

PPAR γ -dependent and PPAR γ -independent effects on the development of adipose cells from embryonic stem cells

C. Vernochet^a, D.S. Milstone^b, C. Iehl^a, N. Belmonte^a, B. Phillips^a, B. Wdziekonski^a, P. Villageois^a, E.Z. Amri^a, P.E. O'Donnell^b, R.M. Mortensen^c, G. Ailhaud^a, C. Dani^{a,*}

^aInstitute of Signaling, Developmental Biology and Cancer Research, CNRS UMR 6543, Centre de Biochimie, 06108 Nice Cedex 2, France

^bDepartment of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

^cUniversity of Michigan Medical School, Ann Arbor, MI, USA

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Abstract Peroxisome proliferator-activated receptor (PPAR) γ was shown to be required for adipocyte formation both *in vivo* and *in vitro*. However, the role of PPAR γ in the initial steps of adipose cell development was not distinguished from its role in the terminal steps. We now show that PPAR γ is expressed early in embryoid bodies (EBs) derived from embryonic stem cells and in E.8.5 mouse embryos. Addition of a specific ligand for PPAR γ in developing EBs over-expressing PPAR γ did not commit stem cells towards the adipose lineage. In differentiated PPAR γ ^{-/-} EBs, only markers characteristic of preadipocytes were found to be expressed. PPAR δ is present in EBs but did not compensate for the lack of PPAR γ in terminal differentiation. Taken together, these results favor a critical PPAR γ -independent phase culminating in preadipocyte formation that precedes a PPAR γ -dependent phase in the development of adipose cells from pluripotent stem cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mouse embryonic stem cell; Adipose cell; Peroxisome proliferator-activated receptor

1. Introduction

Key events of terminal differentiation converting preadipocytes into mature lipid-filled adipocytes have been characterized in recent years. However, the molecular mechanisms regulating the early development of adipose cells are poorly understood, and master genes that commit the progression from stem cells to the adipoblast stage of development have not yet been identified. The best-characterized transcriptional regulators involved in the development of mature adipocytes are members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear hormone receptors. PPARs are ligand-activated transcription factors that bind as heterodimers with members of the retinoid X receptor (RXR) subfamily to PPAR-responsive elements (PPREs) in the promoters of responsive genes. Three subtypes, PPAR α , PPAR δ and PPAR γ , have been identified. PPAR γ is highly expressed in adipocytes and several lines of evidence, first obtained *in vitro*, indicate that PPAR γ plays an important role in adipogenesis. Treatment of preadipocytes with PPAR γ ligands dramatically in-

creases terminal differentiation, and forced ectopic expression of PPAR γ in a variety of mesenchymal cells is sufficient to induce adipogenesis [1]. The requirement of PPAR γ in the development of fat cells has recently been investigated *in vivo* by gene targeting [2–4]. Homozygous PPAR γ -deficient embryos die at midgestation due to a placental defect associated with myocardial thinning [2]. This phenotype revealed a new role of PPAR γ in the development of placenta, but precluded a straightforward approach to investigating the role of PPAR γ in the development of adipose tissue. Blastocyst injection of PPAR γ ^{-/-} embryonic stem (ES) cells showed that these mutant cells did not contribute to the formation of adipose tissue in chimeric animals [4]. Although these studies showed convincingly that PPAR γ is required for the formation of lipid-containing cells *in vivo*, it did not investigate the role of PPAR γ in earlier events relative to adipose cell determination, nor did it indicate at what stage PPAR γ ^{-/-} cells were blocked in the adipocyte developmental program. To address this issue, the role of PPAR γ during the development of ES cells *in vitro* has been investigated.

ES cells are derived from the inner cell mass of 3.5 day blastocysts [5,6]. It is well documented that formation of embryoid bodies (EBs) permits the commitment of pluripotent ES cells into multiple cellular lineages *in vitro* [7]. The differentiation of ES cells can mimic closely the development of early embryos *in vivo* [8]. Therefore, *in vitro* differentiation of ES cells provides an invaluable model both for studying the early steps of development and for the characterization of the role of genes expressed during different developmental programs. We have shown previously that ES cells can differentiate with a high efficiency into fat cells [9–11]. A prerequisite for the commitment of ES cells into the adipose lineage was the treatment of ES cell-derived EBs at an early stage of their differentiation with all-*trans* retinoic acid (RA). Two phases of adipogenesis were identified. The first phase, between day 2 and day 5 after EB formation, consisted of the maintenance of ES cell-derived EBs in suspension and treatment with RA. At this stage EBs contained differentiating cells of ectodermal, endodermal and mesodermal lineages [12]. The second phase consisted of the plating of the RA-treated EBs, which results in the development of differentiated embryonic (DE) outgrowths. The percentage of DE outgrowths containing adipocyte colonies was found to be modulated by adipogenic hormones.

In the studies presented herein, we showed that PPAR γ was expressed early in developing EBs and in E 8.5 embryos. A

*Corresponding author. Fax: (33)-4-92 07 64 04.
E-mail address: dani@unice.fr (C. Dani).

possible role of PPAR γ in the initial step(s) of adipose cell commitment was examined in ES cells over-expressing PPAR γ as well as in PPAR γ -deficient ES cells. The results indicate that there is a PPAR γ -independent phase in which the cells are committed towards the adipogenic lineage, followed by a PPAR γ -dependent phase which is critical for the formation of mature lipid-filled adipose cells from pluripotent stem cells.

2. Materials and methods

2.1. Embryos, ES cell culture and differentiation of EBs

Procedures involving animals conformed to institutional and Federal guidelines and were approved by the appropriate institutional review boards. Matings were established between C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA). CGR8 ES cells [13] were cultivated in the pluripotent state on gelatin-coated plates as previously described [9]. D3 ES cells and PPAR γ ^{-/-} ES cells were cultivated in undifferentiated state on a feeder layer of mitomycin C-inactivated STO cells. Before EB formation, cells were grown on gelatinized plates as described for CGR8 ES cells. The experimental protocol used for differentiation of ES cells into adipocytes has been described previously [9]. Commitment of ES cells into the adipose lineage was initiated by all-*trans* RA treatment. Five-day-old EBs were then plated onto culture plates to allow development of DE cells as outgrowths. Adipocyte differentiation in outgrowths was enhanced by adding 85 nM insulin, 2 nM triiodothyronine (T3) and the thiazolidinedione BRL49653 and CD3986 when indicated. BRL49653 was a gift from SmithKline Beecham and CD3986 was a gift from Galderma (Sophia-Antipolis, France).

2.2. Plasmid constructs

To generate the PPAR γ expressing vector, a 1.5-kb *XhoI/NotI* fragment containing the complete open reading frame of mouse PPAR γ was inserted into the pCAGSIH vector in place of the alkaline phosphatase cDNA fragment. Therefore, the PPAR γ expressing vector consisted of the synthetic CAG promoter [14] driving the PPAR γ -IRES-hygromycin resistance bicistronic gene. This vector was appropriate for gain of function experiments in ES cells as it showed the following features: (i) the CAG promoter has been proven to be efficient in undifferentiated ES cells and in differentiated derivatives ([15] and this paper) and (ii) the bicistronic gene ensured expression of PPAR γ in all stably transfected cells. This assumption has been verified in preliminary experiments (not shown). A polyclonal cell population, containing 10–20 clones, was generated and used for all experiments, avoiding therefore the use of individual clones. The pCAGSIH vector was constructed in Austin Smith's laboratory (CGR, UK) and was also used to generate the control-ES cell population.

2.3. Generation of transgenic ES cell lines and transactivation assays

CGR8 ES cells were transfected with pCAG/PPAR γ and pCAGSIH expressing vectors using the FuGene 6 transfection system (Roche Molecular Biochemicals) according to the manufacturer's protocol. Clones were selected in hygromycin (100 μ g/ml) for 10 days and pooled to generate polyclonal cell populations. For determination of the capacity of transactivation of PPAR γ in control-ES cell and PPAR γ -ES cell populations, cells were stably co-transfected with the PPRE3-tk-firefly luciferase (*luc*) reporter and a neomycin resistance gene expressing vector. Clones were selected in G418 (200 μ g/ml) for 10 days and pooled. The PPRE3-tk-firefly luc reporter contained three copies of the PPRE from the acyl-CoA oxidase promoter [16]. Firefly luciferase activities in EBs (pool of 200 EBs) were determined using the Bright-Glo Luciferase Assay System (Promega, France). Results were normalized with the protein concentrations determined by the Bio-Rad Protein Assay.

2.4. RNA analysis

RNA from embryos was isolated using guanidinium thiocyanate and cesium chloride gradients. Random hexamer-primed cDNA was prepared from 1 μ g of total RNA. PCR used the following oligonucleotides: PPAR γ , forward 5'-CGAGTCTGTGGGATAAAGC-3'; reverse 5'-CAGCGGAAGGACTTATGT-3'; β -actin, forward 5'-TGGAATCCTGTGGCATCCATGAAAC-3', reverse 5'-TAAACGCGACTCAGTAACAGTCCG-3'. PCR conditions were 95°C for

5 min followed by 36 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 40 s. Samples were then heated at 72°C for 10 min and analyzed. RNA from EBs was prepared as previously described [17]. For reverse transcription (RT)-PCR analysis, samples of 500 ng were reverse-transcribed and cDNAs were amplified using the OneStep RT-PCR kit (Qiagen, Germany). RT reactions were denatured for 15 min at 95°C, followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C. Primers for PPAR γ and hypoxanthine guanine phosphoribosyl transferase (HPRT) were included in the same reaction. Sequences of primers used in PCR are as follows: PPAR γ , 5'-GAATGACCAAGTGACTCTGCTCAA-3' and 5'-CAAGCTGAATCACCCAGAGTCC-3'; HPRT, 5'-GCTGGTGGAAAAGGACCTCT-3' and 5'-CACAGGACTAGAACACCTGC-3'. Electrophoretic separation of PCR products was carried out on 2% agarose gels. Northern blot analysis was performed as previously described [9]. Quantification of the hybridization signal was performed using a PhosphorImager apparatus (Fujix Bas 1000) coupled to the MacBas ver2.x bio-imaging analyzer. The *Brachyury* cDNA was obtained from Dr. B. Herrmann (Max-Planck Institut, Freiburg, Germany). The HMGIC cDNA was generated by RT-PCR using RNA prepared from EBs. Sequences of primers and PCR conditions are described in [18].

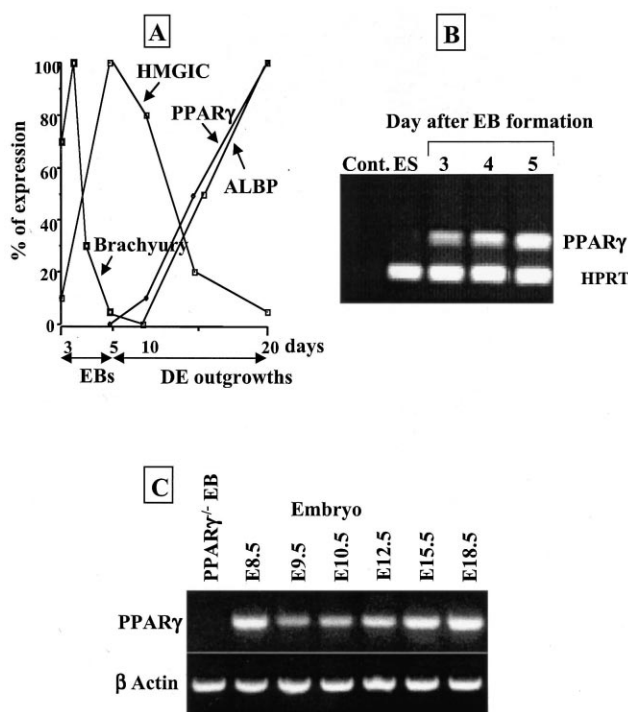


Fig. 1. Expression of PPAR γ in EBs derived from ES cells and in embryos. A: 20 μ g of total RNA was prepared at indicated times and the expression of marker genes was determined by Northern blotting. The results of a representative experiment are shown and are expressed by taking as 100% the maximal signal obtained for each probe. B: Total RNA extracted from pluripotent undifferentiated ES cells (ES) or from EBs at the indicated times was reverse-transcribed and PCR-amplified using primers specific for PPAR γ and the housekeeping gene HPRT. PCR-amplified fragments separated by electrophoresis and stained with ethidium bromide are shown. Cont.: control with no RNA. C: Whole cellular RNA was prepared from embryos at indicated times. 1 μ g was reverse-transcribed using random hexamer-primed cDNA. 5% of the cDNA reaction product was amplified using PPAR γ - and β -actin-specific primer pairs. RNA prepared from PPAR γ ^{-/-} EBs was used as a negative control.

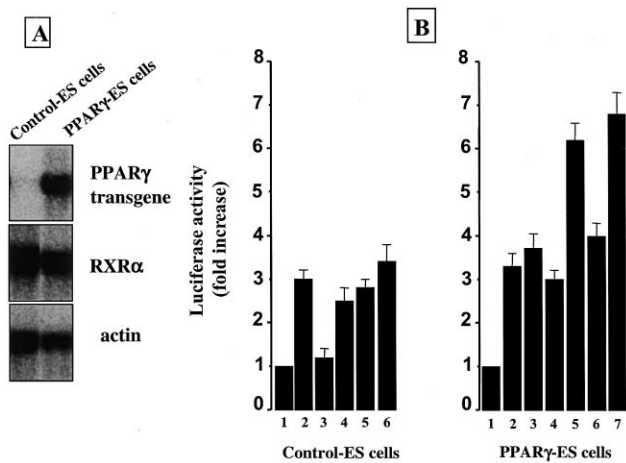


Fig. 2. A: Expression of PPAR γ transgene and RXR α in control and PPAR γ over-expressing EBs. RNAs were prepared from 5-day-old EBs and 20 μ g was subjected to Northern blot analysis. B: Transcription of a PPRE-containing gene in control-ES cells and PPAR γ -ES cells stably transfected with PPRE3-tk-firefly luciferase reporter gene. Luciferase activities were determined 4 h after addition of 1: 0.1% DMSO; 2: 1 μ M PPAR δ ligand CD3986; 3: 1 μ M PPAR γ ligand BRL49653; 4: 10⁻⁷ M all-trans RA; 5: RA+BRL; 6: 10⁻⁶ M 9-cis RA or 7: 9-cis RA plus BRL. Activity was normalized versus protein content. The values represent mean \pm S.E.M. of three independent experiments.

3. Results and discussion

3.1. Generation of a polyclonal ES cell population over-expressing PPAR γ

We have investigated the expression of the endogenous PPAR γ gene during in vitro development of ES cells. Its pattern of expression was compared with that of developmental marker genes such as *Brachyury T*, a marker of nascent mesoderm [19], *HMGIC*, a gene preferentially expressed in undifferentiated mesenchymal cells [20], highly expressed in 3T3-L1 preadipocytes [21] and in adipose tissue [18], and *ALBP*, an adipocyte differentiation-dependent gene [22]. As shown in Fig. 1A, the *Brachyury T* gene was specifically expressed in EBs and corresponded with the permissive period for adipogenic commitment which was defined previously [9]. Expression of *Brachyury T* gene in early differentiating EBs has previously been reported [12]. Expression of the *HMGIC* gene was weak in EBs and peaked early in DE outgrowths at a time when undifferentiated mesenchymal cells were proliferating. Its expression declined thereafter when various terminally differentiated cell types appeared, such as adipose cells characterized by the expression of *ALBP*. Expression of PPAR γ was detected by Northern blotting only in DE outgrowths and paralleled the expression of *ALBP*. However, the greater sensitivity of RT-PCR showed that PPAR γ was also expressed in EBs during the permissive period for adipogenic commitment (Fig. 1B). In mouse embryos, PPAR γ expression was detected by RT-PCR at gestation day 8.5 and beyond (Fig. 1C). To gain insights into the capacity of PPAR γ to commit stem cells into the adipogenic lineage and to study its role in the different steps of adipose cell development, transgenic ES cells were generated. Cells of the pluripotent ES cell line CGR8 were stably transfected with a pCAG/PPAR γ -IRES-hygromycin bicistronic expression vector. A polyclonal cell population, named PPAR γ -ES cell population, was isolated. A polyclonal

cell population, named control-ES cell population expressing an irrelevant cDNA, was also generated (see Section 2). Expression of PPAR γ transgene in early EBs was detected by Northern blotting. RXR α , the partner of PPAR γ required to form functional heterodimers, was expressed at a similar level in control and PPAR γ over-expressing EBs (Fig. 2A). The PPAR γ transgene had the capacity, in 5-day-old EBs, to regulate the transcription of PPRE-firefly luciferase gene under the control of a PPRE. As shown in Fig. 2B, stimulation with a specific PPAR γ ligand, the thiazolidinedione BRL49653 [23], led to detectable luciferase activity in transgenic ES cells only. All-trans RA enhanced transcriptional activity of PPAR γ and activation of RXRs by the 9-cis RA had the same effect as the all-trans RA. Addition of both BRL49653 and all-trans RA or 9-cis RA led to a higher PPAR γ transcriptional activity as expected. Stimulation of EBs with a specific ligand of PPAR δ , the CD3986 also known as GW2433 [24], led to a similar increase in expression of luciferase activity in wild-type and PPAR γ -ES cells. This result is in agreement with our previous data showing expression of PPAR δ in early EBs [9]. Altogether, these results indicated that the intracellular environment in EBs was appropriate for the transgene to activate the transcription of target genes. Therefore, it was possible to study the effect of activation of the transgene PPAR γ on the development of adipose cells.

3.2. Effect of PPAR γ during the development of DE outgrowths

EBs formed from PPAR γ - and control-ES cells were treated with RA to commit them into the adipose lineage. The thiazolidinedione BRL49653 was then added during the phase of differentiation and expression of specific genes for adipocytes, namely *ALBP* and glycerol-phosphate dehydrogenase (GPDH), was investigated by Northern hybridization analysis. *ALBP* and *GPDH* gene expression was increased 2.8 \pm 0.4-fold and 3.5 \pm 0.8-fold respectively in PPAR γ -DE outgrowths compared to control-DE outgrowths (in four independent ex-

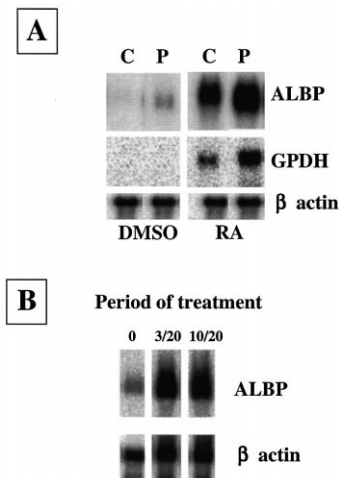


Fig. 3. Effects of over-expression and activation of PPAR γ in DE outgrowths. A: EBs, formed from control-ES cells (C) and PPAR γ -ES cells (P) were stimulated from day 2 to day 5 with either 0.1% DMSO (which is the vehicle of RA) or 0.1 μ M RA. After plating, DE outgrowths were maintained in medium supplemented with 1 μ M BRL49653. B: PPAR γ -DE outgrowths were maintained in differentiation medium in the absence (0) or presence of 0.5 μ M BRL49653 for the indicated period. Total RNA was prepared at day 20 and 20 μ g was subjected to Northern blot analysis.

periments, see Fig. 3A, right panel). In the absence of RA treatment during the critical period for commitment, expression of the *ALBP* and *GPDH* genes remained very low, if detectable at all (Fig. 3A, left panel). These data indicated that RA remained necessary for promoting the initial steps of development of adipose cells despite forced PPAR γ expression. Altogether, these results showed that over-expression and activation of PPAR γ during the differentiation phase of embryonic cells derived from RA-treated EBs led to an enhancement of adipogenesis. Interestingly, the activation of PPAR γ in DE outgrowths at earlier time than day 10 had no effect on the degree of adipocyte differentiation at day 20. As shown in Fig. 3B, exposure of PPAR γ -DE cells to BRL49653 from day 3 or from day 10 led to the same effect on adipocyte differentiation at day 20 as marked by the expression of *ALBP* gene. A similar pattern of expression was obtained for the *GPDH* gene (not shown). Exposure of PPAR γ -DE cells to BRL49653 from day 7 to 10 had no effect on adipocyte differentiation at day 20 and exposure from day 10 was sufficient to achieve maximal response on adipogenesis at day 20 (not shown). These observations confirmed that PPAR γ had a major role in terminal differentiation-related events and suggested that it played no role in the early steps of adipose cell development. We next confirmed this hypothesis by activating PPAR γ during the determination phase of differentiation.

3.3. Consequences of activation of PPAR γ in EBs on the development of adipose cells

PPAR γ -EBs were stimulated from day 2 to day 5 either with RA or BRL49653 and adipogenesis in 20-day-old DE outgrowths was scored. Treatment with RA (10^{-8} M or 10^{-7} M) led to a high level of adipogenesis as expected. In contrast, activation of PPAR γ alone did not promote commitment to the adipose lineage whereas treatment with RA plus BRL49653 did not increase adipogenesis compared to RA

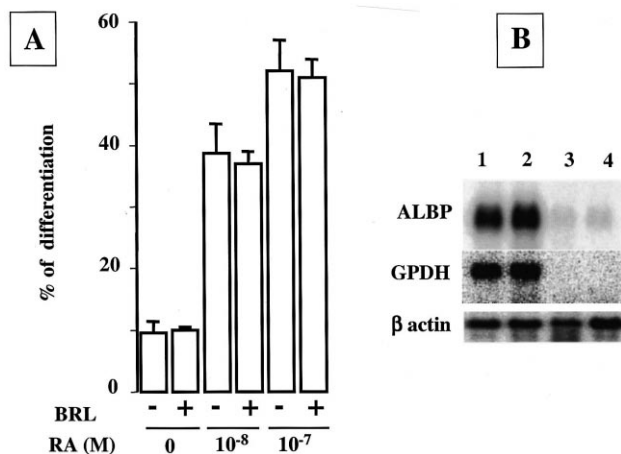


Fig. 4. Lack of effects of PPAR γ during the commitment period of ES cells. A: EBs were treated between days 2 and 5 with 10^{-7} M or 10^{-8} M RA in the presence or in the absence of $1 \mu\text{M}$ BRL49653. Then, DE outgrowths were maintained in medium supplemented with $1 \mu\text{M}$ BRL49653. The percentage of 20-day-old DE outgrowths containing adipocyte colonies was determined. The number of DE outgrowths examined was between 80 and 120 for each condition. Data were the means from two experiments. B: RNA was prepared from 20-day-old DE outgrowths derived from PPAR γ -EBs treated either with 1: $0.1 \mu\text{M}$ RA; 2: $0.1 \mu\text{M}$ RA plus $1 \mu\text{M}$ BRL49653; 3: $1 \mu\text{M}$ BRL49653; 4: 0.1% DMSO.

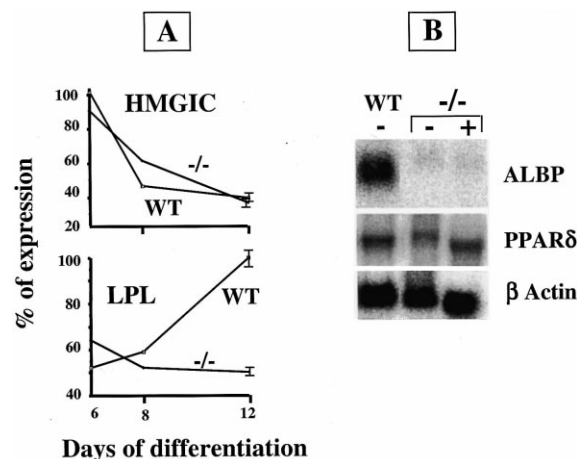


Fig. 5. Expression of differentiation markers in differentiating control- and PPAR $\gamma^{-/-}$ ES cells. A: EBs, formed from D3 ES cells (WT) and from PPAR γ -deficient ES cells ($-/-$), were treated with RA as shown in Fig. 3. RNA was prepared at indicated times. Data represent the average of two experiments and are expressed by taking as 100% the maximal signal obtained for each probe. B: EBs were treated as in A and supplemented (+) or not (-) with $1 \mu\text{M}$ CD3986 from day 7 to 17. Total RNA was prepared at day 17 and $20 \mu\text{g}$ was subjected to Northern blot analysis.

only (Fig. 4A). Activation of PPAR γ together with RXRs was inefficient in promoting adipogenesis (not shown). These results indicated that in the absence of RA the full activation PPAR γ was not able to commit stem cells into the adipogenic lineage. We next analyzed expression of adipocyte differentiation-dependent genes. As shown in Fig. 4B, expression of *ALBP* and *GPDH* genes was high in DE outgrowths derived from RA-treated EBs. No further increase (1.2 ± 0.3 -fold from six independent experiments) was observed after the simultaneous addition of RA and BRL49653 (Fig. 4B, lane 2) whereas their expression remained low in DE outgrowths derived from both BRL49653-treated and untreated EBs (Fig. 4B, lanes 3 and 4 respectively). Expression of *HMGIC* and lipoprotein lipase (*LPL*) genes, two markers expressed in preadipocytes earlier than *ALBP* and *GPDH*, was not modified by activation of PPAR γ in early EBs (not shown) suggesting that early expression of PPAR γ did not lead to the generation of preadipocytes which were blocked prior to terminal differentiation. Altogether, these results demonstrated that activation of PPAR γ could not act as a substitute for the promoting effect of RA on the determination of stem cells towards the adipose lineage and that PPAR γ did not play a role in the initial steps of the adipose cell development.

3.4. PPAR $\gamma^{-/-}$ ES cells treated with RA can be committed into the adipose lineage but are blocked at the preadipose stage of the developmental program

Recently, PPAR $\gamma^{-/-}$ ES cells have been generated and it was shown that they failed to undergo differentiation into adipocytes both in vitro and in vivo. Normal levels of expression of *C/EBP β* and *C/EBP δ* in PPAR γ null ES cells suggested that mutant cells were arrested at the preadipose stage [4]. *C/EBP β* and *C/EBP δ* are two genes expressed in preadipocytes but also expressed in other cell types present in wild-type and mutant differentiating ES cells. Therefore, this result did not indicate whether the lack of PPAR γ was preventing commitment of stem cells towards the adipose lineage or

whether they were blocked at the preadipose stage. To address this issue, the expression of *HMGIC* and *LPL* genes in RA-treated wild-type and PPAR γ -deficient DE outgrowths was investigated. As previously indicated, *HMGIC* expression is high in preadipocytes and does not persist in adipocytes [18]. *LPL* is preferentially expressed in adipose tissue and we have previously shown that during adipose cell differentiation, the emergence of *LPL* characterizes the preadipose stage, its expression being increased during terminal differentiation [25,26]. *LPL* gene is also expressed in skeletal myocytes, but as we have previously reported, differentiation of ES cells into adipocytes inhibits the development of skeletal myocyte lineage [10]. Therefore, studies on the expression of both genes were appropriate for investigating the stage at which adipose cell development was blocked in mutant cells. As shown in Fig. 5A, the kinetics of expression of both preadipocyte markers were paralleled in wild-type and differentiating mutant cells, strongly suggesting that the lack of PPAR γ did not prevent commitment of stem cells into the adipose lineage but led to an arrest at the preadipose stage of the developmental program. The increased expression of the *LPL* gene at day 12 in wild-type cells reflected the appearance of mature adipocytes.

It has recently been reported that ectopic expression of PPAR δ in fibroblasts promotes expression of adipocyte-specific genes [27]. In addition, over-expression of PPAR δ in Ob1771 and 3T3-F442A preadipose cells enhances this process [27,28]. As shown in Fig. 5B, PPAR δ was expressed in wild-type and mutant differentiating outgrowths. However, its stimulation with the specific agonist CD3986 was clearly unable to compensate for the lack of PPAR γ in terminal differentiation of ES cells to adipocytes, as mutant DE outgrowths did not contain adipocytes (not shown) and were not able to express the *ALBP* gene either with or without stimulation (Fig. 5B). These results indicate that, although PPAR γ was not required for the initial steps of adipose cell development, it plays a crucial role in terminal steps of differentiation and that this role could not be replaced by PPAR δ .

In conclusion, a model is proposed in which, in contrast to PPAR γ -dependent differentiation of preadipocytes into adipocytes, commitment of pluripotent stem cells to the adipose lineage is independent of PPAR γ . Altogether, our results indicate that expression of PPAR γ is not rate-limiting in triggering the adipose lineage from pluripotent stem cells. However, one cannot exclude that endogenous ligands and target molecules of PPAR γ , which are not fully identified, represent the rate-limiting step of the process controlled by RA. Further analysis of this possibility requires the characterization of such ligands. Regulation of adipocyte differentiation is an important process if one considers the need to control the development of adipose tissue. The increase in weight of adipose tissue in obesity is due to hypertrophy of fat cells and an increase of the recruitment of new adipocytes from precursors. So far, RA is the only effector able to promote the first steps of adipose cell development. A better understanding of the molecular mechanisms mediating RA effects in early differentiating ES cells and the isolation of RA-specific target genes

should lead to the identification of the regulatory genes involved in adipocyte determination.

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