Adipogenesis is differentially impaired by thyroid hormone receptor mutant isoforms

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Abstract

To understand the roles of thyroid hormone receptors (TRs) in adipogenesis, we adopted a loss-of-function approach. We generated 3T3-L1 cells stably expressing either *TRα1* mutant (*TRα1PV*) or *TRβ1* mutant (*TRβ1PV*). *TRα1PV* and *TRβ1PV* are dominant negative mutations with a frameshift in the C-terminal amino acids. In control cells, the thyroid hormone, tri-iodothyronine (T_3), induced a 2.5-fold increase in adipogenesis in 3T3-L1 cells, as demonstrated by increased lipid droplets. This increase was mediated by T_3 -induced expression of the peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer-binding protein α (C/EBP α), which are master regulators of adipogenesis at both the mRNA and protein levels. In 3T3-L1 cells stably expressing *TRα1PV* (L1- α 1PV cells) or *TRβ1PV* (L1- β 1PV cells), adipogenesis was reduced 94 or 54% respectively, indicative of differential inhibitory activity of mutant TR isoforms. Concordantly, the expression of PPAR γ and C/EBP α at the mRNA and protein levels was more repressed in L1- α 1PV cells than in L1- β 1PV cells. In addition, the expression of PPAR γ downstream target genes involved in fatty acid synthesis – the lipoprotein lipase (*Lpl*) and *aP2* involved in adipogenesis – was more inhibited by *TRα1PV* than by *TRβ1PV*. Chromatin immunoprecipitation assays showed that *TRα1PV* was more avidly recruited than *TRβ1PV* to the promoter to preferentially block the expression of the *C/ebpα* gene. Taken together, these data indicate that impaired adipogenesis by mutant TR is isoform dependent. The finding that induction of adipogenesis is differentially regulated by TR isoforms suggests that TR isoform-specific ligands could be designed for therapeutic intervention for lipid abnormalities.

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Introduction

Thyroid hormone (tri-iodothyronine, T_3) has long been recognized to play a key role in lipid metabolism and thermogenesis. However, the molecular mechanisms by which T_3 regulates lipid homeostasis have not been fully elucidated. The genomic actions of T_3 are mediated by thyroid hormone nuclear receptors (TRs). There are two TR genes, *TRα* and *TRβ*, located on different chromosomes, to encode three major T_3 binding receptors, α 1, β 1, and β 2 (Cheng 2000). These TR isoforms, expressed in a tissue-specific and development-regulated manner, regulate growth, differentiation, and development, and maintain metabolic homeostasis. TRs are ligand-dependent transcription factors that bind to specific DNA sequences known as thyroid hormone response elements (TREs) on the promoter of T_3 target genes. The regulation of the transcriptional activity of TRs is complex. In addition to T_3 , their transcriptional activity is further regulated by a host of corepressors and coactivators (Cheng 2000, O'Malley 2003).

Recent progress in the understanding of TR action on lipid homeostasis has been achieved via genetically engineered mice. Mice deficient in the *Trα* gene exhibit

a lower body temperature than the wild-type mice, indicating that *Trα1* is important in thermogenesis and energy balance (Wikstrom *et al.* 1998). Mice lacking the *Trβ* gene have abnormal cholesterol regulation (Gullberg *et al.* 2002). Mice devoid of all functional TRs display decreased body temperature and basal metabolic rate, and are cold intolerant due to insufficient heat production (Golozoubova *et al.* 2004). These studies revealed that TR isoforms act to regulate different aspects of metabolic homeostasis. More insights into TR actions in lipid metabolism came from the use of loss-of-function approach via knock-in mutations of TR genes in mice. Indeed, knock-in mutant mice harboring different mutations of the *Trα* gene exhibit abnormalities in lipid metabolism. The *TrαIP398H* knock-in mutant mice have increased body fat accumulation and elevated serum levels of leptin, glucose, and insulin (Liu *et al.* 2003). In contrast, the *TrαIR384C* knock-in mutant mice exhibit a lean phenotype with a reduction in fat mass (Sjogren *et al.* 2007). The *Trα1PV* mice that harbor a frameshift mutation in the C-terminal 17 amino acids (Fig. 1) display a lean phenotype, partly due to the reduction in the mass of white adipose tissue (WAT; Kaneshige *et al.* 2001, Ying *et al.* 2007). The reasons for the different

Figure 1 Schematic comparison of the wild-type and mutant TR isoforms. The PV mutation was identified in a patient with resistance to thyroid hormone. It has a C insertion in codon 448 of *TRβ1* that leads to a frameshift mutation in the C-terminal 14 amino acids. The identical mutation was targeted to the *Trα* and *Trβ* genes to encode TRβ1PV and TRα1PV respectively. The different sequences between the wild-type and mutant TR isoforms at the C-terminal region are indicated.

phenotypic manifestations in these three knock-in mutant mice are not clear. The different phenotypes could reflect the differences in the degree of loss of T_3 binding and the potency of dominant negative activity of *Trα1* mutants. *Trα1PV* mice completely lose T_3 binding and exhibit potent dominant negative activity (Kaneshige *et al.* 2001). In contrast, *Trα1P398H* and *Trα1R384C* mice only partially lose T_3 binding activity (Tinnikov *et al.* 2002, Liu *et al.* 2003). The weaker dominant negative activity of *Trα1R384C* is consistent with the restoration of the target gene expression and the rescue of growth retardation in *Trα1R384C* mice when serum thyroxine concentration was increased tenfold (Tinnikov *et al.* 2002).

The expression of *Trα1PV* mutant in WAT impairs adipogenesis via, in part, the transcriptional repression of the master regulator of adipogenesis, peroxisome proliferation-activated receptor γ (*Pparγ*; Ying *et al.* 2007). However, despite *Trβ* expression in WAT (Ying *et al.* 2007), intriguingly, in the *TrβPV* mice that harbor the identical PV mutation in the corresponding C-terminal position (Fig. 1; Kaneshige *et al.* 2000), no apparent changes in WAT are evident (Araki *et al.* 2009), suggesting mutant TR isoforms mediate distinct impairments in adipogenesis.

To dissect the molecular mechanism underlying the distinct phenotypic manifestation in WAT between *Trα1PV* and *TrβPV* mice, we used the 3T3-L1 cell line. This system, developed by Green & Kehinde (1975) in the 1970s, has long been used by investigators as a model cell line to study adipogenesis. To establish the cause–effect relationship, we stably expressed *Trα1PV* or *Trβ1PV* in 3T3-L1 cells and evaluated the alterations in adipogenesis as compared with parental cells that contained only the vector backbone (control cells). Clones with similar abundance of TRα1PV (denoted as L1-α1PV cells) or TRβ1PV (L1-β1PV cells) were used to

elucidate the molecular basis of the distinct phenotypic expression in adipogenesis in WAT observed in *Trα1PV* and *TrβPV* mice (Araki *et al.* 2009). We found that the T_3 -induced adipogenesis was more severely impaired in L1-α1PV cells than in L1-β1PV cells. The expression of two master regulators, PPAR γ and CCAAT/enhancer-binding protein α (C/EBP α), was found to be more repressed in L1-α1PV cells than in L1-β1PV cells. Consistent with the gene expression patterns, the expression of several key lipogenic enzymes was more severely inhibited by TRα1PV than by TRβ1PV. Further analysis indicated that the preferential repression of the *C/ebpα* gene by TRα1PV was mediated, at least in part, by more avid recruitment of TRα1PV to the promoter complex of the *C/ebpα* gene. The finding that TR-mediated adipogenesis is isoform dependent raised the possibility that isoform-specific TR ligands could be considered for therapeutic intervention for lipid abnormalities.

Materials and methods

Cell culture

3T3-L1 cells (ATCC #CL-173) were maintained in DMEM with 10% calf serum as described previously (Ying *et al.* 2007).

Generation of 3T3-L1 cell lines stably expressing TRα1PV or TRβ1PV by retrovirus transduction

Plasmids

Human *Trα1PV* and *Trβ1PV* cDNAs were cloned into pFH-IRESneo plasmid ((Martinez *et al.* 2001), a generous gift of R G Roeder, Rockefeller University, New York, NY, USA) to obtain FH-α1PV and FH-β1PV respectively as described previously (Ying *et al.* 2006). The pMSCVhygro-FH-α1PV or pMSCVhygro-FH-β1PV vector was prepared by inserting FH-α1PV or FH-β1PV cDNA from pFH-IRESneo-α1PV or pFH-IRESneo-β1PV respectively into pMSCVhygro (Clontech). All plasmids were verified by DNA sequencing.

Retrovirus transduction

EcoPack2-293 ecotropic retrovirus packaging cell lines (Clontech) were cultured in DMEM with 10% fetal bovine serum. Recombinant retroviral transduction of 3T3-L1 cells was performed as described (Ge *et al.* 2002). Briefly, EcoPack2-293 cells were seeded at a density of 3×10^6 cells per 10-cm dish. After 24 h, cells were transfected with 22 μ g of retrovirus vector by Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after transfection, the cells were changed into fresh medium. After 12 h, virus-containing supernatant

was collected, filtered through 0.45- μ m membrane, and supplemented with 8 μ g/ml polybrene (Sigma). 3T3-L1 cells were plated at a density of 2×10^5 cells per 10-cm dish 1 day before infection. Cells were incubated with retrovirus for 48 h and split at a density of 2×10^5 cells per 10-cm dish. Cells were selected with 150 μ g/ml hygromycin (Invitrogen) for 14 days. The expression of TR α 1PV or TR β 1PV protein was verified by western blot analysis using anti-FLAG (Sigma–Aldrich), anti-TR β 1 C4 (Bhat *et al.* 1995), or monoclonal anti-PV antibody (#302; Zhang *et al.* 2002).

Induction of adipocyte differentiation

Adipocyte differentiation was induced as described (Ying *et al.* 2007). Briefly, after the 3T3-L1 cells reached confluence, they were incubated with DMEM containing 10% resin-stripped calf serum for 48 h. The cells were treated with DMEM containing 10% resin-stripped fetal bovine serum with 1 μ M dexamethasone (Sigma) and 0.5 mM isobutylmethylxanthine (IBMX) (Sigma) in the presence or absence of 2 nM T₃ for 60 h. Thereafter, cells were subsequently fed every other day with DMEM containing 10% resin-stripped fetal bovine serum with or without 2 nM T₃ until being stained with Oil Red O (Sigma) at 7 days after the initiation of stimulation. Quantification of the staining intensities was carried out by drying the stained plates, adding 0.8 ml isopropanol per 3.5-cm dish, and reading at OD₅₁₀ against the control using a stained dish without cells.

Quantitative real-time RT-PCR

RNAs from control 3T3-L1, L1- α 1PV, and L1- β 1PV cells were extracted using an RNeasy mini kit according to the manufacturer's instructions (Qiagen). DNA was removed by using TURBO DNA-free kit (Ambion, Austin, TX, USA). The determination of mRNA by real-time reverse transcription-PCR (qPCR) was carried out using total RNA (10–50 ng) and primers for *Ppar γ* , *Lpl*, and *ap2* as described previously (Ying *et al.* 2007, Araki *et al.* 2009). The primer sequences for *C/ebp α* are forward TTACAACAGGCCAGGTTTCC and backward CTCTGGGATGGATCGATTGT.

Western blot analysis

Cells (control 3T3-L1, L1- α 1PV, and L1- β 1PV cells) were collected before induction or after induction of adipogenesis. For 100 μ l of packed cell volume, 500 μ l of lysis buffer (10 mM Tris, pH 7.9, 0.1 mM EDTA, 10 mM NaCl, 10 mM dithiothreitol, and protease inhibitors) were used to suspend the cells for 15 min on ice. Subsequently, NP40 was added to make a final concentration of 0.6% to lyse the cells by gentle

vortexing. After centrifugation at 10 000 *g* for 30 s at 4 °C, the supernatant was removed and the pellet was extracted using an extraction buffer (20 mM HEPES/pH 7.9, 0.4 M NaCl, and 1 mM EDTA) by vortexing. The suspension was centrifuged at 16 000 *g* for 10 min at 4 °C. In some experiments, the western blot analysis of lysates was done using cells that were harvested by scraping in buffer (1 $\times$ PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, and 2 mM potassium phosphate monobasic, pH 7.4) and centrifuged at 2300 *g* at 4 °C for 4 min, and then the supernatant had been removed. The pelleted cells were incubated in a lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 $\times$ Halt phosphatase inhibitor cocktail (Product no.: 78 420, ThermoFisher Scientific, Waltham, MA, USA), and 1 $\times$ proteinase tablet cocktail inhibitors (Complete Mini EDTA-free; Roche)) for 10 min on ice, vortexed three times, and spun at 16 100 *g* at 4 °C for 10 min. Supernatant was collected for western blot analysis. The protein concentration of the supernatant was determined by the Bradford method (Pierce Chemical Co., Rockford, IL, USA) with BSA (Pierce Chemical Co.) as the standard. Western blot analysis was carried out as described previously (Ying *et al.* 2006). For the detection of PPAR γ and C/EBP α proteins, nuclear fractions (35 μ g) were separated by SDS-PAGE. The primary antibodies used in the western blot analysis were anti-PPAR γ antibody (1:100 dilution; sc-7273; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-C/EBP α (1:100 dilution; sc-61; Santa Cruz Biotechnology, Inc.).

Chromatin immunoprecipitation

Chromatin isolation from control 3T3-L1, L1- α 1PV, and L1- β 1PV cells was carried out by using a chromatin immunoprecipitation (ChIP) assay kit according to the manufacturer's instructions (Millipore, Billerica, MA, USA). Chromatin solution (1 ml) was immunoprecipitated with the following antibodies: 5 μ l of anti-TR β 1 antibody (Rockland Immunochemicals, Gilbertsville, PA, USA code: 600-401-A38), anti-TR α 1 antibody (1:300 dilution, Rockland Immunochemicals), monoclonal anti-PV antibody (#302; 4 μ g (Zhang *et al.* 2002)), or IgG (2 μ g from kit) for negative control, and anti-ACh3 (2 μ g, from ChIP assay's kit) for positive control. The DNA recovered after immunoprecipitation was used as a template for amplification as described previously (Ying *et al.* 2007). Two percent of the chromatin solution (20 μ l) was used for the input DNA as a control. The primer sequences for the analysis of the TR binding sites for *C/ebp α* were 5'-TAGAGAAGCTGGGCGAAAAGA-3' (forward) and 5'-CCCACTTCCAGCCAACAGTA-3' (reverse). The amplified DNA of 339 bp was analyzed on a 1.5% agarose gel with ethidium bromide staining.

Statistical analysis

All data are expressed as means $\pm$ s.e.m. Statistical analysis was performed with ANOVA, and a *P* value < 0.05 was considered significant.

Results

Impairment of adipogenesis in 3T3-L1 cells by TR mutants is isoform dependent

The observation that adipogenesis of WAT is impaired in *Tr α 1PV*, but not in *Tr β 1PV*, mice prompted us to use 3T3-L1 cells to further elucidate the molecular mechanisms underlying the differential effects of TR mutants on adipogenesis. 3T3-L1 cells have long been used by investigators as a model cell line to study adipogenesis. We, therefore, generated 3T3-L1 cells stably expressing *TR β 1PV* (L1- β 1PV cells) or *TR α 1PV* (L1- α 1PV cells). We chose the clones that stably expressed a similar level of TR mutant isoforms for the study (Fig. 2). Western blot analysis showed that the

Figure 2 3T3-L1 cells stably expressing TR β 1PV and TR α 1PV proteins. (A) 3T3-L1 cells stably expressing TR β 1PV (L1- β 1PV cells) or TR α 1PV (L1- α 1PV cells) were prepared as described in Materials and methods. TR α 1PV (lane 1) and TR β 1PV (lane 2) proteins were detected by monoclonal anti-PV antibody (#302; 2 μ g/ml (Bhat *et al.* 1995)). Lane 3 indicates control cells that were used as negative controls, indicating the specific bands detected in lanes 1 and 2. GAPDH was used as a loading control. (B) Total cellular lysates (30 μ g) were used in the western blot analysis. Endogenous TR α 1 and TR β 1 receptor proteins in L1- α 1PV cells (lane 1), L1- β 1PV cells (lane 2), and 3T3-L1 cells (lane 3) were detected by monoclonal antibody C4 that recognizes the C-terminus of TR β 1 and TR α 1 receptors. GAPDH was used as a loading control.

abundance of TR α 1PV protein in L1- α 1PV cells (lane 1, Fig. 2A) was similar to that of TR β 1PV protein in L1- β 1PV cells (lane 2, Fig. 2A). Lane 3 shows that no TR mutant was expressed in the control cells in which only the vector backbone was transfected (denoted as control cells). Figure 2B shows endogenous TR α 1 and TR β 1 protein abundance in L1- β 1PV, L1- α 1PV, and control cells detected by western blot analysis. Consistent with that found in white adipocytes of wild-type mice (Ying *et al.* 2007), TR α 1 was the major TR isoform detected in these three cell lines. GAPDH was used as loading control in Fig. 2A and B.

To assess whether TR β 1PV and TR α 1PV differentially affected the adipogenesis in 3T3-L1 cells, we compared T₃-enhanced adipogenesis in control cells with that in L1- β 1PV and L1- α 1PV cells (Fig. 3). T₃ induced an increased adipogenesis in control cells, as indicated by an increased accumulation of lipid droplets stained with Oil Red O (compare panel 1 with panel 4, Fig. 3A). This T₃-induced adipogenesis was reduced in L1- β 1PV cells (compare panel 5 with panel 4, Fig. 3A) and L1- α 1PV (compare panel 6 with panel 4, Fig. 3A). The Oil Red O staining intensities were measured, and the quantitative data in Fig. 3B indicate that T₃ induced a 2.5-fold increase in adipogenesis in control cells (bar 2 versus bar 1, Fig. 3B). Importantly, Fig. 3B shows that TR α 1PV was more potent (94% reduction) than TR β 1PV (54% reduction) in the inhibition of the T₃-induced adipogenesis (compare bar 6 with bar 2 and bar 4 with bar 2; Fig. 3B). The reduction of T₃-induced adipogenesis in L1- β 1PV and L1- α 1PV cells could also be visualized by phase contrast microscopy (Fig. 3C). The finding that TR α 1PV was a stronger inhibitor (compare panel f with panel d, Fig. 3C) than TR β 1PV (compare panel e with panel d, Fig. 3C) was also apparent by phase contrast microscopy in that fewer lipid-containing cells (cells with dark intense color) were observed in panel f than in panel e (Fig. 3C). These results are consistent with the findings *in vivo* that impaired adipogenesis in WAT was detected in *Tr α 1PV* mice, but not in *Tr β 1PV* mice (Ying *et al.* 2007, Araki *et al.* 2009).

Repression of key regulators of adipogenesis by TR mutants is isoform dependent

PPAR γ is also a ligand-dependent transcription factor and a member of the nuclear receptor superfamily. Studies have shown that PPAR γ is both necessary and sufficient for adipogenesis, and it is therefore known as a 'master regulator' of adipogenesis (Rosen & MacDougald 2006). To understand the underlying mechanisms by which the two mutant TR isoforms differentially impair adipogenesis in 3T3-L1 cells, we first examined the effect of T₃ on the expression of PPAR γ at the protein level before and after induction

Figure 3 T₃-induced adipogenesis in 3T3-L1 cells is differentially impaired by TR mutant isoforms. Control 3T3-L1, L1-β1PV, and L1-α1PV cells were induced to undergo adipogenesis in the absence (panels 1, 2, and 3) or presence of T₃ (2 nM, panels 4, 5, and 6) as described in Materials and methods. After 7 days, matured adipocytes with lipid droplets were visualized by staining with Oil Red O (A) and by phase contrast microscopy (C). (B) Quantification of relative Oil Red O staining intensities of lipid droplets in matured adipocytes. Data are expressed as mean $\pm$ S.E.M. ($n=6$). The P values are indicated.

of adipogenesis (Fig. 4A). Before induction (day 0), very low PPAR γ protein was observed under experimental conditions in control, L1-β1PV, or L1-α1PV cells whether T₃ was present or not (lanes 1, 2, 5, 6, 9, and 10, Fig. 4A). After induction, both PPAR γ 1 and PPAR γ 2 were induced in control cells (lanes 3 and 4, Fig. 4A). Importantly, their protein abundance was further increased by T₃ ($\sim$ 2-fold; lane 4). The expression of PPAR γ 1 and PPAR γ 2 proteins, however, was strongly decreased in L1-β1PV (lanes 7–8, Fig. 4A) and L1-α1PV cells (lanes 11–12, Fig. 4A). The protein abundance of PPAR γ 1 and PPAR γ 2 in L1-α1PV cells was lower than that in L1-β1PV cells (compare lanes 11–12 with lanes 7–8), indicating that TRα1PV inhibited the expression of PPAR γ 1 and PPAR γ 2 at the protein level more than TRβ1PV.

C/EBP α is the founding member of a family of basic region/leucine zipper (bZIP) transcription factors. It is required for the differentiation of 3T3-L1 preadipocytes; inhibition of C/EBP α blocks adipocyte differentiation, whereas overexpression of this C/EBP family member triggers adipocyte differentiation (Darlington *et al.* 1998, Obregon 2008). C/EBP α acts primarily to keep PPAR γ levels elevated in the cell. Therefore, we

also assessed the effect of T₃ on the expression of C/EBP α at the protein level before and after the induction of adipogenesis (Fig. 4B). Before induction (day 0), the C/EBP α protein level was very low in control, L1-β1PV, or L1-α1PV cells (lanes 1, 2, 5, 6, 9, and 10, Fig. 4B). After induction, C/EBP α was induced in control cells (lanes 3 and 4, Fig. 4B). Its protein level was further increased by T₃ ($\sim$ 2-fold, lane 4, Fig. 4B). In L1-β1PV and L1-α1PV cells, markedly decreased signals were observed (lanes 7–8 and 11–12, Fig. 4B), indicating that mutations of TRs interfere with the expression of C/EBP α . However, a lower expression of C/EBP α protein was observed in L1-α1PV cells than in L1-β1PV cells (compare lanes 11–12 with lanes 7–8, Fig. 4B). Panel C shows the corresponding loading control using GAPDH. Taken together, these results demonstrate that T₃ regulates the expression of PPAR γ and C/EBP α , which are the two master key regulators of adipogenesis.

We further determined the mRNA levels of *Ppar γ* and *C/ebp α* to ascertain whether the expression of these two key regulators is regulated by T₃ and affected by TR mutants at the transcriptional level. Indeed, the expression of *Ppar γ* and *C/ebp α* mRNAs was lower

Figure 4 Abundance of PPAR γ and C/EBP α proteins in 3T3-L1, L1-β1PV, and L1-α1PV cells. Western blot analysis was carried out using nuclear extracts from control 3T3-L1, L1-β1PV, and L1-α1PV cells before induction of adipogenesis (day 0: lanes 1, 2, 5, 6, 9, and 10) and after induction of adipogenesis (day 7: lanes 3, 4, 7, 8, 11, and 12) in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence of T₃ (2 nM; lanes 2, 4, 6, 8, 10, and 12). Western blot analysis was performed using the antibodies against PPAR γ 1/PPAR γ 2 (A), C/EBP α (B), and GAPDH (C). GAPDH was used as a loading control.

(~25–30% reduction) in the absence of T₃ than in the presence of T₃ (lane 2; Fig. 5A and B respectively). Compared with control, the expression of *Pparγ* mRNA was 45% lower in L1-β1PV cells and 75% lower in L1-α1PV cells (bars 3 and 5 respectively, Fig. 5A). Consistently, the mRNA level of *C/ebpα* was 40 and 80% lower in L1-β1PV and L1-α1PV cells respectively compared with the control cells (bars 3 and 5 respectively, Fig. 5B). It is interesting to note that the expression of these two genes was less repressed at the mRNA levels than at the protein level by TR mutants (see Fig. 4). Such discordance could reflect additional post-translational regulation of PPAR γ and C/EBP α . In L1-β1PV and L1-α1PV cells, T₃ had no significant effect on the extent of PV mutant-mediated repression of *Pparγ* and *C/ebpα* mRNAs (Fig. 5A and B respectively). The functional consequences of the decreased expression of *Pparγ* were evident in that its direct target genes – the lipoprotein lipase (*Lpl*) and *aP2*, involved in the lipid synthesis – were concordantly repressed, with a higher extent of repression in L1-α1PV cells than in L1-β1PV cells (compare bars 3 and 5 to bar 1; Fig. 5C and D).

Differential occupancy of mutant TR isoforms on the *C/ebpα* gene promoter

To further understand how TR mutant isoforms differentially impaired adipogenesis, we focused on the study of the *C/ebpα* gene, an essential transcription

factor in the induction of adipogenesis in 3T3-L1 cells (Darlington *et al.* 1998, Obregon 2008). The *C/ebpα* gene is a direct TR target gene with several TREs identified in the promoter of the *C/ebpα* gene (Menendez-Hurtado *et al.* 2000). The finding that TR mutant isoforms differentially repressed the expression of *C/ebpα* (Fig. 5A) prompted us to test the hypothesis that differential recruitment of mutant isoforms to the promoter could underlie differential impairment of adipogenesis by TR mutant isoforms. We, therefore, used ChIP to address this question (Fig. 6). In control cells, when antibodies recognizing specific TR isoforms were used, TRα1 and TRβ1 were found to be recruited to TREs on the promoter of the *C/ebpα* gene (lanes 5 and 6, Fig. 6). When the monoclonal antibody recognizing specifically the PV mutated sequences (#302) was used, only background noise (lane 7) similar to that observed in negative controls (lane 2, no antibody; lane 3, an irrelevant IgG) was seen. In contrast, in L1-β1PV cells, a strong signal was detected when anti-PV antibody was used, indicating the recruitment of β1PV to the TREs on the promoter of the *C/ebpα* gene (lane 14). A concomitant decreased recruitment of TRα1 (compare lane 12: 4.5 ± 0.01 units and lane 5: 5.06 ± 0.18 units) and TRβ1 (compare lane 13: 1.64 ± 0.07 units and lane 6: 2.04 ± 0.14 units) was

Figure 5 Differentially repressed expression of key regulators at the mRNA level in adipogenesis in L1-β1PV and L1-α1PV cells. Total RNA was extracted from control 3T3-L1, L1-β1PV, and L1-α1PV cells after adipogenesis in the presence (closed bars) or absence of T₃ (open bars). Total RNA (10–50 ng) was used for qPCR analyses as described in Materials and methods. The key regulators are indicated. The data are expressed as mean $\pm$ S.E.M. (n=6). **P*<0.05 and ***P*<0.01.

Figure 6 Differential recruitment of TR β 1PV and TR α 1PV to the promoter of the *C/ebp α* gene as determined by ChIP analysis. ChIP assays were performed using control 3T3-L1 (lanes 1–7), L1- β 1PV (lanes 8–14), and L1- α 1PV (lanes 15–21) cells after T₃-induced adipogenesis on day 9. Antibodies used in the chromatin immunoprecipitation were anti-Ac-H3 antibody as a positive control (lanes 4, 11, and 18), anti-TR α 1 antibody (lanes 5, 12, and 19), anti-TR β 1 antibody (lanes 6, 13, and 20), and anti-PV antibody (lanes 7, 14, and 21). The negative controls were no antibody (lanes 2, 9, and 16) as well as an irrelevant IgG (lanes 3, 10, and 17). The chromatin immunoprecipitated and recovered DNA was used as a template for PCR amplification of the receptor-binding region in the promoter of the *C/ebp α* gene. Two percent of the chromatin solution (20 μ l) was used for input DNA as a control. Three separate experiments were performed, and the representative results are shown.

observed, suggesting competition by TR β 1PV with TR α 1 and TR β 1 for TREs on the promoter of the *C/ebp α* gene. The finding that TR α 1PV also competed for wild-type TRs for binding to TREs was also demonstrated in L1- α 1PV cells. A strong signal was detected when anti-PV antibody was used (lane 21: 4.94 ± 0.07 units) with concomitant decreased intensities when anti-TR antibodies were used for TR α 1 (compare lane 19: 1.93 ± 0.05 units and lane 5: 5.06 ± 0.18 units). Furthermore, it is important to point out that the signal intensity detected by anti-PV antibodies in L1- α 1PV cells was stronger than that in L1- β 1PV cells (compare lane 21: 4.94 ± 0.07 units and lane 14: 4.06 ± 0.17 units), indicating a more avid recruitment of TR α 1PV to the promoter of the *C/ebp α* gene. These results suggest that a higher occupancy by TR α 1PV than by TR β 1PV on the promoter of the *C/ebp α* gene, which, in part, could underlie a more severe impairment of adipogenesis by TR α 1 mutations.

Discussion

It has long been known that T₃ plays a critical role in lipid metabolism by regulating genes involved in lipogenesis and lipolysis. The underlying mechanisms, however, have only begun to be unraveled in recent years. Genetically engineered mice are valuable tools to gain insights into the role of TRs in lipid metabolism and energy balance. Mice deficient in TR α 1 exhibit lower body temperature (Wikstrom *et al.* 1998), indicative of the importance of TR α 1 in thermogenesis. Mice deficient in TR β display abnormalities in

cholesterol metabolism (Gullberg *et al.* 2002), demonstrating that TR β plays a major role in cholesterol homeostasis. Significant advances in the understanding of the roles of TR α 1 in adipogenesis came from the mice with knock-in mutations in the TR α gene. Mice that are heterozygous for a TR α 1P398H mutation are obese with increased visceral adiposity and hepatic steatosis (Liu *et al.* 2003). In adipose tissue, the norepinephrine-mediated lipolysis in WAT is blocked, and hormone-sensitive lipase in adipose tissue is decreased. In contrast to the TR α 1P398H mutant mice, mice with a TR α 1R384C mutation are lean and hypermetabolic with reduction in fat depots (Sjogren *et al.* 2007). Similar to the lean phenotype exhibited by TR α 1R384C mutant mice, TR α 1PV mice are dwarfs with reduced WAT mass across a variety of depots (Ying *et al.* 2007). TR α 1PV acts to transcriptionally repress the expression of the *Ppar γ* gene, resulting in impaired adipogenesis. Intriguingly, while TR β is expressed in WAT (Ying *et al.* 2007), no apparent reduction in WAT mass was evident in TR β 1PV mice (Araki *et al.* 2009), indicating distinct phenotypic expression in the adipogenesis in WAT between these two mutant mice. In the present study, the creation of stable 3T3-L1 cell lines expressing a similar level of TR α 1PV or TR β 1PV proteins allowed the dissecting of the differential role of TR isoforms in adipogenesis. Indeed, TR α 1PV was a stronger repressor than TR β 1PV in blocking T₃-mediated adipogenesis in 3T3-L1 cells by inhibiting the expression of two master regulators, PPAR γ and C/EBP α . The stronger repression by TR α 1PV was due, in part, to a stronger recruitment of TR α 1PV than of TR β 1PV to the promoter of the *C/ebp α* gene (Fig. 6). As a result, the expression of the *C/ebp α* gene was repressed, leading to more impaired adipogenesis in L1- α 1PV cells than in L1- β 1PV cells (Fig. 3). Thus, the present study has provided direct evidence to link the mutations of the TR α gene to the impaired adipogenesis in WAT.

Currently, it is not clear how TR α 1PV was preferentially recruited to the promoter complex of the *C/ebp α* gene. TR α 1PV and TR β 1PV differ mainly in the amino-terminal A/B domain (see Fig. 1). Thus, it is reasonable to speculate that the amino-terminal A/B domain of TR α 1PV could affect the folding of the tertiary structure such that TR α 1PV could bind to TREs stronger than TR β 1PV on the promoter of the *C/ebp α* gene. Alternatively, the amino-terminal A/B domain could facilitate the formation of a favorable conformation for TR α 1PV to interact more favorably with other coregulators (e.g. corepressors) to stabilize the TR α 1PV–DNA complex on the *C/ebp α* gene promoter. These two possibilities are not mutually exclusive and could, in fact, act in concordance to strengthen the interaction of TR α 1PV with the promoter to block the expression of the *C/ebp α* gene, thereby impairing adipogenesis in 3T3-L1 cells.

The finding that TR α 1PV had a more deleterious effect than TR β 1PV on the adipogenesis in 3T3-L1 cells provided new insights to understand the observation that WAT mass is reduced in *Tr α 1PV* mice, but not in *Tr β 1PV* mice compared with the wild-type mice (Araki *et al.* 2009). *Tr α 1* is a major TR isoform in WAT; therefore, mutation of a major TR isoform (i.e. *Tr α 1*) results in the loss of function critical for adipogenesis. In *Tr β 1PV* mice, mutation of a minor TR isoform (i.e. *Tr β 1*) cannot completely block the effect of TR α 1, the major TR in WAT. Furthermore, TR β 1PV, as shown in the present study, was not as strong as TR α 1 in interacting with TRE on the promoter of the *C/ebp α* gene promoter, thereby making TR β 1PV less effective in blocking adipogenesis in WAT in *Tr β 1PV* mice. However, besides the preferential interaction of TR α 1PV with the promoter of the *C/ebp α* gene, other mechanisms, yet to be uncovered, could also contribute to the more severe impairment in adipogenesis by TR α 1PV.

As obesity and atherosclerosis have become major health concerns in Western countries, there is an increasing need to develop novel treatment strategies. Thyroid hormone mimetics that have specificity toward TR α 1 or TR β 1 are being developed to derive benefits in treating metabolic diseases but without undesirable effects on other target organs (Baxter & Webb 2009). The finding that TR α 1PV was more effective than TR β 1PV in blocking adipogenesis suggests that TR α 1 could be considered as a potential therapeutic target for decreasing adipose tissue and reducing serum fatty acids. Moreover, 3T3-L1 cells stably expressing *Tr α 1PV* or *Tr β 1PV* could be used as model cell lines to further elucidate the role of T₃ via TR in adipogenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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