PPARγ knockdown by engineered transcription factors: exogenous PPARγ2 but not PPARγ1 reactivates adipogenesis

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To determine functional differences between the two splice variants of PPARγ (γ1 and γ2), we sought to selectively repress γ2 expression by targeting engineered zinc finger repressor proteins (ZFPs) to the γ2-specific promoter, P2. In 3T3-L1 cells, expression of ZFP55 resulted in >50% reduction in γ2 expression but had no effect on γ1, whereas adipogenesis was similarly reduced by 50%. However, ZFP54 virtually abolished both γ2 and γ1 expression, and completely blocked adipogenesis. Overexpression of exogenous γ2 in the ZFP54-expressing cells completely restored adipogenesis, whereas overexpression of γ1 had no effect. This finding clearly identifies a unique role for the PPARγ2 isoform.

Results and Discussion

The mouse PPARγ gene spans >105 kb (Zhu et al. 1995). Coding exons 1 to 6 are conserved between the γ1 and γ2 isoforms (Fig. 1A) and transcription of these is driven by an upstream promoter [P1] that also drives expression of two untranslated γ1-specific exons, A1 and A2. The additional amino acids at the amino terminus of γ2 are encoded by an additional exon, B1, that is uniquely regulated by a separate promoter [P2] lying >63 kb downstream of P1. DNase I digestion of the endogenous PPARγ gene locus in 3T3-L1 cells revealed two DNase I hypersensitive sites in the vicinity of the proximal P2 promoter that represent regions of accessible chromatin at an endogenous locus [Fig. 1B, DHS1 and DHS2]. Two six-finger ZFPs (ZFP54 and ZFP55; Fig. 1C) linked to the KRAB transcriptional repression domain (Margolin et al. 1994) were designed to bind specifically to 18-bp sequences within the DHS1 shown in Figure 1B. Each ZFP bound its cognate site on naked DNA with high affinity [Kd = 20 and 44 pM, respectively].

The ZFP54 and ZFP55 repressor proteins were expressed retrovirally in 3T3-L1 cells to similar levels [Fig. 2A]. Nontransduced wild-type cells and stable pools of infected cells expressing each ZFP, as well as control cells retrovirally transduced to express LacZ, were induced to differentiate and initiate the adipogenic pathway. Total PPARγ mRNA level was determined at 0, 2, and 5 d post induction. Over the 5-d time course PPARγ expression increased ∼5.8- to 6.0-fold in both the wild-type and LacZ control cells [Fig. 2B]. Up-regulation of total PPARγ expression was reduced slightly in the presence of ZFP55 (4.8-fold compared to 6.0-fold in wild type) but completely inhibited by ZFP54. However, a time course analysis of expression of the individual PPARγ isoforms revealed that although ZFP54 effectively inhibited expression of both isoforms, ZFP55 selectively repressed PPARγ2 by ∼50% and had little effect on PPARγ1 compared with wild-type cells [Fig. 2C]. Another ZFP–KRAB fusion protein that binds to a sequence that is absent in the PPARγ gene has no effect on PPARγ expression or adipogenesis [data not shown]. The effects of these ZFPs are confirmed at the protein level whereby PPARγ2 expression is virtually knocked out in the presence of ZFP54, whereas only a low but detectable level of
PPARγ1 remains (Fig. 2D). In the presence of ZFP55 only PPARγ2 expression is reduced substantially by ZFP55. Because P1 and P2 are >63 kb apart, it was predicted that ZFPs targeted to P2 would selectively inhibit PPARγ2 expression. This was the case with ZFP55, which gave 50% knockdown of PPARγ2 only, however ZFP54 suppressed both isoforms almost completely. ZFP54 and ZFP55 bind to opposite strands of the DNA and have slightly different DNA binding affinities, which may contribute to their differential effects on P2.

The promoter specificity exhibited by the two ZFPs facilitates examination of the functional requirements for each PPARγ isoform in adipogenesis. In complete concordance with inhibited mRNA and protein expression, we find that ZFP54 totally blocks the cellular lipid accumulation that is a marker of adipogenesis (Fig. 3). Furthermore, the cells in which only PPARγ2 is selectively repressed by 50% (ZFP55) also show a correspondingly reduced capacity for lipid accumulation.

Figure 1. Structure of PPARγ gene and location of accessible chromatin in P2 promoter. (A) Genomic structure of mPPARγ showing exon splicing [Zhu et al. 1995]. (B) Location of DNase I hypersensitive sites (DHS1 and DHS2) in the proximal P2 promoter, along with positions of ZFPs binding sites. (C) ZFP target sequences and finger designs. (Target sequence) The promoter DNA sequence to which each ZFP was designed. (Finger design) The residues in each position from −1 to +6 of the recognition helix of each finger targeted against the cognate basepair triplet subsite.

Figure 2. Ectopic expression of PPARγ2-specific ZFPs in 3T3-L1 cells. 3T3-L1 cells were infected with retroviral vectors expressing PPARγ2 promoter-specific zinc finger repressors, ZFP54 and ZFP55. The uninfected 3T3-L1 cells (wild type) or cells infected with retroviral vector expressing LacZ gene were used as negative controls. (A) Total RNA was isolated from selected cell lines and determined the levels of ZFP mRNA by Northern blot analysis using a 300 bp C-terminal fragment of ZFP as probe. The equivalence of RNA loading was verified by actin. (B) Total RNA was isolated on indicated days postdifferentiation and subjected to RNase protection assay using a PPARγ-specific riboprobe. Arrows indicate the protected PPARγ and actin mRNAs. The fold-induction was calculated by normalization of PPARγ-specific signal with actin signal and presented as fold-change compared to day-0 of wild-type cells. (C) The real time quantitative RT–PCR (TaqMan) analysis of PPARγ1 and PPARγ2 mRNA expression. 40 ng and 60 ng of reversed transcribed total RNA was used for PPARγ2 and PPARγ1 analysis, respectively. (D) Western blot analysis with a polyclonal antibody against PPARγ. The arrows indicate PPARγ1 and PPARγ2 proteins.
The presence of either ZFPs (Fig. 4A). C/EBP level of C/EBP dance with this prediction, we found that the expression be affected by targeted repression of PPAR. The proximal promoter of PPAR is designed to recognize an 18-bp nucleotide sequence that probability dictates to be unique within the mammalian genome. To further characterize the specificity of PPARγ targeting, we analyzed the effect of ZFP expression on other genes both upstream and downstream of PPARγ in the adipogenic gene cascade. The cell cyclin-dependent kinase inhibitors p21 and p27 play key roles in cell cycle progression [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994].

A key aspect of engineered ZFPs is the high theoretical specificity of DNA binding and gene targeting. Each ZFP is designed to recognize an 18-bp nucleotide sequence that probability dictates to be unique within the mammalian genome. To further characterize the specificity of PPARγ targeting, we analyzed the effect of ZFP expression on other genes both upstream and downstream of PPARγ in the adipogenic gene cascade. The cell cyclin-dependent kinase inhibitors p21 and p27 play key roles in cell cycle progression [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994]. P27 is expressed abundantly in growth-arrested preadipocytes where its expression is decreased transiently on hormonal induction of differentiation but returns to initial levels as the cells exit clonal expansion [Harper et al. 1993; Toyoshima and Hunter 1994].

Functional differences between PPARγ1 and PPARγ2

![Figure 3. Effect of PPARγ2-ZFPs on adipogenesis. Cultured wild-type 3T3-L1 and retrovirally infected cells were induced to differentiate with the standard adipogenic hormones for 10 days. Cell were fixed in 3% formaldehyde and stained with Oil Red O. Stained cells were photographed with Nikon-Diaphot300 microscope (10 × 20) and 3CCD Vida Camera Systems (Optronic Engineering).](Image 102x508 to 282x733)

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![Figure 4. Effects of ZFP54 and ZFP55 on the expression of cyclin-dependent kinase inhibitors, C/EBPs and aP2. Whole cells extracts and total RNA from the experiment shown in Figure 2 were subjected to the following analysis. (A) Western blot analysis of p21 and p27, and C/EBP gene family members (β, α, and γ). Arrows indicate the 42-kD and 30-kD isoforms of C/EBPα. (B) The aP2 gene expression was analyzed by Northern blot.](Image 335x164 to 564x372)

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only PPARγ. Rescue of these cells by exogenous expression of either PPARγ1 or γ2 might be expected to identify isoform-specific functional differences in the capacity to potentiate adipogenesis. Here we used retrovirus to overexpress each PPARγ isoform on the background of ZFP54 expressing 3T3-L1 cells. Data in Figure 5A,B demonstrate the comparable levels of expression of exogenous PPARγ1 and γ2 mRNA and proteins at day 0 in the presence of ZFP54. The exogenously expressed PPARγ1 isoform was completely incapable of inducing adipogenesis in the presence of ZFP54 [Fig. 5C]. In marked contrast, expression of PPARγ2 effectively restored cellular differentiation and lipid accumulation. In addition, PPARγ2 fully restored C/EBPα expression at 7 d postinduction, whereas PPARγ1 was totally ineffective [Fig. 5D]. Neither isoform had any effect on the expression of C/EBPβ or TBP [Fig. 5D]. This result clearly demonstrates for the first time a regulatory function for PPARγ2 in adipogenesis that cannot be achieved by PPARγ1 in the absence of exogenous ligand.

It is unclear how the 30 amino acid residues unique to the PPARγ2 isoform confer additional regulatory function. However, in addition to rendering the constitutive activation function of the PPARγ2 amino terminus up to 10-fold greater than that of PPARγ1 (Werman et al. 1997), clinical studies have identified a human allelic variant of at least one of these residues [Pro12Ala] that has been variously associated with decreased receptor activity, lower body mass index, obesity, improved insulin sensitivity, and decreased risk of type 2 diabetes (Beamer et al. 1998; Deeb et al. 1998; Altshuler et al. 2000).

The effective restoration of the adipogenic cascade by adding back only the product of the targeted gene, PPARγ1, indicates that in this study the use of engineered repressor ZFPs does not result in the general disruption of cellular metabolic processes. This observation, which demonstrates a precise specificity for ZFP targeting, represents an important advance in the rapid establishment of cell-based gene knockdown models and such an approach may, in future, be readily transferable to whole animal transgenic studies.

Materials and methods

Design, synthesis, and DNA binding affinity of zinc finger proteins

Pairs of 3-finger ZFPs were generated as described previously (Zhang et al. 2000) then linked to form each 6-finger ZFP. The DNA binding affinity of each ZFP was determined using a gel shift technique essentially as described earlier (Zhang et al. 2000) except that 10 μM zinc was used.

Plasmids and stable cell lines

The retroviral expression vector pBMN (Garry Nolan Laboratory, Stanford University) was constructed by inserting an internal ribosome entry site [IREs] along with either puro or neom. The retroviral plasmids pBMNpuro-ZFP54 and pBMNpuro-ZFP55 were constructed by inserting ZFP54 and ZFP55 cDNA fragments into pBMN-puro vector at EcoRI and XhoI sites. pBMN/neom-γ1 was generated by inserting the full-length mPPARγ1 cDNA [nucleotide positions −6 to +1415] into pBMN-neo vector at BamHI and NotI sites. pBMN/neom-γ2 construct was derived from mPPARγ2/pSport (a gift from Dr. B. Spiegelman, Harvard Medical School, Boston, MA) by specifically ligating the blunt-ended PPARγ2 cDNA into pBMN/neo vector at the blunt-ended EcoRI site. To generate infectious recombinant viruses, Phoenix-ECO cells (Garry Nolan Laboratory, Stanford University, CA) were cultured in DMEM medium containing 10% FBS. Cells were transfected with 15 μg of retroviral plasmids by Lipofectamine 2000 kit (GIBCO BRL) following the protocol as recommended by the manufacturer. Viral supernatant was harvested at 72 h posttransfection and added onto 75% confluent 3T3-L1 cells in the presence of 8 μg/mL polybrene [Sigma] for 2 h. Infected cells were selected by adding 2
\( \text{μg/mL puromycin (Sigma for 5 d). For double infection, L1/ZFP54 cells were infected with either pBMN/neo-γ1, or pBMN/neo-γ2, and cells were selected with both 600 μg/mL genetin (GIBCO BRL) and 2 μg/mL puromycin for 14 d. Pools of stably infected cells were used in these experiments.} \\

**Cell culture and differentiation**

Uninfected 3T3-L1 and virally infected 3T3-L1 cells were maintained for 2 d postconfluence in DMEM containing 10% CS. Differentiation protocol was conducted as described previously (Camp et al. 2001).

**Mapping of DNase I accessible chromatin regions**

DNase I mapping was performed essentially as described previously (Liu et al. 2001). Undifferentiated 3T3-L1 cell nuclei were partially digested with DNase I and the genomic DNA was extracted followed by Xhol digestion at -1588 bp in the P2 promoter. The probe for Southern blotting of the proximal P2 promoter was an Xba/I EcoRI fragment spanning from -1588 to -1007.

**The real-time quantitative PCR (TaqMan)**

Gene-specific primers and probes were designed using the Primer Express software (Perkin Elmer Life Sciences). The real-time quantitative RT-PCR reaction was performed essentially following the manufacturer’s protocol. Briefly, reaction mixture contained 5.5 mM MgCl\(_2\), 500 μM dNTP, 2.5 μM random hexamers, 200 nM FAM-probe, and 600 nM of target mRNA. The real-time quantitative PCR (TaqMan) analysis was performed as described previously (Camp et al. 1999) with [\(^{32P}\)] dCTP (Amersham) using random primed labeling kit (GIBCO BRL). Northern blot analysis and RNAse protection assay (RPA) were performed as described previously (Camp et al. 1999).

**Immunoblot analysis**

Cells were lysed in HNTG cell lysis buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton, 1.5 mM MgCl\(_2\), and 1 mM EDTA) at 4°C for 15 min, followed by centrifugation at 15,000 rpm at 4°C for 10 min. The supernatant was collected and the protein concentration was determined by BCA protein assay (Pierce). Western blot analysis was carried out as described previously (Camp et al. 1999). Rabbit polyclonal antibodies against C/EBP\(\alpha\), C/EBP\(\beta\), C/EBP\(\delta\), TBP, and monoclonal antibodies against p21 and p27 were purchased from Santa Cruz.

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