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PPAR γ regulates MITF and β -catenin expression and promotes a differentiated phenotype in mouse melanoma S91

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Summary

Melanoma represents one of the most rapidly metastasizing, hence deadly tumors due to its high proliferation rate and invasiveness, characteristics of undifferentiated embryonic tissues. Given the absence of effective therapy for metastatic melanoma, understanding more fully the molecular mechanisms underlying melanocyte differentiation may provide opportunities for novel therapeutic intervention. Here we show that in mouse melanoma S91 cells activation of the peroxisome proliferators activated receptor (PPAR) γ induces events resembling differentiation, such as growth arrest accompanied by apoptosis, spindle morphology and enhanced tyrosinase expression. These events are preceded by an initial transient increase in expression from the Microphthalmia-associated transcription factor gene, (*MITF*) promoter, whereas exposure to a PPAR γ ligand- ciglitazone that exceeds 8 h, causes a gradual decrease of MITF, until by 48 h MITF expression is substantially reduced. Beta-catenin, an MITF transcriptional activator, shows a similar pattern of decline during ciglitazone treatment, consistent with previous reports that activated PPAR γ inhibits the Wnt/ β -catenin pathway through induction of β -catenin proteasomal degradation. We suggest that the PPAR γ -mediated β -catenin down-regulation is likely to be responsible for changes in MITF levels. The data suggest that PPAR γ , besides its well-established role in mesenchymal cell differentiation towards adipocytes, might regulate differentiation in the melanocytic lineage.

Keywords

ciglitazone; differentiation; microphthalmia-associated transcription factor; tyrosinase; spindle morphology

Introduction

There is a common understanding of neoplastic transformation as a process of reversed differentiation or dedifferentiation, resulting in acquisition of embryonic features manifested by intensive proliferation and an anaplastic phenotype (Hendrix et al., 2007). This notion has recently been strengthened by a concept of cancer stem cells as a source of a substantial

fraction of carcinomas, but also tumors of non-epithelial origin, such as melanoma (Grichnik et al., 2006). In melanoma, dedifferentiation has particularly strong connections with the invasive phenotype as it reflects the capacity of neural crest cells for long distance migration during embryogenesis and their plasticity in building connections with various tissues, as well as developing into numerous cell types (Anderson, 1989). The most striking example of anaplastic transformation is uveal melanoma with its ability not only to invade distant organs, but to build mosaic blood vessels together with endothelial cells or even to form sinusoidal channels of loop and network structure within the tissue (Chen et al., 2002; Folberg and Maniotis, 2004; Maniotis et al., 1999). In parallel, there are frequent cases of epithelial-to-mesenchymal transitions (EMT) among cutaneous melanomas, which correlate with malignant invasive behavior (Kuphal and Bosserhoff, 2006; Kuphal et al., 2005). Indeed, the expression of embryonic transcription regulators such as Snail, Slug and Twist, involved in EMT, or Pax3 and Sox10 important for melanocytic lineage development, contribute to the aggressive metastatic phenotype (Gershon et al., 2005; Gupta et al., 2005; Hoek et al., 2004; Rubinfeld et al., 1997). Similarly, Wnt signaling and β -catenin, which play an essential role in embryonic neural crest formation, provide proliferative and survival advantages in melanoma cells, in part through induction of MITF (Garraway et al., 2005; Widlund et al., 2002). Beta catenin, although not capable of direct DNA binding, regulates gene expression through interactions with *bona fide* transcription factors such as the LEF / Tcf family. Constitutive β -catenin expression directly leads to MITF up-regulation (Takeda et al., 2000; Widlund et al., 2002). Recently, a direct interaction between MITF and β -catenin has been demonstrated, suggesting that MITF is able to redirect β -catenin towards melanocyte-specific targets (Schepsky et al., 2006). Whereas concerted expression of numerous transcription factors and modulators in a precise location and timing determine successful embryonic development, their deregulation frequently leads to oncogenic transformation and melanoma.

Unfortunately, the increasing worldwide incidence of melanoma does not go hand in hand with success in treatment for the metastatic disease, and metastases remain the main cause of mortality (Lens and Dawes, 2004). Therefore the differentiation, or even senescence-inducing therapeutic approaches, although incapable of complete disease eradication, may still be valuable by making the tumor cells more controllable and conferring a more predictable behavior (Bennett and Medrano, 2002; Slominski et al., 2001).

Numerous nuclear receptors such as the retinoic acid receptors (RAR), retinoic X receptor (RXR), vitamin D receptor (VDR) and peroxisome proliferator activated receptor (PPAR) are involved in induction of differentiation accompanied by growth arrest and apoptosis (Konopleva et al., 2004; Okuno et al., 2004; Welsh et al., 2003). Retinoid-, rexinoid- (RXR agonists) or vitamin D derivative- based differentiation therapy has been particularly successful in the treatment of acute promyelocytic leukemia and anaplastic thyroid carcinoma (Bruserud et al., 2001; Coelho et al., 2005; Dalhoff et al., 2003). This list includes also application of ligands for PPAR γ , which together with two other PPAR isoforms, α and β , belongs to the steroid hormone receptor superfamily (Escher and Wahli, 2000) and acts as a ligand-modulated transcription factor. PPARs were first discovered to regulate glucose, lipid and amino acid metabolism (Kersten et al., 2001; Patsouris et al., 2004; Pineda Torra et al., 1999), but recently there has been a rapidly growing number of papers revealing their broader function as inflammatory response modulators and potential anticancer agents (Kopelovich et al., 2002).

PPAR γ activation has a potent differentiation-inducing effect on various cancer types, including colon, ovarian and pancreatic cancer, osteosarcoma or leukemia (Haydon et al., 2002; Konopleva et al., 2004; Vignati et al., 2006). Most interestingly, PPAR γ agonists

have been shown to reduce aggressiveness, induce growth arrest and apoptosis and partially reverse EMT in aggressive anaplastic thyroid cancer cells (Aiello et al., 2006).

We have recently reported that fenofibrate, a PPAR α ligand, inhibits metastatic spread from subcutaneous melanoma tumors *in vivo*, and that the PPAR α mediated down-regulation of the Akt pathway results in a reduced metastatic potential *in vitro* (Grabacka et al., 2004, 2006). Here we show that PPAR γ activation in mouse melanoma cells also regulates MITF and β -catenin levels that promotes differentiation manifested by elongated morphology, enhanced tyrosinase activity and is accompanied by growth arrest.

Results

S91 mouse melanoma cells express active PPAR γ and inactive PPAR α

Using specific antibodies for western blotting whole cell extract, PPAR α and γ proteins were detected in mouse melanoma S91 cells, as well as in B16F10 cells, with Liver extract and Brown adipose tissue (BAT) being used as controls (Figure 1A). GRB2 was used as a loading control. Although PPAR expression has previously been reported in melanoma cells as well as in melanocytes (Grabacka et al., 2004, 2006; Kang et al., 2004; Placha et al., 2003), few studies have examined the ability of PPAR to activate transcription in these cells. The ability to transactivate target genes was therefore verified using a construct with the luciferase reporter gene driven by a promoter consisting of three copies of a PPAR response element from the human apolipoprotein AII gene. This promoter is not isoform sensitive and PPAR α or PPAR γ activity can only be distinguished by the use of specific ligands. Incubation with ciglitazone, a PPAR γ agonist, induced a strong response, which was slightly enhanced by 9-cis-retinoic acid (9cRA), which is a ligand for RXR, a PPAR transactivation partner (Figure 1B). 9cRA alone was able to activate the promoter around 2.5 fold, which reflects the recruitment of the endogenous partner by activated RXR, and is consistent with the data showing that 9cRA can transactivate the ApoAII promoter (Vu-Dac et al., 1996). Fenofibrate, an agonist of PPAR α , did not elicit any significant transactivation, suggesting that S91 cell expressed biologically inactive PPAR α . This result highlights the importance of combining expression analysis with functional assays.

PPAR γ activation induces growth arrest in S91 cells

To examine any possible effects of PPAR γ activation of proliferation, cell numbers were determined after incubation with ciglitazone, 9cRA or both (Figure 2A). The results indicated that there was a significant time-dependent inhibition of proliferation with the strongest effect (over 13-fold decrease after 96 h) being observed for the cells treated with both ciglitazone and 9cRA, although ciglitazone or 9cRA alone were also efficient (3- and 8-fold decrease after 96 h, respectively). Cell cycle analysis was performed on the cells treated with both ciglitazone and 9cRA, and revealed a conspicuous increase in the G0/G1 phase population with a concomitant S and G2/M phase decrease and emergence of small sub-G1 fraction that was presumed to contain apoptotic cells (Figure 2B). Induction of differentiation is generally accompanied by growth arrest manifested by an increased fraction of quiescent cells and a presence of apoptosis (Pantazis et al., 1999). The cell cycle pattern of S91 cells with activated PPAR γ is consistent with the results obtained for other neoplastic cell lines and other PPAR γ ligands (Chen and Harrison, 2005; Kopelovich et al., 2002; Yang et al., 2005). Nevertheless, inhibition of proliferation is not sufficient to provide the evidence of induction of differentiation. So, in further experiments we concentrated on the changes in cell morphology and two differentiation markers: MITF and its downstream target, tyrosinase, a key enzyme in melanin biosynthesis.

PPAR γ activation induces melanocytic differentiation markers

One key marker of melanocyte differentiation is the adoption of a dendritic morphology. PPAR γ activation (by ciglitazone alone or with 9cRA) impelled morphology changes in S91 cells, which became spindle shaped with long processes resembling normal primary melanocytes (Figure 3). In contrast, the control cells exhibited a flattened morphology with no such structures. The emerging elongated processes were different from forskolin-induced branched dendrites, suggesting a different underlying mechanism was operating. 9cRA alone did not induce any particular shape changes (Figure 3).

Efficient melanogenesis which requires a set of functional enzymes and melanosomal machinery is a hallmark of melanocytic differentiation. Although the S91 cells remained amelanotic after PPAR γ activation (as verified by electron paramagnetic resonance and spectrophotometric assays, results not shown), we nevertheless determined tyrosinase activity, bearing in mind that frequently pigmentation intensity of melanoma shows little correlation with the expression of pigmentary genes (Eberle et al., 1998). The results showed that DOPA-oxidase activity was significantly increased in the cells treated with ciglitazone (2-fold) and ciglitazone with 9cRA (2.5-fold), but that 9cRA alone had no effect (Figure 4A). Forskolin treatment, which activates the cAMP pathway, induced high tyrosinase activity and was used as a positive control.

We expected that the increased tyrosinase activity was due to increased expression, and consistent with this tyrosinase mRNA levels were upregulated in the ciglitazone treated group and after stimulation with forskolin, however no increase in transcript level was observed in the cells treated with both ciglitazone and 9cRA (Figure 4B). Nevertheless, as expected in both groups where PPAR γ was stimulated, there was an increased amount of the higher molecular weight, glycosylated, therefore mature and enzymatically active forms of tyrosinase (Figure 4C and the data not shown).

Melanocyte differentiation features, including spindle shaped morphology and elevated tyrosinase expression are observable effects triggered by MITF (Carreira et al., 2005, 2006). Therefore, we asked whether activation of PPAR γ could affect MITF promoter activity and expression. Cells were therefore transfected with an MITF-luciferase reporter and incubated with the various ligands for a further 40 h. The results show that PPAR γ activation leads to a remarkable induction of MITF promoter activity (Figure 4D). Since the kinetic of the response to the ligands was different than to forskolin (not shown), the signal recorded for the latter appears low. Since tyrosinase expression is dependent on Mitf, we also asked whether activation of PPAR γ could lead to increased tyrosinase promoter activity. The results indicate that tyrosinase promoter activity followed a pattern similar to that of MITF, being induced by ciglitazone and ciglitazone with 9cRA but not by 9cRA alone (Figure 4E). Given the activation of Mitf expression by C, and C and 9cRA, it seems likely that the effects on tyrosinase expression are a secondary effect of MITF upregulation, consistently with the notion that MITF is a direct transcription activator of tyrosinase.

To gain an insight into the possible mechanism of PPAR γ mediated melanoma cell differentiation, we studied the time course of MITF protein expression in the course of incubation with ciglitazone. The highest MITF level was detected after 4 h but then gradually declined until by 48 h MITF expression was barely detectable (Figure 4F). The MITF promoter is not likely to be a canonical transcriptional target of PPAR γ because we could not identify any potential peroxisome proliferator response elements (PPRE). Nevertheless, PPAR γ is able to influence gene expression through various interactions with transcriptional factors or signaling proteins, for example β -catenin. It has been shown that activated PPAR γ binds β -catenin and induces its proteasomal degradation (Liu and Farmer, 2004; Liu et al., 2006). This provides an interesting link with MITF. The Mitf protein is a

direct target for interaction with β -catenin while the MITF promoter is a target for β -catenin/lymphoid enhancer binding factor 1 (LEF1) (Schepsky et al., 2006; Takeda et al., 2000). We therefore examined β -catenin protein levels after PPAR γ stimulation. The β -catenin expression pattern resembled the one of MITF, especially showed a slight increase between 2 and 4 h incubation with ciglitazone and a subsequent decrease in levels after 8 h (Figure 4F). It was therefore possible that MITF downregulation after longer exposure to ciglitazone results, at least in part, from deficiency of β -catenin-mediated activation of MITF expression. Since β -catenin activity is determined both by its expression levels as well as its cytoplasmic/nuclear localisation we performed immunofluorescence assays using an anti- β -catenin antibody. Immunofluorescent staining showed a substantial decrease in overall β -catenin staining after 48 h incubation with ciglitazone (Figure 4G), which additionally supports the view that decreased levels of active, nuclear β -catenin is likely to be responsible for the decreased MITF expression observed.

Finally we asked the question whether PPAR γ was really responsible for the described effects of its ligands, as previously numerous reports showed that some biological effect of PPAR γ agonists from the class of thiazolidinedione drugs, including ciglitazone, were PPAR γ independent. For this purpose we used a specific PPAR γ inhibitor, GW 9662, and we were able to observe the reversion of tyrosinase upregulation as well as MITF and β -catenin downregulation induced by 72 h incubation with ciglitazone (Figure 4C and G), which clearly indicated that activated PPAR γ was necessary to evoke the effects observed.

Discussion

Here we show the results supporting a novel role of PPAR γ as a regulator of melanocyte differentiation through the influence on MITF and β -catenin. Both proteins are involved in melanocyte maturation during embryonic development and are indispensable for induction of melanocyte differentiation markers (Hemesath et al., 1994; Saito et al., 2003). Nevertheless, they are frequently overactive in melanoma cells, where they acquire the function of oncogenes by promoting survival and proliferation (Larue and Delmas, 2006).

Although it is clear that in the S91 melanoma cells used, PPAR regulates *Mitf* levels, most likely via regulation of β -catenin, it is important to note that the response appears biphasic. An early increase in *Mitf* levels, is followed by up-regulation of the differentiation marker tyrosinase, cell cycle exit, and the adoption of a spindle shaped morphology. However, while we can clearly see an upregulation of *Mitf* protein and promoter activity at early time points, it is difficult to attribute this unequivocally to elevated nuclear accumulation of β -catenin, though this would appear to be the most likely explanation. Increased tyrosinase activity in S91 cells after PPAR γ stimulation was not accompanied by the pigment formation. It might indicate that S91 cells are not capable of melanin biosynthesis at all. Melanoma cells frequently bear the genetic or functional defects in the melanosomal proteins or pigmentary enzymes downstream of tyrosinase, which rule out the melanogenesis (Eberle et al., 1998; Halaban, 2002). By contrast, prolonged stimulation by PPAR γ led to down regulation of *Mitf* expression and consequently may lead to a de-differentiated phenotype, since *Mitf* is a key activator of genes implicated in pigmentation.

Cell morphology and shape are determined by the cytoskeletal structure. Both MITF and β -catenin are implicated in the regulation or interaction with the actin cytoskeleton: MITF upregulates the expression of the diaphanous-related formin *Dial* that controls actin polymerization, and β -catenin is a member of a protein complex with E-cadherin and actin filaments (Carreira et al., 2006; Drees et al., 2005). The spindle shaped morphology acquired by the S91 cells following the incubation with ciglitazone, resembles normal cultured melanocytes and may be the outcome of switching on a differentiation programme.

However, the shape changes were most striking after 72 h incubation with PPAR γ ligands, by which time MITF and β -catenin levels drastically diminished (Figure 4B, C). It is possible however, that the early up-regulation of Mitf expression led to a spindle shaped morphology, but that once established the morphological changes no longer required continued Mitf expression for their maintenance.

The decrease expression of β -catenin induced by long term PPAR γ activation can be explained by the previous reports from Liu and coworkers (Liu and Farmer, 2004; Liu et al., 2006), who showed activated PPAR γ is a member of a multiprotein complex together with GSK3 β that binds the TCF/LEF binding domain of β -catenin and facilitates its phosphorylation on Ser 37 and subsequent ubiquitination leading to proteasomal degradation. Importantly, these processes are independent of the PPAR function as a transcription factor; they take place in the cytoplasm and cause the reduction of cytoplasmic β -catenin and finally prevent its nuclear translocation. This is consistent with the much weaker generalised β -catenin immunostaining in the cells treated with ciglitazone (Figure 4D).

MITF plays a dual role in melanocyte biology as an inducer of opposite responses. On the one hand, it activates proliferative and anti-apoptotic pathways through upregulation of Bcl2 and cyclin-dependent kinase 2 (Cdk2) (Du et al., 2004; McGill et al., 2002) as well as suppressing the expression of p27^{Kip} indirectly via its capacity to regulate Dial (Carreira et al., 2006). On the other hand MITF suppresses the cell cycle by promoting expression or stability of the Cdk inhibitors p16^{Ink4A} and p21^{Cip1} and by cooperation with the tumor suppressor retinoblastoma (pRb1) leading to the terminal differentiation of melanocyte lineage (Carreira et al., 2005; Loercher et al., 2005). The distinction between these contradictory activities is probably based on the level of active MITF, which however may not directly correlate with protein level owing to the many post-translational modifications that have to be taken into account (Carreira et al., 2006; Murakami and Arnheiter, 2005; Steingrimsdottir et al., 2004). In this study, the increase in tyrosinase expression and cell cycle arrest observed most likely reflects the increased Mitf expression obtained after short term PPAR gamma activation. Whether longer term treatment that leads to down regulation of Mitf may lead to a different outcome remain to be investigated. Nevertheless, it cannot be excluded that growth arrest of S91 cells is an MITF-independent effect of PPAR γ activation, because ciglitazone and other PPAR γ ligands have been shown to upregulate Cdk inhibitors, e.g. p21^{Cip1} and p27^{Kip1} in various cancer cell lines (Altieri et al., 1997; Chen and Harrison, 2005; Yang et al., 2005).

Our results reveal a novel and intriguing aspect of PPAR γ activity being a link to melanocyte differentiation pathways. We also demonstrated a putative cross talk with MITF and β -catenin signaling. Further studies focusing on the mechanism of PPAR γ mediated MITF regulation will probably shed new light on the activity and function of PPAR and their ligands in melanocyte biology and pathophysiology of melanoma cells.

Methods

Cell culture

S91 I₃ mouse melanoma cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin (all from Invitrogen, Grand Island, NY, USA). Cells were incubated with: fenofibrate, 25 μ M, forskolin, 20 μ M (Sigma, St Louis, MO, USA), ciglitazone, 25 μ M, GW 9662, 10 μ M (Biomol, Plymouth Meeting, PA, USA), 9-cis-retinoic acid, 1 μ M (ICN Biochemicals, Aurora, OH, USA), all diluted in DMSO, which was added to control groups. Cell growth and survival in various treatment conditions described in the figure captions were evaluated by trypan blue exclusion test. Human

primary melanocytes (a gift from Dr Zalfa Abdel-Malek) were kept in MCDB 153 medium (Sigma, St Louis, MO, USA) with antibiotics as above, 5% FBS, 8 nM PMA, 5 µg/ml insulin, 1 µg/ml transferrin, 1 µg/ml α-tocopherol (all from Sigma) and 0.6 ng/ml bFGF (Invitrogen, Grand Island, NY, USA).

Plasmids

J₃TkpGL3 (a gift from Dr Alicja Józkowicz) – a reporter plasmid with firefly luciferase gene driven by an fragment containing 3 copies of J-site from human apolipoprotein AII promoter (Vu-Dac et al., 1995, 1996), pRL-MCV – a *Renilla* luciferase reporter plasmid for a control of transfection efficiency (Promega), MITFpGL3 and Tyr-pGL3 (both kindly provided by Dr Colin Goding) – plasmids with MITF (–387 → +97) and Tyrosinase (–115 → +80) promoter fragments upstream of firefly luciferase (Bentley et al., 1994; Carreira et al., 1998).

Luciferase assay

The plasmids were transfected into the S91 cells using Cell Line Nucleofector kit R (Amaxa, Gaithersburg, MD, USA) or MetafectenePro reagent (Biontex laboratories, Munich, Germany). Four hours after transfection cells were treated for 40 h with the ligands as indicated in the figures. Luciferase activity was assessed in chemiluminometer, using Dual-Reporter Luciferase assay system (Promega, Madison, WI, USA).

Cell cycle analysis

The cell distribution among the cell cycle phases was determined by a flow cytometric assessment of DNA content. The cells treated with ciglitazone and 9cRA were collected at the indicated time points, washed in PBS and fixed for 30 min in ice cold 85% ethanol, then centrifuged (1600 rpm, 5 min) and the pellets resuspended in 760 µM sodium chloride – sodium citrate buffer (SSC), pH 7.0 with propidium iodide and RNase A, Sigma, St Louis, MO, USA).

Western blot

Western blot assays were performed according to a standard protocol, as described elsewhere (Wang et al., 2005). Aliquots of cell lysates (50 µg each lane) were separated in 8% or 10% SDS–PAGE gels and transferred onto nitrocellulose membranes. The resulted blots were blocked in 5% non-fat milk and probed with the following antibodies: anti-PPAR α (Chemicon, Temecula, CA, USA), anti-PPAR γ, anti-β catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GRB2 (Transduction Laboratories, Lexington, KY, USA), anti-β actin (Sigma, St Louis, MO, USA), anti-tyrosinase (αPEP7, a gift from Dr Vincent Hearnig), anti-MITF (a gift from Dr Heinz Arnheiter).

Immunofluorescent staining

Cells were seeded on the glass slides and cultured for 48 h with DMSO (control) or ligands as indicated in the figure. The slides were fixed in cold methanol for 10 min., washed in PBS and blocked in PBS with 10% FBS (blocking buffer) for 30 min. Mouse monoclonal anti β-catenin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1:100 dilution in blocking buffer were used as primary antibodies. After 1 h incubation, the slides were washed in PBS (5 times) and incubated with FITC-conjugated secondary antibodies (Vector Laboratories), 1:1000 diluted. After final washings, the slides were mounted in the Vectashield mounting medium with DAPI (Vector Laboratories).

RT PCR

After 24 h incubation with ligands cells were collected and total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA, USA) or Total RNA Isolation System (Promega, Madison, WI, USA). Reverse transcription was performed with oligo dT₁₅ primer (Roche, Mannheim, Germany), SuperScript III transcriptase and RNaseOUT ribonuclease inhibitor (Invitrogen, Grand Island, NY, USA). One µg of cDNA was used for the PCR reaction, which was done with Taq polymerase (Roche, Mannheim, Germany) and following primers: tyrosinase forward: 5' GAT TTG AGT GTC TCC GAA AAG AAT A, reverse: 5' CTG ACT CCT GGA GGT AGC TGT AGT C (product size: 994 bp); β-actin forward: 5'CTG GTT GCC AAT AGT GAT GA, reverse: 5' CCA GAT CAT GTT TGA GAC CT (product size: 350 bp). The PCR reaction conditions (30 cycles) for β-actin were: 94°C- 45 s, 55°C- 45 s, 72°C- 1 min, for tyrosinase: 94°C- 30 s, 60°C- 30 s, 72°C- 1 min. The PCR products were visualized on the ethidium bromide agarose gel.

Tyrosinase activity

Cells were treated with the ligands for 72 h, collected and lysed in 20 mM phosphate buffer, pH 6.8 with 1% Triton X-100 and protease inhibitors and frozen in -20°C. After thawing the samples were sonificated on ice 3 × 15 s and centrifuged (6000 rpm, 5 min, 4°C), supernatant collected. The DOPA-oxidase activity was assessed colorimetrically according to Winder and Harris (Winder and Harris, 1991). Briefly, 20 µl of cell extract was added to the reaction mixture containing 5 mM DOPA, 3 mM MBTH in 20 mM phosphate buffer pH 6.9 with 4% N, N-dimethylformamide (Sigma Aldrich, Steinheim, Germany). The absorbance at 505 nm was read every 30 s for 10 min. The reaction specificity for tyrosinase was confirmed using phenylthiourea as a tyrosinase inhibitor.

Statistical analysis

Statistical significance of the differences between groups was tested using the Student's *t* test for homogenous or heterogenous variances, as appropriate, or one way ANOVA (significance level 5%). The shown results come from 3 independent experiments. The plots represent mean values from pooled experiments ±SEM or mean values from a single representative experiment ±SD, as indicated in the figure legends.

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Figure 1. Mouse melanoma cells S91 and B16F10 express transcriptionally active PPAR γ but inactive PPAR α . (A) PPARs protein levels checked by western blot. BAT – brown adipose tissue, positive control for PPAR γ , Liver – positive control for PPAR α . GRB2 - loading control. (B) Transcriptional activity of PPAR α and PPAR γ , determined by luciferase assay. Plot: mean $\pm$ SEM, * $P < 0.05$. Symbols: Ctrl – control, DMSO treated cells, F – Fenofibrate, C – Ciglitazone, 9cRA – 9-cis-retinoic acid.

Figure 2.

PPAR γ activators induce growth arrest in S91 cells. (A) Growth kinetics. Plot: mean $\pm$ SD.

Symbols: Ctrl – control, DMSO treated cells, C – Ciglitazone, 9cRA – 9-cis-retinoic acid.

(B) Cell cycle analysis for S91 cells treated with ciglitazone together with 9-cis-retinoic acid for 24, 48 and 72 h. Time point 0 represents control cells.

Figure 3. PPAR γ activators induce morphological changes. Phase contrast microphotographs from S91 cell culture after 72 h incubation in the indicated conditions. Scale bar: 100 μ m. Symbols: Ctrl – control DMSO treated cells, C – Ciglitazone, 9cRA – 9-cisretinoic acid, Forsk – forskolin, NM – normal human melanocytes.

Figure 4.

PPAR γ activation affects expression of genes involved in melanocyte differentiation. (A) PPAR γ activation increases DOPA-oxidase activity of tyrosinase. The enzyme activity is expressed as: [product mol/l * min⁻¹ * mg of protein⁻¹] * 10⁻¹⁰. Positive control – forskolin treated cells. Plot: mean $\pm$ SEM, *P < 0.05. Symbols: Ctrl – control DMSO treated cells, C – Ciglitazone, 9cRA – 9-cis-retinoic acid, Forsk – forskolin. (B) Tyrosinase mRNA levels (Tyr) checked by RT PCR, β -actin as an internal control, Neg – PCR purity control. (C) Effects of ciglitazone on tyrosinase, MITF and β -catenin protein levels (detected by western blot) are reversed by a specific PPAR γ inhibitor, GW 9662. Forskolin-treated cells are a positive control, β -actin – a loading control. In tyrosinase blot, the arrows indicate the high

molecular weight, glycosylated form and the lower molecular weight, unglycosylated form. (D) MITF and (E) Tyrosinase promoter activities determined by luciferase assay. Plots: mean $\pm$ SEM, *P < 0.05. (F) Time-dependent changes in MITF and β -catenin protein levels (detected by western blot) during the incubation with ciglitazone; β -actin – a loading control. (G) Immunofluorescent detection of β -catenin in S91 cells, the nuclei stained with DAPI. LiCl (30 mM), which blocks β -catenin degradation, is used as a positive control. Symbols: Ctrl – control DMSO treated cells, C – Ciglitazone, 9cRA – 9-cis-retinoic acid, GW – GW 9662, Forsk – forskolin.