A Novel Splicing Variant of Peroxisome Proliferator-Activated Receptor-γ (Pparγ1sv) Cooperatively Regulates Adipocyte Differentiation with Pparγ2

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Abstract

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate expression of a number of genes associated with the cellular differentiation and development. Here, we show the abundant and ubiquitous expression of a newly identified splicing variant of mouse Pparγ (Pparγ1sv) that encodes PPARγ1 protein, and its importance in adipogenesis. The novel splicing variant has a unique 5'UTR sequence, relative to those of Pparγ1 and Pparγ2 mRNAs, indicating the presence of a novel transcriptional initiation site and promoter for Pparγ expression. Pparγ1sv was highly expressed in the white and brown adipose tissues at levels comparable to Pparγ2. Pparγ1sv was synergistically up-regulated with Pparγ2 during adipocyte differentiation of 3T3-L1 cells and mouse primary cultured preadipocytes. Inhibition of Pparγ1sv by specific siRNAs completely abolished the induced adipogenesis in 3T3-L1 cells. C/EBPα and C/EBPδ activated both the Pparγ1sv and Pparγ2 promoters in 3T3-L1 preadipocytes. These findings suggest that Pparγ1sv and Pparγ2 synergistically regulate the early stage of the adipocyte differentiation.

Introduction

Obesity has become a growing worldwide health problem in recent years. An excessive accumulation of white adipose tissue caused by increases in the cell number and size of newly differentiated white adipocytes from preadipocytes is a major cause of obesity. Thus, the elucidation of mechanisms of adipocyte differentiation is essential for understanding the pathogenesis of obesity and obesity-associated diseases.

3T3-L1, a cell line derived from mouse 3T3 fibroblast, has been widely used as a model of adipocyte differentiation [1]. The addition of chemicals and hormones such as dexamethasone or insulin into culture media of 3T3-L1 cells induces the synthesis and accumulation of intracellular triglycerides and changes in their morphology from fibroblast-like to adipocyte-like [2]. During the progression, a number of adipocyte-related genes are up-regulated by a sequential induction of transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and members of the CCAAT/enhancer-binding proteins (C/EBPα, C/EBPβ, and C/EBPδ) [3]. PPARγ is a member of the ligand-dependent nuclear receptor superfamily and plays a pivotal role in adipogenesis and intracellular lipid accumulation. C/EBPs belong to a family of the basic region-leucine zipper (bZIP) transcription factors. C/EBPβ and C/EBPδ are transiently expressed very early during adipocyte differentiation [4], which in turn transactivate gene expression of PPARγ and C/EBPα [5]. Both proteins cooperatively promote downstream adipocyte-related genes such as the adipocyte-specific fatty acid-binding protein gene (FABP4) to develop functional adipocytes.

PPARγ is expressed as at least two splicing variants, the ubiquitously expressed Pparγ1 and adipocyte-specific Pparγ2 [6,7]. Pparγ2 protein that is translated from Pparγ2 mRNA is longer than PPARγ1 (from Pparγ1) by 30 amino acid residues at the N-terminus in mice. PPARγ2 protein has been considered to play a critical role in the adipogenesis, because Pparγ2 mRNA, but not Pparγ1, is abundantly expressed in the adipose tissues. However, PPARγ1 expression also has been observed in adipocytes at similar level to PPARγ2 in the previous reports [8–10], which complicated the role of PPARγ1 in adipogenesis.

In addition to Pparγ1 and Pparγ2, several unique splicing variants of Pparγ have been reported [11,12]. We have recently reported a novel PPARγ splicing variant in humans that is regulated by circadian rhythmic D-site binding protein, DBP [13]. However, the involvement of this splicing variant in adipogenesis has not been uncovered.

In this paper, we report the identification of a novel Pparγ splicing variant, Pparγ1sv, in mice that is synergistically up-regulated with Pparγ2 during adipocyte differentiation of 3T3-L1 cells and mouse primary cultured preadipocytes. Knock-down experiments using siRNA specifically targeting to Pparγ1sv revealed that PPARγ1 protein expressed during adipogenesis is derived from Pparγ1sv mRNA. Thus, this novel splicing variant could explain the induced PPARγ1 protein during adipocyte differentiation. Furthermore, knock-down of Pparγ1sv abolished
the induced adipogenesis of 3T3-L1 cells, indicating that PPARγ1 from Pparγ1sv plays a crucial and synergistic role with PPARγ2 in adipogenesis.

**Materials and Methods**

**Cell Culture, Differentiation, and Staining**

3T3-L1 and ST2 cells were obtained from the Japan Health Science Foundation, Health Science Research Resources Bank (Osaka, Japan) and RIKEN Cell Bank (Tsukuba, Japan), respectively. Mouse primary cultured preadipocytes isolated from white adipose tissues of newborn mice were purchased from Primary Cell Co., Ltd (Hokkaido, Japan). 3T3-L1 and ST2 cells were maintained in DMEM and RPMI1640 (Life Technologies), respectively, supplemented with 10% fetal bovine serum (Sigma-Aldrich) and penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2. Cells were passaged every 3 days. For adipocyte differentiation, we plated cells in 3-cm or 6-cm dishes, allowed them to grow for 95–100% confluency, and then changed the culture medium to DMEM containing 0.25 μM dexamethasone, 500 μM isobutylmethylxanthine, and 1 μM insulin. Primary preadipocytes were cultured in DMEM containing 2.5 μM dexamethasone and 10 μg/ml insulin for two days to start differentiation into adipocytes according to the manufacturer’s instructions. We estimated the adipocyte differentiation by staining intracellular lipid droplets with Oil Red O or quantifying cellular triglycerides content with AdipoRed assay reagent (Lonza).

**Cloning of a Novel Splicing Variant of Mouse Pparγ**

Total RNA was purified from adipocyte-differentiated 3T3-L1 cells (9 days after the chemical induction) using ISOGEN (Nippon Gene). 5'- and 3'-Ready SMART cDNA was synthesized from 1 μg of total RNA using the SMART RACE cDNA synthesis kit according to the manufacturer’s instructions (Takara Bio). The 5'-end of mouse Pparγ cDNA was amplified from 5'-Ready SMART cDNA using mPPARγ5RACE_LP2, 5'-TGGGTCGACCTTCTTGTGGAATGATG3' and Universal Primer Mix (UPM) (Takara Bio). After sequencing the 5'-rapid amplification of cDNA end (RACE) product, full-length cDNA was amplified from 3'-Ready SMART cDNA using mPPARγ-novel5'term, 5'-GGGGCGCTGGACCTCTGCTGGGGATCT-3' and UPM, cloned into pGEM-T Easy vector (Promega) and sequenced.

**Quantitative RT-PCR**

To quantify mouse Pparγ1sv, Pparγ1, Pparγ2, and 18S ribosomal RNA expression in 3T3-L1, ST2, and primary cultured cells by quantitative PCR (qPCR), we used the THUNDERBIRD SYBR qPCR mix (Toyobo) in an ABI Prism 7900 HT sequence detection system (Life Technologies). For analyses of the tissue distribution of Pparγ expression, a part of the cDNA was amplified from 5'-Ready SMART cDNA using mPPARγ-novel5'term, 5'-GGGGCGCTGGACCTCTGCTGGGGATCT-3' and UPM, cloned into pGEM-T Easy vector (Promega) and sequenced.

**Plasmid Constructs and Small Interfering RNAs**

For the assessment of small interfering RNA (siRNA) specificity, the full-length Pparγ1sv cDNA or Pparγ2 cDNA was subcloned into Xho I site between the stop codon of luciferase coding region (luc+) and the poly(A) signal in pGL3-Control vector (Promega) in sense or antisense direction. For the promoter assay of mouse Pparγ, the 5'-flanking region of the Pparγ1sv (−969 to +31), Pparγ1 (−1,529 to +31), and Pparγ2 (−1,473 to +41) were amplified with Advantage 2 DNA polymerase (Takara Bio) from the genomic DNA of 3T3-L1 cells, and were subcloned into pGL3-Basic (Promega). The coding regions of mouse C/EBPα, C/EBPβ, and C/EBPδ were amplified by Advantage 2 DNA polymerase from the cDNA of 3T3-L1 cells, and were subcloned into pGL3.1(-) (Life Technologies) for overexpression in luciferase reporter experiments. All expression vectors were purified using an EndoFree plasmid maxi kit (Qiagen). siRNAs targeting mouse Pparγ1sv, Pparγ1, common sequence of Pparγ, and C/EBPβ mRNAs were purchased from Life Technologies (Stealth RNAi). Target sequences of mRNAs were as follows: 5′-GAUCUGGAAGGCCGAGCCGGGAUUAU-U3′ (si1vs22) and 5′-GGGCGACGCAGCUAUAUUUCUCCC3′ (si1vs30) for Pparγ1sv; 5′-CCAGUGUGUAUAAAGCCAGUCUU-C3′ (si275) and 5′-GGGGUAAACUCUGGGAGAUUCCUGCC3′ (si248) for Pparγ2; 5′-CCAGAGAAGAUCAGACAGCCUCGUAU-3′ (si2common1) and 5′-UCAAGGGUGGGCUUUCCUGUACCCUA-3′ (si2common2) for all transcripts of Pparγ; 5′-CAGCGCCUCUUAGACCCCAUGAGAUU3′ (sic/EBPβ #1) and 5′-CCAGUGGAGUGGGCCACUCCUACU-3′ (sic/EBPβ #2) for C/EBPβ. For siRNA transfections, 5×105 cells/well were seeded onto 6-well culture plates and transfected with the above siRNAs using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s instructions.

**Western Blotting and Antibodies**

Cells cultured in 6-cm dishes were trypsinized and harvested in 1 ml phosphate buffered saline. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo scientific), mixed with 5× sodium dodecyl sulfate (SDS) sample buffer containing 2-mercaptoethanol, heated at 95°C for 3 min, and then loaded onto a 12.5% SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane and incubated in 1% Western blocking solution for all transcripts of Pparγ, 31), and C/EBPα, C/EBPβ, and C/EBPδ (Promega). The coding regions of mouse C/EBPα, C/EBPβ, and C/EBPδ (Promega) were amplified by Advantage 2 DNA polymerase from the cDNA of 3T3-L1 cells, and were subcloned into pGL3.1(-) (Life Technologies) for overexpression in luciferase reporter experiments. All expression vectors were purified using an EndoFree plasmid maxi kit (Qiagen). siRNAs targeting mouse Pparγ1sv, Pparγ1, common sequence of Pparγ, and C/EBPβ mRNAs were purchased from Life Technologies (Stealth RNAi). Target sequences of mRNAs were as follows: 5′-GAUCUGGAAGGCCGAGCCGGGAUUAU-U3′ (si1vs22) and 5′-GGGCGACGCAGCUAUAUUUCUCCC3′ (si1vs30) for Pparγ1sv; 5′-CCAGUGUGUAUAAAGCCAGUCUU-C3′ (si275) and 5′-GGGGUAAACUCUGGGAGAUUCCUGCC3′ (si248) for Pparγ2; 5′-CCAGAGAAGAUCAGACAGCCUCGUAU-3′ (si2common1) and 5′-UCAAGGGUGGGCUUUCCUGUACCCUA-3′ (si2common2) for all transcripts of Pparγ; 5′-CAGCGCCUCUUAGACCCCAUGAGAUU3′ (sic/EBPβ #1) and 5′-CCAGUGGAGUGGGCCACUCCUACU-3′ (sic/EBPβ #2) for C/EBPβ. For siRNA transfections, 5×105 cells/well were seeded onto 6-well culture plates and transfected with the above siRNAs using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s instructions.

Preadipocyte 3T3-L1 cells in the 12-well plate were transiently transfected with 0.6 μg of promoter/luciferase reporter construct (pGL3-Basic), 0.5 μg of overexpression construct (pCDNA3.1), and 0.5 μg of the constitutive Renilla luciferase expression vector (pGL4.74) (Promega) for normalization in a well using Lipofectamine 2000 transfection reagent (Life Technologies). For the assessment of siRNA specificity, 3T3-L1 cells were simultaneously transfected with each of siRNAs, the reporter plasmid pGL3-Control containing the Pparγ1sv or Pparγ2 cDNA, and pGL4.74 for
normalization. Cells were harvested 1 or 2 days after the transfection, and luciferase assays were performed using the Dual-luciferase reporter assay system (Promega). Luminescence was counted for 10 sec using a MiniLumat LB 9506 luminometer (Berthold).

**Results**

**A Novel Mouse Pparγ Splicing variant and its Gene Structure**

We amplified the 5′-ends of mouse Pparγ cDNAs using a reverse primer based on the sequence of exon 1 and a terminal adaptor primer from a cDNA library prepared from adipocyte differentiated 3T3-L1 cells (day 9). The amplified 5′-end products (~350 bp) contained the coding sequence and 5′-UTR of mouse Pparγ cDNAs. We sequenced 22 clones, nine of which contained 5′-end sequences of Pparγ, one contained the 5′-end of Pparγ_1. Remaining 12 clones possessed a unique 5′-UTR that was different from those of Pparγ_1 and Pparγ_2 (Fig. 1A). Full-length cDNAs of the novel splicing variant were then amplified using the 3′-end adapter primer and 5′-end gene-specific primer that was designed based on the sequences of 5′ RACE products. Sequencing of the full-length cDNA showed that the novel splicing variant encoded an identical amino acid sequence of mouse PPARγ1. We have designated this novel splicing variant as Pparγ_1sv. The complete sequence of Pparγ_1sv cDNA was deposited in the DDBJ/EMBL/GenBank database under the accession number AB644275. The transcription initiation site of the novel splicing variant was located on the novel exon C (68 bp) by aligning its complete sequence of information about mouse Pparγ genomic sequence on chromosome 6 (Fig. 1B). Exon C is located far (~60 kb) from exon B of Pparγ_2 whereas it is relatively close (~1 kb) to exon A1 of Pparγ_1 (Fig. 1B). A homology search using the BLAST program revealed that exon C shared 81% sequence identity with porcine exon C (GenBank no. AB121691) and 77% with exon C (or A) of human PPARγ transcript variant 3 (NM_138771). Alignment of the nucleotide sequence of mouse exon C with those of corresponding exons of other mammals are shown in Fig 1C. Additional information about mouse Pparγ_1sv and its homologous transcription variants in other mammals is summarized in Table 1.

**Tissue Distribution and Relative Abundance of Pparγ_1sv in Mice**

We designed unique forward primers for Pparγ_1sv, Pparγ_1, and Pparγ_2, respectively and a common reverse primer for all Pparγ transcripts on exon 1 to quantify their expression levels (Fig. 2A). The relative expression levels of the three transcripts were analyzed by qPCR using normalized cDNAs prepared from 16 mouse tissues and embryos (Fig. 2B). Pparγ_1sv was expressed abundantly in the stomach, placenta, heart, spleen, lung, skeletal muscle, and 17-day mouse embryos. Pparγ_1sv was also abundantly expressed in the white and brown adipose tissues at higher levels than that of Pparγ_2 (Fig. 2C).

**Kinetics of Pparγ_1sv Expression during Adipocyte Differentiation of 3T3-L1 and Primary Cells**

To further clarify the involvement of Pparγ_1sv in adipogenesis, we examined its expression and kinetics in 3T3-L1 and primary cells from white adipose tissue of newborn mice during adipocyte differentiation. Both Pparγ_1sv and Pparγ_2 mRNAs were induced in the early phase (day 1) of adipocyte differentiation of 3T3-L1 cells, and continued to increase up to day 9 (Fig. 3A). Pparγ_1sv and Pparγ_2 mRNA levels were approximately 15- and 234-fold higher at day 9, respectively, than those of cells at day 0 (Fig. 3C). In primary cultured cells, the kinetics of Pparγ_1sv and Pparγ_2 induction were similar to those of 3T3-L1 cells. The expression of Pparγ_1sv and Pparγ_2 significantly increased upon differentiation up to day 6, and reached a plateau at day 9, respectively (Fig. 3B). No appreciable induction of Pparγ_1 mRNA was observed in the course of adipocyte differentiation of both ST2-L1 and primary cells.

Immunoblotting of 3T3-L1 (Fig. 3C) and primary cultured cells (Fig. 3D) with anti-PPARγ antibody revealed that the PPARγ protein was abundant at day 3, expressed at day 6 with similar amount to the PPARγ2 protein, but reduced at day 9. We confirmed adipocyte differentiation of both 3T3-L1 (Fig. 3E) and primary cultured cells (Fig. 3F) by staining intracellular lipid accumulation with Oil Red O.

In ST2 cells, the kinetics of Pparγ_1sv and Pparγ_2 induction were different from 3T3-L1 cells. The expression of Pparγ_1sv and Pparγ_2 slightly increased upon differentiation but was down-regulated at days 6 and 9, respectively (Fig. S1A). This is probably due to the lower extent of adipocyte differentiation of ST2 cells. Upon stimulation with bone morphogenetic proteins, ST2 cells alternatively differentiate into osteoblasts. To assess if the up-regulation of Pparγ_1sv is specific to adipogenesis in ST2 cells, we examined the expression level of Pparγ_1sv in the course of osteoblast differentiation of ST2 cells. Expression levels of both Pparγ_1sv and Pparγ_2 mRNAs were low and not markedly changed during differentiation (Fig. S1B). Alkaline phosphatase staining showed an increase in alkaline phosphatase activity, a hallmark of osteoblastic differentiation, in ST2 cells at 9 days after induction (Fig. S1B, inset photos).

**Pparγ_1sv is Indispensable for Adipogenesis in 3T3-L1**

To evaluate whether the expression of Pparγ_1sv is essential for the adipogenesis, we specifically knocked down Pparγ_1sv mRNA in the early phase of the differentiation. We designed three specific siRNAs for respective targets, Pparγ_1sv (siγ1sv22, siγ1sv30, and siγ1sv38) and Pparγ_2 (siγ2_88, siγ2_48, and siγ2_38) mRNAs. Positions of target sequences for designed siRNAs were indicated in Fig. 4A. 3T3-L1 cells were transfected with either of the siRNAs, and subjected to adipogenic induction in the following day. PPARγ1 and PPARγ2 protein levels were examined 2 days after induction by Western blotting using anti-PPARγ antibody (Fig. 4B). Introduction of all siRNAs for Pparγ_1sv greatly reduced PPARγ1 protein levels relative to differentiated 3T3-L1 cells transfected with negative control siRNA (siControl in Fig. 4B). These results prompted us to evaluate specificity of these siRNAs in quantitative method. For this purpose, we used the luciferase-based reporter system, in which the full-length Pparγ_1sv or Pparγ_2 cDNA was linked to luciferase gene (luc+) in sense or antisense direction (Fig. 4A). We excluded siγ1sv38 and siγ2_88 siRNAs from this validation assay because they showed less specificity in the knock-down of PPARγ1 and PPARγ2 proteins. In Fig. 4C, siγ1sv22 and siγ1sv38 siRNAs for Pparγ_1sv achieved more than 95% knock-down of the reporter gene with
the \textit{Ppar}\textsubscript{1sv} cDNA in sense direction (left upper panel) whereas they had negligible effect on the activity of the reporter with the \textit{Ppar}\textsubscript{2} cDNA in sense direction (left lower panel). On the other hand, si\textsubscript{c2}_8 and si\textsubscript{c2}_48 siRNAs for \textit{Ppar}\textsubscript{2} significantly reduced

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Gene and cDNA structures of the novel splicing variant of mouse \textit{Ppar}. (A) Alignment of 5\textsuperscript{\textprime} end sequences of \textit{Ppar}\textsubscript{1sv}, \textit{Ppar}\textsubscript{1}, and \textit{Ppar}\textsubscript{2} cDNAs. Each initiation codon is outlined, and exon 1, which is common to all three, is underlined. (B) Gene structure of N-terminal exons and common exon 1 of mouse \textit{Ppar}\textsubscript{1} on chromosome 6. Distances between two exons and exon lengths are indicated as numbers of nucleotides. Arrows indicate the positions of the transcription initiation site of each transcription variant. (C) Multiple alignment of nucleotide sequences of mouse exon C and corresponding exons in other mammals. Distances between the 5\textsuperscript{\textprime} end of each cDNA and exon C or corresponding exons of other mammals are indicated as numbers of base pairs.
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\end{figure}

\begin{table}
\centering
\caption{Summary of \textit{Ppar}\textsubscript{1sv} and other mammalian cDNAs that contain the unique exons illustrated in Fig. 1C.}
\begin{tabular}{|l|l|l|l|}
\hline
\textbf{Gene name} & \textbf{Source} & \textbf{Unique exon} & \textbf{Accession \#} \\
\hline
\textit{Ppar}\textsubscript{1sv} & Mouse & C & AB644275 \\
\textit{PPAR}\textsubscript{1}, \textit{PPAR}\textsubscript{7} tv*2 & Rat & ND** & AF156665, NM_001145366 \\
\textit{PPAR}\textsubscript{1c} & Pig & A' & AB097928 \\
\textit{PPAR}\textsubscript{1}, \textit{PPAR}\textsubscript{6}, \textit{PPAR}\textsubscript{7} & Monkey & A1 & AY048694, AY048699, AY048700 \\
\textit{PPAR}\textsubscript{7} tv*3 & Human & C, A' & AB472042, NM_138711 \\
\hline
\end{tabular}
\end{table}

*transcription variant, **not defined.
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the activity of the reporter with Ppar\(_c2\) cDNA (Fig. 4C, left lower panel) whereas no or little effect on the reporter with the Ppar\(_c1sv\) cDNA (left upper panel). Both si\(_c\) common siRNAs effectively knocked down the expression of the reporter gene with Ppar\(_c1sv\) or Ppar\(_c2\) in sense direction (Fig. 4C, left upper and left lower panel). Neither of siRNAs affected the activity of the reporter with Ppar\(_c1sv\) or Ppar\(_c2\) in antisense direction (Fig. 4C, right upper and right lower panel). These results confirmed that each siRNA could suppress its target mRNA with high specificity. We thus concluded that depletion of one PPAR\(_c\) isoform affect the other’s protein level in 3T3-L1 cells during adipocyte differentiation.

We next examined the effect of knock-down of Ppar\(_c1sv\) mRNA on the adipogenesis by Oil Red O staining at day 9 (Fig. 4D) and quantitation of intracellular triglycerides at day 6 (Fig. 4E). Both results showed that knock-down of Ppar\(_c1sv\) completely (by si\(_c1sv22\)) or substantially (by si\(_c1sv30\)) inhibited the lipid accumulation as observed in Ppar\(_c2\) knock-down cells, implying the importance of Ppar\(_c1sv\) in adipogenesis. We further characterized si\(_c1sv\)- and si\(_c2\)-transfected cells by analyzing the expression of the adipocyte-related proteins C/EBP\(_\beta\), C/EBP\(_\delta\), FABP4 (aP2), and DLK (pref-1) by Western blotting (Fig. 4F). Transfection of si\(_c1sv22\) or si\(_c1sv30\) resulted in no apparent change in protein levels of C/EBP\(_\beta\) at days 2 and 6 compared with those of the control cells whereas a slight inhibition in protein levels of C/EBP\(_\delta\) at day 6. Induction of FABP4, an adipogenic marker protein, was markedly inhibited in si\(_c1sv22\) and si\(_c2\) cells at day 6, but neither in siControl nor in si\(_c1sv30\) cells. The expression of DLK, a preadipocyte marker, was drastically down-regulated upon differentiation in siControl cells. It was also decreased but could be detected at a very low level in all si\(_c1sv\), si\(_c2\), and si\(_c\) common knock-down cells at day 2 (Fig. 4F).

Ppar\(_c1sv\) Expression is Dependent on C/EBP\(_\beta\) and C/EBP\(_\delta\) in 3T3-L1 Cells

The significant induction of mouse Ppar\(_c1sv\) mRNA during adipocyte differentiation raised a question of how transcription of Ppar\(_c1sv\) is regulated. C/EBP\(_\beta\) and C/EBP\(_\delta\) are induced within a day during adipogenesis of 3T3-L1 cells [4]. This in turn activates expression of Ppar\(_\gamma\) and C/EBP\(_\beta\). The two reciprocally stimulate each other by forming a positive feedback loop, and synergistically promote the downstream gene expression required to accomplish adipogenesis. The promoter of Ppar\(_\gamma\) contains two C/EBP\(_\beta\) recognition elements, and Ppar\(_\gamma\) is directly up-regulated by C/EBP\(_\beta\) and C/EBP\(_\delta\) [14]. To clarify whether C/EBPs up-regulate Ppar\(_c1sv\) as well, we performed a luciferase reporter assay using the Ppar\(_c1sv\) promoter (−969 to +50) that had been subcloned into the
luciferase reporter vector pGL3-Basic. The reporter construct was co-transfected with the expression vector harboring either of the coding sequence of C/EBP\textsubscript{a}, C/EBP\textsubscript{b}, or C/EBP\textsubscript{d}. C/EBP\textsubscript{a} and C/EBP\textsubscript{b} have several isoforms, which include full-length and N-terminally truncated proteins [15]. In differentiating 3T3-L1 cells, we detected three C/EBP\textsubscript{b} isoforms, full-length (p34) and two N-terminally truncated C/EBP\textsubscript{b} proteins (p30 and p20). Of the three, C/EBP\textsubscript{b}(p30) was the dominant isoform (Fig. 5A). As shown in Fig. 5B, C/EBP\textsubscript{b}(p30) and C/EBP\textsubscript{d} markedly increased the promoter activities of \textit{Ppar\textsubscript{c}1sv} and \textit{Ppar\textsubscript{c}2}, while full-length C/EBP\textsubscript{b}(p34) did not. Overexpression of C/EBP\textsubscript{b} gave a slight but significant increment ($P<0.05$) in both \textit{Ppar\textsubscript{c}1sv} and \textit{Ppar\textsubscript{c}2} promoter activities compared to control cells, respectively. \textit{Ppar\textsubscript{c}1} promoter activity was not altered by co-transfection with each of the overexpression vectors (Fig. 5B).

We next examined the effect of C/EBP\textsubscript{b} knock-down on the expression levels of \textit{Ppar\textsubscript{c}1sv} and \textit{Ppar\textsubscript{c}2} mRNAs after the induction of adipocyte differentiation. Each of two discrete siRNAs targeting C/EBP\textsubscript{b} were transfected into 3T3-L1 cells. As shown in Fig. 5C, expression of both \textit{Ppar\textsubscript{c}1sv} and \textit{Ppar\textsubscript{c}2} mRNAs were markedly inhibited in C/EBP\textsubscript{b} knock-down cells at day 3 of induction. Protein levels of PPAR\textsubscript{c}1 and PPAR\textsubscript{c}2 were also significantly suppressed at days 2 and 6 of induction by transfecting C/EBP\textsubscript{b} siRNA (#1) relative to those of control siRNA (Fig. 5D).

### Discussion

In this study, we analyzed 5'-ends of mouse \textit{Ppar\textsubscript{c}} cDNAs and isolated full-length cDNA of the novel splicing variant, \textit{Ppar\textsubscript{c}1sv}, that encodes PPAR\textsubscript{c}1 protein. PPAR\textsubscript{c}1 was remarkably up-regulated with an induced adipogenesis. Knock-down of \textit{Ppar\textsubscript{c}1sv} in adipocytes resulted in the substantial reduction of the PPAR\textsubscript{c}1 protein and intracellular lipid accumulation, indicating an indispensable role of \textit{Ppar\textsubscript{c}1sv} in adipocyte differentiation.

Several \textit{Ppar\textsubscript{c}} splicing variants except for \textit{Ppar\textsubscript{c}1} and \textit{Ppar\textsubscript{c}2} have been identified in humans [12,13,16–18], monkeys [19], and pigs [11]. Mouse \textit{Ppar\textsubscript{c}1sv} and corresponding splicing variants in the above 3 mammals and rats share a unique exon (named C in mouse) (Table 1), which implies that the expression of this splicing variant is ubiquitous in mammals.

The expression profiling of three \textit{Ppar\textsubscript{c}} transcripts showed that their different abundance in mouse tissues (Figs. 2B and 2C). In several tissues, \textit{Ppar\textsubscript{c}1sv} is expressed at higher levels than the others. For example, expression level of \textit{Ppar\textsubscript{c}1sv} in spleen was 3.3 and 5.3 times higher than those of \textit{Ppar\textsubscript{c}1} and \textit{Ppar\textsubscript{c}2}, respectively.
Thus, \textit{Ppar\textsubscript{1}sv} could be a major transcript and contribute to the PPAR\textgamma protein expression the most in those tissues. While localization of \textit{Ppar\textsubscript{1}sv} in mouse embryo is undetermined, \textit{Ppar\textsubscript{1}sv} is dramatically up-regulated during the late stages of fatal development (15- and 17-day, Fig. 2B), implying that \textit{Ppar\textsubscript{1}sv} is deeply involved in cell differentiation in embryo.

**Figure 4. Effect of knock-down of Ppar\textsubscript{1}sv on adipogenesis of 3T3-L1 cells.** (A) Locations of the targeting sites of siRNAs for \textit{Ppar\textsubscript{1}sv}, \textit{Ppar\textsubscript{2}}, and both transcripts (common). For the validation of siRNAs using a luciferase reporter gene, the full-length \textit{Ppar\textsubscript{1}sv} or \textit{Ppar\textsubscript{2}} cDNA was inserted into Xho I site in pGL3-Control in sense or antisense direction. (B) Evaluation of siRNAs for \textit{Ppar\textsubscript{1}sv} or \textit{Ppar\textsubscript{2}}. 3T3-L1 cells were transfected with each of siRNA, and subjected to adipogenic induction in the following day (day 0). Expression of PPAR\textgamma and PPAR\textbeta proteins at day 2 was detected using PPAR\textgamma antibody with Lamin B1 as a control for nuclear extracts. siControl is negative control siRNA. Arrows indicate the positions of PPAR\textgamma and PPAR\textbeta proteins. (C) Validation of siRNA specificity. The relative luciferase activities were obtained from 3T3-L1 cells 1 day after transfection with pGL3-Control containing the \textit{Ppar\textsubscript{1}sv} or \textit{Ppar\textsubscript{2}} cDNA in sense or antisense direction and each of two respective siRNAs for \textit{Ppar\textsubscript{1}sv} and \textit{Ppar\textsubscript{2}}. The values represent the mean of triplicate measurements. The activity of siControl cells is defined as 100%. (D) 3T3-L1 cells transfected with siRNAs were subjected to adipogenic induction and stained by Oil Red O at day 9. Microscopic (x100) observations are shown. (E) Quantitative measurement of intracellular triglycerides in siRNA knock-down cells at days 0 and 6 using AdipoRed reagent. Data represent the mean of 4 replicate assays. * \textit{P}<0.01 compared to siControl cells at day 6. (F) Protein expression levels of PPAR\textgamma, C/EBP\beta, C/EBP\alpha, Lamin B1 (control for nuclear extracts), FABP4 (aP2), DLK (pref-1), and \textalpha-tubulin (control for cytosolic extracts) in \textit{Ppar\textsubscript{1}sv} or \textit{Ppar\textsubscript{2}} knock-down cells at days 2 and 6 detected by the corresponding antibodies.

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Thus, \textit{Ppar\textsubscript{1}sv} could be a major transcript and contribute to the PPAR\textgamma protein expression the most in those tissues. While localization of \textit{Ppar\textsubscript{1}sv} in mouse embryo is undetermined,
pathological analyses of PPARγ-deficient mice revealed PPARγ functions in multiple tissues such as the adipose tissue, the placenta, and the developing heart during pre- and postnatal development [20]. Recently, overlapping and distinct functions of PPARγ1 and PPARγ2 in prostate epithelial cells have been reported [21]. We are presently generating Pparγ1sv- and/or Pparγ1-deficient mouse to assess the specific roles of each isoform in development, which will provide some answers to the meaning and importance of the production of multiple transcripts in Pparγ.

To date, PPARγ2 protein but not PPARγ1 protein is thought to play an essential role in adipogenesis, because Pparγ2 mRNA is up-regulated during the initiation of adipocyte differentiation whereas Pparγ1 is not. In this study, we showed that Pparγ1sv is highly expressed in the white and brown adipose tissues (Fig. 2C), which indicates considerable expression of not only PPARγ2 but also PPARγ1 protein in the adipose tissues. In fact, both PPARγ1 and PPARγ2 proteins drastically increased during adipogenesis of 3T3-L1 and primary cultured cells (Figs. 3C and 3D). We showed that Pparγ1sv is markedly up-regulated during adipocyte differentiation (Figs. 3A and 3B). The knock-down of Pparγ1sv using siRNAs resulted in significant suppression of PPARγ1 protein during adipocyte differentiation of 3T3-L1 cells (Fig. 4B). These results strongly support that PPARγ1 proteins expressed during adipogenesis is derived from Pparγ1sv mRNA. Knock-down of Pparγ1sv also greatly inhibited the accumulation of intracellular triglyceride (Fig. 4E) and the induction of an adipocyte marker FABP4 (siγ1sv#2 in Fig. 4F) in 3T3-L1 cells. Incomplete adipocyte differentiation of siγ1sv- and siγ2-transfected cells was also confirmed by the partial expression of a preadipocyte marker, DLK at day 2 (Fig. 4F). It was likely that inhibition of adipocyte differentiation evaluated by lipid accumulation and marker proteins was dependent on the abundance of PPARγ1 and PPARγ2 proteins in siγ1sv- and siγ2-treated cells. We thus concluded that the up-regulation of PPARγ1 proteins originated

Figure 5. Luciferase reporter assay for the Pparγ1sv promoter in 3T3-L1 cells. (A) Western blotting of 3T3-L1 cells during adipocyte differentiation (days 0–9) and NIH/3T3 cells transfected with the overexpression construct containing no insert (control) or coding region of C/EBPβ isoforms (p34 and p30) detected by anti-C/EBPβ antibody. (B) The luciferase reporter construct, the pGL3-Basic, containing the Pparγ1sv, Pparγ1, or Pparγ2 promoter was co-transfected with the C/EBP overexpression construct to 3T3-L1 cells. Cells were harvested 2 days after transfection and assayed using Dual-luciferase reporter assay reagents. The values represent the mean of triplicate measurements. The activity obtained from cells transfected with empty vector is defined as 1. (C) Effect of C/EBPβ depletion by siRNA on Pparγ expression. Each of two discrete C/EBPβ siRNAs (siC/EBPβ #1 and #2) was transfected to 3T3-L1 cells. Cells were harvested at days 0 and 3 of differentiation, and expression of Pparγ1sv (black bar) and Pparγ2 (gray bar) mRNAs was evaluated by qPCR. Values were normalized to those of 18S rRNA. The RNA expression of siControl cells at day 3 is defined as 100%. (D) Immunoblotting of the nuclear extracts of siC/EBPβ #1-treated cells detected by antibodies specific to each of C/EBPβ, PPARγ, or Lamin B1 (control).

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from Ppars during adipogenesis is indispensable to accomplish the differentiation process.

C/EBPβ and C/EBPδ play key roles in the early phase of the adipogenic molecular cascade. Expression of both proteins is enhanced during the initial few hours of differentiation in 3T3-L1, which in turn activate expression of Pparγ2 and C/EBPδ. We have found that C/EBPβ and/or C/EBPδ also activated the Pparγ1sw promoter (Fig. 5B). Unexpectedly, N-terminally truncated C/EBPβ (p30) significantly activated the both Pparγ1 and Pparγ2 promoters (Fig. 5B), but full-length C/EBPβ isomorph (p34) did not. We demonstrated that the major product in adipogenesis of 3T3-L1 cells was p30 (Fig. 5A). Therefore, it is possible that p30 and C/EBPδ directly initiate the synergistic expression of Pparγ1sw and Pparγ2 mRNA in the early period of adipogenesis.

Intriguingly, inhibition of either Pparγ transcript by specific siRNA resulted in suppression of both PPARγ proteins (Fig. 4B). Validation of designed siRNAs using the luciferase reporter system showed their highly effective and specific knock-down properties (Fig. 4C). These results imply that expression level of PPARγ1 protein could affect that of PPARγ2 and vice versa during adipogenesis of 3T3-L1 cells. One possible explanation is the direct up-regulation of the Pparγ transcription by PPARγ proteins. It has been demonstrated that Pparγ2 gene expression is regulated by binding of the Pparγ2/RXRα heterodimer to the Pparγ2 promoter during adipocyte differentiation of 3T3-L1 [8]. Although direct interaction of the PPARγ1 protein to the Pparγ2 promoter has not been clarified, it is probable that depletion of the PPARγ1 protein by siRNA targeting to Pparγ1 caused reduction in the amount of the PPARγ2/RXRα heterodimer, which resulted in less activation of the Pparγ2 promoter and down-regulation of the Pparγ2 protein (Fig. 6, arrow with an asterisk). On the other hand, binding of the PPARγ2/RXRα heterodimer to the regions of the Pparγ1sw promoter (Fig. 6, arrow with a sharp) was not observed [8]. We could not identify the consensus sequence for PPARγ and RXR binding in the Pparγ1sw promoter (~1 kb). Therefore, down-regulation of Pparγ1sw by the introduction of Pparγ2-specific siRNA might be involved in downstream factors that are regulated by PPARγ1 protein and activate the Pparγ1 promoter.

The present study suggests the importance of Pparγ1sw in the adipocyte differentiation and a real need to elucidate a detailed mechanism of the Pparγ1sw regulation and the precise function of PPARγ1 protein in cell differentiation.

Supporting Information

Figure S1 Relative expression of three PPARγ transcripts during adipogenic and osteoblastic differentiation of ST2 cells. (A) Confluent ST2 cells were cultured in RPMI1640 medium supplemented with 10% FBS, 0.25 µM dexamethasone, 500 µM isobutylmethylxanthine, 1 µM insulin, and 1 µM rosiglitazone to induce adipocytic differentiation. Cells were harvested at the indicated time and analyzed by real-time RT-PCR. (B) ST2 cells were cultured in RPMI1640 medium supplemented with 10% FBS and 100 ng/ml BMP-4 (Wako, Japan) to induce osteoblastic differentiation. Cells were analyzed by real-time RT-PCR or fixed with 10% formalin for 20 min and stained using an alkaline phosphatase staining kit (Primary Cell Co., Ltd; TIF).

Figure 6. Schematic model for the transcriptional control of Pparγ1 in adipocyte differentiation. C/EBPβ and C/EBPδ directly transactivate both Pparγ1sw and Pparγ2 genes, which are in turn translated to PPARγ1 and PPARγ2 proteins, respectively. Pparγ1sw enhances the expression of downstream genes involved in adipogenesis, forming a positive feedback loop with C/EBPs. Arrows with an asterisk and a sharp are speculative feedback pathways for Pparγ up-regulation by PPARγ proteins.

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Author Contributions

Conceived and designed the experiments; YT TN II. Performed the experiments; YT. Analyzed the data; YT TN II. Contributed reagents/materials/analysis tools; YT TN II YS TA MI SK. Wrote the paper; YT TN.

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