FEBS Letters 510 (2002) 94–98 FEBS 25620

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

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Received 2 October 2001; revised 28 November 2001; accepted 28 November 2001

First published online 6 December 2001

Edited by Ned Mantei

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 PPARγ in the initial steps of adipose cell development was not distinguished from its role in the terminal steps. We now show that PPARγ 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 PPARγ in developing EBs over-expressing PPARγ did not commit stem cells towards the adipose lineage. In differentiated PPARγ$^{-/-}$ EBs, only markers characteristic of preadipocytes were found to be expressed. PPARδ is present in EBs but did not compensate for the lack of PPARγ in terminal differentiation. Taken together, these results favor a critical PPARγ-independent phase culminating in preadipocyte formation that precedes a PPARγ-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, PPARα, PPARδ and PPARγ, have been identified. PPARγ is highly expressed in adipocytes and several lines of evidence, first obtained in vitro, indicate that PPARγ plays an important role in adipogenesis. Treatment of preadipocytes with PPARγ ligands dramatically increases terminal differentiation, and forced ectopic expression of PPARγ in a variety of mesenchymal cells is sufficient to induce adipogenesis [1]. The requirement of PPARγ in the development of fat cells has recently been investigated in vivo by gene targeting [2–4]. Homozygous PPARγ-deficient embryos die at midgestation due to a placental defect associated with myocardial thinning [2]. This phenotype revealed a new role of PPARγ in the development of placenta, but precluded a straightforward approach to investigating the role of PPARγ in the development of adipose tissue. Blastocyst injection of PPARγ$^{-/-}$ 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 PPARγ is required for the formation of lipid-containing cells in vivo, it did not investigate the role of PPARγ in earlier events relative to adipose cell determination, nor did it indicate at what stage PPARγ$^{-/-}$ cells were blocked in the adipocyte developmental program. To address this issue, the role of PPARγ 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 PPARγ was expressed early in developing EBs and in E 8.5 embryos. A

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0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)03235-5

possible role of PPARγ in the initial step(s) of adipose cell commitment was examined in ES cells over-expressing PPARγ as well as in PPARγ-deficient ES cells. The results indicate that there is a PPARγ-independent phase in which the cells are committed towards the adipogenic lineage, followed by a PPARγ-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 PPARγ$^{-/-}$ 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 PPARγ expressing vector, a 1.5-kb *Xho*I/*Not*I fragment containing the complete open reading frame of mouse PPARγ was inserted into the pCAGSIH vector in place of the alkaline phosphatase cDNA fragment. Therefore, the PPARγ expressing vector consisted of the synthetic CAG promoter [14] driving the PPARγ-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 PPARγ 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/PPARγ 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 μg/ml) for 10 days and pooled to generate polyclonal cell populations. For determination of the capacity of transactivation of PPARγ in control-ES cell and PPARγ-ES cell populations, cells were stably co-transfected with the PPREx3-tk-firefly luciferase (luc) reporter and a neomycin resistance gene expressing vector. Clones were selected in G418 (200 μg/ml) for 10 days and pooled. The PPREx3-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 μg of total RNA. PCR used the following oligonucleotides: PPARγ, forward 5′-CGAGTCTGTGGGGATAAAGC-3′; reverse 5′-CAGCGGGAAGGACTTTATGT-3′; β-actin, forward 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, reverse 5′-TAAAACGCAGCTCAGTAACAGTCCG-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 PPARγ and hypoxanthine guanine phosphoribosyl transferase (HPRT) were included in the same reaction. Sequences of primers used in PCR are as follows: PPARγ, 5′-GAATGACCAAGTGACTCTGCTCAA-3′ and 5′-CAAGCTGAATCACCCAGAGTCC-3′; HPRT, 5′-GCTGGTGAAAAGGACCTCT-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].

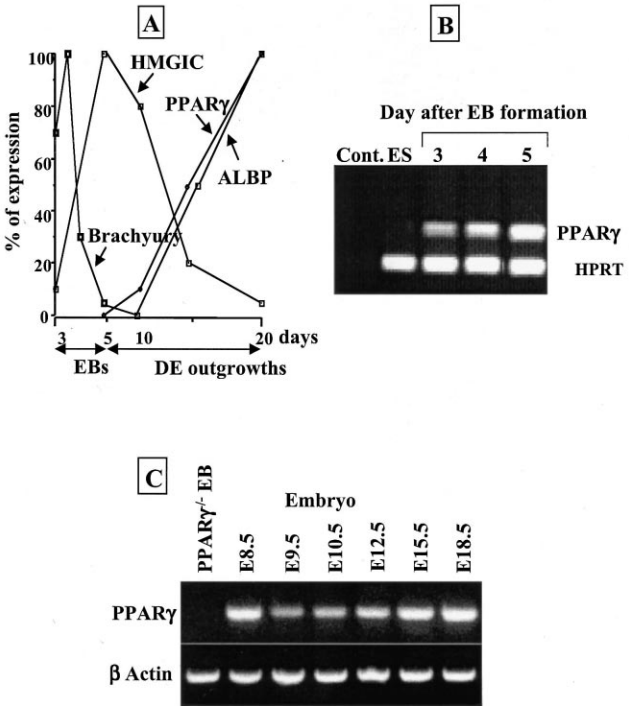

Fig. 1. Expression of PPARγ in EBs derived from ES cells and in embryos. A: 20 μg of total RNA was prepared at indicated times and the expression of marker genes was determined by Northern blotting. The results of a representative experiment are shown and are expressed by taking as 100% the maximal signal 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 PPARγ 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 μg was reverse-transcribed using random hexamer-primed cDNA. 5% of the cDNA reaction product was amplified using PPARγ- and β-actin-specific primer pairs. RNA prepared from PPARγ$^{-/-}$ EBs was used as a negative control.

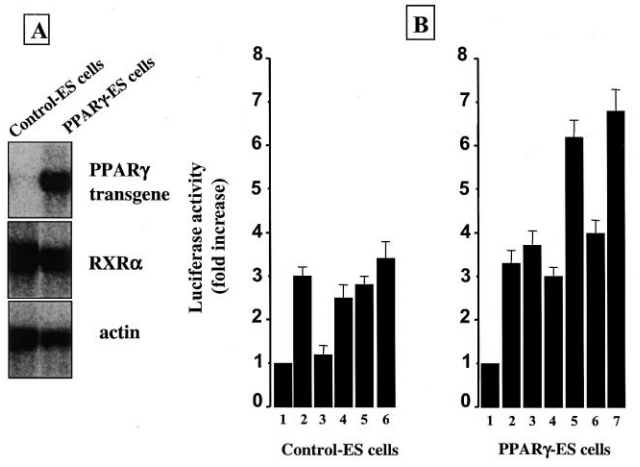

Fig. 2. A: Expression of PPARγ transgene and RXRα in control and PPARγ over-expressing EBs. RNAs were prepared from 5-day-old EBs and 20 μg was subjected to Northern blot analysis. B: Transcription of a PPRE-containing gene in control-ES cells and PPARγ-ES cells stably transfected with PPREx3-tk-firefly luciferase reporter gene. Luciferase activities were determined 4 h after addition of 1: 0.1% DMSO; 2: 1 μM PPARδ ligand CD3986; 3: 1 μM PPARγ 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 ± S.E.M. of three independent experiments.

3. Results and discussion

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

We have investigated the expression of the endogenous PPARγ 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 PPARγ was detected by Northern blotting only in DE outgrowths and paralleled the expression of ALBP. However, the greater sensitivity of RT-PCR showed that PPARγ was also expressed in EBs during the permissive period for adipogenic commitment (Fig. 1B). In mouse embryos, PPARγ expression was detected by RT-PCR at gestation day 8.5 and beyond (Fig. 1C). To gain insights into the capacity of PPARγ 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/PPARγ-IRES-hygromycin bicistronic expression vector. A polyclonal cell population, named PPARγ-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 PPARγ transgene in early EBs was detected by Northern blotting. RXRα, the partner of PPARγ required to form functional heterodimers, was expressed at a similar level in control and PPARγ over-expressing EBs (Fig. 2A). The PPARγ 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 PPARγ ligand, the thiazolidinedione BRL49653 [23], led to detectable luciferase activity in transgenic ES cells only. All-*trans* RA enhanced transcriptional activity of PPARγ 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 PPARγ transcriptional activity as expected. Stimulation of EBs with a specific ligand of PPARδ, the CD3986 also known as GW2433 [24], led to a similar increase in expression of luciferase activity in wild-type and PPARγ-ES cells. This result is in agreement with our previous data showing expression of PPARδ 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 PPARγ on the development of adipose cells.

3.2. Effect of PPARγ during the development of DE outgrowths

EBs formed from PPARγ- 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 ± 0.4-fold and 3.5 ± 0.8-fold respectively in PPARγ-DE outgrowths compared to control-DE outgrowths (in four independent ex-

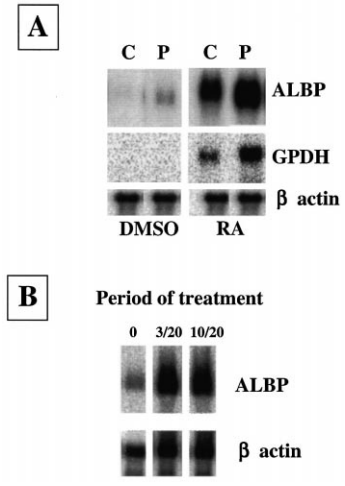

Fig. 3. Effects of over-expression and activation of PPARγ in DE outgrowths. A: EBs, formed from control-ES cells (C) and PPARγ-ES cells (P) were stimulated from day 2 to day 5 with either 0.1% DMSO (which is the vehicle of RA) or 0.1 μM RA. After plating, DE outgrowths were maintained in medium supplemented with 1 μM BRL49653. B: PPARγ-DE outgrowths were maintained in differentiation medium in the absence (0) or presence of 0.5 μM BRL49653 for the indicated period. Total RNA was prepared at day 20 and 20 μg 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 PPARγ expression. Altogether, these results showed that over-expression and activation of PPARγ during the differentiation phase of embryonic cells derived from RA-treated EBs led to an enhancement of adipogenesis. Interestingly, the activation of PPARγ 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 PPARγ-DE cells to BRL496543 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 PPARγ-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 PPARγ 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 PPARγ during the determination phase of differentiation.

3.3. *Consequences of activation of PPARγ in EBs on the development of adipose cells*

PPARγ-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 PPARγ alone did not promote commitment to the adipose lineage whereas treatment with RA plus BRL49653 did not increase adipogenesis compared to RA

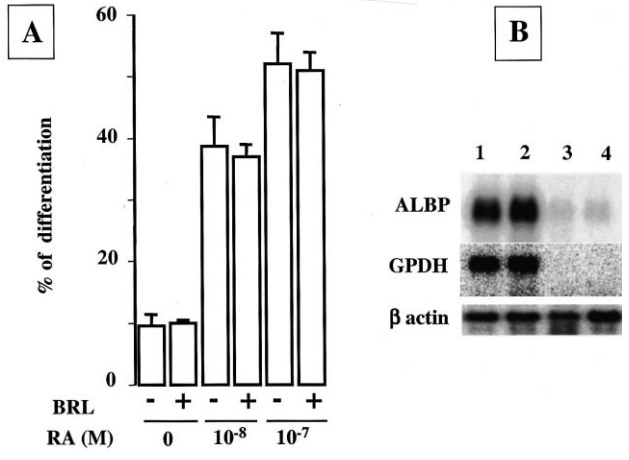

Fig. 4. Lack of effects of PPARγ during the commitment period of ES cells. A: EBs were treated between days 2 and 5 with 10^{-7} M or 10^{-8} M RA in the presence or in the absence of 1 μM BRL49653. Then, DE outgrowths were maintained in medium supplemented with 1 μM BRL49653. The percentage of 20-day-old 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 PPARγ-EBs treated either with 1: 0.1 μM RA; 2: 0.1 μM RA plus 1 μM BRL49653; 3: 1 μM BRL496543; 4: 0.1% DMSO.

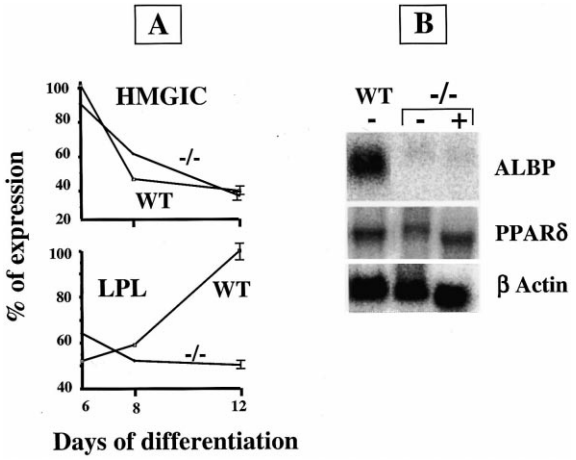

Fig. 5. Expression of differentiation markers in differentiating control- and PPARγ$^{-/-}$ ES cells. A: EBs, formed from D3 ES cells (WT) and from PPARγ-deficient ES cells (−/−), were treated with RA as shown in Fig. 3. RNA was prepared at indicated times. Data represent the average of two experiments 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 μM CD3986 from day 7 to 17. Total RNA was prepared at day 17 and 20 μg was subjected to Northern blot analysis.

only (Fig. 4A). Activation of PPARγ together with RXRs was inefficient in promoting adipogenesis (not shown). These results indicated that in the absence of RA the full activation PPARγ 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 PPARγ in early EBs (not shown) suggesting that early expression of PPARγ did not lead to the generation of preadipocytes which were blocked prior to terminal differentiation. Altogether, these results demonstrated that activation of PPARγ could not act as a substitute for the promoting effect of RA on the determination of stem cells towards the adipose lineage and that PPARγ did not play a role in the initial steps of the adipose cell development.

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

Recently, PPARγ$^{-/-}$ 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/EBPβ and C/EBPδ in PPARγ null ES cells suggested that mutant cells were arrested at the preadipose stage [4]. C/EBPβ and C/EBPδ 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 PPARγ 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 PPARγ-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 PPARγ 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 PPARδ in fibroblasts promotes expression of adipocyte-specific genes [27]. In addition, over-expression of PPARδ in Ob1771 and 3T3-F442A preadipose cells enhances this process [27,28]. As shown in Fig. 5B, PPARδ 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 PPARγ 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 PPARγ 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 PPARδ.

In conclusion, a model is proposed in which, in contrast to PPARγ-dependent differentiation of preadipocytes into adipocytes, commitment of pluripotent stem cells to the adipose lineage is independent of PPARγ. Altogether, our results indicate that expression of PPARγ is not rate-limiting in triggering the adipose lineage from pluripotent stem cells. However, one cannot exclude that endogenous ligands and target molecules of PPARγ, 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.

Acknowledgements: We thank Dr. Evan Rosen for advice on RT-PCR of embryos. This work was supported by the Centre National de la Recherche Scientifique (Grant UMR6543), by a special grant from the Bristol Myers Squibb Foundation (to G.A.), by the Association pour la Recherche Contre le Cancer (Grant 9982 to C.D., and a fellowship to B.P.) and by National Institutes of Health, USA Grants HL5409 and HL30628 (to D.S.M.).

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