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Altered PPARγ expression inhibits myogenic differentiation in C2C12 skeletal muscle cells

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Abstract

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily known to regulate adipocyte differentiation. However, its role in skeletal muscle differentiation is not known. To investigate possible involvement of PPARγ in skeletal muscle differentiation, we modulated its expression in C2C12 mouse skeletal muscle cells by stable transfection with sense or antisense plasmid constructs of PPARγ cDNA. Phenotypic observations and biochemical analysis of different myogenic markers showed that altered expression of PPARγ inhibited the formation of myotubes, as well as expression of muscle-specific myogenic proteins including myogenin, MyoD and creatine kinase activity. Together, we show that critical expression of PPARγ is required for skeletal muscle cells differentiation.

Key words: myogenesis, PPARγ, skeletal muscle cells

Introduction

The development of skeletal muscle is a highly regulated process in which pluripotent mesodermal cells give rise to myoblasts that subsequently withdraw from the cell cycle and differentiate to form myotubes through temporally distinct sequence of events. Muscle cells undergo a distinct and well-characterized series of biochemical and morphological changes during the process of differentiation i.e. myogenesis. The process leads to the induction of several differentiation-linked genes specifically expressed in muscle cells. At molecular level, myogenesis is controlled by a family of myogenic regulatory factors, which includes myogenin, muscle creatine kinase and MyoD that are expressed with a well-defined time course [1–3]. Myoblasts align and fuse to form multinucleated myotubes. The endocrine factors and signal transduction pathways that coordinate skeletal muscle cell differentiation and expression of muscle-specific proteins [4, 5] have been extensively studied [3, 6–8]. However, the mechanisms and molecules that are temporally required for regulating the process of skeletal muscle differentiation are not fully understood [7].

Peroxisome proliferator-activated receptors (PPARs) are members of nuclear receptor superfamily [9] and regulate a large number of genes that are essential for lipid and metabolic homeostasis and energy balance [10]. PPARγ is expressed predominantly in mature adipocytes and its role as a key transcriptional factor controlling adipocyte differentiation has been demonstrated [11, 12]. Although very little amount of PPARγ is detectable in skeletal muscle cells

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(5–10% of the expression observed in fat cells) [13–17], its physiological role in muscle is suggested by the fact that insulin sensitizers such as thiazolidinediones confer action on muscle as PPARγ ligand, the main site for insulin-stimulated glucose uptake [18–20]. Role of PPARγ in skeletal muscle differentiation is not clear. Earlier studies with genetic manipulation of PPARγ failed because complete elimination of functional PPARγ gene by knockout resulted in embryonic lethality [20–22] making PPARγ an essential gene for their survival. Role of PPARγ in the urothelial differentiation programme has been proposed [23]. Recently, an interaction between MyoD and PPARγ has been reported indicating a connection between PPARγ and MyoD [24]. However, whether PPARγ could mediate myogenic differentiation of skeletal muscle cells remains to be established.

To elucidate a functional link between PPARγ expression and the induction of myogenic differentiation, we tested whether modulation of PPARγ expression can affect skeletal muscle cells differentiation. Results suggest that critical expression of PPARγ is required in the normal myogenic differentiation of skeletal muscle cells. To the best of our knowledge, this is the first report, which establishes the relationship between skeletal muscle cell differentiation as a function of PPARγ expression.

Materials and methods

Materials

C2C12 Mouse skeletal muscle cell line was kindly provided by Dr. H. Blau, Stanford University, School of Medicine, Stanford, USA and Dr. J. Dhawan, CCMB, India. Dulbecco's modified Eagle's medium (DMEM), horse serum and trypsin-EDTA were purchased from Gibco BRL, USA. Foetal calf serum (FCS) was from Biological Industries, Israel. Rabbit polyclonal PPARγ antibody, anti-myogenin antibody and anti-MyoD antibody were purchased from Santa Cruz Biotechnology, USA. Nitrocellulose membranes, TEMED, acrylamide, bisacrylamide and glycine were purchased from Bio-Rad, USA. All other reagents, unless attributed specifically, were purchased from Sigma, USA. All the plasticwares were purchased from Tarsons, India.

Plasmid constructs

For sense and antisense constructs of PPAR-γ, a copy of ∼1.5 kb mouse PPAR-γ gene was isolated from pCMX-mPPARg cDNA clone (kind gift from Ronald M. Evans, The Salk Institute for Biological Studies, San Diego, CA, USA) and inserted into the cloning site of the plasmid pCDNA3.1neo^r (Invitrogen) in sense and antisense orientation with respect to CMV promoter (sense, pCDNA3.1-mPPARγ/+ and antisense, pCDNA3.1-mPPARγ/−). Plasmids were expanded in *Escherichia coli* (strain DH5α) and isolated with Wizard Plus Midiprep DNA isolation kit (Promega, Madison, WI, USA).

Transfection

C2C12 skeletal muscle cells in the exponential growth phase were transfected with PPAR-γ sense or antisense plasmid construct using TransFast transfection reagent (Promega, Madison, WI, USA), as described previously [6]. Briefly, transfection reagent was incubated with plasmid DNA constructs in serum-free DMEM at room temperature for 15 min. This transfection mixture was applied to the proliferating cells and incubated for 1 h at 37 °C. Following incubation, DMEM with 15% FCS was overlaid on the plate and further incubated at 37 °C. Selection drug (G-418) was applied to a final concentration of 400 μg ml^{-1} after 24 h incubation and were maintained in a medium containing G-418 until proliferating cells in the control plate had died (approximately after 8–10 subculture). At this stage all the proliferating cells from the plates containing transfected cells were trypsinized, washed, and a part of the cells was continued with the subculturing without G418 and another part was subjected to differentiation, according to the procedure described below. After differentiation, the cells were lysed and tested for the degree of expression of PPARγ protein. When expected results were obtained (over expression in case of PPARγ/+ transfected cells and under expression in PPARγ/− transfected cells) in the differentiated cells, the proliferated cells, which were being parallaly subcultured, were grown in bigger culture, frozen as stocks of stably transfected cell lines. As and when required cells were thawed from the stocks, subcultured 2–3 times, tested for degree expression of PPARγ in respective stable cell line and subjected to experimentation.

Cell culture and induction of differentiation

The C2C12 skeletal muscle cell lines (wild type and transfectants) were cultured as described previously [25]. Briefly, they were maintained in DMEM supplemented with 15% FCS and antibiotics (penicillin 100 IU ml^{-1}, streptomycin 100 μg ml^{-1}) in 5% CO_2 at 37 °C. When the cells achieved 70% confluency, they were differentiated in 2% horse serum for 3 days. Under these conditions, cells were found to be healthy, viable and not undergoing necrosis or apoptosis, as

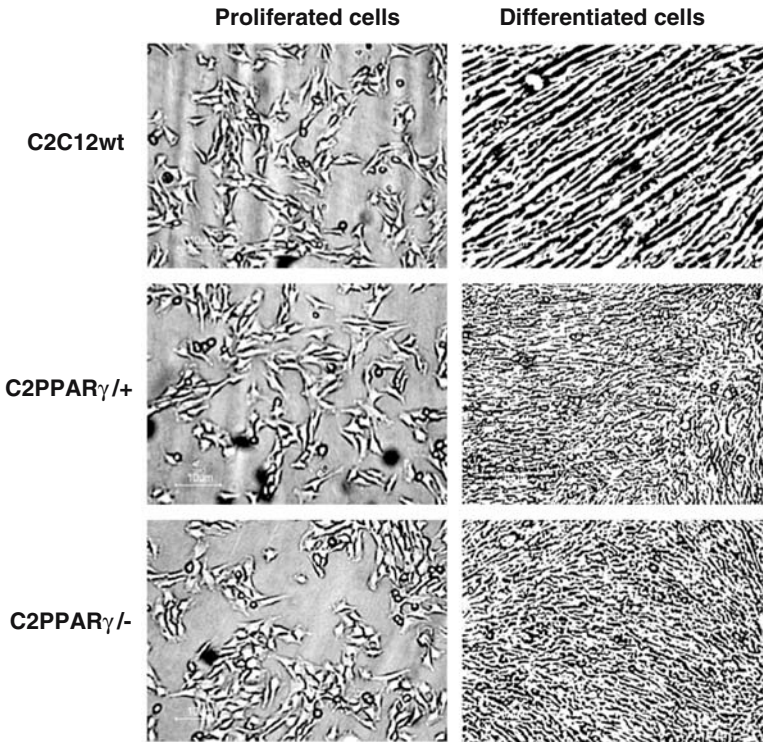

Fig. 1. State of differentiation due to modulation of PPARγ expression in C2C12 myoblasts. C2C12wt, C2PPARγ/+ and C2PPARγ/− cells were proliferated for 48 h after subculture. When cells reached to 70% confluency, they were differentiated for 3 days in differentiation medium containing 2% horse serum and photographed.

observed under phase contrast microscope (Fig. 1) and trypan blue staining (data not shown). Moreover, all three kinds of cells were found to be capable of significant amount of glucose uptake [26].

Cell counting for determination of fusion index

Fusion index of the myotubes were calculated as described previously [27] with minor modifications. Briefly, cells were grown over cover slips, fixed with paraformaldehyde (3%, w/v) and stained with 10 μg ml^{-1} propidium iodide (PI). The total number of nuclei and the number of nuclei incorporated in myotubes were scored in 10 microscopic fields/dish chosen at random. The fusion index was calculated as the percentage of nuclei incorporated in the myocytes relative to the total number of nuclei.

Preparation of cell lysates and Western-immunoblotting

Cells were lysed and protein expression levels were analyzed by Western-immunoblot analysis, as described previously [25]. Equal amount of protein samples were resolved by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) and incubated with the indicated primary antibodies for 12–16 h, followed by 1 h incubation with alkaline phosphatase conjugated secondary antibody. The protein bands were visualized with BCIP/NBT as substrate.

Creatine kinase (CK) assay

The CK assay was performed, as described previously [6]. Briefly, cells growing on 35 mm Petri plates were washed twice with ice-cold PBS. To each Petri plate, 40 μl of ice-cold 0.05 M glycylglycin (pH 6.75) was added. Petri plates were kept at −20 °C for 24 h. The cells were scrapped after thawing on ice, centrifuged at 16,000 × g for 10 min at 4 °C and subjected to CK assay. The assay contained 10 mM glucose, 2.5 mM magnesium acetate, 0.5 mM ADP, 5 mM AMP, 0.4 mM NADP, 10 mM creatine phosphate, 1 U/ml hexokinase, 0.5 U/ml glucose-6-phosphate dehydrogenase and freshly added 1.54 mg/ml dithiothreitol in 0.1 M glycylglycin (pH 6.75). The reaction was initiated by

adding 1 μg of protein in a total assay volume of 50 μl. The change in absorbance was measured at 340 nm up to 5 min using Lambda Bio 10 UV/vis spectrometer (Perkin Elmner, USA).

Immunofluorescence microscopy

Immunofluorescence microscopy studies were carried out, as described previously [26] and visualized under florescence microscope (Nikon E600, Japan).

Densitometric analysis

Densitometric analyses of the Western blots were done using Gel Doc 2000 equipped with Quantity One 1-D analysis software (Bio-Rad, USA) as described previously [26]. The relative values of the samples were determined by giving an arbitrary value of 1.0 to the respective control samples of each experiment, keeping the background value as 0.

Statistical analysis

The data are expressed as mean ± standard error of mean (s.e.m.). For comparison of two groups, *p*-values were calculated by two-tailed unpaired Student's *t*-test. In all cases, $p < 0.05$ was considered to be statistically significant.

Results

C2C12 is well-characterized cell culture model used to study skeletal muscle differentiation. Under conditions permissive for differentiation, such as low serum concentration, C2C12 myoblasts undergo differentiation to form myotubes. We have previously established cell lines derived from C2C12 myoblasts after stable transfection with sense (C2PPARγ/+) or antisense (C2PPARγ/−) plasmid constructs of mouse PPARγ in order to study its role in insulin resistance [26]. By Western-immunoblot analysis, we have shown that stable transfection of C2C12 skeletal muscle cells with sense or antisense plasmid constructs of PPARγ significantly modulated the level of PPARγ expression with 28.57% increase in C2PPARγ/+ cells or 23.07% decrease in C2PPARγ/− cells as compared to C2C12wt [26]. Using these cell lines, present studies were designed to analyze the potential effect(s) of PPARγ expression on skeletal muscle cell differentiation.

To investigate the effect(s) of PPARγ expression in skeletal muscle differentiation, wild type and transgenic C2C12 myoblasts (C2C12wt, C2PPARγ/+ and C2PPARγ/−) were incubated under conditions permissive for differentiation and cultures were examined daily. When cultured in DMEM growth medium containing 15% FCS, all the three cell lines mentioned above proliferated normally (Fig. 1). When these cells were incubated for 3 days in differentiation medium containing 2% horse serum, extensive myotube formation was observed in C2C12wt cells adopting a spindle shaped morphology and membrane fusion to form multinucleated myotubes, as expected under these conditions (Fig. 1). Similar results were obtained in cells that contained vectors without the inserted gene (data not shown). In contrast, C2C12 cells either overexpressing (C2PPARγ/+) or inhibited PPARγ (C2PPARγ/−) expression exhibited a marked reduction in their ability to form myotubes (Fig. 1).

To visualize the nuclei, cells were stained with propidium iodide (Fig. 2A). In C2C12wt cells, majority of them were multinucleated (Fig. 2A) with average number of nucleus in each myotubes being 13.2 ± 1.28 (Fig. 2B, lane 1) and the fusion index was calculated to be 38.75 ± 1.6 (Fig. 2C, lane 1). However, in C2PPARγ/+ or C2PPARγ/− cells, majority of them were mononucleated (Fig. 2A, B) and the fusion index was decreased to 3.8 ± 0.92 and 4.2 ± 1.4 respectively (Fig. 2C, lanes 2 and 3). Thus, data shows that altered expression of PPARγ repressed the myogenesis process in C2C12 cells.

To further establish these observations, the phenomenon was investigated biochemically by the determination of myogenin and MyoD expression levels and creatine kinase activity, as they are known muscle differentiation markers [27–29]. During differentiation, these myogenic factors activates muscle-specific genes and coordinate withdrawal from the cell cycle [4]. It has been well established that expression of myogenin, an early marker for the entry of myoblasts into the differentiation pathway occurs only in post-mitotic myocytes and is expressed between 8 and 24 h of the *in vitro* initiation of differentiation [2, 7, 30, 31]. Therefore, our initial interest was to determine whether myogenin expression is affected due to alteration of expression of PPARγ in C2C12 skeletal muscle cells. To this end, all the three cell lines (C2C12wt, C2PPARγ/+ and C2PPARγ/−) were incubated for 24 h under conditions for differentiation (DMEM containing 2% horse serum) and the level of myogenin expression was examined by Western-immunoblotting (Fig. 3). Myogenin expression was not detected in proliferating myoblasts of all the three cell lines (data not shown), which was expected [2, 32, 33]. C2C12wt cells differentiated in 2% horse serum medium showed high level of myogenic expression (Fig. 3, lane 1). In contrast, the level of myogenin expression was very low in C2PPARγ/+ (6.4% of C2C12wt) (Fig. 3, lane 2 compared to lane 1; $p < 0.01$) or in C2PPARγ/− cells (4.4% of C2C12wt) (Fig. 3, lane 3 compared to lane 1; $p < 0.01$).

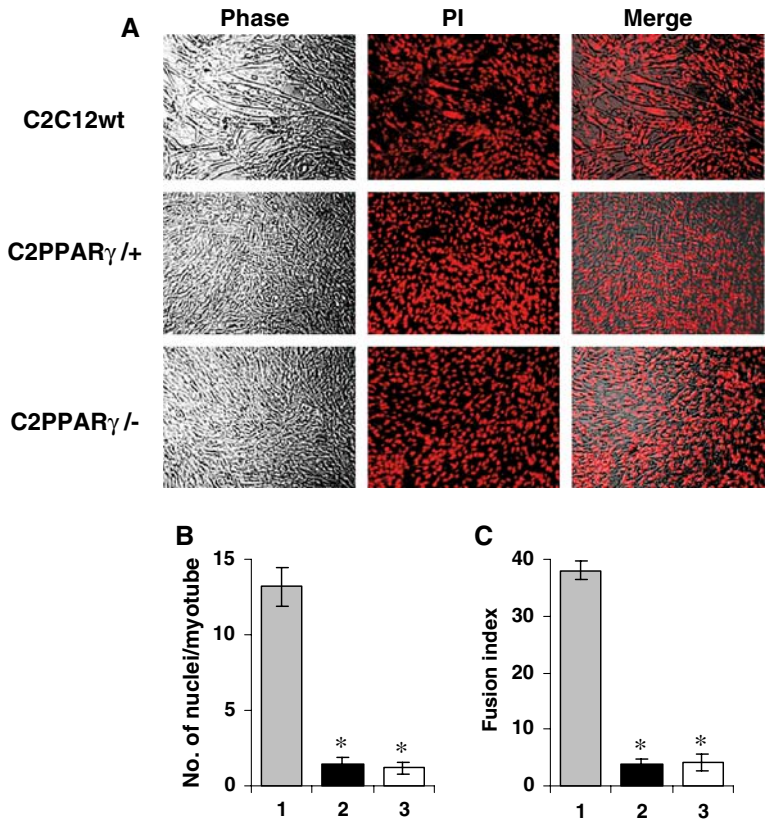

Fig. 2. Determination of nuclear fusion in differentiated cells. (A) C2C12wt, C2PPARγ/+ and C2PPARγ/− cells were grown on coverslips and differentiated for 3 days. Cells were fixed, permeabilized and stained with PI (10 μg/ml). At least 10 microscopic fields were photographed and a representative figure is shown. (B) Average number of nuclei per myotubes were counted and presented. lane 1: C2C12wt; lane 2: C2PPARγ/+; lane 3: C2PPARγ/−. (C) Fusion index were calculated as described in 'material and methods'. lane 1: C2C12wt; lane 2: C2PPARγ/+; lane 3: C2PPARγ/−. Data are mean ± standard error of mean (s.e.m.) of three independent experiments (*$p < 0.01$).

Proliferating myoblast showed no detectable CK activity (data not shown); however, CK activity was detectable after day 1 of culture in differentiation medium. In C2C12wt cells, CK activity increased by about 3.5-fold from day 1 to day 3 when incubated in differentiation medium (Fig. 4, lanes 7 compared to lane 1, $p < 0.01$), which was consistent with our previously reported results [25]. However, when C2PPARγ/+ or C2PPARγ/− cells were incubated in differentiation medium, the CK activity was constantly lower than that detected in C2C12wt cells for the entire 3 days period of differentiation (Fig. 4). C2PPARγ/+ cells showed 3.5-fold and 4.5-fold less activity than C2C12wt after day 2 and day 3, respectively (Fig. 4, lane 5 compared to lane 4 and lane 8 compared to lane 7; $p < 0.01$), whereas C2PPARγ/− cells showed 3.3-fold and 4.1-fold less activity than C2C12wt after day 2 and day 3, respectively (Fig. 4, lane 6 compared to lane 4 and lane 9 compared to lane 7; $p < 0.01$).

To determine the degree of MyoD expression, all the three cell lines were incubated in the differentiation medium for 3 days. Cells were lysed and Western immunoblotted using anti-MyoD antibody (Fig. 5A). Marked reduction of MyoD expression was observed in C2PPARγ/+ or C2PPARγ/− cells as compared to C2C12wt cells (Fig. 5A, lanes 2 and 3 compared to lane 1; $p < 0.01$). The expression of MyoD was also monitored by immunofluorescence microscopy. Cells growing on coverslips were incubated in differentiation medium for 3 days and subjected to immunofluorescence microscopy analysis probing anti-MyoD antibody and visualized by FITC labeled secondary antibody. Immunofluorescent image showed that the expression of MyoD was greatly inhibited in cells with altered PPARγ expression (C2PPARγ/+ or C2PPARγ/−) (Fig. 5B). Thus, data show that alteration of PPARγ expression in C2C12 cells repressed the myogenesis process.

These observations suggest that the critical level of expression of PPARγ is required for normal differentiation of skeletal muscle cells. Any alteration in the PPARγ expression in skeletal muscle may cause faulty differentiation.

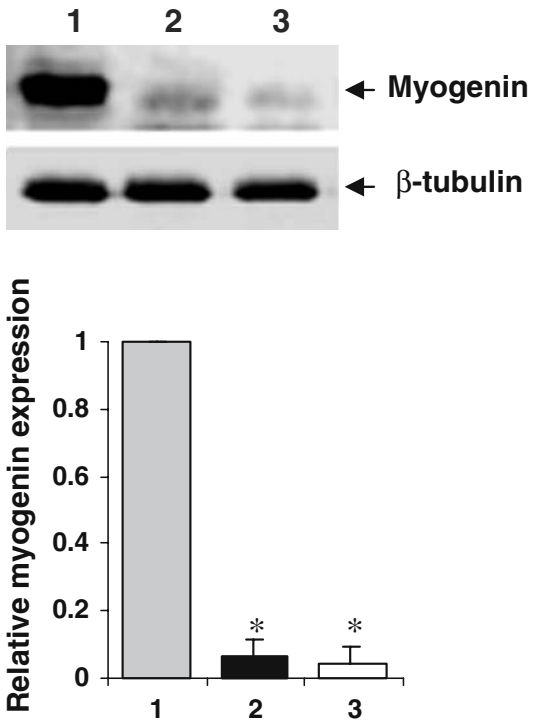

Fig. 3. Determination of myogenin expression during myogenic differentiation of C2C12 skeletal muscle cells as a function of PPARγ expression. Cell lysates (40 μg) were resolved in 10% SDS-PAGE, transferred to nitrocellulose paper, immunoblotted with anti-myogenin antibody or β-tubulin (as an indicator of loading control) Experiments were performed three times and one representative blot has been shown. lane 1: C2C12wt; lane 2: C2PPARγ/+; lane 3: C2PPARγ/−. Data are mean ± standard error of mean (s.e.m.) (*$p < 0.01$).

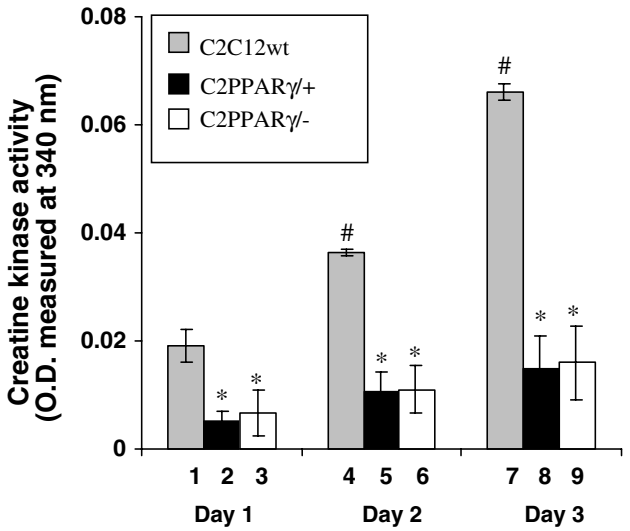

Fig. 4. Creatine kinase activity during myogenic differentiation of C2C12 skeletal muscle cells as a function of PPARγ expression. C2C12wt, C2PPARγ/+, C2PPARγ/− cells were allowed to differentiate for 3 days. Creatine kinase activities were recorded daily for 3 days of differentiation. Data are mean ± standard error of mean (s.e.m.) of three independent experiments ($^{\#}p < 0.01$ compared with lane 1; *$p < 0.01$ compared with respective controls within the group).

Discussion

Physiological characterization of PPARγ deficiency in mice has been limited to the study of heterozygous PPARγ+/− mice or tissue-specific deletions; because PPARγ−/− mice die during intra-uterine development owing to defects in the placenta [34, 35]. Muscle-specific PPARγ deficient mice have been shown to develop insulin resistance [16, 17]. PPARγ is required in mature white and brown adipocytes for their survival in the mouse [36]. It has been shown *in vitro* that PPARγ is required for differentiation of adipose cells from embryonic stem cells and from embryonic fibroblasts [37]. These studies have led to re-interpret the role of PPARγ in the control of cellular physiology and prompted us to investigate whether PPARγ is required for skeletal muscle cell development and differentiation.

Recently, we have shown that inhibition of muscle PPARγ expression caused insulin resistance *in vitro* [26]. Overexpression of PPARγ sensitized the cells to insulin causing more glucose uptake even under insulin resistant conditions [26]. However, the cells with alerted PPARγ expression could not differentiate properly. In cultured cell systems, it has been possible to observe the activation of inappropriate programme of gene expression. In this study, alteration in the expression of PPARγ completely blocked muscle differentiation and prevented transcriptional activation of muscle-specific genes by the myogenic activators myogenin, MyoD and creatine kinase, which are directly linked to cell cycle regulating proteins and are regulators of skeletal muscle differentiation. The ability to form myotubes was completely lost if the expression of PPARγ was altered. This suggests PPARγ's role as myogenic transcriptional regulators. To date there are no report of the role of PPARγ in skeletal muscle differentiation. It appears from present studies that critical expression of PPARγ is necessary to maintain differentiation in skeletal muscle cells. Long-term expression or activation of PPARγ may result in modulation of different subsets of differentiation related genes. The mechanism whereby PPARs may participate in different cellular processes is still not clear but may involve different substrates, which needs to be addressed. This suggests PPARγ's role as myogenic transcriptional regulators.

The three PPAR subtypes identified PPARα, PPARδ (also called β), and PPARγ (γ1, γ2, and γ3 isoforms) exhibit unique as well as overlapping tissue-specific expression and ligand activation profiles, and regulate physiological processes [38–40]. They are encoded by separate genes and

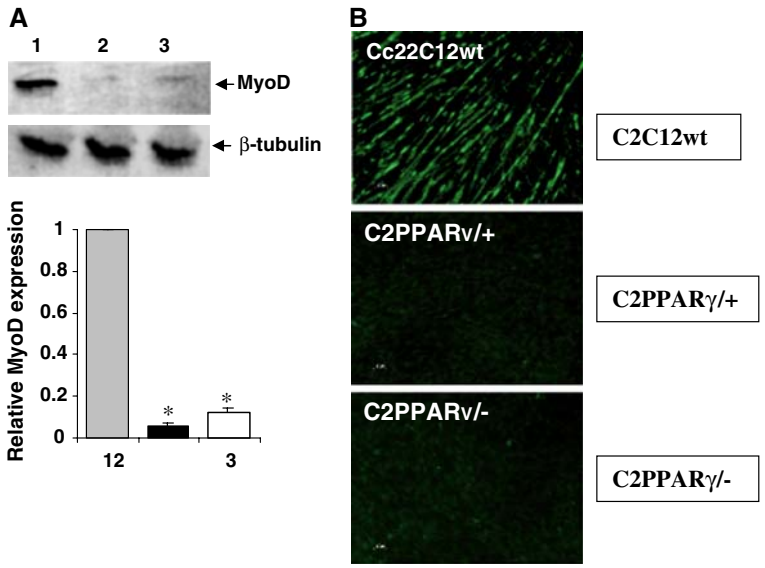

Fig. 5. Determination of MyoD expression during myogenic differentiation of C2C12 skeletal muscle cells as a function of PPARγ expression. (A) Cell lysates (40 μg) were resolved in 10% SDS-PAGE, transferred to nitrocellulose paper, immunoblotted with anti-MyoD antibody or β-tubulin (as an indicator of loading control). lane 1: C2C12wt; lane 2: C2PPARγ/+; lane 3: C2PPARγ/−. Data are mean ± standard error of mean (s.e.m.) (*$p < 0.01$). (B) Immunofluorescence microscopy photograph of C2C12wt, C2PPARγ/+ and C2PPARγ/− cells after 3 days of differentiation. Experiments were performed three times and representative blots or photographs have been shown.

exhibit distinct tissue distribution in animals [41–42]. However, the normal function of PPARs remains unclear. PPARγ is expressed predominantly in mature adipocytes and its role as a key transcriptional factor controlling adipocyte differentiation has been demonstrated [43–46]. Peroxisome proliferation (PP) family of nuclear receptors are PPARα, PPARγ, and PPARδ. PP is mediated by PPARα, that regulate the expression of genes associated with lipid metabolism and adipocyte differentiation. PPARγ is involved in adipogenesis and differentiation, but the events do not directly involve peroxisomes and peroxisome proliferation. PPARs heterodimerize with 9-*cis*-retinoic acid receptor (RXR), and bind to PP response element(s) (PPREs) on the target gene promoter to initiate transcriptional activity. There are tissue- and species-specific responses that depend on relative abundance of PPAR, PPRE, the degree of competition and/or cross-talk among nuclear transcription factors, pharmacokinetics and modulations of coactivators and corepressors on ligand-dependent transcription of PPARs [47]. Zhu and Reddy [47] have identified PPAR coactivators like steroid receptor coactivator-1 (SRC-1) and PPAR-binding protein (PBP). PPARγ coactivator-1α (PGC-1α), a coactivator at the transcriptional level is specifically expressed in skeletal muscle. The expression of PGC-1α in muscle is regulated by two transcription factors, MEF2 and FKHR, implicated in terminal differentiation of muscle [48, 49]. In both C2C12 and Sol8 myoblasts, Chang *et al*. [49] observed that myogenic bHLH proteins, MyoD, activated PGC-1α expression *in vivo*, which in turn was activated during terminal muscle differentiation. These results directly implicated the activation of the key fibre-type and metabolic switch gene PGC-1α. PGC-1α and PGC-1β regulate PPARα [50]. Identification of additional coactivators that may be responsible for cell-specific transcriptional activation of PPAR-mediated target genes will be necessary to gain more insight into the responses regulated by PPAR. We have provided evidences for a cellular control component that regulate muscle-specific proteins. PPARγ conceivably participates through a variety of known and/or yet unknown coactivators, as discussed above. It may affect the myogenic factors in the regulatory networks that convey inhibitory signals in myoblasts to the important myogenic control factors present in the nucleus. In this connection, it is important to assemble a complete picture of signaling pathways triggered by proliferative and differentiation factors, and the mechanisms that coordinate these processes leading to muscle differentiation. This understanding, together with greater physiological knowledge of the impact of certain genetic manipulations *in vivo*, is essential to design future approaches for the treatment of skeletal muscle diseases and for the prevention of or recovery from muscle loss in situations such as cachexia, muscle wasting or sarcopenia. From a pathophysiological point of view, it may be essential to ensure that PPARγ expression is not altered as it may alter sequence of events in the repair of injured muscles during

subsequent differentiation of myotubes. To the best of our knowledge, this is the first report, which establishes the relationship between skeletal muscle cell differentiation as a function of PPARγ expression.

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