogenic lineages, upon the stimulation with distinct growth and differentiation cues. The lineage-specific differentiation is a multiple-stage and well-coordinated process regulated by master regulators, such as PPAR $\gamma$  and C/EBP $\beta$  for adipogenesis and Runx2 and Osterix for osteogenesis. Osteogenic differentiation can be staged by measuring alkaline phosphatase (early marker) and osteocalcin and osteopontin (late markers). Expression of FABPs and production of lipids are indicators of terminal adipogenic differentiation.

the use of agonists or antagonists of the nuclear receptor superfamily, including vitamin D3, thyroid hormone, glucocorticoids, sex hormones, retinoids, and orphan receptors [14–18]. One interesting subgroup of the nuclear receptor superfamily is peroxisome proliferator-activated receptors (PPARs), which play a role in both promoting tumorigenesis and inducing terminal differentiation and apoptosis. Antitumor activity of PPAR $\gamma$  agonists has been shown in tumor cells derived from liposarcoma, colon cancer, breast cancer, leukemia, gastric cancer, nonsmall cell lung cancer, and prostate cancer [19–31]. Furthermore, PPAR $\gamma$  agonists have the potential to induce terminal differentiation in osteosarcoma cells [2, 9, 32]. In this review, we focus on the functional role of PPARs and their cross-talk with other nuclear receptors in osteogenic differentiation and tumorigenesis and the potential use of PPAR $\gamma$  agonists as chemotherapeutic and/or chemopreventive agents for human OS.

## 2. PPARs and Their Ligands

PPARs are ligand-activated transcription factors that achieve functionality after forming a heterodimer with the 9-cis retinoid X receptor (RXR). The subsequent transcriptional activity is modulated by nuclear receptor coactivators and corepressors [33], such as C/EBP PGC1 ( $\alpha/\beta$ ), PRIP, N-Cor, SRC-1, p300, Hsp-72, and PBP [34–39]. The ligands include the synthetic thiazolidinediones and fibrates and endogenous

fatty acids and eicosanoids [40, 41]. Upon ligand binding and heterodimerization with RXR, PPARs recognize PPAR response elements (PPREs) containing the direct repeat sequence (DR-1) AGGTCA [33, 34]. PPARs can also repress gene transcription through interfering with NF $\kappa$ B, STAT, and AP-1 signaling pathways [41–43]. Three subtypes of PPARs have been identified, PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  [16, 44]. PPAR $\alpha$  is found in liver, brown fat, kidney, heart, and skeletal muscle. PPAR $\beta$  (also known as PPAR $\delta$ ) is expressed in the gut, kidney, brain, skeletal muscle, and heart [34, 45]. PPAR $\gamma$  is expressed primarily in adipose tissue and to a lesser extent in large intestine, kidney, prostate, cartilage, osteoblasts, epithelial cells, and monocytes [46].

PPARs play a role in diabetes, atherosclerosis, obesity, the inflammatory response, and cancer [47]. PPARs regulate the expression of many genes associated with lipid storage,  $\beta$ -oxidation of fatty acids, terminal differentiation of preadipocytes, and modulation of the body's response to insulin and glucocorticoids [16, 47–50]. PPAR $\alpha$  functions primarily in lipid catabolism, lipoprotein metabolism, and inflammation, as its expression increases with stress, glucocorticoid expression, exercise, and fasting [47, 51, 52]. PPAR $\alpha$  knockouts develop normally, however, exhibit hepatomegaly from lipid accumulation and liver tumors, impaired wound healing, prolonged inflammatory responses, and increased adipose tissue [53]. PPAR $\beta$  has a relatively diverse range of functions, including  $\beta$ -oxidation of fats, tumorigenesis [54], vascular integrity [55], and bone

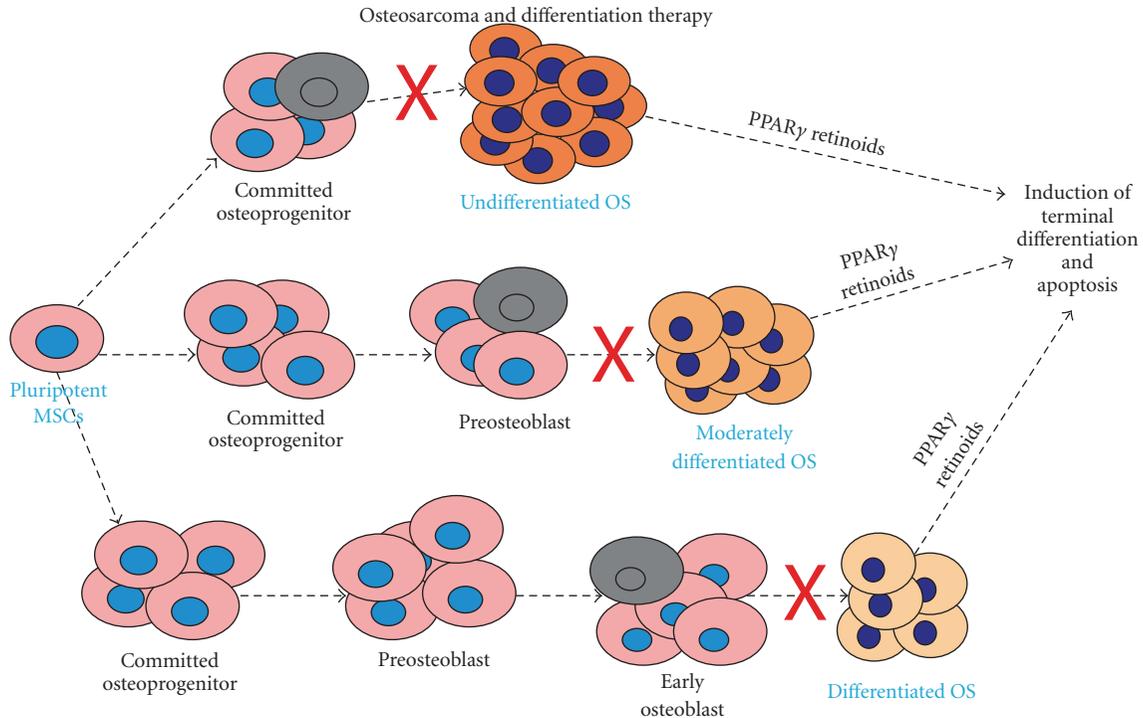


FIGURE 2: Osteosarcoma (OS) development and nuclear receptor agonist-mediated differentiation therapy. OS can be regarded as a differentiation disease, which is caused by disruptions of the terminal osteogenic differentiation. The stage and nature of differentiation defects may determine the aggressiveness of OS tumors. PPAR $\gamma$  agonists and/or retinoids have been shown to inhibit OS proliferation, induce apoptosis, and promote osteogenic differentiation. Thus, these agents can be used as differentiation therapy, in combination with conventional chemotherapy, for OS treatment.

metabolism [16]. PPAR $\beta$  knockouts have fatal placental defects secondary to abnormal vascular development, and those that survive become small, but healthy adults [53]. PPAR $\gamma$  affects the storage of fatty acids in adipose tissue, while also opposing TNF $\alpha$  and IL-6 production in inflammatory responses and insulin sensitization [47, 49, 50, 56, 57]. PPAR $\gamma$  knockouts are embryo-lethal as the placenta fails to implant and develop properly, and those that survive display severe metabolic, intestinal, hepatic, and adipogenic abnormalities [53].

### 3. Formation of PPAR and RXR Heterodimeric Receptor Complexes

PPARs consist of 4 domains, AB, C, D, and E. The AB domain function has not been clearly elucidated. The C domain represents the DNA binding domain (DBD), whereas the D domain contains the DBD carboxyl group along with the hinge connecting the C and E domains. The E domain (LBD) has a variety of functions, including ligand binding, hormone transactivation, and dimerization interface [16, 34, 45]. The three-dimensional structure of the LBD domain is very well conserved amongst the thyroid hormone receptor (TR $\alpha$ 1), retinoic acid receptor (RAR), and retinoid X receptor (RXR) [58–60]. RXR is a promiscuous receptor and able to heterodimerize with RAR, PPAR, VDR, TR, and orphan receptors. This enables the competition among

multiple hormones and other ligands to exert a variety of effects within the same tissue. It has been demonstrated that high concentrations of thyroid hormone inhibit the ability of PPAR $\gamma$  to heterodimerize with RXR and, therefore, blocks transcriptional activation (Figure 3) [61]. Transrepression occurs through sequestration of the coactivators CBP and SRC-1 by the PPAR/RXR heterodimers, preventing their utilization in other signaling pathways [62].

A unique association with PPAR and RXR is the direct repeat responsive elements (DR) and the PPAR response elements (PPRE) associated with the heterodimer. DR1 is a repetition of 2 core motifs consisting of AGGTCA spaced apart from one another by one nucleotide in the promoter of multiple target genes (Figure 3) [63]. These motifs are recognized by two zinc finger-like motifs in the DBD region of PPAR. These PPREs produced by the PPAR:RXR heterodimer are different from those recognized by the vitamin D receptor (DR3), thyroid hormone receptor (DR4), and retinoic acid receptor (DR2, DR5) [34, 64, 65]. The importance of PPAR $\gamma$ :RXR interaction is seen in Familial Partial Lipodystrophy, an autosomal dominant condition associated with metabolic syndrome, characterized by dyslipidemia, abnormal adipose tissue distribution, and a number of metabolic abnormalities [66]. This syndrome is associated with multiple missense and nonsense mutations of PPAR $\gamma$  that affect its ability to dimerize with RXR and bind coactivators.

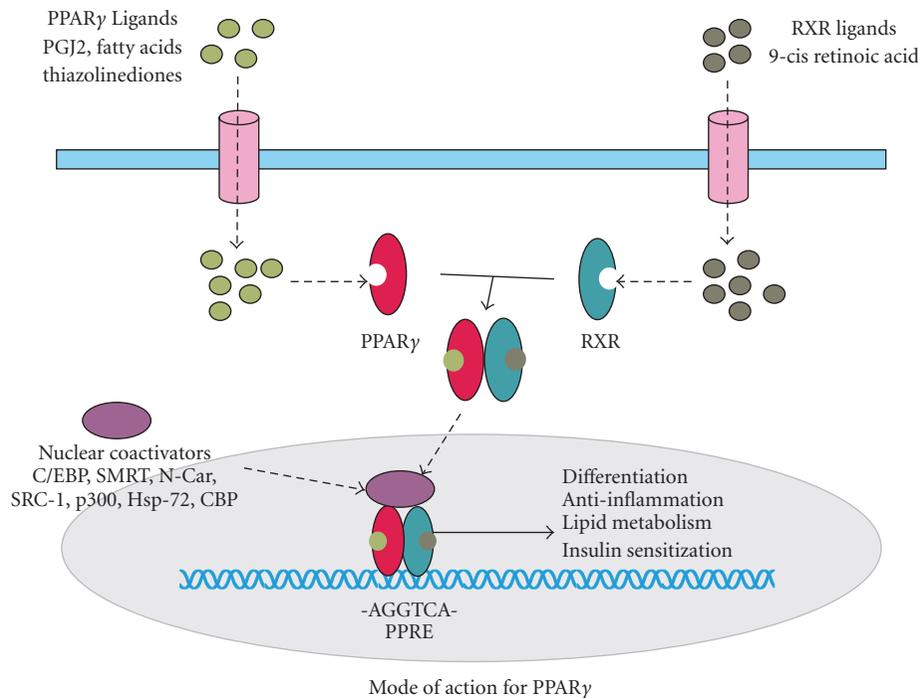


FIGURE 3: Current mode of action for PPAR $\gamma$ . PPAR $\gamma$  and RXR form a heterodimer, which is activated by the respective ligands. The activated PPAR $\gamma$ /RXR heterodimer will be translocated into nucleus and regulates downstream target genes in concert with nuclear receptor coactivators.

#### 4. Diverse Functions of PPAR $\gamma$

PPAR $\gamma$  is of particular interest because of its roles in adipogenesis, atherosclerosis, inflammation, proliferation, differentiation, and apoptosis [67]. The PPAR $\gamma$  gene contains 3 promoters, producing 2 different proteins, PPAR $\gamma$ 1 and PPAR $\gamma$ 2, which likely contributes to this diverse range of functions [55]. PPAR $\gamma$  expression in vascular endothelium and smooth muscle cells leads to an inhibition of MMPs, downregulation of Angiotensin II type 1 receptor, and alteration of macrophage invasion [68–71]. In inflammatory responses, its strongest association occurs with the ligand prostaglandin J2 (PGJ2) [16, 42, 72]. This eicosanoid metabolite binds directly to PPAR $\gamma$ , leading to its activation in the inflammatory response cascade. Furthermore, the insulin sensitizing effects of PPAR $\gamma$  are demonstrated using the synthetic antidiabetic therapy thiazolidinediones [42, 72, 73]. Thus, the promiscuity of its ligand binding and a variety of associated nuclear proteins enables the diversity of PPAR $\gamma$  functions in many tissues.

For adipogenesis, PPAR $\gamma$  binds to fatty acids and their derivatives, such as linoleic acid and docosahexaenoic acid (DHA) [34, 43]. These ligands activate PPAR $\gamma$ , stimulating preadipocytes to differentiate and initiate the steps required in lipid storage. PPAR $\gamma$  effects are carried out through target genes, such as aP2, lipoprotein lipase (LPL), acyl-Coa synthetase (ACS), and CD36 [74, 75]. Overexpression of PPAR $\gamma$  in fibroblasts initiates the adipogenic cascade, while PPAR $\gamma$  knockout mice are unable to form adipocytes or adipose tissue [48, 49, 76]. In humans, an activating

mutation in PPAR $\gamma$  leads to increased adipogenesis and obesity [77]. Another mutation decreasing PPAR $\gamma$  activity results in lower body mass index [78, 79].

Adipose tissue targets of PPAR $\gamma$  include LPL and fatty acid transporter FATP in stimulating fatty acid uptake, malic enzyme in NADPH synthesis for lipogenesis, phosphoenolpyruvate carboxykinase in gluconeogenesis, and ACS in FA esterification [16, 80–82]. PPAR $\gamma$  promotes adipocyte differentiation of MSCs through various signaling pathways [83–85]. MSCs can differentiate into either osteogenic or adipogenic lineage, depending on the differentiation cues (Figure 1). It has been reported that osteogenesis and its signaling cascade are inhibited by PPAR $\gamma$  activation [86–89]. However, the extent of PPAR $\gamma$ -associated adipogenic stimulation or osteogenic inhibition depends on the nature of the ligands [90]. Such ligand-dependent regulatory functions of differentiation may explain the shift away from osteogenesis in the aging process, due to an increase in the number of bone marrow adipocytes, oxidized LDL metabolites, and fatty acids metabolites.

#### 5. Role of PPAR $\gamma$ in Osteogenesis and Adipogenesis of MSCs

Osteogenesis and adipogenesis appear to originate from the same progenitor bone marrow mesenchymal stem cells (MSCs) [91–93]. MSC differentiation into osteoblast or preadipocytes occurs through a complex regulation of events [91–94] (Figure 1). Bone morphogenetic proteins (BMPs)

play an important role in this differentiation process and subsequent bone formation [2, 93, 95–101]. BMP-2, BMP-6, and BMP-9 regulate targets associated with osteoblast differentiation, while BMP-2, BMP-4, and BMP-7 appear to be associated with preadipocyte differentiation [93, 101–110]. Mice with BMP2-regulated Schnurri-2 knockout showed a reduction in white fat mass [111].

PPAR $\gamma$  can stimulate adipocyte differentiation of MSCs. PPAR<sup>+</sup> blasts (progenitor cells) have been noted in hematopoietic lineages, and PPAR $\gamma$  is able to induce terminal differentiation in monocytes and adipocytes [48, 50, 112, 113]. Overexpression of PPAR $\gamma$  in fibroblastic cells initiates the adipogenic cascade, while PPAR $\gamma$  knockout mice were unable to form adipocytes or adipose tissue [48, 49, 76]. In humans, an activating mutation in PPAR $\gamma$  leads to increased adipogenesis and obesity, while inactivating mutation results in a lower body mass [77–79]. To demonstrate its importance, no factor has been shown to be able to induce adipogenesis in the absence of PPAR $\gamma$  and almost all pathways involved in adipogenesis involve regulation of PPAR $\gamma$  [114].

Furthermore, in a review by Giaginis et al. PPAR $\gamma$  agonists were found to have a remarkable role regulating bone turnover [115]. However, while PPAR $\gamma$  seems to shift the differentiation pathway away from osteoblastogenesis and towards osteoclastogenesis, this is not always the case. Giaginis et al. reviewed studies focusing on both the synthetic and natural PPAR $\gamma$  ligand effects on osteoblast and osteoclast formation, as well as apoptosis and overall bone formation. They found divergent results, as it appears there are other factors that contribute to bone turnover regulated by PPAR $\gamma$ . For example, since natural ligands are found in both the diet and the inflammatory cascade, perhaps these processes determine the final outcome of PPAR $\gamma$ -regulated bone turnover. Its effect in clinical studies poses a similar paradox, while some patients receiving synthetic PPAR $\gamma$  agonists for Diabetes Mellitus type II experienced bone loss, others were noted to have a decrease in bone resorption markers [115].

PPAR $\gamma$  plays an intriguing role in both adipogenesis and osteogenesis. Earlier reports indicate that homozygous PPAR $\gamma$  deficient progenitor cells spontaneously differentiate into osteoblasts via increased osteoblastogenic factors *in vitro*, and heterozygous PPAR $\gamma$  deficiency results in increased *in vivo* bone formation [87]. However, recent studies have demonstrated that osteogenic BMPs can effectively induce adipogenic differentiation [101, 116, 117]. PPAR $\gamma$  has been shown to be significantly upregulated by osteogenic BMPs [101, 118]. Overexpression of PPAR $\gamma$ 2 promotes the osteogenic BMP-induced osteogenesis and adipogenesis [101]. Silencing PPAR $\gamma$ 2 expression leads to an inhibition of adipogenic differentiation as well as stimulation of osteogenic differentiation and osteoid matrix mineralization [101]. However, it remains to be elucidated how BMP-induced MSC differentiation into osteogenesis and adipogenesis diverges.

The regulation underlying these effects could be secondary to the nuclear competition between PPAR $\gamma$  and other members of its nuclear receptor superfamily. Regulation of the osteogenic promoter, osteocalcin, by glucocorticoids,

vitamin D, and thyroid hormone, occurs through the same nuclear pathway as PPAR $\gamma$  [119–121]. In addition, PPAR $\gamma$  activation by fatty acids and their derivatives might lead to a slowing of osteoblast differentiation, which would explain the tendency to shift to adipogenesis. These findings are intriguing as recent studies have indicated that aging activates adipogenesis and suppresses osteogenesis, possibly through the increased availability of these fatty acids and a decrease in metabolic production of many nuclear hormones. These would shift the signaling towards PPAR $\gamma$ , which might explain part of the mechanism underlying osteoporosis [122, 123].

**5.1. Side Effects of PPAR $\gamma$  Ligands.** In the treatment of Diabetes Mellitus (DM) type II with synthetic PPAR $\gamma$  ligands, the most common side effects observed have been headaches, gastrointestinal symptoms (nausea, diarrhea), and susceptibility to infections. Troglitazone has been withdrawn from the market secondary to its hepatic toxicity; however, this appears to be drug specific and not universal amongst PPAR agonists.

In a review by Mudaliar and Henry about the clinical use of glitazones, side effects include edema, weight gain, and mild drops in hematocrit. Rarely, increases in liver enzymes are observed. These synthetic PPAR $\gamma$  agonists induce the cytochrome P450 isoform CYP3A4 in the liver, affecting the metabolism of many other drugs. These drugs have been shown to increase plasma volume, thus, leading to edema and a dilutional drop in hematocrit. While the mechanism has not been elicited, PPAR $\gamma$  agonists antagonize the vasoconstriction induced by hyperinsulinemia, by sensitizing cells to the effects of insulin. Therefore, they relax vascular smooth muscles and decrease peripheral blood pressure. While there is no mention of increased peripheral adipose tissue, the propensity of PPAR $\gamma$  to induce lipid storage might underlie the observed weight gain [124].

Theoretically, a shift away from osteogenesis and towards adipogenesis might also promote osteoporosis and increase fracture risk. This has been demonstrated in a prospective study of over 80,000 patients being treated for DM type II [125]. Furthermore, competition for the RXR heterodimer might decrease the effects of other nuclear receptors in the superfamily, having a variety of effects on many different tissues. While recently there was a report suggesting increased fracture risk in patients receiving PPAR $\gamma$  agonists, there has been relatively little other evidence supporting any of these notions [122].

## 6. Molecular Biology of Osteosarcoma

The molecular pathogenesis underlying OS development is poorly understood. OS is associated with aberrations in p53 and Rb expression [1, 2, 126–128]. Other genetic alterations associated with OS development include p16<sup>INK4a</sup>, c-Myc, Fos-Jun, MDM2, CDK4, and cyclin D [1, 2, 126]. Altered cell signaling pathways in OS include Wnt, sonic hedgehog, TGF $\beta$ /BMP families, and IGF2 [1, 2, 126]. Mutations in DNA helicase increase OS risk and MMP expression leads

to a more aggressive OS tumor, while lack of telomerase activity is associated with a favorable prognosis [129–131]. The identifiable risks associated with OS include exposure to the FBJ or SV40 virus, beryllium oxide chemical, and radiation [132–134].

Disruption of the osteogenic differentiation pathway from MSCs is thought at least in part to be responsible for OS tumorigenesis [2, 7, 9, 10] (Figure 2). By preventing the differentiation of MSCs, the proliferative capability of preosteoblasts increases the risk for malignant transformation. It has been well established that early progenitor cells have similar characteristics to a variety of tumor cells. For example, progenitor cells of the hematopoietic system share similar leukocyte receptors to leukemic cells. Furthermore, tumor cells share many of the antiapoptotic and self renewal machinery with stem cells [135]. It has been proposed that a small subset of cancer cells, known as cancer stem cells, act in a similar manner to adult stem cells, with proliferative and regenerative capabilities that enable tumors to survive and grow [135]. In colon cancer cells, preneoplasia and neoplasia have the same nuclear morphotypes that 5–7-week-old fetal gut stem cells possess but are not found in the adult colonic crypt cells [136]. Osteosarcoma cells display similar characteristics to undifferentiated osteoblasts [7–10]. In OS cell lines, early osteogenic markers, such as CTGF, are high while late markers such as Runx2, Alkaline Phosphatase, Osteopontin, and Osteocalcin are low [7].

Therefore, investigation into the induction of terminal differentiation in cells with such differentiation defects has increased in recent years. A similar process has been reported in Ewing's Sarcoma, in which silencing the EWS/FLI-1 in Ewing's sarcoma cells leads to their recovery of MSC capability to differentiate into osteogenic lineages [137]. Accordingly, overexpression of this oncogene causes MSCs to remain in an undifferentiated state and promotes tumor growth [138]. Rb mutations occur in many OS tumors [1, 2, 126–128]. Rb coactivates the osteoblast differentiating agent Runx2 and loss of function of Rb stalls terminal osteoblast differentiation [139]. Furthermore, it has been recently shown that OS cells are refractory to BMP-induced osteogenic differentiation, whereas osteogenic BMPs promote OS growth *in vivo*, which can be overcome by introducing key osteogenic regulator Runx2 [7]. These findings suggest that the late stages of osteogenic differentiation may be preserved [7, 8]. Therefore, anti-OS therapies may be developed by promoting osteogenic terminal differentiation [2, 7]. Differentiation agents would add another dimension to the current chemotherapeutic cocktails focusing on the inhibition of the cell cycle.

**6.1. Molecular Biology Relating to the Differentiation Status in Tumors.** It appears that the differentiation status not only is responsible for the development of OS but also may predict its malignant potential. From the early 1970s when the idea of differentiation was first proposed, to more recently when differentiation agents are used for certain cancer phenotypes, it has been observed that this process is associated with many morphological changes in the respective cells. These changes

leading to a well-differentiated cell include repression of responsiveness to growth factors, withdrawal from the cell cycle into a state of quiescence, and a decreased ability to re-initiate proliferation [140]. For example, as adipocytes differentiate, they progressively become less responsive to mitogenic growth factors MIX and PDGF, eventually repressing the expression of proto-oncogenes c-jun and junB [141, 142]. The more differentiated the adipocyte, the less responsive it is to growth factors. Terminal adipocyte differentiation is accompanied by expression of proteins that repress RNA expression, along with induction of p21, leading to irreversible loss of proliferative potential [140, 143]. When breast cancer's estrogen receptors were first discovered and evaluated, the notion was proposed that the cancers with estrogen receptors represent a well-differentiated class of tumors that undergo clonal evolution and eventually lose their receptor status when they become poorly differentiated [144]. Another example occurs when Simian Virus 40 large T antigen transforms cells to increase their responsiveness to growth factors and become undifferentiated [145].

The fundamental idea behind differentiation therapy for tumors is that by inducing terminal differentiation, the tumor cells lose their proliferative phenotypes. Differentiation causes cells to lose their proliferative potential and repress their responsiveness to growth factors, while at the same time possibly increasing their susceptibility to apoptosis, induction of tumor suppressors, repression of oncogenes, inhibition of angiogenesis, and induction of cytotoxic agents. As cells become more differentiated, these changes make them less aggressive and more responsive to other chemotherapeutic agents.

**6.2. Clinical Examples of Therapeutic Success by Induction of Terminal Differentiation.** Inhibition of tumor growth through differentiation therapy has been demonstrated in clinical cases of hematologic and breast tumors. Induction of terminal differentiation was first shown to be successful in treating AML with low-dosage araC [146]. Recently, there have been many more therapeutic interventions that have focused on overcoming the uncontrolled cell proliferation through terminal differentiation [8, 9, 11–13]. One possibility deals with the nuclear receptor superfamily associated with vitamin D3, thyroid hormone, glucocorticoids, sex hormones, retinoids, and orphan receptors [14–17]. Treatment focusing on counteracting hormone dependent activation of these nuclear receptors is seen in therapies such as tamoxifen for breast cancer [18]. In this and other examples, regulation of the nuclear receptor leads to differentiation, causing the cells to lose their proliferative properties and antiapoptotic tendencies.

## 7. Role of PPARs in Tumorigenesis and Differentiation

PPARs play an important role in tumorigenesis and differentiation (Table 1). PPAR $\alpha$  is responsible for hepatocarcinogenic effects in rodents [147]. PPAR $\beta$  was identified as a downstream target of the APC/ $\beta$ -catenin pathway,

associated with human colon tumors [54]. PPAR $\beta$  has been shown as a target for nonsteroidal anti-inflammatory drug- (NSAID-) induced chemopreventive effects in colon cancer [45, 54]. High dose of NSAIDs, such as sulindac and indomethacin, displays chemopreventive effects in the familial adenomatous polyposis mouse model. It downregulates Cox-2 expression in humans, leading to a decrease in intestinal polyps, inhibition of cell cycle progression, and induction of apoptosis in colorectal tumor cells [45, 148, 149]. NSAIDs can disrupt the ability of PPAR $\beta$  to bind to its peroxisome proliferator response elements (PPREs) *in vitro*, while PPAR $\beta$  overexpression was able to rescue NSAID induced apoptosis in colon tumor cells [45]. These findings may explain the correlation between dietary fat consumption and colon cancer incidence, since fatty acids can serve as ligands for PPAR $\beta$ .

PPAR $\gamma$  has shown promise in therapy promoting terminal differentiation and apoptosis in a variety of malignancies, including liposarcoma, breast cancer, leukemia, gastric cancer, non-small cell lung cancer, and prostate cancer [19–27]. In humans, the treatment of end stage prostate cancer with PPAR $\gamma$  synthetic ligand troglitazone leads to prostate specific antigen (PSA) stabilization [150]. Although these results are promising, there are many examples of contradictory roles of PPAR $\gamma$  in tumorigenesis. It has been observed that while PPAR $\gamma$  agonists inhibit growth and induce apoptosis in both breast tumor cells and leukemic cells, administration of PPAR $\gamma$  antagonists enhanced this tumor growth inhibitory effect [151, 152]. The fusion protein EWSRI/NR4A3 in extraskeletal chondrosarcomas activates PPAR $\gamma$  expression [153]. In fibrosarcoma cells, the synthetic PPAR $\gamma$  agonist ciglitazone induces tumor cell invasion through the generation of ROS and ERK [154].

This controversy is best exemplified by the role of PPAR $\gamma$  in colorectal tumorigenesis. PPAR $\gamma$  agonists have been shown to promote mouse intestinal tumors, while loss of function mutations of PPAR $\gamma$  has been identified in human colon tumors [30, 45, 150]. Synthetic PPAR $\gamma$  agonists promote the development of colon tumors in mice with a mutation in the tumor suppressor APC [29, 30]. This leads to increased levels of B-Catenin. Furthermore, mice diets high in saturated fats promotes tumorigenesis [155]. PPAR $\gamma$  activation by Fas could explain the link between high fat diets and colon cancer. However, one study disputed the PPAR $\gamma$  agonist role in tumor promotion, as it showed that PPAR $\gamma$  agonists were able to induce differentiation and inhibit human tumors from growing in nude mice [31]. Furthermore, PPAR $\gamma$  agonists are able to induce differentiation, cell cycle arrest, and apoptosis in human colon cancer cell lines [31]. The anti-inflammatory effects of PPAR $\gamma$  lead to a reduced number of cancer precursor foci in inflammatory bowel disease [156]. One possible explanation for the antagonistic role of PPAR $\beta$  and PPAR $\gamma$  in colon tumorigenesis may be competition for RXR heterodimerization. The cellular proliferation caused by PPAR $\beta$ , induced by specific ligands, may lead to overexpression and inhibition of PPAR $\gamma$  heterodimerization, and subsequently contribution to human colon cancer development.

These contradictory results might be explained by species specific effects, where a combination of the different factors within each animal leads to different PPAR $\gamma$ -associated signaling outcomes. Another example of the differences between species occurs in hepatic cancers. PPAR $\gamma$  agonists are seen as potent carcinogens in rodents, but not seen in humans or primates [157, 158]. Furthermore, PPAR $\gamma$  agonist rosiglitazone seems to enhance carcinogenic effects of the urinary bladder in rodents, while treatment of diabetes with pioglitazone in humans does not seem to increase the incidence of these or any other tumors [159].

While the mechanisms underlying PPAR $\gamma$  action are not fully established, it has been shown that PPAR $\gamma$  can inhibit the cell cycle, which is accomplished at least in part through downregulating the protein phosphatase PP2A upon PPAR $\gamma$  activation [160]. The PPAR $\gamma$  ligands can also inhibit the G1/S transition by inhibiting Rb phosphorylation [161]. Furthermore, PPAR $\gamma$  upregulates the CDK inhibitors p18 and p21 [162]. PPAR $\gamma$  ligand PGJ2 induces both CDK p21 and the proapoptotic Bax but downregulates the anti-apoptotic Bcl-xL [163]. Synthetic PPAR $\gamma$  agonist treatment in human pancreatic cancer and bladder cancer cell lines resulted in G1 cell cycle arrest secondary to p21 induction [164, 165]. Further insight into the cross-talk between these different mechanisms will guide future antitumor therapies.

## 8. Antitumor Activity of PPAR $\gamma$ Agonists in Osteosarcoma

Increasing evidence suggests that activation of PPAR $\gamma$  may be explored as a possible intervention in osteosarcoma (Table 2). PPAR $\gamma$  agonists are thought to induce terminal differentiation in adipogenesis. OS cells share many characteristics to undifferentiated osteoblasts [7–10]. Therefore, modulators that are able to promote the differentiation of these immature osteoblasts should have similar effects on the OS cells. PPAR $\gamma$  agonist rosiglitazone has been shown to inhibit osteoblast proliferation, leading to decreased osteogenesis [88, 166]. A recent study showed PPAR $\gamma$  to be a critical mediator underlying doxorubicin resistance in OS cell lines [167]. The chemoresistant OS cell lines were shown to have an increased expression of IL-8, which induces the antiapoptotic KLF2 [167, 168]. KLF2 is thought to negatively regulate the PPAR $\gamma$ -induced expression of C/EBP and ADD1/SREBP, suggesting that the drug resistance may occur through the inhibition of PPAR $\gamma$ -induced apoptosis. Furthermore, the NSAID-Associated Gene 1 (NAG-1), which is associated with NSAID-induced apoptosis, has been upregulated by PPAR $\gamma$  in canine OS cell lines [169].

PPAR $\gamma$  agonists and 9-cis-retinoic acid have the capability to induce osteoblastic differentiation of OS cells and inhibit OS proliferation [9, 32]. After exposure to these agents, not only did the OS cells show decreased proliferative capabilities and susceptibility to apoptosis but they also expressed increased differentiation markers, such as alkaline phosphatase. Therefore, it appears that such agents would be useful in preventing recurrence and metastasis after surgical removal of osteosarcoma. The results are further supported

TABLE 1: Basic features of the three PPAR isoforms.

	Location	Ligands	Coactivators	Primary function	Knockout
PPAR $\alpha$	liver, brown fat, kidney, heart, skeletal muscle	fibrates fatty acids (e.g., oleic acid, palmitic acid) eicosanoids (e.g., arachidonic acid)	p300, c/EBP, SRC-1, PBP, PGC-1, PRIP	lipid catabolism, inflammatory responses, lipoprotein metabolism	hepatomegaly, liver tumors, impaired wound healing, prolonged inflammatory responses, increased adipose tissue
PPAR $\delta$	gut, kidney, brain, heart, skeletal muscle	fatty acids, NSAIDS (antagonist)	SRC-1, PBP	fatty acid $\beta$ -oxidation, bone metabolism, tumorigenesis, vascular integrity	fatal placental defects from abnormal vasculature, small healthy adults
PPAR $\gamma$	adipose tissue, cartilage, osteoblasts, epithelial cells, prostate, large intestine, monocytes, kidney	thiazolinediones eicosanoids (e.g., 15d-PGJ2, 15-HETE) fatty acids (e.g., DHA, linoleic acid)	p300, c/EBP, SRC-1, PBP, PGC-1, PRIP	adipogenesis, inflammatory response, insulin sensitization, differentiation	embryo-fatal, placenta fails to implant and develop, severe metabolic, hepatic, intestinal, adipogenic, abnormalities

by the ability of PPAR $\gamma$  to induce apoptosis in chondrosarcoma cells [177]. However, one study by Lucarelli et al. showed that treatment of human osteosarcoma cells with the PPAR $\gamma$  agonist troglitazone promotes the *in vitro* survival via reduction in apoptosis of the malignant cells [170]. Although the mechanisms accounting for this difference are not known, it is likely that this molecular complexity results from the nuclear cross-talk and interplays between PPAR $\gamma$  and other nuclear receptor hormones. It is plausible that the effects of PPAR $\gamma$  agonists on OS are largely dependent on which step in the differentiation pathway the defect has occurred (Figure 2). Downstream defects may be resistant to PPAR $\gamma$  agonists-induced terminal differentiation of its upstream counterparts. The specific factors that participate in the nuclear signaling and transcriptional regulation, along with the differentiation molecules associated with OS tumorigenesis, have yet to be fully elicited.

To the best of our knowledge, there have been no studies examining the effects of PPAR $\gamma$  on the metastatic potential in OS. However, our notion of the potential for PPAR $\gamma$  to reduce metastatic potential of OS is supported by examples in other tumors. Rosiglitazone has been shown to decrease the number of lung metastasis of mammary tumors in mice [178]. Dietary administration of PPAR $\gamma$  ligands linoleic acid and conjugated linoleic acid inhibited peritoneal metastasis of colorectal tumors in nude mice [179]. Furthermore, Pioglitazone inhibited colon tumor liver metastasis in mice, possibly by downregulating Cox-2 and cyclin D1 [180]. These anti-inflammatory and other possible antiangiogenic effects of PPAR $\gamma$  extend its potential as a chemotherapeutic agent beyond differentiation. Overexpressing PPAR $\gamma$  in nonsmall cell lung cancer cells inhibited tumor number and metastasis [181]. Due to its ability to inhibit angiogenesis, tumor cell invasion, and inflammation, it therefore follows that PPAR $\gamma$  would inhibit metastasis. In an analysis of primary breast tumors, PPAR $\gamma$  expression was more often

in low-grade than high-grade tumors, associated with a more favorable survival, and decreased in tumor relapses [182]. PPAR $\gamma$  ligand troglitazone inhibited growth and liver metastasis of papillary thyroid tumors [183].

## 9. Synergistic Antitumor Activity between PPAR $\gamma$ Agonists and Retinoids in Osteosarcoma

Receptors for retinoids include retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs are activated by all-trans retinoic acid, a vitamin A metabolite, and heterodimerizes with RXR after ligand binding. RARs have been implicated in early embryonic morphogenesis, including development of the forebrain, hindbrain, and body axis, as well as, early signaling associated with the pancreas, heart, eye, lung, and genitourinary tracts [184–186]. Furthermore, it is able to induce the differentiation of many cancer cells and is used as an effective therapy in the treatment of acute promyelocytic leukemia by differentiating cells that express the PML-RAR $\alpha$  fusion protein [9, 32, 45, 187–189].

RXRs are activated by 9-cis retinoic acid. Beyond the ability of RXR to heterodimerize with many members of the nuclear receptor superfamily, such as PPARs, RXRs can form homodimers. RXRs play important roles in signaling pathways associated with development and carcinogenesis. We have recently demonstrated that exogenous expression of RAR $\alpha$  induces ligand-independent myogenic differentiation from progenitor cells [190]. We have also found that all-trans retinoic acid and 9-cis retinoid acid can effectively induce the differentiation of mouse fetal liver-derived hepatic progenitor cells [191]. RXRs are overexpressed in breast ductal carcinomas, its ablation leads to prostate and skin hyperplasia, and its overexpression sensitizes tumors to retinoid family differentiation agents [192–195]. A synthetic

TABLE 2: Potential effects of nuclear receptor ligands on osteosarcoma tumors.

	Ligand	Experimental Setup	Effect	Mechanism	Reference
PPAR $\gamma$	Troglitazone	<i>in vitro</i> proliferation and apoptosis assays	inhibited proliferation	promotion of apoptosis, induce differentiation (alkaline phosphatase)	Haydon 2002, 2007 [9, 32]
	Ciglitazone	<i>in vitro</i> proliferation and apoptosis assays	minimal effect	unknown	Haydon2002 [32]
	Troglitazone	<i>in vitro</i> MTT proliferation and apoptosis assays	increased proliferation	inhibition of apoptosis	Lucarelli 2002 [170]
	Pioglitazone and PG J(2)	<i>in vitro</i> MTT and apoptosis assays of Chondrosarcoma Cells	inhibition of proliferation	promoted apoptosis	Nishida [171]
Retinoic Acid	9-cis retinoic acid	<i>in vitro</i> proliferation and apoptosis assays	inhibited proliferation	promotion of apoptosis	Haydon 2002, 2007 [9, 32]
Estrogen	17-beta estradiol, Ospemifene	U2OS expressing ER, <i>in vitro</i> apoptosis assays	opposed Etoposide-induced cell death	oppose increases in IL-6 and decreases in OPG, preventing osteoclast activation	Kallio 2008 [172]
	Tamoxifen, Raloxifene	U2OS expressing ER, <i>in vitro</i> apoptosis assays	no effect on Etoposide-induced cell death	unknown	Kallio 2008 [172]
	17-beta estradiol, SERMS (genistein, daidzein)	U2OS expressing ER, <i>in vitro</i> cell cycle, proliferation, apoptosis assays	inhibit proliferation, promote apoptosis	decrease EGFR, increased osteoblast maturation markers	Salvatori 2009 [173]
Vitamin D	1-alpha hydroxyvitamin D3	oral administration in Dunn murine OS model	inhibits tumor growth and metastasis	increased necrosis, no cell cycle mitotic index effects	Hara 2001 [174]
	Prolactin + 1,25 (OH) <sub>2</sub> Vitamin D3	RT-PCR, western blots	PRL inhibits VDR expression in response to 1,25 (OH) <sub>2</sub> Vitamin D3	VDR expression is dependent on BRCA1 expression	Deng [175]
	calcitriol	OS cells with increased RXR degradation, treated with calcitriol	increased expression of RXR restores anti-proliferative effects of calcitriol	calcitriol induced degradation is dependent on RXR expression	Prufer 2002 [176]

retinoid bexatrone has been developed for use in chemotherapeutic cocktails in mice mammary tumors, as well as human cutaneous T-cell lymphoma, with partial responsiveness in nonsmall cell lung cancer [196–198].

A comprehensive analysis of the possible synergistic effects between PPAR $\gamma$  and retinoids has been carried out in a panel of OS cell lines (Table 2) [9, 32]. As a single agent, PPAR $\gamma$  ligand troglitazone was shown to be the most effective in inducing cell death, followed by 9-cis retinoic acid [9, 32]. The strong synergistic effect on the induction of cell death was observed when both troglitazone and 9-cis retinoic acid or ciglitazone and 9-cis retinoic acid were administered to osteosarcoma cells [9, 32]. Troglitazone was shown to effectively induce alkaline phosphatase activity, a well-characterized hallmark for osteoblastic differentiation [9, 32]. These findings suggest that PPAR $\gamma$  and/or RXR

ligands may be used as efficacious adjuvant therapeutic agents for osteosarcoma as well as potential chemopreventive agents for preventing the recurrence and metastasis of osteosarcoma after the surgical removal of the primary tumors.

## 10. Other Nuclear Receptors in Osteosarcoma

Except for PPARs and retinoid receptors, several members of the nuclear receptor superfamily are also involved in the cell signaling and differentiation processes associated with OS (Table 2). Estrogens and selective estrogen receptor modulators (SERMs) are able to induce terminal differentiation in osteosarcoma cell lines through the downregulation of EGFR [173]. EGFR is a critical mediator of cell proliferation and differentiation, whose expression decreases over the

course of osteoblast differentiation and maturation [199, 200]. The stimulation of estrogen receptors leads to the downregulation of EGFR in OS, resulting in cell cycle inhibition and apoptosis. Alternatively, the estrogen 17  $\beta$ -estradiol protected osteosarcoma cells expressing estrogen receptors from etoposide-induced apoptosis [172]. However, the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene have no antiapoptosis effects.

Vitamin D receptor (VDR) has also been shown to play a role in OS cell lines and their responsiveness to therapeutic interventions. VDR is overexpressed on some OS cell lines and administration of 1, 25-dihydroxyvitamin D<sub>3</sub> inhibits tumor growth and metastasis, while promoting terminal differentiation [174, 201]. Interestingly, it has been reported that the upregulation of VDR in OS cell lines is dependent on the tumor suppressor BRCA1 [202]. Although a synthetic VDR ligand calcitriol is able to exert antiproliferative effects in OS cells, this process is dependent on the expression of RXRs. Degradation or downregulation of RXRs causes OS resistance to the antitumor effect of calcitriol [176]. Thus, the anti-OS activity of VDR is dependent on many other nuclear proteins, including BRCA1 and RXRs.

## 11. Concluding Remarks

OS is the most frequent primary bone sarcoma, comprising approximately 20% of all bone tumors and about 5% of pediatric tumors overall. OS tumors display a broad range of genetic and molecular alterations, including the gains, losses, or arrangements of chromosomal regions, inactivation of tumor suppressor genes, and the deregulation of major signaling pathways. However, except for p53 and/or RB mutations, most alterations are not constantly detected in the majority of osteosarcoma tumors. Recent studies strongly suggest that OS may be regarded as a differentiation disease that is caused by genetic and epigenetic disruptions of osteoblast terminal differentiation. It has been well established that PPARs and retinoids play an important role in regulating osteogenic differentiation of MSCs. Increasing evidence indicates that PPAR agonists and/or retinoids can inhibit cell proliferation and induce apoptosis in cancer cells, including OS cells. PPAR agonists and/or retinoids may also inhibit OS growth by promoting osteoblastic terminal differentiation. One of the future directions is to develop safe and effective PPAR agonists and/or retinoid derivatives. These agents can be then used as adjuvant therapeutic drugs for OS therapy. Meanwhile, more thorough investigations will be needed to examine the potentially beneficial and/or adverse effects of PPAR $\gamma$  ligands on different cells in bone and bone marrow microenvironment. Furthermore, these agents can be used as chemopreventive agents for the patients with OS who undergo the resection of the primary bone tumors in order to prevent local recurrence and/or distal pulmonary metastasis.

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## Review Article

# Anticancer Role of PPAR $\gamma$ Agonists in Hematological Malignancies Found in the Vasculature, Marrow, and Eyes

**P. J. Simpson-Haidaris,<sup>1,2,3</sup> S. J. Pollock,<sup>4</sup> S. Ramon,<sup>2</sup> N. Guo,<sup>5</sup> C. F. Woeller,<sup>4</sup>  
S. E. Feldon,<sup>5</sup> and R. P. Phipps<sup>4,5,6</sup>**

<sup>1</sup> Department of Medicine/Hem-Onc Division, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

<sup>2</sup> Department of Microbiology and Immunology, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

<sup>3</sup> Department of Pathology and Laboratory Medicine, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

<sup>4</sup> Department of Environmental Medicine, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

<sup>5</sup> Department of Ophthalmology, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

<sup>6</sup> The Lung Biology and Disease Program, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

Correspondence should be addressed to P. J. Simpson-Haidaris, [pj\\_simpsonhaidaris@urmc.rochester.edu](mailto:pj_simpsonhaidaris@urmc.rochester.edu)

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The use of targeted cancer therapies in combination with conventional chemotherapeutic agents and/or radiation treatment has increased overall survival of cancer patients. However, longer survival is accompanied by increased incidence of comorbidities due, in part, to drug side effects and toxicities. It is well accepted that inflammation and tumorigenesis are linked. Because peroxisome proliferator-activated receptor (PPAR)- $\gamma$  agonists are potent mediators of anti-inflammatory responses, it was a logical extension to examine the role of PPAR $\gamma$  agonists in the treatment and prevention of cancer. This paper has two objectives: first to highlight the potential uses for PPAR $\gamma$  agonists in anticancer therapy with special emphasis on their role when used as adjuvant or combined therapy in the treatment of hematological malignancies found in the vasculature, marrow, and eyes, and second, to review the potential role PPAR $\gamma$  and/or its ligands may have in modulating cancer-associated angiogenesis and tumor-stromal microenvironment crosstalk in bone marrow.

## 1. Introduction

Peroxisome proliferator activated receptors (PPARs) are a subfamily of the larger nuclear hormone receptor superfamily of transcription factors [1, 2]. Three distinct but closely related isoforms designated PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$  make up the family. PPAR $\gamma$  functions are further delineated by two isoforms PPAR $\gamma$ 1 and PPAR $\gamma$ 2, which arise due to alternative promoter usage accompanied by alternative splicing and/or polyadenylation of the primary transcript (recently reviewed in [3]). PPARs are best known for their roles in lipid homeostasis and energy metabolism including cholesterol and triglyceride turnover [4], obesity [5], metabolic syndrome [6–9], and diabetes [5, 10, 11];

however, since their discovery, the PPARs and/or PPAR agonists have been implicated in a broader spectrum of biological processes playing protective and homeostatic roles such as promoting wound healing [12, 13] and, for the most part, countering the effects of aging [14], cardiovascular disease [15, 16], inflammation and immune responses [17–19], thrombosis and hemostasis [7, 8, 17–21], pathological angiogenesis [22–32], and cancer [24, 25, 31–41].

A number of naturally occurring ligands activate PPAR $\gamma$  (Table 1), such as unsaturated fatty acids and eicosanoids [42], 15-deoxy- $\Delta$ -12-14-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), and components of oxidized low density lipoproteins (LDLs) [43]. The affinity of PPAR $\gamma$  for many of the endogenous ligands is low and, in some cases the physiological relevance

TABLE 1: PPAR- $\gamma$  ligands.

Natural ligands	Ref.
lysophosphatidic acid	[349]
nitrolinoleic acid	[350]
9-hydroxyoctadecadienoic acid	[351, 352]
13-hydroxyoctadecadienoic acid	[351, 352]
15-hydroxyeicosatetraenoic acid	[353]
prostaglandin D2	[351, 353–357]
15-deoxy- $\Delta$ 12,14-prostaglandin J <sub>2</sub> (15d-PGJ <sub>2</sub> )	[351, 353–357]
Synthetic Ligands	Ref
Thiazolidinedione family (TZDs)	[18, 44, 313, 353, 354, 358, 359]
ciglitazone	
pioglitazone	
rosiglitazone	
troglitazone	
TZD 18	
Nonsteroidal anti-inflammatory drugs	[353, 360, 361]
indomethacin	
ibuprofen	
flufenamic acid	
fenoprofen	
L-tyrosine-based	[351, 352]
GW-7845	
GW-1929	
diindolylmethane analogs	[351, 362]
triterpenoid 2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid (CDDO)	[46, 351]
CDDO C-28 methyl ester derivative (CDDO-Me)	[214, 363, 364]
CDDO C-28 imidazole (CDDO-Im)	[50]
1,1-bis[3'-(5-methoxyindolyl)]-1-( <i>p-t</i> -butylphenyl) methane (DIM #34),	[365]

of the ligand needs to be determined. However, it is well accepted that 15d-PGJ<sub>2</sub> is the most potent endogenous ligand for PPAR $\gamma$ . The thiazolidinediones (TZDs) are a class of synthetic ligands with high affinity for PPAR $\gamma$  that are used for their antidiabetic effects to sensitize cells to insulin [44]. Nonsteroidal anti-inflammatory drugs such as ibuprofen and indomethacin are low affinity PPAR $\gamma$  ligands [45]. Furthermore, the synthetic triterpenoid, 2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid (CDDO), and derivatives are high affinity ligands for PPAR $\gamma$  [46] (Table 1).

Two overarching principles should be kept in mind when weighing the plethora of therapeutic benefits touted for PPAR $\gamma$  agonists. First, PPAR $\gamma$  agonists evoke both PPAR $\gamma$ -dependent and PPAR $\gamma$ -independent effects, thus therapeutic benefits ascribed to certain PPAR $\gamma$  ligands do not necessarily require interaction with the PPAR $\gamma$  ligand binding domain. Although PPAR $\gamma$ -independent effects induced by 15d-PGJ<sub>2</sub> and CDDO are due in part to the electrophilic nature of these ligands [47–50], PPAR $\gamma$ -independent effects induced

by TZDs are through a number of signaling pathways including inhibition of Bcl-2/Bcl-xL function, proteasomal degradation of cell cycle- and apoptosis-regulatory proteins, and transcriptional repression [51]. Second, PPAR $\gamma$  agonists have been shown to have paradoxical physiological effects, likely due to tissue-specific and/or context-dependent regulatory signaling events.

Recently, we reviewed the role of PPAR $\gamma$  and its ligands in the treatment of hematological malignancies, which is summarized in Tables 1 and 2 [3]. The purpose of this paper is twofold: first to highlight the potential uses for PPAR $\gamma$  agonists in anticancer therapy with special emphasis on their role when used as adjuvant or combined therapy in the treatment of hematological malignancies, and second, to review the potential role PPAR $\gamma$  and PPAR $\gamma$  ligands may have in modulating cancer-associated angiogenesis and tumor-stromal microenvironment crosstalk in bone marrow—two pathophysiological events associated with most all types of cancer including hematological malignancies.

TABLE 2: PPAR $\gamma$  and PPAR $\gamma$  ligands as potential therapy for hematological malignancies.

Hematological malignancy/cell line	PPAR $\gamma$ agonist	Comments	Ref
HL-60	troglitazone	Inhibited cell proliferation by G1 arrest; induced differentiation to monocytes	[366]
HL-60	15d-PGJ <sub>2</sub> , troglitazone	Inhibited cell proliferation; induced caspase-dependent apoptosis	[367]
HL-60, K562	15d-PGJ <sub>2</sub> , troglitazone	Induced apoptosis through Bax/Bcl-2 regulation	[368]
Mono Mac 6, U937	15d-PGJ <sub>2</sub> , troglitazone	Induced apoptosis; downregulated cyclooxygenase-2	[369]
HL-60	15d-PGJ <sub>2</sub>	PPAR $\gamma$ -independent TRAIL-induced apoptosis	[370]
Jurkat, PC3	15d-PGJ <sub>2</sub>	PPAR $\gamma$ -independent TRAIL-induced apoptosis	[371]
EoL-1, U937, KPb-M15	troglitazone	Inhibited cell proliferation by G0/G1 arrest	[372]
HL-60, K562	15d-PGJ <sub>2</sub> , troglitazone	Inhibited cell growth, adhesion, and invasion through Matrigel; inhibited MMP-2 and MMP-9 expression	[230]
AML	DIM #34	Inhibited cell growth; induced apoptosis through PPAR $\gamma$ -dependent and independent mechanism	[365]
HL-60, U937, AML, CLL	rosiglitazone, 15d-PGJ <sub>2</sub> , CDDO	Inhibited cell growth, induced differentiation, induced apoptosis when combined with RXR-selective ligands	[373]
HL-60	Thiazolidinedione	Inhibited cell proliferation by G0/G1 arrest; induced apoptosis; induced differentiation	[374]
U937	troglitazone	Inhibited cell proliferation by G1 arrest	[375]
NB4	15d-PGJ <sub>2</sub> , pioglitazone	Inhibited cell proliferation; induced differentiation and lipogenesis when combined with specific RXR ligands	[376]
HL-60, AML	CDDO-Me	Induced cell differentiation; induced apoptosis	[214, 363, 364]
HL-60	CDDO	Induced apoptosis; induced differentiation and increased phagocytosis at sub-apoptotic doses	[377]
APL, NB4, MR2	CDDO	Enhanced all- <i>trans</i> -retinoic acid-induced differentiation and apoptosis	[378]
AML	CDDO	Induced apoptosis in a caspase-dependent and independent manner	[379]
U937	CDDO-Im	Inhibited cell proliferation; induced differentiation through PPAR $\gamma$ -independent mechanism	[50]
U937	CDDO, CDDO-Me, CDDO-Im	Induced apoptosis by increasing reactive oxygen species and decreasing intracellular glutathione	[380]
THP-1	rosiglitazone	Inhibited 9- <i>cis</i> retinoic acid-induced cell growth	[381]
THP-1	troglitazone, rosiglitazone	Inhibited MCP-1-induced migration	[382]
K562, KU812, KCL22, BV173, SD1, SupB-15	TZD18	Inhibited cell growth through a PPAR $\gamma$ -independent mechanism; inhibited proliferation; induced apoptosis	[359, 383]
K562	troglitazone, pioglitazone	Inhibited cell proliferation and erythroid phenotype; downregulated GATA-1	[384]
B-ALL	15d-PGJ <sub>2</sub> , pioglitazone	Inhibited cell growth by G1 arrest; induced apoptosis partially dependent on caspase signaling	[385]
UTree-O2, Bay91, 380	troglitazone	Inhibited cell growth by G1 arrest; induced apoptosis; downregulates c-myc expression	[386]

TABLE 2: Continued.

Hematological malignancy/cell line	PPAR $\gamma$ agonist	Comments	Ref
U266, RPMI 8226, BL-41, HS-Sultan	15d-PGJ <sub>2</sub>	Induced apoptosis; downregulation of NF- $\kappa$ B-dependent antiapoptotic proteins	[387]
Jurkat, J-Jahn, T-ALL	15d-PGJ <sub>2</sub> , PGD2	Induced apoptosis through PPAR $\gamma$ -dependent mechanism	[388]
Karpas 299	15d-PGJ <sub>2</sub> , GW7845, rosiglitazone	Induced cell death at high ligand concentration but promoted cell survival at low doses	[389]
CTCL and Sezary syndrome cell lines: MJ, Hut78, and HH	CDDO	Induced apoptosis through a PPAR $\gamma$ -independent mechanism by decreasing antiapoptotic protein Bcl-xL and activating caspase 3	[390]
GRANTA-519, Hbl-2, JeKo-1	15d-PGJ <sub>2</sub> , rosi-glitazone, pioglitazone	Induced apoptosis and downregulation of cyclin D1	[391]
CLL B cells	CDDO	Induced apoptosis in part by activation of caspase-8	[392]
CLL B cells, Jurkat	CDDO	Induced apoptosis through the intrinsic pathway	[393]
DLBCL	CDDO	Inhibited proliferation; induced apoptosis through a PPAR $\gamma$ -independent mechanism	[47]
Primary B lymphocytes, Ramos, OCI-Ly19 DLBCL	CDDO, CDDO-Im, Di-CDDO	Induced apoptosis through a mitochondrial dependent pathway	[394]
ANBL6, RPMI 8226	15d-PGJ <sub>2</sub> , ciglitazone	Induced apoptosis via caspase activation and mitochondrial depolarization	[208]
LP-1, U-266, RPMI 8226-S, OPM-2, IM-9	rosiglitazone, pioglitazone, 15d-PGJ <sub>2</sub>	Inhibited tumor cell growth	[395]
Waldenstrom's macroglobulinemia	rosiglitazone, ciglitazone	Inhibited cell growth; induced apoptosis	[396]
multiple myeloma (MM) drug sensitive MM.1S or drug resistant MM.1R cells, KAS6/1, ANBL-6	15d-PGJ <sub>2</sub> , troglitazone	Inhibited cell adhesion to BMSCs and adhesion-triggered IL-6 production; overcame resistance to dexamethasone (MM.1R cells)	[212]
MM cells, U266, RPMI 8226, bone marrow mononuclear cells	CDDO, CDDO-Im	Induced apoptosis by disruption of mitochondrial membrane potential	[397]
Dexamethasone-resistant MM.R1, RPMI 8226/LR-5, RMPI 8226/Dox-40, U266	CDDO-Im	Induced apoptosis; decreased MM adhesion-triggered IL-6 production	[398]
RPMI 8226, JJN3	CDDO-Im	Inhibited Stat3 and Stat5 phosphorylation; induced Stat inhibitors SOCS-1 and SHP-1	[399]
Normal human B cells and B lymphoma cells (Daudi, Ramos, Raji)	rosiglitazone, pioglitazone, 15d-PGJ <sub>2</sub>	Inhibited cell proliferation; induced apoptosis	[209]
MM cell lines (RPMI 8226 and U266); BMSCs, HS-5	PPAR $\gamma$ over-expression; ciglitazone	PPAR $\gamma$ overexpression inhibited proliferation and induced apoptosis in MM cells; inhibited IL-6 production in BMSCs	[207]
B cell lymphoma (Raji, Ramos cell lines)	PPAR $\gamma$ siRNA	Silencing of PPAR $\gamma$ induced cell proliferation and cell differentiation; PPAR $\gamma$ knockdown enhanced NF- $\kappa$ B activity in Ramos cells	[206]

## 2. Tumor-Stromal Microenvironment Crosstalk and Tumor-Associated Angiogenesis

**2.1. Cancer Stem Cell Theory and Tumor Dormancy.** A key issue of debate in cancer biology is whether tumor growth is caused by a substantial proportion of the tumor cells or exclusively by an infrequent subpopulation of cells termed cancer stem cells (CSCs) [52]. Regardless of the cancer type, most patients who have experienced many years of disease-free survival after successful treatment of the primary tumor ultimately die from metastatic disease. Patients who relapse must harbor cancer cells for years or even decades until the cancer cells overcome the regulatory mechanisms that keep the tumor in check. Dormant cancer cells are defined by a prolonged absence of or a balance in either proliferation or apoptosis, resulting in essentially a perpetual state of quiescence that protects them from conventional cytotoxic drugs, which only target actively proliferating cells. It is unknown whether dormant cancer cells represent a specialized subpopulation of cells programmed to stay dormant, an unspecialized population of cells not able to grow in the new microenvironment, or a combination of both [53]. CSCs are usually slowly cycling cells and thus insensitive to cytotoxic drugs as well [54, 55]. Dormant cancer cells are inferred to be CSCs or tumor initiating cells, as some prefer to call them [56]. Nonetheless, the relative frequency of CSCs varies as a function of both the tumor type and the specific experimental system used [57]. To date, published data most strongly support the presence of CSCs in hematologic malignancies such as leukemia [58], and in three major solid tumor types, including aggressive brain, breast, and colon cancers [59, 60]. Moreover, the existence of treatment resistant tumor cells following disease relapse has bolstered the theory that CSCs exist [56]. Thus, new approaches to target CSCs are actively being sought.

Although little evidence is available to suggest whether PPAR $\gamma$  agonists could be used to specifically target CSCs while sparing normal hematopoietic stem cells, a few studies have been reported. Chearwae and Bright [61] demonstrated that PPAR $\gamma$  agonists inhibit the proliferation of brain CSCs by inducing cell cycle arrest and apoptosis, which was associated with upregulated expression of PPAR $\gamma$  and inhibition of signal transducer and activator of transcription (Stat)-3 signaling. Saiki and colleagues [62] showed that pioglitazone inhibits the growth of human leukemia cell lines and primary leukemia cells while sparing normal stem cells. Preclinical testing has identified additional cancer therapeutics that selectively target leukemic stem cells but not normal stem cells, including idarubicin with the proteasome inhibitor, parthenolide (known as feverfew), and TDZD-8 [63]. These agents target the NF- $\kappa$ B pathway, a critical link in the well-established association between inflammation and carcinogenesis. In that PPAR $\gamma$  agonists inhibit both NF- $\kappa$ B- and Stat3-mediated transactivation of target genes and both of these transcription factors play a prominent role in cancer progression (see Section 2.8 and references therein), it is a likely extension to consider a role for PPAR $\gamma$  agonists to target CSCs.

**2.2. Tumor-Associated Angiogenesis.** Regardless of the type of cancer, once a primary tumor becomes established, it needs to develop its own blood supply for nutrient delivery and removal of toxic waste. The process of angiogenesis, that is the formation of new blood vessels from existing vasculature, involves complex interplay among cancer and stromal cell-secreted factors, extracellular matrix (ECM) constituents, and endothelial cells (ECs) (Figure 1). The adult vasculature is composed of quiescent ECs lining blood vessels and, with the exception of reproduction; the process of angiogenesis begins only in response to a broad array of tissue injury.

Several isoforms of VEGF-A/165 are produced by alternative mRNA processing of the primary transcript, and these isoforms differ primarily in their ability to adhere to heparin or heparan sulfate proteoglycans (HSPGs) found both in the ECM or on the surface of stromal and tumor cells [72]. The VEGF gene family encodes VEGF isoforms A-F and placenta growth factor (PLGF) with at least three cognate receptors, VEGFR1/Flt-1, VEGFR2/Flk-1/KDR, VEGFR3/Flt-4 and two coreceptors, neuropilin (NRP) and HSPGs. VEGF-A/165 (hereafter designated VEGF) signaling through VEGFR2 is the major isoform responsible for pathological angiogenesis and induction of vascular permeability in tumors [73, 74], which leads to enhanced transendothelial migration of cancer cells during intravasation and extravasation [75]. VEGF-C and VEGF-D bind to VEGFR2 as well as VEGFR3 and are important for lymphangiogenesis and cancer metastasis to lymph nodes and spread through the lymphatic system [76–78].

VEGF production and intracrine signaling through VEGFR2 by ECs is essential for vascular homeostasis but is dispensable for angiogenesis as shown in EC-specific VEGF knockout mice [79]. Intracrine VEGF signaling requires expression of both VEGF and VEGFRs by the same cell and resistance to VEGF inhibitors that fail to penetrate the intracellular compartment. Hematopoietic stem cell survival also involves a VEGF-dependent internal autocrine loop mechanism [80]. Although it was originally thought that VEGFR expression was restricted to ECs, it is now apparent that other cell types express functional VEGFRs. Furthermore, VEGF is an autocrine growth factor for VEGFR-positive human tumors, including Kaposi sarcoma, melanoma, breast, ovarian, pancreatic, thyroid and prostate carcinomas, and leukemia [81–87]. Thus, in VEGFR-expressing tumors, VEGF inhibition may directly inhibit tumor cell growth as well as tumor-associated angiogenesis [83]. A host of proangiogenic factors play a role in pathological angiogenesis [64]; however, since most anticancer therapeutic strategies target the VEGF signaling pathway [64, 88], this paper focuses thereon.

**2.3. Tumor-Stromal Microenvironment.** Paget's "seed and soil" hypothesis emphasizes the importance of the interaction between the tumor cell ("seed") and its environment ("soil") for metastasis to occur (reviewed in [89]). The stroma of the tumor microenvironment consists of several components including growth factors, chemokines, matrix glycoproteins and proteoglycans, proteases, and host cells

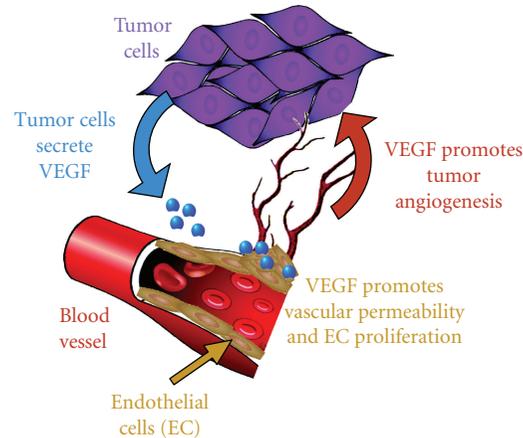


FIGURE 1: *Molecular mechanisms of tumor-associated angiogenesis.* Angiogenesis is essential for the persistence of solid tumor growth and, only recently, has it been appreciated that angiogenesis plays a role in progression of hematological malignancies as well. Cancer-associated angiogenesis in solid tumors begins once the tumor mass reaches a critical size such that the hypoxic environment inside the tumor leads to cancer cell-specific expression of proangiogenic factors including VEGF to shift the balance from endogenous antiangiogenic factors to tumor supplied proangiogenic factors—the angiogenic switch. Once proangiogenic factors overwhelm antiangiogenic factors, new blood vessels form in response to VEGF-induced endothelial permeability by EC sprouting, migration into the tumor mass, and proliferation from existing blood vessels—molecular mechanisms also induced by VEGF [64–67]. The tumor integrity of the vasculature is compromised in that it remains leaky with poor cell-to-cell adhesion, is abnormally branched and not well supported by pericytes (mural cells), the vascular smooth muscle cells that stabilize normal blood vessels [67, 68]. The chronic immaturity of tumor vessels has led Dvorak to characterize a tumor as a “wound that never heals” [69]. Notwithstanding, these features make tumor vessels viable targets for antitumor therapies. Benjamin et al. [70] demonstrated that removal of growth factors leads not only to the cessation of new vessel growth, but also to regression of the immature tumor vasculature [71].

that influence the behavior of cancer cells (reviewed in [90–102]). Host ECs, pericytes, macrophages, dendritic cells, lymphocytes, adipocytes, and fibroblasts/myofibroblasts present in the tumor microenvironment participate in the metastatic process (Figure 2). Initiation of new blood vessel formation requires activation of matrix metalloproteinases (MMPs) leading to degradation of the basement membrane, sprouting of ECs, and regulation of pericyte attachment for vessel stabilization. Activated fibroblasts, myofibroblasts, play an important role in synchronizing these events [94]. Furthermore, the topography of the ECM mediates vascular development and regulates the speed of cell migration during angiogenesis [103].

Chronic inflammation is associated with cancer initiation and progression [104–106]. Vascular ECs play a pivotal role in regulating leukocyte recruitment during inflammation [90]. Thus, in most cases, cancers exploit inflammation and recruited inflammatory cells for their own benefit [91]. Although activated inflammatory cells in the tumor microenvironment play important roles in cancer initiation, progression, angiogenesis, and metastasis [92], they are not the most numerous. Cancer-associated fibroblasts, which resemble myofibroblasts of healing wounds, are the most abundant cells of the tumor stroma [93], and contribute significantly to chronic inflammation, production of proangiogenic factors, and metastasis [94].

**2.4. Inhibitors of Angiogenesis.** As discussed above, angiogenesis is the hallmark pathology in tumor growth, progression,

and metastasis. Inhibiting tumor angiogenesis adds to the arsenal of treatment options for a number of solid tumor types [111, 112], and recently has been proposed for hematological malignancies as well [107, 113–119]. Endogenous inhibitors of angiogenesis are critical for tight regulation of pathological angiogenesis; however, in response to malignant transformation the putative “angiogenic switch” bypasses this tight regulation to promote tumor progression [120]. Whereas radiation and chemotherapy target killing of the tumor cells, antiangiogenic therapy is primarily directed against tumor blood vessels. Endostatin [121, 122], angiostatin [122], and TSP-1 [123] are among a host of well-known endogenous inhibitors of angiogenesis [98, 124]. TSP-1 is a large molecular weight glycoprotein that inhibits the proliferation and migration of ECs by interacting with CD36 expressed on the cell surface; CD36 is a PPAR $\gamma$  target gene. Small molecules based on a CD36-binding peptide sequence from TSP-1 are being tested for cancer treatment. One analog, ABT510, exhibits potent proapoptotic activity in vitro, while clinically it is very well tolerated with therapeutic benefits against several malignancies reported in phase II clinical trials [125–129].

Targeting VEGF-induced angiogenesis is in current use as monotherapy or combination therapy to treat a wide variety of cancers [130–132]. Bevacizumab (Avastin) and ranibizumab (Lucentis) are FDA-approved humanized monoclonal antibodies that recognize and block VEGF signaling in cancer and age-related macular degeneration (AMD) [130–134]. Additional, but not all-inclusive VEGF inhibitors

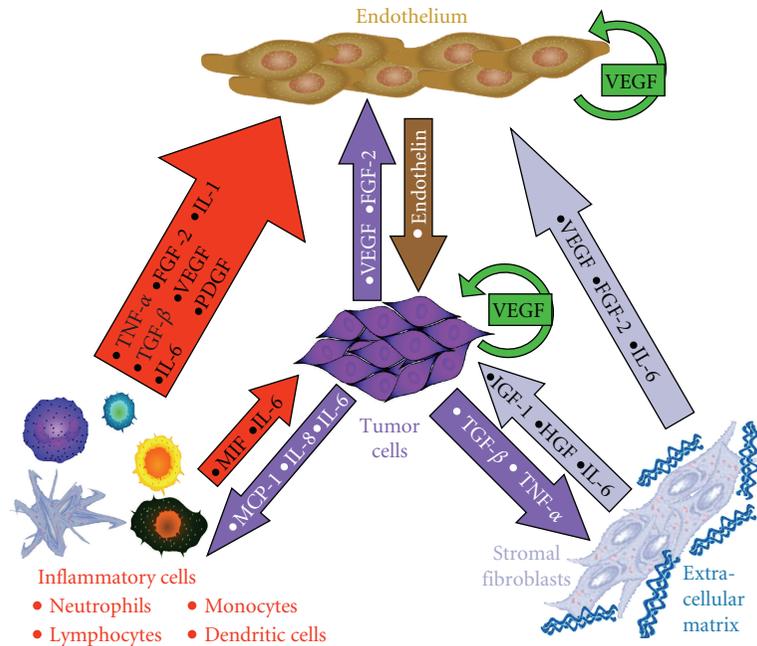


FIGURE 2: *Tumor-associated angiogenesis is sustained through stromal microenvironment crosstalk.* Most tumors are associated with the activation of tumor-promoting innate immune responses involving neutrophils, macrophages, and NK cells. Specific (adaptive) antitumor immune responses involving T- or B-lymphocytes are less efficient in suppressing tumor growth. Increased formation of blood and lymphatic vessels in bone marrow and lymph nodes provide oxygen and nutrients to malignant cells. Stromal cells, including ECs, inflammatory cells, and fibroblasts/myofibroblasts, produce cytokines and growth factors that act in a paracrine fashion to promote malignant cell proliferation or survival. In turn, malignant cells produce angiogenic factors and express their cognate receptors establishing functional autocrine loops to perpetuate their survival including signaling through the VEGF pathway [85–87, 107]. The secreted factors produced by and in response to those secreted by stromal and tumor cells include, but are not limited to VEGF, FGF-2, PDGF, IGF-1, HGF, TGF- $\alpha$ , TGF- $\beta$ , TNF- $\alpha$ , IL-8, MCP-1/CCL2, MIF, IL-6, and IL-1 [95]. The potent vasoconstrictor peptide endothelin-1 has been implicated in the pathophysiology of atherosclerosis and its complications [108], as well as tumor angiogenesis and lymphangiogenesis [109, 110]. Proteases important for invasion through the basement membrane and remodeling of the ECM, such as plasminogen [96] and MMPs, including MMP-2 and MMP-9 [97], and their inhibitors, PAI-1/2 and TIMPs, respectively, are produced by stromal and tumor cells. Downregulation of endogenous inhibitors of angiogenesis such as thrombospondin (TSP)-1 occurs in the stromal compartment as well to favor angiogenesis, cancer cell growth, and metastasis [98]. In recent years, it has been recognized that a better understanding of the tumor-stromal microenvironment crosstalk may lead to elucidation of new therapeutic strategies for cancer therapy [99–102].

(direct or indirect) are the RNA aptamer, pegaptanib; VEGF receptor decoy, VEGF-Trap (Aflibercept); small interfering RNA-based therapies, bevasiranib, and AGN211745; rapamycin, sirolimus; tyrosine kinase inhibitors including vatalanib, pazopanib, imatinib (Gleevec), TG100801, TG101095, AG013958, and AL39324; soluble VEGFRs; proteasome inhibitors, bortezomib (Velcade); thalidomide and derivatives.

At present, established therapies have been very successful in reducing the vision loss associated with AMD [135, 136]; however, a number of reports on the clinical outcomes of antiangiogenic therapy with VEGF inhibitors have shown equivocal results [88, 137–141]. Unfortunately, no significant survival benefit has been demonstrated in anti-VEGF monotherapy trials. When anti-VEGF inhibitors are used in combination with standard chemotherapeutic approaches for solid tumors, such treatment does not prolong survival of cancer patients for more than a few months [137–141], except as shown in phase II and phase III clinical trials for metastatic colon cancer and metastatic breast cancer where median survival over chemotherapy alone was extended

~15–26 months (reviewed in [142]). Although different classes of VEGF-targeted therapies inhibit primary tumor growth, recent studies surprisingly report that treatment with VEGF inhibitors leads to more invasive and metastatic tumors [139, 143]. Most patients who initially respond to VEGF-targeted therapy will develop resistance, and the molecular and cellular mechanisms promoting resistance are poorly understood [137, 138]. Thus, resistance or refractoriness of tumor ECs to treatment with VEGF inhibitors limits the utility of long-term treatment [143]. These findings indicate that new studies and molecular approaches are needed to overcome the lack of sensitivity or resistance of tumor ECs to antiangiogenic therapies.

**2.5. Targeting Transcription Factor Signaling Pathways Activated in Angiogenesis.** Although VEGF is upregulated in response to many inducers activated in cancer, only two major transcription factors have been identified for its promoter, hypoxia inducible factor (HIF)-1 and Stat3 [144]. Both HIF-1 production and Stat3 activity are upregulated in many types of cancer. VEGF is strongly induced by the

hypoxic tumor microenvironment before the tumor becomes vascularized, and thus, is important in hypoxic regulation of angiogenesis [145, 146]. HIF-1 is composed of the constitutively expressed HIF-1 $\beta$  subunit (aka the aryl hydrocarbon nuclear translocator/ARNT [146]) and an O<sub>2</sub>- and growth factor-regulated HIF-1 $\alpha$  subunit. HIF-1 $\alpha$  is also constitutively expressed but rapidly degraded under normoxia due to hydroxylation at two proline residues within the central degradation domain. Hydroxylation increases the affinity of HIF-1 $\alpha$  for the tumor suppressor protein von Hippel-Lindau (pVHL) E3 ligase complex, which mediates ubiquitination and proteasomal degradation of HIF-1 $\alpha$  thereby preventing formation of an active HIF-1 heterodimer [147]. Because the HIF hydroxylases have an absolute requirement for oxygen, hydroxylation is suppressed under hypoxic conditions allowing the HIF-1 $\alpha$  subunit to accumulate, translocate to the nucleus, and heterodimerize with HIF-1 $\beta$  to activate transcription of target genes [148].

Activation of the Jak/Stat3 pathway by IL-6 through its high affinity receptor, IL-6R $\alpha$ , and its binding partner, gp130, is a well-known inflammatory response evoked by the acute phase response of innate immunity [149, 150]. Stat3 is a latent transcription factor whose maximal activation requires both tyrosine (Y-705) and serine (S-727) phosphorylation. Inhibition of Stat3 activation blocks HIF-1 and VEGF expression in vitro and inhibits tumor growth and angiogenesis in vivo [151]. Activation of Stat3 signaling by various mitogens is prevalent in different types of cancers. Furthermore, when Stat3 is inhibited, tumor cells will no longer express proangiogenic mediators in response to IL-6R signaling. Because Stat3 is constitutively active in many types of cancers, it is considered oncogenic [152, 153]. Therefore, Stat3 is an apt upstream target for inhibiting tumor VEGF expression and angiogenesis [151].

NF- $\kappa$ B transcription factor links inflammation and tumorigenesis, and its activation allows both premalignant and malignant cells to escape apoptosis [154]. NF- $\kappa$ B signaling occurs in essentially all aspects of cancer progression from uncontrolled growth, evasion of apoptosis, tumor cell invasion through stromal compartments and into the blood stream, and sustained angiogenesis [104, 154]. Constitutive NF- $\kappa$ B activation is found in lymphoid and myeloid malignancies, including preneoplastic conditions, emphasizing its role in malignant transformation [155, 156]. More than 200 genes involved in cell survival, apoptosis, cell growth, immune responses and inflammation are transactivated by NF- $\kappa$ B [157]. NF- $\kappa$ B is sequestered in the cytoplasm by inhibitor proteins such as I $\kappa$ B $\alpha$  [104, 154–156]. Upon activation, proteasomal degradation of I $\kappa$ B $\alpha$  releases NF- $\kappa$ B, which then translocates to the nucleus to bind to the  $\kappa$ B response element in promoter regions of target genes. Thus, small inhibitory molecules that target these various steps are continually being sought for cancer treatment. PPAR $\gamma$  agonists have anti-inflammatory properties that are conferred, in part, through their ability to inactivate transcription factors that regulate inflammation including Stat3, NF- $\kappa$ B, and AP-1 [158–160]. The potential for PPAR $\gamma$  agonists as inhibitors of Stat3 and NF- $\kappa$ B survival signaling in hematological malignancies is discussed in Section 2.8.

*2.6. Angiogenesis and Targeted Antiangiogenic Therapy in Hematological Malignancies.* Since hematological malignancies originate in bone marrow and lymphatic organs and do not form solid tumor masses, it was generally believed that angiogenesis would not be as critical for cancer progression as in solid tumors. In the recent years, however, the importance of angiogenesis and lymphangiogenesis in hematological malignancies has been recognized and discussed in detail in a number of excellent reviews and references therein [113, 114, 116–119]. Because PPAR $\gamma$  agonists are being tested as inhibitors of angiogenesis, it is important to understand the role of angiogenesis and associated signal transduction pathways in the progression of hematological malignancies. Increased bone marrow microvessel density (MVD), an in vivo measure of tumor-associated angiogenesis, is found in hematological malignancies [161], confirming the importance of angiogenesis for malignant progression.

In general, increased MVD correlates with increased disease burden and poor prognosis or treatment outcome [118]. A number of antiangiogenic agents have been used to treat hematological malignancies as discussed in the review articles cited above. For example, thalidomide, well known as a potent teratogen causing stunted limb growth, has gained favor as an inhibitor of angiogenesis in multiple myeloma (MM) [162–167]. Thalidomide and similar immunomodulatory drugs and proteasome inhibitors (e.g., bortezomib) exert their effects directly by induction of apoptosis of MM cells or indirectly by inhibiting production of cytokines and proangiogenic factors, including VEGF, by bone marrow stromal cells (BMSCs) [162, 168]. The angiogenic activity of MM ECs correlates with downregulated expression of the endogenous antiangiogenic factor, endostatin [169]. Increased MVD in bone marrow correlates with shorter overall disease-free survival in AML, and elevated VEGF mediates both autocrine and paracrine signaling in support of leukemia cell survival and induction of angiogenesis [86, 87, 113, 161].

Angiogenesis in chronic lymphocytic leukemia (CLL) occurs in both marrow and lymph nodes [170]. Increased vascularity leads to elevated production of hematopoietic growth factors by new vessel ECs, which stimulates expression of VEGF and VEGFRs by CLL cells for autocrine signaling to promote survival [113, 170]. Elevated levels of VEGF are found in the serum of patients with chronic myeloid leukemia (CML), which correlates with worse survival [171]. Non-Hodgkin lymphoma (NHL) cells secrete VEGF and express VEGFRs, which also contribute to autocrine and paracrine signaling [172]. A phase II clinical trial of bevacizumab (Avastin) therapy in patients with relapsed, aggressive NHL showed a median increase in disease-free survival by 5.2 months [115], suggesting that anti-VEGF therapy is a limited but viable target for treatment. Antiangiogenic therapy would likely be more efficacious if combined with active chemotherapy regimens [115, 173]. Increased MVD in lymph nodes and elevated VEGF are statistically correlated with a greater tumor burden in Hodgkin lymphoma in newly diagnosed patients [174, 175]. Survival after treatment of diffuse large-B-cell lymphoma is adversely affected in patients whose tumor

stroma show elevated MVD, indicating that differences in the tumor microenvironment play a critical role in treatment outcomes [176]. However, the role of angiogenesis varies in lymphoma subtypes due to heterogeneity in expression of proangiogenic factors [113, 177].

In addition to agents targeting VEGF-VEGFR signaling directly, a number of agents have been developed to target the tumor microenvironment (reviewed in [99–102]), including ECM modulators, tyrosine kinase inhibitors, and immunomodulators, many of which indirectly target cancer angiogenesis. Nonetheless, autocrine VEGF signaling to promote malignant cell survival appears to be a common theme in hematological malignancies [85–87, 107, 113, 170, 172, 178], suggesting that anti-VEGF/VEGFR targeted therapy would promote direct killing of tumor cells, as well as inhibit angiogenesis associated with several types of hematological malignancies. It should be noted that antiangiogenic therapy in combination with conventional therapy for metastatic colon cancer and metastatic breast cancer significantly increased survival [142]; these cancers represent two of the three solid tumors (the third being brain cancer) for which published data most strongly support the presence of CSCs [59, 60]. In that CSCs have been documented in hematologic malignancies such as leukemia [58], it is interesting to speculate that patients with hematological malignancies other than leukemias may benefit from adding antiangiogenic therapy to standard treatments if CSCs could be identified in the malignant population of cells.

*2.7. Effects of PPAR $\gamma$  and PPAR $\gamma$  Ligands on EC Functions and Angiogenesis.* The endothelium releases a balance of bioactive factors that regulate vasoconstriction and relaxation to facilitate vascular homeostasis [179]. During homeostasis, the endothelium also inhibits platelet and leukocyte adhesion to the vascular surface and maintains the balance between prothrombotic and profibrinolytic activities. Several common conditions with a predisposition to atherosclerosis, including hypercholesterolemia, hypertension, diabetes, and stroke, are associated with endothelial dysfunction, leading to a proinflammatory and prothrombotic endothelium [180]. For more than a decade investigators have studied the effects of PPAR $\gamma$  ligands on EC functions with a particular interest in determining whether they could be used to inhibit cancer cell growth (reviewed in [25, 31, 181, 182]) and cancer-associated angiogenesis (reviewed in [23, 25, 31, 181–184]). The functions that PPAR $\gamma$  ligands target during angiogenesis include induction of apoptosis, inhibition of EC proliferation, downregulation of proangiogenic factors, and as inhibitors of the inflammatory events that trigger and perpetuate pathological angiogenesis (Table 3). In addition to targeting tumor angiogenesis, PPAR $\gamma$  ligands have direct effects on cancer cells due to their ability to promote apoptosis, inhibit cell proliferation or induce differentiation [3, 71, 185–188]. However, to date, disappointing results have been obtained in phase II clinical trials using the PPAR $\gamma$  ligand troglitazone to inhibit progression of treatment-refractory metastatic breast cancer [189], chemotherapy-resistant metastatic colorectal cancer [190], and prostate cancer [191]. In recent years, the focus has shifted from treating

the tumor to targeting the signaling pathways that drive aberrant cell proliferation and survival and tumor-associated angiogenesis. Such targets have the potential for greater specificity together with reduced systemic toxicity [104].

*2.8. Therapeutic Potential of PPAR $\gamma$  and PPAR $\gamma$  Ligands to Target Angiogenic Signaling Pathways in Treatment of Hematological Malignancies.* It has been suggested that PPAR $\gamma$  functions as a tumor suppressor gene [204]; therefore, it is important to understand the complexity of signal transduction pathways and molecular players affected by PPAR $\gamma$  that promote tumor growth, cancer-associated angiogenesis, and metastasis. MM, a progressive hematological malignancy of plasma cells, remains largely incurable with survival averaging 3–5 years despite conventional and high-dose therapies; therefore, novel treatment approaches are desperately needed. MM is characterized by excessive numbers of abnormal plasma cells in the bone marrow and overproduction of intact monoclonal immunoglobulin (IgG, IgA, IgD, or IgE) or Bence Jones protein (free monoclonal  $\kappa$  and  $\lambda$  light chains). Common clinical manifestations of MM are hypercalcemia, anemia, renal damage, increased susceptibility to bacterial or viral infection, and impaired production of normal immunoglobulins (<http://www.themrf.org/living-with-multiple-myeloma/newly-diagnosed-patients/what-is-multiple-myeloma/definition.html>). Lytic lesions are often found in the bone including the pelvis, spine, ribs, and skull. Furthermore, neovascularization in bone marrow parallels disease progression of MM [205].

Our laboratory has shown that normal and malignant B cells, including MM, express PPAR $\gamma$  [206–210], and that certain PPAR $\gamma$  ligands can induce apoptosis in MM cells [207, 208]. Because PPAR $\gamma$  ligands also have PPAR $\gamma$ -independent effects, we examined the functional consequences of PPAR $\gamma$  overexpression in human MM [207]. PPAR $\gamma$  overexpression in myeloma cells decreased cell proliferation, induced spontaneous apoptosis even in the absence of exogenous ligand, and enhanced their sensitivity to PPAR $\gamma$  ligand-induced apoptosis. Apoptosis was associated with the downregulation of anti-apoptotic proteins XIAP and Mcl-1 as well as induction of caspase-3 activity [207]. IL-6 mediates growth and survival of human myeloma cells through the MEK/MAPK and Jak/Stat signaling pathways, and IL-6 confers protection against dexamethasone-induced apoptosis via activation of the protein tyrosine phosphatase, SHP2 [211]. Binding of MM cells to BMSCs triggers expression of adhesive molecules and secretion of IL-6, promoting MM cell growth, survival, drug resistance, and migration. Furthermore, PPAR $\gamma$  overexpression-induced cell death of myeloma cells is not abrogated by coculture with BMSCs [207]. Overexpression of PPAR $\gamma$  in myeloma cells and BMSCs inhibited both basal and myeloma cell adhesion-induced IL-6 production by BMSCs. These results indicate that PPAR $\gamma$  negatively controls MM growth and viability, in part, through inhibition of IL-6 production by BMSCs [207]. Wang et al. [211] showed that myeloma cells express PPAR $\gamma$  and that the PPAR $\gamma$  agonists, 15d-PG $_2$  and troglitazone, abolish IL-6-inducible myeloma cell proliferation and promote apoptosis

TABLE 3: Effects of PPAR $\gamma$  agonists on endothelial cell function and angiogenesis.

Ref.	Goal of Study	Results	Pro- or antiangiogenic activity as shown	Direct or Indirect Effects
[192]	To determine whether PPAR $\gamma$ ligands induce EC proliferation or influence cytokine-induced proliferation in vitro.	PPAR $\gamma$ ligands troglitazone and pioglitazone negligibly affected basal EC proliferation in vitro; troglitazone and pioglitazone significantly inhibited FGF-2-induced EC proliferation.	• Antiangiogenic	Not reported
[22]	To determine effects of PPAR $\gamma$ ligands on in vitro and in vivo angiogenesis and EC proliferation.	15d-PGJ <sub>2</sub> , BRL49653, or ciglitazone, dose-dependently suppresses HUVEC differentiation into tube-like structures and cell proliferation; 15d-PGJ <sub>2</sub> downregulated VEGFR1, VEGFR2 and uPA and increased PAI-1 mRNA expression in vitro; 15d-PGJ <sub>2</sub> inhibited angiogenesis in vivo.	• Antiangiogenic activity • Anti-cell proliferation and anti-cell differentiation activity	Not reported
[193]	To determine whether human ECs express PPAR $\gamma$ and if PPAR $\gamma$ regulates PAI-1 expression in EC.	ECs expressed functionally active PPAR $\gamma$ ; PPAR $\gamma$ ligands (15d-PGJ <sub>2</sub> ) and oxidized linoleic acid regulated PAI-1 expression in ECs.	• Antiangiogenic activity by inhibiting fibrinolysis (fibrin induces angiogenesis)	Not reported
[194]	To determine the antiangiogenic effects of PPAR $\gamma$ agonists on CNV in vitro and on experimental laser photocoagulation-induced CNV in vivo.	PPAR $\gamma$ ligands troglitazone and rosiglitazone inhibited VEGF-induced migration and proliferation of human RPE cells and bovine CECs and tube formation of CEC in a dose-response manner; troglitazone inhibited CNV in rat and monkey eyes.	• Antiangiogenic activity in the eye • Anti-cell proliferation activity	Not reported
[195]	To determine whether PPAR $\gamma$ ligands inhibit cancer cell growth and cancer-associated angiogenesis.	PPAR $\gamma$ expressed in tumor EC; rosiglitazone suppressed primary tumor growth and metastasis; rosiglitazone inhibited bovine capillary EC but not tumor cell proliferation; rosiglitazone decreased VEGF production by tumor cells in vitro; rosiglitazone suppressed angiogenesis in vivo and in a variety of primary tumors.	• Antiangiogenic activity • Anti-EC but not tumor cell proliferation activity	Direct and indirect
[196]	To determine whether PPAR $\gamma$ ligands regulate PPAR $\gamma$ and CD36 gene expression in microvascular and large vessel EC in vitro and modulate TSP-1 peptide ABT510 antiangiogenic activity in tumor-associated endothelium in vivo (mouse tumor models).	15d-PGJ <sub>2</sub> , troglitazone, and rosiglitazone induced PPAR $\gamma$ and CD36 gene expression in EC in vitro and inhibited angiogenic endothelial functions in vitro and neovascularization in vivo in an additive manner; ABT510 and PPAR $\gamma$ ligands enhanced synergistically the antiangiogenic and antitumor effects of TSP-1 peptide ABT510.	• Antiangiogenic activity • Anti-proliferation activity in EC • Anti-invasion activity of EC • Cooperative inhibition of EC angiogenic functions • Synergistic inhibition of tumor angiogenesis	Direct for in vitro activities

TABLE 3: Continued.

Ref.	Goal of Study	Results	Pro- or antiangiogenic activity	Direct or Indirect Effects
[197]	To determine whether PPAR $\gamma$ agonists modulate bone marrow-derived bipotential APCs to promote endothelial lineage differentiation and re-endothelialization after vascular intervention.	Rosiglitazone promoted differentiation of bone marrow-derived APCs toward the endothelial lineage and attenuated restenosis after angioplasty in C57/BL6 mice; rosiglitazone inhibited APC differentiation toward smooth muscle cell lineage.	<ul style="list-style-type: none"> <li>• Proangiogenic activity</li> <li>• Anti-inflammatory</li> <li>• Promoted lineage-specific differentiation</li> </ul>	Not reported
[198]	To determine the efficacy of pioglitazone to inhibit corneal neovascularization.	PPAR $\gamma$ ligand pioglitazone decreased MVD in a VEGF-induced neovascularization in a rat cornea model.	<ul style="list-style-type: none"> <li>• Antiangiogenic activity in the eye</li> </ul>	Not reported
[24]	To determine whether PPAR $\gamma$ ligands can inhibit angiogenesis in A549 lung cancer cell xenograft in vivo and which signaling pathway is involved in vitro.	PPAR $\gamma$ ligands troglitazone and pioglitazone significantly inhibited A549 primary tumor growth in SCID mice, likely due to inhibition of cancer-associated angiogenesis; in vitro studies on A549 cells suggested PPAR $\gamma$ ligands inhibit chemokine expression and inhibit NF- $\kappa$ B activity; the transcription factor necessary for chemokine expression.	<ul style="list-style-type: none"> <li>• Antiangiogenic activity</li> <li>• Inhibited NF-<math>\kappa</math>B transcription factor activity</li> </ul>	Direct and indirect
[199]	To determine effects of PPAR $\gamma$ ligands on VEGF expression by human endometrial cells.	PPAR $\gamma$ ligands rosiglitazone and 15d-PGJ <sub>2</sub> repressed VEGF gene expression through a PPRE in the VEGF promoter.	<ul style="list-style-type: none"> <li>• Antiangiogenic activity</li> <li>• Identified PPRE in VEGF promoter</li> </ul>	Not reported
[200]	Because endothelial precursor cell (EPC) function is impaired in type 2 diabetic patients and EC dysfunction can be ameliorated by treatment with TZDs, this study asked whether TZDs affect the number and function of EPCs.	Rosiglitazone improved number and migratory activity of EPCs from type 2 diabetic patients; rosiglitazone increased the CD133+ subpopulation of CD34+ cells (stem cells); rosiglitazone increased circulating levels of VEGF; effects may be due to increased bioavailability of NO by Akt-dependent phosphorylation of eNOS—a pathway that is activated by VEGF or the insulin signaling cascade.	<ul style="list-style-type: none"> <li>• Proangiogenic activity</li> <li>• Akt survival pathway activated</li> <li>• Elevated CD133+/CD34+ stem cells towards EC lineage (VE-cadherin+ and CD31+)</li> </ul>	Not reported
[201]	To determine whether TZDs increase the number of bone marrow-derived EPCs in mice and the signaling pathways activated.	Treatment of mice with pioglitazone upregulated bone marrow and circulating EPCs; pioglitazone prevented apoptosis of human and mouse EPCs in a PI3K-dependent manner in vitro.	<ul style="list-style-type: none"> <li>• Proangiogenic activity</li> <li>• PI3K activated</li> <li>• Anti-apoptotic</li> </ul>	Not reported; indirect activation of PI3K-Akt not activated by pioglitazone
[27]	To study the effect of PPAR $\gamma$ agonists on VEGF- and FGF-2-induced angiogenesis and EC migration.	Pioglitazone and rosiglitazone inhibited the proangiogenic effects of FGF-2 and VEGF in the chick chorioallantoic membrane model angiogenesis; pioglitazone and rosiglitazone inhibited VEGF- and FGF-2-induced EC migration.	<ul style="list-style-type: none"> <li>• Antiangiogenic in vivo</li> <li>• Inhibited EC migration</li> </ul>	not reported
[28]	To determine whether activation of PPAR $\alpha$ and PPAR $\gamma$ stimulates angiogenesis.	PPAR $\alpha$ agonist WY14643 and PPAR $\gamma$ agonist GW1929 induced EC tube formation in EC/interstitial cell cocultures by increasing VEGF production; WY14643 and GW1929 induced angiogenesis in murine corneal angiogenesis model and Akt activated in vitro.	<ul style="list-style-type: none"> <li>• Proangiogenic activity</li> <li>• Induced VEGF production</li> <li>• Prosurvival</li> </ul>	Direct for both PPAR $\alpha$ and PPAR $\gamma$

TABLE 3: Continued.

Ref.	Goal of Study	Results	Pro- or antiangiogenic	Direct or Indirect Effects
[30]	To investigate the impact of diabetes on ischemia-induced collateral vessel growth, and tested the hypothesis that PPAR $\gamma$ agonists augment collateral flow to ischemic tissue.	Pioglitazone ameliorated endothelial dysfunction and enhanced blood flow recovery after tissue ischemia in diabetic mice; pioglitazone restored VEGF levels that were reduced by ischemic injury; Activation of eNOS essential for pioglitazone to promote angiogenesis in ischemic tissue.	<ul style="list-style-type: none"> <li>• Proangiogenic activity</li> <li>• Induced VEGF production</li> </ul>	Not reported
[202]	To determine effects of rosiglitazone on gastric cancer cell cycle, proliferation, migration, and invasion; endothelial capillary tube formation (an in vitro measure of angiogenesis).	Rosiglitazone inhibited gastric cancer cell growth, caused G1 cell cycle arrest and induced apoptosis in a dose-dependent and PPAR $\gamma$ -dependent manner; rosiglitazone inhibited gastric cancer cell migration, invasion, and expression of MMP-2 in a dose-dependent manner in a PPAR $\gamma$ -independent manner; rosiglitazone reduced VEGF-induced “angiogenesis” of HUVEC in a dose- and PPAR $\gamma$ -dependent manner.	<ul style="list-style-type: none"> <li>• Antiangiogenic activity</li> <li>• Antitumor cell proliferation activity</li> <li>• Anti-invasion</li> <li>• Proapoptotic</li> </ul>	Not reported
[32]	To determine the effects of PPAR $\gamma$ ligands on pancreatic cancer-associated angiogenesis, VEGF expression, and tumor growth in vitro and in vivo.	Rosiglitazone inhibited pancreatic carcinoma growth both in vitro and in vivo; rosiglitazone suppressed xenograft tumor angiogenesis by downregulating VEGF expression; 15d-PGJ <sub>2</sub> , 9-cis-RA, and their combination inhibited VEGF mRNA expression in PANC-1 cells in a dose- and time-dependent manner; MVD was decreased in rosiglitazone-treated mice.	<ul style="list-style-type: none"> <li>• Antiangiogenic activity</li> <li>• Antitumor cell proliferation activity</li> </ul>	Not reported
[203]	To determine whether adipose tissue angiogenesis was stimulated by rosiglitazone using an assay to study angiogenic sprout formation ex vivo.	Obesity and TZD treatment in vivo induced angiogenic sprout formation from adipose tissue fragments, but not from aorta rings; rosiglitazone induced expression of VEGF-A, VEGF-B, and ANGPTL4; ANGPTL4 stimulated EC growth and capillary tube formation; ANGPTL4 alleviated the growth inhibitory actions of rosiglitazone on ECs in the presence or absence of VEGF likely causing a net expansion of the capillary network in adipose tissue in response to PPAR $\gamma$ activators.	<ul style="list-style-type: none"> <li>• Proangiogenic activity in adipose tissue</li> <li>• Induced VEGF production</li> <li>• Induced ANGPTL4 expression</li> </ul>	Indirect likely via a PPAR $\gamma$ -stimulated adipocyte-specific factor ANGPTL4 capable of overcoming direct antiangiogenic effect of rosiglitazone on ECs

in a PPAR $\gamma$ -dependent manner. These PPAR $\gamma$  agonists also reduced cell-cell adhesion between BMSCs and MM cells and overcame resistance to dexamethasone-mediated apoptosis in the MM.1R cell line through a PPAR $\gamma$ -dependent mechanism [212]. Taken together, the results of these studies demonstrate that PPAR $\gamma$  agonists can be used to inhibit IL-6-dependent crosstalk between myeloma cells and BMSCs [207, 211, 212], validating novel therapeutic strategies that target the tumor-stromal microenvironment.

Dankbar and colleagues [205] demonstrated that biologically active VEGF is expressed and secreted by myeloma cell lines and plasma cells isolated from the marrow of patients with MM. However, the myeloma cells did not express or only weakly expressed VEGFR1 and VEGFR2, indicating that autocrine VEGF signaling in MM is unlikely. In contrast, they demonstrated that BMSCs abundantly express VEGFR2 and that such expression could be stimulated in response to IL-6. In addition, exposure of BMSCs and microvascular ECs to VEGF induced a time- and dose-dependent increase in IL-6 secretion. They showed that IL-6-stimulated VEGF expression in and secretion from myeloma cell lines and in plasma cells purified from the marrow of patients with MM as well. Thus, this study confirms that paracrine interactions between myeloma and marrow stromal cells triggered by VEGF and IL-6 represent feasible signal transduction pathways to target for treatment of MM [205].

PPAR $\gamma$  ligands are known to inhibit or repress the activity of a number of transcription factors important in innate immunity, inflammation and cancer, including Stat3 and NF- $\kappa$ B [158, 159]; therefore, targeted inhibition of Stat3 and NF- $\kappa$ B activity with PPAR $\gamma$  agonists is a relevant avenue of investigation for new cancer therapeutics [213]. Wang and colleagues [211] showed that 15d-PGJ<sub>2</sub> and troglitazone significantly inhibited Stat3 binding to its cognate response element and inhibited Stat3 binding to the promoters of c-MYC and MCL-1 thereby preventing transactivation of these Stat3 target genes. Whereas 15d-PGJ<sub>2</sub> promotes direct binding of PPAR $\gamma$  to Stat3 forming a complex such that Stat3 is no longer capable of binding to the type II IL-6 response element on promoters of Stat3 target genes, troglitazone induces the redistribution of the corepressor NCoR/SMRT from PPAR $\gamma$  to Stat3, which leads to repression of Stat3 transactivation of target genes [211] (Figure 3(a)). In contrast, 15d-PGJ<sub>2</sub> and troglitazone did not affect the expression of IL-6R or activation by phosphorylation of the downstream signaling molecules Jak/Stat3, MAPK, and PI3K/Akt in myeloma cells [211].

PPAR $\gamma$  and its ligands effectively blocked IL-6 transcription and secretion from BMSCs that is induced in response to myeloma cell adhesion [212]. Such inhibition occurs through competition between PPAR $\gamma$  and NF- $\kappa$ B for the coactivator PGC-1, which is recruited from p65/p50 complexes by ligand-activated PPAR $\gamma$  (Figure 3(b)). Direct complex formation between PPAR $\gamma$  and C/EBP $\beta$  also prevents transactivation of the IL-6 promoter. The natural PPAR $\gamma$  ligand, 15d-PGJ<sub>2</sub>, has a PPAR $\gamma$ -independent effect on NF- $\kappa$ B by decreasing phosphorylation of IKK and I $\kappa$ B to prevent activation of NF- $\kappa$ B [212]. Prolonged treatment with the PPAR $\gamma$  ligand CDDO-Me inactivates

Erk signaling in AML cells effectively inhibiting cell growth [214]. In vitro studies show that CDDO-Me inactivates Stat3, Src, and Akt; reduces expression of the c-MYC gene; promotes accumulation of cells in the G2-M phase of the cell cycle; and, abrogates invasive growth and induction of apoptosis of mammary cells [215]. Furthermore, mammary cell growth and lung metastases were completely eliminated in mice treated with CDDO-Me starting one day after tumor implantation; tumor growth was significantly inhibited when started after 5 days. Thus, CDDO-Me may have therapeutic potential for hematological malignancies and solid tumors through inactivation of Stat3 [215].

Bortezomib (Velcade, formerly PS-341) is a proteasome inhibitor that is used for antiangiogenic therapy in various cancers including MM [216]. Bortezomib targets myeloma cells and also inhibits the binding of myeloma cells to BMSCs. Furthermore, intravenous bortezomib, with or without dexamethasone, is well tolerated and effective in treating patients with relapsed or refractory MM [216]. Because bone marrow angiogenesis plays an important role in the pathogenesis and progression of MM and bortezomib inhibits angiogenesis, Roccaro and colleagues [217] tested the effects of bortezomib on the angiogenic phenotype of MM patient-derived ECs (MMECs). At clinically relevant concentrations, bortezomib inhibited the proliferation of MMECs and human umbilical vein endothelial cells (HUVECs) in a dose-dependent and time-dependent manner. Bortezomib also inhibited angiogenesis as measured by capillary tube formation on Matrigel in vitro and in the chick embryo chorioallantoic membrane assay in vivo [217]. Furthermore, binding of drug sensitive MM cells (MM.1S) to MMECs triggered their proliferation, which was prevented by bortezomib. Bortezomib also triggered a dose-dependent inhibition of VEGF and IL-6 production by and secretion from MMECs and abrogated IL-6 triggered signaling cascades via caspase-dependent downregulation of gp130 in MM [218]; gp130 is the signaling component of the high affinity IL-6R complex that activates Stat3. These data provide mechanistic insight on the antiangiogenic effects of bortezomib on MMECs in the bone marrow microenvironment [217] and support the concept that adding antiangiogenic agents as adjuvant or combination therapy with standard therapy would be more efficacious in treating patients with relapsed or refractory MM [219], and perhaps other hematological malignancies as well.

Although inhibiting IL-6 signaling through its high affinity receptor promotes apoptosis of MM cells when cocultured with BMSCs, some myeloma cells survive suggesting that the marrow microenvironment stimulates IL-6-independent pathways that exert a prosurvival effect [220]. BMSCs stimulate MAPK signaling in myeloma cells through IL-6R-independent mechanisms thereby circumventing the need for Stat3-mediated signaling in response to IL-6 for myeloma cell survival. Chatterjee et al. [220] went on to show that disruption of both the IL-6R/Stat3 and MAPK signaling pathways led to significantly more apoptosis of MM cell lines and primary MM cells even in the presence of BMSCs than singly inhibiting each signaling pathway. These results suggest that combined targeting of different and independently

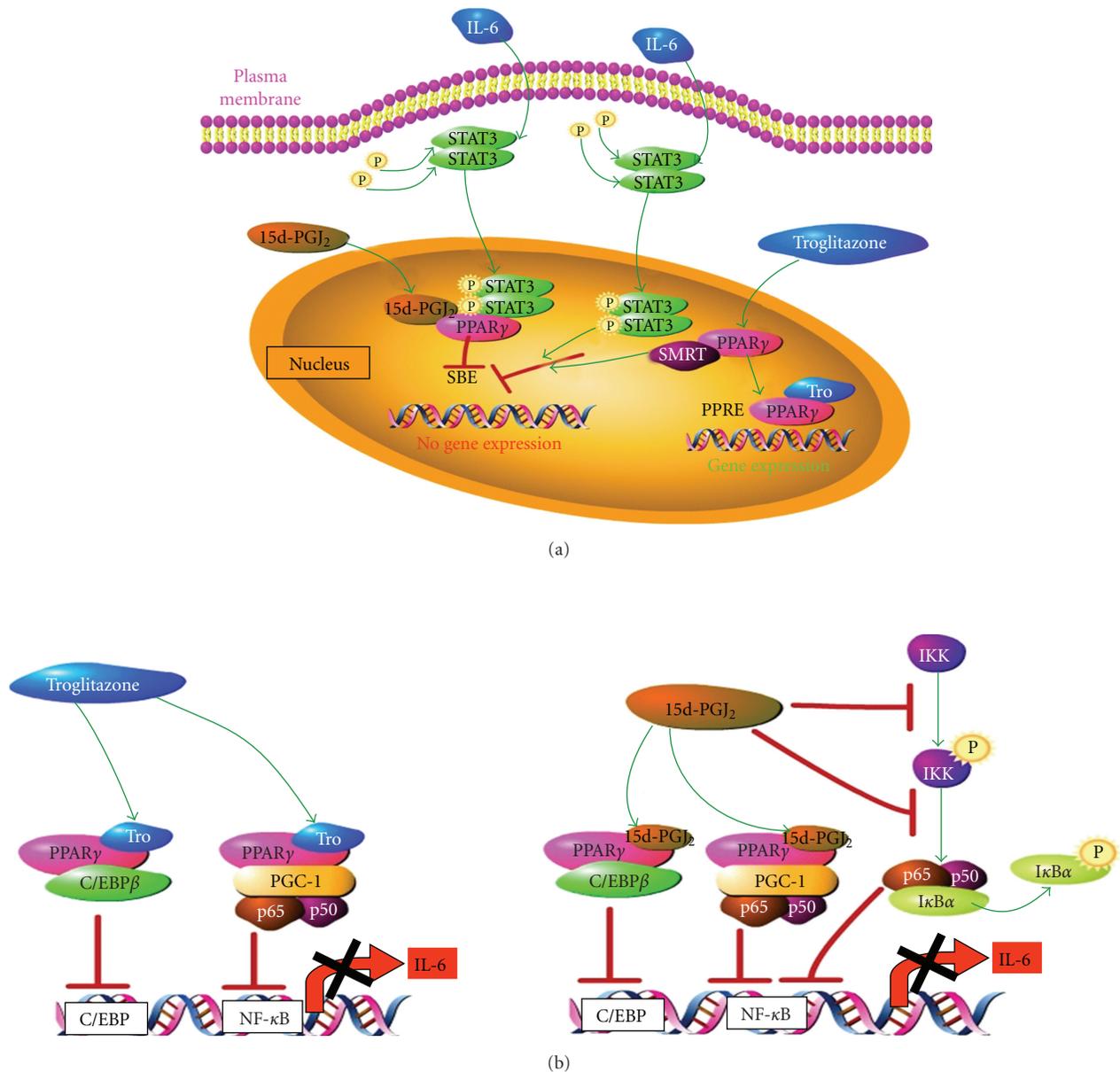


FIGURE 3: PPAR $\gamma$  agonists inhibit Stat3-mediated IL-6 gene expression in myeloma cells. Inactivation of IL-6-activated Stat3 by PPAR $\gamma$  agonists occurs in a PPAR $\gamma$ -dependent manner; however, the molecular mechanisms by which two distinct PPAR $\gamma$  agonists (15d-PGJ<sub>2</sub> and troglitazone) suppress IL-6-activated Stat3 in MM cells differ as shown in (a) [211]. Direct complex formation between phosphorylated Stat3 and PPAR $\gamma$  activated by 15d-PGJ<sub>2</sub> prevents Stat3 binding to its cognate response element (SBE) on the promoters of target genes ((a), left). This mode of transcriptional inactivation does not require binding of the activated PPAR $\gamma$  transcription factor to DNA in the promoter region and, thus, can occur in the absence of a PPRE. However troglitazone activated PPAR $\gamma$  promotes redistribution of the corepressor SMRT from PPAR $\gamma$  to phosphorylated Stat3 so that Stat3 can no longer recruit the transcriptional machinery necessary for gene expression ((a), right) [211]. High levels of IL-6 are found in MM and promote myeloma cell proliferation and survival and indirectly promote tumor-associated angiogenesis. The PPAR $\gamma$  agonists troglitazone and 15d-PGJ<sub>2</sub> have been shown to inhibit transcription of the IL-6 promoter driven by C/EBP $\beta$  and NF- $\kappa$ B [212]. Troglitazone-activated PPAR $\gamma$  binds to C/EBP $\beta$  preventing binding to its cognate response element on the IL-6 promoter, which is the major mechanistic pathway of troglitazone-mediated downregulation of IL-6 expression. In addition activated PPAR $\gamma$  competes with NF- $\kappa$ B for the PGC-1 coactivator, which leads to decreased NF- $\kappa$ B binding to the  $\kappa$ B response element on the IL-6 promoter contributing to inhibition of IL-6 gene expression, albeit to a lesser extent than inhibition of C/EBP $\beta$  ((b), left). A slightly different mechanistic emphasis on PPAR $\gamma$ -mediated inhibition of IL-6 gene expression occurs in response to 15d-PGJ<sub>2</sub>. Although 15d-PGJ<sub>2</sub>-activated PPAR $\gamma$  inhibits C/EBP $\beta$ -mediated transactivation of the IL-6 promoter similarly to troglitazone-activated PPAR $\gamma$ , the predominant mode of inhibition is through 15d-PGJ<sub>2</sub>-activated PPAR $\gamma$  using the coactivator PGC-1 as a bridging protein to interact with NF- $\kappa$ B to prevent transactivation of the IL-6 promoter. Furthermore, 15d-PGJ<sub>2</sub> inactivates NF- $\kappa$ B by inhibiting phosphorylation of IKK and I $\kappa$ B independently of PPAR $\gamma$  activation ((b), right). The schematics in this figure were adapted from [211, 212].

activated pathways is required to efficiently induce apoptosis of MM cells in the marrow microenvironment [220].

It should be kept in mind that anti-VEGF/VEGFR-targeted therapy could occur through a number of mechanistic pathways, such as direct inhibition of VEGF-induced angiogenesis or indirectly through mechanisms that inhibit expression of additional proangiogenic factors, promote apoptosis, or induce tumor dormancy [88, 221]. Rather than target the VEGF-signaling pathway, it may be possible to alter the phenotype of the angiogenic endothelium. The angiogenic EC phenotype is characterized by marked downregulation of CD36/fatty acid translocase (FAT) [222]. CD36 is a glycoprotein associated with normal and pathologic processes including scavenger receptor functions, lipid metabolism and fatty acid transport, cell adhesion, angiogenesis, modulation of inflammation, activation of TGF- $\beta$ , atherosclerosis, diabetes, and cardiomyopathy [223]. PPAR $\alpha$  regulates expression of CD36 in mouse liver and PPAR $\gamma$  regulates its expression in mouse adipose tissues [224, 225]. Furthermore, statins and PPAR $\gamma$  ligands together have an additive effect on upregulation of CD36 production by potentiating the transcription of the CD36 gene in monocytes [226]. CD36 is the cellular receptor for TSP-1 on microvascular endothelium and is necessary for its antiangiogenic, proapoptotic activity, making CD36 an attractive target for development of therapeutic agents [227].

Vascular endothelium expression of CD36 is sporadic however, with lower levels of expression in larger vessels [196, 228]. As discussed in Section 2.4, loss of endogenous inhibitors of angiogenesis in favor of proangiogenic factors produced by tumors leads to tumor-associated angiogenesis. A small peptide (ABT510) derived from TSP-1 type 1 repeats binds to CD36 and blocks tumorigenesis by reversing the “angiogenic switch” [229]. Huang et al. [196] demonstrated that 15d-PGJ<sub>2</sub>, troglitazone, and rosiglitazone potentiate the antitumor activity of AB510 in a CD36-dependent manner. Furthermore, these ligands upregulated EC expression of PPAR $\gamma$  and CD36 [43, 196], which likely leads to the synergistic inhibition of tumor-associated angiogenesis and induction of EC apoptosis in vivo [196]. Importantly, lower doses of PPAR $\gamma$  agonists could be used in combination with AB510 to significantly reduce tumor-associated angiogenesis and promote EC apoptosis. This study provides compelling evidence that PPAR $\gamma$  ligands could be useful as adjuvant or combination therapy in treatment of tumor angiogenesis.

Another important molecular mechanism to target for intervention of cancer progression in hematological malignancies is regulation of stromal matrix remodeling by proteases [193, 230]. PAI-1 production by ECs inhibits plasmin-mediated proteolytic degradation of the ECM. PPAR $\gamma$  ligands upregulate expression and release of PAI-1 from ECs [193], which would inhibit degradation of tumor-associated fibrin leading to EC migration, proliferation, and angiogenesis [231]. PPAR $\gamma$  ligands inhibit the adhesion of the myeloid leukemia HL-60 and K562 cells to the ECM as well as their invasion through Matrigel [230]. In addition, 15d-PGJ<sub>2</sub> and troglitazone in both the HL-60 and K562 cell lines significantly inhibited MMP-9 and MMP-2 expression and proteolytic activities. The results of this study suggest

that PPAR $\gamma$  ligands may inhibit leukemic cell adhesion to and invasion through the ECM as well as regulate angiogenesis by inhibiting matrix remodeling that favors cancer cell invasion and EC migration [230].

*2.9. MicroRNAs and PPAR $\gamma$  Agonists in Hematological Malignancies.* MicroRNAs (miRNAs) are short noncoding RNAs that function as negative regulators of the stability and/or translation of specific target mRNAs [232–234]. Typically, miRNAs target a cluster of genes instead of one specific gene, and a single miRNA can have more than 100 targets [233, 235]. Regulation of gene expression by miRNAs is increasingly being accepted as a pivotal point in cell function, either in normal development or disease states (recently reviewed in [234, 236–238]). Mature miRNAs derive from primary miRNA transcripts that are either transcribed from their own promoter regions [239] or processed introns spliced from pre-mRNAs [240]. Primary miRNAs are first processed in the nucleus by the RNase III endonuclease, Drosha, to form pre-miRNAs [241]. Pre-miRNAs are exported out of the nuclear compartment into the cytoplasm by exportin-5 [242]. Once in the cytoplasm, the pre-miRNA is further processed by another RNase III endonuclease, Dicer [243]. Finally, the mature miRNA is loaded onto the Argonaute (Ago) protein and incorporated into the ribonucleoprotein complex, RISC (RNA induced silencing complex) [244], which directs the miRNA to its target mRNA. Mature miRNAs primarily bind to transcripts through imperfect Watson-Crick base pairing to conserved miRNA binding sites in the 3′ untranslated region (UTR) of target mRNAs [234, 245]. The ability of miRNAs to regulate the expression of numerous genes at once often leads to pleiotropic effects and can modulate multiple cellular pathways.

There is growing evidence that dysfunctional expression of miRNAs is a common feature of malignancy in general and hematological malignancy in particular [233, 246]. Aberrant miRNAs have been documented in almost all hematological malignancies [247]. For example, Calin and colleagues [248] first implicated miRNAs in hematological malignancies when they demonstrated that miR-15 and miR-16 are frequently deleted or downregulated in CLL associated with deletions on chromosome 13q14. Deletion or downregulated expression of miR-15a and miR-16 on chromosome 13 is also found in MM cells [249]; deletion of chromosome 13 predicts significantly reduced survival in patients with MM [250]. In 2005, another group reported that the polycistronic precursor transcript of the miR-17~92 cluster, which encodes seven different miRNAs, is overexpressed in human B cell lymphomas and acts as an oncogene [251]. The miR-17~92 cluster is amplified and/or overexpressed in other hematological malignancies including AML [252, 253] and MM [246], as well as cancers of epithelial origin such as lung [254], thyroid [255], and hepatocellular [256] carcinomas. Overexpression of miR-21 occurs in MM [246, 257] and other cancers including glioblastoma [258] and breast cancer [259]. Thus, there is enormous hope that miRNA research will provide breakthroughs in the understanding of cancer pathogenesis and in the development of new prognostic markers [260].

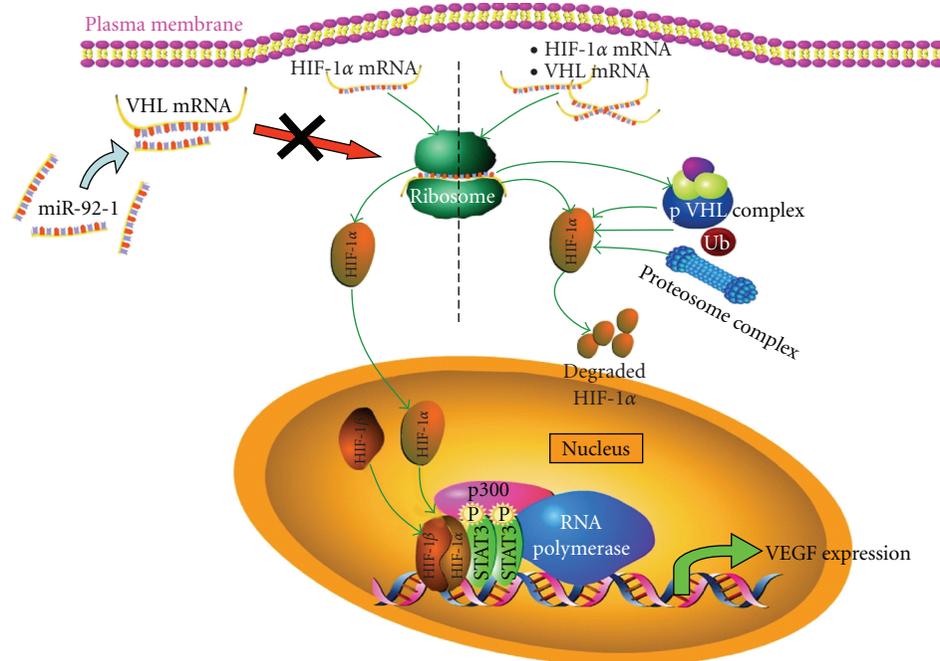


FIGURE 4: Autocrine production of VEGF in CLL B cells is regulated by miRNA-92-1 inhibition of pVHL production. Expression of high levels of VEGF by tumor cells is critical to promote and sustain the angiogenesis needed for cancer progression. Under normal oxygen tension, the HIF-1 $\alpha$  subunit of the transcription factor, HIF-1, is constitutively produced and rapidly degraded by pVHL-induced proteasomal degradation, which prevents transcription of the VEGF gene. In solid tumors, HIF-1-induced VEGF expression occurs when tumor growth exceeds the dimensions where existing blood vessels can feed the tumor and carry away waste products. The resulting hypoxia leads to stabilization of HIF-1 $\alpha$  and activation of the HIF-1 heterodimer resulting in high VEGF production by tumor cells. Although solid tumors do not develop in hematological malignancies, angiogenesis is an important process of disease progression. CLL B cells constitutively express high levels of VEGF and VEGFRs leading to autocrine signaling and increased resistance to apoptosis. Recently, Ghosh et al. [273] discovered that HIF-1 is stabilized in CLL B cells due to low levels of pVHL as a result of miR-92-1 overexpression and subsequent repression of translation of the VHL transcript. Therefore, HIF-1 accumulates and translocates to the nucleus where it forms an active complex with the transcriptional coactivator p300 and phosphorylated Stat3 and, together with the basal transcription machinery, transactivates the VEGF promoter. PPAR $\gamma$  agonists could potentially inhibit overexpression of VEGF by inhibiting Stat3 signaling in CLL B cells. The schematic in this figure was adapted from [273].

Kuehbacher et al. [261] recently reviewed miRNAs that possess proangiogenic or antiangiogenic function. The miR-17~92 cluster, let-7f, and miR-27b possess proangiogenic functions, in part, by inhibiting expression of TSP-1 and CTGF. A role for miR-221 and miR-222 in blocking angiogenesis is suggested by their ability to inhibit EC migration, proliferation, and angiogenesis in vitro. In addition, miR-21 is implicated in the invasive and metastatic properties of colon and breast cancer cell lines by targeting multiple tumor suppressor genes, such as PTEN, TPM1, and MASPIN [259, 262, 263]. Moreover, miR-21 overexpression, which occurs in MM as discussed below, is associated with advanced clinical disease, lymph node metastasis and poor prognosis for overall survival in breast cancer [264]. The Sessa group demonstrated that a functional miRNA biogenesis pathway is required for angiogenesis [265, 266]. Inactivation of Dicer, the miRNA processing enzyme, impairs angiogenesis induced by multiple stimuli such as VEGF, and during tumorigenesis and wound healing [266]. VEGF also induces the expression of several proangiogenic miRNAs including the miR-17~92 cluster [266]. Furthermore, miR-130a

functions in angiogenesis by inhibiting expression of two antiangiogenic homeobox transcription factors, HOXA5 and GAX [267].

Although the mechanisms regulating expression of miRNAs are only beginning to be understood [234, 236–238, 268, 269], key regulators of the biosynthetic pathway are often abnormally expressed in hematological malignancies [270]. Recently, Löffler and colleagues [257] demonstrated that survival of IL-6-dependent MM cells involves Stat3-mediated induction of miR-21. Two bona fide IL-6 type II-response elements for Stat3 binding are located upstream of the miR-21 genes of various vertebrate species [257]. Stat3 regulates transactivation of several anti-apoptotic genes such as survivin, Bcl-2, and Mcl-1. Löffler et al. [257] suggest that Stat3 induction of miR-21 represents a “slow-acting yet long-lasting” survival stimulus to complement the immediate induction of anti-apoptotic proteins. The cancers in which miR-21 is overexpressed contain constitutively activated Stat3 for survival or growth [257]. These results suggest that miR-21 is important for the oncogenic potential of Stat3 in the pathogenesis of MM and other malignancies.

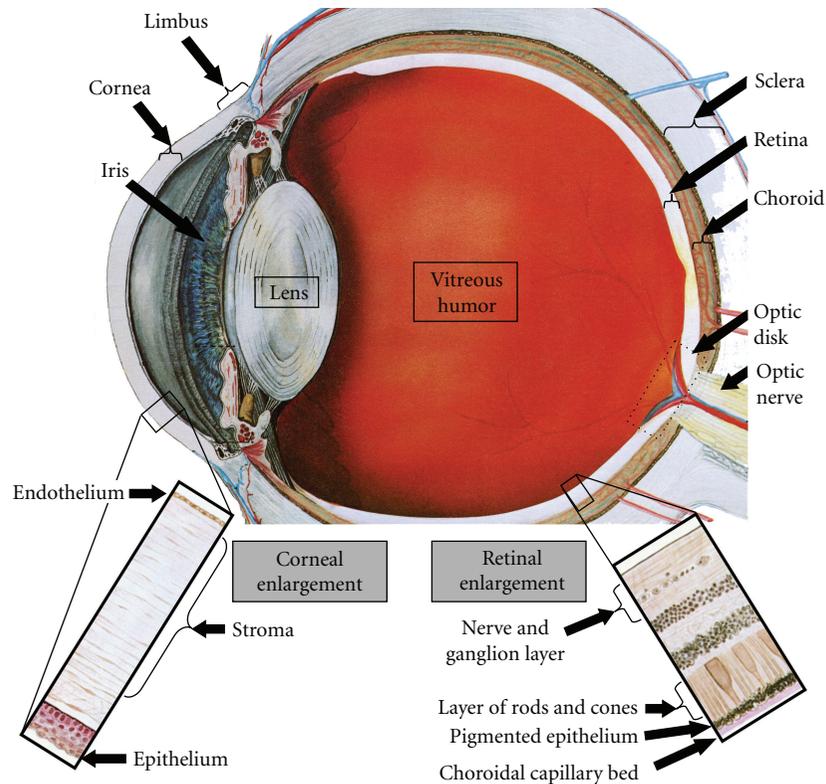


FIGURE 5: PPAR $\gamma$  is broadly expressed in the eye providing a pharmacological target for treating ocular angiogenesis. PPAR $\gamma$  expression is found in the retina including RPE cells, REC, pericytes [287], and ganglion cells. In the cornea, PPAR $\gamma$  is most prominently localized in the epithelial and endothelial layers. Excessive angiogenesis is a pathological hallmark of a number of eye diseases, and anti-VEGF/VEGFR strategies are used therapeutically to treat ocular neovascularization. Manifestations of hematological malignancies in the eye have been documented for leukemia, lymphoma, and multiple myeloma. The potential benefits of PPAR $\gamma$  agonist therapy to inhibit tumor-associated angiogenesis could also be applied to treatment of neovascular eye diseases.

IL-6-mediated activation of Stat3 is also important for transformation of nonmalignant breast epithelial cells to self-renewing mammospheres that contain CSCs [271]. Inflammation in cancer leads to elevated IL-6 production by two mechanisms: Src-mediated activation of NF- $\kappa$ B leading to transactivation of the IL-6 gene, and rapid degradation of let-7 miRNA, which is a direct inhibitor of IL-6 expression [271]. Let-7 is downregulated in some cancers including Burkitt lymphoma [272] thereby leading to elevated IL-6 production, likely due to activation of the oncogenic NF- $\kappa$ B-IL-6-Stat3 inflammatory pathway. In that the PPAR $\gamma$  agonist CDDO-Me inactivates Src and Stat3 in cancer cells [215], further investigation of the efficacy of various PPAR $\gamma$  ligands as anticancer agents is certainly warranted.

Recently, Roccaro and colleagues [249] identified a multiple myeloma-specific miRNA signature characterized by downexpression of miR-15a and miR-16 and overexpression of miR-222, miR-221, miR-382, miR-181a, and miR-181b in bone marrow-derived CD138<sup>+</sup> MM cells. Both miR-15a and miR-16 regulate proliferation and growth of plasma cells by inhibiting Akt and MAPK cell survival signaling pathways. However, both miR-15a and miR-16 are deleted on chromosome 13 associated with MM [249] thereby preventing normal repression of cell proliferation during cancer

progression. Pichiorri et al. [246] also identified an miRNA signature associated with MM pathogenesis. Overexpression of miR-21, the miR-106b~25 cluster, and miR-181a and miR-181b was found in MM and monoclonal gammopathy of undetermined significance (MGUS) samples. On the other hand, selective upregulation of miR-32 and the miR-17~92 cluster was identified only in MM cells. Expression of suppressor of cytokine signaling (SOCS)-1, involved in negative feedback regulation of Jak/Stat signaling, is downregulated by miR-19a and miR-19b thereby leading to sustained IL-6-mediated MM cell proliferation [246]. Furthermore, miR-19a, miR-19b, miR-181a, and miR-181b antagonists suppress human MM tumor cell growth in nude mice, suggesting that miRNAs that modulate the expression of proteins critical to myeloma pathogenesis, including the IL-6-regulated Stat3 pathway, are potential targets for development of new therapeutic strategies for treatment [246].

The Stat3-regulated gene, HIF-1 $\alpha$ , is constitutively expressed under normoxia in CLL B cells, most likely as a result of low production of pVHL, which is responsible for HIF-1 $\alpha$  degradation. Ghosh and colleagues [273] demonstrated that overexpression of miR-92 in CLL B cells targets the VHL transcript to repress its translation (Figure 4). Furthermore, stabilized HIF-1 $\alpha$  forms an active complex

with the transcriptional coactivator p300 and activated Stat3 on the VEGF promoter, which likely explains the anomalous autocrine VEGF secretion from CLL B cells [273]. In that PPAR $\gamma$  agonists inhibit the IL-6-regulated Stat3 signaling cascade, a role for PPAR $\gamma$  agonists in regulating expression of miRNAs critical to the pathogenesis of hematological malignancies may be an important avenue of future scientific investigations.

Recently, miRNAs have emerged as epigenetic regulators of metabolism and energy homeostasis [274]. It is clear that there is an obesity epidemic in the United States [275]. Increased body weight is associated with increased mortality for most all types of cancers including hematological malignancies [276]. Additional studies have confirmed that obesity puts patients at a moderate increased risk of developing MM [276–279], and that this risk may be higher in women than men [279]. An important link between obesity and MM is elevated expression of IL-6 in adipose tissue [280] and bone marrow [207], which also leads to elevations in circulating IL-6. Lin et al. [274] demonstrated that the miR-27 gene family is downregulated during adipogenic differentiation. Furthermore, overexpression of miR-27 specifically inhibits adipocyte formation and expression of PPAR $\gamma$  and C/EBP $\alpha$ , the two master transcriptional regulators of adipogenesis. Although PPAR $\gamma$  and C/EBP $\alpha$  mRNA and protein levels were markedly reduced by miR-27a or miR-27b, it was not a direct miRNA effect [274]. Expression of miR-27 is increased in fat tissue of obese mice and is regulated by hypoxia, an important extracellular stress associated with both obesity and cancer. During adipogenesis the expression of miR-27b, an important regulator of angiogenesis, is downregulated in human adipogenic stem cells, and PPAR $\gamma$  mRNA expression increases concomitantly with decreasing miR-27b expression [281]. Both miR-27a and miR-27b directly bind RXR $\alpha$  mRNA and regulate RXR $\alpha$  translation in rat hepatic stellate cells [282]. It is well known that RXR $\alpha$  heterodimerizes with PPAR $\gamma$  to activate numerous genes required for adipogenesis and energy metabolism. These data suggest that miR-27 represents a new class of adipogenic inhibitors and their downregulation may play a role in the pathological development of obesity [274]. Furthermore, in that PPAR $\gamma$  is a master regulator of adipogenesis and target of insulin sensitizing drugs, it is reasonable to consider that the beneficial effects of PPAR $\gamma$  agonists in cancer treatment may be exerted through regulation of energy homeostasis, at least in part, by modulation of miRNA expression. Indeed, the anticarcinogenic activity of one of the triterpenoids is due to repression of oncogenic miR-27a [283].

All known forms of the human PPAR $\gamma$  mRNA contain numerous miRNA binding sites in the 3'UTR as predicted through different bioinformatic algorithm databases (TargetScan [284], miRanda [285], PicTar [286]). The miRNA binding sites for miR-27a/b, miR-130a/b, miR-301, miR-34a/b in the PPAR $\gamma$  3'UTR are conserved in human, chimp, mouse, and rat. Notably, two conserved binding sites for miRNAs in the PPAR $\gamma$  3'UTR are for miR-27b and miR-130a that have angiogenic or proliferative functions. It would be interesting to determine whether these miRNAs suppress PPAR $\gamma$  function during angiogenesis and/or tumor growth.

This could lead to identification of novel targets that may induce PPAR $\gamma$  expression leading to the anticancer functions of cell differentiation and loss of proliferation. However, a role for PPAR $\gamma$  agonists in miRNA-based therapeutic strategies to treat cancer awaits further clarification by new research endeavors.

### 3. Anticancer Role of PPAR $\gamma$ Agonists as Adjuvant or Combination Therapy in Hematological Malignancies of the Eyes

**3.1. Ocular Hematological Malignancy.** Ocular lymphoma is relatively uncommon, accounting for 5–10% of all extranodal lymphomas [288]. However, it is one of the most common orbital malignancies and it is increasing in incidence because of its association with the acquired immunodeficiency syndrome (AIDS) [289]. Ocular lymphoma can be divided into intraocular and adnexal disorders, the former, including malignant lymphoid cells, invade the retina, vitreous body, or optic nerve head; the latter include conditions affecting the eyelid, the conjunctiva, the lacrimal gland, and the orbit [290]. Primary intraocular lymphoma (PIOL) is a subset of primary central nervous system lymphoma. It is usually a large B-cell NHL [291]. PIOL typically presents as a vitritis that is unresponsive to corticosteroid therapy. Diagnosis of PIOL requires pathologic confirmation of malignant cells in specimens of the cerebrospinal fluid, vitreous, or chorioretinal biopsies. The extranodal marginal zone lymphoma (mucosaassociated lymphoid tissue lymphoma) is the dominant lymphoma subtype in the orbit and ocular adnexa. Extranodal marginal zone lymphoma is considered to be the neoplastic counterpart of the marginal zone cells in reactive follicles [292]. Although optimal therapy has yet to be determined [293], it is believed that PIOL should be treated with a combination of chemotherapy and radiation.

Ocular involvement is common in patients with acute leukemia and has been described in up to half of patients at the time of diagnosis [294]. Eye involvement may be due to leukemic infiltration of various ocular tissues or as a result of one of the secondary complications of the disease [295]. These complications include anemia, thrombocytopenia, and leukostasis, which can lead to retinal hemorrhaging and ischemia [294]. Hemorrhaging in the retina is the most striking feature of ocular leukemia. Furthermore, retinal microaneurysms, capillary closure, and neovascularization have been documented in individuals with chronic leukemia [296, 297]. The treatments include chemotherapy, radiation, or bone marrow transplantation. Ocular findings may be the first manifestation of MM [298]. It may also occur as one of the extramedullary manifestations of the disease or as the first sign of insufficient chemotherapy. MM causes ocular pathology by direct infiltration or as extramedullary plasmacytomas resulting in the displacement or compression of tissues causing hyperviscosity syndrome and immunoglobulin light chain deposition in ocular tissues. Virtually any ocular structure can be affected, including the conjunctiva, cornea, sclera, lens, retina, optic nerve, lacrimal glands, and orbit [298] (Figure 5).

**3.2. Ocular Neovascularization.** Ocular angiogenesis or ocular neovascularization, the abnormal growth of blood vessels in the eye, is the hallmark of the vast majority of eye diseases that cause a catastrophic loss of vision including diabetic retinopathy, AMD, retinopathy of prematurity, and vein occlusion retinopathy [299, 300]. The new vessels may grow into nearly all mature ocular tissue and affect the cornea, iris, retina, and optic disk [301]. They are structurally weak, both leaking fluid and lacking structural integrity. Moreover, the resultant hemorrhage, exudate, and accompanying fibrosis often cause blindness [302].

The cornea is a highly organized transparent tissue located in the anterior part of the eye and it is normally avascular. However, under certain conditions, such as corneal trauma, chemical burns, infection, and inflammation, the development of new blood vessels starts from the vessel of the limbal area (Figure 5). Newly formed blood vessels cover the corneal surface [303], which can lead to severe or permanent visual impairment [302]. The choroid is the layer of blood vessels and connective tissue between the sclera and retina and supplies nutrients to the inner parts of the eye [304]. Choroidal neovascularization (CNV) is associated with many other conditions, such as AMD, inflammatory, infectious, degenerative, hereditary, congenital disorders, tumors, trauma, and a few miscellaneous ocular disorders [302]. In CNV, neovascular channels grow from the choroidal vasculature and extend into the subretinal space leading to local tissue damage. Activation and migration of choroidal ECs (CECs) and retinal pigment epithelial (RPE) cells into the CNV membranes play an important role in the development of the lesion [305]. The mammalian retina is a light sensitive tissue lining the inner surface of the eye, which is composed of multiple cell-types organized within defined layers. It has a dual blood supply from the central retinal artery and the choroidal blood vascular system [304]. Neovascularization of the retina is a critical part of the disease process associated with retinopathy in diabetes, prematurity, and sickle cell disease [302].

**3.3. Expression of PPAR $\gamma$  in the Eye and Effects on Ocular Neovascularization.** PPAR $\gamma$  expression in the mammalian eye has been reported prominently in retina [306, 307] including RPE cells [194, 308, 309], retinal capillary ECs (REC) [310, 311], retinal pericytes [287], and retinal ganglion cells [312]. PPAR $\gamma$  is most prominently localized in the epithelial and endothelial layers of the cornea [198]. PPAR $\gamma$  is also found in CECs [194] and in orbital fibroblasts [313, 314]. The broad expression of PPAR $\gamma$  in the eye provides a pharmacological target for treating ocular angiogenesis.

In vivo alkali-burned mouse cornea experiments showed that neovascularization and scar formation are suppressed by introduction of PPAR $\gamma$  gene expression. PPAR $\gamma$  overexpression suppressed monocyte/macrophage invasion and suppressed the generation of myofibroblasts, as well as upregulation of inflammation/scarring-related growth factors (TGF- $\beta$ , CTGF, and VEGF) and MMPs in a healing cornea. In vitro experiments showed that overexpression of PPAR $\gamma$  suppressed epithelial cell expression of MMP-2/-9 and TGF- $\beta$ 1,

inhibited cell migration, and suppressed myofibroblast generation upon exposure to TGF- $\beta$ 1. Thus, adenoviral-driven expression of the PPAR $\gamma$  gene led to inhibition of the anti-inflammatory and antifibrogenic responses induced in an alkali-burned mouse cornea, and also inhibited activation of ocular fibroblasts and macrophages in vitro [12]. In a VEGF-induced neovascular rat cornea model, intrastromal implantation of the PPAR $\gamma$  ligands pioglitazone [198] or 15d-PGJ<sub>2</sub> [22] resulted in decreasing MVD, indicating inhibition of ocular angiogenesis. Furthermore, systemic oral administration of rosiglitazone and troglitazone significantly inhibits vessel growth in a dose-dependent fashion in a model of FGF-2-induced mouse corneal neovascularization [195].

PPAR $\gamma$  ligands troglitazone and rosiglitazone inhibit VEGF-induced cell proliferation and migration in bovine CECs and human RPE cells in vitro. Troglitazone also inhibits VEGF-induced tube formation (neovascularization) of CECs [194]. Troglitazone pretreatment can significantly prevent TGF- $\beta$ -induced epithelial-mesenchymal transition of human RPE cells, and retard cell migration [315]. In vivo, laser photocoagulation induced CNV was markedly inhibited by intravitreal injection of troglitazone in rat and monkey eyes. The lesions showed significantly less fluorescein leakage and were histologically thinner in the troglitazone-treated animals without apparent adverse effects in the adjacent retina or in control eyes [194], indicating that the PPAR $\gamma$  ligands are logical for therapy to suppress vascular permeability in the eye.

PPAR $\gamma$  agonists, troglitazone, rosiglitazone, Pioglitazone, RWJ241947, and 15d-PGJ<sub>2</sub>, inhibit proliferation of human REC and pericytes in vitro through a PPAR $\gamma$ -independent pathway [316]. TZDs downregulate cyclin E (S-phase cyclin) and cyclin A (G2/M-phase cyclin) resulting in cell cycle arrest [316]. Troglitazone and rosiglitazone inhibit VEGF-induced proliferation and tube formation by bovine REC in collagen gels, and inhibit VEGF-induced REC migration in a dose-dependent manner [311]. Retinal angiogenesis is induced in newborn mice by oxygen-induced ischemic injury; however, intravitreal injection of troglitazone or rosiglitazone markedly reduced development of retinal neovascular tissue [311]. In the chick chorioallantoic membrane model of angiogenesis, pioglitazone and rosiglitazone significantly inhibit EC migration as well as the proangiogenic effects of FGF-2 and VEGF [27]. Rosiglitazone may delay the onset of proliferative diabetic retinopathy, possibly because of its antiangiogenic activity [317].

Taken together, these studies demonstrate that PPAR $\gamma$  ligands are potent inhibitors of angiogenesis in vivo and in vitro, and suggest that PPAR $\gamma$  may be an important molecular target for inhibiting angiogenesis. The use of PPAR $\gamma$  ligands to prevent pathological angiogenesis holds great potential as a novel therapeutic for neovascularized eye diseases. It may also apply to other neovascularization-related diseases, including hematological malignancies of the eye. However, future clinical investigations should consider analysis of the potential benefits of PPAR $\gamma$  agonist treatment along with ongoing evaluation of potential cardiac risk in studies where the risk-benefit profiles are deemed appropriate [317].

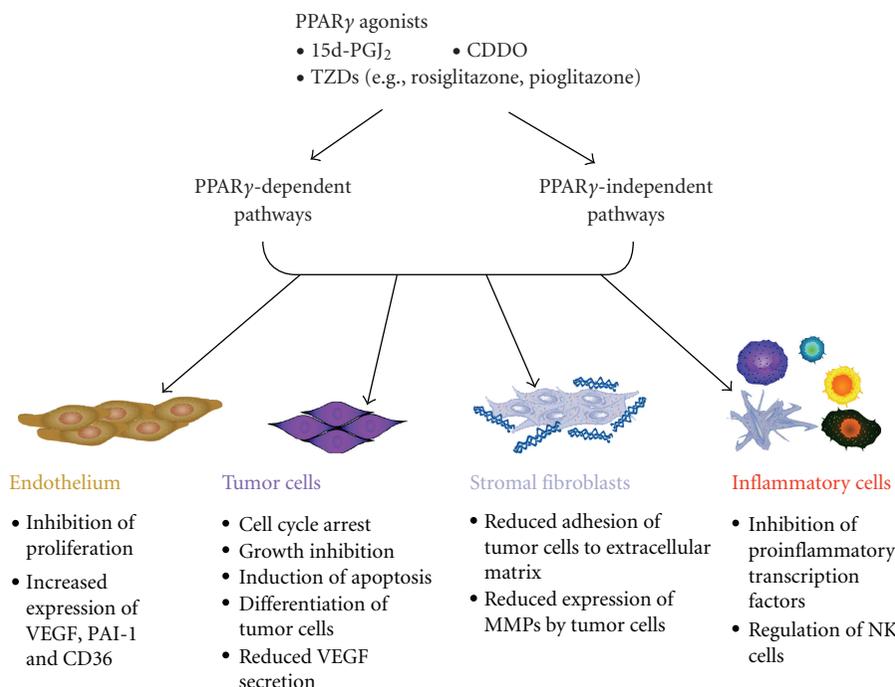


FIGURE 6: Direct and indirect effects of PPAR $\gamma$  agonists on tumor and stromal cells. “Off-target” (PPAR $\gamma$ -independent) effects of PPAR $\gamma$  agonists frequently occur when the agonists are used at high concentrations (much higher than needed to activate PPAR $\gamma$  by ligand binding) and in response to electrophilic PPAR $\gamma$  agonists such as 15d-PGJ<sub>2</sub> and CDDO, which can promote covalent bond formation with cellular proteins in a redox-sensitive manner to modulate signal transduction pathways. PPAR $\gamma$  agonists have been shown to affect almost every stage of tumor progression from inhibition of uncontrolled tumor growth, induction of apoptosis, inhibition of tumor cell adhesion and invasion through stromal compartments into or out of the blood stream, and inhibition of tumor-associated angiogenesis. PPAR $\gamma$  agonists induce expression of tumor-inhibiting molecules such as CD36, the EC receptor for TSP-1, as well as promote the differentiation of tumor cells, which tends to reduce their invasive and metastatic capabilities. The schematic in this figure was adapted from [181].

#### 4. The Paradox of PPAR $\gamma$ as a Molecular Target in Anticancer Therapy

The aforementioned studies examining the role of PPAR $\gamma$  ligands for treatment of hematological, ocular, and solid malignancies is by no means a complete review of the available literature. The list of off-target effects of PPAR $\gamma$  agonists continues to grow [51]. Furthermore, many of the published studies suggesting that PPAR $\gamma$  ligands exert antitumor properties did not determine whether the effects required ligand activation of the PPAR $\gamma$  transcription factor per se (Table 3). Many human cancer cell lines express high levels of PPAR $\gamma$ , which when treated with high concentrations of TZDs, undergo cell cycle arrest, apoptosis, or differentiation, suggesting a link between PPAR $\gamma$  signaling and their antitumor activities. In contrast, mounting evidence refutes the dependence of the antitumor effects of TZDs on PPAR $\gamma$  activation [25, 51, 318]. Of note, the off-target effects of PPAR $\gamma$  ligands usually occur at much higher concentrations than those required for ligand-dependent PPAR $\gamma$  effects, and there is no correlation between the expression levels of PPAR $\gamma$  in cancer cells and their sensitivity to TZDs [25, 51, 318]. Indeed, PPAR $\gamma$  agonists exert pleiotropic effects on signal transduction pathways involved in cell proliferation, survival and differentiation [25, 51, 71, 188, 318–322] (Table 3 and Figure 6).

Currently, two PPAR $\gamma$  agonists belonging to the TZDs remain on the market, rosiglitazone (Avandia) and pioglitazone (Actos). In 2000, troglitazone (Resulin) was removed from the market due to severe hepatotoxicity. Moreover, the incidence of delayed drug-induced liver injury that progresses after discontinuation of drug therapy, and whether such injury is specific to just troglitazone or TZDs as a class of drugs, remains unknown [323]. Additional adverse effects associated with TZDs used for insulin sensitizing therapy include edema, weight gain, macular edema, and heart failure [323, 324]. TZDs may cause hypoglycemia when combined with other antidiabetic drugs as well as decrease hematocrit and hemoglobin levels. Furthermore, an increased risk of bone fracture is linked to TZD therapy [324, 325]. When considering the use of PPAR $\gamma$  agonists as adjuvant or combination therapy in hematological malignancies, it will be important to design appropriate preclinical studies that assess the severity of these side effects in the context of each type of cancer. For example, increased edema is associated with increased vascular permeability. The loss of endothelial barrier integrity leads to increased vascular permeability, enhanced transendothelial migration, and metastatic spread of cancer cells [75]. Thus, the potential for TZDs to promote rather than prevent the metastatic spread of cancer should be considered. The malignant proliferation of plasma cells in MM produces skeletal lesions

leading to bone pain and pathologic fractures such as vertebral compressions [326]. In that TZDs are associated with increased risk of bone fractures; the use of TZDs for treatment of MM must be evaluated as well.

Evidence suggesting that the effects of TZDs on improving endothelial-dependent vascular function and decreasing inflammatory biomarkers independently of insulin-sensitizing effects came from studies reporting the effects of TZDs in diabetic and nondiabetic individuals with atherosclerosis [327–329]. In general, PPAR $\gamma$  agonists inhibit tumor-associated angiogenesis by inhibiting FGF-2- and VEGF-induced EC growth, invasion and migration in vitro and in vivo [27, 192], downregulate expression of VEGF by tumor cells [195, 199] and VEGFRs by EC [32], and decrease tumor-associated MVD [24, 32, 198] and EC tube formation [202], measures of angiogenesis in vivo and in vitro, respectively. TZDs inhibit pathological angiogenesis associated with diabetic retinopathy [287, 317], as well as choroidal and retinal neovascularization [194, 198, 311], and suppress primary tumor growth and metastasis by inhibiting angiogenesis [35] (Table 3). Interestingly, in contrast to these reports, TZDs increase VEGF expression in human vascular smooth muscle cells [330] and promote angiogenesis after ischemia [331]. Additional reports suggest that PPAR $\gamma$  ligands are capable of promoting angiogenesis by inducing VEGF expression [28, 30, 203].

Huang and colleagues [30] have suggested that pioglitazone has different effects on pathological angiogenesis compared to ischemia-induced collateral vessel growth [332]. TZDs promote differentiation of EPCs/APCs towards the endothelial lineage [197, 200, 201], consistent with the idea that PPAR $\gamma$  ligands have differential effects on angiogenesis needed for restoration of homeostasis in cardiovascular disease or diabetes compared to pathological angiogenesis associated with cancer progression. The role of PPAR $\gamma$  and its ligands in inhibiting or promoting angiogenesis is likely context dependent (Section 2.7 and Table 3) [30, 332]; thus, the use of PPAR $\gamma$  ligands alone or in addition to antiangiogenic agents for treatment of hematological malignancies will require a better understanding of the effects of PPAR $\gamma$  agonists on EC function during pathological angiogenesis.

Many studies have demonstrated beneficial effects of PPAR $\gamma$  agonists on atherosclerosis and ischemia reperfusion injury by reducing inflammation, preventing restenosis after percutaneous coronary intervention, and in some instances, preventing myocardial infarction and cardiovascular death. Recently, however, a number of review articles have discussed the “rosiglitazone debate” about whether taking rosiglitazone puts patients at a higher overall risk of cardiovascular death. The higher risk is based on findings derived from meta-analyses of existing clinical trial data, the release of FDA safety warnings that rosiglitazone increases cardiac ischemic risk, manufacturer updates on TZD labels with a black-box warning for heart failure, as well as warnings and precautions about coadministration of rosiglitazone with nitrate or insulin [333–336]. TZDs are known to induce salt and water retention, which exacerbate the risk of congestive heart failure in patients with type 2 diabetes. Rosiglitazone is a more potent agonist of PPAR $\gamma$  than pioglitazone, thus

increased fluid retention and salt imbalance may explain the higher risk of heart failure with this TZD [336]. However, even though treatment with rosiglitazone may, in general, be associated with a higher incidence of cardiovascular events, some studies suggest that there is no increase in all-cause or cardiovascular mortality observed with rosiglitazone treatment [333, 335]. Clearly, prospective randomized trials need to include outcomes measures to determine whether the TZDs and other such compounds under development put patients at a higher overall risk of cardiovascular death.

As cancer treatments improve, the number of patients who reach the 5-year benchmark of disease-free survival continues to grow. However, adverse effects of anticancer therapy may confound long-term survival. For example, as methods for detecting and treating breast cancer improve, survival of breast cancer patients is increasing but the side effects of adjuvant therapy, including cardiotoxicity, remain clinically important [337]. Agents commonly used for the treatment of breast cancer, including anthracyclines and trastuzumab, have been associated with cardiotoxicity [338], which ranges from subclinical to life-threatening pathology and even fatal results [339]. Imatinib (Gleevec) inhibits the continuously active tyrosine kinase, Bcr-Abl, which results from the translocation of chromosomes 9 and 22 and is effective for the treatment of CML as well as ALL; however, cardiotoxicity is a potentially serious side effect of this drug as well [340]. In that the TZD class of PPAR $\gamma$  agonists is associated with adverse cardiovascular events, additional studies on the efficacy of PPAR $\gamma$  agonists and other lead compounds as adjuvant or combination therapy to treat cancer should be designed to look at the cardiovascular risks and benefits in addition to their efficacy in treating the primary disease.

## 5. Conclusions

The goal to find a cure for all types of cancer is a major initiative of both public and private grant funding institutions and foundations. Thus, forwarding thinking researchers are exploring strategies to identify molecular expression profiles of cancer subtypes and CSCs, to optimize tumor imaging methods to identify cancer micrometastases, as well as to develop more-specific, less toxic drugs through medicinal chemistry to provide tailored therapy to treat and cure cancer in individual patients. However, metastatic disease remains the major cause of morbidity and mortality in both solid tumors and hematological malignancies. Because tumor-associated angiogenesis is critical for cancer progression and metastatic disease, the initiative to identify molecular targets and new or improved chemotherapeutic or biologic agents to inhibit angiogenesis is a high priority area of research in cancer medicine.

Specific areas of research where PPAR $\gamma$  agonists may be further examined for efficacy in treatment of angiogenesis in hematological malignancies as well as comorbidities that affect quality of life for long-term cancer survivors include signal transduction pathways (e.g., Jak/Stat, PI3K/Akt, PTEN, mTOR) [181, 341, 342], aberrant/oncogenic miRNAs [246, 257, 261, 283, 343–345], targeting CSCs while

sparing normal hematopoietic stem cells, and correcting dysregulated metabolic pathways due to drug side effects such as hyperglycemia, hypertension, gastrointestinal toxicity, coagulation disorders, and depression associated with the neurotoxicity of chemotherapeutic drugs [341, 346–348]. Moreover, limitations in the experimental design of published studies should be carefully evaluated. A significant number of studies continue to use troglitazone as a PPAR $\gamma$  agonist despite its having been pulled from the marketplace due to hepatotoxicity. In vitro experiments examining the efficacies of candidate drugs as inhibitors of angiogenesis need to reflect the complexity of the tumor microenvironment in keeping with the in vivo context. For example, large vessel ECs isolated from the veins of human umbilical cords (HUVECs) are frequently used to study angiogenesis by capillary tube formation in 2D-matrix configurations in vitro; however, in vivo tumor-associated angiogenesis occurs in a complex environment composed of multiple cell types including microvessel ECs and matrix constituents in a 3D-configuration. It will also be important to determine whether the therapeutic effects of PPAR $\gamma$  agonists are due to off-target interactions. In conclusion, we hope that this paper has provided a conceptual framework upon which future studies will be designed to unravel the pleiotropic effects of PPAR $\gamma$  in the context of the stromal microenvironment during tumor angiogenesis, growth and metastasis in hematological malignancies.

## Abbreviations

15d- PGJ <sub>2</sub> :	15-deoxy- $\Delta$ -12-14-prostaglandin J <sub>2</sub>	CTGF:	Connective tissue growth factor
Ago:	Argonaute	DIM #34:	1,1-bis[3'-(5-methoxyindolyl)]-1-(p-t-butylphenyl) methane
AIDS:	Acquired immunodeficiency syndrome	DLBCL:	Diffuse large B cell lymphoma
Akt/PKB:	v-akt murine thymoma viral oncogene homolog/protein kinase B	EC:	Endothelial cell
AMD:	Age-related macular degeneration	ECM:	Extracellular matrix
AML:	Acute myeloid leukemia	EPC:	Endothelial precursor cell
ANGPTL4:	Angiopoietin-like factor-4	FAT:	Fatty acid translocase
AP-1:	Activator protein 1	FGF-2:	Fibroblast growth factor-2
APC:	Angiogenic precursor cell	gp130:	Glycoprotein 130
APL:	Acute promyelocytic leukemia	HIF:	Hypoxia inducible factor
ARNT:	Aryl hydrocarbon nuclear translocator	HSF:	Hepatocyte stimulatory factor
B-ALL:	B type acute lymphoblastic leukemia	HSPG:	Heparan sulfate proteoglycan
Bcr-Abl:	Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1/Philadelphia chromosome	HUVEC:	Human umbilical vein endothelial cell
BMSC:	Bone marrow stromal cell	IGF-1:	Insulin-like growth factor
C/EBP:	CAAT enhancer binding protein	I $\kappa$ B:	Inhibitor of $\kappa$ B
CAM:	Chorioallantoic membrane	LDL:	Low density lipoprotein
CDDO-Im:	CDDO C-28 imidazole	MAPK:	Mitogen-activated protein kinase
CDDO-Me:	CDDO C-28 methyl ester derivative	MASPIN:	Mammary serine protease inhibitor (tumor suppressor gene)
CDDO:	2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid	MCP-1/CCL2:	Macrophage chemotactic protein
CEC:	Choroidal endothelial cell	MIF:	Macrophage inhibitory factor
CLL:	Chronic lymphocytic leukemia	miRNA:	MicroRNA
CML:	Chronic myeloid leukemia	MM:	Multiple myeloma
CNV:	Choroidal neovascularization	MMEC:	Multiple myeloma derived endothelial cell
CSC:	Cancer stem cell	MMP:	Matrix metalloproteinase
CTCL:	Cutaneous T cell lymphoma	mTOR:	Mammalian target of the rapamycin
		MVD:	Microvessel density
		NF- $\kappa$ B:	Nuclear factor $\kappa$ B
		NHL:	Non-Hodgkin lymphoma
		NOD/SCID:	Nonobese diabetic/severe combined immune deficiency
		NRP:	Neuropilin
		p300/CBP:	Transcriptional coactivator protein/cAMP-response element-binding protein (CREB) binding protein
		PAI:	Plasminogen activator inhibitor
		PDGF:	Platelet derived growth factor
		PGC-1:	PPAR $\gamma$ coactivator-1
		PI3K:	Phosphatidylinositol 3-kinase
		PIOL:	Primary intraocular lymphoma
		PLGF:	Placenta growth factor
		PPAR:	Peroxisome proliferator-activated receptor
		PPRE:	PPAR $\gamma$ response element
		PTEN:	Phosphatase and tensin homolog (tumor suppressor gene)
		pVHL:	Protein von Hippel-Lindau
		REC:	Retinal capillary endothelial cell
		RISC:	RNA induced silencing complex
		RPE:	Retinal pigmented epithelial
		SBE:	Stat3 Binding Element
		SMRT/NCoR:	Silencing mediator for retinoid and thyroid hormone receptors/nuclear receptor corepressor
		SOCS:	Suppressor of cytokine signaling

Src: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian); aka, p60-Src  
 STAT: Signal transducer and activator of transcription  
 TGF- $\alpha/\beta$ : Transforming growth factor  
 TIMP: Tissue inhibitor of metalloproteases  
 TNF- $\alpha$ : Tumor necrosis factor  
 TPM1: Tropomyosin 1 (tumor suppressor gene)  
 Tro: Troglitazone  
 TSP: Thrombospondin  
 TZDs: Thiazolidinediones  
 UTR: Untranslated region  
 VEGF: Vascular endothelial growth factor  
 VEGFR: VEGF receptor  
 VPF: Vascular permeability factor.

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