RESEARCH COMMUNICATION

C/EBP α induces adipogenesis through PPAR γ : a unified pathway

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PPAR γ and C/EBP α are critical transcription factors in adipogenesis, but the precise role of these proteins has been difficult to ascertain because they positively regulate each other's expression. Questions remain about whether these factors operate independently in separate, parallel pathways of differentiation, or whether a single pathway exists. PPAR γ can promote adipogenesis in C/EBP α -deficient cells, but the converse has not been tested. We have created an immortalized line of fibroblasts lacking PPAR γ , which we use to show that C/EBP α has no ability to promote adipogenesis in the absence of PPAR γ . These results indicate that C/EBP α and PPAR γ participate in a single pathway of fat cell development with PPAR γ being the proximal effector of adipogenesis.

Received September 27, 2001; revised version accepted November 9, 2001.

Adipogenesis is the process by which undifferentiated precursor cells differentiate into fat cells. This has become one of the most intensively studied developmental processes for at least two reasons: the increasing prevalence of obesity in our society has focused attention on many aspects of fat cell biology, and the availability of good cell culture models of adipocyte differentiation has permitted detailed studies not possible in other systems. Experiments using these in vitro models of adipogenesis, which include the 3T3-L1 and 3T3-F442A lines, have illustrated the transcriptional cascade that promotes fat cell differentiation (Rosen et al. 2000). Representatives of several transcription factor families have been implicated in this process, including the CCAAT/enhancer binding proteins C/EBP α , C/EBP β , and C/EBP δ ; the

nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPAR γ); and the basic helix-loop-helix protein ADD1/SREBP1c. Studies in adipogenic cell lines have shown that hormonal induction of differentiation is rapidly followed by expression of C/EBP β and C/EBP δ (Cao et al. 1991; Yeh et al. 1995). Within the next day or so, levels of these proteins peak and then begin to drift downward, coincident with a rise in C/EBP α and PPAR γ . These latter factors induce gene expression changes characteristic of mature adipocytes and remain elevated for the life of the cell. In the present model of the transcriptional cascade leading to adipogenesis, C/EBP β and C/EBP δ induce low levels of PPAR γ and C/EBP α , which are then able to induce each other's expression in a positive feedback loop that promotes and maintains the differentiated state. This model is consistent with gain-of-function data showing that the addition of either PPAR γ or C/EBP α can promote adipogenesis in fibroblast cell lines (Lin and Lane 1994; Tontonoz et al. 1994).

Loss-of-function studies have shown convincingly that PPAR γ is required for adipogenesis in vivo and in vitro, and cells lacking PPAR γ express greatly reduced levels of C/EBP α (Barak et al. 1999; Kubota et al. 1999; Rosen et al. 1999). Similarly, fibroblasts lacking C/EBP α have reduced adipogenic potential, and express reduced levels of PPAR γ (Wu et al. 1999). Importantly, adding PPAR γ back to C/EBP α ^{-/-} fibroblasts with a retroviral vector restores their capacity to accumulate lipid and activate markers of adipogenesis, including the endogenous PPAR γ gene (Wu et al. 1999). These C/EBP α ^{-/-} adipocytes are normal in almost every way with the important exception that they do not show insulin sensitivity.

Importantly, because the converse manipulation has not been performed, it is not known whether the presence of exogenously applied C/EBP α is sufficient to promote adipogenesis in the absence of PPAR γ . The reasons for this omission are largely technical, and relate to the fact that PPAR γ ^{-/-} embryos die at embryonic day E9.5–E10, prior to the stage where the establishment of embryonic fibroblasts is generally considered feasible (Barak et al. 1999; Kubota et al. 1999). Additionally, the protocols for differentiating adipocytes directly from ES cells are cumbersome (Dani et al. 1997; Rosen et al. 1999) and are not amenable to retroviral expression of C/EBP α or other factors, as the LTRs of most available retroviruses are rapidly and irreversibly silenced after ES cell infection (Cherry et al. 2000).

It has therefore been difficult to delineate the relationship between C/EBP α and PPAR γ in adipogenesis. Two competing models consistent with the available data are illustrated in Figure 1. In Figure 1A, a model is depicted in which PPAR γ and C/EBP α induce each other's expression and can each act independently to promote fat cell differentiation. Figure 1B presents an alternative model in which PPAR γ is the direct regulator of adipogenesis, whereas the major role of C/EBP α is centered on maintaining expression of PPAR γ and promoting full insulin sensitivity.

To ascertain which model is more likely, we have created a fibroblast cell line that lacks PPAR γ . We use these cells to show that the adipogenic action of C/EBP α is entirely dependent on PPAR γ .

[Key Words: Adipogenesis; PPAR γ ; C/EBP α ; PPAR γ null fibroblasts]
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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.948702>.

Figure 1. Two models of adipogenesis. Competing models are shown consistent with available data on the relative roles of PPAR γ and C/EBP α in adipogenesis. See text for details.

Results and Discussion

Generation of immortalized cell lines heterozygous or null for PPAR γ

Mice carrying floxed alleles of PPAR γ were generated (data not shown). The PPAR γ null allele was generated by crossing PPAR γ -floxed mice with the EllaCre transgenic mouse line as outlined previously (Hayhurst et al. 2001). Offspring that lacked exon 2 were selected by Southern blotting, and the null allele was maintained in the heterozygous state with the active PPAR γ -floxed allele. Matings were established between mice that were heterozygous null at the PPAR γ locus (+/-) and animals carrying a single allele of PPAR γ with loxP sites flanking exon 2 (flox/-). At E12.5, embryos were harvested, minced, and trypsinized to generate mouse embryonic fibroblasts (MEFs). Embryonic heads were genotyped by PCR, and were found to represent the expected ratios of flox/+, flox/-, and +/- offspring (-/- embryos die at E9.5–E10.5, and are not seen by E12.5). MEFs from flox/+ and flox/- embryos were passaged repeatedly until they passed through crisis using the classic 3T3 protocol of Todaro and Green (1963). After immortalization, cells were expanded and infected with one of two different adenoviruses. One adenovirus expressed the Cre recombinase and green fluorescent protein (GFP) separated by an internal ribosome entry site (IRES) sequence, whereas the second virus expressed only GFP. After infection, cells were trypsinized and sorted by fluorescence detection; cells expressing GFP were collected, replated, and expanded. As shown in Figure 2B, Southern blotting showed that cells (flox/+ or flox/-) infected with the Cre-producing adenovirus completely lost their floxed alle-

les. This result was confirmed with genomic PCR (not shown), which showed the complete absence of a band associated with a residual floxed exon 2. Northern analysis of cells treated with conditions selected to induce adipogenesis (see Materials and Methods and below) shows the presence of PPAR γ mRNA in -/- cells, but RT-PCR of this RNA shows that all of it is associated with loss of exon 2 (data not shown). Western blot analysis of similarly treated cells reveals no PPAR γ protein (Fig. 2C); the expected translation product of PPAR γ mRNA lacking exon 2 is ~10 kD and does not appear on Western blotting, perhaps because of protein instability. One distinct advantage of this approach is that the PPAR γ ^{-/-} and flox/- cells were immortalized as a single line prior to exposure to Cre recombinase. This ensures that any biological changes associated with transformation are present in both flox/- and -/- cells, which in turn increases our confidence that any differences noted between these cell lines specifically reflects the presence or absence of PPAR γ .

Ectopic PPAR γ can stimulate adipogenesis in PPAR γ ^{-/-} fibroblasts, but C/EBP α cannot

Flox/- fibroblasts can be differentiated into adipocytes with low efficiency (<2%) in the presence of dexamethasone, methylisobutylxanthine, and insulin (DMI), in addition to the synthetic PPAR γ agonist troglitazone. In contrast, however, the PPAR γ ^{-/-} fibroblasts are not competent to undergo adipogenesis at all; we have never seen even a single fat cell develop in any experiment. This result is consistent with earlier observations made by our group and others showing that PPAR γ is absolutely required for adipogenesis in vitro and in vivo (Barak et al. 1999; Kubota et al. 1999; Rosen et al. 1999).

Figure 2. Generation of PPAR γ ^{-/-} fibroblasts. (A) Scheme showing strategy used to make PPAR γ ^{-/-} cells. PPAR γ flox/- (or flox/+) embryos were harvested at day 12.5. Embryonic fibroblasts were plated and passaged repeatedly using a classic 3T3 protocol. After immortalization, cells were split into two aliquots and infected with one of two different adenoviruses expressing Cre recombinase and GFP (Ad-Cre-IRES-GFP) or GFP alone (Ad-X-IRES-GFP). Cells were sorted by fluorescence to pick GFP-expressing cells. (B) Southern blot showing complete loss of floxed alleles in cells that received Ad-Cre-IRES-GFP, whereas floxed alleles were retained by cells that received Ad-X-IRES-GFP. (C) Immunoblot of PPAR γ in PPAR γ flox/- and -/- cells exposed to a prodifferentiative regimen.

Figure 3. PPAR γ restores adipogenesis in PPAR $\gamma^{-/-}$ cells, but C/EBP α does not. (A) Dishes containing PPAR γ flox/− or −/− cells were infected with retroviruses expressing PPAR γ 2, C/EBP α , or vector only. Cells were exposed to a prodifferentiative regimen with or without troglitazone, and stained with oil red O after 7 d. (B) Microscopic view of cells in A.

We next examined whether the block in differentiation seen in PPAR $\gamma^{-/-}$ cells is specifically caused by the lack of PPAR γ by infecting them with a PPAR γ 2-expressing retrovirus. Ectopic expression of PPAR γ in flox/− cells resulted in a dramatic increase in adipogenic potential when DMI alone was used as the inducing cocktail. The effect was even more pronounced when troglitazone was added to the cells, suggesting that at some level of PPAR γ expression, the amount of endogenous ligand for PPAR γ becomes limiting. Importantly, retroviral introduction of PPAR γ was able to rescue the adipogenic potential of PPAR $\gamma^{-/-}$ cells, indicating that the lack of adipogenesis seen in vector-treated cells truly reflects the absence of this nuclear hormone receptor, and not some unanticipated disruption of the adipogenesis machinery at a site distinct from PPAR γ .

To ascertain the adipogenic potential of C/EBP α in the absence of PPAR γ , this transcription factor was added to the PPAR γ flox/− and −/− cells using the same retroviral delivery system. C/EBP α enhanced the extent of adipogenesis in PPAR γ flox/− cells, consistent with the gain-of-function effects seen in the past with this protein (Fig. 3A,B). C/EBP α , however, failed to induce any lipid accumulation whatsoever in the PPAR $\gamma^{-/-}$ cells. This lack of adipogenic action is not caused by any impairment of C/EBP α expression in these cells, because immunoblot analysis reveals high levels of the 42-kD C/EBP α protein (Fig. 4A).

Gene expression analysis confirms the findings shown

at the level of cell morphology and lipid accumulation, that is, that C/EBP α can support adipocyte-specific gene expression only when PPAR γ is also present. The adipocyte-selective fatty-acid-binding protein aP2 is a direct target gene of PPAR γ . Ectopic high-level expression of PPAR γ is able to promote aP2 expression at day 0, before fat cell differentiation has even begun (Fig. 4B). The lack of aP2 expression in PPAR $\gamma^{-/-}$ fibroblasts in the presence of C/EBP α reflects the lack of adipogenesis in these cells. A similar pattern is observed for adipisin, another fat-cell-specific protein induced during adipogenesis. Recent data indicate that C/EBP α is a major contributor to the expression of adipisin in fat cells (Chen et al. 2000); the fact that we do not detect adipisin message in PPAR $\gamma^{-/-}$ cells even when high levels of C/EBP α are present indicates that other factors present in mature adipocytes must be required as well. PPAR γ itself is unlikely to be the missing factor, in part because we do not see induction of adipisin message in PPAR γ -expressing cells before the onset of adipogenesis (Fig. 4B), and because activation of PPAR γ by thiazolidinedione drugs has actually been shown to decrease adipisin expression in mature fat cells. PPAR γ also must be present in order for C/EBP α to maximally induce its own mRNA expression.

An enhanced model of the adipogenic transcriptional cascade

Studies using NIH-3T3 fibroblasts have indicated that expression of either PPAR γ or C/EBP α is sufficient to induce adipogenesis (Lin and Lane 1994; Tontonoz et al. 1994). Regardless of which factor is employed, the phenotype of the differentiated cells appears to be very simi-

Figure 4. Expression of C/EBP α , PPAR γ , and other adipocytic markers in PPAR γ flox/− and −/− cells. (A) Immunoblot analysis of C/EBP α in PPAR γ flox/− and −/− cell lines. (B) Northern analysis of multiple adipocytic markers in PPAR γ flox/− and −/− cell lines. (V) Viral message, (E) endogenous message.

lar. The mechanistic interpretation of this result is difficult, however, because ectopic expression of either factor results in enhanced endogenous expression of the other. Fibroblasts that lack C/EBP α have been generated previously, and have been shown to be deficient in both PPAR γ expression and adipogenic potential; when PPAR γ is replaced, however, these cells are competent to undergo nearly all aspects of fat cell differentiation with the exception of insulin sensitivity (El-Jack et al. 1999; Wu et al. 1999). This result opened the possibility that PPAR γ and C/EBP α are largely redundant in adipogenesis. In the present study, PPAR $\gamma^{-/-}$ fibroblasts were generated and used to show that such is not the case.

In light of the data presented above, what role does C/EBP α play in adipogenesis? Clearly, C/EBP α is required for adipogenesis as shown by loss-of-function studies *in vivo* and *in vitro*. The fact that C/EBP α null fibroblasts undergo adipogenesis when PPAR γ is replenished, however, strongly supports the argument that the role of C/EBP α in fat cell differentiation is limited to the induction and maintenance of PPAR γ levels. Indeed, this is consistent with observations that C/EBP α can induce PPAR γ 2 expression by direct binding to specific sites in the PPAR γ promoter (Elberg et al. 2000). Interestingly, recent studies on C/EBP α null mice indicate that the development of white adipose tissue (WAT), but not brown adipose tissue (BAT), is dependent on C/EBP α (Linhardt et al. 2001); we would hypothesize based on our data that PPAR γ levels in WAT are more dependent on C/EBP α than PPAR γ levels in BAT.

In addition to maintaining PPAR γ expression in developing adipocytes, C/EBP α is critical in the establishment of insulin sensitivity. This effect is mediated in part by direct transcriptional induction of insulin receptor and IRS-1 levels, and in part by a poorly characterized post-receptor mechanism (Wu et al. 1999). Additionally, other genes typical of the differentiated state (e.g., adiponin and leptin) are strongly promoted by the direct actions of C/EBP α (Chen et al. 2000). C/EBP α , then, plays several important roles in adipocyte function, despite not being directly responsible for the process of adipogenesis *per se*.

Another possibility is that PPAR γ may be permissive for C/EBP α action, perhaps directly through protein-protein interactions (although no evidence exists for such an interaction) or alternatively through induction of a co-factor critical for C/EBP α . We believe that the PPAR $\gamma^{-/-}$ fibroblasts will be invaluable to screen for these and other factors that act downstream of PPAR γ in adipogenesis; such studies are already underway.

Materials and methods

Generation of adenoviral constructs

To create the Ad-Cre-IRES-GFP virus, the pLEP plasmid (Wang et al. 2000) was cleaved with *Hind*III and *Not*I, and a cDNA encoding Cre recombinase (kindly provided by M. Murakawa, MGH Nessel Gene Therapy Center) was inserted. A second fragment, encoding the internal ribosomal entry site sequences derived from the encephalomyocarditis virus and a codon optimized green fluorescent protein (kindly provided by E.C. Park, MGH Nessel Gene Therapy Center) was inserted at the *Not*I site (Fukumura et al. 1998). This plasmid and the plasmid pREP7 were both cleaved by *P*-*Not*I, ligated to each other using DNA ligase, and then packaged in phage packaging extracts (MaxPlax, Epicentre Technologies) as described (Wang et al. 2000). Cosmid DNA was isolated from *Escherichia coli* transduced with the packaged DNA and human 293 embryonic kidney cells were transfected with 10 μ g of this DNA follow-

ing cleavage by *I*-*Ceu*I. Virus propagation, purification, and plaque assay were performed using established adenoviral protocols (Graham and Prevec 1991).

Generation of PPAR γ flox/- and -/- cell lines

PPAR γ flox/- mice were mated to PPAR γ +/- mice. At E12.5, embryos were harvested. Primary MEFs were generated by removing the heads of the embryos (saved for PCR genotyping), scratching out the viscera with a forceps, and trypsinizing the bodies after mincing. The resulting slurry was plated in 75-cm² flasks. Cells derived from flox/- and flox/+ embryos were passaged by plating 3×10^5 cells per 60-mm dish every 3 d as described (Todaro and Green 1963). After crisis and expansion, cells were split into two aliquots and infected with adenovirus Ad-Cre-IRES-GFP or Ad-X-IRES-GFP at an m.o.i. of 100,000:1. Forty-eight hours after infection, cells were flow-sorted on a Cytomation Mo-Flo by exciting cells with a 488-nm laser and collecting at 530/480 bp. The most intensely GFP-expressing cells were replated, expanded, and frozen.

Cell culture

Cells were cultured in DME with 10% FBS at 10% CO₂. After retroviral infection and selection (see below), cells were allowed to grow to confluence in either 100-mm dishes or 6-well plates. Once confluence was reached, cells were exposed to a prodifferentiative regimen including dexamethasone (1 μ M), insulin (5 μ g/mL), and isobutylmethylxanthine (0.5 mM) with or without 10 μ M troglitazone. After 2 d, cells were maintained in medium containing insulin until ready for harvest at day 7.

Retroviral infections

Retroviruses were constructed in pMSCV vectors (Clontech) using either puromycin or hygromycin selectable markers. Viral constructs were transfected into 293EBNA cells using FuGene (Roche) along with plasmids expressing gag-pol and the VSV-G protein. Supernatants were collected after 48 h, and either used immediately or frozen at -80°C for later use. Viral supernatants were added to PPAR γ flox/- or -/- cells for 4 h; selection with puromycin (2 μ g/mL) or hygromycin (175 μ g/mL) was started 48 h later. Cells were selected, expanded, and studied immediately or frozen for later use.

Northern and Western blots

For Northern analysis, cells were grown to confluence and treated with a prodifferentiative regimen as noted above. Cells were lysed in Trizol and processed according to the manufacturer's instructions. For each sample, 10 μ g of total RNA was loaded onto formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized with the appropriate ³²P-labeled probe in Ultrahyb (Ambion). Blots were stripped in boiling 0.1% SDS between different probes.

For Western analysis, cells were grown to confluence and treated with a prodifferentiative regimen as noted above. Lysis buffer (PBS with 1% Triton X-100 and complete protease inhibitor tablets; Boehringer Mannheim) was added, and the cells were triturated in Eppendorf tubes on ice. After shaking at 4°C for 10 min, lysates were spun at 14,000g, and supernatants were collected and snap-frozen in liquid nitrogen. Protein concentrations were determined with a BioRad assay, and 200 μ g of total protein was TCA-precipitated and run on 10% SDS-PAGE. Transfer was performed using a semidry apparatus onto PVDF, and equal loading was ascertained by Ponceau staining. Membranes were blocked in 5% milk prior to incubation with primary antibody (PPAR γ : E-8; C/EBP α : sc-61, Santa Cruz). After washing in TBST and exposing to secondary antibody, blots were developed with enhanced chemiluminescent reagent and exposed to film.

Acknowledgments

We thank the Dana-Farber Flow Cytometry Core Facility, and Eric Smith for help with the figures. E.D.R. was supported by NIH DK02535. B.M.S. was supported by NIH R37DK31405.

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Genes Dev. 2002, **16**:

Access the most recent version at doi:[10.1101/gad.948702](https://doi.org/10.1101/gad.948702)

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