PPAR Research

The Molecular Basis of PPAR Function

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Contents

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Editorial

The Molecular Basis of PPAR Function

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Over the past four and a half years, fifteen issues of PPAR Research have been published and an additional five are currently in various phases of production. Collectively, these issues have covered a large array of physiological functions of PPARs in health and disease, including their functions in diverse cell and tissue types, their roles in a host of clinical conditions, and their rich pharmaceutical potential. Yet, one crucial overarching selection seems to have escaped attention amid this exciting variety—the fundamental molecular mechanisms that underlie PPAR action. PPARs are first and foremost transcription factors that execute intricate regulation of gene networks, and are in turn subject to complex fine-tuning and control. Inevitably, thorough understanding of their cellular and organismal functions and full capitalization on their pharmaceutical power require detailed understanding of these molecular underpinnings.

While the list of potential subplots is far too long to fit into a single issue, the current issue has begun to fill this coverage gap. Three review articles and one research manuscript tackle the subject of PPAR target genes. First is a comprehensive review of PPARα target genes by Rakhshandehroo et al. Although the primary focus of their review is hepatic targets, many of the discussed features are applicable to the physiology of PPARα in other tissues. Next, Bugge and Mandrup review two complementary aspects of PPARγ-regulated genes. The first concerns the role of the N-terminal ligand-independent activation domain, also known as the A/B or AF-1 domain, in determining PPAR subtype specificity and in modulating target activation in adipocytes. The second focuses on recent insights into genomewide PPARγ actions in adipocytes and macrophages gained from chromatin immunoprecipitation studies. In the third review, Costa et al. discuss population-wide sequence variations of PPARγ and their potential impact on target gene expression, and review the application of massive parallel sequencing for the identification of PPARγ cistromes (the collection of genomic sequences bound by PPARγ) and transcriptomes. In the second section of this issue, the research article by Le Bouter et al. analyzes gene expression changes in adipose tissue, liver, and muscle of leptin receptor deficient diabetic obese mice treated with the PPARγ agonist and insulin sensitizer rosiglitazone, in order to establish the molecular basis of its therapeutic physiological effects.

Two papers by Viswakarma et al. and Pyper et al., one a review and the other a research manuscript, concern transcription cofactors of PPARs. Their thorough review summarizes no less than twelve types of PPAR coactivators, which, ironically, do not account for the fullest gamut of known PPAR coactivators. Nothing is better to illustrate the complexity of this ever-growing subplot than this group's accompanying research article, which describes the identification and initial characterization of yet a new PPAR coactivator, a subunit of the mediator complex termed PRIC295.

In the course of executing their physiological roles, PPARs are both regulated by and intersect with diverse signaling pathways. Three review articles touch on three of these many types of interactions. Charoensuksai and Xu review the effects of circadian signaling on the expression and activities of PPARs, Moreno et al. summarize the interactions between nutrient signaling and PPARs, whereas Takada et al. expand on the interaction of PPARγ with downstream effectors of cytokine and Wnt signaling in mesenchymal stem cells.

While this volume is a solid start, it did not go the full distance and many of the molecular bases of PPAR function remain to be reviewed. Some topics, such as the structure-function relationships of PPARs or their posttranslational regulation won no coverage in this issue. Among the touched upon topics, some additional reviews are crucial for rounding out the coverage, such as target genes of PPARβ/δ or additional signaling crosstalk of PPARs. And so, it would be great to see a fresh team of guest editors pick the topic up where we left off and cover the next expanse of this literature gap.

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Research Article

Peroxisome Proliferator-Activated Receptor Alpha Target Genes

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The peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated transcription factor involved in the regulation of a variety of processes, ranging from inflammation and immunity to nutrient metabolism and energy homeostasis. PPARα serves as a molecular target for hypolipidemic fibrates drugs which bind the receptor with high affinity. Furthermore, PPARα binds and is activated by numerous fatty acids and fatty acid-derived compounds. PPARα governs biological processes by altering the expression of a large number of target genes. Accordingly, the specific role of PPARα is directly related to the biological function of its target genes. Here, we present an overview of the involvement of PPARα in lipid metabolism and other pathways through a detailed analysis of the different known or putative PPARα target genes. The emphasis is on gene regulation by PPARα in liver although many of the results likely apply to other organs and tissues as well.

1. Introduction

Nutrient metabolism and energy homeostasis are tightly controlled by numerous regulatory systems involving specific transcription factors. The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors and play an important role in nutrient homeostasis [1–3]. Three different PPAR subtypes are known: PPARα, PPARβ/δ, and PPARγ. All PPARs share the same molecular mode of action via formation of heterodimers with the nuclear receptor RXR, followed by binding to specific DNA-response elements in target genes known as peroxisome proliferator response elements (PPREs). PPREs are characterized by a common core sequence consisting of a direct repeat of the consensus sequence AGGTCA interspaced by a single nucleotide [1, 4]. Expression of PPARα and PPARβ/δ is found ubiquitously, whereas PPARγ is mainly expressed in adipose tissue, macrophages, and colon [5, 6]. Activation of transcription by PPARs is dependent on a number of different steps including ligand binding to PPAR, binding of PPAR to the target gene, removal of corepressors and recruitment of coactivators, remodeling of the chromatin structure, and finally facilitation of gene transcription [7]. This paper will focus exclusively on PPARα.

PPARα was first discovered in the early 1990s and since then has been identified as the master regulator of hepatic lipid metabolism [8]. In addition, PPARα has been shown to govern glucose metabolism, lipoprotein metabolism, liver inflammation, amino acid metabolism, and hepatocyte proliferation (specifically in rodents). Synthetic agonists of PPARα lower plasma triglycerides and raise plasma high-density lipoprotein (HDL) levels and are thus used clinically in the treatment of dyslipidemia [2, 9–11].

In recent years, the advent of microarray technology has allowed the study of whole genome expression profiles. Accordingly, a wealth of new information has become available about the role of specific transcription factors in regulation of gene expression. Combined with data collected using more established methods, microarray has permitted the generation of a comprehensive picture of the impact of PPARα on gene expression, thereby providing key insight into the functional role of PPARα. The present paper is aimed at providing a detailed and updated overview of PPARα

target genes in different biological processes and to highlight possible differences between mouse and human.

Although the presence of a functional PPRE is often used as a criteria for designating direct PPARα target genes, we did not apply this criteria very stringently in our analysis as the in vivo functionality of most of the identified PPREs remains uncertain. Recent studies indicate that the standard approach to screen for PPREs in the 1-2 kb region upstream of the transcriptional start site (TSS) is at odds with accumulating evidence that PPARs often bind quite distant from the TSS [12–14]. In those cases, contact with the basal transcription machinery is expected to be established via DNA looping. Thus, the absence of a PPRE in the 1-2 kb region upstream of the TSS cannot be used as a criterion to disqualify target genes. Other aspects that need to be taken into account include correspondence in gene function with better established PPAR targets and the timing of gene induction following PPARα activation.

2. PPARα Tissue Expression Profile in Mouse and Human

High expression levels of PPARα expression are found in liver and specifically in the parenchymal cell population. Expression of PPARα in nonparenchymal liver cells such as Kupffer cells is much lower [15, 16]. Other tissues with high PPARα mRNA levels are heart, kidney, intestine, and brown adipose tissue, all of which are characterized by an elevated rate of fatty acid catabolism [17]. PPARα expression has also been detected in immune cells such as the peripheral blood mononuclear cell population, and specifically in T-cells and macrophages [18–22]. Evidence suggests that mice and humans share similar PPARα tissue expression profiles [6, 17] (Figure 1). In the past, the importance of PPARα in human liver was questioned based on data showing approximately 10-fold lower PPARα mRNA levels in human liver compared with mouse liver [23]. A recent study using more advanced methodology revealed similar PPARα expression in mouse and human liver and in mouse and human hepatocytes, thus strongly arguing against this notion [24]. Given that PPARα has been most extensively studied in liver, most of the information on PPARα target genes presented here relates to hepatic gene regulation.

3. PPARα Structure in Mouse and Human

Analogous to other nuclear receptor superfamily members, PPARα has a domain structure consisting of an N-terminal activating function-1 (AF-1) domain, a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) [25, 26]. The N-terminal domain can be phosphorylated leading to changes in transcriptional activity and even ligand binding of the receptor [27]. The DBD is responsible for physical interaction with DNA and allows PPARα to bind to specific PPREs as a heterodimer with RXR [28]. The LBD harbors the ligand-binding pocket crucial for dimerization with RXR and contains the activating function-2 involved in physical interactions with coregulatory proteins [7, 29, 30]. Comparison of human and murine PPARα shows 85% identity at the nucleotide level and 91% identity at the amino acid level. Data have indicated that there is some genetic heterogeneity in the functional coding sequence of human PPARα that translates into functional differences in receptor activity. One identified variant of the human PPARα gene produces a protein that is mutated within the PPARα DNA-binding domain. This L162V gene variant exhibits greater ligand-induced activity compared to the wild-type receptor [31, 32]. While there is some evidence for a link between the L162V polymorphism and metabolic parameters such as plasma lipid levels, these correlations are not always found [32–37]. Interestingly, the effect of L162V polymorphism has been suggested to be modulated via gene-drug and gene-nutrient interactions [38–40]. The V227A polymorphism was found in Japanese population and has been associated with altered serum lipid levels and nonalcoholic fatty liver disease [41–44]. In addition to polymorphic variants, a truncated splice variant of human PPARα has been described that negatively interferes with wild-type PPARα activity [45].

4. PPARα Ligands

PPARα serves as receptor for a structurally diverse set of compounds. The most important class of synthetic PPARα ligands is the fibrates, including gemfibrozil, bezafibrate, clofibrate, fenofibrate, and Wy14643 [2, 9–11, 46]. This class of drugs is used in the treatment of dyslipidemia primarily associated with type 2 diabetes mellitus. In addition, PPARα is activated by plasticizers, insecticides, and other rodent hepatic carcinogens. Natural ligands of PPARα include a variety of fatty acids as well as numerous fatty acid derivatives and compounds showing structural resemblance to fatty acids, including acyl-CoAs, oxidized fatty acids, eicosanoids, endocannabinoids, and phytanic acid [47–53]. Endogenous ligand activation of PPARα in liver was initially suggested to occur primarily during fasting as large amounts of free fatty acids are released into the bloodstream and enter the liver [54, 55]. However, compelling evidence indicates that hepatic PPARα is not activated by plasma free fatty acids, whereas it can be activated by dietary fatty acids and fatty acids generated via de novo lipogenesis [56–60]. Recently, it was shown that the effects of dietary unsaturated fatty acids on hepatic gene expression are almost exclusively mediated by PPARα and mimic the effect of synthetic PPARα agonists [61].

5. PPARα and Hepatic Lipid Metabolism

Regulation of lipid metabolism is mainly coordinated by liver, which actively metabolizes fatty acids as fuel and continuously produces very low-density lipoproteins (VLDLs) particles to provide a constant supply of fatty acids to peripheral tissues. Disturbances in these pathways are the basis for hepatic steatosis and alterations in plasma lipoprotein levels. Many aspects of hepatic lipid metabolism are under control of PPARα, including fatty acid uptake through membranes, fatty acid activation, intracellular fatty acid trafficking, fatty

Table 1: *List of PPARα target genes in different biological processes in liver.* Genes regulated by *PPARα* in mouse are shown in black. Genes regulated in human and mouse are shown in red. Genes regulated only in human are shown in bold font, and genes with detected functional PPRE are shown in italic font.

Lipid metabolism	Lipid/hormone transport	Adipor2 [24, 62], Cd36 [24, 62–64], **LEPR** [24, 62], *Slc27a1* [62, 64–67], **SLC27A2** [24, 62, 63, 68], **SLC27A4** [24, 62]
	Acyl-CoA formation/hydrolysis/binding	Acot1 [24, 62, 69], Acot7 [62], **ACOT12** [24, 62], ***ACSL1*** [24, 62, 70–72], **ACSL3** [24, 62], Acsl4 [24, 62, 63, 73], **ACSL5** [24, 62, 63], **ACSM3** [24, 62], Acss2 [62], ***FABP1*** [24, 62, 74, 75], Fabp2 [24, 62, 63], **FABP3** [24, 62], Fabp4 [62, 68], Fabp5 [62]
	Mitochondrial β-oxidation/oxidative phosphorylation	**ACAA2** [24, 62, 68], Acadl [24, 62, 70, 74, 76], ***ACADM*** [24, 55, 62, 70], **ACADS** [24, 54, 62, 70], **ACADVL** [24, 62, 63, 70], Acad8 [62], Acad9 [62], Acad10 [62], Acot2 [24, 62, 69], Acot9 [62], Acot10 [62], ***CPT1A*** [24, 54, 55, 62, 77–79], Cpt1b [24, 62], ***CPT2*** [24, 62, 70, 80], Crat [24, 62, 74], Dci [24, 62, 65, 68, 76], Decr1 [24, 62, 76, 81], ETFA [24], Etfb [24, 62], **ETFDH** [24, 62], **HADHA** [24, 62, 63, 68, 82], **HADHB** [24, 62, 63, 68], *Hadh* [24, 62, 68, 74, 76, 83], Hadh2 [62], Hibch [24, 62], ***SLC25A20*** [24, 62, 84], **SLC22A5** [24, 62, 85, 86], **TXNIP** [24, 62], Ucp2 [24, 62, 87–89], Ucp3 [62]
	Ketogenesis/ketolysis	Acat1 [24, 62, 68], Bdh [62], ***FGF21*** [24, 62, 90–92], Hmgcl [62], ***HMGCS2*** [24, 62, 93–95]
	Peroxisomal β-oxidation	**ABCD2** [24, 62, 96], **ABCD3** [24, 62, 96], **ACAA1A** [24, 62, 68, 76], Acaa1b [24, 62], Acot3 [24, 62], Acot4 [24, 62], Acot5 [24, 62], Acot8 [62, 97], ***ACOX1*** [24, 55, 62, 68, 70, 98, 99], Crot [24, 62, 74], Decr2 [24, 62, 68, 100] **ECH1** [24, 62, 63, 68], *Ehhadh* [24, 62, 101, 102], HACL1 [24], **HSD17B4** [24, 62, 103, 104], Peci [24, 62, 65, 100], Pex11a [24]
	Microsomal (ω-hydroxylation)	ALDH3A1 [24], *Aldh3a2* [24, 62, 105], **ALDH9A1** [24], *Cyp4a1* [74, 106–109], Cyp4a3 [55, 74, 108], ***Cyp4a10*** [24, 62, 68, 84, 106, 110], Cyp4a12a [24, 62, 65], Cyp4a14 [24, 62, 68, 110, 111], Cyp4f15 [24], Cyp4x1 [24]
	Lipogenesis	Acaca [62], **ACACB** [62], **AGPAT2** [62], Agpat3 [24, 62], Agpat5 [62], Agpat6 [62], Dgat1 [62, 112], ELOVL5 [62, 113, 114], **ELOVL6** [24, 62, 113, 114], Elovl7 [62], **FADS1** [24, 62, 113], *Fads2* [62, 113, 115], Fasn [62, 112], **GPAM** [24, 62, 65], *Hsd17b12* [62], *Lpin2* [24, 56, 62], **MLYCD** [24, 62], Mogat1 [24, 62], ***MOD1*** [24, 62, 70, 116], *Scd1* [24, 62, 117, 118], Scd2 [62, 68], Slc25a10 [62, 100], Srebf1 [24, 62, 119]
	Lipases/lipid droplet proteins	***ADFP*** [24, 62, 120], Ces1 [62], Ces3 [24, 62], Cidea [24, 62, 121], **CIDEC** [24, 62, 121], *Gos2* [24, 62, 122, 123], Lipa [24, 62], Lipe [24, 62, 68], Mgll [24, 62, 63, 68], Oxpat/Lsdp5 [24, 62, 124, 125], Plin1 [24], **PNPLA2** [24, 62], **S3-12** [24, 62]
	Lipoprotein uptake and metabolism	***ANGPTL4*** [24, 62, 126, 127], **APOA1** [128–133], *APOA2* [24, 134], *APOA5* [24, 135, 136], **APOCIII** [137–139], **LIPC** [24, 62], Lipg [62], *Lpl* [62, 64, 65, 140], Lrp4 [24, 62], **PCTP** [24, 62], *Pltp* [62, 65, 141, 142], Mttp [24, 62, 143], **VLDLR** [24, 62]
	Cholesterol/Bile transport and metabolism	**ABCA1** [24, 62, 144], **ABCB4** [24, 62, 94, 144], Abcb11 [62], Abcg5 [62, 144], Abcg8 [62, 144], Cav1 [24], ***CYP7A1*** [24, 62, 145–147], *Cyp8b1* [62, 148], Cyp27a1 [145], FXR [62], *LXR* [144, 149], Npc1 [62], Rab9 [24, 62], Scarb2 [62], Slc10a1 [94], *Slc10a2* [62, 150]
Other pathways	Glucose/Glycerol transport and metabolism	**AQP3** [24, 62, 151], Aqp7 [62], Aqp9 [62, 151], Fbp2 [24, 62], **G6PC** [24], *Gpd1* [24, 62, 151], Gpd2 [62, 151], **GYK** [24, 62, 151], Gys-2 [152], Ldha [62], Pcx [62], PCK1 [24], Pdk1 [24], **PDK4** [24, 62, 153, 154]
	Biotransformation	**AKR1B10** [24], AKR1C3 [24], CYP1A2 [24], Cyp2a5 [110], CYP2B6 [24], CYP2C8 [24], CYP2C9 [24], Cyp2c11 [155], Cyp2c12 [155], Cyp2c29 [110], **CYP2J2** [24], CYP3A5 [24], **CYP3A7** [24], **CYP3A11** [24, 110], Cyp3a43 [24], **EPHX2** [24, 156], Gsta3 [157], MGST3 [24], ***UGT1A9*** [158]
	Amino Acid metabolism	**ABAT** [24, 159], Acmsd [159], **AGXT2** [24, 160], Arg1 [160], **ASL** [24, 160], Ass1 [160], **CBS** [24, 159], **CPS-1** [24, 160], Cth [159], Got1 [160], Got2 [62, 64, 160, 161], Gls [160], **GLS2** [159], **GPT** [24, 159], Hal [159], Hpd [159], **OAT** [24, 159], **ODC1** [24, 62, 159], **OTC** [24, 62, 160], **PAH** [24, 159], **PSAT1** [24, 62, 159], Tat [159, 162]
	Inflammation	Apcs [163], Birc3 [163], Cebpb [164], **Cd68** [24, 165], Crp [24, 163], Cxcl10/IP10 [165], **FGB** [24, 155, 166, 167], Emr1 (F4/80) [156], Icam-1 [24, 165], Ifi47 [24, 163], Igtp [163], Nfkbia [168–170], Il-1β [165], Il-1r1 [111], Il1rn [163], Il1rap [163], Il-6 [111, 171], Il-6ra [111, 163], Il18 [163], Lcn2 [163, 165], Lifr [163], Ccl2 [165], Ccl3 [165], Mt1 [24, 163], Mt2 [163, 165], Orm2 [163], Orm3 [24, 163], Nfkb1 [24, 164], Pla1a [24, 163], Saa2 [163], Saa4 [24, 163], Stat1 [165], Stat2 [163], Stat3 [163], Steap4 [24, 163], Stress induced protein [163], Tnfα [165], Traf2 [163], Vcam-1 [24, 165]

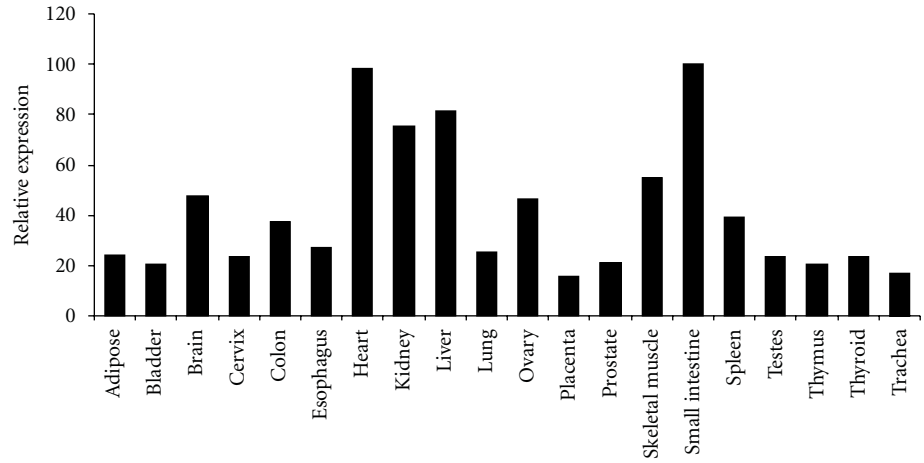

Figure 1: *Expression profile of PPARα in human tissues.* The FirstChoice Human Total RNA Survey Panel (Ambion) was reverse transcribed and used for qPCR using primers specific for human PPARα. Expression levels are expressed relative to small intestine, which showed the highest expression level (100%).

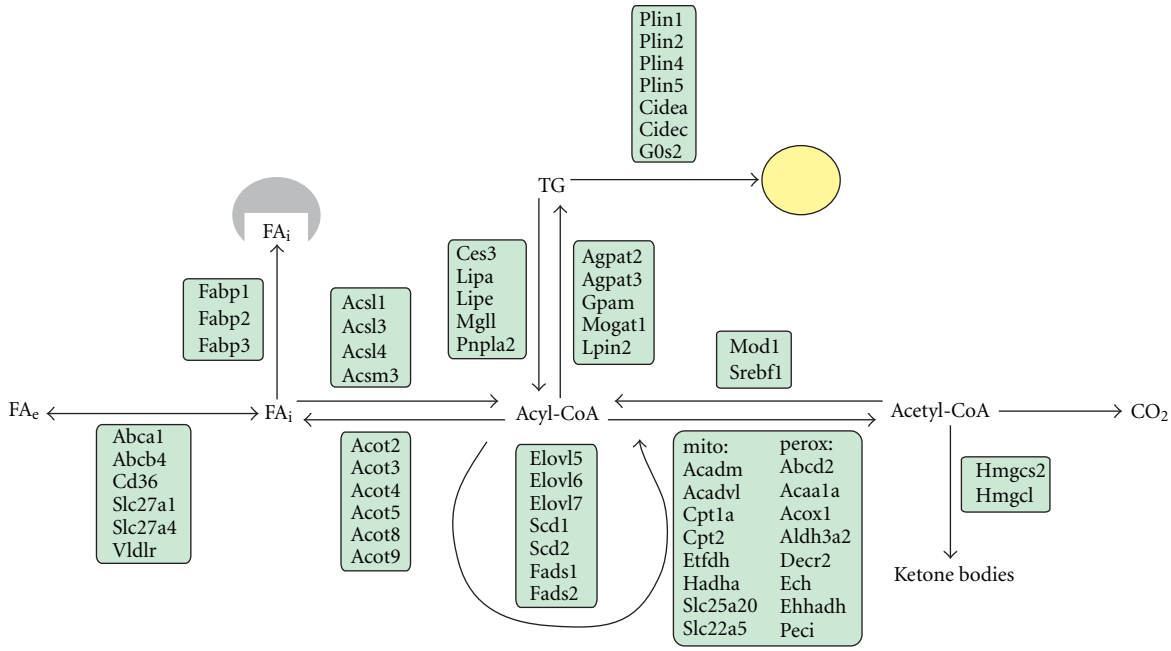

Figure 2: Schematic representation of PPARα target genes in different aspects of hepatic lipid metabolism.

acid oxidation and ketogenesis, and triglyceride storage and lipolysis (Figure 2). It has been suggested that part of the effect of PPARα on hepatic ketogenesis may be mediated by induction of the PPARα target fibroblast growth factor 21 [90–92]. A detailed discussion of the specific genes within the various lipid metabolic pathways that are targeted by PPARα is provided below (Table 1).

5.1. Peroxisomal Fatty Acid β-Oxidation. The first link between PPARα and fatty acid catabolism was established by the identification of the Acyl-CoA oxidase gene, encoding the rate-limiting enzyme in peroxisomal long-chain fatty acid oxidation, as a direct PPARα target gene [98, 172]. Peroxisomes are known to be involved in many aspects of lipid metabolism, including synthesis of bile acids and plasmalogens, synthesis of cholesterol and isoprenoids, alpha-oxidation, glyoxylate and H_2O_2 metabolism, and beta-oxidation of very-long-straight-chain or branched-chain acyl-CoAs. The beta-oxidation of straight-chain acyl-CoAs starts with a reaction catalyzed by acyl-CoA oxidase 1 (Acox1) followed by one of two enzymes carrying both enoyl-CoA-hydratase and 3-hydroxyacyl-CoA dehydrogenase activity (L-bifunctional enzyme, Ehhadh; D-bifunctional enzyme, Hsd17b4) and finally peroxisomal 3-ketoacyl-CoA thiolase (Acaa1a, Acaa1b). All genes mentioned above represent PPARα targets [24, 55, 62, 68, 70, 76, 98, 99, 101–104]. Additionally, genes involved in peroxisomal fatty acid uptake (Abcd2 and Abcd3), conversion of fatty acid

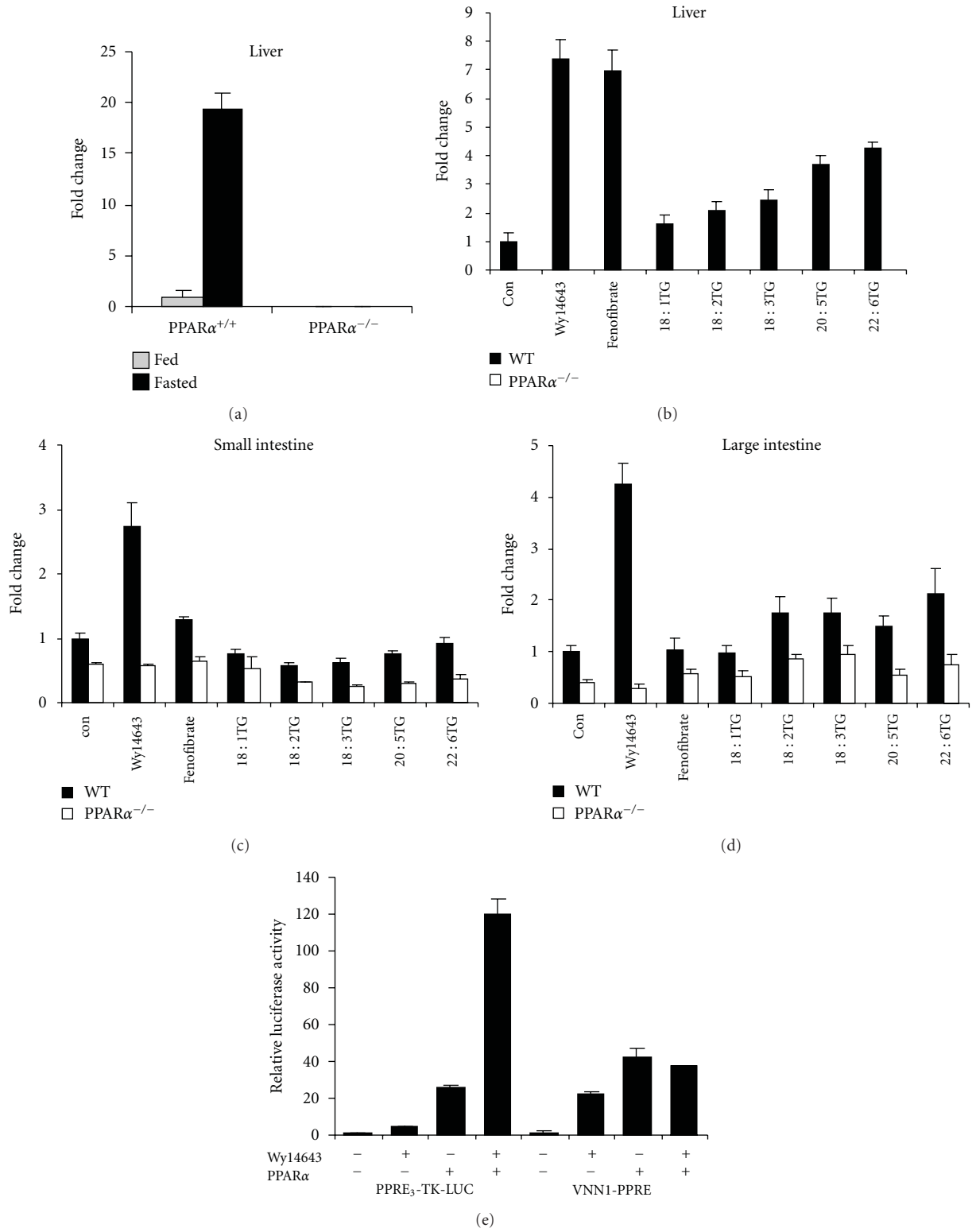

Figure 3: *Vanin-1 likely represents a direct PPARα target gene.* (a) Vnn1 expression in livers of ad libitum fed and 24 h fasted wildtype and PPAR$\alpha^{-/-}$ mice. (b) Vnn1 expression in liver, (c) small intestine, and (d) large intestine of wildtype and PPAR$\alpha^{-/-}$ mice 6 h after administration of a single oral dose of Wy14643 (4 mg), fenofibrate (4 mg), and synthetic triglycerides triolein, trilinolein, trilinolenin, trieicosapentaenoin, or tridocosahexaenoin (400 mL). (e) HepG2 cells were transiently transfected with reporters (PPRE)3-TK-LUC or PPRE-Vnn1-LUC (PPRE present in intron 3-4 of the Vnn1 gene cloned into pGL3-promoter) and PPARα expression plasmid (pSG5). After transfection, cells were treated with WY14643 (50 μM) for 24 hours followed by determination of luciferase and β-galactosidase activities in the cell lysates. Luciferase activities were normalized to β-galactosidase, and the relative luciferase activity of the cells treated with DMSO was set to 1. Error bars represent SEM.

to acyl-CoA (Crot), and numerous thioesterases (Acots) that convert acyl-CoAs back to fatty acids have been reported to be regulated by PPARα [24, 62, 74, 96, 97]. Activation of PPARα using synthetic agonists is known to cause massive proliferation of peroxisomes in rodents via induction of a large set of genes encoding peroxisomal fatty acid oxidation enzymes, as well as genes involved in peroxisomal biogenesis (Pex genes). Chronic exposure to these so-called peroxisome proliferators can also induce liver cancer in rodents [173]. In contrast, activation of PPARα in humans does not seem to induce hepatocellular carcinomas, suggesting a species specific response to PPARα activation. Initially, it was believed that the differential response was due to the lack activation of Acox1 and other peroxisomal genes by PPARα in humans [71, 174, 175]. However, recent data indicate that PPARα is able to induce a significant number of genes involved in peroxisomal fatty acid oxidation in human primary hepatocytes, including Acox1 [24]. Also, PPARα-mediated induction of the Pex11a gene involved in peroxisome proliferation is observed in both species [24].

5.2. Mitochondrial Fatty Acid β-Oxidation. The crucial role of PPARα in mitochondrial fatty acid oxidation is illustrated by the phenotype of fasted PPAR$\alpha^{-/-}$ mice, which exhibit hypoketonemia, hepatic steatosis, and elevated plasma free fatty acid levels [54, 55, 176]. It is now evident that virtually every enzymatic step within the fatty acid oxidative pathway is under control of PPARα. Specifically, PPARα induces genes controlling fatty acid import into the mitochondria (Cpt1, Cpt2, Slc25a20, Slc22a5), as well as the major enzymes within the β-oxidation pathway, including various acyl-CoA dehydrogenases (Acad, step 1), mitochondrial trifunctional enzyme (Hadh, step 2–4), and genes involved in β-oxidation of unsaturated fatty acid (Dci, Decr) [24, 54, 55, 62, 63, 65, 68, 70, 74, 76–86].

Additionally, synthesis of ketone bodies via mitochondrial HMG-CoA synthase (Hmgcs2) and HMG-CoA lyase (Hmgcl) is governed by PPARα [24, 62, 93–95], as is the expression of genes encoding electron transferring flavoprotein and the corresponding dehydrogenase (Etfa, Etfb, Etfdh) [24, 62]. The latter proteins mediate the transfer of electrons from Acyl-CoA dehydrogenases to the membrane-bound electron transfer flavoprotein ubiquinone oxidoreductase, allowing further entry into the oxidative phosphorylation pathway [177, 178]. Finally, PPARα induces uncoupling proteins Ucp2 and Ucp3, which have been proposed to function as an outward transporter of nonesterified fatty acid anions from the mitochondrial matrix [24, 62, 87–89].

5.3. Microsomal Fatty Acid ω-Hydroxylation. Cyp4A enzymes are members of the cytochrome P450 monoxygenase superfamily and catalyze microsomal ω-hydroxylation of fatty acids [106, 179]. Studies using PPAR$\alpha^{-/-}$ mice have shown that hepatic expression of Cyp4a genes is almost completely dependent on PPARα (Cyp4a10, Cyp4a12, Cyp4a14 in mice, Cyp4a1, Cyp4a3 in rat, Cyp4a11 in human) [55, 62, 74, 84, 106–111]. Furthermore, expression is extremely sensitive to PPARα ligand activation, indicating that Cyp4a genes may serve as PPARα marker genes. Although previous studies performed in human primary hepatocytes could not show regulation of Cyp4a by human PPARα, our microarray data revealed significant induction of Cyp4a11 by Wy14643 in primary human hepatocytes [24, 68, 180, 181]. ω-hydroxylation of saturated and unsaturated fatty acids may lead to the generation of high-affinity PPARα ligands, including hydroxyeicosatetraenoic acids (HETEs), thus creating a positive feedback loop [182]. Alternatively, induction of ω-oxidation by PPARα has been suggested to promote the degradation of the PPARα agonist leukotriene B4 as part of a feedback mechanism aimed at controlling the duration of the inflammatory response [53].

5.4. Hepatic Lipogenesis. Whereas PPARα is mostly known for its ability to induce fatty acid oxidation, growing evidence points to a role of PPARα in regulation of lipogenesis. A functional PPRE was identified in the promoter of a limited number of lipogenic genes including Δ6 desaturase (Fads2), malic enzyme (Mod1), phosphatidate phosphatase (Lpin2), and Δ9 desaturase (Scd1) [56, 115–117]. Gene expression profiling showed that chronic in vivo treatment of mice with PPARα agonist causes the upregulation of a large set of lipid biosynthetic genes [62]. However, regulation is much less pronounced in primary hepatocytes, suggesting an indirect mechanism. Consistent with this notion, induction of lipogenic genes by chronic PPARα activation was completely abolished in SREBP1$^{-/-}$ mice [183]. The effect of PPARα agonists on SREBP targets has been attributed to increased activation of SREBP1c via enhanced proteolytic cleavage [112]. Such a mechanism may also lead to increased SREBP1 mRNA via an autoloop regulatory circuit [184]. Alternatively, it is possible that PPARα is recruited to promoters of SREBP targets and stimulates SREBP activity [12]. Interestingly, in rat FAO hepatoma cells, it was found that PPARα activation reduced expression of lipogenic genes, including Fasn, Gpam, and SREBP1c, while Insig1 expression was increased by PPARα [185]. The reason for the discrepancy is not clear.

In contrast to de novo fatty acid and cholesterol synthesis, synthesis of triglycerides may be directly targeted by PPARα. Several genes within these pathways are upregulated by PPARα activation, including Gpam, various Agpat genes, Mogat1, Dgat1, and Lpin2 [24, 62, 65, 112]. Induction of genes involved in triglyceride synthesis from fatty acids may reflect a broader role of PPARα in the hepatic response to fasting aimed at neutralizing large amounts of incoming adipose tissue-derived free fatty acids.

5.5. Fatty Acid Uptake and Binding. Before they can be metabolized in the liver, fatty acids have to be transferred across the cell membrane. Several proteins are involved in fatty acid transport across the plasma membrane, a number of which carry both fatty acid transporter and acyl-CoA synthetase activity. Studies have shown that the fatty acid transport proteins Slc27a1, Slc27a2, and Slc27a4 are upregulated by PPARα in liver [24, 62, 64–68, 101].

Slc27a1 is not expressed and not regulated by PPARα in isolated primary hepatocytes, suggesting regulation occurs in liver macrophages (Kupffer cells). So far, the only fatty acid transporter for which a PPAR response element has been identified is Slc27a1. PPARα agonists also markedly induce hepatic expression of the fatty acid transporter/scavenger receptor Cd36, which is expressed in various liver cell types [24, 62–64]. Additionally, expression of numerous acyl-CoA synthetases is induced by PPARα [24, 62, 63, 70–73]. Currently, limited information is available about the cellular localization and the structure/function relationship of acyl-CoA synthetase enzyme [186].

The Fabp gene family comprise a group of high-affinity intracellular fatty acid-binding proteins. Interestingly, Fabp1 was one of the first PPARα target genes identified [74, 75, 187, 188]. Recent studies indicate that Fabp1 may be involved in partitioning of FA to specific lipid metabolic pathways [189]. Other Fabp genes induced by PPARα activation in mouse liver include Fabp2, Fabp3, Fabp4, and Fabp5 [24, 62, 63]. Induction of Fabp4 (A-FABP, aP2) upon PPARα activation likely occurs via its expression in Kupffer cells. Fabp4 expression in hepatocytes is correlated with acquisition of a steatotic phenotype concurrent with upregulation of PPARγ mRNA [190].

5.6. Lipases and Lipid Droplet Proteins. PPAR$\alpha^{-/-}$ mice exhibit elevated hepatic TG accumulation, especially under fasting conditions [54, 191, 192]. Conversely, treatment with PPARα agonists lowers hepatic triglyceride levels in models of hepatic steatosis and can prevent the fasting-induced increase in liver TG [193, 194]. The antisteatotic effect of PPARα has mainly been attributed to stimulation of fatty acid oxidation, which would decrease the availability of fatty acids for TG storage.

Recently, hepatic lipid droplets were shown to be targeted by autophagy, which ultimately leads to TG hydrolysis via lysosomal acid hydrolase (Lipa). Which other lipases importantly contribute to intracellular lipolysis of hepatic TG stores remains unclear, but lipases active in adipocytes likely play a role, including Ces3, Lipe, Pnpla2, Mgll, and perhaps Pnpla3 [195–200]. With the exception of Pnpla3, all of the above genes are induced by short-term treatment with PPARα agonist in mouse hepatocytes. Regulation of Pnpla2 was also observed in human hepatocytes. Pnpla2 and Lipe were previously classified as direct target genes of PPARγ in adipose tissue, suggesting that they are direct target of PPARα as well [201, 202]. Thus, apart from induction of fatty acid oxidation, PPARα activation may also decrease hepatic TG storage by stimulating the TG hydrolysis pathway.

Lipid droplets are coated with one or more members of the perilipin family of proteins: perilipin (Plin1), Adrp/adipophilin (Plin2), Tip47 (Plin3), S3-12 (Plin4), and Oxpat/Lsdp5 (Plin5). Adrp and Lsdp5 have been identified as target genes of PPARα in liver [120, 124]. A recent study suggests that Adrp could serve as potential mediator of the effect of PPARα on VLDL production. Adrp induction by PPARα may diminish VLDL production by favoring fatty acids storage in cytosolic lipid droplets rather than directing through VLDL assembly [203]. Besides Adrp, expression of S3-12 and perilipin, which are known as PPARγ target genes in adipose tissue, is induced by PPARα agonist in human hepatocytes [24, 204]. Perilipin expression in human liver is correlated with development of steatotic liver [205].

Two recently identified lipid droplet-associated proteins that are not part of the perilipin family are Cidec (FSp27) and Cidea [206, 207]. Both proteins promote TG accumulation and are targets of PPARγ in adipocytes [208, 209]. In addition, they are regulated by PPARα in mouse liver, although the kinetics of induction of the two genes seems to be quite different [121]. Cidec but not Cidea upregulation by PPARα agonist could be confirmed in human primary hepatocytes [24].

Interestingly, the G(0)/G(1) switch gene 2 (G0s2) was recently identified as an inhibitor of Pnpla2 activity and located to lipid droplets in adipocytes stimulated with β-adrenergic receptor agonist [122]. Previously, G0s2 was shown to be a direct PPARα target gene in mouse liver and PPARγ target in adipocytes [123]. Whether G0s2 associates with lipid droplets in hepatocytes remains to be further investigated. Similar to the induction of triglyceride synthesis, regulation of numerous lipid droplet proteins by PPARα reflects a broader role of PPARα in the hepatic response to fasting aimed at deflecting large amounts of incoming adipose tissue-derived free fatty acids towards storage in lipid droplets.

6. PPARα and Lipoprotein Metabolism

Clinical studies in humans have provided ample evidence that fibrate drugs effectively lower fasting plasma triglycerides (TG) and raise plasma HDL [210–213]. At the molecular level, fibrates act as synthetic agonist for PPARα, indicating an important role of PPARα in the control of lipoprotein metabolism. PPARα lowers plasma TG in part by reducing very low-density lipoprotein (VLDL) production [194]. Traditionally, this effect of PPARα was ascribed to induction of genes involved in fatty acid oxidation and the concomitant reduction in lipid availability for VLDL production. However, this paper has made it evident that in addition to its role in fatty acid catabolism, PPARα influences multiple aspects of intracellular lipid trafficking and metabolism, some of which may oppose hepatic TG lowering. Furthermore, expression of Mttp, which is involved in the lipidation of apoB100 to form a nascent VLDL particle, is positively regulated by PPARα [214]. Thus, the precise target genes underlying the suppressive effect of PPARα agonist on hepatic VLDL production remain to be fully elucidated.

In addition to suppressing VLDL production, PPARα agonists are known to stimulate clearance of TG-rich lipoproteins [194]. Clearance of TG-rich lipoproteins VLDL and chylomicrons is mediated by the enzyme lipoprotein lipase (LPL), which is attached to the capillary endothelium of muscle and adipose tissue. Expression of Lpl in liver is restricted to Kupffer cells and upregulated by PPARα agonists [140, 215]. In contrast, no evidence is available indicating a stimulatory effect of PPARα on Lpl expression in heart

and skeletal muscle, which account for the major share of plasma TG clearance [140, 216]. LPL activity is mostly regulated posttranslationally via altered secretion from liver of LPL-modulating factors, including apolipoprotein C-III (Apoc3), apolipoprotein A-V (Apoa5), Angiopoietin-like protein 3 (Angptl3), and Angiopoietin-like protein 4 (Angptl4). Firstly, PPARα agonists downregulate the expression of LPL inhibitor APOC3, supposedly via mechanisms involving the transcription factors REV-ERBα, HNF4α, or FOXO1 [137, 217–219]. Secondly, PPARα agonists increase hepatic expression and plasma levels of APOA5, which is a positive regulator of LPL [220]. A functional PPAR responsive element has been identified in the promoter of the human APOA5 gene, classifying APOA5 as a direct PPARα target gene [135, 136]. Thirdly, PPARα upregulates hepatic expression and plasma levels of Angptl4, which acts as inhibitor of LPL activity by converting active LPL dimers to inactive monomers [126]. The DNA response element conferring PPAR regulation was located to intron 3 of the Angptl4 gene [127]. Finally, PPARα stimulates hepatic expression of the VLDL receptor (Vldlr) [24, 62]. The functional significance of Vldlr regulation in liver is unclear, as Vldlr is most highly expressed in adipose tissue, heart, and skeletal muscle, where it plays an auxiliary role in plasma TG hydrolysis by LPL. Recently, Vldlr was shown to be under control of PPARγ in adipocytes [221]. Thus, it appears that both pro- and antilipolytic pathways are activated by PPARα. Under conditions of pharmacological PPARα activation, the prolipolytic actions of PPARα dominate, as illustrated by the stimulation of plasma TG clearance.

PPARα agonists raise plasma HDL levels in humans, which is most likely achieved via species specific mRNA induction of apolipoprotein A-I (Apoa1) and A-II (Apoa2) [128, 175, 222–224]. Apoa1 gene expression is not induced by PPARα in rodents due to the presence of disabling mutations within the PPAR-response element [129]. In fact, PPARα activation in mouse downregulates Apoa1 mRNA expression and plasma concentrations through an indirect pathway involving the PPARα-dependent induction of the nuclear receptor REV-ERBα, a negative regulator of transcription [129, 130, 225].

The impact of PPARα in HDL metabolism likely extends beyond regulation of apolipoproteins. Evidence suggests that both PPARα and PPARβ/δ stimulate expression of endothelial lipase (Lipg) in liver [62, 226]. Endothelial lipase mainly carries phospholipase activity and its overexpression was shown to significantly reduce plasma HDL cholesterol levels [227–229]. Since Lipg is expressed in endothelial cells, macrophages, and hepatocytes, regulation of hepatic Lipg by PPARα and PPARβ/δ may be mediated by different cell types. In as much as PPARα agonists raise plasma HDL levels, the physiological relevance of Lipg induction by PPARα remains to be established.

In our recent publication, the PPARα agonist Wy14643 modestly induced hepatic lipase (Lipc) expression in primary human hepatocytes [24]. Hepatic lipase exhibits both phospholipase and triglyceride hydrolase activity and hydrolyzes triglycerides and phospholipids of chylomicron remnants, IDL, and HDL [230]. Whether Lipc represents a direct target gene of PPARα in human remains unclear. Other genes involved in lipoprotein metabolism that are regulated by PPARα include phosphatidylcholine transfer protein (Pctp). Induction of Pctp mRNA by PPARα is conserved in primary human hepatocytes [24]. Pctp encodes a steroidogenic acute regulatory-related transfer domain protein that binds with high affinity to phosphatidylcholines. In a recent publication, a role for Pctp in the metabolic response to PPARα was proposed [231]. Overall, it is evident that PPARα governs multiple aspects of plasma lipoprotein metabolism.

7. PPARα and Glucose/Glycerol Metabolism

Although PPARα has mostly been linked to fatty acid metabolism, studies in mice have yielded considerable evidence for a role of PPARα in hepatic glucose metabolism. Indeed, fasted PPAR$\alpha^{-/-}$ mice display severe hypoglycemia [54, 55, 176]. Several mechanisms may account for the hypoglycemia, including decreased hepatic glucose production and increased peripheral glucose utilization. Genes involved in gluconeogenesis that have been identified as PPARα targets include phosphoenolpyruvate carboxykinase (Pck1), pyruvate carboxylase (Pcx), and lactate dehydrogenase A [62]. Interestingly, regulation of Pck1 by PPARα was only observed in human hepatocytes [24]. Pyruvate carboxylase was identified as direct target of PPARγ in adipocytes [232].

PPARα was shown to have a specific role in the metabolic conversion of glycerol in liver by directly upregulating expression of genes such as Gpd1, Gpd2, Gyk, Aqp3, and Aqp9 [151]. Besides governing glucose production, PPARα may also alter glucose utilization in numerous tissues via induction of pyruvate dehydrogenase kinase isoform 4 (Pdk4) [153, 154, 233–236]. Pdk4 phosphorylates and inactivates pyruvate dehydrogenase, thereby limiting carbon flux through glycolysis. Synthesis of glycogen is also affected in PPAR$\alpha^{-/-}$ mice, which may be mediated in part via defective regulation of Gys2 [152]. It is noteworthy that in contrast to studies in mice, human trials generally do not support an effect of PPARα activation on plasma glucose levels. Consistent with these data, it was found that upregulation of genes involved in the glycolysis/gluconeogenesis pathway by Wy14643 was uniquely observed in mouse hepatocytes and not human hepatocytes [24].

8. PPARα and Hepatic Cholesterol/Bile Metabolism

It has been demonstrated that PPARα activation increases efflux of cholesterol to HDL. Formation of nascent HDL is mediated by Abca1-dependent lipidation of newly-secreted Apoa1. Expression of Abca1 is upregulated by PPARα agonists in both human and mouse hepatocytes, as well as in mouse intestine [24, 237]. Presently, it is not clear if this effect of PPARα activation is mediated via LXRα, as was shown previously in macrophages [21]. Other genes involved in cholesterol uptake and transport that were shown to be under control of PPARα include Abcg5, Abcg8, Cav1, Npc1, and Rab9 [24, 62, 144].

While PPARα is known to govern specific genes involved in bile acid synthesis, the overall impact on bile acid homeostasis remains somewhat ambiguous. Expression of Cyp7a1, which represents the rate-limiting enzyme in bile acid synthesis, is markedly downregulated in PPAR$\alpha^{-/-}$ mice in fasting condition [62]. Paradoxically, synthetic PPARα agonists reduce Cyp7a1 expression in both mice and human [145, 238–240]. In agreement with the latter observation, fibrate treatment leads to decreased bile acid synthesis. To what extent the changes in Cyp7a1 expression reflect direct regulation by PPARα is unclear as PPARα also influences the expression of other nuclear hormone receptors involved in regulation of Cyp7a1 such as FXR and LXR. It has also been suggested that PPARα can antagonize LXR signaling and LXR-dependent activation of Cyp7a1 gene promoter [241–243].

Other genes involved in bile acid synthesis that are regulated by PPARα include Cyp27a1 which is downregulated by PPARα agonists in a PPARα-dependent manner [145], and Cyp8b1 which is upregulated by PPARα [62, 148]. Recently, CYP7b1 expression was shown to be suppressed by PPARα in a sex-specific manner, which was shown to occur via sumoylation of the LBD of PPARα [244]. Finally, PPARα stimulates expression of the hepatobiliary phospholipid transporter Abcb4 [24, 62, 94, 144].

9. PPARα and Amino Acid Metabolism

Accumulating evidence supports a role for PPARα in regulation of amino acid and urea metabolism [159, 160, 162, 245]. Studies in mice have shown that PPARα governs metabolism of amino acids by suppressing expression of genes involved in transamination (Aspartate amino transferase (Got1), Alanine amino transferase (Gpt), Alanine glyoxylate aminotransferase (Agtx2), and deamination (Glutaminase (Gls)), as well as numerous genes that are part of the urea cycle (Cps1, Otc, Ass1, and Asl) [160, 161, 245]. In agreement with these data, PPAR$\alpha^{-/-}$ mice exhibit increased plasma urea levels [160]. Several of the above genes were also downregulated by PPARα agonist in primary human hepatocytes, suggesting that regulation of nitrogen metabolism by PPARα is at least partially conserved between mice and human [24].

At the present time, the mechanism behind downregulation of nitrogen metabolism by PPARα remains elusive. It has been proposed that PPARα may modulate the activity of other transcription factors that are directly involved in amino acid homeostasis, including HNF4α and C/EBPα [160]. However, concrete evidence supporting such a mechanism is lacking.

Whereas PPARα activation decreases hepatic aminotransferase expression in mice, PPARα agonists were shown to increase expression of Gpt in human hepatocytes and HepG2 cells, which occurred via direct regulation of the gene promoter [161, 246]. The observed increase in plasma alanine amino transferase activity in patients treated with fibrates may thus be related to direct regulation of Gpt transcription, rather than drug-induced liver injury.

10. PPARα and Inflammation

Besides regulating numerous metabolic pathways, PPARα also governs inflammatory processes, which is mainly achieved by downregulating gene expression via a mechanism generally referred to as transrepression. The first clue towards anti-inflammatory effects of PPARα came from the observation that PPAR$\alpha^{-/-}$ mice exhibit a prolonged inflammatory response in the ear swelling test [53]. The anti-inflammatory effects of PPARα are likely explained by interference of PPARα with the activity of many proinflammatory transcription factors including signal transducer and activator of transcription (Stat), Activator protein-1 (AP-1), and NF-κB [247]. Specifically, it has been shown that activated PPARα binds to c-Jun and to the p65 subunit of NF-κB, thereby inhibiting AP-1- and NF-κB- mediated signaling [248]. Additionally, PPARα induces the inhibitory protein IκBα, which normally retains NF-κB in a nonactive form, leading to suppression of NF-κB DNA-binding activity [168]. Suppression of fibrinogen gene expression by PPARα activation is likely mediated by interference with the transcription factor CAATT/enhancer-binding protein (C/EBP) via sequestration of the coactivator glucocorticoid receptor-interacting protein 1/transcriptional intermediary factor 2 (GRIP1/TIF2) [166]. Finally, recent data indicate that activated PPARα may downregulate gene expression by causing the loss of STAT1 and STAT3 binding to DNA [12].

Specific genes downregulated by PPARα include a number of acute phase genes such as fibrinogen, serum amyloid P-component, lipocalin 2, metallothioneins, and serum amyloid A2, which were shown to be suppressed by the PPARα agonist Wy14643 in wild-type mice but not PPAR$\alpha^{-/-}$ mice [163]. Similarly, in humans fenofibrate treatment has been shown to decrease plasma levels of several acute phase proteins including C-reactive protein, fibrinogen-α and -β and interleukin 6 [166, 249, 250]. With the exception of the sIl-1 receptor antagonist and Vanin-1, to our knowledge no inflammatory genes have been identified as direct positive targets of PPARα [163].

The Vanin-1 (Vnn1) gene encodes a glycosylphosphatidylinositol-linked membrane-associated pantetheinase that generates cysteamine from pantothenic acid. Studies suggest that Vanin1 may promote inflammation. Mice lacking Vnn1 showed decreased NSAID- or Schistosoma-induced intestinal inflammation, which was associated with higher glutathione levels [251]. Other evidence indicates that Vanin-1 stimulates production of inflammatory mediators by intestinal epithelial cells and thereby controls the innate immune response, possibly by antagonizing PPARγ activity [252]. Epithelial Vanin-1 was also found to regulate inflammation-driven cancer development in a colitis-associated colon cancer model [253]. Evidence presented in Figure 3 demonstrates that Vnn1 likely represents a direct target gene of PPARα. Expression of Vnn1 in mouse liver was markedly increased by fasting in wildtype but not PPAR$\alpha^{-/-}$ mice (Figure 3(a)). Negligible Vnn1 expression was detected in PPAR$\alpha^{-/-}$ mouse liver. Moreover, hepatic Vnn1 expression was significantly induced by 6 h treatment with dietary fatty acids and by the synthetic PPARα agonists

Wy14643 and fenofibrate (Figure 3(b)). Additional data lend strong support to the importance of PPARα in Vnn1 gene regulation in small and large intestine (Figures 3(c) and 3(d)), although the results are not quite as striking as in liver. Finally, it was shown that two adjacent and partially overlapping PPREs located around 4 kb downstream of the transcription start site of the mouse Vnn1 gene were functional in a luciferase reporter assay in HepG2 cells (Figure 3(e)). PPARα transfection and Wy14643 markedly increased luciferase activity, although for reasons that remain unclear, no synergism between the two treatments was observed. Overall, these data suggest that Vnn1 represents a direct PPARα target gene.

The ability of PPARα to stimulate fatty acid oxidation and suppress hepatic inflammation has led to the exploration of PPARα agonists as a therapeutic option for nonalcohol fatty liver disease and specifically nonalcoholic steatohepatitis (NASH). Several studies in mice have shown that PPARα activation can reduce or even reverse the progression of steatohepatitis [193, 254–259]. The inhibitory effect of PPARα on progression of steatosis to steatohepatitis may be mediated in part by COX2 (Ptgs2), a candidate gene involved in steatohepatitis development that is suppressed by PPARα [260]. In the absence of PPARα, liver steatosis and inflammation are enhanced in mice chronically fed a HFD [165]. Whether the effects of PPARα on NASH are primarily related to changes in hepatic TG content or occur via direct suppression of inflammatory genes and markers remains unclear.

11. PPARα and Biotransformation

The detoxification of endogenous and exogenous molecules is generally divided into three distinct biotransformation phases. The phase I reaction involves the introduction of a polar group into the xenobiotic molecule and is catalyzed by members of the cytochrome P450 (CYP) superfamily [179, 180, 261]. Phase II enzymes are responsible for covalent linkage of the absorbed chemicals or products of the phase I reactions with compounds such as glutathione, glucuronic acid, or amino acids and are carried out by sulfotransferases, UDP-glucuronosyltransferases (UGTs), glutathione-S-transferases (GSTs), and N-acetyltransferases [261]. The third phase corresponds to elimination of the conjugated molecule from cells and their excretion into bile or urine via specific transporters, mainly members of the superfamily ATP-binding cassette transporter proteins [262, 263]. Studies have shown that peroxisome proliferators modulate exclusively the Cyp4a class of monooxygenases (involved in the metabolism of biologically important compounds such as fatty acids, see Section 5.3) in mouse while regulating various other Cyp genes in human hepatocytes, including members of the Cyp1a, Cyp2a, Cyp2c, and Cyp2e subfamilies [24]. Our recent microarray data confirmed the human specific regulation of Cyp genes belonging to classes 1-3 by PPARα in primary human hepatocytes. Interestingly, we also observed a significant induction of another subfamily member of Cyp4 enzymes, Cyp4x1, by PPARα in human primary hepatocytes which was not conserved in mouse [24]. Cyp4x1 has been shown to be involved in oxidation of anandamide, which represents one of the endocannabinoids. Besides upregulation of gene expression, a number of genes involved in phase I biotransformation are downregulated by PPARα in mice, including Cyp2a5, Cyp2c11, Cup2c12, and Cyp2c29 [110, 155].

With respect to phase II biotransformation, PPARα has been shown to downregulate Glutathione-S-transferase A [GSTA], possibly leading to decreased biliary excretion of glutathione conjugates [157, 264, 265]. In contrast, expression of UDP-glucuronosyltransferase 1A (Ugt1a9), which participates with other UGT enzymes in glucuronidation of bilirubin, arachidonic, and linoleic acid metabolites, is under direct stimulatory control of PPARα [158]. Overall, it is evident that PPARα is a major regulator of biotransformation enzymes and governs the expression of numerous cytochrome P-450 and conjugating enzymes. However, only a small portion of the regulation seems to be conserved between rodents and humans.

12. Conclusion

In 2010, we are celebrating the 20th anniversary of the discovery of PPARα by Isseman and Green. PPARα was initially isolated as a novel nuclear hormone receptor that serves as molecular target of a diverse class of rodent hepatocarcinogens. Since then it has become clear that PPARα can be activated by a large variety of endogenous and synthetic agonists including fibrate drugs. In fact, PPARα is nowadays considered as a crucial fatty acids sensor that mediates the effects of numerous fatty acids and fatty acid derivatives on gene expression. Furthermore, over the years PPARα has emerged as a crucial transcriptional regulator of numerous metabolic and inflammatory processes. Although PPARα has mostly been connected with stimulation of fatty acid oxidation, it is now evident that the effects of PPARα are much more widespread and cover numerous aspects of nutrient metabolism and energy homeostasis, including metabolism of lipoproteins, glucose/glycerol, cholesterol and bile acids, xenobiotics, and amino acids. Certainly, PPARα merits the classification as a master regulator of hepatic intermediary metabolism. Until recently, much confusion surrounded the effects of PPARα activation in human liver. Recent studies indicate that at least in terms of lipid metabolism, the function and specific target genes of PPARα are generally well conserved between mouse and human. One of the major challenges lying ahead is to gain better understanding of the molecular mechanism underlying downregulation of gene expression by PPARα, to improve insight into the specific mechanisms and pathways of endogenous PPARα activation, and to better link the functional consequences of PPARα activation to induction of specific PPARα target genes.

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Review Article

Molecular Mechanisms and Genome-Wide Aspects of PPAR Subtype Specific Transactivation

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The peroxisome proliferator-activated receptors (PPARs) are central regulators of fat metabolism, energy homeostasis, proliferation, and inflammation. The three PPAR subtypes, PPARα, β/δ, and γ activate overlapping but also very different target gene programs. This review summarizes the insights into PPAR subtype-specific transactivation provided by genome-wide studies and discusses the recent advances in the understanding of the molecular mechanisms underlying PPAR subtype specificity with special focus on the regulatory role of AF-1.

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) constitute a subgroup of the nuclear receptor (NR) family. The founding member of the family, PPARα, was identified in 1990 and named by its ability to become activated by chemicals known to induce peroxisome proliferation in rodents [1]. Subsequently, the two other PPAR subtypes, PPARβ/δ and PPARγ, were identified by homology screens [2, 3]. The three PPAR subtypes are encoded by distinct genes located on different chromosomes (reviewed by [4]). Alternate promoter usage and splicing give rise to two different protein isoforms from the PPARγ gene called PPARγ1 and PPARγ2, with the latter containing 30 additional amino acids at the N-terminus (Swiss-Prot http://www.expasy.org/). All three PPAR subtypes can be activated by a large variety of fatty acids and fatty acid metabolites, such as hydroxylated eicosanoids, prostaglandins, and leukotrienes, and by many synthetic compounds. PPARα is specifically activated by fibrates and other hypolipidemic drugs, whereas PPARγ is activated by the insulin-sensitizing, antidiabetic thiazolidinedione drugs [4].

The PPARs play important regulatory roles in numerous cellular processes related to metabolism, inflammation, differentiation, proliferation, and atherosclerosis (reviewed by [5, 6]). The three subtypes display dissimilar patterns of tissue distribution and activate both overlapping and distinct sets of target genes. Most notably, whereas PPARα [7, 8] and -β/δ [9] are potent activators of genes involved in lipid oxidation, PPARγ stands out by its additional ability to activate lipogenic genes and adipocyte differentiation [10, 11]. In fact, PPARγ is obligate for adipocyte differentiation and is sufficient to transform many nonadipogenic cell lines into adipocyte-like cells [12, 13]. As a reflection of these subtype-specific properties, PPARα and PPARβ/δ are highly expressed in tissues with high β-oxidation rates such as liver, muscle, heart, and brown adipose tissue. By contrast PPARγ is highly expressed in adipose tissue, and PPARγ2 is fat selective, whereas the PPARγ1 isoform is expressed at low levels in several tissues, including colon, spleen, liver, and muscles. PPARβ/δ is the most ubiquitously expressed subtype with the highest levels found in the intestines and keratinocytes (see [14] and reviewed by [5]).

Like most NRs, the PPAR protein structure consist of four domains: The N-terminal A/B-domain containing the ligand-independent activation function 1 (AF-1), the C-domain, which is the DNA-binding domain (DBD), the D-domain, also called the hinge region, and finally the E-domain, commonly referred to as the ligand binding domain (LBD). The E-domain contains the ligand-dependent AF-2, which is highly dependent on the C-terminal helix 12. While the A/B -and D-domains are only poorly conserved

between the PPAR subtypes, the C -and E-domains share a high degree of sequence and structural homology (reviewed by [4]). In fact, the C-domains are completely interchangeable between the PPAR subtypes and appear to have no effect on specificity [15, 16]. The PPARs bind DNA as obligate heterodimers with members of the retinoid X receptor (RXR) family of nuclear receptors to modified direct repeat 1 elements (DR1) with the consensus sequence 5′-AACTAGGNCA A AGGTCA-3′. The PPARs occupy the 5′ extended half site of the binding site [17].

Given the important role of the PPARs in regulation of metabolism, inflammation, differentiation, and cellular growth, a large number of specific and potent synthetic ligands have been generated. This has spurred a huge interest in understanding the molecular mechanisms of PPAR transactivation and in genome-wide approaches to identify new PPAR target genes. These studies have provided the field with important insights into how different ligands modulate the transactivation capacity of the PPARs and to what extent the individual PPAR domains are involved in ensuring subtype-specificity by enabling or preventing transactivation of specific subsets of target genes. This paper focuses on the recent advances in understanding PPAR subtype-specific transactivation as seen from a molecular and a genome-wide perspective. In particular, the regulatory role of the AF-1 in maintaining PPAR subtype-specificity and transactivation capacity is reviewed.

2. Molecular Mechanisms of PPAR Subtype Specific Transactivation

The ability of the individual PPAR subtypes to induce very different cellular fates is intriguing because they share a high degree of sequence- and structural homology and activate overlapping sets of target genes. Nevertheless, the PPARs maintain a high degree of subtype-specificity when expressed in a given cell line at comparable levels [11, 18, 19] and adenoviral expression of PPARγ1 in mouse liver leads to induction of several genes which are not readily activated by PPARα, that is, genes involved in lipid accumulation and adipogenesis [20]. These results indicate that although the chromatin setting ultimately determines the accessibility of the PPAR response elements, intrinsic properties of the individual PPAR subtypes are key determinants of the gene programs that can be activated. Mechanisms maintaining subtype-specificity are of significant general interest because subtype selective gene regulation is a recurrent theme among transcription factor families, and therefore several attempts have been made to address this issue. These studies reported that the PPAR subtypes differ only little in their ability to transactivate artificial promoter reporter constructs in transient transfections [21–24] and display limited specificity in their binding to naked DNA containing target gene PPAR response elements (PPREs) in mobility shift assays, although poorly conserved PPREs preferentially bind PPARγ:RXR heterodimers [25]. By contrast, work from our laboratory has shown that in the endogenous chromatin setting, the binding level of a particular PPAR subtype to a given genomic PPRE generally correlates with its potential to transactivate the corresponding target gene, although exceptions clearly exist [11].

2.1. The LBD and AF-2. Because of the obvious therapeutic potential for modulating PPAR transactivation through the administration of ligands that bind to the E-domain, the cofactor interaction surfaces and the molecular mechanisms underlying activation of the AF-2 have been extensively studied. The recent publication of an almost complete structure of the DNA bound PPARγ:RXRα heterodimer [26] represents a major breakthrough in the understanding of the positioning of the PPARγ and RXR domains relative to each other and their interactions. Unfortunately, the PPARγ and RXRα A/B-domains could not be crystallized, most likely due to their high mobility and lack of internal structure. However, the overall impression from this study is that the PPARγ LBD is the centerpiece of the complex, around which all other domains from both PPARγ and RXRα are arranged [26]. This accentuates previous descriptions of extensive interdomain cross-talk in the PPARs [27, 28].

The PPARγ LBD, is composed of 13 α-helices and a small four-stranded β-sheet that forms a large (approx. 1300 Å^3) T-shaped hydrophobic ligand-binding pocket typical for the promiscuous NRs, such as PPARs and the pregnane X receptor (PXR), that bind many different ligands with low affinity [29]. The ligand-binding pocket of PPARβ/δ is narrower than those of PPARγ and PPARα, which appears to be a major determinant of ligand binding, as it prohibits binding of TZDs and severely decreases the affinity of PPARβ/δ towards fibrate ligands due to the bulky acid and alkyl groups on these compounds [30]. PPARα contains the most lipophilic ligand binding pocket, which potentially explains why PPARα, as opposed to PPARβ/δ and PPARγ, binds a variety of saturated fatty acids [31]. An additional layer of ligand binding specificity is imposed by the size and charge of the amino acids lining the ligand binding pocket [30]. Ligand-binding affects the stability of the PPARs with liganded PPARα being transiently stabilized [32] while proteasomal degradation of PPARγ is increased upon ligand binding [33].

The structural basis for AF-2-mediated transcriptional activity is a ligand-induced conformational change in the LBD, causing the most C-terminal helix 12 to fold up against the core [30]; thereby generating an activation surface, often described as a charge clamp, onto which coactivators can dock. Many coactivators bind to the NR E-domains through a motif with the consensus sequence LXXLL, which facilitates direct interaction with the charge clamp. In contrast, corepressors often have a LXXXIXXX(I/L) motif that interacts with an overlapping surface but is unable to fit into the charge clamp (reviewed by [34]). The variations in the primary sequences of the PPAR LBDs results in slight differences in the cofactor interaction surfaces and ligand-induced conformations of these domains [35, 36] and PPAR subtype-specificity is thought to be partly imposed by differential affinity of the receptors towards the individual cofactors [37, 38]. Thus, the cofactor expression pattern in a specific cellular context may favor

transcriptional activation of one PPAR subtype over the others.

Probably, the transcriptional activity of the PPARs should not be regarded as determined by a static positioning of helix 12 in either the "on" or "off" position. Rather, it appears that ligand-binding shifts the equilibrium of the different helix 12 positions in the receptor population towards the more active conformations (reviewed by [39]) [40]. Helix 12 is absolutely necessary for the activity of AF-2, and PPAR mutants with helix 12 partly or completely deleted are dominant negative inhibitors of PPAR signaling [41, 42]. Interestingly, the requirement for helix 12 does not appear to equalize a requirement for ligand-binding. Recently, it has been demonstrated that a natural PPARγ mutant that is impervious to activation by virtually all known agonists has intact adipogenic potential [43]. Indeed, the PPARs display high basal transcriptional activity that can be explained by the AF-1 in the A/B-domain and the presence of endogenous ligands. In addition, ligand-independent recruitment of coactivators to the AF-2 has been observed in overexpression and *in vitro* studies, indicating that in addition to the AF-1, the AF-2 also contributes to ligand-independent transactivation. Perhaps, this is possible because the shift in the positioning of helix 12 upon ligand-binding is not as pronounced for the PPARs as for the steroid NRs [44, 45].

2.2. The Elusive Structure of the PPAR A/B-Domain. Compared to the AF-2, the mechanisms for AF-1-mediated transcriptional activity are less well-understood despite several publications pointing to an important role for the A/B-domains in maintaining PPAR subtype-specificity [15, 16, 46]. The fact that it so far has proven impossible to crystallize a NR A/B-domain indicates that these domains are poorly structured, a notion that has been confirmed by experiments using deuterium exchange mass spectrometry [26], circular dichroism spectroscopy, nuclear magnetic resonance spectroscopy [47, 48], or limited proteolysis [49, 50]. It has been suggested that secondary structure formation is an important step in AF-1-mediated transactivation and both the PPARα [51] and glucocorticoid receptor (GR) [47] AF-1 display α-helical characteristics in the presence of trifluoroethanol, a strong α-helix stabilizing agent. Furthermore, mutational analyses of the PPARα [51], GR [52, 53], and hepatocyte nuclear factor 4 (HNF-4) [54] AF-1 domains have shown that conservation of the hydrophobic amino acids, that potentially could be involved in α-helix formation is especially important for the transcriptional activity, while mutation of individual acidic amino acids made less of an impact, suggesting that α-helix formation is an important step in AF-1-mediated gene activation. Interestingly, it was recently shown that mitogen-activated protein kinase (MAPK) phosphorylation of serine 211 in the glucocorticoid receptor A/B-domain induces formation of secondary or tertiary structure in this region which facilitates interaction between the AF-1 and coregulators thereby leading to enhanced transcriptional activity [55]. Another model proposes that, as individual coactivators offer different surfaces for unstructured activation-domains to fold up on, distinct conformations could be induced by the different coactivators, thereby resulting in differential transcriptional activity or specificity [56]. Several of the cofactors reported to interact with NR A/B-domains have no sequence or known structural homology, and this model offers an attractive explanation for how that is possible.

2.3. PPAR Transcriptional Activity Is Regulated by Modification of the A/B-Domain. It is well-established that posttranslational modifications of the PPAR A/B-domains influence the transcriptional activity of both the AF-1 and AF-2 through various mechanisms. The PPARα and $-\gamma$ A/B-domains, but not the PPARβ/δ A/B-domain, are modified by phosphorylation. MAPK- phosphorylation of serine 12 and 21 in the PPARα A/B-domain, enhances the transcriptional activity by transiently increasing receptor stability through reduced ubiquitination [32]. Oppositely, phosphorylation of serine 76 by glycogen synthase kinase 3 (GSK3) leads to increased ubiquitination and degradation of PPARα [93]. MAPK mediated phosphorylation of serine 82 in the PPARγ1 A/B-domain (corresponding to serine 112 in PPARγ2) has been shown to inhibit both ligand-dependent and independent transactivation [94], the former by decreasing the ligand-binding affinity of the receptor [27]. Interestingly, it was recently published that the phosphorylated PPARγ AF-1 domain is bound by the peptidyl-prolyl isomerase Pin1, whereby both polyubiquitination and the transcriptional activity of PPARγ is inhibited, possibly due to the decreased turnover rate of the receptor [95]. Oppositely, it has also been reported that S112 phosphorylation of PPARγ2 by a constitutively active MAPK kinase (MEK) [96] or by cyclin-dependent kinase 9 (Cdk 9) residing in the positive transcription elongation factor b complex (P-TEFb) results in increased transcriptional activity [71]. Thus, it appears that the cellular and/or molecular context determines the transcriptional effect of PPARγ A/B-domain phosphorylation. Mice homozygous for the S112A mutation are indistinguishable from the wild types on a normal diet, but they are significantly more glucose tolerant in the setting of diet-induced obesity [97], an effect analogous to the outcome of PPARγ activation by TZD treatment. In line with phosphorylation of S112 decreasing the insulin sensitizing actions of PPARγ, humans carrying a P115Q mutation, that blocks phosphorylation of serine 114 (corresponding to serine 112 in mice), are extremely obese but are also less insulin-resistant than expected based on their degree of obesity [98]. In addition to affecting the activity of PPARγ through regulation of MAPK, MEK has also been reported to interact directly with PPARγ and promote its nuclear export [99]. Recently, it was furthermore reported that phosphorylation of serine 16 and 21 of PPARγ by Casein-kinase-II likewise promotes shuttling of PPARγ from the nucleus to the cytosol [100]. Besides phosphorylation, PPARγ transactivation is also repressed by conjugation of small ubiquitin-related modifier (SUMO) to lysine 107 in the A/B-domain [101].

2.4. The PPAR A/B-Domain Is Involved in the Recruitment of Cofactors. In addition to regulating the PPAR transcriptional

Table 1: Cofactors regulating PPAR activity.

Coactivator	Enzymatic activity	Interaction	Reference
Bifunctional enzyme (BFE)	Dehydrogenase	α (A/B)	[57]
BRG1-associated factor 60c (BAF60c)	None	γ (A/B)	[58]
Coactivator-associated arginine methyltransferase 1 (CARM1)	HMT	SRC-1-3	[59]
Constitutive coactivator of PPARγ (CCPG)	None	γ (D)	[60]
CREBP- binding protein (CBP)	HAT	α,γ (A/B, LBD, SRC)	[61]
Hydrogen peroxide-inducible clone 5 protein (Hic5)	None	γ	[62]
LIM domain-only protein (LMO4)	None	γ (D, LBD)	[63]
Lipin 1	None	α, PGC-1	[64]
Mediator subunit 1 (MED1)	None	α, β/δ,γ (LBD)	[65]
Mediator subunit 14 (MED14)	None	γ (A/B)	[66]
Multiple Endocrine Neoplasia type 1 (MEN1)	None	γ (LBD)	[67]
Multiprotein bridging factor 1 (MBF-1)	None	γ (D, LBD)	[68]
Murine double minute 2 (MDM2)	Ubiquitin ligase	α, β/δ (A/B)	[69]
p300	HAT	α,γ (A/B, LBD, SRC)	[61]
Poly ADP-ribose polymerase 2 (PARP-2)	ADP-ribose polymerase	γ	[70]
Positive transcription elongation factor b complex (P-TEFb)	Kinase	γ	[71]
PPARα-interacting complex 285 (PRIC285)	DNA helicase	α, β/δ, γ (DBD)	[72, 73]
PPARα-interacting complex 320 (PRIC320)	DNA helicase	α	[74]
PPAR-interacting protein (PRIP)	None	α,γ (LBD)	[75]
PPARγ coactivator 1α (PGC-1α)	None	α,γ (DBD)	[76]
PPARγ coactivator 2 (PGC-2)	None	γ (A/B)	[15]
PR domain containing 16 (PRDM16)	None	α,γ	[77]
PRIP-interacting protein with methyltransferase domain (PIMT)	HMT	PRIP, CBP, MED1	[78]
Protein arginine N-methyltransferase 2 (PRMT2)	HMT?	γ	[79]
Steroid receptor coactivator-1 (SRC-1)	HAT	α, β/δ, γ (LBD)	[80]
Steroid receptor coactivator-2 (SRC-2)	HAT	α,γ (LBD)	[81]
Steroid receptor coactivator-3 (SRC-3)	HAT	α, β/δ,γ (LBD)	[81]
Tat interactive protein (Tip60)	HAT	γ (A/B)	[82]
Thyroid hormone receptor interacting protein 3 (TRIP3)	None	γ (LBD)	[83]
Corepressor	**Enzyme activity**	**Interaction**	**Reference**
Histone deacetylase 1 (HDAC1)	HDAC	NCoR, SMRT	[84]
Histone deacetylase 3 (HDAC3)	HDAC	NCoR, SMRT	[85, 86]
Insulin-like growth factor-binding protein-3 (IGFBP-3)	None	γ	[87]
Nuclear receptor corepressor 1 (NCoR)	None	α, β/δ, γ	[88]
Receptor-interacting protein 140 (RIP140)	None	α,γ (LBD)	[89]
Scaffold attachment factor B1 (SAFB1)	None	α, β/δ, γ	[90]
Silencing mediator of retinoid and thyroid receptors (SMRT)	None	α, β/δ, γ	[88]
Silent mating type information regulation 2 homolog 1 (SIRT1)	HDAC	NCoR, PGC-1	[91]
Tribbles homolog 3 (TRB3)	None	γ (A/B)	[92]

Histone methyltransferase (HMT), Histone acetyltransferase (HAT), and Histone deactylase (HDAC).

activity by affecting receptor stability, cellular compartmentalization, and perhaps interdomain communication in response to the posttranslational modification status, the PPAR A/B-domains are involved in recruiting a handful of cofactors. The PPARγ AF-1 is the most well-described of the three PPAR A/B-domains and the coactivators Tat-interacting protein 60 (Tip60) [82] and PPARγ coactivator-2 (PGC-2) [15] but also the corepressor *tribbles* homolog 3 (TRB3) [92] are recruited to PPARγ exclusively through binding to the A/B-domain. Both PPARα and PPARγ have been shown to bind the histone acetyl transferase (HAT) coactivators p300 and CREBP-binding protein (CBP) through interaction surfaces in both the A/B-and E-domains in GST-pulldown studies [61]. The significance of the A/B-domain interaction was previously unknown, but we have recently shown that recruitment of p300 and CBP is compromised by deletion of the PPARγ A/B-domain specifically on the PPREs of the target genes that required AF-1 activity

to become fully activated [46]. The SWI/SNF chromatin remodeling complex BRG1-associated factor 60c (BAF60c) subunit which interacts directly with PPARγ likewise appear to have the potential to bind both the A/B- and E-domains, but the AF-1 interaction is stronger and ligand-independent [58]. PPARα has been shown to be coactivated by binding of the target gene product bifunctional enzyme (BFE) to the A/B-domain [57], and the ubiquitin ligase murine double minute 2 (MDM2) is bound by the N-terminal of all three PPAR subtypes [69]. In addition, we have recently demonstrated that the Mediator complex is tethered to the PPARγ A/B-domain through the MED14 subunit and that MED14 is required for full transcriptional activation of PPARγ subtype-specific genes by PPARγ [102]. A complete list of the cofactors currently known to interact with the PPARs is shown in Table 1.

2.5. The A/B-Domains Play an Important Role in Maintaining PPAR Subtype-Specificity. Because the A/B-domain is the least conserved region among the PPARs, it has long been suspected that subtype-specificity, and target gene selectivity is completely or partly mediated through this domain. This hypothesis has been investigated by deleting the A/B-domain or by constructing chimeric PPARs where domains have been swapped between the subtypes.

Despite the undisputed observation that the PPARα and -γ A/B-domains are potent transactivators when expressed as GAL4-fusion proteins [51] there has been controversy regarding the physiological importance of the activity of the PPAR A/B-domains. Deletion of the A/B-domain was reported to have no effect [82, 103] or to significantly decrease PPAR-mediated expression from a reporter plasmid in transient transfections [51]. Interestingly, deletion of the A/B-domain affected the transcriptional activity of PPARα differentially depending on the target gene promoter used in the reporter construct. One study employed the acyl-CoA oxidase promoter and found that the A/B-domain contributed significantly to the transcriptional activity of PPARα [51], while another study showed that luciferase expression driven by the cytochrome P450 4A6 promoter was completely unaffected by deletion of the AF-1 [103]. We have recently on a global scale shown that deletion of the PPARγ A/B-domain selectively decreases the transactivation potential of PPARγ on the highly subtype-specific target genes. We found that out of 257 PPARγ-induced genes only 25 are dependent on the PPARγ A/B-domain to become fully activated in the presence of the TZD rosiglitazone. The A/B domain dependent genes are the highly PPARγ selective target genes many of which are involved in lipid storage. Notably, in the absence of synthetic agonist, transactivation of this subgroup of genes in particular relies almost exclusively on the PPARγ A/B-domain [46].

The importance of the PPAR A/B-domains in maintaining subtype-specificity has been indicated by several studies showing that these domains are not interchangeable. Thus, although both the α and γ A/B-domains contain potent activation functions [51], they cannot functionally substitute for each other as evidenced by the observation that a chimera consisting of the PPARα A/B-domain and the PPARγ CDE-domains is able to transactivate the PPARγ selective target genes similar to that of PPARγCDE [46]. However, the A/B-domains of PPARγ and PPARα can impose a partial subtype-specific activation in the context of a noncognate receptor. Spiegelman and coworkers showed that swapping the PPARγ A/B-domain on to the nonadipogenic PPARβ/δCDE conferred adipogenic potential to this receptor subtype [15]. Subsequently, Tontonoz and coworkers reported that the PPAR A/B-domains function to restrict target gene activation in the context of the cognate receptor and showed that A/B-domain deleted PPARβ/δ (PPARβ/δCDE) can induce adipogenesis. This study thereby raised the question as to what degree the adipogenic potential of the PPARγ A/B-domain-PPARβ/δCDE chimera used in the Spiegelman study arose by the addition of the PPARγ A/B-domain or the lack of the PPARβ/δ A/B-domain [13, 16]. In agreement with the A/B-domains conferring subtype-specificity to the PPARs in part by limiting nonselective target gene activation, we have shown that compared to the full length receptors, the A/B-domain deleted PPARαCDE and -γCDE are far better transactivators of the noncognate highly subtype selective PPAR target genes normally activated by the opposite subtype. However, reminiscent of the Spiegelman data we also found that addition of the PPARα A/B-domain greatly enhances the ability of PPARγCDE to activate a PPARα specific target gene [46]. Thus, it appears that the A/B-domains contribute to maintaining PPAR subtype-specificity by both potentiating activation of the highly subtype selective target genes, and by restricting nonselective target gene activation exclusively in the context of the cognate CDE domains. By contrast, the A/B-domain plays only a minor role in the activation of the target genes shared between the subtypes (Figure 1).

3. Genome-Wide Approaches to Mapping PPAR Subtype-Specific Transactivation

The PPAR transcriptome in cells and tissues has been mapped by ectopically expressing a particular subtype and/or treating with a specific agonist and mapping the changes in gene expression using array technology. More recently the combination of chromatin immunoprecipitation (ChIP) with microarray analysis (ChIP-chip), high throughput sequencing (ChIP-seq), or with pair end-tagging sequencing (ChIP-PET) has allowed the mapping of the PPAR cistrome in cells and tissues following various treatments. These global techniques have led to important insights into the role of the different PPAR subtypes in the regulation of metabolism and differentiation and into the action of PPAR agonists.

3.1. Expression Array Studies. Although microarray studies of NIH-3T3 fibroblast overexpressing PPARβ/δ have confirmed that this PPAR subtype rightfully is recognized as being an inducer of genes involved in energy expenditure and β-oxidation of fatty acids [16] it appears that at least in insulin-resistant (*db/db*) mice, activation of this pathway by PPARδ-specific agonists is limited to the muscles. Administration of PPARβ/δ agonist ameliorates both muscle and liver insulin

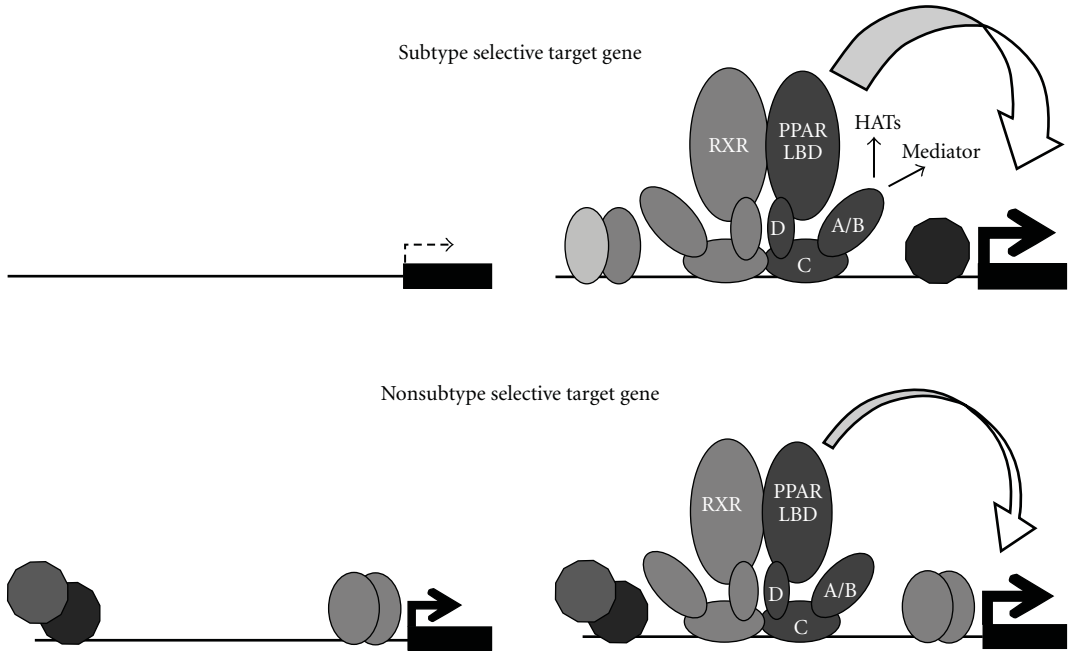

Figure 1: **The PPAR A/B-domains potentiate transactivation of the highly subtype selective target genes.** Illustration of the differential requirement for the PPAR A/B-domains in the transactivation of highly subtype selective and non-subtype selective target genes. The PPAR subtype specific target genes are generally expressed at very low levels in the absence of PPARs, but expression is dramatically increased upon introduction of full length exogenous PPAR. The activities of the PPAR A/B-domains are necessary to obtain this potent induction of the highly subtype specific target genes, presumably by facilitating recruitment and tethering of histone acetylase complexes (HATs) and the Mediator complex. Conversely, non-subtype selective PPAR target genes are usually already expressed at high levels in the absence of PPARs, and their expression levels are only increased by a few fold in response to ectopic PPAR expression. The PPAR A/B-domains appear to be dispensable for transactivation of this group of target genes.

resistance in *db/db* mice by lowering the hepatic glucose output, increasing glucose disposal, and inhibiting fatty acid release from the adipose tissue. Surprisingly however, the expression arrays only detected induction of carnitine palmitoyltransferase 1 (*Cpt1*), a key gene in fatty acid β-oxidation, in the muscles, whereas the pathways responsible for the increased glucose disposal appeared to be hepatic fatty acid synthesis and pentose phosphate shunt that generates NADPH to provide reducing power for lipid synthesis [104]. Consistent with previous reports [105], PPARβ/δ agonist treatment did lead to increased β-oxidation rates in the muscles, suggesting that PPARβ/δ promote insulin sensitivity by consuming glucose through induction of hepatic fat-production in combination with a counterbalancing fat burning in muscle [104]. A recent study comparing hepatic gene regulation between wild type and PPARβ/δ -or PPARα knockout mice showed that while PPARα expression is highly upregulated during fasting, *Ppar*β/δ mRNA is downregulated. In accordance with this finding, the differences in gene expression between the wild type and PPARβ/δ knockout mice were more pronounced in the fed state but surprisingly a relatively large subset of genes were downregulated in a PPARβ/δ-dependent manner during the fast. Interestingly, there is only limited overlap between the hepatic genes regulated in a PPARα or PPARβ/δ-dependent manner in the fed state, while a large proportion of the target genes appear to be regulated by both subtypes in the fasted state. It is evident that some of the differential effects on liver gene expression in the two knockout mouse models may be due to indirect effects imposed by other tissues; however, in agreement with the general perception of PPARβ/δ being an inhibitor of inflammation, pathway analyses showed that several gene sets involved in these processes were enriched in the knockout mice in the fed state. In the fasted state, the electron transport and oxidative phosphorylation pathways were decreased and in both metabolic states deletion of PPARβ/δ resulted in downregulation of genes involved in lipoprotein metabolism and carbohydrate metabolism, which included glycogen metabolism, glycolysis, gluconeogenesis, and the pentose phosphate pathway [106]. In agreement with the conclusion from the *db/db* mouse study indicating that PPARβ/δ is an important regulator of glucose disposal and utilization [104], these changes in gene expression resulted in significantly increased fasting plasma glucose levels in the PPARβ/δ knockout mice [106]. Besides studies of the PPARα knockout mice [106, 107], other approaches to determine the PPARα transcriptome includes overexpression studies in fibroblasts [16], exposure of hepatoma cell lines to synthetic PPARα agonist [108], and *in vivo* examinations of the alterations in the gene expression pattern of mouse and monkey liver [109, 110] and mouse intestine [111] in response to agonist treatment. These studies unanimously

report that PPARα is the major inducer of β-oxidation in these tissues. In addition, it has also been reported that PPARα function to repress amino acid catabolism [112].

The PPARγ transcriptome of the adipogenic 3T3-L1 cell line has been characterized in several expression array studies because of the high endogenous expression level of PPARγ and the observation that white adipose tissue is essential for the insulin sensitizing effects of the TZD PPARγ agonists [113, 114]. Studies using these agonist to drive the differentiation of preadipocytes show that the TZDs are potent activators of adipogenesis and induce or enhance the expression of adipocyte specific markers and genes involved in lipid storage and transport, but also lipid hydrolysis and oxidation [115, 116]. Interestingly however, when mature adipocytes are exposed to TZDs for a few hours up to a couple of days, it leads to decreased expression of both PPARγ itself and of lipid storage and adipokine genes while fatty acid activation and degradation is induced [116, 117]. The global effects of TZD treatment on gene expression has also been investigated in macrophages [118], colon cancer cells [119], aorta [120], and dendritic cells [121] with regulation of genes involved in lipid metabolism and inflammation being a consistent finding in these studies.

Other approaches to annotate the PPARγ transcriptome include analysis of NIH-3T3 fibroblasts transduced with retrovirus encoding PPARγ2 [16, 122] and adenoviral overexpression of PPARγ1 in the liver of PPARα knockout mice for 2–6 days [20]. The latter resulted in hepatic steatosis, thus underscoring the lipogenic potential of PPARγ. In order to increase the chances of identifying genes targeted directly by PPARγ as opposed to genes being differentially expressed as a consequence of secondary regulation, we made use of acute adenoviral expression of the PPARs in NIH-3T3 fibroblasts, which have very low levels of endogenous PPARs [11]. This system allows us to induce rapid expression of the PPARs and subsequently evaluate the immediate effects on target gene activity at the mRNA level within 8 h after transduction, whereby secondary effects (e.g., induction of endogenous PPARs) on gene expression were minimized. By combining this cellular system with expression array analysis, we found that ectopic PPARγ2 expression in combination with TZD treatment acutely activated a large number of known and novel target genes in the NIH-3T3 cells. Both expression of genes involved in lipid anabolic and catabolic pathways were induced but the net outcome was lipid accumulation [46]. These results corroborate previous findings from our lab that when expressed at adipocyte levels, PPARγ2 is a strong transactivator capable of activating most PPAR target genes, even if other subtypes expressed at the same level would be better activators [11].

3.2. ChIP-Chip and ChIP-Seq Studies. The generation of genome-wide profiles of PPAR, RXR, and cofactor occupancy using the ChIP-chip, ChIP-seq, and ChIP-PET technologies has significantly increased the understanding of PPAR-mediated transactivation. First, these studies have shown that most target genes have multiple PPAR binding sites not only in the proximal promoter region but also in introns and at distant positions up- and downstream of the gene. Notably, about 50% of all PPAR:RXR-binding sites are found in introns. This distributions of binding sites reflects that of most other nuclear receptors [123–125] and of the several other transcription factors [126–128]. While these studies provide invaluable help in determining the position of binding sites, they also complicate the picture of functional PPREs and underscore the weaknesses of traditional promoter and enhancer characterization, where often only the proximal promoter or genomic sequences immediately upstream of the transcription start site are cloned in front of a luciferase reporter.

The first PPAR cistrome to be published was that of PPARγ in differentiating and mature 3T3-L1 adipocytes as mapped by ChIP-seq [129] and ChIP-chip [130]. Subsequently, the PPARγ cistrome in 3T3-L1 cells has also been mapped by others using ChIP-chip [131], ChIP-seq [132], and ChIP-PET [133]. The mappings revealed between 2730 and 7000 genomic PPARγ binding sites, depending on the method and false discovery rate employed. Notably, all genes encoding proteins involved in fatty acid handling and storage as well as lipolysis were found to have adjacent PPARγ:RXR binding sites, but surprisingly this was also true for most genes encoding enzymes involved in steroid metabolism [133], glycolysis, the pentose phosphate pathway, and the TCA cycle [129, 131]. Interestingly, a significant overlap between PPARγ:RXR binding sites and binding sites of CCAT/enhancer binding protein (C/EBP) α and -β was found [129, 130] indicating that the cooperativity between PPARγ and members of the C/EBP family in the regulation of adipocyte gene expression (reviewed previously [134]) takes place on a much larger scale than previously anticipated.

A recent study profiling the PPARγ cistrome in primary mouse macrophages found that there was only very limited overlap between the genomic sites bound by the receptor in this cell type and in adipocytes. Interestingly, the transcription factor PU.1, which is involved in monocyte development and not expressed in adipocytes, was enriched at the macrophage specific sites. This study thus addresses the intriguing question of how cell type-specific PPARγ transactivation is achieved at the level of chromatin binding and suggests that tissue-specific factors may play a role in facilitating PPARγ binding to the tissue selective binding sites [132].

So far, genome-wide cistromes are not available for other PPAR subtypes, but recently PPARα binding sites in a human hepatoma cell line was mapped by ChIP-chip using an array covering promoter regions from 7.5 kb upstream to 2.5 kb downstream of the transcription start site. This study showed increased binding of PPARα to 4220 genomic regions in response to agonist treatment [135]. The group of genes assigned to these binding sites that were upregulated as determined by microarray analysis, clustered as being involved in sterol and lipid biosynthetic pathways, which is surprising given the general perception of PPARα as an inducer of lipid degradation. The downregulated genes were involved in innate and humoral immune response, which is consistent with the well-described anti-inflammatory activity of PPARα (Reviewed previously [5]).

4. Summary and Perspectives

As described in this paper, several molecular mechanisms conferring subtype-specificity to the PPARs or leading to preferential activation of a specific PPAR subtype in a certain cellular context have been elucidated. (1) The PPAR subtypes bind to the individual genomic PPREs with differential affinity. (2) The PPARs are activated by different ligands. (3) The PPAR subtypes display differential affinity towards various cofactors. (4) PPAR transcriptional activity is modulated by subtype-specific posttranslational modifications. (5) The PPAR A/B-domains potentiate subtype-specific activation of target genes while restricting nonselective target gene activation.

Most likely, PPAR subtype-specificity is maintained through the concerted effects of the regulatory mechanisms exerted by the individual PPAR domains or communicating PPAR and RXR domains. However, the current knowledge strongly suggests that the relative importance of these contributions is differential and that especially the A/B-domains are important mediators of PPAR subtype-specificity. Future studies should aim at pinpointing the exact sections of the A/B-and E-domains, and potentially the D-domain, that are involved in maintaining PPAR subtype-specificity and to fully elucidate the molecular mechanisms underlying this activity.

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Review Article

PPARG: Gene Expression Regulation and Next-Generation Sequencing for Unsolved Issues

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Peroxisome proliferator-activated receptor gamma (PPARγ) is one of the most extensively studied ligand-inducible transcription factors (TFs), able to modulate its transcriptional activity through conformational changes. It is of particular interest because of its pleiotropic functions: it plays a crucial role in the expression of key genes involved in adipogenesis, lipid and glucid metabolism, atherosclerosis, inflammation, and cancer. Its protein isoforms, the wide number of PPARγ target genes, ligands, and coregulators contribute to determine the complexity of its function. In addition, the presence of genetic variants is likely to affect expression levels of target genes although the impact of *PPARG* gene variations on the expression of target genes is not fully understood. The introduction of massively parallel sequencing platforms—in the Next Generation Sequencing (NGS) era—has revolutionized the way of investigating the genetic causes of inherited diseases. In this context, DNA-Seq for identifying—within both coding and regulatory regions of *PPARG* gene—novel nucleotide variations and haplotypes associated to human diseases, ChIP-Seq for defining a PPARγ binding map, and RNA-Seq for unraveling the wide and intricate gene pathways regulated by PPARG, represent incredible steps toward the understanding of PPARγ in health and disease.

1. Introduction

Gene transcription requires an elaborate network of intra- and extracellular signals, such as hormones, xenobiotics, micro- and macronutrients (lipid metabolites, vitamins, ions, etc.) and drugs, that converge to the nucleus following different pathways, resulting in the expression of each gene in each tissue. It is a current assumption that transcription is mostly shaped by environmental factors, acting both via direct and indirect mechanisms. Translating exogenous and endogenous signals which affect gene transcription, into a cellular physiological response requires the coordinated action and the fine tuning of transcription factors (TFs) acting at DNA level, including those belonging to the nuclear receptor (NR) superfamily [1, 2].

The human NR superfamily comprises 48 ligand-inducible transcription factors that respond to a variety of *stimuli* and are able to modulate their transcriptional activity through conformational changes [3]. The most extensively studied members of this TF superfamily are the Peroxisome proliferator-activated receptors (PPARs, also known as nuclear receptor family 1C, NR1C). Crystallographic studies have shown that all NRs superfamily members, and among them PPARs, share common structural features which include a poorly conserved N-terminal A/B domain (with a potential transactivation domain AF-1), a highly conserved DNA binding domain (DBD), with two zinc finger motifs, a C-terminal region containing the ligand-binding domain (LBD) and confer the ligand-dependent transactivation function (AF-2), and a length-variable hinge region between the DBD and LBD [4].

PPARs function as heterodimers with retinoid X receptor (RXR, NR2B), and their TF activity is regulated by the binding of ligands, the interactions with coregulators (both activator and repressor proteins), and DNA-binding sites [5].

In a basal state, the PPARs-RXR complex is bound to corepressors and is transcriptionally inactive. The binding of endogenous or synthetic ligands to the AF-2 domain promotes a conformational change, which results in the release of corepressors, allowing the recruitment of—and the interaction with—coactivators [6]. These proteins either possess or recruit proteins with, histone acetyltransferase (HAT) activity allowing the RNA polymerase II complex to bind and initiate transcription of target genes [4, 7].

PPAR genes are expressed in different organs, such as reproductive and major insulin target organs—liver, white and brown adipose tissue (WAT and BAT, resp.), and skeletal muscle—cardiac tissue, and others [8]. They have been implicated in different biological pathways ranging from lipid and glucose homeostasis and insulin sensitization, to control of cell proliferation/differentiation, tissue injury and wound repair, inflammation, and immunity [9].

PPARs occur in three different isotypes, termed α (NR1C1), β/δ (NR1C2), and γ (NR1C3), encoded by three separate genes, localized on human chromosome 22q12-q13.1 [10], 6p21.2-p21.1 [11], and 3p25.2 [12], respectively, and expressed in a tissue-specific manner. All three isotypes are able to bind, with different affinities, the same consensus response element on DNA, named peroxisome proliferator response element (PPRE) [13]. Despite their substantial homology and evidence of shared transcriptional targets, the physiological functions of each PPAR are unique.

PPARγ, the best studied member of the PPAR family, is induced during the differentiation of preadipocytes into adipocytes and is expressed most abundantly in WAT and BAT [14]. PPARs have a great relevance in the human physiology, but they are also involved in the etiology of many human diseases, and, in this contest, PPARγ is of particular interest because of its pleiotropic functions: it plays a dominant role in the control of the expression of a plethora of genes related to a wide spectrum of physiological processes, such as adipose cell differentiation, metabolism, atherosclerosis, inflammation, and cancer.

Alternative promoters usage and mRNA splicing give rise to at least seven PPARγ isoforms: the 5′ end of the mRNA consists of alternately spliced exons A1, A2, B, C, and D in various combinations. Each splice variant differs only in the 5′-UTR: the exons at the 5′ end account for little or none of the final translated PPARγ protein [15, 16]. In particular, the well-studied PPARγ1 and PPARγ2 have distinct N-terminal portions, differing by the presence of extra 28 (mouse) -30 (human) amino acids for PPARγ2 isoform [17, 18]. PPARγ1, whose expression can be regulated by multiple promoters (γ1, γ3, and γ4), is expressed in all PPARγ-expressing tissues and cells whereas PPARγ2 is almost exclusively found in adipose tissue [19, 20], where it exerts a pronounced adipogenic activity.

Two *PPARG* gene 3′ splice variants—lacking almost the entire LBD— γORF4 and PPAR$\gamma 1_{tr}$, have been identified as dominant negative *versus* PPARγ wild type [21, 22]; hence they are not able to promote the transactivation of *PPARG* target genes.

The significant number of PPARγ isoforms, as well as for other NRs, strongly suggests that splicing plays an important role in the nuclear receptor functioning. Moreover, the large number of PPARγ target genes, ligands, and coregulators (both coactivators and corepressors) confers additional complexity to PPARγ function. In addition, alterations in the PPARγ trans-activating ability have to be analyzed in the light of environmental factors, genetic background, and the interactions among them [23].

This paper summarizes the transcriptional regulation exerted by PPARγ on key target genes and the effects of the most frequent *PPARG* gene nucleotide variations on its function, also approaching to the next generation sequencing (NGS) technologies that will allow an unprecedented level of accuracy and completeness to the study of PPARγ and other transcription factors. Indeed, this paper describes in detail how these novel technologies will allow to identify novel genetic variants and polymorphisms (SNPs) in PPARG gene, to draw high-resolution binding map of PPARγ across the genome, and to understand the transcriptional regulation of PPARγ-modulated genes.

2. PPARG and Gene Expression Regulation (Target Genes)

PPARγ controls several arrays of biological processes by modulating the expression of specific target genes mainly through a ligand-dependent mechanism [24]. PPARγ ligands include a surprisingly diverse set of natural ligands [25] such as prostaglandin PGJ2, linolenic, eicosapentaenoic, docosahexaenoic, and arachidonic acids, and synthetic ligands, such as the thiazolidinediones (TZDs), L-tyrosine-based compounds, several nonsteroidal anti-inflammatory drugs, and a variety of new chemical classes.

The PPARs, and PPARγ among these, like many nonsteroid members of the NR family, function as obligate heterodimers with RXRs [26]. The heterodimers are able to bind PPRE, consisting of direct repeats of the canonical AGGTCA half-site separated by one base pair (DR1) together with the upstream specificity element AAACT [13, 27]. Typically, RXRs do not function alone but rather serve as master regulators of several crucial regulatory pathways, in combination to different NRs' partners.

Recently this issue has been better elucidated through the use of standard chromatin immunoprecipitation (ChIP) coupled with massive sequencing on NGS platform (described more in detail in Section 4.2 entitled "*Transcription Factors and ChIP-Seq*") [28]. In this study the authors profiled PPARγ- and RXR-binding sites throughout adipogenic differentiation (Figure 1). They identified differential spatial and temporal recruitment of PPARs and RXR to target sites during adipogenesis; in particular, at the onset of differentiation the DNA occupancy by RXR alone was detected. Interestingly, immediately afterwards, many of these sites become occupied by RXR and PPARδ, lowly expressed into adipocytes. Moreover, through the early days of differentiation, they observed a different temporal and compositional pattern of occupancy with a switch between PPARδ and PPARγ, which becomes the main RXR partner throughout the adipogenesis, coinciding with a significant increase in

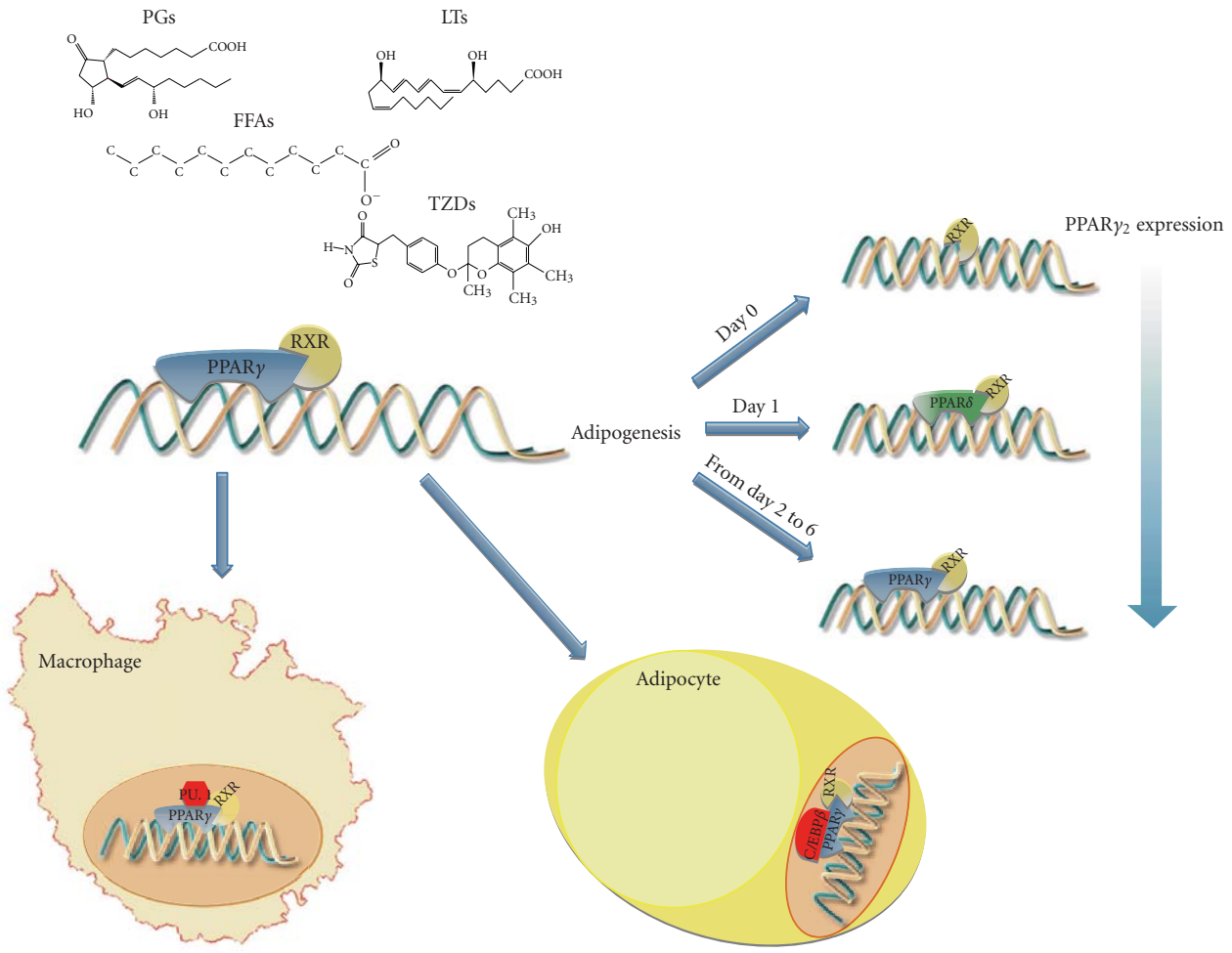

Figure 1: Novel insight into PPARG world trough new approaches. In the presence of ligands (upper), PPARγ binds to its PPRE as heterodimer with RXR to activate or repress target genes' expression. The figure summarizes novel molecular mechanisms of PPARγ obtained through ChIP-seq. PPARγ- and RXR-binding sites detected by ChIP-seq reveal different spatial and temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis (right panel, study from [28]). *PPARγ in Adipocytes and Macrophages:* tissue-specific regulatory regions employ cell-type-specific coregulators, C/EBPβ in adipocytes and PU.1 in macrophages (lower panel; ChIP-Seq study from [31]).

both PPARγ1 and PPARγ2 expression [28–30]. The binding of RXR alone—in the early stage of differentiation—on the target sites later bound by PPARγ:RXR complex has been hypothesized to serve as a signature needed for subsequent PPARγ-dependent binding and/or activation of transcription for target genes [28].

The modulation of transcription depends on the recruitment of cofactors able to remodel the chromatin structure making it more accessible to the basal transcription machinery recruitment and assembly at the core promoter of target genes [32, 33]. Indeed, it has been widely assumed that chromatin accessibility to the transcriptional machinery, through histone modifications (acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, deimination, ADP ribosylation, and proline isomerization) represents, a very relevant process into gene expression regulation [34–37]. In light of this, the different temporal—and compositional pattern—of occupancy on these binding sites, observed by Nielsen and colleagues (2008) [28] is likely to be required for the chromatin remodeling to such *loci*, rendering these regions accessible for PPARγ:RXR binding and the subsequent transactivation of target genes.

Although PPARγ:RXR heterodimer controls the expression of many inducible genes, transcription is regulated both globally and locally by different factors. Determining which cell-specific coactivators/corepressors are recruited by PPARγ in different cell types, and how these may contribute to chromatin modifications and differential gene expression, represents a crucial issue for fulfilling our gap towards the understanding of PPARγ biology and function.

The currently assumed dogma, mostly referred to all TFs, is that the cell-type-specific trans-activating ability is due to the cooperative binding to other cell-type-selective factors, which specifically "drive" the TF to its target genes.

However, although it is well known that PPARγ is able to modulate target genes' expression in some cell types but

not others, the molecular mechanisms underlying its ability are not yet well elucidated. Differential binding of PPARγ to the PPRE of target genes or its differential activity at DNA level (i.e., in chromatin remodelling) has been claimed as the putative mechanisms accounting for the cell-type specificity of its action [38].

A very recently published work of Lefterova and colleagues (2010) [31] has provided novel intriguing insight into the molecular basis of cell-type-specific gene expression in primary mouse adipocytes and macrophages. The authors, by using ChIP-Seq (see Section 4.2), identified the molecular signatures of PPARγ binding, disclosing distinct macrophage- and adipose-specific PPARγ-binding sites overall the genome. Moreover, they shed light on the cell-specific expression of PPARγ target genes, demonstrating the tight and well-regulated cooperation of PPARγ and other crucial cell-type-specific proteins (PU.1 and C/EBPb, nearby macrophage- and adipocyte-specific target genes, resp.) (see Figure 1). "PPARγ dances with different partners" [38], and all the biological processes PPARγ-modulated can be thus attributed to a differential recruitment of coactivators and corepressors functioning as scaffolds for chromatin remodelling enzymes.

The coactivators of PPARγ include well-established cofactors such as p300/CBP, p160, and PGC-1 (PPARγ coactivator-1), as well as TRAP220 (thyroid hormone receptor-associated protein 220 or PBP, PPARγ-binding protein) [39, 40], ARA70 (Androgen Receptor-Associated protein) [41], and PRIP (PPARγ-interacting protein, ASC-2/RAP250 /TRBP/NRC) [42].

In the absence of ligand, PPARγ recruits corepressors such as silencing mediator for retinoic and thyroid hormone receptors (SMRT) and the nuclear receptor corepressor (N-Cor), which bind repressive enzymes such as histone deacetylase enzymes (HDAC), and particularly HDAC3 [43] or the histone methyl transferase (HMT) SUV39H1, which specifically methylates histone H3 at lysine 9 (H3K9) [44]. RIP140 (receptor-interacting protein) may also be a component in the corepressor complex [45, 46]. The ability of PPARγ to repress transcriptional responses to diverse signaling pathways is an essential aspect of its biological activities, but mechanisms determining the specificity and functional consequences of the process known as transrepression remain poorly understood. However, PPARγ can also influence gene expression independently of its binding to the PPRE. Indeed, PPARγ-dependent repression of inflammatory gene expression occurs through interference with the action of NF-kB via transrepression [47]. Moreover, the activity of other transcription factors, for example, AP-1 and STAT-1, can be inhibited by PPARγ via direct interaction or by competition for limiting supplies of coactivators [48].

PPARγ transactivation ability is induced by ligand-dependent and independent mechanisms. The AF-1 domain of PPARγ is the ligand-independent activation domain that regulates the specificity of PPARγ transcriptional activity during adipogenesis [49]. The presence of an extra 30 aminoacids in the AF-1 domain of PPARγ2 isoform that makes it a better transcriptional activator than PPARγ1 [50]. Indeed, it was shown that PPARγ2 is about 10 times more active than PPARγ1 in ligand-independent transcriptional activation, through this domain [50, 51]. Thus, PPARγ1 and PPARγ2 may have different functions, with PPARγ1 being used when the ligand is abundant whereas PPARγ2 would be crucial under conditions of low ligand concentration, such as it might occur in early adipocyte differentiation [51]. However, the ligand-independent transactivation through the AF-1 domain, common to PPARs, is poorly understood and beyond the scope of this paper.

2.1. PPARG-Modulated Pathways: Obesity and Inflammation. The biological activities of PPARγ are very wide but it is generally acknowledged as a transcriptional regulator of lipid and glucose metabolism, since it is highly expressed in adipocytes and controls the expression of several adipocyte-specific genes involved in lipid synthesis and storage, insulin signalling, and adipokine production [52, 53].

PPAR$\gamma^{-/-}$ mice models, with selective ko in three metabolic tissues (adipose tissue, skeletal muscle, and liver), have revealed that PPARγ is a master regulator of adipogenesis; PPARγ deficiency and/or partial disruption in any of these tissue severely affects whole body lipid homeostasis, altering insulin sensitivity. The essential role of PPARγ in adipogenesis was revealed by inactivation of both PPARγ1 and PPARγ2 in the adipose tissue [54, 55].

PPARγ2 depletion was shown to dramatically diminish adipose tissue (WAT) mass—due to a strongly reduced adipocyte differentiation observed also *in vitro*—providing protection against high-fat-diet induced weight gain and to determine an impairment of insulin sensitivity [56]. In this context, a common aminoacid polymorphism (Pro12Ala) in PPARγ2 (described in detail in the next section) has been associated with type 2 diabetes and has been suggested to induce a modest impairment of transcriptional activation due to decreased DNA-binding affinity [57].

Conflicting results have been reported by Medina-Gomez and colleagues (2005) [58]. Although they observed a clear *in vitro* defect in fat cell differentiation, they demonstrated that PPARγ2-depletion is directly linked to insulin resistance, without alteration of *in vivo* adiposity, even in presence of a high-fat diet. The possible explanation of a residual presence of fat depots in these ko mice strongly suggested that PPARγ1 was able to initiate, at least in part, adipocyte differentiation. In addition, it has been shown a global deregulation in the repartitioning of lipids in these mice models. A complex cross-talk between these metabolically active tissues (liver, adipose tissue, and muscle) appears to be essential for energy balance.

Other studies have demonstrated that mouse models of heterozygous PPARγ (PPAR$\gamma^{-/+}$), with a decreased *PPARG* gene expression, show improved insulin sensitivity compared to wt mice [59, 60] although the reduced *PPARG* gene expression was associated with decreased metabolic rate and physical activity [61]. Reduction of *PPARG* gene expression in the PPARG$^{-/+}$ mouse model is associated with a mild decrease in PPARγ protein levels [62], suggesting that modulation of PPARγ protein levels, rather than mRNA itself, may play a role in determining PPARγ activity in adipocytes. Indeed, regulation of PPARγ protein translation

is expected to be tightly regulated. Althoguh a moderate decrease of PPARγ protein may protect against high-fat diet-induced insulin resistance, its complete lack in adipocytes is deleterious to lipid and glucose metabolism as well as insulin sensitivity in the presence of a high fat diet, as shown in most, but not all, studies of adipose-specific PPARγ knockout mouse models [63].

A considerable role for PPARγ in macrophage lipid metabolism has been also clearly demonstrated [64]. The involvement of PPARγ in regulating lipid metabolism in macrophages was initially suggested by the discovery of CD36, member of scavenger receptor family that mediates uptake of oxidized LDL, as a PPARγ target gene in macrophages [65].

PPARγ has a similar function in macrophages and adipocytes as it modulates lipid homeostasis in both cell types via regulation of genes including *LPL* (lipoprotein lipase), *ACAT* (acetyl coenzyme A acetyltransferase) and *PLA* (phospholipase A) genes, and the levels of *FFAs* (free fatty acids), *PGs* (prostaglandins), and *LTs* (leukotriens). PPARγ-deficient mice have provided clues to an antiatherogenic role of PPARγ since these mice showed a significantly impaired lipid homeostasis in the arterial wall and enhanced atherosclerosis development [66, 67]. The molecular mechanisms underlying the antiatherogenic properties of PPARγ involve stimulation of cholesterol efflux from macrophages into the plasma and inhibition of monocyte recruitment into the developing atherosclerotic lesion [67]. Interestingly, macrophage-specific ablation of PPARγ resulted in high rates of insulin resistance suggesting that macrophage PPARγ may exert a protective role in obesity [68].

Indeed, it is becoming always more evident a functional link between macrophage activity, inflammation, adipose tissue, and type 2 diabetes mellitus (T2DM) [69, 70].

In a physiological state, macrophages residing in fat mass are responsible for keeping in the adipose tissue an anti-inflammatory environment, conferring an adequate degree of insulin sensitivity. In pathological conditions, such as obesity, adipose tissue is continually under metabolic stress, leading to the constitutive activation of stress and inflammatory pathways, resulting in macrophage accumulation within the adipose tissue. Proinflammatory macrophages infiltrate adipose tissue, exacerbating local inflammation and giving rise to insulin resistance [31]. In this scenario, even though PPARγ is not required for macrophage differentiation or phagocytic activity, its deficiency is associated with the constitutive onset of an inflammatory milieu, in turn resulting in an enhanced susceptibility to diet-induced obesity, glucose intolerance, and insulin resistance [31].

All these findings indicate the crucial role of PPARγ in adipocytes as well as macrophages, although, to date, only two studies [28, 31] have analyzed in-depth the localizations and mechanism of PPARγ recruitment within this cells, trying to address these quite complicated but fundamental questions.

The recent technological advances—such as high-throughput sequencing methods and innovative techniques for following the three-dimensional interactions of chromosomes in the nucleus—allow to rapidly uncover new layers of complexity within PPARγ world. By using these approaches, it would be of interest to analyze the selective pattern of PPARγ activity within specific cell types, with the final aim to understand how its alterations may affect human health.

Several studies have been performed about *PPARG* gene and its main isoforms, namely, PPARγ1 and -γ2, even though other variants have been disclosed [16, 21, 22]. In the near future it would be of great relevance to address also the role of newly described isoforms in physiologic as well as pathologic conditions.

However, the phenotypic effects described for human PPARγ variants, and various mouse models with altered expression of PPARG mRNA, and often conflicting results from different studies so far performed, unequivocally depict a highly complex picture of PPARγ functions and biology.

3. PPARG Target Genes: Polymorphisms, Haplotypes, and Gene Expression

PPARG gene nucleotide variations, and their possible phenotype consequences, have been widely and conversely analyzed in the last two decades [7, 23, 71, 72]. Since PPARγ is a transcriptional factor involved in the regulation of several target genes in many tissues, the primary consequence of a genetic variant is likely to be an alteration of expression levels of target genes.

Although the impact of common single nucleotide polymorphisms (SNPs) in *PPARG* gene on the expression of its target genes is not fully understood, an SNP and/or a combination of them (haplotype) may affect the *PPARG* transcript itself and in turn its ability to regulate gene expression [23].

What does really happen to PPARγ activity in the presence of a DNA polymorphism and/or mutation? Few studies have directly considered the real effect of *PPARG* variants on the *PPARG* expression itself and of its target genes, evaluating the alteration of its binding affinity to PPRE, the promoter efficiency, and other factors that may affect its transactivation ability [73–84].

Indeed, most of the studies about nucleotide variations in *PPARG* have mainly focused on the association between a DNA variant and a specific phenotype (such as predictors of diabetes, obesity, and BMI) [57, 85–89] or related biochemical markers (plasma levels of hormones, peptides, or metabolites) demonstrated—or just supposed—to be transcriptionally regulated by PPARγ itself [78, 90–102].

The most widely studied SNP in PPARG gene [57, 72, 73, 83], Pro12Ala, occurs in PPARγ2 isoform and has been very often associated to clinical consequences and several alterations of physiological metabolic status [57, 72, 73, 85–87, 89, 103]. About the direct effect of this polymorphism on PPARγ activity, some functional studies have revealed that Pro12Ala confers to PPARγ2 a decreased binding affinity to PPRE and a reduced transactivation ability, both in a luciferase reporter gene assay and in TZD-induced adipogenesis [73, 75].

It has been also shown that in human adipose tissue there were no significant differences in the basal expression levels

of some PPARγ target genes (*UCP-2, LPL, p85aPI3K,* and *PPARγ1*) between obese Pro12Ala and Pro12Pro carriers, except for a reduction of about 40% observed for *p85aPI3K* gene in the omental fat [78].

To explain the observed discrepancies, between *in vitro* and *in vivo* studies, Kolehmainen et al. speculated that subjects Ala12 homozygous have more relevant differences in gene expression activation compared to Ala12 heterozygous; moreover, it must be considered the interaction of genetic and environmental factors and observed tendency for a higher expression of PPARγ2 in the subcutaneous fat depots of Pro12Ala carriers [78].

In addition, Heikkinen and colleagues (2009) [83] have recently highlighted the importance of metabolic context in modulating Pro12Ala effects, reporting or confirming several associations between this PPARG variant and phenotype traits (Table 1). They have shown that in WAT of Ala/Ala mice some genes were downregulated, whereas a great number of genes were upregulated in muscle. Furthermore, they have interestingly suggested that Pro12Ala might be implicated in G protein function, in sensitization of adiponectin signaling and altered cofactors recruitment [83].

To investigate how Pro12Ala might influence gene expression of molecular targets and in turn the response to exogenous *stimuli*, the functional properties of N-terminal domain should be also considered. In particular, this SNP occurs at position 12 in the N-terminal region of PPARγ2 and shows different transactivation ability than PPARγ1, differing only in its N-terminus. As mentioned above, the additional residues at N-terminus of PPARγ2, encoded by the exon B, confer a trans-activating ability up to tenfold greater than PPARγ1, indicating that γ2 isoform is more potent to induce the expression of target genes in the absence of activating ligands [50]. Pro to Ala amino acid change might affect the secondary structure of the protein and consequently its functionality [110]. Indeed, it has been recently shown that proline residues, although counteracting α-helix formation**,** fit well only into N-terminal of α-helices, positively modulating the proteins' stability [111].

The direct relationship between PPARγ transcriptional ability and an SNP in the regulatory region of *PPARG* gene, C-2821T, was reported by Muller and colleagues (2003) [79] in the Pima Indians population. This polymorphism, in strong linkage disequilibrium (LD) with Pro12Ala, falls within a putative E2-box in a binding site for δEF1, a transcriptional repressor. Since it has been shown that C-2821T confers to PPARγ an increased transcriptional ability [79], this SNP might be responsible for a decreased binding affinity between δEF1 and E2-box or for a reduced complex stability. Although the mechanism by which these alleles in LD ($-$2821T and Ala12) function remains uncertain, taken together these findings suggest that Ala12 may alter PPARγ2 transactivation ability, and $-$2821T may alter transcription of PPARγ2 isoform [79]. Other nucleotide variations, most of them gain- or loss-of-function mutations, have been described in *PPARG* gene.

A functional study about a rare gain-of-function PPARγ2 mutation, Pro115Gln, highlighted the relevance of phosphorylation at Ser 114 in reducing PPARγ activity; this variation in the ligand independent activation domain of PPARγ affects phosphorylation and renders PPARγ constitutively active, according to increased body mass index (BMI) observed in obese individuals [71, 108].

Another *PPARG* nucleotide variation, affecting PPARγ function, occurs in the same domain: a rare frameshift mutation, [A553ΔAAAiT]fs.185[stop186], resulting in a truncated protein in the DBD [76]. Within the same family, this premature stop codon was found in all individuals with insulin resistance and metabolic syndrome (MS), carrying also a similar mutation ([C1984ΔAG]fs.662[stop668]) in PPP1R3A (protein phosphatase1- regulatory subunit 3) [7, 76, 82]. This frameshift is a loss of function mutation that affects heterodimers formation and PPARγ interaction with PPRE in target gene promoters, resulting in a failed transactivation [76].

It has been shown, in *vitro,* that four rare mutations in the LBD of PPARγ result in a reduced PPARγ trans-activating ability in the presence of a synthetic ligand, affecting its ability to recruit cofactors, ligands, and RXRα: Pro495Leu (also called Pro467Leu), Val318Met (also called Val290Met), Phe388Leu, and Arg425Cys (Table 1) [7, 71, 74, 77, 81, 109]. The first two mutations affect two helices critical for the recruitment of ligand and cofactors and have dominant-negative activity against wild-type PPARγ. The latter, in contrast, are haploinsufficient mutations, occurring in a hydrophobic region that interacts with RXRα and ligands [7, 71].

In a more recent study, other rare mutations, occurring in DBD—Cys114Arg, Cys131Tyr, and Cys162Trp—and in LBD $-$315Stop and Arg357X—of PPARγ, have been described. These variants encode proteins unable to bind DNA, which lack the transactivation ability and show a dominant negative activity consisting in the competitive recruitment of coactivators with wild-type PPARγ (see Table 1) [82].

Furthermore, we recently reported a novel dominant negative mutation in PPARγ LBD, Ser289Cys, associated with colorectal cancer, dyslipidemia, hypertension, and overweight, but no with T2DM. The formation of an S-S bridge, between Cys289 and Cys285, might impede agonist positioning, explaining the demonstrated reduction of transactivation ability of mutant protein [84].

Although some studies have demonstrated the functional impact of *PPARG* nucleotide variations on protein activity and/or stability and on its ability to trans-activate target genes, most of *PPARG* variants have been associated with clinical effects [71, 88, 89] or plasma levels of a protein without investigating *PPARG* expression, isoform abundance, and mRNA levels of target genes. These non-functional association studies do not prove—allowing just to hypothesize—the altered expression of *PPARG* target genes. Moreover, it has been demonstrated that gene-gene and gene-environment interactions (i.e., diet, exercise, and age of onset of the disease) may greatly affect the contribution of a specific SNP to the resulting phenotype.

Taken together, these considerations contribute to explain the conflicting results about *PPARG* nucleotide variations obtained in different populations [57, 73, 78, 85–89, 104, 108, 110, 112–115].

Table 1: Nucleotide variations within coding and regulatory regions of PPARG.

Variant	Disease/trait	Outcome/Association	References
Pro12Ala	T2DM Insulin resistance	Conflicting results about association to T2DM and insulin resistance. When in LD with C1431T no protection from T2DM development	[57, 73, 83, 85, 87, 89, 102, 104, 105]
	Cardiac disease	Decreased incidence of cardiac disease	[103]
	HDL	Higher HDL cholesterol	[73]
	BMI	Reduction of BMI and fat and lean mass in nonobese (potentiated when in LD with C1431T) and BMI increase in obese individuals	[73, 83, 86, 91, 106]
	LPL	Reduced LPL activity and levels.	[83, 93]
	Leptin	Increased leptin levels	[92, 100]
	Adiponectin	Reduced adiponectin levels	[94, 96, 98, 101]
	Resistin	Reduced resistin levels	[97, 102]
	Bone features	Increase of total bone area and bone mineral content in Ala/Ala mice.	[83]
C1431T	BMI T2DM Leptin Resistin	Increased BMI and fat mass. Reduced risk of T2DM. Increased leptin levels. Increased resistin levels.	[88, 90, 91, 102, 107]
Pro115Gln	BMI	Increased BMI in obese individuals	[108]
[A553ΔAAAiT]	Insulin resistance T2DM Hypertension	In association to 662stop668 mutation in *PPP1R3A* is responsible of variable hyperinsulinemia, T2DM, hyperlipidemia, hypertension, and dyslipidemia.	[76]
Pro495Leu and Val318Met	Insulin resistance T2DM Blood pressure Partial lipodystrophy Protein plasma levels	Severe insulin resistance, TD2M, and early-onset hypertension. Dyslipidemia, preservation of abdominal fat with selective loss of gluteal and limb subcutaneous fat; inability to trap and store NEFA in the postprandial state, hepatic steatosis; reduced adiponectin plasma levels.	[74, 76]
Phe388Leu	Partial lipodystrophy and related features.	Lipodystrophy and dyslipidemia less severe, with absence of fat depots on the upper arms, phlebectasia of limb veins and of hepatic steatosis. Atherosclerosis, polycystic ovarian disease, increased C-peptide concentration, higher insulin resistance.	[77]
Arg425Cys	Partial lipodystrophy T2DM	Diabetes mellitus and hypertriglyceridemia previous to the development of limb and facial lipoatrophy; loss of subcutaneous fat, except for sc truncal fat. Hirsutism in a female carrier.	[109]
Cys114Arg Cys131Tyr Cys162Trp 315Stop Arg357X	Partial lipodystrophy and related features.	Reduced body fat, partial lipodystrophy of limb and gluteal depots, insulin resistance, hepatic steatosis, severe dyslipidemia, increased triglycerides levels, low HDL levels. Not for all: early-onset hypertension, cutaneous eruptive Xanthomata, pancreatitis.	[82]
Ser289Cys	Colorectal cancer	Colonic lesions, reduced restraint of cell proliferation both in vitro and in vivo, interference with the inflammatory pathway in tumor tissues and proximal normal mucosa	[84]
A-2819G	T2DM and diabetic retinopathy	Association with T2DM and proliferative retinopathy in diabetic females.	[88]
C-689T C-681G	BMI LDL	Increased BMI. Increased LDL levels.	[95, 99]
A-14G	Partial lipodystrophy MS	−14G associated with familial partial lipodystrophy subtype 3 (FPLD3). It has been found MS and a relative reduction of gluteal and extremities'fat.	[80]

For instance, Pro12Ala has been often associated with several diseases and phenotype effects [7, 71, 72], such as increased protection from T2DM onset and insulin resistance, decreased incidence of cardiac disease, higher HDL cholesterol, reduction of BMI in nonobese individuals [57, 73, 85, 87, 103], and increased BMI in obese individuals [86, 91]. A recent study in Russian population supports the association of Pro12Ala with improved insulin sensitivity and the protection from T2DM [89]. Moreover, a recent meta-analysis of 60 association studies also confirms the association between Ala12 allele and reduced T2DM risk [110].

In contrast, two recent conflicting studies in the Indian population have shown that Pro12Ala contributes to T2DM development [105] and do not exhibit any association with MS, T2DM, and obesity, respectively [102]. Gene-environment and gene-gene interactions might strongly contribute to the different Pro12Ala effects observed in the studied populations [23, 116].

This SNP has been also associated to altered plasma levels of LPL, leptin, adiponectin, and resistin. Indeed, it was shown, *in vivo*, that Ala12 allele is associated with a reduced LPL activity in postheparin plasma [93]; higher leptin levels were observed in Pro12Ala compared to Pro12Pro carrier women [92]. The effect of Pro12Ala on increased leptin levels is likely to be supported by a study in women with functional hyperandrogenism (FOH), in which the authors demonstrated that Ala allele was more frequent in FOH women than in healthy controls (36% versus 28%) and that leptin levels were higher in nonobese FOH women compared to controls [100].

Also the association between Pro12Ala and adiponectin plasma levels seems controversial: in the Japanese population Ala12 allele is associated with reduced serum adiponectin levels [94, 96] whereas no significant effect of this polymorphism on serum adiponectin was observed in polycystic ovary syndrome, healthy women, and in Asian Indians [98, 101].

In a study by Wang et al. (2004) in [97], it has been reported that Ala12 allele might affect the expression of a gene *RETN* encoding another adipose tissue-related molecule, the resistin, in the Chinese population; both heterozygous and homozygous Ala12 carriers showed lower plasma resistin levels compared to homozygous Pro12 carriers [97]. On the opposite, a recent report in an Indian population described no statistically significant differences in resistin plasma levels between Pro12 and Ala12 carriers (both heterozygous and homozygous) [102].

Pro12Ala has been described in linkage disequilibrium (LD) with another common *PPARG* variant, C1431T; this silent SNP, occurring in the exon 6, is also known as His477His and C161T of exon 6 [71]. It has been observed that when Pro12Ala is in LD with C1431T SNP, its protective effect on T2DM development disappears [87], while the consequences on BMI are potentiated [106].

The lack of functional findings within the above-described association studies and possible influence of ethnicity, environmental and genetic factors are likely to explain the controversial results so far reported. Moreover, due to LD between polymorphisms, determining the relative contribution of each SNP on the resulting phenotype is quite difficult.

For instance, different studies report that 1431T allele is associated with an increased BMI in obese Finns [91], a reduced risk of diabetes in a large Asian population [107], and not at all associated with T2DM, obesity, and BMI alteration [88].

About its effects on plasma proteins levels, C1431T has been associated with increased leptin levels [90]. Also Valve et al. [91] observed higher leptin levels in the obese women with C1431T than other obese women studied; this polymorphism was associated with increased fat mass, and, albeit in this study, the authors hypothesize that higher leptin levels were entirely due to increased adipose tissue mass and not directly linked to PPARγ-dependent transcriptional regulation [91].

Moreover, also resistin levels were significantly increased in individuals carrying C1431T whereas the Pro-C haplotype was more frequent in groups with lower resistin levels. In contrast, Pro-T and Ala-T haplotypes showed increased frequency in groups with higher resistin levels although statistically not significant [102].

Moreover, nucleotide variations in putative regulatory regions of PPARG have been associated, with different extents, to human diseases. Indeed, we recently identified A-2819G SNP in PPARG promoter and observed a significant association with T2DM and proliferative retinopathy in diabetic females whereas no linkage disequilibrium with Pro12Ala nor association with obesity was observed [88]. It has been hypothesized that this SNP might alter *PPARG* transcript abundance influencing in turn the expression levels of some PPARγ targets involved in the eye physiology [88].

Other three variants in PPARG putative promoter have been identified: A-14G, C-681G, and C-689T [71], even though their impact on PPARG transcription and function has not been completely elucidated. These polymorphisms may possibly affect the expression of some PPARγ molecular targets, since C-681G and C-689T were associated with increased plasma LDL levels and A-14G with a decreased activity of PPARγ4 promoter [80, 95, 99].

The introduction of massively parallel sequencing platforms, which have offered to researchers the possibility to identify, in a single experiment, point mutations and/or gross genomic rearrangements, within coding and yet unexplored regulatory regions of disease-causing genes, will surely represent a powerful tool to systematically discover variations in *PPARG* gene, possibly giving a causal link to human diseases.

4. Next-Generation Sequencing Technologies and Transcription Factors: ChIP-Seq, Targeted Resequencing, and RNA-Seq

Any genetic information is conveyed from DNA to proteins via mRNA, through a complex and finely regulated process. Unraveling how these genomic information are then translated into gene regulation has been for many decades

an intriguing field, fulfilled by many advances, speculations, and scientific debate. To achieve this tuned regulation, the concerted action of multiple *cis*-acting proteins, able to specifically bind *cis*-regulatory elements, such as promoters and enhancers, is needed [2, 117]. Moreover, since the basal transcriptional activity, resulting from the binding of so-called general TFs to the *core* promoter, is usually low, different site-specific TFs participate to the recruitment and/or the stabilization of general TFs' complexes, increasing the cell transcriptional rate. Moreover, histone-modifying enzymes may be recruited by other factors—binding to distal enhancer regions—and determine a favourable chromatin environment and a subsequent transcriptional enhancement. On the other hand, the transcription can be negatively modulated through the binding of repressive factors to distal silencer regions or the competition with TFs themselves.

To understand PPARγ-mediated phenomena in a specific cell/tissue/organ one cannot ignore the consideration that PPARγ is a transcription factor. Its mechanistic understanding represents a prerequisite for fine-tuning the therapeutic activities of *PPARG*.

More generally, several human diseases have been directly linked to alterations in the gene expression caused by defects in the structure and/or function of a key transcriptional regulator [2] although it is arguable that many other "TF-disease associations" still remain to be identified. Expanding our understanding of how site-specific TFs contribute to gene expression regulation, and in turn how alterations in both TF structure and activity may account for a specific disease phenotype, appears to be a crucial endpoint.

In this context, the specific case of PPARγ is not an exception, rather it is likely to be one of the most representative candidate genes in "TF-disease" association studies, for its involvement in many physiological and also pathological processes [7].

To this aim, the introduction of massively parallel sequencing platforms in the 2004, coupled with the recent advances in chromatin immune-precipitation (ChIP) followed by sequencing (ChIP-seq), has clearly revolutionized the way we approach to—and also study—different biological phenomena [118–121]. Although all the sequencing platforms commercially available use different sequencing chemistry and methodological procedures, also varying in the number of sequenced reads, read length and error characteristics, they all are based on the generation of libraries to sequence, and the miniaturization of individual sequencing reactions [121]. Unlike previously used tag-based sequencing methods, such as Serial and Cap Analysis of Gene Expression (SAGE and CAGE, resp.), Polony Multiplex Analysis of Gene Expression (PMAGE), NGS libraries do not require a prior step of cloning before sequencing. Moreover, a common feature of NGS platforms is the template binding to a solid surface or support (immobilization by primer or template) or its indirect immobilization (by linking a polymerase to the support) [122]. However, whatever are the sequencing chemistry and the methodological procedures used, a single NGS platform can generate a large amount of data up to 2 gigabases (Gb) of sequence reads per day, shifting the effort of researchers from biology to bioinformatics.

These platforms have been quickly applied to many scientific contexts, giving rise to many "Seq" protocols, specifically developed and suited for a particular research branch, from transcriptomics (RNA-Seq) to the targeted resequencing for the identification of disease-causing nucleotide variations (CNV-Seq and DNA-Seq), including DNA-protein interaction studies (ChIP-Seq) and genome-wide profiling of epigenetic marks (Methyl-Seq).

Although it is beyond any doubt that NGS platforms have changed the way we think about many scientific issues, one of the broadest and useful applications of this technology is towards the identification of the genetic causes of inherited diseases, both mendelian and multifactorial.

In light of this, deeply investigating *PPARG*—from DNA variations to gene expression and its regulation—will surely enhance our understanding about its involvement in health and disease. Identifying novel nucleotide variations, both point mutations and gross genomic rearrangements, within coding regions and yet unexplored intronic and regulatory regions of *PPARG* by targeted resequencing (on NGS platforms) will be the first brick towards building a more complete and detailed view of *PPARG* function and activity (summarized in Figure 2). In addition, the possibility to identify the exact position of its binding sites and thus draw a complete high-resolution binding map across the genome (by ChIP-Seq) [123, 124], combined with the large amount of useful whole transcriptome data obtained by RNA-seq, will provide an unprecedented level of accuracy and complexity than ever done (see Figure 1) [125].

4.1. NGS for the Targeted Resequencing. The whole genome resequencing of affected individuals' genomes by the use of NGS platforms is likely to represent the most powerful approach to identify single nucleotide variants and/or genomic rearrangements (insertions, deletions, and copy number variations) within disease-causing genes. Nonetheless, it is clear that such genome-wide approach cannot be used for a routine mutational screening in wide number of affected individuals, due to the high computational and economic effort required, particularly considering that there are few research groups in big companies and/or large corporations, as well as big public and private world leading research institutions, able to sustain these costs.

Thus, targeted resequencing of a small number of candidate genes or disease *loci* appears to be the only reliable way to obtain the high accuracy of NGS data at the accessible costs of a standard array analysis. On the other hand, it appears crucial to have efficient and cost-effective capture methods to enrich the sample with "high-value" genomic regions to sequence in order to avoid off-target sequencing.

In light of this, different techniques have been recently developed allowing researchers to enrich their sample of target genomic regions to be further sequenced. Multiplex PCR amplification of specific target regions was first used for candidate gene approaches, to enrich the samples with regions of interest, further processed to prepare libraries prior to sequencing [126–128]. Another approach is the capture-by-hybridization [129]. Efficient array-based

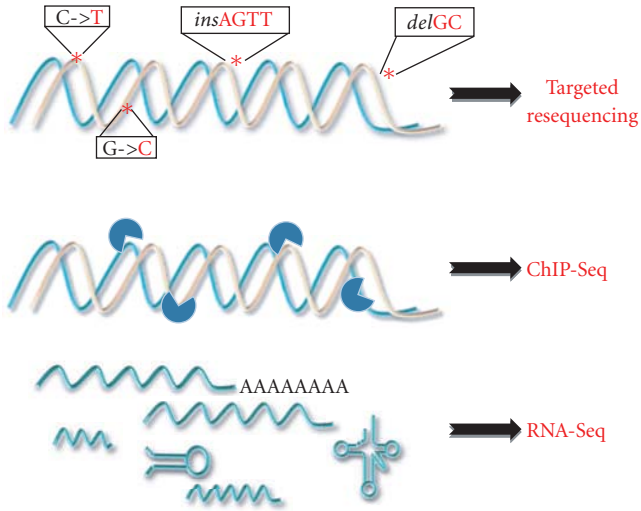

Figure 2: Innovative approaches by using next generation sequencing technologies. Next generation sequencing can be applied to many scientific contexts: targeted resequencing for the identification of disease-causing nucleotide variations for both coding and unexplored regulatory regions of genes (CNV-Seq and DNA-Seq); ChIP-Seq, for DNA-protein interaction studies coupling chromatin immunoprecipitation (ChIP) and massively parallel sequencing; RNA-Seq, for whole transcriptome studies, including expression levels of known and yet unknown transcripts (both coding and non-coding), differential splicing, allele-specific expression, RNA editing, and fusion transcripts (see review [121]).

capture approaches (custom *in-situ* synthesized oligonucleotide microarrays) have been successfully used to enhance the sequencing template enrichment [130–133]. Companies, such as NimbleGen, have recently developed microarrays for the capture-by-hybridization of thousands of predefined genomic regions, mainly coding regions (exons), widely used for targeted resequencing experiments [134]. Several research groups have clearly shown that the above-described capture methods are playing a crucial role in driving targeted resequencing applications of NGS platforms [129].

Since most of human genetics studies have so far mainly focused on protein-coding exons, these regions usually represent high-value targets for targeted resequencing, even though this approach can be—and we believe in most cases it must be—extended to gene regulatory regions (upstream the translation start sites and introns). Indeed, the identification of nucleotide variations in putative or already known regulatory sequences, within non-coding genomic regions, is therefore of great relevance for future research. This approach appears to be very promising above all for the study of TFs binding sites, since their involvement in human disease, both mendelian and multifactorial [135]. Barrio and colleagues (2009) [135] first identified, by target resequencing of a genomic region encompassing about 20kb, non-coding variations associated with two kinds of red cell aplasia, demonstrating that non-coding *RPS19* gene sequence variants contribute to the high clinical variability observed in red cell aplasia. They hypothesized that specific alleles in these non-coding regions may alter the binding of regulatory proteins and/or TFs, possibly altering or removing an important stimulus for hematopoiesis [135].

In this context, the possibility to have high enrichment for the both coding and unexplored regulatory regions of *PPARG,* coupled to the targeted resequencing on NGS platforms, will represent a very powerful approach for researchers. Indeed, it is likely to allow the identification of all the potential risk-conferring variations, within its coding regions, of putative novel single nucleotide variations (mutations and SNPs) and insertions/deletions or other genomic rearrangements, possibly associated to human diseases. It will also allow gaining further insights into the genomic architecture of its regulatory regions, offering the possibility to rapidly and accurately identify potential sources of variation responsible for the alteration of its mRNA levels. Moreover, the specific enrichment of target regions, followed by targeted resequencing, could also be performed on well-known PPARγ-regulated genes in specific pathways.

Indeed, since several studies performed on PPARγ target genes have not unequivocally shown a clear correlation between SNPs and the related human diseases, by using these approaches it will be easier to identify specific alleles in non-coding regions of target genes and verify whether these nucleotide variations are responsible for the alteration of known PPRE—and in turn of PPARγ binding to these elements—finding a direct and functional link to the disease.

4.2. Transcription Factors and ChIP-Seq. Thanks to the introduction of NGS platforms, widely used approaches of chromatin immuno-precipitation followed by microarray (ChIP-chip) have been flanked—and in many cases substituted—by ChIP-seq protocols. Indeed, in ChIP-seq, the DNA fragments of interest (i.e., binding sites for a TF) are directly sequenced instead of being hybridized on a chip-array. Thanks to the high resolution, coverage, the wider dynamic range, and the absence of hybridization-based artifacts, ChIP-Seq allows now researchers to improve both quantity and quality of produced data. Moreover, fundamental advances toward a

more accurate definition of the consensus sequences for the binding of TFs have been done [136].

To date, this novel approach, which couples in a single experiment a standard ChIP assay to the large-scale massive sequencing of target genome regions, allows researchers to obtain a more complete map of TFs-DNA interactions [2]. Drawing a precise map of TFs binding sites, core transcriptional machinery, and other DNA-binding proteins is a crucial step towards the identification of gene regulatory networks underlying physiological as well as pathological processes [136].

In particular, since PPARγ acts in combination with RXR as heterodimer and requires the cooperation with many different tissue-selective factors, understanding the differential spatial and temporal recruitment of PPARγ:RXR complex to target genes is likely to improve our knowledge about PPARγ biology.

In a recent study, Nielsen et al. [28] by ChIP-Seq on NGS platform (Illumina, Roche) obtained a PPARγ- and RXR-binding sites map during the adipocytes differentiation of 3T3-L1 cells [28]. In particular, they sequenced a total of about 86 million of fragments (divided for the six days of the analysis on the adipocytes) derived from PPARγ ChIP assay and about 50 million derived from RXR ChIP. They demonstrated that spatial and temporal recruitment of PPARγ and RXR to target genes varied during adipogenesis (Figure 1). More in detail, they observed that in the very early stages of adipocyte differentiation, coinciding with the very low levels of PPARγ at day 0, only nine PPARγ target sites were detectable, and however this number remained low at day 1. In contrast, a high DNA occupancy by RXR alone was detected. More interestingly, going on with the differentiation process, most of these sites become occupied by PPARδ:RXR complexes. A subsequent switch—starting at day 2—between PPARδ and PPARγ, which becomes the main RXR partner throughout the adipogenesis, coincided with a significant increase in both PPARγ1 and PPARγ2 expression [28–30]. They identified >5000 high-confidence PPARγ:RXR-binding sites in adipocytes coinciding to the majority of induced genes. *In silico* analysis allowed to observe that binding occurs in the proximity of genes involved in lipid and glucose metabolism. The highest number (about 7000) of PPARγ:RXR-binding sites was observed at day 6. This genome-wide ChIP-Seq analysis allowed to confirm the binding of PPARγ:RXR heterodimer to well-established PPREs in already known target genes. In addition, novel target sites in introns of different genes were also identified.

ChIP-Seq was also recently used by Lefterova and colleagues (2010) [31] to address a critical issue affecting several reports about PPARγ function, the specificity of action, that is, how PPARγ modulates target genes in some cell types but not others. By using this innovative approach, the authors determined which cell-type-specific cofactors are recruited by PPARγ in mouse macrophages and adipocytes [31]. Indeed, it has been widely demonstrated the PPARγ transactivation ability on target genes, with characteristic cell-type specific patterns of gene modulation, but the molecular basis of such a specificity has not yet been fully understood.

As generally assumed for other TFs, it has been postulated that its cell-type specificity might be due to a differential binding to consensus sequences in the regulatory regions of target genes or a differential ability to recruit chromatin remodelling enzymes [38]. The authors identified a specific molecular signature of PPARγ binding, by massively sequencing—overall the mouse genome—the regions directly bound by PPARγ. This analysis revealed that PPARγ cooperates with some cell-type-specific factors, PU.1 and C/EBPβ, in the defining the specificity of action for PPARγ in each cell type (macrophages and adipocytes, resp.) (Figure 1). PPARγ in macrophages binds uniquely at genomic sites located in the proximity of immunity-related genes and specifically colocalizes with PU.1 in areas of open chromatin and in presence of histone acetylation whereas, in preadipocytes, the presence of a repressive histone signature excludes PPARγ from macrophage-specific sites. In this case it has been shown that PPARγ is able to open the chromatin and increase histone acetylation at adipocyte-specific genomic sites. This paper demonstrates that, at least in these cell types, the transcriptional regulation exerted by PPARγ is attributed to a differential recruitment of specific cofactors functioning as scaffolds for chromatin remodelling enzymes.

Above described works have clearly shown the great potential of sequencing-based ChIP assays, which do not require *a priori* information about the genomic position of TFs binding sites and allow to generate high-resolution binding maps in response to a specific *stimulus* [123, 124]. However, as demonstrated in a recent work by Reddy and colleagues (2009) [125], coupling ChIP-Seq to RNA-Seq (described in detail in the next paragraph) for studying the response of a TF to a specific drug allows to examine well-known models at much greater depth and detail. In particular, they obtained a comprehensive map of glucocorticoid receptor binding to DNA overall the genome by ChIP-Seq, and measured related changes in gene expression by RNA-Seq, in response to treatment with dexamethasone [125].

We firmly believe that combining a sequencing-based ChIP assay to high-throughput transcriptome analysis by RNA-Seq on NGS platforms, above all for inducible transcription factors (and PPARγ among them), will surely provide a complete, accurate, and reliable source of useful of data, enabling to complete, piece by piece, the intricate puzzle of PPARγ functions.

4.3. Discovering the Transcriptional Landscape through RNA-Seq. Since the end of the 90s the term "transcriptome" was used to describe the identity of each expressed gene in a specific cell type and/or tissue/organ/organism, and of its related transcriptional levels [137]. It was first believed to consist of 80–90% of ribosomal RNA (rRNA), 5–15% of transfer RNA (tRNA), and the remaining fraction of messenger RNA (mRNA), with most of the genome consisting of untranscribed and genetically inert regions.

In contrast, recent evidences have shown that both intragenic and intergenic sequences cannot be any longer considered as "junk DNA", but they represent one of the

main driving force accounting for diversity and biological complexity of all living organisms [121]. Indeed, several studies have demonstrated an unexpected level of complexity of the eukaryotic transcription, showing its pervasive nature with almost the full length of nonrepeat regions of the genome being transcribed [138, 139].

Hence, interpreting the complexity of a whole transcriptome is likely to be a crucial endpoint for unraveling the role of functional elements of a genome, and, in light of this, the introduction of NGS platforms has provided researcher a powerful tool for analysis in a single experiment.

Indeed, the rapid diffusion of RNA-Seq protocols has raised the possibility to quantify the differential expression of transcripts in both physio- and pathological conditions and to identify and characterize all the transcripts (both protein-coding and non-coding) expressed within a specific cell and/or tissue—at a particular development stage or after an endogenous or exogenous *stimulus*—correctly determining the splicing and the structure of genes. Unlike hybridization-based gene expression methods (microarray) and tag-based sequencing (i.e., CAGE and SAGE), RNA-Seq does not require prior knowledge of any gene sequence (as occurs for microarrays) or laborious and time-consuming steps for the cloning and sequencing (as occurs for existing tag-based approaches) (reviewed in [121]).

Several recent studies have clearly demonstrated the advantages of using RNA-Seq in the interrogation of transcriptomes under multiple conditions, such as cell proliferation, differentiation, and various environmental stress [140–148].

In this context, due to the crucial role of PPARγ as TF involved in many cellular pathways, investigating the PPARγ-dependent regulation of target genes expression via RNA-Seq in a single experiment represents a great challenge.

Whereas previously described ChIP-Seq allows to draw a binding map of PPARγ to PPRE, activating or repressing target gene expression, directly identifying (by RNA-Seq) the gene expression response to PPARγ-modulating drugs (agonists such as TZD), or in particular development conditions (during adipogenesis), will provide researchers the opportunity to directly measure its ability to modulate the transcription of specific genes in a cell/tissue specific manner.

Since high-throughput sequencing has definitely proved to be a powerful and quantitative method to sample the transcriptomes at single nucleotide resolution [149], the use of RNA-Seq is likely to shed a new light on the specificity of action of PPARγ in different cell types or tissue, in both physiological and pathological conditions. Several unsolved questions about the "real" impact of PPARγ on the regulation of target gene expression—in several conditions—can now be fruitfully addressed by the use of NGS.

5. Concluding Remarks

Over the past years, PPARs, and especially PPARγ, have emerged as crucial transcription factors modulating the expression of genes involved in several important pathways and biological processes and, noteworthy, in human diseases. Despite the huge knowledge in the field, future research efforts will undoubtedly reveal novel mechanisms through which PPARγ integrates these complex physiological and pathological pathways. Particular attention should be given to the question of how the selective effects of PPARγ are achieved in different cell types. It will also be of great importance to understand the subtle mechanisms dictating this selectivity of action through the study of its different isoforms, genetic variations, and their recruited cofactors able to remodel the chromatin structure. Knowing all the PPARγ targets is a prerequisite for a full understanding of the metabolic defects that occur in people with *PPARG* mutations and/or variation and will help in the interpretation of effects—and also side effects—that can occur with PPARγ agonists already in clinical use. Thus, to have a complete picture of PPARγ functions and implications, studying altogether these aspects, through the use of massively parallel sequencing platforms, will provide a way to better characterize the actions of *PPARG* products and agonists.

Acknowledgments

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Research Article

Coordinate Transcriptomic and Metabolomic Effects of the Insulin Sensitizer Rosiglitazone on Fundamental Metabolic Pathways in Liver, Soleus Muscle, and Adipose Tissue in Diabetic db/db Mice

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Rosiglitazone (RSG), developed for the treatment of type 2 diabetes mellitus, is known to have potent effects on carbohydrate and lipid metabolism leading to the improvement of insulin sensitivity in target tissues. To further assess the capacity of RSG to normalize gene expression in insulin-sensitive tissues, we compared groups of 18-day-treated db/db mice with increasing oral doses of RSG (10, 30, and 100 mg/kg/d) with untreated non-diabetic littermates (db/+). For this aim, transcriptional changes were measured in liver, inguinal adipose tissue (IAT) and soleus muscle using microarrays and real-time PCR. In parallel, targeted metabolomic assessment of lipids (triglycerides (TGs) and free fatty acids (FFAs)) in plasma and tissues was performed by UPLC-MS methods. Multivariate analyses revealed a relationship between the differential gene expressions in liver and liver trioleate content and between blood glucose levels and a combination of differentially expressed genes measured in liver, IAT, and muscle. In summary, we have integrated gene expression and targeted metabolomic data to present a comprehensive overview of RSG-induced changes in a diabetes mouse model and improved the molecular understanding of how RSG ameliorates diabetes through its effect on the major insulin-sensitive tissues.

1. Introduction

Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus, is a chronic disease that affects more than 100 million people worldwide, and its prevalence is soaring in western countries driven by high-fat diets and sedentary lifestyles. This pathology is characterized by resistance to the effects of insulin in peripheral tissues, which is manifested as reduced insulin-stimulated glucose uptake into skeletal muscle and adipose tissue, defective insulin-dependent suppression of hepatic glucose output, and reduced insulin secretion from pancreatic β-cells. Insulin-sensitising drugs, such as RSG, are widely used in clinical practice to improve diabetes alteration in glucose metabolism. Thiazolidinediones (TZDs) are already known to decrease blood glucose concentration, to improve liver and muscle insulin sensitivity [1, 2], and to have significant impact on adipose tissue by inducing adipose differentiation, lipogenesis, and TG

storage [3–6]. The mechanisms of action of TZDs are mediated through binding and activation of the peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor that has a regulatory role in lipid metabolism and in cell differentiation, particularly in adipocytes [7, 8]. PPARγ is also expressed in several other tissues, including muscle, liver, pancreas, heart, and spleen [9]. Among the multiple actions of TZDs is the normalisation of blood glucose level, by increasing glucose uptake and decreasing hepatic glucose production [10]. Moreover, TZDs induce shifts in systemic lipid profiles, with a decrease in FFAs and TGs levels in the circulation, partly by improving adipocyte function [11]. Taken together, these effects on carbohydrate and lipid metabolism are associated with the improvement of insulin sensitivity of peripheral tissues. However, TZDs actions are also accompanied by increased adipogenesis and lipid accumulation in tissues [12, 13]. RSG is a prototype of the TZD chemical class developed for the treatment of type 2 diabetes mellitus, and some of these effects are known to be mediated via gene transcriptional regulation [14–17]; however, the relationship between the RSG-mediated gene expression regulation in insulin-dependent tissues and the subsequent physiological changes remains unclear.

Diabetic db/db mice, which have a defect in the leptin receptor, reproduce many of the metabolic disturbances present in patients with type II diabetes mellitus, including hyperglycaemia, hyperinsulinemia, hyperlipidemia, and insulin resistance [18]. To address the question of whether RSG-mediated normalisation of blood and tissue biological characteristics in treated db/db is accompanied by normalized gene expression in three major insulin-dependent tissues, liver, IAT (inguinal adipose tissue), and skeletal muscle, we analysed transcriptomic profiles of RSG-treated diabetic mice db/db and compared them with those of untreated non diabetic littermates db/+. Multivariate analysis was then used to establish relationships between the RSG-regulated gene expression profiles and metabolomic data obtained from the insulin-sensitive tissues or blood samples.

This study provides a comprehensive evaluation of RSG-induced changes in genome-wide expression and its relationships with RSG-mediated physiological changes in three main insulin-sensitive tissues known to be involved in lipid and glucose homeostasis.

2. Materials and Methods

2.1. Animals and Treatment. An eight-week old male BKS-Cg-*m*+/+ Lepr*db/J* diabetic (db/db) mice and their nondiabetic strain (db/+) were obtained from Charles River Laboratories (L'Arbresle, France). Three animals were housed per cage and acclimatised for 1 week under standard light (12 h light/dark), feeding (db/+: standard laboratory chow (A03, Safe, France); db/db: 5K52, Safe, France). Mice were randomly divided into 5 different groups (n = 6/group) as follows: vehicle- (1% HEC-) treated nondiabetic db/+ and diabetic db/db mice (control db/db mice), and diabetic db/db mice treated orally with the PPARγ agonist RSG (Syntheval, Caen, France) at 3 separate doses (10, 30, 100 mg/kg/day) for 18 days. Body weight was measured daily, and blood was collected by orbital sinus puncture before and after treatment (18h after the last treatment) to assess glucose levels. All experimental procedures were in accordance with the International European Ethical Standards (86/609-EEC) and the French National Committee (decree 87/848) for the care and use of laboratory animals.

2.2. Tissue and Blood Analyses. At the end of treatment, mice were euthanized by cervical dislocation. Liver, IAT, and soleus muscle were immediately taken, weighed, flash frozen in liquid nitrogen, and kept at –80°C. Blood samples were drawn into heparin-containing tubes (1.5 I.U. heparin for 100 μL of blood), and plasma was aliquoted and stored at 4°C. Blood glucose, Hb, and HbA1c levels were determined using the automatic analyser COBAS system (Roche, Basel, Switzerland). FFA and TG Liver and adipose tissues (50 mg aliquots) were extracted with $CHCl_3$. After evaporation, the organic dry extract was reconstituted in CH_3CN/iPrOH (1/1). Plasma (50 μL) was extracted with a mixture of $CHCl_3$/MeOH (2/1). FFA and TG analyses were performed by UPLC-MS using a C18 BEH Acquity analytical column, a gradient of CH_3CN, iPrOH, and formic acid (0.1%) (0.5 mL/min, 50°C), and a 4000 QTRAP (Applied Biosystems, Foster City, CA, USA) and LCT premier (Waters).

2.3. RNA Preparation. Total RNA was prepared from frozen liver, soleus, and IAT, using Trizol reagent (Invitrogen, France) and was further purified by ammonium acetate precipitation according to standard protocols. Purified total RNA concentration and 260/280 nm or 260/230 nm ratios were determined using a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech, France). Then, total RNA integrity was checked by microelectrophoresis on acrylamide gel (Agilent 2100 Bioanalyzer, Agilent, Santa Clara, CA, USA).

2.4. Microarray Hybridization and Data Analysis. Total RNA (500 ng) samples were labelled with cyanine Cy5- or Cy3-CTP dyes using Agilent Low RNA Input Linear Amplification Kit (Agilent Technology, PN 5184-3523), according to the manufacturer's protocols. Following in vitro transcription, 825 ng of test sample (individual db/+: n = 6) or RSG-treated db/db cRNA were mixed with 825 ng of reference sample (pooled: n = 6 db/db cRNA). The samples were hybridized (dye-swap replicates) on 4 × 44 k whole-Genome 60-mer oligo-microarrays (Agilent technologies, PN G4122F) for 17 hours at 65°C. Microarrays were scanned using a dynamic auto focus microarray scanner (Agilent technology). Raw data were normalised using local background subtraction and local Lowess dye normalisation. Data were analysed using Rosetta Resolver Gene Expression Analysis System v6.0 (Rosetta Inpharmatics LLC, Seattle, WA). Dye-swap replicates were precombined before any statistical analysis. For each group, statistically significant regulated sequences were defined as the sequences whose expression in the db/+ or in the RSG-treated db/db samples was statistically different from the expression in the untreated

db/db (reference), as calculated by Rosetta error-weighted model [19]. For this study a P-value < .001 was considered to be significant, and a fold-change value cutoff was ≥ |1.3|. Ingenuity Pathway Analysis application (Ingenuity Systems, CA, USA) was used to allow functional interpretation of the data. Promoter gene sequences were investigated for the presence of proximal (~500 bp from initiation site) PPAR response element (PPRE) in their promoters with MatInspector (Genomatix; GmbH) [20]. The partial least-squares (PLSs) projection to latent structure analysis [21] was performed using Simca-P12 software (Umetrics, Umea, Sweden).

2.5. Real-Time RT-PCR. Total RNA (1 μg) from each animal (n = 6/group) and from pooled reference db/db was reverse transcribed into cDNA using a transcription kit (Applied Biosystems, PN 4368813). PCR reactions were then proceeded in microfluidic cards using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Each microfluidic card was preloaded with predesigned Taqman probes and primers for genes and RNA 18S reference gene. cDNA (100 ng) was mixed with 2X Taqman Universal PCR master Mix (Applied Biosystems, PN 4324020) and loaded in each well (n = 3). The following temperature profile was used: 10 min at 94.5°C, followed by 40 cycles of 97°C for 30 sec and 59.7°C for 1 min. Normalised data with RNA 18S were analysed using the threshold cycle (Ct) relative quantification method [22], and the ΔΔCt method was used to compare the amounts of RNA in test and reference groups.

2.6. Immunocytochemistry. Adipose tissue was fixed in 4% neutral buffered formaldehyde, and cryostat sections of 7 μm were prepared. The labelling was performed using a Discovery XT automat (Ventana Medical Systems, Tucson, USA). Sections were incubated 1h30 at 37°C with Alexa fluor 488-conjugated anti-OxPhos Complex IV subunit I antibody (1/100 dilution, Molecular Probes, PN A21296). Immunolabelling was visualized and imaged using an LSM 510 confocal microscope (Carl Zeiss SAS, France).

3. Results

3.1. Effects of RSG on Physiological Characteristics. At the beginning of the experiment the RSG-treated and control db/db groups of mice were comparably obese and diabetic. Blood glucose concentrations and body weights for these two groups were similar and were both significantly different from db/+ nondiabetic mice (Table 1). Eighteen days of dose-dependent treatment with RSG resulted in a reversion of hyperglycaemia. Indeed, a reduction of blood glucose concentrations and HbA1c were observed for all RSG doses tested. Blood glucose normalisation was obtained from 30 mg/kg (0.98 ± 0.12 versus 1.1 ± 0.1 g/L in db/+); HbA1c levels from db/db-treated mice were all significantly above the db/+ control mice, showing only a partial normalisation of this parameter (Table 1).

3.2. Targeted Metabolomic Assessment of Plasma and Tissue Lipids. Diabetic db/db mice displayed higher plasma trioleate concentrations than db/+. RSG-treated db/db mice showed a clear decrease in the levels of the two principal plasma TGs, trilinoleate and trioleate. When compared to db/+, TG normalisation was achieved from 10 mg/kg RSG treatment (16.8 ± 6.5 versus 26.8 ± 10.2 nmol/mL and 6.7 ± 1.6 versus 11.8 ± 3.4 nmol/mL for trilinoleate and trioleate, respectively). Plasma FFA concentrations showed a tendency to be higher in db/db than db/+ mice, but the difference remained significant only for linoleic acid. RSG treatment further decreased plasma FFA concentrations compared to both db/db and db/+, but in this latter case it did not achieve the significance threshold (Table 1).

In the liver of diabetic db/db mice, both TG and FFA levels reached a higher concentration compared to db/+. From 10 mg/kg the RSG treatment of db/db further increased trioleate and oleic and palmitic acids. In parallel, the liver weight of RSG-treated db/db mice increased (+86%, +64%, and +46% at 10, 30, and 100 mg/kg RSG, resp.) versus the control db/db mice.

As expected, IAT weight was higher in both RSG-treated and control db/db mice compared to the db/+ mice (2.10 ± 0.06 in untreated db/db mice; 1.99 ± 0.11, 2.09 ± 0.13, and 2.16 ± 0.09 g in treated db/db mice at 10, 30, and 100 mg/kg RSG, respectively *versus* 0.17 ± 0.02 g in db/+ mice). In contrast, the TG concentrations relative to adipose tissue mass in db/+ were markedly higher than in RSG-treated or untreated db/db. FFA adipose tissue concentrations remained basically unchanged either between db/db and db/+ or following RSG treatment except for oleic acid the levels of which in db/db with or without RSG treatment far exceeded those of db/+.

Soleus muscle weight was unaltered by both pathology and treatment (Table 1), although fat accumulation was observed for control db/db versus RSG-treated db/db during the soleus muscle dissection.

3.3. Transcriptomic Profile of the Insulin-Sensitive Tissues of db/+ and RSG-Treated db/db. Changes in global gene expression were assessed in three insulin-sensitive tissues, liver, IAT, and soleus muscle, involved in glucose and lipid metabolism using whole-mouse genome microarrays. Differences in gene expression between db/+ or RSG-treated db/db versus the control db/db group were filtered according to a P-value < .001 and a fold change ≥ |1.3| (Rosetta error- weighted model [19]). One gene could be represented by different gene (probes) sequences. Gene alteration between nondiabetic db/+ versus diabetic db/db mice (see Supplementary Table 1 in material available online at doi: 10.1155/2010/679184) was determined in the liver (6539 regulated sequences), in the soleus muscle (7895 regulated sequences), and in the IAT (13207 regulated sequences). RSG treatment of db/db mice elicited significant changes in the expression of a comparable number of genes to that observed between db/+ and the diabetic control db/db reference group, namely in the liver (5567, 4788, and 4549 regulated sequences at 10, 30, and 100 mg/kg RSG, resp.), in the IAT (5710, 9005, and 10335

Table 1: Animal characteristics, blood and tissue analysis: db/db were compared by Student's "t" test versus db$^+$, $^{\#}P < .05$; $^{\#\#}P < .01$; $^{\#\#\#}P < .001$. RSG-treated groups were compared by ANOVA and Dunnett test versus untreated db/db, $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$, or versus db/+, $^{\dagger}P < .05$; $^{\dagger\dagger}P < .01$; $^{\dagger\dagger\dagger}P < .001$. $n = 6$ animals otherwise specified.

	db/db				db/+
Parameters (units)	Untreated	RSG 10 mg/kg	RSG 30 mg/kg	RSG 100 mg/kg	Untreated
Body weight 0d	$38.9 \pm 0.4^{\#\#\#}$	39.4 ± 0.6	39.8 ± 0.7	39.5 ± 1.1	24.6 ± 0.7
Body weight (g) 18d	$40.6 \pm 0.7^{\#\#\#}$	$46.2 \pm 0.9^{**}$	$47.8 \pm 0.6^{***}$	$46.8 \pm 2.1^{**}$	25.9 ± 0.4
Liver weight (g)	$1.75 \pm 0.03^{\#\#\#}$	$3.25 \pm 0.17^{***\,\dagger\dagger\dagger}$	$2.83 \pm 0.20^{**\,\dagger\dagger\dagger}$	$2.47 \pm 0.27^{*\,\dagger\dagger}$	1.37 ± 0.05
Inguinal Adipose weight (g)	$2.09 \pm 0.06^{\#\#\#}$	$1.99 \pm 0.11^{\dagger\dagger\dagger}$	$2.09 \pm 0.13^{\dagger\dagger\dagger}$	$2.16 \pm 0.09^{\dagger\dagger\dagger}$	0.17 ± 0.02
Soleus muscle weight (mg)	6.67 ± 1.33	6.00 ± 0.85	5.05 ± 0.5	4.79 ± 0.36	6.13 ± 1.82
Blood Glucose (mmol/l) 0d	$16.9 \pm 1.3^{\#\#\#}$	$16.3 \pm 1.3^{\dagger\dagger\dagger}$	$15.8 \pm 0.9^{\dagger\dagger\dagger}$	$16.4 \pm 1.1^{\dagger\dagger\dagger}$	5.8 ± 0.4
Blood Glucose (mmol/l) 18d	$15.1 \pm 0.9^{\#\#\#}$	$8.0 \pm 0.7^{***\dagger\dagger}$	$5.4 \pm 0.6^{***}$	$4.1 \pm 0.5^{***}$	5.1 ± 0.5
HbA1c %	$7.14 \pm 0.28^{\#\#\#}$	$6.16 \pm 0.34^{\dagger\dagger\dagger}$	$5.76 \pm 0.32^{**\dagger\dagger\dagger}$	$5.52 \pm 0.23^{**\dagger\dagger\dagger}$	3.61 ± 0.13
Plasmatic triglycerides (nmol/mL)					
Trilinoleate ($n = 3 - 5$)	49.6 ± 2.3	$16.8 \pm 6.5^{***}$	$10.2 \pm 3.0^{***}$	$23.2 \pm 5.4^{**}$	26.8 ± 10.2
Trioleate ($n = 3 - 5$)	$19.7 \pm 1.4^{\#}$	$6.7 \pm 1.6^{***}$	$3.2 \pm 0.9^{***}$	$8.0 \pm 2.5^{***}$	11.8 ± 3.4
Plasmatic FFA (nmol/mL)					
Palmitic acid	91.8 ± 5.5	70.4 ± 7.0	$51.4 \pm 7.8^{***}$	$64.9 \pm 7.0^{*}$	85.9 ± 10.5
Linoleic acid	$83.8 \pm 4.5^{\#}$	70.5 ± 6.0	$43.0 \pm 10.2^{*}$	54.5 ± 12.7	65.3 ± 4.4
Oleic acid	91.1 ± 12.0	72.1 ± 5.4	$38.4 \pm 12.1^{**}$	$47.4 \pm 13^{*}$	70.2 ± 8.9
Liver triglycerides (nmol/g)					
Trilinoleate	$116 \pm 24^{\#\#}$	$76.0 \pm 21.9^{\dagger}$	$43.2 \pm 8.9^{*}$	$33.5 \pm 4.4^{**}$	20.0 ± 3.9
Trioleate	$1033 \pm 314^{\#}$	$10240 \pm 1489^{***\,\dagger\dagger\dagger}$	$7746 \pm 1603^{**\,\dagger\dagger\dagger}$	2708 ± 846	59 ± 13
Liver FFA (nmol/g)					
Palmitic acid	481 ± 49	$583 \pm 43^{\dagger\dagger}$	$573 \pm 47^{\dagger}$	$702 \pm 67^{**\,\dagger\dagger\dagger}$	366 ± 13
Linoleic acid	$289 \pm 27^{\#}$	236 ± 7	253 ± 17	$331 \pm 46^{\dagger\dagger}$	209 ± 11
Oleic acid	$484 \pm 87^{\#}$	$1031 \pm 110^{**\,\dagger\dagger\dagger}$	$997 \pm 82^{**\,\dagger\dagger\dagger}$	$945 \pm 148^{*\,\dagger\dagger\dagger}$	186 ± 18
IAT triglycerides (nmol/g)					
Trilinoleate	5965 ± 2373	$3670 \pm 1177^{\dagger\dagger}$	$5343 \pm 1542^{\dagger}$	$4118 \pm 674^{\dagger}$	15524 ± 4320
Trioleate	7766 ± 1962	6515 ± 2729	15382 ± 5458	11836 ± 3059	16016 ± 5333
IAT FFA (nmol/g)					
Palmitic acid	236 ± 61	265 ± 24	302 ± 67	$462 \pm 36^{*}$	424 ± 92
Linoleic acid	$329 \pm 53^{\#}$	328 ± 25	454 ± 97	$570 \pm 57^{*}$	558 ± 81
Oleic acid	$281 \pm 48^{\#\#}$	$310 \pm 32^{\dagger}$	$416 \pm 110^{\dagger\dagger}$	$514 \pm 56^{\dagger\dagger\dagger}$	65 ± 10

regulated sequences at 10, 30, and 100 mg/kg RSG, resp.), and in the soleus muscle (3413, 3644, and 3620 regulated sequences at 10, 30, and 100 mg/kg RSG, resp.).

3.4. Functional Interpretation of the Changes in Transcriptomic Profiles. To interpret the biological alteration, differentially expressed genes (db/+ or RSG-treated db/db versus control db/db reference group, Supplemental Table 1) with known gene symbol (HUGO) were submitted to Ingenuity Pathway analysis. Each gene symbol was mapped to its corresponding gene in the Ingenuity Pathways Knowledge database, and biological functions and diseases were assigned to the pattern of gene expression. Functions were listed from most significant to least, and the horizontal line shows the cutoff value for significance ($P < .05$, adjusted Benjamini-Hochberg; Supplemental Figure 1). As expected, either in the nondiabetic db/+ (diabetes effect given as reciprocal (db/+ versus db/db), see below) or in the RSG-treated db/db mice, the changes in expression mainly involved genes related to lipid and carbohydrate metabolism not only in the liver but also in the IAT and the soleus muscle (Supplemental Figure 1). Moreover, groups of genes were also enriched in several functions related to cell signalling, movement, or development in liver, adipose tissue, and soleus muscle. These results could be explained by diabetes-induced morphological and/or growth alteration of hepatocytes, adipocytes, and myocytes in db/db mice as well as in db/db under RSG treatment. However, in the attempt to link transcriptomic and metabolomic data we focussed our study on genes involved in lipid and carbohydrate metabolism and potentially associated with diabetes. This list included 506 gene sequences involved in glycolysis, gluconeogenesis, TG and FFA metabolism, and pentose phosphate synthesis, as well as genes involved in mitochondrial functions, that is, β-oxidation, citrate cycle, and oxidative phosphorylation. Thereafter, to facilitate direct comparison with RSG treatment effect, we used the reciprocal form of the diabetes

Table 2: Model parameters from multivariate analysis (PLS) based on liver, IAT and muscle gene expression. R^2X: % of variation of X that explained Y; $R^2_{Y\text{cum}}$: % of variation of Y explained by the model; $Q^2_{Y\text{cum}}$: % of variation of Y predicted by the model. Cum are for all PLS components.

	PLS component	R^2X(cum)	R^2Y(cum)	Q^2(cum)	*P*-value
Liver					
Oleic 275 gene sequences	1	0.67	0.78	0.76	3.06E-07
Oleic 40-top gene sequences	1	0.94	0.80	0.80	5.00E-08
Trioleate 275 gene sequences	2	0.74	0.86	0.80	3.11E-06
Trioleate 40-top gene sequences	2	0.72	0.89	0.85	2.30E-07
Weight 275 gene sequences	2	0.77	0.96	0.95	4.92E-11
Weight 40-top gene sequences	1	0.75	0.96	0.93	1.80E-14
Liver+IAT+Soleus					
Glycemia 846 gene sequences	4	0.71	0.94	0.73	1.00E+00
Glycemia 40-top gene sequences	3	0.86	0.84	0.68	7.00E-03

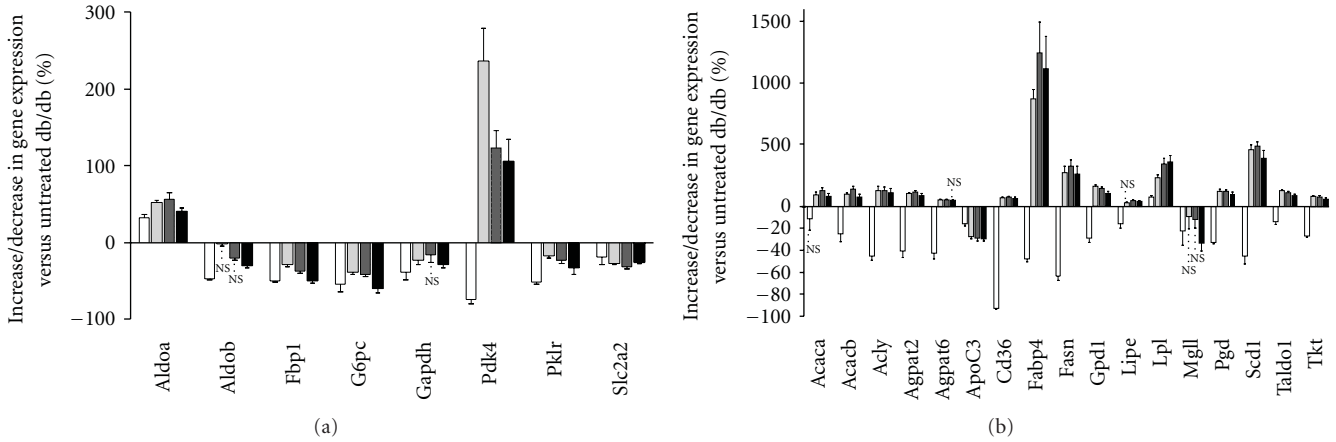

Figure 1: Expression profiles of key genes involved in liver glucose and lipid metabolism. Differentially expressed genes in db/+ (the white square) as well as 10 (the off-white square), 30 (the grey square), and 100 (the black square) mg/kg RSG-treated db/db mice were measured *versus* untreated db/db and plotted as the mean (% increase/decrease of db/db control) ±SEM ($n = 6$). NS; $P \geq .001$ otherwise $P < .001$. (a) Genes related to glucose metabolism: Aldoa, Aldob, aldolase 1A, 2B; Fbp1, fructose bisphosphatase 1; G6pc, glucose-6-phosphatase, catalytic; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Pdk4, pyruvate dehydrogenase kinase, isoenzyme 4; Pklr, pyruvate kinase liver and red blood cell; Slc2a2, solute carrier family 2 (facilitated glucose transporter), member 2. (b) Genes related to FA transport, FA and TG synthesis, and pentose pathway: Acaca, Acacb, acetyl-Coenzyme A carboxylase alpha, beta; Acly, ATP citrate lyase; Agpat2, Agpat6, 1-acylglycerol-3-phosphate O-acyltransferase 2, 6; Apoc3, apolipoprotein C-III; Cd36, CD36 antigen; Fabp2, fatty acid-binding protein 2, intestinal; Fabp4, fatty acid-binding protein 4, adipocyte; Fasn, fatty acid synthase; Gpd1, glycerol-3-phosphate dehydrogenase 1 (soluble); Lpl, lipoprotein lipase; Mgll, monoglyceride lipase; Pgd, phosphogluconate dehydrogenase; Scd1, stearoyl-Coenzyme A desaturase 1; Taldo1, transaldolase 1; Tkt, transketolase.

effect, that is, untreated nondiabetic db/+ versus diabetic mice db/db. Consequently, RSG-induced normalisation of gene expression is defined as genes that were significantly changed by RSG (RSG-treated db/db versus db/db) in the same direction as nondiabetic (db/+ versus db/db) mice.

3.5. Effect of RSG on the Liver Metabolic-Related mRNA Expression. In the liver among the 506 gene sequences 276 were differentially expressed in either db/+ versus db/db (Supplemental Table 2) or RSG-treated db/db versus db/db. Supplemental Figure 2(a) displays the microarray

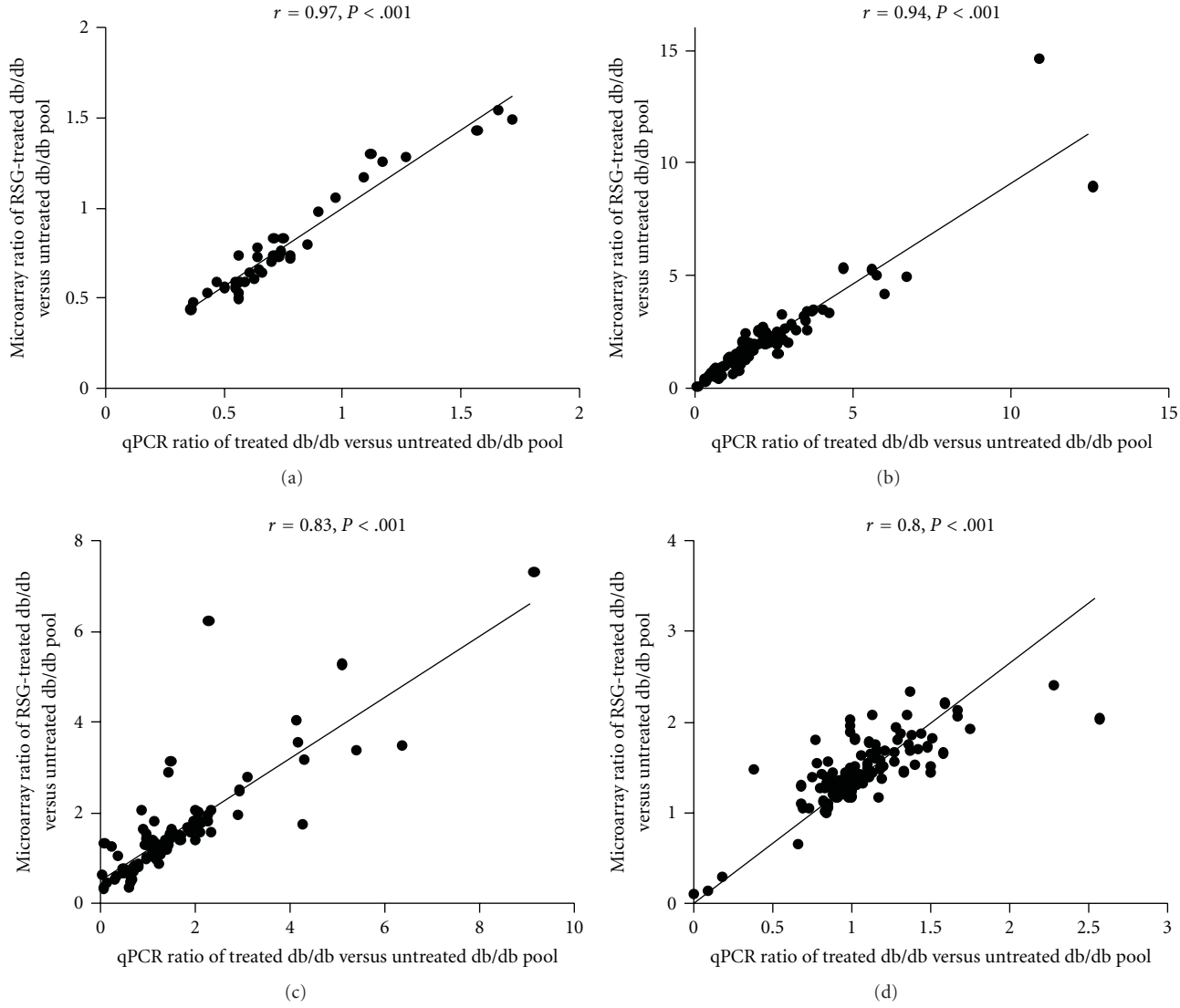

Figure 2: Correlation plot between microarray and qPCR data. Genes involved in (a) liver carbohydrate metabolism, (b) liver FA and TG metabolism, (c) IAT FA and TG metabolism, and (d) IAT citrate cycle and oxidative phosphorylation. Microarray ratio of differentially expressed genes in db/+ and RSG-treated db/db *versus* untreated db/db mice were plotted on the y-axis and qPCR ratio data on the x-axis. Correlations were assessed using Pearson's correlation coefficient (r) and P-value <.001.

data overview of differentially expressed genes between lean db/+ or RSG-treated mice and the diabetic db/db strain involved in glucose metabolism. As shown in Figure 1(a), remarkably, RSG treatment resulted in a partial or complete normalisation in mRNA expression of genes encoding for glycolysis/gluconeogenesis key enzymes such as Glut-2, Gapdh, Pklr or Fbp1, and G6pc. These effects were particularly pronounced at 100 mg/kg of RSG. The only exception is Pdk4 mRNA expression which is downregulated in db/+ mice but increased in RSG-treated db/db mice when compared to the control db/db. Real-time PCR was used to confirm the differential expression of genes involved in glucose metabolism (Supplemental Table 3). Figure 2(a) shows that microarray ratio measurements were strongly correlated to that obtained by PCR ($r = 0.97$; $P < .001$; number of xy pairs = 23).

Contrary to gluconeogenesis-related genes, which seemed to be normalised by RSG treatment, microarray data overview shows that a large number of genes involved in lipid metabolism was regulated in the opposite direction to db/+ (Supplemental Figures 2(b) and 1(b)). This included genes that were downregulated in db/+ but were all markedly upregulated in RSG-treated mice when compared to db/db. For example, at 10 mg/kg RSG-mediated upregulation of genes implicated in lipid metabolism synthesis (Figure 1(b)), such as the lipid transporters Cd36 (+62%, $P < .001$), the fatty acid binding protein Fabp4 (+870%, $P < .001$), the key enzymes in FFA and TG synthesis such as the ATP citrate lyase (Acly, +124%, $P < .001$), the acetyl-CoA-carboxylase (Acaca, +87%, $P < .001$), the fatty acid synthase (FAS, +270%, $P < .001$), and the stearoyl-Coenzyme A desaturase (Scd1, +455%, $P < .001$) or was involved in the pentose

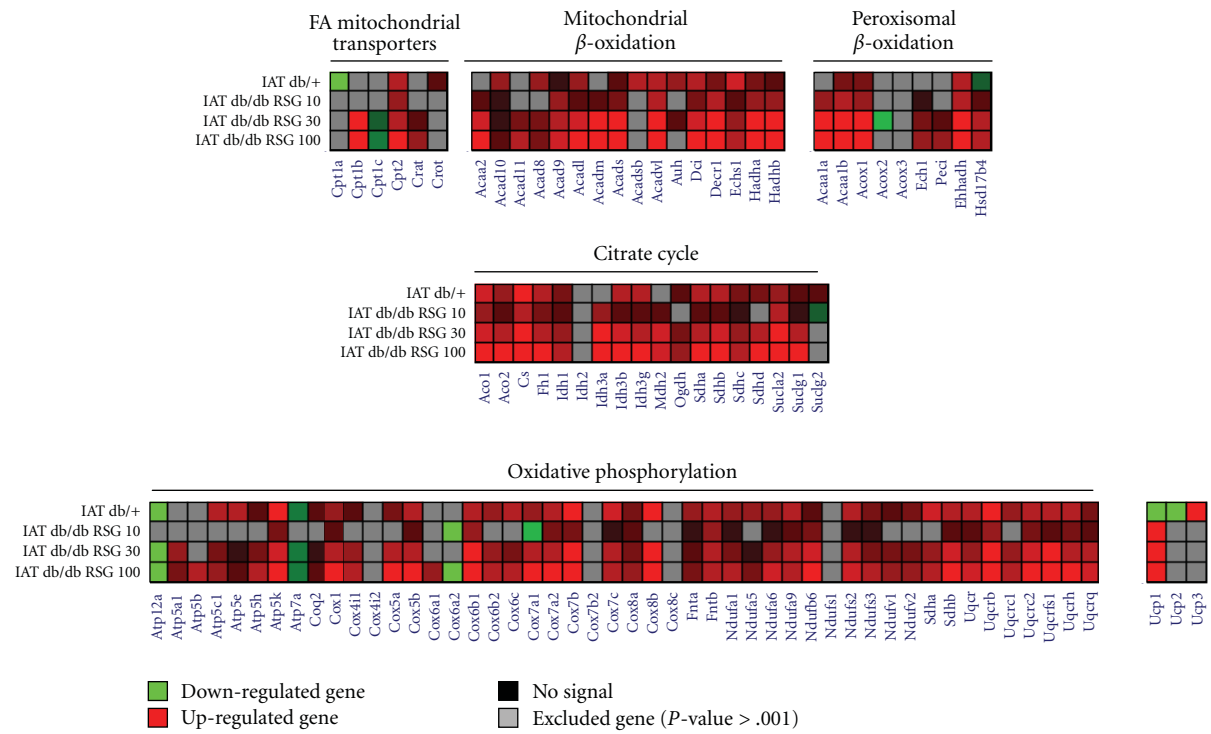

FIGURE 3: Two-dimensional representation of differentially expressed genes involved in IAT mitochondrial and peroxisomal functions. Ratio for differentially expressed genes in db/+ and RSG-treated db/db *versus* untreated db/db were plotted. Each coloured box represents differential expression ratio ranging from bright green (lowest) to bright red (highest). Missing value are in grey when P-value $\geq$ .001. Genes involved in mitochondrial transport of FA, in mitochondrial and peroxisomal β-oxidation and citrate cycle and in oxidative phosphorylation were shown.

phosphate pathway such as the transketolase (Tkt, +74%, $P < .001$) and phosphogluconate dehydrogenase (Pgd, +117%, $P < .001$). All these results were confirmed by real-time qPCR with a very strong correlation (Supplemental Table 3 and Figure 2(b); $r = 0.94$, $P < .001$; number of xy pairs = 155). The increased expression of genes encoding lipogenic enzymes and the pentose phosphate pathway could account for the increase in liver TG, FFA content, and weight liver (Table 1).

3.6. Effect of RSG on IAT Metabolic-Related mRNA Expression. In adipose tissue, among the 506 gene sequences 391 were differentially expressed in either db/+ versus db/db or RSG-treated db/db versus db/db (Supplemental Table 2). Numerous upregulated genes in db/+ or RSG-treated db/db relative to db/db diabetic mice (Supplemental Figure 3 and Figure 3) potentially stimulate lipid metabolism and mitochondrial functions.

Contrary to the liver, RSG treatment in IAT regulated the majority of metabolism-related genes in the same direction as observed in db/+ mice when compared to db/db. Indeed, genes involved in glucose metabolism, FFA and TG metabolism, pentose phosphate synthesis, and mitochondrial function were upregulated and/or normalised by RSG-treatment, some of them with a clear dose effect (Figures 4(a), 4(b), 4(c)). RSG treatment (10–100 mg/kg) partially normalised mRNA expression (Figure 4(a)) of two key genes involved in glucose metabolism such as the glucose transporter Glut4 (Slc2a4, +45%, $P < .001$ in 30 mg/kg RSG group and +114%, $P < .001$ in db/+ group) and the pyruvate dehydrogenase kinase 4 the downregulation of which stimulates glucose metabolism (Pdk4, 37%, $P < .001$ in 30 mg/kg RSG group and no change in db/+ group). On one hand, we observed partial normalisation of genes encoding FFA or TG metabolic enzymes including Acaca (+163%, in 30 mg/kg RSG group and +474%, in db/+ group, $P < .001$) and Fasn (+240%, in 30 mg/kg RSG group and +674%, in db/+ group, $P < .001$) or the pentose phosphate pathway gene Tkt (+86%, in 30 mg/kg RSG group and +409% in db/+ group, $P < .001$) (Figure 4(a)). On the other hand, for almost all genes involved in mitochondrial β-oxidation, citrate cycle, and oxidative phosphorylation, the level of upregulated genes observed in RSG-treated mice far exceeded that of db/+ regulated genes (Figures 4(b), 4(c)). All these results were validated with qPCR technique with strong correlation (Supplemental Table 3 and Figure 2(c); $r = 0.83$, $P < .001$; number of xy pairs = 96; Figure 2(d); $r = 0.80$, $P < .001$; number of xy pairs = 156).

3.7. Mitochondrial Renewal in db/db IAT Treated with RSG. The gene expression changes observed in adipose tissue suggested a mitochondrial dysfunction in db/db diabetic mice, but the RSG treatment appears to normalise a part of these alterations. To test whether diabetes and RSG

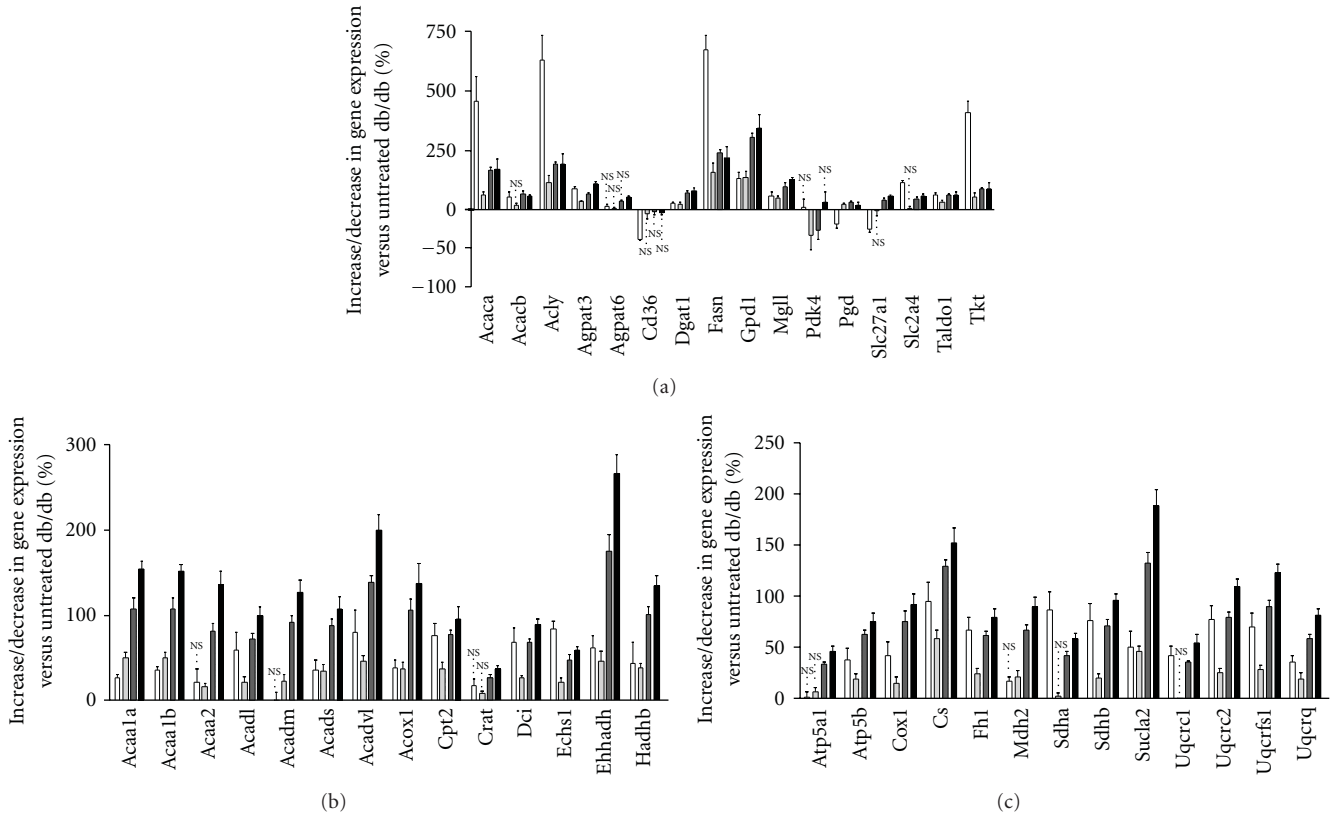

Figure 4: Expression profiles of key genes involved in IAT FA metabolism. Differentially expressed genes in db/+ (the white square) as well as 10 (the off-white square), 30 (the grey square), and 100 (the black square) mg/kg RSG-treated db/db mice were measured *versus* untreated db/db and plotted as the mean (% increase/decrease of db/db control) ±SEM ($n = 6$). NS; $P \geq .001$ otherwise $P < .001$. (a) Genes related to FA transport, glucose, FA and TG synthesis and pentose pathway*: Agpat3, 1-acylglycerol-3-phosphate O-acyltransferase 3; Dgat1, diacylglycerol O-acyltransferase 1; Slc27a1, solute carrier family 27 (fatty acid transporter), member 1; Slc2a4, solute carrier family 2 (facilitated glucose transporter), member 4. (b) Genes related to mitochondrial and peroxisomal β-oxidation: Acaa1a, Acaa1b, acetyl-Coenzyme A acyltransferase 1A, 1B; Acaa2, acetyl-Coenzyme A acyltransferase 2; Acadl, acyl-Coenzyme A dehydrogenase, long chain; Acadm, acyl-Coenzyme A dehydrogenase, medium chain; Acads, acyl-Coenzyme A dehydrogenase, short chain; Acadvl, acyl-Coenzyme A dehydrogenase, very long chain; Acox1, acyl-Coenzyme A oxidase 1, palmitoyl; Cpt2, carnitine palmitoyltransferase 2; Crat, carnitine acetyltransferase; Dci, dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase); Echs1, enoyl-Coenzyme A hydratase, short chain 1 mitochondrial; Ehhadh, enoyl-Coenzyme A, hydratase/3-hydroxyacyl-Coenzyme A dehydrogenase; Hadhb, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase, beta subunit. (c) Genes related to citrate metabolism and oxidative phosphorylation: Atp5a1, ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1; Atp5b, ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit; Cox1, M. musculus mRNA for mitochondrial gene for subunit I of cytochrome c oxidase; Cs, citrate synthase; Fh1, fumarate hydratase 1; Mdh2, malate dehydrogenase 2, NAD (mitochondrial); Sdha, succinate dehydrogenase complex, subunit A, flavoprotein (Fp); Sdhb, succinate dehydrogenase complex, subunit B, iron sulfur (Ip); Sucla2, succinate-Coenzyme A ligase, ADP-forming, beta subunit; Uqcrc1, Uqcrc 2, ubiquinol-cytochrome c reductase core protein 1, 2; Uqcrfs1, ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1; Uqcrq, ubiquinol-cytochrome c reductase, complex III subunit VII. *gene abbreviations given in Figure 1 were not mentioned.

also affected mitochondrial biogenesis and functioning at cellular level, the mitochondrial content of adipocytes was determined by using a monoclonal antibody specific for OxPhos Complex IV subunit I (Cox1), a mitochondrial membrane-bound protein complex. We have compared control db/db versus 30 mg/kg RSG-treated db/db adipocytes mitochondrial content, to avoid any possible side effects of the 100 mg/kg dose. As shown in Figure 5, signal intensity was stronger in adipocytes treated with RSG than in control db/db diabetic mice ($P < .05$). These results confirmed the transcriptional upregulation of Cox1 (+74%; $P < .001$) observed at 30 mg/kg dose and suggested that RSG induced mitochondrial biogenesis in adipocytes, as previously described [23, 24].

3.8. Effect of RSG on Metabolic-Related mRNA Expression in Soleus Muscle. In soleus muscle among the 506 regulated gene sequences 186 were differentially expressed either in db/+ versus db/db or RSG-treated db/db versus db/db (Supplemental Table 2). Soleus muscle plays an important role in both carbohydrate and FFA metabolism. It is well established that in obesity there is a dysfunction in the capacity of skeletal muscles to store glycogen. Also, increased

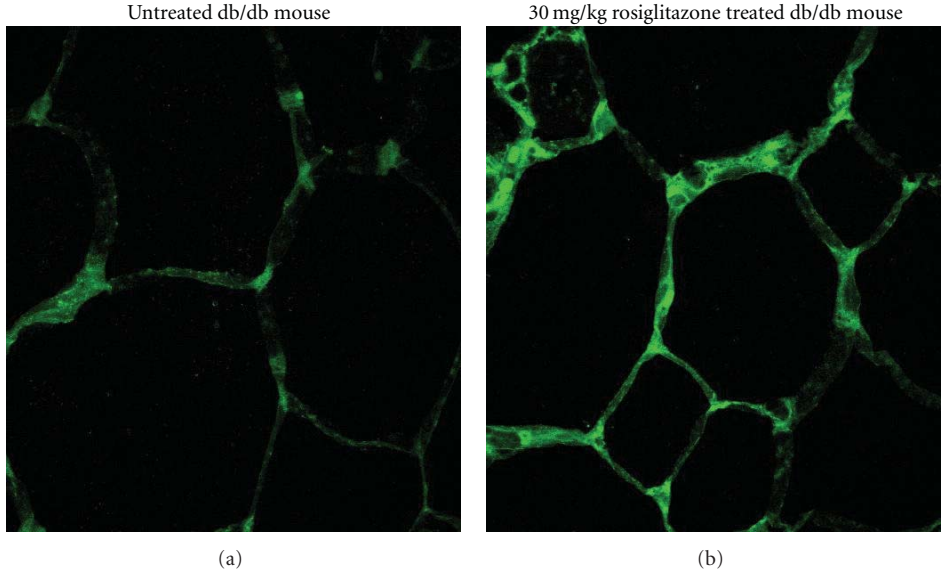

Figure 5: Immunochemical detection in db/db and RSG-treated db/db of the IAT OxPhos complex IV subunit I. IAT from untreated db/db (a) and from 30 mg/kg RSG-treated db/db (b) were isolated, fixed on slide, and stained with anti-OxPhos complex IV subunit I. Nuclei were identified with Hoechst 33342.

TG storage is positively correlated with markers of insulin resistance. Supplemental Figure 4 displayed microarray data overview of differentially expressed genes between lean db/+ or RSG-treated db/db mice and the untreated diabetic db/db involved in glucose and FFA metabolism. Fewer genes were regulated by RSG treatment in muscle in comparison to the liver and IAT. However, similarly to the differential gene expression observed between the db/+ and db/db diabetic mice, RSG induced an upregulation of key genes involved in carbohydrate metabolism such as Pkm2 (+44%, +45%, and +42% at 10, 30, and 100 mg/kg RSG, resp.) and HK1 (+57%, +60%, and +69%). Moreover, RSG treatment resulted in the repression of genes involved in lipid transport (Figure 6(a)), like Cd36 (42%, 30%, and 11% at 10, 30, and 100 mg/kg RSG, resp.) and Fabp4 (59%, 35%, and +30%). RSG also decreased the expression of genes involved in FA and TG metabolism like Acaca (25%, 40%, and 16% at 10, 30, and 100 mg/kg RSG, resp.) and MgII (32%, 14%, and +33%) (Figure 6(a)). It is noteworthy that the changes induced in gene expression by 10 mg/kg RSG tended to be most similar to db/+ versus db/db than the 30 and 100 mg/kg doses (see Figure 6(a), Acaca, Fabp4 or Mgll). This tendency seemed to be specific to soleus muscle and suggests possible toxic or secondary effects of high RSG concentrations. Taken together, these results suggest a decrease in FFA uptake and an increase in glucose utilization in soleus muscle of RSG-treated animals.

3.9. PLS Analysis. Multivariate statistical approach (PLS) has been applied in order to establish potential relationships between the changes in transcriptional profile and metabolic parameters under RSG treatments (Table 1). This method utilizes a linear regression model on latent structure to find correlations between two data matrices (X and Y). The significant differentially expressed sequences (Supplemental Table 2) involved in metabolism were defined as predictor variables (X) and FFA, TG (tissues and plasma) and glucose concentrations were defined as observation variables (Y). The models were retained based on $R^2{}_{Ycum}$ (% of explained sum of squares), $Q^2{}_{Ycum}$ (% of predictive sum of squares) and P-value. No model based on differential gene expression using data from IAT (391 sequences) and soleus (186 sequences) could be obtained. However, the predictor variables corresponding to differentially expressed sequences in liver (276) enable us to derive significant PLS models regarding the Y variables such as oleic acid and trioleate tissue concentrations (Table 2) as well as the liver weight. Figures 7(a), 7(b) show the correlation between the observed trioleate concentrations or liver weight and the predicted ones obtained from PLS models built using the 40 best sequence predictors ($R^2{}_{Ycum} = 0.89$ and 0.96, resp.). Among the 40 sequence predictors for liver trioleate concentrations and weight, 20 are common. Removing redundancy among the 20 predictors led to 16 genes from which 13 displayed PPRE in their proximal promoters as determined by MatInspector from Genomatix suite (see Section 2 and Supplemental Table 4). An interesting observation is that using the combination of differential gene expression from the three tissues (853 gene sequences) we were able to find a PLS model that made the link between predictor gene sequences and the blood glucose concentrations. Correlation between observed and predicted glucose using the 40 best predictors led to an acceptable model ($P = .007$ and $R^2{}_{Ycum} = 0.83$; Figure 7(c)) but predictive potency of the model remained rather weak ($Q^2{}_{Ycum} = 0.68$). Among the 40 predictors 35 belong to the IAT, 4 to the liver, and 1 to the soleus. A great deal of IAT mRNA- (24/35 sequences) encoded proteins involved in the mitochondrial

Table 3: Differential expression of the 40 best predictor gene sequences from PLS analysis of combined tissues: liver, IAT, and soleus. x: MatInspector-predicted PPRE.

Tissues	Gene name	Function	Sequence description	PPRE	db/+	RSG 10 MK	RSG 30 MK	RSG 100 MK
					F. C.	F. C.	F. C.	F. C.
S	Cyp4a12a	Fatty acid metabolism	cytochrome P450, family 4, subfamily a, polypeptide 12a		1.72	1.25	1.27	1.46
TA	Dci	Fatty acid metabolism	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	x	1.51	1.28	1.68	1.85
TA	Mcat	Fatty acid metabolism	malonyl CoA: ACP acyltransferase (mitochondrial)		1.57	1.29	1.54	1.66
TA	Ppargc1b	Fatty acid metabolism	peroxisome proliferative activated receptor, gamma, coactivator 1 beta		1.63	−1.06	1.37	1.83
TA	Ppargc1b	Fatty acid metabolism	peroxisome proliferative activated receptor, gamma, coactivator 1 beta		2.11	1.10	1.67	2.33
TA	Ppargc1b	Fatty acid metabolism	peroxisome proliferative activated receptor, gamma, coactivator 1 beta		1.41	−1.10	1.30	1.80
TA	Dlat	Gluconeogenesis	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	x	1.77	1.49	2.10	2.72
TA	Ldhb	Gluconeogenesis	lactate dehydrogenase B		1.76	1.07	1.36	1.81
TA	Pdhb	Gluconeogenesis	pyruvate dehydrogenase (lipoamide) beta		2.19	1.69	2.49	3.05
TA	Pdhb	Gluconeogenesis	pyruvate dehydrogenase (lipoamide) beta		2.04	1.65	2.32	2.82
L	Pfkfb2	Gluconeogenesis	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	x	−1.16	−1.72	−1.59	−1.56
L	Ppargc1a	Gluconeogenesis	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	x	−1.32	−1.53	−1.61	−1.59
L	Ppargc1a	Gluconeogenesis	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha		−1.64	−1.71	−1.75	−1.75

Table 3: Continued.

Tissues	Gene name	Function	Sequence description	PPRE	db/+	RSG 10 MK	RSG 30 MK	RSG 100 MK
TA	Atp5h	Mitochondrial respiratory chain	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	x	1.21	1.07	1.26	1.41
TA	Atp5h	Mitochondrial respiratory chain	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d		1.23	1.06	1.26	1.47
TA	Cox6b1	Mitochondrial respiratory chain	cytochrome c oxidase, subunit VIb polypeptide 1		1.54	1.39	1.82	2.16
TA	Cox6b2	Mitochondrial respiratory chain	cytochrome c oxidase, subunit VIb polypeptide 2		1.35	1.08	1.37	1.59
TA	Cox7a1	Mitochondrial respiratory chain	cytochrome c oxidase, subunit VIIa 1	x	1.37	−1.52	1.45	1.98
TA	Cox7b	Mitochondrial respiratory chain	cytochrome c oxidase, subunit VIIb	x	1.96	1.33	1.87	2.15
TA	Ndufa1	Mitochondrial respiratory chain	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	x	1.44	1.13	1.42	1.52
TA	Ndufa1	Mitochondrial respiratory chain	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1		1.35	1.09	1.31	1.46
TA	Uqcrb	Mitochondrial respiratory chain	ubiquinol-cytochrome c reductase binding protein	x	1.81	1.40	1.82	2.14
TA	Uqcrfs1	Mitochondrial respiratory chain	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1		1.57	1.24	1.87	2.25
TA	Uqcrh	Mitochondrial respiratory chain	ubiquinol-cytochrome c reductase hinge protein		1.89	1.43	1.81	1.97
TA	Uqcrh	Mitochondrial respiratory chain	ubiquinol-cytochrome c reductase hinge protein		1.65	1.28	1.57	1.83
TA	Fntb	Steroid biosynthesis	farnesyltransferase, CAAX box, beta	x	1.48	1.43	1.55	1.49
L	Hmgcs2	Steroid biosynthesis	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	x	−1.32	−1.09	−1.28	−1.37
TA	Agpat3	Triglyceride	1-acylglycerol-3-phosphate O-acyltransferase 3	x	1.81	1.31	1.65	2.07

Table 3: Continued.

Tissues	Gene name	Function	Sequence description	PPRE	db/+	RSG 10 MK	RSG 30 MK	RSG 100 MK
TA	Aco1	Tricarboxylic acid cycle (mit oxidation)	aconitase 1	x	1.77	1.37	1.70	1.94
TA	Aco2	Tricarboxylic acid cycle (mit oxidation)	aconitase 2, mitochondrial	x	1.38	1.18	1.66	1.98
TA	Aldh5a1	Tricarboxylic acid cycle (mit oxidation)	aldhehyde dehydrogenase family 5, subfamily A1	x	1.60	1.33	1.65	1.69
TA	Dlst	Tricarboxylic acid cycle (mit oxidation)	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	x	1.43	1.16	1.63	1.83
TA	Fh1	Tricarboxylic acid cycle (mit oxidation)	fumarate hydratase 1	x	1.56	1.28	1.63	1.87
TA	Idh3b	Tricarboxylic acid cycle (mit oxidation)	isocitrate dehydrogenase 3 (NAD+), beta	x	1.49	1.18	1.73	1.91
TA	Idh3g	Tricarboxylic acid cycle (mit oxidation)	isocitrate dehydrogenase 3 (NAD+), gamma	x	1.67	1.18	1.59	1.91
TA	Mdh1	Tricarboxylic acid cycle (mit oxidation)	malate dehydrogenase 1, NAD (soluble)		1.93	1.17	1.91	1.95
TA	Mdh1	Tricarboxylic acid cycle (mit oxidation)	malate dehydrogenase 1, NAD (soluble)		1.63	1.44	2.01	2.17
TA	Sdhb	Tricarboxylic acid cycle (mit oxidation)	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)		1.60	1.21	1.67	1.97
TA	Sdhc	Tricarboxylic acid cycle (mit oxidation)	succinate dehydrogenase complex, subunit C, integral membrane protein	x	1.32	1.10	1.35	1.48
TA	Sdhd	Tricarboxylic acid cycle (mit oxidation)	succinate dehydrogenase complex, subunit D, integral membrane protein	x	1.29	1.11	1.57	1.90

respiration and tricarboxylic acid cycle (Table 3) and 3 out of 4 genes from liver are involved in gluconeogenesis. Finally, among the 32 unique genes (corresponding to the 40 gene sequences), 21 presented a PPRE in their promoter regulatory sequences.

These observations underline the link between RSG-mediated PPARγ activation and expression of genes involved in IAT mitochondrial function, liver gluconeogenesis, and regulation of blood glucose.

4. Discussion

In this paper, for the first time we have attempted to establish a link between metabolic status of RSG-treated diabetic mice (db/db) and RSG-mediated transcriptomic changes in the three major insulin-dependent tissues including liver, adipose tissue, and skeletal muscle.

As expected, numerous genes regulated by RSG and identified in this study fall into key metabolic pathways involved in carbohydrate and lipid metabolism. PPARγ

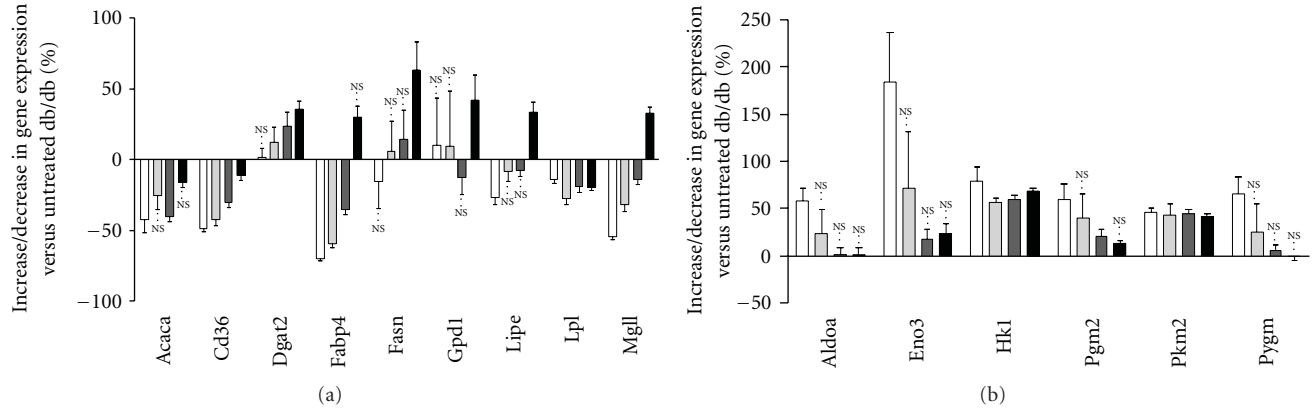

Figure 6: Expression profiles of key genes involved in Soleus muscle glucose and lipid metabolism. Differentially expressed genes in db/+ (the white square) and 10 (the off-white square), 30 (the grey square), and 100 (the black square) mg/kg RSG-treated db/db mice were measured *versus* untreated db/db and plotted as the mean (% increase/decrease of db/db control) ±SEM ($n = 6$). NS; $P \geq .001$ otherwise $P < .001$. (a) Genes related to glucose metabolism*: Eno3, enolase 3, beta muscle; Hk1, hexokinase 1; Pkm2, pyruvate kinase, muscle; Pgm2, phosphoglucomutase 2; Pygm, muscle glycogen phosphorylase. (b) Genes related to lipid metabolism: Dgat2, diacylglycerol O-acyltransferase 2; Lipe, lipase hormone sensitive; *gene abbreviations given in Figures 1, and 4 were not mentioned.

agonist also regulates many more genes in adipose tissue than in liver or skeletal muscle, as might be expected, based on PPARγ expression levels in the respective tissues. Despite this difference, our data showed that PPARγ activation has coordinated effects on fundamental metabolic pathways in each of these tissues, including glucose and lipid metabolism in skeletal muscle, gluconeogenesis in liver, as well as lipogenesis, TG storage, and mitochondrial function in adipose tissue. Gene transcription modulation by RSG could be classified into two types, namely, those which counteract diabetes-induced alteration and correlated to the modulation observed in db/+ versus db/db and those which accentuate diabetes induced alteration and are inversely correlated. The first group could explain the glucose and lipid-lowering actions of PPARγ agonists, and the second group could be linked to TZDs compound-mediated side effects.

4.1. Liver. One of the most interesting observations is that RSG treatment normalised glucose homeostasis in db/db mice (result presented here in and in [15]) and the mRNA expression of gluconeogenic key enzymes such as the G6pc, Fbp1, and the glucose transporter Glut-2. These results suggest that transcriptional regulation of G6pc and Fbp1 mRNA play a role in RSG-mediated decreased gluconeogenesis and blood glucose normalisation. This observation is in agreement with previous studies showing an increase in G6pc and Fbp1 activities in db/db mice and the subsequent increase in glucose production by the db/db liver [25].

In human, hepatic gluconeogenesis is known to be significantly enhanced in type 2 diabetes and normalised by RSG [10, 26]. Therefore, the transcriptional status of G6Pc and Fbp1, related to RSG-mediated gluconeogenesis normalisation, deserves to be further investigated in humans.

In rodents one of the thiazolidinedione treatment side effects is associated with liver dysfunction. RSG-treated db/db mice have shown a rise in their liver weight and steatosis appearance, as previously shown [12]. Indeed, the potent antihyperglycaemic effect of RSG was accompanied by an increase of *de novo* synthesis of fatty acids when compared to either db/+ or db/db. Lipid metabolome analysis was concordant with our gene expression profiling obtained in liver and corroborate with the results obtained in a previous study [13]. A rise in hepatic expression of genes belonging to numerous steps of the TG synthesis in liver was observed under RSG treatment including those that encode for FA transporters (Lpl, Cd36, Fabp2, 4), TG (Gpd1) and FFA synthesis (Acly, Fasn, Acaca, Gpd1, Agpat2, 6, Scd1) and are likely associated to the RSG-mediated increase in hepatic trioleate levels. This result is in contrast with the RSG effects in humans and normal mice, where chronic RSG treatment reduced liver fat [27, 28]. However db/db is a leptin signalling deficient paradigm, and numerous publications demonstrated in wild-type mice that leptin decreases hepatic *de novo* synthesis of FA through the decrease in mRNA, proteins, or enzymatic activities of FFA and TG metabolism enzymes including Acly, Acaca, Fas, Scd-1, or Agpat [29–33]. Therefore, mice lacking leptin signalling are not the best paradigm to foresee PPAR agonist secondary effects in human liver.

4.2. Inguinal Adipose Tissue. In adipose tissue, mitochondria are not only the major site of fatty acid oxidation but may also play a critical role in lipogenesis by providing key intermediates for TG synthesis. Impaired mitochondria may lead to the lack of ATP and subsequent reduction in lipid metabolism [23]. This dysfunction could be linked to a reduced ability of glucose utilization, participating in high blood glucose levels in diabetic mice. A decrease in ATP may also impair the synthesis and secretion of adipokines, which were previously shown to be associated with diabetes [34]. In our study, RSG significantly induced a dose-dependent increase in a number of genes implicated in

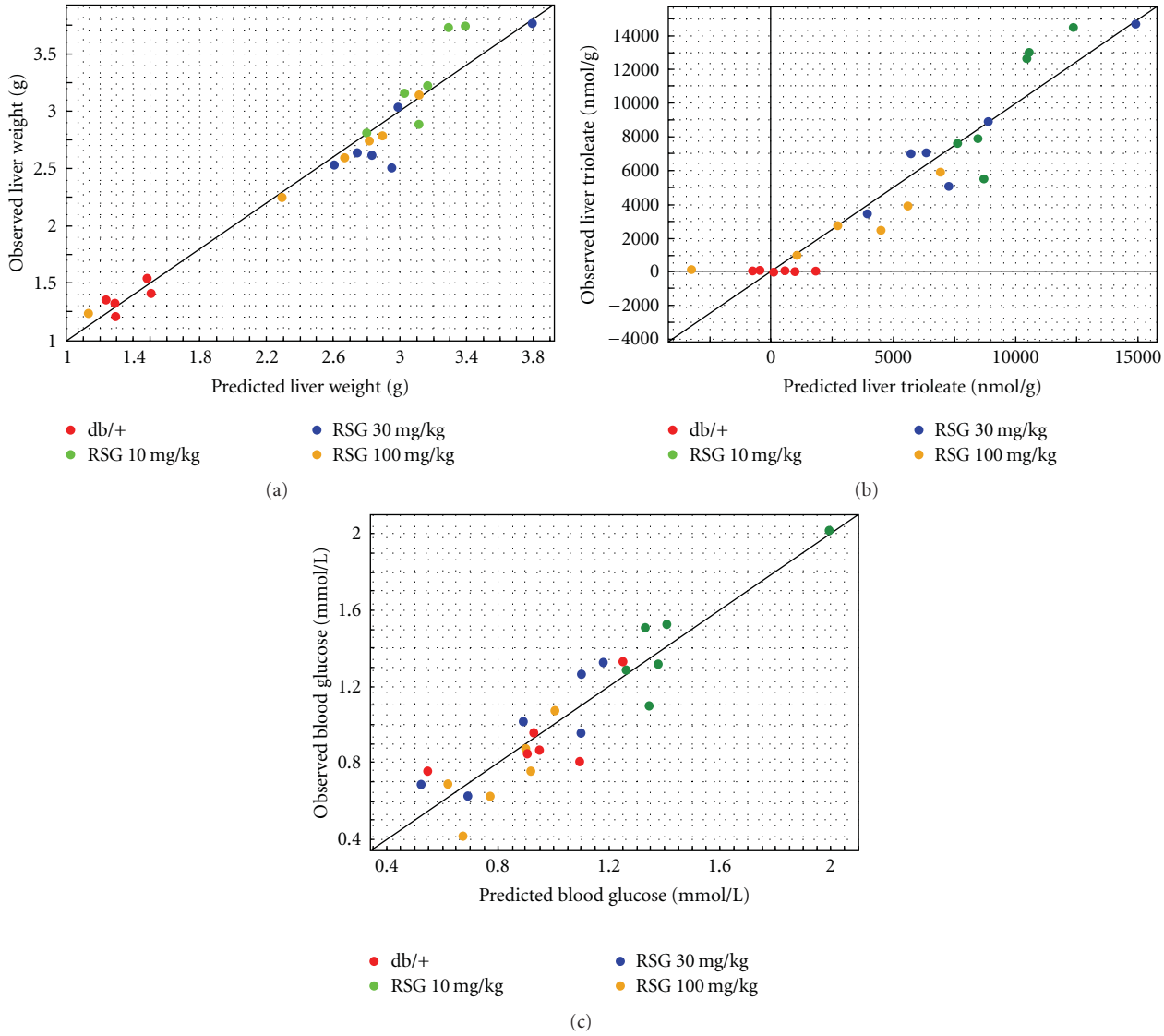

Figure 7: Generation of PLS models to predict physiological changes from multivariate gene expression data. Results show the correlations between the actual liver weight (a), the liver trioleate concentrations (b), the blood glucose level (c) and the predicted parameters from the PLS model. The normalised logs (ratio) of genes involved in glucose and lipid metabolism were used as predictor variables (X) and physiological parameters as response variables (Y). All variables were centred and scaled to unit variance, before the PLS analysis was performed.

mitochondrial activities. Genes encoding for enzymes involved in β-oxidation, citrate cycle, and oxidative phosphorylation were upregulated in a similar manner to that observed in db/+ versus db/db mice. Moreover, we have observed an upregulation of PPARγ coactivator PGC1α (data not shown), which is known to potently activate mitochondrial biogenesis in adipose and muscle tissues [24, 35]. Combined with immunocytochemistry results, these data showed that RSG treatment led to an increase in mitochondrial biogenesis. The effects of PPARγ agonist on both mitochondrial number and morphology were previously observed in adipose tissues of rat and dog treated with RSG [36]. RSG is also known to induce adipocyte differentiation and mitochondrial biogenesis [37]. These new small adipocytes are insulin sensitive and possess a higher lipid metabolism capacity that could explain the RSG-increased mRNA of genes involved in lipid transport and oxidation. The IAT is composed of both brown adipose tissue (BAT) and white adipose tissue (WAT). Our data show the upregulation of BAT markers Ucp1 or Cpt1b in IAT of treated mice, as described by others [34]. This observation supports the view of RSG converting WAT to BAT, transforming IAT into fat-oxidizing machinery. They also suggest that decreasing the exposure of peripheral tissues to lipids may improve the whole-body insulin sensitivity.

4.3. Soleus Muscle. Normalisation of hyperglycaemia by RSG-decreased hepatic glucose synthesis and output seems to be combined with better use of glucose in soleus muscle.

Indeed, RSG-treated mice showed an increased expression of genes coding for key enzymes involved in glycolysis, like Hexokinase1 Hk1, Enolase Eno3, or Pyruvate kinase Pkm2, similar to our observation in db/+ versus db/db. RSG treatment of db/db also resulted in a coordinated decrease in the expression of some genes involved in fatty acid transport and metabolism in muscle. It is noteworthy that the expression of these genes was upregulated in liver and to a lesser extent in IAT in response to RSG, suggesting that PPARγ activation promotes a flux of fatty acids into hepatic and adipose tissues and away from muscle. Altogether, these data suggest a decreased reliance on fatty acids and an increased reliance on glucose as an energy source in muscle, as previously described [2, 38]. Surprisingly, the RSG-mediated gene expression normalisation in muscular cells was observed mainly at 10 mg/kg dose, whereas the highest doses induced the expression of genes related to glucose and lipid pathways in opposite direction to that measured in db/+ versus db/db, suggesting a possible adverse effect at higher drug concentrations in this tissue.

4.4. PLS Models. Taking advantage of the experimental design including both transcriptomic and metabolomic approaches, we applied a multivariate linear regression model (PLS) in order to predict the metabolic parameters (i.e., blood glucose and FFA and TG from blood and tissues) based on regulated genes (predictor (X) variables). Only the variables X from liver allowed us to derive models that significantly predicted some of the measured biological parameters such as liver oleic acid and trioleate as well as the liver weight. It is worth noticing that among the 40 best predictors 20 (16 genes) are common between trioleate and the liver weight model. The fact that among these 16 genes 13 display a PPRE and therefore are supposed to be regulated by PPAR agonist reinforces the predictory status of these 20 gene sequences. Obviously more experiments should be undertaken with different RSG dose treatments to confirm these predictors. Moreover, no significant model could be built with differential gene expression data from liver and blood metabolic parameters, and no model could be obtained with IAT or soleus muscle regarding both tissue and blood metabolic parameters. However, the most interesting observation is that a model was validated with blood glucose levels and the combination of gene expression data from the 3 tissues. When looking at the 40 best predictors, we found that a large majority of gene sequences implied IAT-expressed genes (35/40), and among the 35, 25 encoded mitochondrial proteins involved in energy cell production and 4 gene sequences were originated from the liver and are involved in gluconeogenesis. In that case we may hypothesise that those blood glucose predictors are linked to molecular mechanisms because; (i) mitochondrial dysfunction is linked to diabetes type 2 and thiazolidinediones were known to improve the diabetes status by the production of more functional adipocytes; (ii) in db/db mice high levels of blood glucose partially is due to gluconeogenesis [25] and RSG by decreasing mRNA encoding gluconeogenic enzymes may indeed regulate blood glucose.

Therefore, PLS reveals not only IAT/liver predictors for blood glucose regulation but also a potential molecular mechanism that could explain in part the glucose regulation. Whether or not this mechanism is due to direct PPARγ-induced transcriptional effects or PPAR interaction with the mitochondrial protein such as MitoNEET [39] that subsequently activate mitochondrial functions remains to be investigated, but a majority of genes in the top 40 predictor sequences (21/32 genes) displayed predicted PPRE in their respective promoter.

5. Conclusion

To our knowledge, this is the first study comparing gene expression profiles between db/+ and RSG-treated db/db with control diabetic db/db in liver, muscle, and adipose tissues. The combination of transcriptomic and metabolomic approaches led to a comprehensive molecular portrait and hypothesis on the dose-dependent effects of RSG in db/db diabetic mice and highlights the role of the respective insulin-dependent tissues. This approach could be useful in the future to discriminate between selective PPAR modulators regarding their specific molecular profiles in relation to specific target tissues.

Abbreviations

FFAs:	Free fatty acids
RSG:	Rosiglitazone
IAT:	Inguinal adipose tissue
PPAR:	Peroxisome proliferator-activated receptor
PPRE:	PPAR response element
PLSs:	Partial least squares
TZD:	Thiazolidinedione
TGs:	Triglycerides
UPLC-MS:	Ultra performance liquid chromatography-mass spectrometry.

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Review Article

Coactivators in PPAR-Regulated Gene Expression

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Peroxisome proliferator-activated receptor (PPAR)α, β (also known as δ), and γ function as sensors for fatty acids and fatty acid derivatives and control important metabolic pathways involved in the maintenance of energy balance. PPARs also regulate other diverse biological processes such as development, differentiation, inflammation, and neoplasia. In the nucleus, PPARs exist as heterodimers with retinoid X receptor-α bound to DNA with corepressor molecules. Upon ligand activation, PPARs undergo conformational changes that facilitate the dissociation of corepressor molecules and invoke a spatiotemporally orchestrated recruitment of transcription cofactors including coactivators and coactivator-associated proteins. While a given nuclear receptor regulates the expression of a prescribed set of target genes, coactivators are likely to influence the functioning of many regulators and thus affect the transcription of many genes. Evidence suggests that some of the coactivators such as PPAR-binding protein (PBP/PPARBP), thyroid hormone receptor-associated protein 220 (TRAP220), and mediator complex subunit 1 (MED1) may exert a broader influence on the functions of several nuclear receptors and their target genes. Investigations into the role of coactivators in the function of PPARs should strengthen our understanding of the complexities of metabolic diseases associated with energy metabolism.

1. Introduction

The foundation for the discovery and designation of the PPAR subfamily of nuclear receptors in the early 1990s is the cumulative work over the preceding 25 years with peroxisome proliferators, a group of structurally diverse chemicals that induce characteristic and predictable pleiotropic responses including the transcriptional activation of genes involved in the fatty acid oxidation [1–6]. The PPAR subfamily consists of three members namely PPARα (NR1C1), PPARβ (also known as δ) (NR1C2), and PPARγ (NR1C3) with a high degree of sequence conservation across the species [1, 2, 7–9]. All three PPARs in the human and mouse are encoded by separate genes that are on different chromosomes [9]. PPARγ has two isoforms, PPARγ1, and an N-terminal 30 amino acid extended form PPARγ2, both encoded by the same gene using two distinct promoters and alternate splicing [10, 11]. All three members of PPAR subfamily function as sensors for fatty acids and fatty acid derivatives and control metabolic pathways involved in energy homeostasis [12, 13]. PPARs display high levels of homologies at the protein level, but exhibit distinct and noninterchangeable functional roles in mammalian energy metabolism [9]. PPARα is expressed in tissues with high fatty acid oxidation activities, which include liver, kidney, small intestine, heart, and skeletal muscle, consistent with its predominant functional role in regulating lipid catabolism. In the liver, PPARα is the master regulator of mitochondrial, peroxisomal, and microsomal fatty acid oxidation systems where it is activated by synthetic peroxisome proliferators and in addition senses the influx of fatty acids during fasting to upregulate the fatty acid burning capacity [14]. PPARα also plays a role in lipoprotein synthesis, inflammatory responses and the development of cancer in the rodent liver [15–19]. PPARβ is ubiquitously expressed with relatively higher levels found in brain, adipose tissue, and skin [20]. Activation of PPARβ also induces expression of genes required for fatty acid oxidation and energy dissipation

in skeletal muscle and adipose tissue which in turn lead to improved lipid profiles and reduced adiposity [21]. In the liver, PPARβ can be activated by plasma free fatty acids influxed during fasting conditions [22]. PPARγ which is expressed at a relatively high level in adipose tissue serves as an essential regulator for adipocyte differentiation and promotes lipid/energy storage in mature adipocytes by increasing the expression of several key genes in this pathway [23]. These two important functions of PPARγ, namely adipogenesis and fat storage in adipocytes account for the insulin sensitizing effects of the anti-diabetic thiazolidinediones [24]. In summary, PPARα and PPARβ participate in energy burning, whereas PPARγ is critical in regulating adipocyte differentiation and energy storage by adipocytes [11, 25, 26].

2. Transcriptional Regulation of PPARs

PPARs are ligand-activated transcription factors similar to other members of the nuclear hormone receptor superfamily [7, 8]. PPARs are nuclear in location, where they remain heterodimerized with the 9-cis retinoic acid receptor, RXRα (NR2B) [13] and bind to the upstream cis-acting regulatory regions termed as peroxisome proliferator response element (PPRE) of target genes [9, 27]. The canonical PPRE consists of two direct repeats AGGTCA separated by a single nucleotide so-called DR-1 element [28]. The two half-sites are distinguishable by their 5′ and 3′ positioning on the DR1 element whereby the DNA binding domain of PPAR binds 5′ half-site while RXR binds to the 3′ half-site [29, 30]. In addition to core DR-1 sequence, PPRE element contains an additional AAACT motif at the 5′ upstream region [30]. The hinge region of PPAR forms extensive interaction with the upstream AAACT element [30]. In the absence of ligand, the unliganded PPAR-RXR heterodimer remains bound to the nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), two well-characterized corepressors (Figure 1) that are mostly present in the corepressor complex [31]. Both NCoR and SMRT directly interact with the Sin3 complex to form a multisubunit repressor complex [32]. SMRT functions as a platform protein and facilitates the recruitment of histone deacetylases (HDACs) to the DNA promoters bound by specific interacting transcription factors [32]. Another corepressor, Receptor Interacting Protein 140 (RIP140) also known as NRIP1 (nuclear receptor interacting protein 1) directly recruits HDAC and represses the activity of various nuclear receptor including PPARs by competing with their coactivators [33–35]. In the absence of ligand activation of nuclear receptor, the corepressor protein complex is known to subdue target gene transcription by causing the deacetylation of histones [31].

The nuclear receptor-regulated transcriptional activation of target genes depends on the binding of a cognate ligand to the receptor (activator). For example, activation of PPARα-RXR heterodimer by a PPARα ligand triggers conformational changes in the receptor which releases the corepressor complex and recruits cofactor complexes to the promoter region of target genes (Figure 1) to initiate transcription [38, 39]. Transcription coactivators increase gene transcription via the acetylation of histones and through the recruitment and stabilization of the transcriptional complexes, mainly the Mediator complex which interacts directly with activator proteins and pol II [40–42]. During the past 15 years, more than 300 cofactors (coactivators, coregulators, corepressors etc.) have been identified but the in vivo physiological regulatory functions of many of these molecules in receptor/gene/cell-specific transcription remain to be explored [43]. This paper summarizes the current state of knowledge about the roles of coactivators and coactivator associated proteins (Table 1), with special emphasis on p160/SRC family members and PPAR-binding protein (PBP/PPARBP)/thyroid hormone receptor-associated protein 220 (TRAP220)/mediator complex subunit 1 (Med1), in the functioning of PPARs.

3. Coactivators for PPAR Function

Transcriptional activation of PPAR-regulated genes is enhanced by coactivators. Most coactivators possess one or more LXXLL motifs (L: leucine and X: any amino acid) some of which may make contact with a coactivator-binding groove in the ligand-binding domain of nuclear receptor [44]. The assembly or preassembled coactivator complexes facilitate the liganded PPAR to achieve transcriptional activation of specific target genes in a tissue/cell-specific manner [45, 46]. Once coactivators are recruited to a liganded nuclear receptor they remodel chromatin structure by the intrinsic histone acetyltransferase (HAT) or methyl transferase activities [46]. In order to achieve this, steroid receptor coactivator (p160/SRC) family of proteins, which possess HAT activity, are recruited to the activation function 2 (AF-2) domain of the nuclear receptor and complex with the universal coactivator cyclic-AMP responsive element binding protein (CREB)-binding protein (CBP) and its homologue p300 [47–49]. CBP and p300 also exhibit potent HAT activity [50].

The second category of coactivators, with no known enzymatic functions, participates in the formation of a well-known multisubunit protein complex, variously called TRAP/DRIP/ARC/Mediator complex, consisting of 15–30 proteins [36, 37, 41, 57, 73–77]. Mediator complex, which is anchored by PBP (PPARBP)/TRAP220/DRIP205/Med1 facilitates interaction with poll II of the basal transcription machinery [41, 73, 75]. Members of this Mediator complex appear to be devoid of intrinsic enzymatic activities [46], but play an important role in connecting CBP/p300 bound coactivators with pol II containing preinitation complex [76]. Disruption of CBP/p300 and Med1 genes in the mouse results in embryonic lethality around E11.5 days, indicating that deletion of these pivotal anchoring coactivators affects the integrity of the cofactor complexes, thus altering the function of many nuclear receptors and most likely of other transcription factors [77–79].

A number of other coactivators and coactivator-associated proteins that possess enzymatic activities like

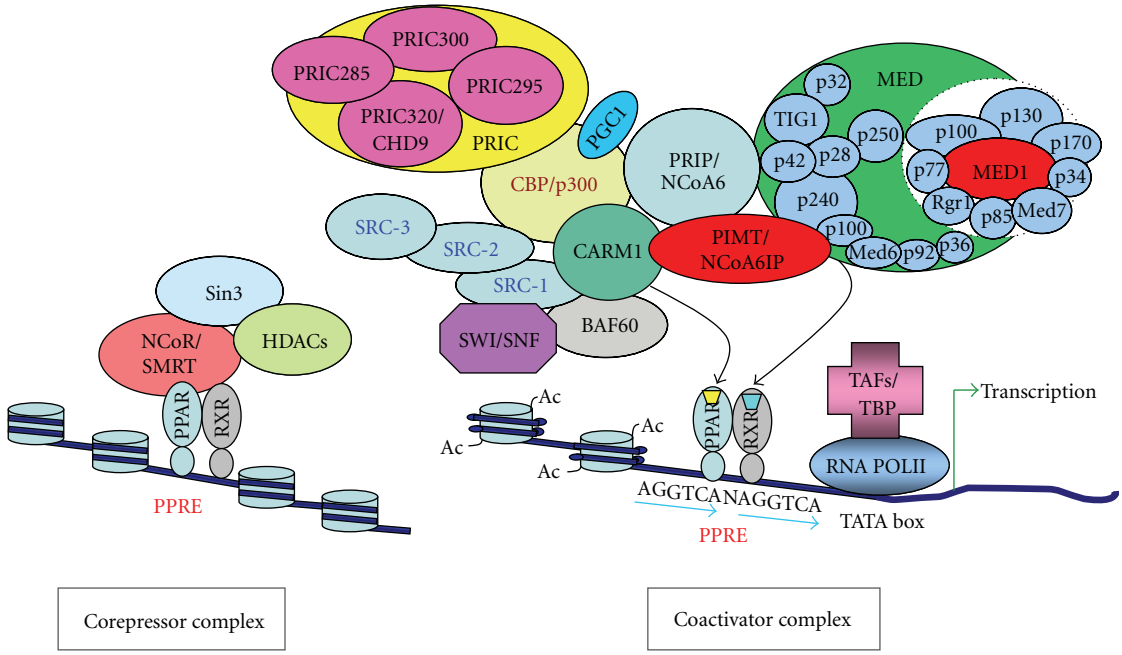

Figure 1: A schematic representation of ligand-dependent recruitment of coactivators for PPAR-regulated target gene transcription. In the absence of ligand, the PPAR-RXR heterodimer recruits corepressors, which in turn, assemble additional components of a repressor complex including histone deacetylase (HDAC). When ligand (yellow trapezium representing PPAR ligand, and blue trapezium representing 9-cis-retinoic acid as RXR ligand) binds, conformational changes in PPAR-RXR induce dissociation of corepressor complex. Active transcriptional complex assembles with coactivator proteins either sequentially or preassembled subcomplex modules. PPAR binds to peroxisome proliferator response element (PPRE) and assemble coactivator complexes that acetylate (SRCs, p300) or methylate (CARM1) nucleosomes for chromatin remodeling. Mediator components [36, 37] contact PPARs and facilitate the recruitment of the basal transcription machinery (TATA-box-binding protein [TBP]/TBP-associated factors [TAFs]) to form linkage with RNA polymerase II for transcription of specific target genes.

Table 1: Some known coactivator and coactivator associated proteins that regulate PPAR function.

Coactivator proteins	Enzyme activity	Function	References
SRC-1/NCoA-1	HAT	Histone acetylation	[51, 52]
SRC-2/TIF2/GRIP1	HAT	Histone acetylation	[53]
SRC3/pCIP/AIB1	HAT	Histone acetylation	[54, 55]
CBP/p300	HAT	Histone acetylation followed by recruitment of p160/SRCs	[56]
MED1/TRAP220/PBP	None	Anchor for Mediator complex	[57, 58]
PGC-1α	None	Recruit coactivator with HAT activities	[59]
PGC-1β/PERC	None	Recruit coactivator with HAT activities	[60]
PRIP/NCoA6	None	Recruit ASC complex	[61, 62]
PRIC285	Helicase	Chromatin remodeling by histone displacement and nucleosomal sliding	[63]
PRIC320/CHD9	Helicase	Chromatin remodeling by histone displacement and nucleosomal sliding	[64]
SWI/SNF	ATPase	ATP dependent mobilization of nucleosome	[65, 66]
BAF60a/SMARCD1	None	Recruit SWI/SNF complex	[67, 68]
BAF60c/SMARCD3	None	Recruit SWI/SNF complex	[69]
Coactivator-associated proteins			
PIMT/NCoA6IP	Methyltransferase	Methylation of caps of snRNAs and snoRNAs	[70]
CARM1/PRMT4	Methyltransferase	Potentiate SRCs by methylation of Histone H3	[71]
CoAA	None	RNA splicing	[72]

methyltransferase (CARM1) [71], helicase (PRIC285, p68) [63, 80], and ATP dependent chromatin remodeling properties (PRIC320, SWI/SNF) [63, 80] or those without any enzymatic activities such as PPARγ coactivator-1α (PGC-1α), PGC-1β, and BAFs [81, 82] have been identified in the active PPAR transcriptional complex, referred to as PRIC (PPARα-receptor interacting cofactor) complex [64, 80]. Some other important coactivators such as PRIP (peroxisome proliferator-activated receptor-interacting protein)/ASC2/AIB3/RAP250/NCoA6 [61, 83–85] and PIMT (PRIP-interacting protein with methyltransferase domain/NCoA6IP) [70] are also identified, which serve as linkers between the initial HAT complex of CBP/p300 and p160 coactivators and the downstream mediator complex [76]. CCPG (constitutive coactivator of PPARγ) is identified as a novel coactivator for PPARγ [86]. As mentioned above, sequential or combinatorial recruitment of various multisubunit coactivator proteins on the liganded nuclear receptor leads to the establishment of a stable preinitiation complex with multiple possible configurations on to the target gene promoter.

4. p160/SRC Family of Coactivators with HAT Activity

p160/SRC family of coactivators consists of three members, namely, SRC-1/NCoA-1, SRC-2/TIF2 (transcriptional intermediary factor 2)/GRIP1 (glucocorticoid receptor interacting protein 1), and SRC-3/pCIP (CBP-interacting protein)/RAC3 (receptor-associated coactivator-3)/ACTR/AIB1 (amplified in breast cancer-1)/TRAM-1(thyroid hormone receptor activator molecule 1) [51–55]. These proteins are required for mediating the transcriptional function of nuclear receptors and other transcription factors in a ligand-dependent manner [87, 88]. All three p160/SRC family members contain bHLH and PAS domains, which are involved in protein-protein interactions. These coactivators also contain three LXXLL motifs, that mediate recognition of, and binding to AF-2 region of a variety of nuclear receptors [89]. They possess HAT activity and are part of the first multiprotein coactivator complex with CBP/p300 on DNA bound-liganded nuclear receptors and participate in the acetylation of histones and remodel chromatin structure to unravel DNA for transcription [76, 88]. SRC-1 interacts with many nuclear receptors including PPARγ and PPARα, and the X-ray crystal structure of SRC-1 and the liganded PPARγ complex revealed that binding is between highly conserved glutamate and lysine residues in the PPARγ ligand binding domain and the backbone atoms of the LXXLL helices of SRC-1 [29]. Protein-protein interactions between PPARα and SRC-1 and SRC-3 have been documented and these interactions appears to be ligand independent [52, 54]. p160/SRC family members exhibit strong sequence homology and somewhat similar functions under in vitro transactivation conditions. But gene knockout mouse models have provided valuable insights into the in vivo functional properties of these molecules [75, 88]. These mouse models include SRC-1$^{-/-}$, SRC-2$^{-/-}$ and SRC-3$^{-/}$ single gene disruptions and SRC-1$^{-/-}$/SRC-2$^{-/-}$ and SRC-1$^{-/-}$/SRC-3$^{-/-}$ double nulls [50, 90–95].

Mice lacking SRC-1 were generated to delineate its role in estrogen receptor, progesterone receptor, and PPARα signaling [90, 92]. SRC-1 null mice are viable and fertile but show somewhat subdued response to sex hormonal stimuli after orchiectomy or ovariectomy [90, 96]. However, SRC-1$^{-/-}$ mice when challenged with PPARα ligands, such as Wy-14,643 or ciprofibrate, display the characteristic robust pleiotropic responses, including hepatomegaly, hepatic peroxisome proliferation and PPARα-target gene activation [92]. These responses appear essentially similar to those exhibited by SRC-1$^{+/+}$ littermates indicating that SRC-1 is not essential for PPARα signaling in liver [92]. Likewise, as shown here, studies with SRC-2 and SRC-3 null mice also revealed that PPARα target gene activation in liver is not dependent on these coactivators. Hepatic responses of SRC-1, SRC-2 and SRC-3 null mice following Wy-14,643 administration appear similar to those of wild-type mice treated with a PPARα ligand (Figure 2). Histological evaluation of liver sections, processed to visualize peroxisomal catalase, show similar increases in the number of peroxisomes in hepatic parenchymal cells of wild-type and SRC null mice treated with a peroxisome proliferator (Figures 2(a)–2(h)). To further investigate the influence of SRC family on PPARα function, we evaluated the changes in fatty acid-metabolizing enzymes in the liver of SRCs null and wild-type mice by Northern and Western blot analyses (Figures 2(i) and 2(j)). Northern blot analysis of total liver RNA shows similar basal levels of peroxisomal fatty acyl-CoA oxidase 1 (ACOX1), enoyl-CoA hydratase/L-3hydroxyacyl-CoA dehydrogenase bifunctional protein (L-PBE), peroxisomal 3-ketoacyl-CoA thiolase (PTL), and microsomal cytochrome P450 fatty acid ω-hydroxylase CYP4A1 in the livers of both wild-type and SRC-1, -2 and -3 null mice (Figure 2(i)). Massive increases in hepatic mRNA levels of these enzymes were noted in all SRC null mice treated with a PPARα ligand (Figure 2(i)). The increases appear similar to that noted in the livers of Wy-14,643 treated wild-type mice (Figure 2(i)). Western blot analysis reveals increases in the content of fatty acid oxidation enzyme proteins in liver of intact and SRC null mice (Figure 2(j)). The expression levels of hepatic peroxisomal fatty acid β-oxidation enzymes ACOX1, L-PBE, D-PBE, PTL, and SCPx (sterol carrier protein x) were increased significantly after Wy-14,643 administration in both wild-type and SRC-1, -2, and -3 null mice (Figure 2(j)). Furthermore, no significant differences in the magnitude of increases are observed in hepatic mitochondrial enzymes short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and long-chain acyl-CoA dehydrogenase (LCAD) among wild-type and SRCs null treated mice (Figure 2(j)). Together, these data indicate that no member of p160/SRC family of coactivators (SRC-1, SRC-2 and SRC-3) is required for PPARα-mediated transcriptional activation in vivo [90].

Although the single gene-knockout mice have demonstrated that loss of individual members of p160/SRC family of coactivators is redundant for PPARα function in liver, it remains to be ascertained if deletion of two or all three

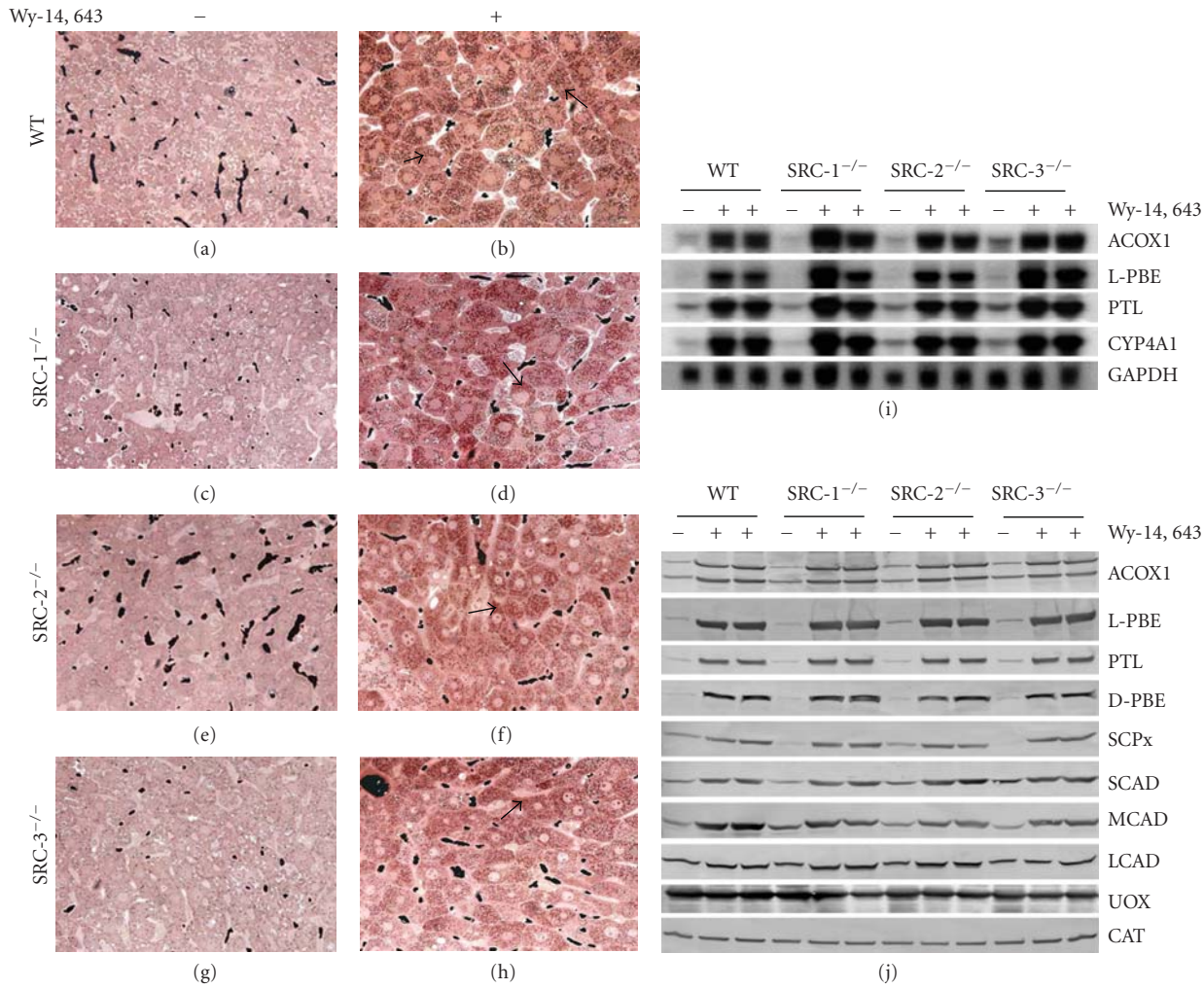

Figure 2: (a–h) Peroxisome proliferation in liver cells of wild-type (WT), SRC-1$^{-/-}$ SRC-2$^{-/-}$ and SRC-3$^{-/-}$ mice treated with Wy-14,643 for 4 days. Liver sections were processed for the cytochemical localization of peroxisomal catalase by using alkaline 3′,3′-diaminobenzidine substrate. Control diet (upper panels; a, c, e, g). Wy-14,643 diet (lower panels; b, d, f, h). Peroxisomes appear as brown dots (arrows) distributed throughout the cytoplasm in these 0.5 μm thick sections. All mice, wild-type and SRC nulls displayed extensive peroxisome proliferation after treatment with Wy-14, 643 indicating that these coactivators are not required for PPARα-regulated pleiotropic responses including fatty acid oxidation. (i) Northern blot analysis to confirm changes in mRNA expression of peroxisomal and microsomal fatty acid metabolizing enzymes in wild-type and SRC nulls after 4-days treatment with PPARα ligand Wy-14, 643. All genes are regulated by PPARα. Fatty acyl-CoA oxidase-1 (ACOX1), peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (L-PBE), and peroxisomal thiolase (PTL) represent peroxisomal β-oxidation system while CYP4A1 is involved in microsomal ω-oxidation of fatty acids. GAPDH is used as an indicator of RNA loading. (j) Western blot analysis from the above-mentioned livers was used to verify the degree of expression of peroxisomal and mitochondrial fatty acid metabolizing enzymes in wild-type and SRC knockout mice. Liver homogenates (20 μg) from each group of mice were run on 4–20% SDS PAGE gel and immunoblotted using antibodies for peroxisomal (ACOX1, L-PBE, PTL, D-PBE, and SCPx) and mitochondrial (SCAD, MCAD, and VLCAD) fatty acid metabolizing enzymes. No difference in the induction was observed between SRC nulls and wild-type for the β-oxidation enzymes.

of these coactivators affects the PPARα signaling. SRC-1 is required for the assembly of a complex that includes CBP/p300 for enhancing the coactivator function of PPARγ coactivator-1 (PGC-1) [95]. It appears that the docking of PGC-1 to PPARγ stimulates an apparent conformational change in PGC-1 that permits binding of SRC-1 and CBP/p300, resulting in a large increase in transcriptional activity, and this transcriptional enhancement function of PGC-1 fails to manifest in SRC-1 null cells [97]. SRC-1 null mice exhibit partial impairment of PPARγ function with decreased PGC-1 regulated thermogenic activity in brown adipose tissue [95]. The lower energy expenditure in SRC-1 null mice predisposes to higher sensitivity to obesity upon high fat feeding [88, 95, 98]. Fatty acid oxidation in brown fat is also decreased due to partial impairment of PPARγ/PPARα function in the absence of SRC-1.

SRC-2 has been implicated in a broader range of physiological processes including reproduction, mammary

morphogenesis, uterine function and energy metabolism by affecting the regulation of adaptive thermogenesis [99]. SRC-2 null mice are viable, but the fertility of both sexes is impaired [94, 100]. These mice are resistant to diet-induced obesity and displayed enhanced adaptive thermogenesis [95]. In white adipose tissue, disruption of SRC-2 increases leptin expression as well as that of genes involved in lipolysis. Additionally, these mice manifest a decreased potential for fatty acid storage [95, 101]. Disruption of SRC-2 gene in the mouse reduces PPARγ function in white adipose tissue resulting in a lesser degree of fat accumulation [76, 95]. This enhances the function and development of brown adipose tissue, leading to increased levels of uncoupling protein 1, PGC 1α and ACOX1, promoting energy expenditure [76, 87, 95]. Gene knockout studies have demonstrated that SRC-3 is required for normal growth, puberty and mammary gland development [93]. SRC-3 null mice also show reduced body weight and adipose tissue mass with a significant decrease in PPARγ expression. At molecular level, SRC-3 interacts with the transcription factor CAAT/enchancer-binding protein(C/EBP) to control gene expression through PPARγ. These results imply that SRC-3 exerts a key role in adipocyte differentiation in vitro and in vivo, and that this regulation of adipogenesis is upstream of PPARγ [101, 102]. SRC-3 gene is often amplified or overexpressed in many types of cancers [93, 94].

The redundancy of p160/SRC family of coactivators in the energy balance or expenditure function of PPARs noted in SRC single-knockout mice suggests the existence of possible cooperative effects among the three members of the SRC family. To illuminate the physiological functions affected by double deletion of these coactivators, mice deficient in both SRC-1 and SRC-2 [100], or deficient in both SRC-1 and SRC-3 [50] have been generated. Most SRC-1/SRC-2 double null mice die at birth, generally before the weaning stage [100]. SRC-$2^{-/-}$ mice are protected against obesity and display enhanced adaptive thermogenesis, whereas SRC-$1^{-/-}$ mice are prone to obesity due to reduced energy expenditure. Together, these two members of SRC family control energy balance between white and brown adipose tissues through regulating PPARγ activity [101].

Most SRC-1 and SRC-3 double null mice also die before birth and surviving combined-knockout mice are lean and resistant to high-fat diet induced obesity [50]. These mice exhibit a developmental arrest in interscapular brown adipose tissue and defective thermogenesis due to a deficiency in the regulation of selective PPARγ target genes involved in adipogenesis and mitochondrial uncoupling. It is of interest that these double null mice consume more food because of lower leptin levels, but remain lean mostly due to a higher basal metabolic rate and enhanced physical activity [50]. Taken together, SRC-1 and SRC-3 play critical roles in energy balance by mediating both energy intake and energy expenditure [75].

To investigate the changes in gene expression profiles, microarray analysis has been used with the RNA from livers of SRC-1, SRC-2, and SRC-3 single null animals [98]. The overall pattern of altered hepatic gene expression in the SRC-1 null mice was one of upregulation as compared to wild-type mice. SRC-2 null mice appeared an overall downregulation compared to wild-type mice. In SRC-3 deficient mice, a minimal change of gene expression in liver was observed. All these data suggest that changes in gene expression for each SRC member show specific and nonoverlapping expression patterns and that the three members of SRC family play a key role in coregulating energy homeostasis and obesity [76, 98]. It is clear from experiments in mice that all three members of the SRC family contain both redundant and distinct functions and that each individual SRC contains the capacity to regulate different biological functions [53].

5. CBP/p300 with HAT Activity

CBP and p300, generally referred to as CBP/p300, are universal coactivators that link transcriptional activators to the basal transcription apparatus and provide a scaffold to integrate multiple cofactors. CBP was first identified as a protein that binds CREB (cAMP response element-binding protein), whereas p300 was cloned as an adenovirus oncoprotein E1A-associated protein [56]. Subsequent studies established that CBP and p300 are significantly related and that human CBP resembles the human p300 more closely [103, 104]. These proteins are well conserved amongst mammals and homologs of CBP/p300 have been found in *Drosophila*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* [105]. No CBP/p300 homologs are found in prokaryotes or yeast [104]. CBP/p300 proteins possess HAT activity and the HAT domain has the ability to recruit other proteins such as p160/SRC family members with HAT activity to further enhance the acetylation potential of the coactivator complex to remodel chromatin structure for efficient gene transcription [76, 106–108]. CBP/p300 proteins share several conserved regions, which constitute most of their known functional domains such as bromodomain [109], cysteine/histidine-rich domains, a KIX domain to which transcription factor CREB binds, glutamine- and proline-rich domain, receptor-interacting domain, and SRC-1 interaction domain [108]. The C-terminal glutamine-rich domain of CBP/p300 forms contacts with other coactivators, most notably those involved in nuclear hormone-receptor signaling pathways. The complexity and breadth of CBP/p300 interactions attest to the unique involvement of CBP/p300 in the transcriptional control as universal and versatile cointegrators.

CBP and p300 directly interact with the ligand-binding domain of several nuclear receptors including PPARs [110]. C-terminal of PPARα that corresponds to AF-2 domain (residues 448–468) is required for the interaction with N-terminal region of p300 spanning aa 39–117, and also the N-terminal fragment of CBP encompassing aa 1–115 in a ligand dependent manner. Fragments of both p300 and CBP that interact with PPARα contain one LXXLL motif [110]. Interaction of CBP and p300 with the ligand-binding domain of PPARα or PPARβ was demonstrated in human intestine-like Caco2 cell line [111]. Induction of conformational change and transactivation potential of PPARβ

was considerably lower than that of PPARα in response to arachidonic acid, as well as other polyunsaturated fatty acids [112]. Arachidonic acid enhanced binding of p300 to PPARα but not to PPARβ. Additionally, arachidonic acid induced in vitro binding of both PPARα-RXRα and PPARβ-RXRα heterodimers to several PPREs [113, 114]. CBP, which is highly expressed in brown fat, also coactivates PPARα-dependent regulation of the UCP-1 gene promoter in the HepG2 cells in the presence of PPARα ligand, Wy-14,643 [115]. Presence of CBP in PRIC complex that interacts with full-length PPARα in the presence of ciprofibrate and leukotriene B4 also substantiate the coactivator status of CBP [63].

p300 interacts with the N- and C-terminal regions of PPARγ in a ligand-independent and -dependent manner, respectively [116]. Leu-311, Asn-312, and Thr-316 in helix 4 of PPARγ ligand-binding domain are involved in PPARγ binding with CBP [117]. Deletion of A/B-domain of PPARγ compromises recruitment or stabilization of CBP- and p300-containing cofactor complexes on a subset of target genes involved in lipid storage [118]. Both PPARγ and CBP are expressed in preadipocytes and differentiated adipocytes suggesting that CBP serves as a physiologically relevant coactivator for PPARγ signal transduction [119]. p300 also transactivates PPARγ in the presence of natural ligand 15-deoxy-Δ12, 14-prostaglandin J2, but troglitazone, a synthetic PPARγ ligand, failed to induce PPARγ interactions with p300. CBP/p300 also increases the transcriptional activity of PPARγ through PGC-1 which stimulates an apparent conformational change in PGC-1 that permits binding of SRC-1 and CBP/p300 resulting transactivation of target genes [91]. Recruitment of CBP/p300 and PGC-1α was shown recently on PPARγ/RXRα heterodimer bound to the promoter of UCP-1 gene after the activation of β-adrenergic receptor in Jhdm2a knockout mice [120]. Jhdm2a is a H3K9-specific demethylase that directly regulates PPARα and Ucp1 expression [120]. PPARγ recruits CBP to the aP2 gene promoter as evidence by chromatin immunoprecipitation and in vitro immunoprecipitation assay in the MEFs induced for adipogenic differentiation [121]. Recently, it has been shown that beraprost, a synthetic analogue of prostacyclin serves as a ligand for PPARβ that enhances transcriptional activation of p21/p27 by increasing CBP nuclear translocation, which contributes to the vasoprotective action in rat aortic smooth muscle cells [122].

6. PBP (PPARBP)/TRAP220/DRIP205/MED1

PPAR-binding protein (PBP/PPARBP) was first cloned through yeast two-hybrid system using Gal4-PPARγ as bait to screen a mouse liver cDNA library and identified as a nuclear receptor coactivator with 2 LXXLL motifs [58]. Subsequently, PBP was shown as a critical component of TRAP/DRIP/ARC/Mediator complex [57, 73, 74, 123, 124] and it is variously referred to as PBP (PPARBP)/TRAP220/DRIP205/Med1 subunit of Mediator complex [41]. The Mediator complex was first discovered in yeast and was shown to be essential for pol II dependent transcription [41]. The mammalian Mediator complex consists of ~31 subunits and PBP/TRAP220/Med1 is the prominent member of this complex [73, 75]. Med1 binds to several nuclear receptors such as PPARα, RARα, TRβ1, RXR, VDR, FXR, ERα and GR via two conserved LXXLL motifs in a ligand-dependent manner [58, 74, 121, 123–125], Med1 also interacts with a variety of other transcriptional factors, including tumor suppressor p53, five GATA family members, p300, PGC-1 and C/EBPβ [46, 79, 126–129]. These interactions imply a major role for Med1 in nuclear receptor mediated cellular proliferation, differentiation and homeostatic regulation [76, 130].

Med1 serves as an anchor for the Mediator complex and facilitates the linkage between HAT containing CBP/p300 and p160/SRC protein complex and pol II basal transcription machinery in regulating transcription [41, 46]. Phosphorylation of Med1 by mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK-ERK) promotes its association with Mediator [131, 132]. Med1 is widely expressed in many tissues of adult mice, including brain, heart, lung, liver, kidney, adipose tissues, and the most prominent being the testis [76, 133]. Recently, it has been reported that Med1 is a target for miR-205. miR-205 interacts with a specific target in the 3′-UTR sequence of Med1 and silences its expression in human trophoblasts exposed to hypoxia [134].

Med1 ablation leads to embryonic lethality at midgestation, day 11.5 postcoitum (E11.5), which is attributed, in part, to defects in the development of placental vasculature, similar to those encountered in PPARγ [78, 135–137]. Embryonic development of the heart, eye, vasculature and the hematopoietic system is altered in Med1 null mice. This phenotype is similar to that in mice deficient in members of GATA, a family of transcription factors that modulates differentiation of adipocytes, megakaryocytes and erythrocytes [79].

As indicated above, Med1 was first identified as a PPARγ coactivator and it plays an important role in the PPARγ signaling pathway [58, 78, 136]. Med1 and PPARγ interaction requires two LXXLL nuclear receptor recognition motifs present in Med1 [138]. Med1 modestly increases the transcriptional activity of PPARγ, and a truncated form of Med1 (aa 487–735) acts as a dominant negative repressor [74, 123]. It has been shown that the deletion of 12 amino acids from the extreme carboxyl terminus of PPARγ results in the abolition of Med1-PPARγ interaction [58]. However, deletion of the PPARγ A/B-domain does not affect Med1 recruitment [118]. To study the role of Med1 in PPARγ-mediated adipogenesis in vitro, Med1$^{+/+}$ and Med1$^{-/-}$ MEFs were isolated from E10.0 littermate embryos and infected with a retroviral vector driving PPARγ [139]. Disruption of TRAP220/Med1 in MEFs is refractory to PPARγ-stimulated adipogenesis but not MyoD-stimulated myogenesis [139]. Surprisingly however, a conserved N-terminal region of Med1 that lacks the LXXLL motifs but gets incorporated into Mediator fully supports PPARγ-stimulated adipogenesis [138]. A direct interaction between PPARγ and the mediator complex through Med1 is not essential

for PPARγ-stimulated adipogenesis and for PPARγ target gene expression in cultured fibroblasts [138]. Furthermore, PPARγ target gene expression and recruitment of Mediator to a PPARγ response element on the aP2 promoter in undifferentiated MEFs do not require Med1 [138]. These findings imply that the presence of alternative mechanisms for Mediator recruitment, possibly through intermediate cofactors or other cofactors that are functionally redundant with Med1 [138].

To further study the role of Med1 in specific tissues in vivo, mice carrying floxed Med1 alleles were generated for conditional null mutation [140, 141]. Conditional deletion of Med1 gene in liver results in the abrogation of PPARα ligand-induced pleiotropic effects, indicating that Med1 is essential for PPARα signaling and fatty acid oxidation [140]. Med1 deficiency in liver parenchymal cells results in the near abrogation of PPARα ligand-induced peroxisome proliferation, liver cell proliferation, and induction of PPARα-regulated genes. In contrast, scattered residual $\text{Med1}^{+/+}$ hepatocytes that escape Cre-mediated excision of floxed alleles in Med1 liver nulls, show DNA synthesis and were markedly hypertrophic with peroxisome proliferation in response to PPARα ligands (Figures 3(a) and 3(b)). $\text{Med1}^{-/-}$ hepatocytes are refractory for PPARα ligand-induced peroxisome proliferation [140]. Moreover, $\text{Med1}^{\Delta\text{Liv}}$ mice, chronically exposed to PPARα ligand Wy-14,643, show a striking proliferative response and clonal expansion of residual $\text{Med1}^{+/+}$ hepatocytes (Figures 3(c) and 3(d)) but no proliferative expansion of $\text{Med1}^{-/-}$ hepatocytes occurs and these Med1 null hepatocyte appeared hypoplastic (boxed areas in Figures 3(c)–3(e)) as compared to hyperplastic large $\text{Med1}^{+/+}$ hepatocytes.

Surprisingly, the Med1 liver conditional null mice develop liver tumors on long-term exposure to PPARα ligand, but all tumors developing in $\text{Med1}^{\Delta\text{Liv}}$ mice reveal Med1 expression and no tumors developed from $\text{Med1}^{-/-}$ hepatocytes [142]. These data suggest that Med1 plays a key role in PPARα ligand-induced liver tumor development and that cells deficient in Med1 do not give rise to tumors [142]. Furthermore, initiation by a genotoxic carcinogen diethylnitrosamine followed by phenobarbital promotion in $\text{Med1}^{\Delta\text{Liv}}$ mice results in a failure of Med1 null hepatocytes to undergo proliferation. As in the case of Wy-14,643 treatment, all hepatocellular carcinomas developing in $\text{Med1}^{\Delta\text{Liv}}$ mice are Med1 positive [143]. Liver tumors that develop in $\text{Med1}^{\Delta\text{Liv}}$ mouse livers are transplantable in athymic nude mice and these maintain $\text{Med1}^{\text{fl/fl}}$ genotype. These observations imply that Med1 is essential for the development of hepatocellular carcinoma in the mouse [143]. The failure of Med1 null hepatocytes to develop liver tumors following PPARα ligand administration or after prolonged promotion with phenobarbital, which is an activator for nuclear receptor constitutive androstane receptor (CAR), implies that coactivator Med1 is a critical component of PPARα and CAR signaling and thus participates in the neoplastic process [142–144]. Med1 deficient livers fail to develop hepatic steatosis induced by glucorcorticoid receptor (GR) agonist [145] and also fail to develop hepatic steatosis when induced by PPARγ overexpression (unpublished data). In addition, using a conditional null mutation, it has been shown that Med1 is required for mammary gland development [146], and is also essential for the growth of Notch4-immortalized mammary cells by activating SOX10 expression [147]. Earlier studies have demonstrated the Med1 is either overexpressed or amplified in several breast carcinomas implying that Med1 plays a role in ER signaling and cancer [125, 148]. More recently, Med1 has been shown to play an important coregulatory role in prostate cancer cell proliferation and survival [149]. However, decrease of Med1 expression in human melanoma cells increases their tumorigenic phenotype and the reason for this discordancy is unclear [150].

In summary, using conditional knockout mice, it has been established that Med1 subunit is essential for the signaling of nuclear receptors PPARγ, PPARα, CAR and GR [139–145]. Evidence indicates that Med1-deficiency does not lead to the disintegration of the Mediator complex as originally speculated but it is possible that Mediator complex devoid of Med1 subunit may be impaired in its ability to recruit pol II to transduce the transcriptional signal [151].

7. PGC-1 Family in Coactivation of PPAR

PGC-1 family of coactivators, with three members, plays a critical role in the maintenance of mitochondrial function, thermogenesis and energy homeostasis [59]. The first member of the PGC-1 family was identified as a PPARγ-interacting protein from brown fat cDNA library using yeast two-hybrid screen and is now termed PGC-1α [59]. Thereafter, two related coactivators, PGC-1β (also termed PERC) and PGC-1-related coactivator (PRC) were discovered through searches of new data base entries [60, 152]. PGC-1α and PGC-1β share similar tissue distribution with highest levels of expression in brown fat, heart and slow-twitch skeletal muscle [59, 152], and their mRNA levels are induced significantly in the adult liver following fasting [152, 153]. Expression of PGC-1α mRNA is also elevated in brown fat after cold exposure, whereas PGC-1β does not respond [59, 152]. Less is known about the expression patterns and biological roles of PRC [60].

In addition to PPARγ, PGC-1α also coactivates a variety of other nuclear receptors, including PPARα [154], PPARβ [21], TRβ [59], ERα [59], GR [155], FXR [156], LXR [157], HNF4 [152] and RAR, but not RXRα [59]. Cotransfection experiments in cells show that PGC-1α increases the PPARα-mediated transcriptional activity and that AF2-LXXLL interaction is necessary for the coactivation of PPARα by PGC-1α [154]. Furthermore, overexpression of PPARα and PGC-1α in 3T3L1 cells cooperatively induces the expression of mitochondrial fatty acid β-oxidation enzyme system genes and increases cellular palmitate oxidation rates [154]. PPARα-driven mitochondrial biogenic response reveals that expression of PGC-1α is activated in wild-type mice but not in PPARα-deficient mice [158]. PGC-1α promotes expression of mammalian tribbles homolog TRB-3 through PPARα and knockdown of hepatic TRB-3 expression improves glucose tolerance, whereas hepatic overexpression of TRB-3

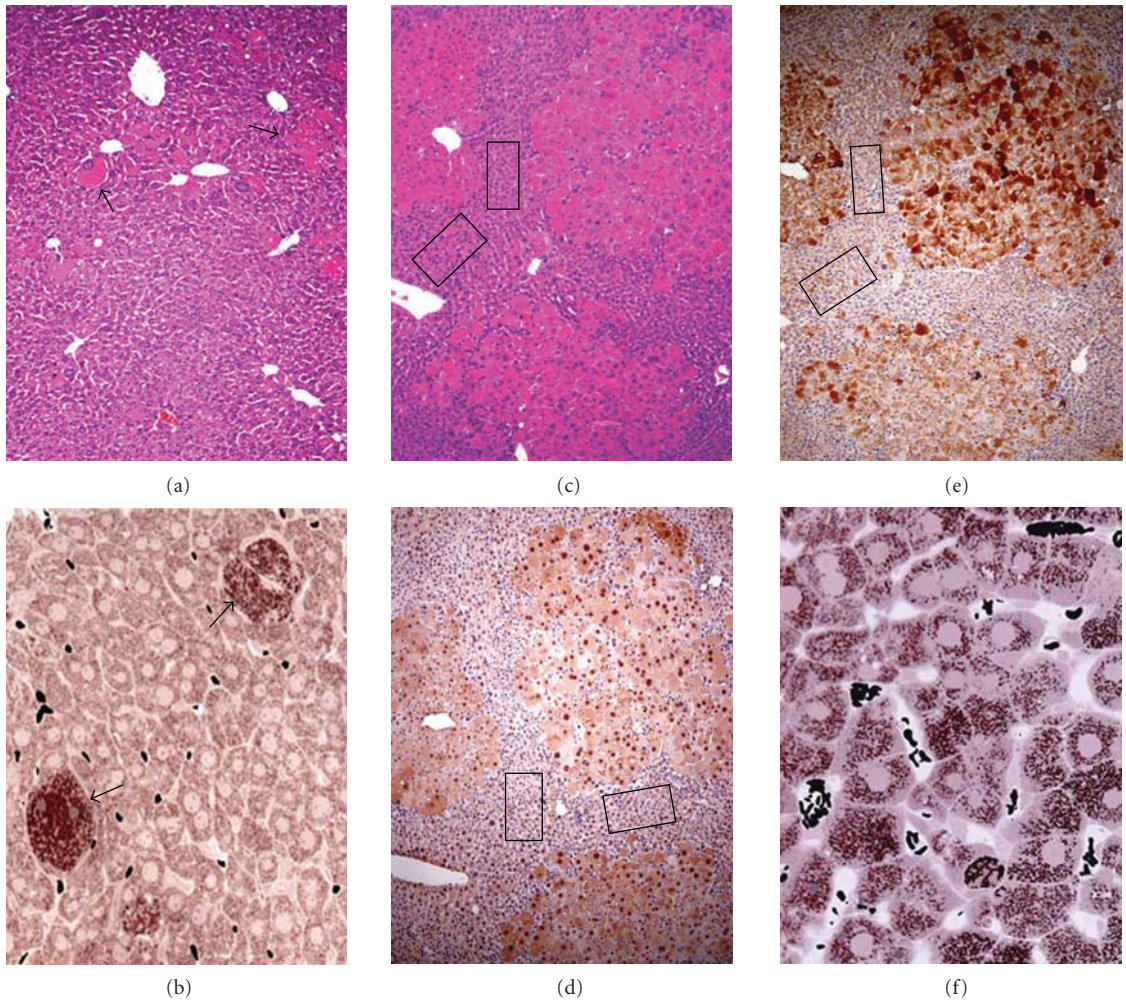

Figure 3: Effects of Med1 and PRIP deletion on PPARα ligand-induced pleiotropic responses in liver. Med1$^{\Delta Liv}$ mice treated with Wy-14,643 (0.125% wt/wt) for 2 weeks (a, b) show an occasional hepatocyte that escaped Alb-Cre mediated deletion of Med1 floxed alleles. These Med1 positive hepatocytes respond to the peroxisome proliferative effects of PPARα-ligand (arrows indicate to Med1$^{+/+}$ cells with numerous peroxisomes) but not the majority of Med1$^{-/-}$ hepatocytes. Chronic treatment of Med1$^{\Delta Liv}$ mice with 0.02% Wy-14,643 for 5 months (c–e) results in clonal expansion of residual Med1$^{fl/fl}$ cells as demonstrated by H&E staining (c). In contrast, the adjacent hepatocyte lacking Med1 are generally smaller than normal hepatocytes (see boxed area in c). Immunohistochemical localization of Med1 reveals that expanding colonies of large hepatocytes are Med1 positive (nuclear Med1 in panel d). These cells also show abundant cytoplasmic expression of L-PBE, the second enzyme of the peroxisomal fatty acid β-oxidation system (panel e), whereas the smaller Med1 null hepatocytes (*boxed areas*) fail to show L-PBE induction (panel e). Disruption of coactivator PRIP in hepatocytes does not interfere with PPARα ligand-induced peroxisome proliferation as evidenced by the abundant catalase positive peroxisomes (brown dots) in all hepatocytes (panel f). Compare this panel (f) with panel (b) in which only an occasional Med1$^{+/+}$ cell responds to PPARα ligand.

reversed the insulin-sensitive phenotype of PGC-1-deficient mice [159].

PGC-1α utilizes a domain rich in proline residues to bind to a region that overlaps the DNA binding and hinge region of PPARγ [59]. The strong interaction of PGC-1α and PPARγ is mediated through both hydrophobic and specific polar interactions. Mutations within the context of the full-length PGC-1α indicate that the first PGC-1α motif is necessary and sufficient for PGC-1α to coactivate PPARγ in the presence or absence of rosiglitazone [160]. Thiazolidinediones and rexinoids induce PGC-1α gene expression in brown and white adipocytes by a PPARγ-dependent pathway. This is attributed to the presence of a PPARγ-responsive element in the distal region of the PGC-1α gene promoter that binds PPARγ/RXR heterodimers [161]. The interaction between PGC-1α and PPARβ depends on the LXXLL motif in PGC-1α (aa 142–146) and this interaction is enhanced in the presence of PPARβ agonist GW501516 [21]. Tetradecylthioacetic acid, a pan-PPAR ligand, induces hepatic fatty acid oxidation in PPAR$\alpha^{-/-}$ mice possibly through PGC-1α mediated PPARβ coactivation [162]. Pharmacological activation of PPARβ induces fatty acid oxidation and this depends upon PGC-1α as the induction is completely abolished in the absence of both PGC-1α and PGC-1β [163]. To study

the role of PGC-1α in vivo, PGC-1α knockout mice have been generated [164, 165], which show normal embryonic development, suggesting that PGC-1α driven coactivation is not critical for PPARγ and PPARβ functions in the maintenance of placental adequacy [164, 165]. Interestingly, PGC-1$\alpha^{-/-}$ mice survive with modestly blunted postnatal cardiac growth, suggesting that PGC-1α is essential for the maintenance of maximal, efficient cardiac mitochondrial fatty acid oxidation, ATP synthesis, and myocardial lipid homeostasis [165, 166].

Hepatic PGC-1β overexpression results in the attenuation of changes induced by Wy-14,643, a PPARα ligand [167]. PGC-1β poorly activates the expression of gluconeogenic genes in hepatocytes or liver in vivo. The reduced ability of PGC-1β to induce gluconeogenic genes is due, in part, to its inability to physically associate with and coactivate HNF4α and FOXO1 [153]. PGC-1β null mice are viable and fertile and show no overt phenotype. However, PGC-1β deficient mice display an altered expression in a large number of nuclear-encoded genes governing mitochondrial functions in multiple tissues including heart, skeletal muscle, brain, brown adipose tissue, and liver [168]. PGC-1α null mice appeared hyperactive in comparison to somewhat sluggish PGC-1β null mice. When acutely exposed to cold, these mice develop abnormal hypothermia and morbidity [168]. Furthermore, high-fat feeding induced hepatic steatosis and increases serum triglyceride and cholesterol levels in the mutant mice [168].

8. PRIP/NCoA6

Nuclear receptor coactivator PRIP (PPAR-interacting protein) [168], also referred to as activating signal cointegrator-2(ASC-2) [83]/nuclear receptor activating protein 250 (RAP250) [61]/nuclear receptor coregulator (NRC) [84]/thyroid hormone receptor (TR)-binding protein (TRBP) [85], was cloned by different groups using yeast two-hybrid screens with a nuclear receptor as bait. PRIP (NCoA6) was identified as a ligand-dependent nuclear receptor-interacting protein. PRIP forms large steady-state complex of approximately 2MDa (ASC-2 complex [ASCOM] with retinoblastoma-binding protein RBQ-3, α/β-tubulins and subset of Trithorax-related proteins [169]. PRIP, like MED1 gene is also amplified and overexpressed in breast, colon and lung cancers [170, 171]. PRIP and PRIP-interacting protein with methyltransferase domain (PIMT/NCoA6IP) appears to serve as a linker between CBP/p300-anchored and Mediator complexes. PRIP contains two LXXLL motifs, one at the N-terminal region (aa 892 to 896) plays a pivotal role for ligand-dependent interactions with a wide spectrum of nuclear receptors including PPARs, TRs, RXRs, ERs, GR, VDR and RARs [64, 81, 83] and the second LXXLL located at the C-terminal does not bind with PPARs, RAR, RXRs, and GR but does bind with LXRα and LXRβ [85, 172–174]. These two LXXLLs interact with LXRs and other nuclear receptors and regulate insulin secretion and maintain β-cell function [175].

PRIP acts as a strong coactivator for PPARγ, and a truncated form of PRIP (aa 786–1132) acts as a dominant-negative repressor [62]. It is worth noting that PRIP is also detected in the transcriptionally active PPARα interacting cofactor (PRIC) complex isolated from rat liver [63]. PRIP protein expression has been detected in many tissues, especially in the reproductive organs such as testis, prostate and ovary [76, 176]. In the testis and ovary, PRIP immunostaining shows intense staining of the nuclei of Sertoli and follicular granulosa cells respectively [177]. Mice with disrupted PRIP/RAP250/NRC/AIB3 gene die at embryonic stage E11.5 and E12.5 days. PRIP mutant embryo mortality has been attributed to placental dysfunction including the failure of labyrinthine development, the dilation of maternal blood sinuses, the massive erythrophagocytosis by trophoblastic cells, alteration in trophoblast population and the formation of fewer blood vessels in extra-embryonic membrane covering the embryo [76, 85, 170, 173, 178, 179]. In addition, developmental abnormalities in heart, liver, and the nervous system have been noted [85, 173, 178]. Interestingly, MEFs derived from PRIP/NRC null embryos display growth retardation and apoptosis [176]. Further studies with heterozygous PRIP/NRC$^{+/-}$ show a spontaneous wound healing deficiency, suggesting that PRIP/NRC is important in maintaining integrity during wound healing [176, 180]. Haploid inactivation of PRIP/AIB3 in AIB3$^{+/-}$/PyMT bitransgenic mice cause inhibition of cell proliferation mediated by PPARγ/RXR [181].

PRIP null MEFs are also resistant to PPARγ stimulated adipogenesis [121]. This defect occurs because of apparent disruption of the linkage between the CBP/p300 anchored subunit complex and the MED1-dependent mediator complex [121]. In order to investigate the physiological role of PRIP in vivo, conditional knockout mice have been generated [182]. Conditional PRIP null mutation in the mouse mammary gland results in defective mammopoiesis, similar to that encountered in Med1 deficient mammary glands [146, 168]. To further understand the function of PRIP in mammary gland tumorigenesis, a mammary tumor cell line with the PRIP$^{loxP/loxP}$ genotype was established and disruption of the PRIP gene in these cells has been shown to abrogate their tumorigenic potential. PRIP deficiency substantially reduced the expression of FOS gene [171]. Liver-specific disruption of the PRIP gene fails to affect the induction of PPARα-regulated pleiotropic responses, including hepatomegaly, hepatic peroxisome proliferation (Figure 3(f)), and induction of genes involved in the fatty acid oxidation systems [182]. These results are dissimilar to those encountered with liver specific MED1 gene disruption [140], indicating that PRIP is not essential for PPARα target gene activation in liver [182].

9. PRIC285

PRIC complex isolated from the rat liver nuclear extract using full-length GST-PPARα fusion protein in the presence of a PPARα ligand comprises of ~25 subunits [63]. A protein complex similar to this was also obtained using

PPARα ligand (ciprofibrate)-affinity matrix [64]. MALDI-TOF analysis of the components of PRIC complex showed identities of many already known genes identified in yeast two-hybrid screens known to be involved in transcriptional regulation and some novel proteins [63]. This complex includes CBP/p300, p160/SRC-1, MED1, PRIP, PIMT and novel coactivators designated as PRIC285, PRIC295, and PRIC320 based on estimated molecular size (see [63, 64], unpublished data). PRIC285 is a component in the PRIC complex isolated using ciprofibrate or LTB4 as ligand in GST PPARα pull down system [63]. Subsequently, a longer isoform has been cloned using human PPARγ as bait in yeast two-hybrid screen and has been referred to as PPARγ-DBD-interacting protein 1 (PDIP1)-α [183]. PRIC285 is expressed in multiple human tissues such as skeletal muscle, colon, spleen, liver, kidney, heart, lung, pancreas, small intestine, thymus, prostate, ovary, peripheral blood, and placenta [63]. PRIC285 has been detected in several human cancer lines such as HeLa, colorectal adenocarcinoma SW480, melanoma, HepG2, medulloblastoma HTB185, and DU145 (prostate) [63, 183].

The human PRIC285 gene, which spans ~16.1 kb, is located on chromosome 20 at position 20q13.33 and it encodes a protein of 2080 amino acid with an estimated molecular mass of 285 kDa [63]. PRIC285 contains five LXXLL signature motifs at aa 506–510; 549–553; 604–608; 1443–1447; 1660–1664 [183]. It appears that none of these LXXLL motifs of PRIC285 is needed for interaction with PPARs as demonstrated by mutating LXXLL motifs [183]. PRIC285 binds to the DBD-hinge (DBD-H) of the PPARs through its C-terminal region mapped at aa 1675–1823 [183]. Comparison of the amino acid sequences flanking core LXXLL motifs in PRIC285 with those identified in other coactivators revealed that this configuration did not fit well with the proposed alignment rules [184]. Other than LXXLL signature motifs human PRIC285 also displays amino acid sequence homologous to RnaseB (RNB) and UvrD/REP motifs, a superfamily I DNA helicase [63, 183]. It is found to be transcriptional coactivator for the PPARα, PPARδ/β and PPARγ in transfected cells. Cotransfection of PPARα and PRIC285 into HEK293 cells stimulates transcription of PPRE-TK-Luc gene in the presence of ciprofibrate, a PPARα specific ligand. This interaction between PRIC285 and PPARα was also shown by the colocalization in nucleus of 293 cells [63]. Transactivation of PPARα by PRIC285 also occurs after treatment with a different PPARα ligand, fenofibrate [183]. Human PRIC285 has been shown to enhance PPARγ-mediated transactivation of DR1 reporter gene using the synthetic PPARγ ligand troglitazone. PRIC285 also coactivates PPARβ in the transactivation assay where CV-1 cells were treated with PPARβ ligand cyclic prostaglandin [183].

To assess the biological significance of PRIC285, we have generated whole-body gene knockout mice using two-*loxP* and two-*frt* system and characterized them for PPARα-mediated transcriptional activation in vivo [185]. Mice homozygous for PRIC285 mutation (PRIC285$^{-/-}$) are apparently healthy and fertile and show no consistent phenotypic differences when compared to their wild-type floxed littermates (PRIC285$^{fl/fl}$). When challenged with PPARα ligands, such as Wy-14,643 or ciprofibrate, no differences were observed in the magnitude of pleiotropic responses, which include hepatomegaly, peroxisome proliferation in hepatocytes, and increased levels of PPARα target genes such as peroxisomal and mitochondrial β-oxidation enzymes [185]. The role of PRIC285 in PPARγ mediated adipogenesis in the liver has been examined using PRIC285 null mice. Adenovirally driven PPARγ gene when injected through tail vein induced hepatic steatosis in both PRIC285 null and wild type floxed littermates to delineate the role of the coactivator PRIC285 in hepatic steatosis. No discernible differences in the PPARγ-mediated hepatic adipogenic steatosis in the normal and mutant PRIC285 mouse liver has been noted [L. Bai, unpublished]. These results may point to a functional redundancy of PRIC285 in the general transcriptional machinery as far as PPARα and PPARγ are concerned. The discordance between in vitro and in vivo results of PRIC285 function reflects the complexity and redundancy in that loss of a single component of a multisubunit protein complex could be compensated in vivo by other members of this mega complex. Nonetheless, it would be a challenge for the immediate future to assess the role of PRIC285 in the signaling of other nuclear receptors in vivo using the whole-body or conditional deletion.

10. PRIC320/CReMM/CHD9: ATP Dependent Chromatin Remodeling Activity

PRIC320 was identified in high molecular weight protein complex isolated using ciprofibrate coupled AH-sepharose affinity pulldown from the rat liver nuclear extract [64]. PRIC320 is also known as chromodomain helicase DNA binding protein 9 (CHD9)/Chromatin Related Mesenchymal Modulator (CReMM). It is a member of the CHD (chromodomain-helicase-DNA-binding) family of proteins that interacts with nucleosomes and plays a role in chromatin remodeling to modulate transcription [64, 186–188]. Members of the CHD family of enzymes belong to the SWI/SNF2 (SWItch/Sucrose Nonfermentable) superfamily of ATP-dependent chromatin remodelers. PRIC320 displays two tandem N-terminal chromodomains (aa 692–752; 774–826) that function as interaction surfaces for a variety of chromatin components. It also contains SNF2-like ATPase/DEAD-like helicase domain (aa 879–1028) located in the central region of the protein structure [189, 190]. A C-terminal cluster of domains such as paired BRK (Brahma and Kismet; aa 2483–2532; 2557–2601) domains, a SANT-like domain, and a DNA-binding domain are also present in PRIC320 [64, 191, 192]. The SNF2-like ATPase/DEAD-like helicase domain contains a conserved set of amino acid motifs that has been found in proteins involved in many of cellular processes including chromatin assembly, transcription regulation, DNA repair, DNA replication, development and differentiation [69, 193]. PRIC320 contains five LXXLL signature motifs that mediate interaction with nuclear receptors [64].

Two isoforms of PRIC320 designated as PRIC320-1 and PRIC320-2 that encode aa 2882 and 1995, respectively, have been identified in the human [64]. PRIC320-1 with an estimated molecular weight of 320 kDa contains all five LXXLL motifs located at aa 868–872; 1036–1040; 2031–2035; 2706–2710; and 2778–2782, whereas PRIC320-2 with an estimated molecular weight of 240 kDa contains distal four LXXLL motifs. The gene encoding human PRIC320 is mapped to long arm of chromosome at 16q12.2 (Ensembl; www.ensembl.org). PRIC320 transcript is present in various human tissues though at very low levels. PRIC320 mRNA has been detected in cancer cell lines such as HL-60, HeLa, Burkitt's lymphoma Raji and colorectal and lung carcinoma [64]. Cancer cell lines such as HL-60, HeLa cells, are shown to express two isoforms of mRNA 11.5 kb and 10.5 kb corresponding to PRIC320-1 and PRIC320-2. In mice, expression of PRIC320 is higher in brain, followed by heart, kidney, and skeletal muscle [64].

PRIC320 interacts with PPARα and functions as a coactivator in vitro [64]. Full length PPARα fused to GST interacts with both PRIC320 isoforms in a ligand dependent manner whereas interaction with PPARγ appeared minimal [64]. This selectivity for PPARs indicates a differential role of PRIC320 in the regulation of downstream target genes. The recognition in PRIC320/CHD9 of chromatin remodeling function and nuclear receptor coactivator function is suggestive of the multiple roles played by these nuclear receptor cofactors.

11. SWI/SNF: ATP-Dependent Chromatin Remodeling Complex

The SWI/SNF (mating type switch/Sucrose Nonfermenting) families of chromatin remodeling complexes mobilize nucleosomes and function as master regulators of transcription factor function. Although the precise mechanisms by which SWI/SNF modifies chromatin structure remains unclear, this process involves a conformational change of nucleosome and chromatin-remodeling in an ATP-dependent manner [65, 66]. SWI/SNF complex contains one or two possible ATPases, BRM (Brahma) or BRG1 (Brahma-Related gene 1) [194]. Chromatin remodelling represents an important step in adipocyte differentiation. C/EBPα, which is known to interact with the pol II-associated general transcription factors TBP/TFIIB, also interacts with BRM of the human SWI/SNF complex [195]. PPARγ depends on a specific BRG1-containing SWI/SNF complex to activate adipogenesis under in vitro conditions [196]. SWI/SNF complex and TFIIH are recruited on the promoter of PPARγ to transactivate PPARγ [197]. The docking of SWI/SNF complex on PPARγ promoter occurs through the subunit BAF60c (BRG1/Brm-associated factor subunit c) [69]. Recently, an interaction between SWI/SNF complexes and PPARα was demonstrated through BAF60a. SWI/SNF also plays a role in the regulation of the hepatic lipid metabolism through the fatty acid oxidation [67].

12. BAF(s) Family

The BAF (BRG1/Brm-associated factor) family represents the accessory subunits of SWI/SNF complexes that act as the connection between transcription factors and SWI/SNF complexes. Several BAFs been identified which include BAF250, BAF170, BAF155, BAF60, BAF57 and BAF53a [198–200]. Recent studies have implicated the BAF60 family members, including BAF60a, BAF60b and BAF60c, in mediating the interaction between the SWI/SNF complexes and target transcription factors. BAF60a or SMARCD1 (SWI/SNF related, matrix associated, actin-dependent regulator of chromatin subfamily d, member 1), a protein of 60 kDa, is known to be the connection between SWI/SNF and GR [68]. More recently, BAF60a was identified as a molecular link between SWI/SNF complexes and hepatic lipid metabolism. Adenoviral expression of BAF60a has been shown to stimulate fatty acid β-oxidation in primary hepatocytes culture to ameliorate hepatic steatosis in vivo. PGC-1α mediates the recruitment of BAF60a to the PPRE and enhances the transcription of PPARα regulated fatty acid oxidation system genes [67]. BAF60a is considered as a regulator of hepatic lipid metabolism. BAF60c or SMARCD3 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily d, member 3), which is also a 60 kDa protein, binds to several nuclear receptors, including PPARγ, ERα, and RORα [69]. Recently, a new regulator of PPARγ, the subunit BAF60c2 has been identified in a yeast two-hybrid screen of a human adipose tissue cDNA library. BAF60c2 represents a new isoform of BAF60c, which allows the recruitment of SWI/SNF to the nuclear receptor. Two isoforms BAF60c1 and BAF60c2 are localized primarily in the nucleus and are expressed in a wide variety of tissues. BAF60c proteins interact in a ligand-independent manner with PPARγ and enhance its transcriptional activity [69].

13. PIMT

PIMT (NCoA6IP, TGS1) was first isolated as a PRIP (NCoA6)-interacting protein [70]. PIMT is an RNA methyltransferase and was cloned from a human liver cDNA library using PRIP as bait in yeast two-hybrid assay. Human PIMT protein contains 852 amino acids. It has a methyltransferase motif at the C- terminus and an RNA-binding domain at the N-terminus [70]. PIMT is an evolutionarily conserved protein found in C. elegans, Arabidopsis thaliana, and yeast [70]. PIMT serves as a linker between multiprotein complexes anchored by CBP/p300 and PBP/MED1. PIMT enhances Med1mediated transcriptional activity of the PPARγ which was increased by PRIP [70, 79, 201]. Consistent with its RNA methyltransferase function, PIMT homologue in yeast known as trimethylguanosine synthase1 (TGS1), plays a role in the formation of the 2, 2, 7-trimethylguanosine (m3G) 5′-cap structure of snRNAs and small snoRNAs [202]. In Drosophila it is designated as DTL (Drosophila Tat-like) where it is important in development [203]. PIMT is localized predominantly to the nucleus. It is expressed in most adult tissues and in all embryonic stages in the mouse

[76]. Inhibition of PIMT by siRNA in HeLa cells results in G2/M phase arrest [204].

In order to investigate the biological functions of this gene in mammalian development and growth, PIMT gene knockout mice have been generated [205]. Heterozygous ($PIMT^{+/-}$) mice grow normally and are indistinguishable from their wild-type ($PIMT^{+/+}$) littermates. Disruption of both PIMT alleles results in early embryonic lethality due to apoptosis and decreased proliferative potential of the blastocyst cells [205]. PIMT deficient embryos die shortly after implantation and then resorbed. $PIMT^{fl/fl}$ MEFs treated with adenovirus expressing Cre showed defective wound healing and G2 phase arrest of cell cycle. These results suggest that PIMT is important for early embryonic development of mice [205].

14. CARM1

Protein methylation is involved in regulating protein-protein interactions that affect key cellular events, including regulation of transcription [206]. Proteins can be methylated irreversibly on the side-chain nitrogens of the amino acids arginine, lysine, and histidine in a reaction with S-adenosylmethionine (AdoMet) [207]. Coactivator-associated arginine methyltransferase (CARM1)/protein arginine Nmethyltransferase 4 (PRMT4) is identified as a binding partner of SRC-2/GRIP1 (glucocorticoid receptor-interacting protein 1) [71]. Recently, CARM1 shown to promote adipocyte differentiation by coactivating PPARγ using cDNA microarray and serial analysis of gene expression (SAGE) [208]. CARM1 also stimulates transcriptional activation by nuclear receptors in combination with the p160 family of coactivators [71]. The p160/SRC coactivators recruit CBP/p300 and CARM1 via two activation domains, AD1 and AD2 [71]. AD1 binds CBP or p300, whereas AD2 has been shown to activate transcription through the recruitment of the arginine methyltransferase CARM1. The ternary complex of p160-CARM1-CBP/p300 functions synergistically to enhance transcriptional activation by nuclear receptors. CARM1 efficiently methylates three arginine residues (R714, R742 and R768) spanning aa 685–774 of CBP which are also conserved in p300 to transactivate SRC-2/GRIP-1 [209]. Other than these three methylated arginine residues, CARM1 also methylates KIX domain of CBP/p300 to block the interaction with KID domain of CREB (Cyclic AMP response element binding protein) [210]. Methylation is an irreversible process but peptidyl deiminase 4 removes methylated arginine from the p300 (Arg-2142) which is localized in the p160-binding domain to inhibit the bimolecular interactions between p300 and GRIP1. The functional significance of the methylation and demethylimination of the arginine residue of p300 may be a key mechanism in p300/CBP-p160-CARM1 coactivator synergy. Methylation of p300/CBP by CARM1 promotes a conformational change that allows the p300-p160 interaction in the complex and facilitates additional steps in transcriptional activation [211].

Although CARM1 methylates CBP/p300 to enhance protein-protein interaction in the activated nuclear receptor complex, it also methylates histone tail, preferentially H3 which in turn relaxes chromatin to generate a docking site for coactivators and other transcriptional factors on the promoter of the target genes [71, 212, 213]. Methylation of the Q-rich domain of SRC-3 through CARM1 has an antagonizing activity on ER-mediated transcriptional activation [214]. During estrogen signaling methylation of SRC-3/AIB1 by CARM1 attenuates the transcriptional response by dissociating SRC-3/CARM1 coactivator complex from the ER receptor and thus completing a dynamic equilibrium of receptor-mediated coactivator assembly and disassembly at the promoter [214]. Mice deficient in CARM1 die at the perinatal stage emphasizing that CARM1 is crucial during late embryonic development or immediately after birth [215]. Methylation of CBP/p300 was shown to be abolished in the CARM1 knockout embryos and cells. Thus, it appears that CARM1 mediated methylation is needed for interaction between p/160 family of proteins and CBP/p300 to maintain general transcript integrity.

15. Coactivator Activator (CoAA) with RNA Splicing Activity

The Coactivator activator (CoAA) was first identified as a protein associated with PRIP/TRBP (thyroid hormone receptor-binding protein) in a yeast two-hybrid screen [72]. CoAA functions as a general activator of transcription for several nuclear receptors and stimulates transcription through its interaction with the C-terminal of PRIP/TRBP [72]. CoAA interacts with both PRIP/TRBP and p300 in vitro. The PRIP-interaction domain on CoAA protein is localized at the central region, which is encoded by exon 2 of CoAA gene [72]. In addition, CoAA potently coactivates transcription mediated by multiple hormone-response elements and acts synergistically with PRIP/TRBP and CBP. Thus, CoAA appears to function as a coactivator associated protein. Apart from participating in PRIP/TRBPmediated transcription, CoAA also regulates alternative splicing in a promoter-dependent manner [72]. The N-terminal region of the CoAA protein contains two RNA recognition motifs (RRMs) at amino acid position 3–68 and 81–144. Both RRMs are composed of two conserved ribonucleoprotein (RNP) consensus motifs that regulate posttranslational RNA splicing [216]. Coactivator modulator (CoAM), a splice variant is generated as a result of alternative splicing of exon 2 of the CoAA. CoAM, which lacks a PRIP/TRBP-interacting domain, represses both PRIP/TRBP and CBP action suggesting that CoAM may modulate endogenous CoAA function [72]. In conclusion, CoAA and PIMT, both capable of interacting with PRIP/TRBP/NCoA6 appear to function as regulators of RNA processing.

16. Conclusion

During the past 15 years, using yeast 2 hybrid screening [51, 52, 58, 59, 84], affinity pulldown of nuclear extracts via covalently bound ligand to the sepharose matrix [63], GST-receptor pulldown [64] and proteomic approaches [217],

over 300 nuclear receptor transciption cofactors have been identified. Transcriptional control is a multistep process, a fact reflected in the diversity of the coregulators, and their intrinsic enzyme activities. These coregulators are possibly organized into stable, preformed multiprotein complexes, the modular character of which may facilitate the efficient assembly of functionally diverse complexes by a liganded nuclear receptor. In addition, the modular character of these complexes provides the potential for different activators to assemble diverse configurations of regulatory complexes at their cognate cis-acting elements. Emerging genomic and proteomic approaches promise to advance the characterization of coactivator proteins and their physiological functions. It should be worth noting that of the many cofactors, about 165 coactivators have been implicated to date in various human diseases [218]. Gene knockout mouse models have clearly established that Med1 is necessary for PPARα and PPARγ functions and that SRC-1, SRC-2, and SRC-3 are redundant for PPARα function. It is anticipated that further studies of nuclear receptor coregulators and their complexes will yield significant insights into the basis of the complexity of signaling by PPARs and their ligands.

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Research Article

PRIC295, a Nuclear Receptor Coactivator, Identified from PPARα-Interacting Cofactor Complex

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The peroxisome proliferator-activated receptor-α (PPARα) plays a key role in lipid metabolism and energy combustion. Chronic activation of PPARα in rodents leads to the development of hepatocellular carcinomas. The ability of PPARα to induce expression of its target genes depends on Mediator, an evolutionarily conserved complex of cofactors and, in particular, the subunit 1 (Med1) of this complex. Here, we report the identification and characterization of PPARα-interacting cofactor (PRIC)-295 (PRIC295), a novel coactivator protein, and show that it interacts with the Med1 and Med24 subunits of the Mediator complex. PRIC295 contains 10 LXXLL signature motifs that facilitate nuclear receptor binding and interacts with PPARα and five other members of the nuclear receptor superfamily in a ligand-dependent manner. PRIC295 enhances the transactivation function of PPARα, PPARγ, and ERα. These data demonstrate that PRIC295 interacts with nuclear receptors such as PPARα and functions as a transcription coactivator under *in vitro* conditions and may play an important role in mediating the effects *in vivo* as a member of the PRIC complex with Med1 and Med24.

1. Introduction

Lipid metabolism in mammals is a complex process regulated by diverse factors including the members of the nuclear receptor subfamily known as peroxisome proliferator-activated receptors (PPARs). There are three isoforms of PPAR known as PPARα, PPARβ/δ, and PPARγ [1–3]. PPARα, the initial isoform identified, is centrally involved in the pleiotropic responses induced in rat and mouse liver by treatment with peroxisome proliferators [4–7]. Peroxisome proliferators are structurally diverse chemicals and include compounds such as clofibrate, bezafibrate, nafenopin, and others along with phthalate ester plasticizers, certain herbicides, pesticides, industrial solvents, and leukotriene D_4 receptor antagonists [8, 9]. The pleiotropic responses induced following treatment with a peroxisome proliferator include the lowering of serum lipids, the proliferation of hepatic peroxisomes, and the coordinated induction of fatty acid oxidation genes involved in the mitochondrial and peroxisomal β-oxidation, and microsomal ω-oxidation pathways [10]. Additionally, rats and mice chronically exposed to peroxisome proliferators develop a high incidence of hepatocellular carcinomas even though these compounds are nongenotoxic and considered a paradigm for epigenetic carcinogenesis [5, 6, 11]. PPARα also exerts a significant role in the process of inflammation [12–15]. All these responses, including the development of hepatocellular carcinomas, are receptor mediated and achieved through selective activation of PPARα [16–18].

PPARs heterodimerize with retinoid X receptor-α (RXRα) and bind to peroxisome proliferator response elements (PPREs) present in the promoter region of target genes [19–22]. In the absence of cognate ligand, transcription of target genes is repressed by corepressor proteins such as silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) [23], nuclear receptor corepressor (NCoR) [24, 25], and receptor interacting protein 140 (RIP140) [26] bound to the PPAR-RXR heterodimers. Upon ligand binding, PPARs undergo a conformational change causing the dissociation of corepressor proteins and then allowing for

the orchestrated recruitment of coactivator proteins [27, 28]. These coactivator proteins enhance transcription through various means including histone acetyltransferase activity, methyltransferase activity or mediating the interaction of the activated PPAR-RXR heterodimer with the basal transcription machinery of the cell [29–35].

Coactivators and coactivator-associated proteins known to be involved in PPARα-mediated transactivation include three members of the SRC (steroid receptor coactivator)/p160 family of proteins [29, 32, 36], CBP (CREB-binding protein)/p300 [35, 37, 38], PBP (PPARγ-binding protein)/MED1/TRAP220/DRIP205 [30, 39–45], PRIP/NCoA6/ASC2/RAP250/TRBP/NRC [46–50], PIMT/NCoA6IP [31], CARM1[51], PRIC285 [33], PRIC320/CHD9/CReMM [34, 52], PGC-1α [53], PGC-1β, and others [54, 55]. Each of these coactivator proteins contains one or more conserved LXXLL (L = leucine, X = any amino acid) motifs which are necessary for the interaction with the activation function-2 (AF-2) domain present at the C-terminal end of a nuclear receptor [56]. Although several cofactors have been shown to interact with PPARs and function as transcriptional regulators under *in vitro* conditions, there is limited data on the *in vivo* functions of these molecules in PPAR-regulated target gene transcription. To date, deletion of Med1 in liver has been shown to abrogate the ability of PPARα to activate transcription of target genes as well as block other pleiotropic effects of receptor activation including liver regeneration and the development of hepatocellular carcinoma [57]. Furthermore, Med1 deletion also affects PPARγ-regulated adipogenesis in mouse embryonic fibroblasts and the adipogenic steatosis induced by PPARγ overexpression in liver [58, 59]. Med1 was first cloned using PPARγ as bait in the yeast two-hybrid system and subsequently detected in the PPARα-interacting cofactor (PRIC) complex of approximately 25 proteins isolated from rat liver nuclear extracts [30, 33]. The identities of proteins in the PRIC complex [33] revealed several coactivators previously identified in yeast two-hybrid screens such as Med1 [30], CBP [35], SRC-1 [29], Med24/TRAP100 [60], PIMT [31], and PGC-1 [53]. The PRIC complex also included some novel proteins including PRIC285 and others [33]. Subsequently, this approach was modified to use ciprofibrate, a synthetic PPARα agonist, to pull down a complex of proteins from rat nuclear extract and this resulted in the identification of PRIC320/CHD9 and some other high molecular weight proteins [34]. In this study, we present data to show that one of the hitherto uncharacterized high molecular weight proteins, designated PRIC295, contains 10 LXXLL coactivator motifs and interacts with PPARα in a ligand-dependent manner. PRIC295 significantly enhances the transcriptional activity of PPARα *in vitro*. We further demonstrate that PRIC295 interacts with the Med1 and Med24 subunits of the Mediator complex suggesting that PRIC295 may be important for nuclear receptor signaling *in vivo*.

2. Materials and Methods

2.1. Preparation of Liver Nuclear Extracts and Ciprofibrate-Sepharose Beads for Affinity Pulldown. Liver tissue was harvested from 5 male F-344 rats sacrificed 1 hour after an intragastric dose of ciprofibrate (250 mg/kg body weight). Nuclei were isolated and extract prepared as described elsewhere [33, 34]. Ciprofibrate was immobilized on AH-Sepharose 4B by carbodiimide reaction as previously described [61]. Animals were housed under a 12 hour light/12 hour dark cycle and maintained in individual cages with standard rodent chow and water *ad libitum*. All animal procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Northwestern University.

2.2. Affinity Pulldown and Matrix-Assisted Laser Desorption/Ionization-Time of Flight. Ciprofibrate-sepharose beads were blocked with 1% fatty-acid free bovine serum albumin and washed with buffer containing 0.5% NaCl. Nuclear extract (~10 mg protein) was allowed to interact with Sepharose beads with immobilized ciprofibrate overnight at 4°C. After washing, bound proteins were eluted from the beads by boiling and resolved by SDS-PAGE on a 3%–8% Tris-acetate gel or 4%–20% acrylamide gel as described previously [34]. Gels were stained with silver nitrate to visualize the resolved proteins and selected bands were cut and processed for matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis using a Voyager DE Pro at the IMSERC Laboratory at Northwestern University. Observed peaks were identified by the use of MS-FIT (Protein Prospector, University of California, San Francisco). To isolate PRIC295 interacting proteins, nuclear extracts were allowed to interact with GST-ΔPRIC295$^{840–1815}$ (F2) protein and the bound proteins were subjected to MALDI-TOF analysis.

2.3. PRIC295 Cloning and Plasmid Constructs. The human full-length cDNA (KIAA0219) was purchased from the Kazusa DNA Research Institute, Chiba, Japan. Full-length expression vectors were made by restriction digestion of the cDNA and ligation into the pcDNA3.1(+) expression vector (Invitrogen). Smaller fragments of the cDNA were amplified using the high fidelity rTth polymerase (Applied Biosystems) and ligated into pcDNA3.1(+), pGEX-5X-1 (GE Healthcare), or pM (Clontech) vectors at the designated sites to give the plasmids pcDNA-ΔPRIC295$^{1–915}$ (Fragment1—F1), pcDNA-ΔPRIC295$^{840–1815}$ (F2), and pcDNA-ΔPRIC295$^{1740–2671}$ (F3). These fragments were also ligated into pGEX-5X-1 and pM. All final vectors were confirmed by sequencing at the Genomics Core Facility at Northwestern University. *In vitro* translated proteins were made using the TnT T7 quick-coupled transcription/translation kit from Promega according to the manufacturer's protocol and radiolabeled with L-^{35}S-methionine (Perkin Elmer). Other plasmids including pGEX-PPARα, pCMX-PPARα, pGEX-PPARγ, pCMX-PPARγ, pGEX-ERα, pcDNA-ERα, pGEX-CAR, pGEX-TRα, pGEX-RXRα, pCMX-RXRα, CMV-RL, pGL3-3xPPRE-LUC, PGL3-3xERE-LUC, GST-Med1, and cloned fragments of Med1 fused to GST have all been previously described [30, 33, 62].

2.4. Northern Blotting and Quantitative Real-Time PCR. Northern blotting for human PRIC295 mRNA expression was performed using a multiple tissue blot purchased from Clontech and the 3′-terminal 1 kb of the PRIC295 cDNA as a probe. Total RNA for quantitative PCR was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was done using an ABI 7300 (Applied Biosystems). Samples were tested in triplicate and normalized with 18S ribosomal RNA. Specific PCR products were measured by melting curve analysis and relative gene expression changes were measured using the comparative C_T method, $X = 2^{-\Delta\Delta CT}$. PRIC295 qPCR primers were designed from exons 27 and 28.

2.5. GST-Pulldown with PRIC295 and Nuclear Receptors. GST and GST-fusion proteins were purified using glutathione sepharose 4B beads (GE Healthcare) and incubated with *in vitro* synthesized PRIC295 labeled with ^{35}S-methionine (or fragments of PRIC295). Binding was allowed to take place on a gentle shaker at 4°C for 2 hours, then bound protein was washed, eluted, and resolved by SDS-PAGE and analyzed by autoradiography as described earlier [30, 46]. Quantification of binding was performed using ImageJ software available from the NIH.

2.6. Transactivation Assays. The ability of PRIC295 to enhance transcriptional activation mediated by PPARα, PPARγ, or ERα was measured by transfecting HeLa cells with appropriate plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Plasmids used in these assays were pCMX-PPARα, pCMX-PPARγ, pcDNA-ERα, pCMX-RXRα, pGL3-3xPPRE-Luc, pGL3-3xERE-Luc, and pcDNA3.1-PRIC295. Experiments were conducted in triplicate and luciferase expression was assessed using the dual-luciferase reporter assay system from Promega according to the manufacturer's protocol. Ligands used in these experiments (Wy-14,643 for PPARα, rosiglitazone for PPARγ, and 17-β-estradiol for ERα) were added at a concentration of 100 μM. Transcriptional activity of PRIC295 was further examined by transfecting HeLa cells with pM-ΔPRIC295$^{1-915}$ (F1), pM-ΔPRIC295$^{840-1815}$ (F2), or pM-ΔPRIC295$^{1740-2671}$ (F3) which express chimeric proteins containing fused fragments of PRIC295 with the DNA-binding domain (aa 1–147) of the yeast Gal4 transcription factor [63]. These cells were also transfected with the Gal4-TK-Luc plasmid containing the c-terminal portion of the Gal4 protein and the thymidine kinase promoter upstream of the luciferase reporter gene. The ability of chimeric Gal4-PRIC295 proteins to activate transcription of the luciferase reporter gene was analyzed by comparison to cells transfected with Gal4-TK-luc and empty pM vector [64].

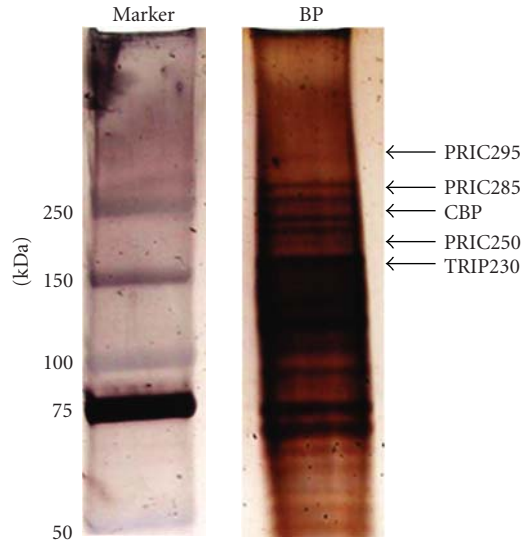
2.7. Immunofluorescence, Immunoblotting, and Coimmunoprecipitation. HeLa cancer cells were transfected with pCMX-PPARα and pcDNA-PRIC295-3xFLAG using lipofectamine 2000 reagent. Cells were fixed 48 hours post-transfection with 4% paraformaldehyde for 20 minutes and stained with anti-PPARα (Santa Cruz Biotechnology Inc.—sc-9000) and anti-FLAG monoclonal antibody M2 (Sigma). Fluorescence microscopy and digital image photographs were obtained using a Nikon Eclipse E600 microscope equipped with a Spot RT slider digital camera and image analysis software (Diagnostic Instruments). Immunoblotting was performed to ascertain the presence of PPARα, Med1, or Med24/TRAP100 in the PRIC complex of proteins isolated using ciprofibrate-Sepharose and proteins that bind PRIC295 in the GST-pulldown assay. For coimmunoprecipitation, HeLa cells were transfected with pcDNA3.1-PRIC295-3xFLAG and pCMX-PPARα expression vectors as previously described. Nuclear extract was made and interacting proteins were purified using resin covalently bound with the anti-FLAG M2 antibody (Sigma). Proteins were subjected to SDS-PAGE, then immunoblotted using anti-FLAG, anti-Med1 (Santa Cruz Biotechnology, Inc.—sc-5334), or anti-PPARα.

2.8. Statistical Analysis. Statistical significance of the difference between transfected groups of cells used in the luciferase assays were calculated by one-tailed Student's *t*-test using the available functions in Microsoft Excel 2007

3. Results and Discussion

3.1. Identification of PRIC295. To identify rat liver proteins that interact with peroxisome proliferators we initially used an affinity chromatography approach that involved ciprofibrate immobilized on AH-Sepharose 4B by carbodiimide reaction [61]. This procedure resulted in the isolation of peroxisome proliferator-binding proteins that ranged in apparent Mr of 31,000–79,000 [61], but they remained uncharacterized due to the limited availability and applicability at that time of MALDI-TOF technology [65]. The general availability of MALDI-TOF mass spectrographic technology in recent years enabled the analysis of liver nuclear proteins that bind to GST-fused full-length PPARα [33], or to AH-Sepharose-ciprofibrate affinity matrix [34]. In the present study, we describe the identification of a member of the PRIC complex from ciprofibrate-bound proteins (Figure 1(a)) which has been designated as PRIC295 based on molecular mass. Mass spectrographic analysis of one of the high molecular weight protein bands revealed several peptide fragments that matched the human KIAA0219 protein (accession number D86973, reference sequence NM_006836) (Figure 1(b)). Human PRIC295 shares 94% homology with the rat and 93% homology with the mouse orthologues. Other proteins identified by MALDI-TOF in the PRIC complex include PRIC285, CREB-binding protein (helicase CBP), PRIC250, and thyroid receptor-interacting protein 230 (TRIP230) (Figure 1(b)). In this complex, we also identified previously known PPARα cofactor proteins that include PRIC285 and PRIC320 (data not shown).

3.2. Molecular Characterization of PRIC295. The gene encoding PRIC295 is located on human chromosome 12q23.24 and consists of 58 exons. The nucleotide sequence data



(a)

Protein	Peptide sequences	m/z	Accession no.
PRIC295	ISSAGCLGELCAFLTEEELSAVLQQCLLADVSGIDWMVR	4383.139	D86973
	QIGSVIRNPEILALAPVLLDALTDPSR	2871.631	
	ETVLRGLMELHMVLPAPDTDEK	2494.269	
	YSSDVQEMILSSATADR	1872.865	
	ENVNSLLPVFEEFLK	1777.938	
PRIC285	EALEKPFTVIQGPPGTGKTIVGLHIVFWFHK	3446.899	Q9BYK8
	HSRLFWVQIVLWRQGFYYPLGIVR	3033.673	
CBP	KLDTGQYQEPWQYVDDVWLMFNNAWLYNRK	3820.822	Q92793
	FLTLARDKHWEFSSLR	2192.125	
PRIC250	SVFYLLMKMFVTSSHLQLKSLTK	2701.483	AB011111
	FQEQSQLLYLLDVVRNGIR	2291.251	
TRIP230	ISSTSHTQDVVYLQQQLQAYAMEREKVFAVLNEK	3954.007	Q15643
	VENLVDQLNKSQESNVSIQK	2272.179	

(b)

Figure 1: PRIC295 in ciprofibrate bound protein complex. (a) Ciprofibrate-binding protein complex isolated from rat liver nuclear extracts analyzed on a 3%–8% Tris-acetate silver stained gel. Lane 1 marker; lane 2 BP, binding proteins. Selected high molecular weight bands (arrows) were excised from the gel and digested with trypsin. MALDI-TOF analysis was conducted on the marked bands yielding peptide-matching protein identities, in descending order from highest molecular weight to lowest, of PRIC295, PRIC285, CBP, PRIC250, and TRIP230. (b) Mass spectrometric and limited sequence analysis data of proteins identified by MALDI-TOF with their associated peptide fragment sequences, m/z ratios and corresponding accession numbers.

for PRIC295 are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK006575. The 8,666 bp long transcript has an open reading frame of 8013 bp that encodes a 2,671 aa protein of approximately 295 kDa (accession number NP_006827.1). PRIC295 contains 10 LXXLL coactivator motifs located at aa L_1,465–469; L_2,1160–1164; L_3,1461–1465; L_4,1499–1503; L_5,1596–1600; L_6,1839–1843; L_7,1926–1930; L_8,2045–2049; L_9,2330–2334; L_{10},2594–2598 (Figure 2(a)). The LXXLL motifs are significant in coactivator and activator (receptor) interactions. In PRIC295 these motifs are evolutionarily conserved across many different species (Figure 2(b)). Sequences flanking these conserved LXXLL motifs have also been shown to be influential in determining the specificity of the interaction between coactivators and activators. Three of the LXXLL motifs, namely, L_2, L_7, and L_{10}, present in PRIC295 possess a conserved proline in the -2 position relative to the first leucine of the LXXLL motif (Figure 2(b)). The presence of proline at this position is similar to the biologically important LXXLL motifs known to interact with PPARα such as those found in Med1 [30].

In addition to LXXLL motifs, the PRIC295 sequence also reveals the presence of 24 HEAT repeats (see Table 1 in Supplementary Material available online at doi:10.1155/2010/173907) which may play a role in protein-protein interactions as well as energy production and conversion (NCBI—Conserved Domain Database—COG1413) (Figure 2(a)) [66]. HEAT repeat domains are composed of approximately 50 hydrophobic and charged amino acids conserved at particular positions within the motif [67]. These motifs are believed to be important in mediating protein-protein interactions though the exact mechanism by

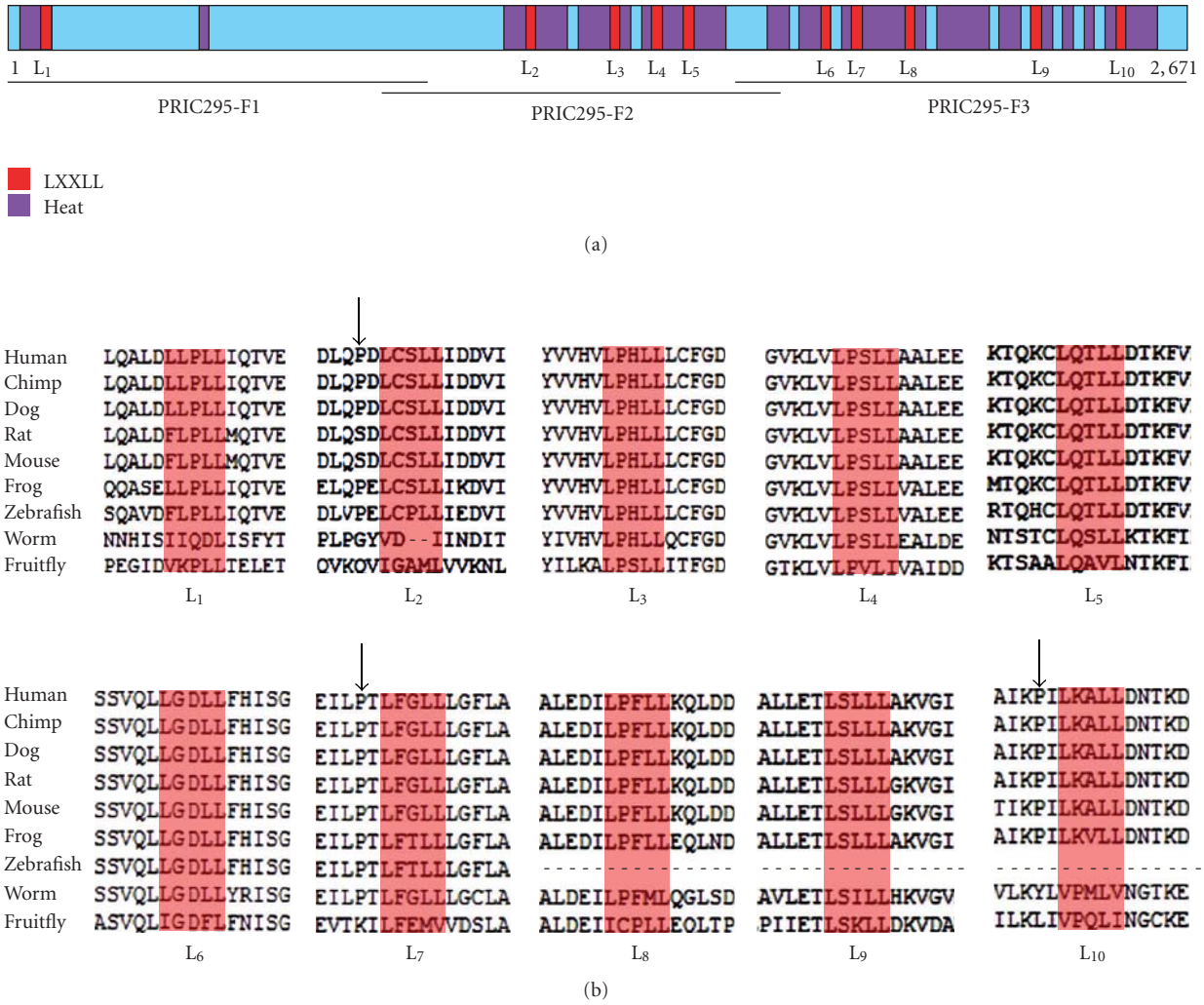


Figure 2: PRIC295 structural features. (a) Schematic representation of the PRIC295 protein to indicate the locations of LXXLL motifs in red color and HEAT repeat domains in purple color. (b) Alignment of the orthologous PRIC295 genomic sequences of selected species at the loci of all 10 of the LXXLL motifs contained in the human PRIC295 protein. LXXLLs are numbered L_1 to L_{10} beginning from the N-terminal region: L_1aa465–469; L_2aa1160–1164; L_3aa 1461–1465; L_4aa1499–1503; L_5aa1596–1600; L_6aa1839–1843; L_7aa1926–1930; L_8aa2045–2049; L_9aa2330–2334; L_{10}aa2594–2598. The model organism to which each sequence corresponds is indicated at the left side of the sequence. The location of conserved proline at—2 of L_2, L_7, and L_{10} are indicated using an arrow.

which this occurs is not clear. The presence of HEAT repeats in PRIC295 places it in a class of HEAT repeat proteins known to be involved in gene transcription and translation, but also have other functions [67]. It is possible that the HEAT repeats in PRIC295 may be important in mediating interactions between different transcription cofactors within the PRIC complex.

3.3. Tissue Distribution of PRIC295 Transcripts. Northern blot analysis revealed that the PRIC295 mRNA transcript is 8.5 kb in length and is expressed in many different human tissues with the highest expression observed in brain, heart, skeletal muscle, and placenta (Figure 3(a)). In thymus, spleen, liver, and small intestine PRIC295 expression was evident whereas in lung and colon the transcript was barely detectable. The expression of PRIC295 in different mouse tissues was evaluated by qPCR and the data show that it is expressed robustly in testes, white adipose tissue, brain, heart, and kidney (Figure 3(b)). Embryonic expression of PRIC295 mRNA in the mouse was also evaluated using the qPCR approach and the data suggest higher levels of expression during developmental stages E15.5 and E16.5. By E18.5 the expression level was low (Supplementary Figure 1). *In situ* hybridization data for PRIC295 mRNA localization during various developmental stages in the mouse should provide clues about the expression in tissues at different developmental stages.

3.4. Interaction of PRIC295 with Nuclear Receptors. Interactions between PRIC295 and PPARα and some other members of the nuclear receptor superfamily were analyzed by GST-pulldown assays. These were performed using

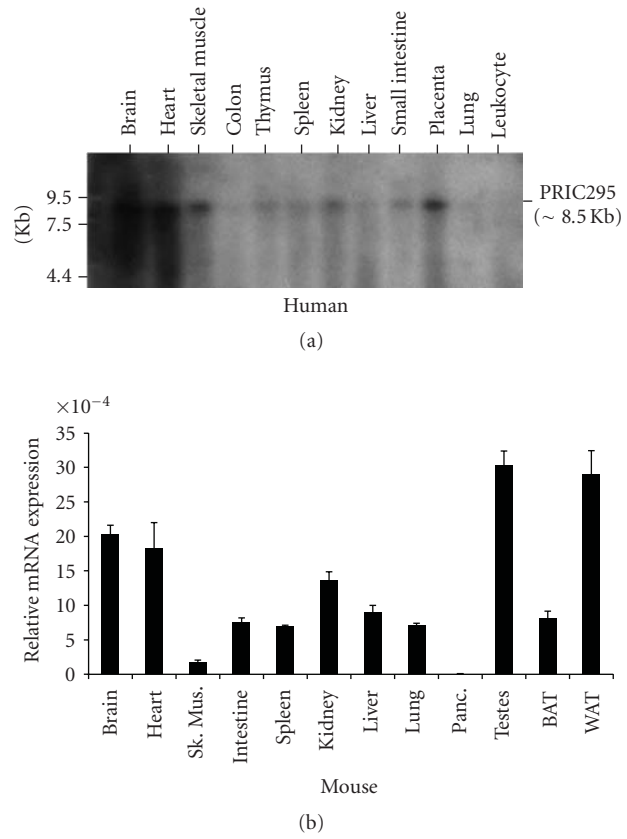


Figure 3: Expression of PRIC295 in human and mouse tissues. (a) Northern blot analysis of PRIC295 mRNA expression using a human multiple tissue blot (Clontech). Blot contains 2 μg of polyA RNA in each lane from tissues indicated. PRIC295 transcript (~8.5 kb) is expressed in nearly all tissues. (b) Quantitative PCR data showing the expression of PRIC295 mRNA in several different mouse tissues. Primers were designed to amplify from the exon 27-28 region of the cDNA sequence. Samples were normalized with 18S RNA. RNA from 5 different C57B6/J males was pooled and samples were run in triplicate. Standard deviations are shown.

bacterially-expressed GST-fusion nuclear receptor proteins and *in vitro* translated pcDNA-PRIC295 (Figure 4(a)). Full-length PRIC295 bound *in vitro* to PPARα, PPARγ, RXRα, CAR, ERα, and TRα and addition of receptor specific ligand (10 μM to 100 μM) enhanced this binding (Figure 4(a); Supplementary Figure 2). Ligands used for each receptor were pirinixic acid (Wy-14,643) for PPARα, rosiglitazone for PPARγ, 9-cis-retinoic acid for RXRα, TCPOBOP for CAR, 17-β-estradiol for ERα, and triiodothyronine (T3) for TRα. PRIC295 did not bind to GST alone, as expected (Figure 4(a)).

To further investigate which region of PRIC295 protein might be involved in receptor interactions, additional GST-pulldown assays were undertaken using the GST-fusion receptor proteins and *in vitro* translated PRIC295 fragments designated ΔPRIC295$^{1–915}$ (F1), ΔPRIC295$^{840–1815}$ (F2), and ΔPRIC295$^{1740–2671}$ (F3) (Figure 4(b)). Fragment F1 contains 1 LXXLL (L_1), fragment 2 contains 4 LXXLLs (L_2–L_5), and fragment 3 contains 5 LXXLLs (L_6–L_{10}) (Figure 2(a)). The fragment ΔPRIC295$^{1–915}$ (F1) with 1 LXXLL (L_1) bound strongly to GST-PPARγ, GST-RXRα, and GST-TRα, while ΔPRIC295$^{840–1815}$ (F2) bound strongly to GST-PPARα, GST-CAR, and GST-ERα. In general, F1 and F2 bound more avidly than F3 to these receptors. Although PRIC295 fragments F1 and F2 bound to PPARα, surprisingly, there was no perceptible interaction of this receptor with fragment ΔPRIC295$^{1740–2671}$ (F3) although F3 contains 5 LXXLLs (Figures 2(a) and 4(b)). The relative binding levels were digitally quantified (Supplementary Figure 3). These observations suggest that PRIC295 might exert a role in mediating the transcription of target genes of several members of the nuclear receptor superfamily. Since different nuclear receptors exhibited different affinities for interacting with the 3 different fragments of PRIC295, it is possible that different LXXLL motifs may determine the binding to a given nuclear receptor. In this regard, fragment 1 (F1) which has only one LXXLL (L_1) may be important biologically as this fragment binds more readily than the other two fragments. Additional studies are needed to modify this first LXXLL and selected others for binding specificity.

3.5. PRIC295 Enhances PPARα, PPARγ, and ERα-Mediated Transcription in Mammalian Cells. In order to examine the functional relevance of the interaction between PRIC295 and nuclear receptor proteins, we transiently coexpressed PRIC295 with pCMX-PPARα/pCMX-RXRα (Figure 5(a)), with pCMX-PPARγ/pCMX-RXRα (Figure 5(b)), or with pcDNA-ERα (Figure 5(c)) in HeLa cells. These cells were concurrently transfected with luciferase reporters pGL3-3xPPRE-LUC for PPARα and PPARγ, or pGL3-3xERE-LUC for ERα in order to measure the ability of PRIC295 to transactivate receptor-mediated transcription. PRIC295 clearly increased the transcriptional activation of the luciferase reporter gene in a ligand-dependent manner when cotransfected with pCMX-PPARα, pCMX-PPARγ, or pcDNA-ERα (Figures 5(a)–5(c)). PRIC295 also enhanced PPARα/RXRα-mediated transcriptional activation of pGL3-3xPPRE-LUC in a dose-dependent manner (Figure 5(d)). PRIC295 also enhanced PPARα/RXRα-mediated transcription in the presence of the RXRα ligand 9-cis-retinoic acid (Supplementary Figure 4). These results confirm that PRIC295 functions as a coactivator for nuclear receptors PPARα, PPARγ, and ERα.

To further confirm the transactivational activity of PRIC295, a Gal4-binding assay was performed using the yeast Gal4-DBD fused to the PRIC295 fragments, ΔPRIC295$^{1–915}$ (F1), ΔPRIC295$^{840–1815}$ (F2), or ΔPRIC295$^{1740–2671}$ (F3). These chimeric proteins also showed the ability to significantly enhance transcription of the luciferase reporter under the direction of the thymidine kinase promoter fused to the c-terminal of Gal4. Cells transfected with the chimeric PRIC295-F1 fragment showed a statistically significant (P-value = .000748 for Gal4-DBD-F1 and .001733 for Gal4-DBD-F2) increase in relative luciferase activity as compared to cells transfected with only the Gal4-DBD (Figure 6). Gal4-DBD-F3 exhibited only a modest increase in the activity (not illustrated). These data clearly demonstrate the activity of PRIC295 as a coactivator

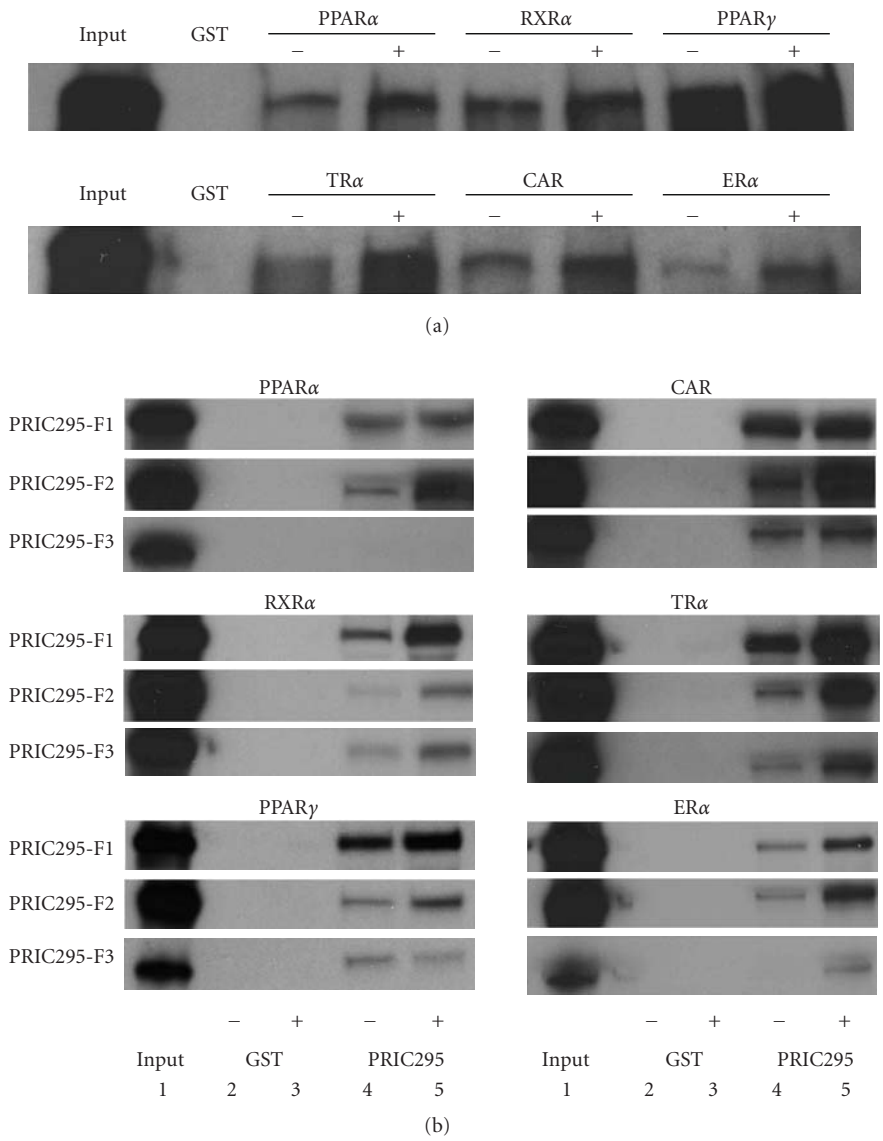


Figure 4: PRIC295 interactions with nuclear receptors. (a) *In vitro* interaction of radiolabeled full-length PRIC295 with nuclear receptors PPARα, RXRα, PPARγ, TRα, CAR, and ERα. PRIC295 was radiolabeled using ^{35}S-methionine (Perkin Elmer) and incubated with each shown receptor fusion protein in GST-pulldown assay. (−) minus indicates *in vitro* binding interaction in the absence of cognate ligand and (+) plus indicates the presence of receptor-specific ligand during the *in vitro* binding interaction. Ligands used: Wy-14,643 for PPARα; 9-cis-retinoic acid for RXRα; rosiglitazone for PPARγ; triiodothyronine (T3)TRα; 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) for CAR; 17-β-estradiol for ERα. All ligands were used at a concentration of 100 μM. (b) Interaction of radiolabeled PRIC295 fragments ΔPRIC295$^{1–915}$ (F1), ΔPRIC295$^{840–1815}$ (F2), and ΔPRIC295$^{1740–2671}$ (F3) with GST-fusion nuclear receptor proteins. Loading for each lane is indicated at the base of each panel. Lane 1 input; lane 2 GST alone without ligand; lane 3 GST alone with receptor-specific ligand; lane 4 GST-receptor fusion in the absence ligand; lane 5 in the presence of receptor-specific ligand. All ligands used are as previously described in (a).

protein though the exact mechanism by which PRIC295 exerts this influence is presently unclear.

3.6. PRIC295 Binding Partners. We have previously demonstrated that coactivator-binding protein PIMT (NCoA6IP) interacts with PRIP (ASC-2/NCoA6), CBP, p300, and Med1 to presumably form a PIMT complex [62]. PRIP/ASC-2 also interacts with other cofactors to form a steady complex described as ASCOM (for ASC-2 complex) [68]. ASCOM contains histone H3-lysine-4 (H3LK4) methyltransferase MLL3 or its paralogue MLL4 [68]. Interaction between ASCOM and the ATPase-dependent chromatin remodeling complex Swi/Snf have also been demonstrated and these interactions promote the binding of these complexes to nuclear receptor target genes [69]. Since coactivator and coactivator-associated protein interactions are being recognized with increasing frequency, we decided to investigate whether PRIC295 associates with other cofactors. For this

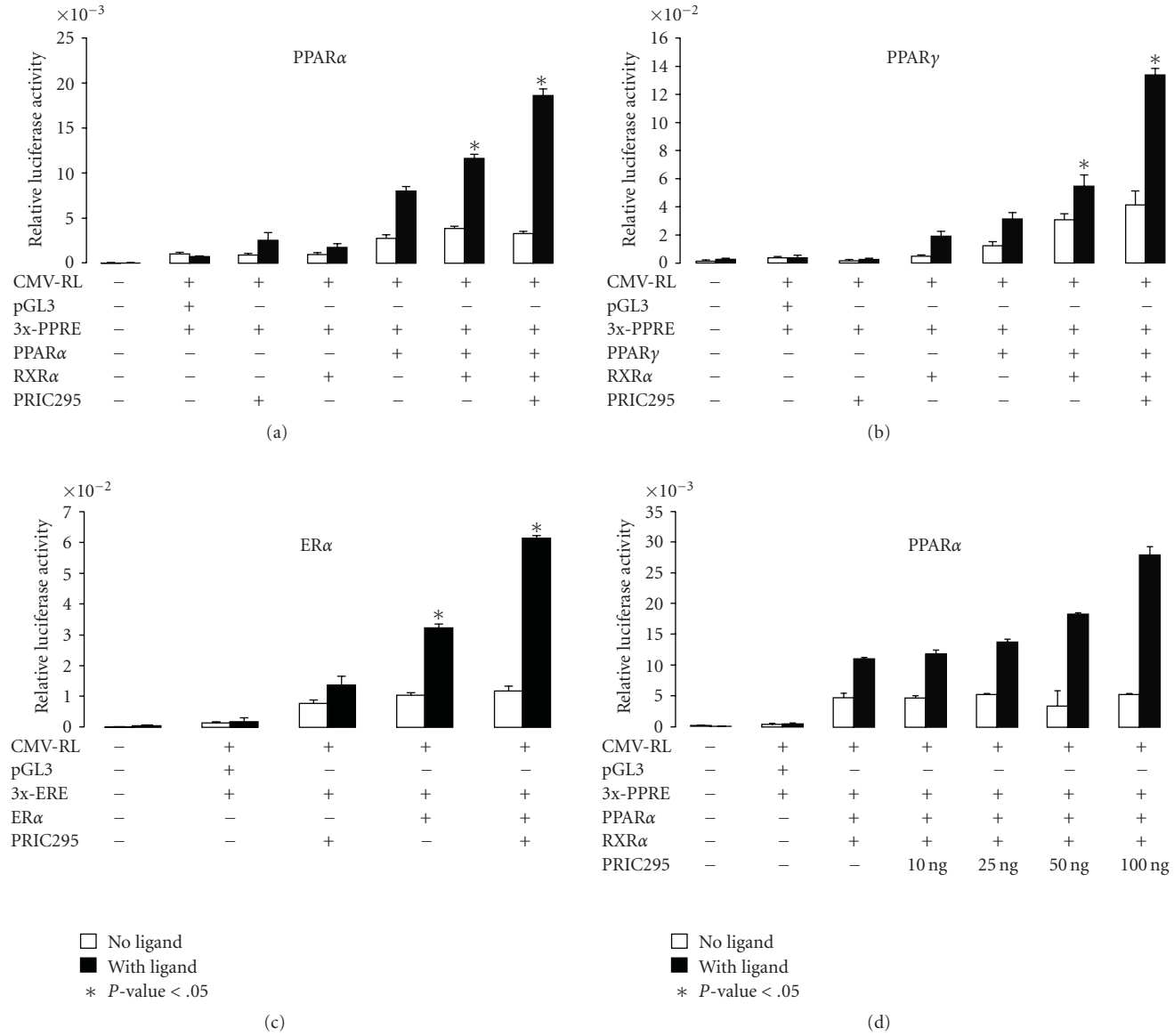


Figure 5: PRIC295 functions as a transcriptional coactivator for nuclear receptors. Data are shown for PPARα (a), PPARγ (b), and ERα (c). pGL3-3xPPRE-LUC was used as a reporter for PPARα and PPARγ whereas 3xERE was used as reporter for ERα. HeLa cells were transfected with 100 ng of pcDNA-PRIC295, pCMX-PPARα, pCMX-pCMX-PPARγ, pCMX-ERα, pCMX-RXRα, pGL3-3xPPRE-LUC (or empty pGL3-LUC vector), and 50 ng of CMV-RL plasmid as indicated (+ or −). Each column shown is the mean relative luciferase unit value for triplicate experiments. Open bar represents activity in the absence and dark bar in the presence of respective ligand (see Figure 4). All experiments were normalized against the expression of *Renilla* luciferase (CMV-RL) as an internal control. Statistical significance (P-value) is indicated at the base of the panel and calculated by comparing transfected groups marked with an *. (d) Transcriptional activation of PPARα with the addition of the increasing amounts of PRIC295. All other plasmids were transfected using the same amounts indicated above.

purpose, we chose to use ΔPRIC295$^{840–1815}$ (F2) region fused to GST at the N-terminus of the protein to affinity pulldown potential PRIC295 binding partners from rat liver nuclear extracts (Figure 7(a)). PRIC295-F2 was chosen because this fragment with 4 LXXLLs generally bound to all nuclear receptors analyzed in this study, and also we could not successfully express full length PRIC295 as GST-fusion protein. MALDI-TOF mass spectrographic analysis of selected PRIC295 F2 fragment bound peptides (Figure 7(a)) resolved by SDS-PAGE revealed the presence of PRIC285 [Q9BYK8] [33], Med12L [Q86YW9], Med24 [Q99K74] [60], PRMT1 [Q63009] [70], C/EBPβ [P17676] [71], Med20 [Q9H944] [72], and ZNRD1 [Q6MFY5] [73] among others (Figure 7(b)). To confirm some of these potential interactions, we performed immunoblot analysis for the presence of PPARα, Med24, and Med1 in the ciprofibrate bound protein complex (Figure 7(a)). These immunoblots established the presence of Med24 and Med1 among the proteins pulled down using ciprofibrate-Sepharose slurry that appear to form PRIC295 complex (PRIC295COM).

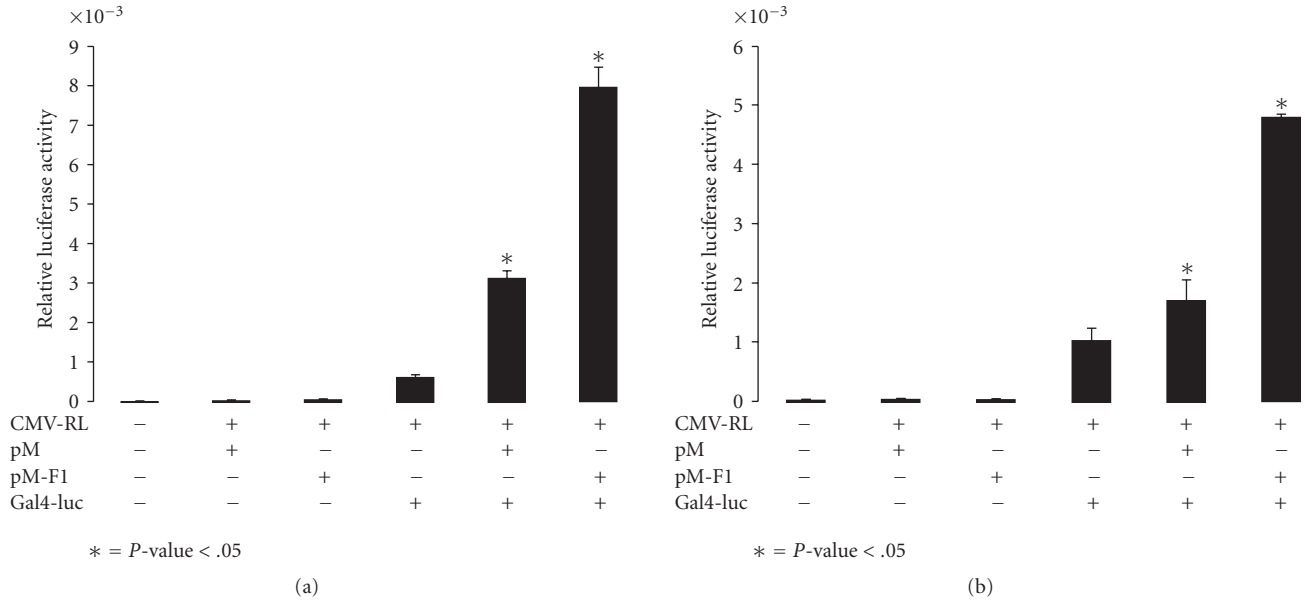


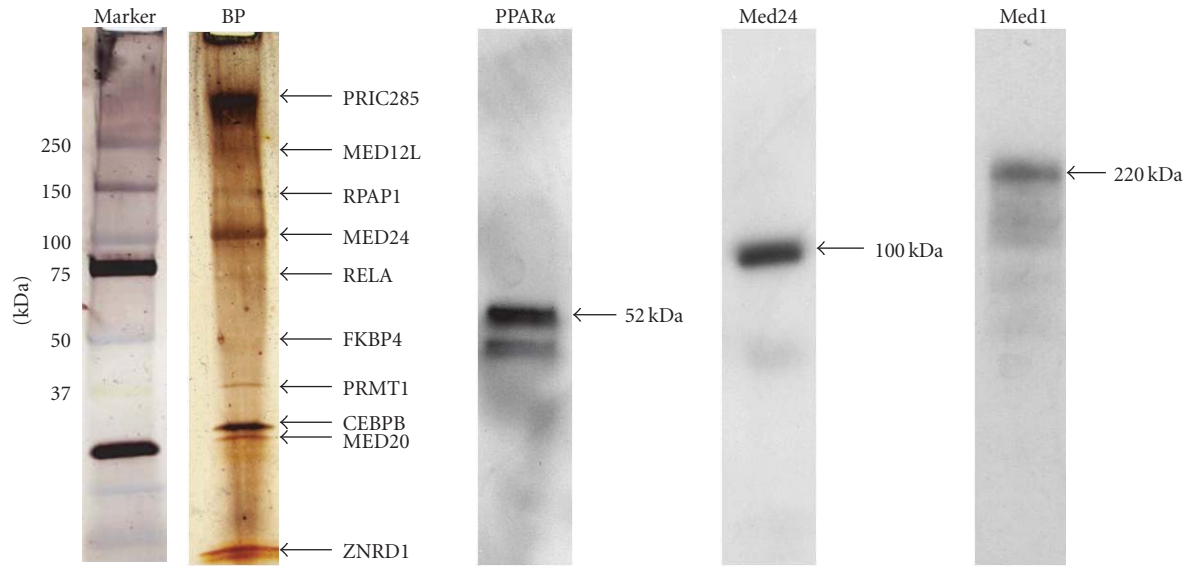
Figure 6: PRIC295 functions as a coactivator protein in Gal4-binding assay. Transfection of chimeric Gal4-DBD-PRIC295-F1(E) or Gal4-DBD-PRIC295-F2(F) significantly enhanced transcription of the luciferase reporter gene. Transfection of chimeric Gal4-DBD-PRIC295-F3 did not significantly enhance the transcription of the luciferase reporter (F3; not illustrated). HeLa cells were transfected with 100 ng of pM-ΔPRIC295$^{1–915}$ (F1) (a), pM-ΔPRIC295$^{840–1815}$ (F2) (b), Gal4-TK-Luc and 50 ng of CMV-RL as indicated (+ or −). Each column shown is the mean relative luciferase unit value for triplicate experiments. Statistical significance was calculated by comparison of transfected groups marked with an ∗.

3.7. PRIC295 Localizes to the Nucleus and Interaction between PRIC295 and PPAR, Med24, and Med1 in Intact Cells. To confirm the subcellular localization of PRIC295, pcDNA-PRIC295-3xFLAG was transfected into HeLa cells. Immunofluorescence microscopy revealed the nuclear localization of FLAG-tagged PRIC295 (Figure 8(a); panel PRIC295-3xFLAG). We also cotransfected these cells with pCMX-PPARα and using anti-PPARα antibodies we confirmed the expression of this receptor in the nucleus (Figure 8(a); panel PPARα). When PRIC295 and PPARα images were merged most nuclei were colored yellow indicating a high degree of overlap in these cells that express both proteins (Figure 8(a); merged image) but further high resolution studies are needed to determine if these two proteins are colocalized in the nucleus. Analysis of several cells indicated that some cells were expressing PRIC295 while others only PPARα (Figure 8(a); one red and one green nucleus in addition to several yellow colored nuclei). DAPI was used to stain all nuclei (Figure 8(a), DAPI).

We used immunoprecipitation followed by immunoblotting to establish the *in vivo* interaction of PRIC295 with PPARα, Med24, and Med1 (Figure 8(b)). For this purpose, we transfected cells with PRIC295-3xFLAG and immunoprecipitated with FLAG antibodies and immunoblotted the precipitates with antibodies against PPARα, Med24, and Med1 (Figure 8(b)). Clearly, this approach established the presence of coimmunoprecipitated PPARα, Med24, and Med1 (Figure 8(b)). These observations demonstrate that PRIC295 interacts *in vivo* not only with PPARα but also with two members of the Mediator complex in living cells. Further studies will be necessary to analyze nuclear proteins that bind to all three PRIC295 fragments, F1, F2, and F3, to fully delineate the PRIC295COM.

3.8. GST-Pulldown to Establish PRIC295 Interaction with Med1 and Med 24 In Vitro. Further experimentation was performed to confirm the interaction of PRIC295 with Mediator complex proteins Med1 and Med24 *in vitro* (Figures 9(a)–9(c)). *In vitro* translated and radiolabeled full-length PRIC295 was allowed to interact with GST-Med1 fragments (Figure 9(a)). Fragments covering Med1 aa 1–740 (not shown), 740–860, 860–980, 980–1055, 1055–1130, 1130–1250, 1250–1370, and 1370–1470 failed to bind with full-length PRIC295 (Figure 9(a)). However, PRIC295 bound strongly with a fragment from the C-terminal of Med1 containing amino acids 1470–1570 (Figure 9(a)). Coomassie-stained GST-Med1 fusion proteins used in these assays are shown in Supplementary Figure 5. Analysis of this region of Med1 showed a lack of any known conserved domains or binding sites. This Med1 fragment is devoid of any LXXLL motif. In order to ascertain the region of the PRIC295 that binds to Med1 aa 1470–1570, GST-pulldowns were performed using *in vitro* translated PRIC295 fragments F1, F2, and F3 and found that F1 interacts strongly with Med1 but the other PRIC295 fragments revealed minimal binding (Figure 9(b)). Finally, GST-pulldowns also showed that PRIC295 interacts with Med24 (Figure 9(c)).

During the past 15 years, several proteins have been identified which interact with PPARs and other nuclear



(a)

Protein	Peptide sequences	m/z	Accession number
PRIC285	RDGVLDVEARRQGAAFYAPGRE	2148.303	Q9BYK8
	KVPEEVLRPGTLFTVELLPKQLPDLRK	2859.6342	
MED12L	RCILAYLYDLYVSCSHLRSKF	2375.1886	Q86YW9
	RYSFVCNTLMNVCMGHQDAGRI	2375.9988	
	RYVLRTICQQEWVGEHCLKEPERL	2858.4076	
RPAP1	RLARHSLESAMRVLECPRL	2041.043	Q3T1I9
	RAPSAEQVVPSPDAPEGAVPCETPSSKD	2607.2243	
MED24	KTMDADHSKSPEGLLGVLGHMLSGKS	2480.2272	Q99K74
	KRHREDIEDYVSLFPVEDMQPSKL	2690.2879	
RELA	RELSEPMEFQYLPDTDDRHRI	2378.0718	Q04206
	KICRVNRNSGSCLGGDEIFLLCDK V	2696.2906	
FKBP4	KVHALRLASHLNLAMCHLKL	2084.1368	P30416
	KVGEVCHITCKPEYAYGAAGSPPKI	2505.1901	
PRMT1	KVVLDVGSGTGILCMFAAKAGARKV	2321.2468	Q63009
	KRNDYVHALVAYFNIEFTRCHKR	2667.3249	
CEBPB	RLVAWDPACLPLPPPPPAFKS	2086.1194	P17676
	KSMEVANFYYEADCLAAAYGGKA	2330.0104	
MED20	RHDAVYGPADTMVQYMELFNKI	2329.0628	Q9H944
	KLEMLGAEKQGTFCVDCETYHTAASTLGSQGQTGKL	3705.6829	
ZNRD1	RQMRSADEGQTVFYTCINCKF	2319.412	Q6MFY5
	KLGTVIPMSVDEGPESQGPVVDRRCSRC	2855.567	

(b)

Figure 7: PRIC295-interacting proteins. (a) PRIC295-interacting proteins were isolated using GST-ΔPRIC295$^{840–1815}$ (F2) (pGEX-ΔPRIC295$^{840–1815}$) to pull down interacting proteins from rat liver nuclear extract. Selected bands (lane 2-BP; arrows) were excised from the 4–20% Tris-HCl gel and digested with trypsin to release peptide fragments. (b) Peptide fragments derived from excised bands were subjected to MALDI-TOF analysis. Proteins matching the identities with peptide fragments are shown with some matching peptide sequences, m/z ratios, and accession numbers. In (a), immunoblotting of ciprofibrate-binding protein complex reveals the presence of PPAR*α* (lane 3), Med24/TRAP100 (lane 4), and Med1 (lane 5).

receptors and serve as transcription cofactors/coregulators [18, 55, 74]. We have been using the MALDI-TOF approach to identify and characterize PPAR*α* or its ligand binding proteins referred to as PRIC complex [33, 34]. Using this mass spectrography-based technique, we now report the identification of a novel coactivator, PRIC295. PRIC295 revealed a strong, ligand-dependent interaction not only with PPAR*α*, but with several other nuclear receptor proteins. Of considerable interest is that this protein contains several LXXLL and HEAT repeat motifs that appear to be important in protein-protein interactions and in transcription [67]. This may indicate that PRIC295 has a role in mediating the transcription of target genes for several members of the nuclear receptor superfamily, though further investigation of this interaction and its role with those receptor proteins *in vivo* is warranted.

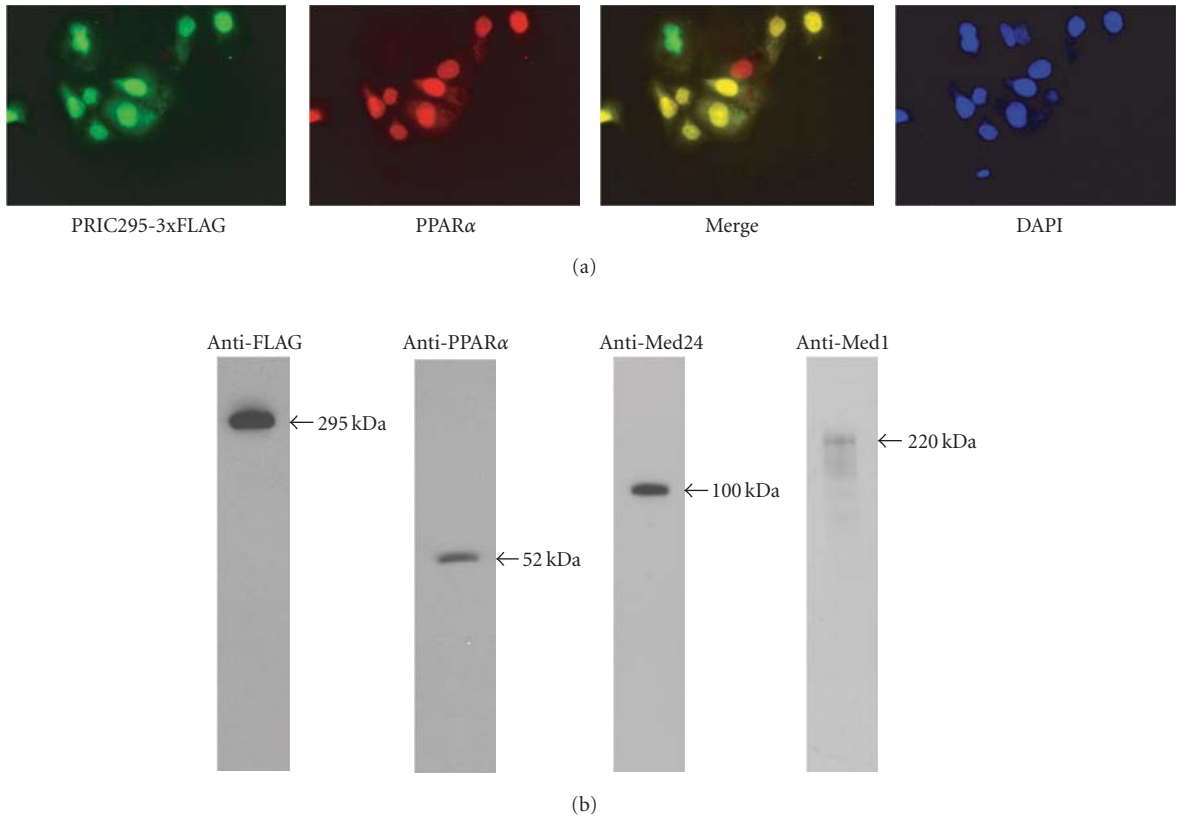


Figure 8: Intracellular localization of PRIC295. (a) Immunofluorescence microscopy to visualize intracellular localization of PRIC295-3xFLAG and PPARα in HeLa cancer cells after transfection with pcDNA-PRIC295-3xFLAG and pCMX-PPARα. Anti-FLAG M2 monoclonal antibody was used for PRIC295-3xFLAG (green color), and anti-PPARα antibody was used to stain PPARα (red color). DAPI nuclear stain is for the visualization of cells in the field. Merged image of PRIC295-3xFLAG (green) and PPARα (red) reveals yellow coloration of nuclei that coexpress both proteins. The presence of a green and red cell in the merged panel is an indication that these cells are not likely transfected with both plasmids. (b) Immunoprecipitation data showing purified PRIC295-3xFLAG protein and coimmunoprecipitated proteins PPARα, Med24/TRAP100, and Med1. Cells were transfected with pcDNA-PRIC295-3xFLAG only.

This study also utilized MALDI-TOF procedures to delineate PRIC295 binding protein complex (PRIC295COM). Analysis revealed PRIC295 binding proteins that included PRIC285, Med12L, Med20, and Med24 suggesting that PRIC295 plays a potentially important role in transcription. For example, it should be noted that PRIC285 contains a UvrD helicase motif and has been previously shown to enhance transcriptional activation mediated by PPARα [33]. The association of PRIC295 with Med12L, a putative homologue of Med12 subunit of Mediator complex, and with Med1, Med20, and Med24, which are members of the Mediator complex suggests that PRIC295 may be involved as a platform protein in the formation of the large transcription complex [33, 34, 75, 76]. While Med1 has been studied extensively (see [77]; Viswakarma et al., PPAR Research in press) less is known about Med24 and Med20 [77]. Med24 was initially identified as a cofactor important for mediating transcription by thyroid hormone and vitamin D receptors which interacts with and localizes together with Med1/TRAP220 [60]. Med24 may also have additional roles in gene regulation outside of its role as a member of the Mediator complex [78]. Med20 was first identified as a human homologue of the *Drosophila* TRF-proximal protein (hTRFP) and was shown to be able to enhance the transcriptional activity of RNA Pol II [72]. Med20, together with Mediator complex subunits Med8 and Med18 plays an important role in the proper folding and formation of one of the subunits of the Mediator complex [79]. PRMT1 is an arginine methyltransferase which is identified in the complex of PRIC295-interacting proteins. PRMT1 was identified by its interaction with the TIS21 and BTG1 proteins and has been shown to have important roles in RNA processing and transcription [70, 80]. PRMT1 potentiates the activity of PGC-1α, a known coactivator of PPARs [81]. The role of PRMT1 in RNA processing and transcription has been shown to be important part in mediating viral infection [82]. C/EBPβ is a transcription factor that is known to be involved in processes mediated by PPARs including adipogenesis and the induction of endoplasmic reticulum stress [71, 83, 84]. In this study, we have identified C/EBPβ in the complex of proteins interacting with PRIC295. Zinc ribbon domain-containing 1 (ZNRD1) was also among the complex of PRIC295-interacting proteins. ZNRD1 has a role in regulating ERCC1 and Bcl-2 which are important in

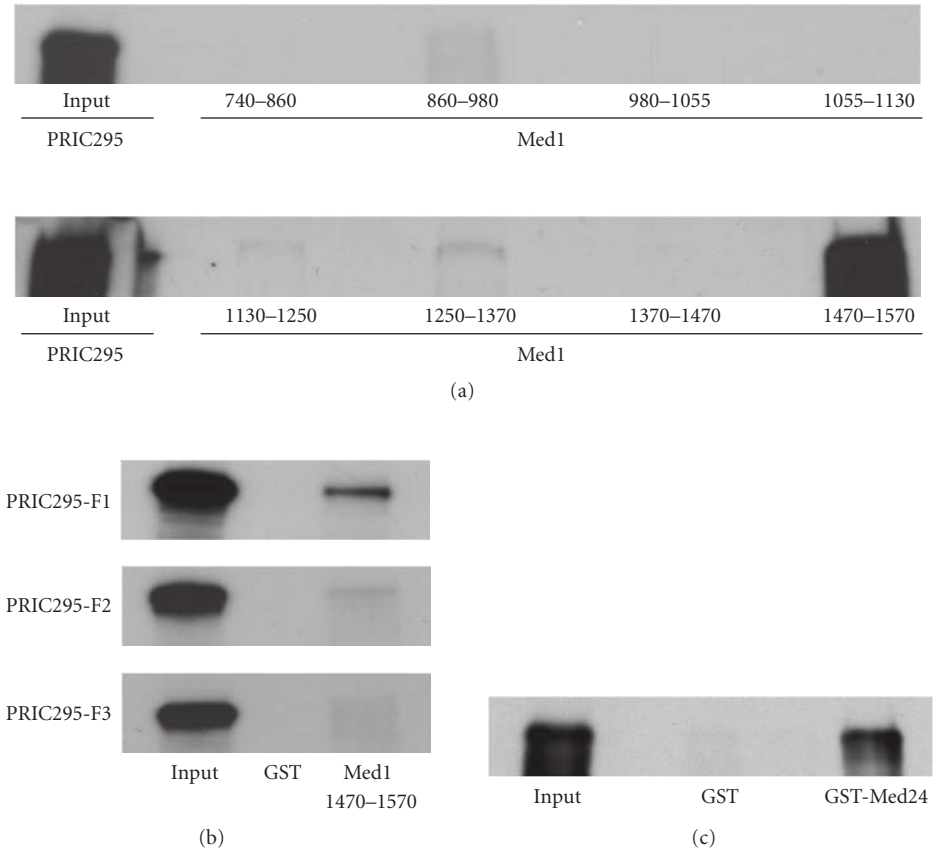


Figure 9: PRIC295 interacts with Med1. (a) *In vitro* translated and radiolabeled PRIC295 protein interacts with the known PPARα coactivator, Med1, as assessed by GST-pulldown approach using GST-fusion protein fragments of the PBP protein. Interaction between PRIC295 and GST-ΔPBP6$^{1470–1570}$ appears robust. (b) GST-pulldown of radiolabeled ΔPRIC295$^{1–915}$ (F1), ΔPRIC295$^{840–1815}$ (F2), and ΔPRIC295$^{1740–2671}$ (F3), respectively, using GST-ΔPBP$^{1470–1570}$ showing a strong binding interaction occurring between ΔPRIC295$^{1–915}$ (F1) and GST-ΔPBP$^{1470–1570}$.

cancer progression though the mechanism by which it does this is presently unclear [85]. ZNRD1 also appears to have an influence in the progression of HIV infection [86].

Evidence suggests that certain coactivators are essential for the effective transcriptional activation of PPARα target genes [57, 87]. Conditional deletion in the mouse liver of the coactivator MED1 results in impaired liver regeneration following partial hepatectomy [57]. Absence of Med1 results in the abrogation of PPARα ligand induced pleiotropic responses including hepatocarcinogenesis [57, 87]. Deficiency of Med1 also prevents acetaminophen-induced hepatotoxicity [87]. On the other hand, absence of SRC-1, SRC-2, and SRC-3 had no effect on PPARα signaling (see [88]; Viswakarma et al., PPAR Research in press). Med1 is essential for the interaction of the activated PPARα/RXRα heterodimer with RNA polymerase II and the basal transcription machinery of the cell [89]. In addition, germ-line deletion of Med1 results in embryonic lethality illustrating the important roles that coactivator proteins may be playing in other tissues [90–92]. Given this information, the interaction of PRIC295 with Med1, Med24, and some other members of the Mediator complex of proteins is of particular interest

4. Conclusions

In the recent past, many new coactivator proteins that are involved in the transcriptional regulation of the expression of PPARα target genes have been discovered and studied. These coactivators grant a way to regulate the transcriptional ability of PPARα and other nuclear receptors in a tissue and cell-specific manner. Our studies show that PRIC295 is a nuclear receptor coactivator which interacts with PPARα and several other nuclear receptors and may play a larger role in the transcription of target genes through nuclear receptors. The association of proteins such as Med1, Med24, and Med20, the members of Mediator complex with PRIC295 suggests the existence *in vivo* of a complex of proteins designated PRIC295COM.

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of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK006575. This work was supported by NIH Grants DK083163 (to J. K. Reddy) and DK054030.

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Review Article

PPARs in Rhythmic Metabolic Regulation and Implications in Health and Disease

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The circadian rhythm, controlled by a complex network of cellular transcription factors, orchestrates behavior and physiology in the vast majority of animals. The circadian system is comprised of a master clock located in central nervous system with 24-hour rotation and periphery clocks to ensure optimal timing of physiology in peripheral tissues. Circadian expression of peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily and key mediators of energy homeostasis and metabolism, is regulated by clock genes. PPARs serve as sensors of nutrient and energy/metabolism status to temporally entrain peripheral clock. Metabolism and circadian clocks are tightly intertwined: clock genes drive metabolism, and various metabolic parameters affect clock genes, producing a reciprocal feedback relationship. Due to PPARs' robust relationship with energy status and metabolism, the aberration of PPARs in the biological clock system leads to abnormal expression of genes in metabolic pathways, thus, contributing to etiology of metabolic syndrome. Studying PPARs' functions under the context of the mammalian circadian system could advance our understanding of how energy and metabolic status are maintained in the body, which may ultimately lead to rhythmic medical treatment against metabolic syndrome.

1. Introduction

1.1. The Mammalian Circadian Clock. The behavior and physiology of the vast majority living organisms oscillate in a 24-hour cycle in response to the light-dark cycle. It is estimated that 3%–20% of mammalian genes are under circadian regulation in a tissue-dependent manner [1]. Gene expression microarray analysis of mouse prefrontal cortex revealed that approximately 10% of transcripts demonstrate a diurnal expression rhythm [2]. Among the rhythmic genes identified, many have functions in biosynthetic and metabolic processes [1]. In addition to rhythmic transcriptional regulation of gene expression, various biological processes including hormone secretion, blood pressure, sleep-awake pattern, blood glucose and lipid level, body temperature, and metabolism exhibit circadian oscillation [3]. This endogenous response has clear implications in health and disease. For instance, the occurrence of certain cardiovascular diseases such as stroke, unstable angina, and myocardial infarction is markedly more frequent in specific parts of the day [4–6].

In mammals, the internal biological clocks are composed of central and peripheral components whose function is to coordinate biological processes to maintain synchrony with the environmental cycles of light and nutrients [7–9]. The central clock or master clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus [10]. The role of the central clock is to coordinate peripheral oscillators situated in various peripheral tissues such as the liver, the kidney, the heart, and muscles in such a way that normal circadian rhythm is maintained at the organismal level [10, 11].

Light is a major daily external photic resetting signal of the circadian system in mammals. Through the retinohypothalamic tract, light resets the master clock in the SCN triggering neural and humoral signaling that subsequently synchronizes the peripheral clock [8]. Although the pathways to synchronize an organism at the systemic level remain poorly understood, a model to explain this event at the cellular level has been established. Transcription factors, activators, and repressors are now emerging as clock components. They form feedback regulatory loops resulting

in rhythmic expression of each component and lead to cascades of gene expression with 24-hour periodicity. The key players are two transcription factors: brain and muscle Arnt-like protein 1(Bmal1) and CLOCK/NPAS2 [7], which form heterodimers to constitute the central loop (Figure 1, (2)). CLOCK has intrinsic acetyl acetyltransferase transferase activity [8] and acetylates its heterodimer partner Bmal1 [8]. The acetylated Bmal1/CLOCK heterodimer then binds to the E-box (5′-CACGTG-3′) enhancer elements of Period genes (Per1, Per2, and Per3) and Cryptochrome gene (Cry1 and Cry2) promoters (Figure 1, (3)) [8, 12, 13] to activate transcription of Per and Cry [14]. The products of these genes interact to form repressor complexes which translocate into the nucleus and inhibit Bmal1-CLOCK/NPAS2 activity, thus resulting in repression of their own transcription [7]. To sustain the negative feedback loop, multiple components are subjected to posttranslational regulations. For example, Per and Cry proteins are regulated by ubiquitin-proteasome pathways [15], and CLOCK acetyltransferase activity can be reversed by SIRT1 deacetylase (Figure 1, (1)) [8].

In addition to central transcription factors regulating the central loop, another feedback loop line tunes the activity of the core clock components (Figure 1) [16]. Bmal1 expression is controlled by orphan nuclear receptors: retinoid-related orphan receptor alpha (RORα) and Rev-erbα (NR1D1) [7, 8]. RORα and Rev-erbα are closely related and recognize similar response elements (RORE) [7]. As a result, they compete for common RORE in the Bmal1 promoter and trigger opposing responses [7]. RORα interacts with peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) resulting in histone acetylation and subsequent Bmal1 transcription activation (Figure 1, (8)) whereas Rev-erbα interacts with corepressors leading to histone deacetylation and Bmal1 transcription inhibition (Figure 1, (12)) [14]. This RORα/Rev-erbα interplay is believed to be responsible for Bmal1 rhythmic expression. Reciprocally, Bmal1 and CLOCK regulate Rev-erbα by binding to the E-box in Rev-erbα promoter (Figure 1, (4)) [7]. Moreover, the promoter of Rev-erbα also contains RORE rendering it to be negatively controlled by itself and positively controlled by RORα (Figure 1, (5) and (6)) [7].

A recent study by Schmutz et al. has identified a novel role of Per2 in the regulation of circadian rhythm; that is acting as cofactors for two nuclear receptors. Per2 was shown to interact with Rev-erbα at Bmal1 promoter to repress Bmal1 expression in liver in a Rev-erbα-dependent manner [17]. In the liver of *Rev-erb*$\alpha^{-/-}$*/Per2* mutant mice, Bmal1 rhythmic mRNA expression was completely abolished [17]. In a different phase, Per2 can be brought to the PPRE region of Bmal1 promoter by PPARα and functions as a coactivator to enhance Bmal1 expression. Taken together, Per2 appears to be a modulator of Bmal1 expression by two circadian mechanisms: rhythmic repression mediated by Rev-Erbα and rhythmic activation mediated by PPARα.

Attempts have been made to identify novel regulators of the biological clocks. A recent study employing genome-wide RNAi screening has identified ~1000 genes of which knockdown resulted in amplitude reduction, and hundreds of genes of which knockdown altered oscillation period length or increased amplitude [18]. Pathway analysis of newly identified genes has shown that many of these genes participate in insulin signaling, hedgehog signaling, cell cycle regulation, and folate metabolism [18], exemplifying the involvement of biological clocks in the regulation of cellular metabolism, growth, and development. Several reviews have discussed the molecular mechanisms of biological clocks in great details [3, 8, 13], thus are referred to for further reading.

1.2. PPARs Overview. Peroxisome proliferator-activated receptors (PPARs) are a subfamily of the nuclear receptor superfamily of transcriptional factors [19]. The PPAR subfamily constitutes three members: PPARα, PPARβ/δ and PPARγ [19–21]. Despite a high degree of homology observed among PPARs, each isoform possesses distinct biological activities [20] and is expressed in different tissues [19, 21, 22]. PPARα is mainly expressed in the liver, the kidney, and the heart, and is primarily involved in lipid metabolism [22–24]. PPARγ is a master regulator of adipogenesis and fat storage, which regulates adipocyte differentiation and insulin sensitivity in adipose tissue [22–24]. PPARβ/δ is found in a broad range of tissues with relatively high expression in brain, adipose tissue, and skin, but its function awaits further exploration [22–24]. Taken together, PPARs are key mediators of energy homeostasis, and lipid and glucose metabolism although they have also been associated with other biological processes including development, differentiation, inflammation, atherosclerosis, wound healing, and tumor formation [19, 21]. To date, PPARα and PPARγ are reported to possess significant clinical value. PPAR isoform-specific agonists, specifically fibrates for PPARα and thiazolidinedione for PPARγ, are currently prescribed as lipid and glucose-lowering drugs, respectively [23]. Interestingly, recent data have shown that expression of all three forms of PPARs displays circadian rhythm [22]. Hence, the interdependence of circadian and metabolic systems jointly regulated by PPARs appears to be disease relevant and thus, is the focus of this paper.

2. Reciprocal Regulation: Interplay between Central Circadian System and PPARs in Peripheral Tissues

PPARs act as molecular links between clock genes and specific rhythmic metabolic outputs. Emerging evidence has shown that, in peripheral tissues, PPARs and the core clock genes cross-regulate each other at transcription level. On one hand, the circadian expression of PPARα is regulated by core clock genes and clock-controlled genes (Figure 1, (10)) [25–28]. On the other hand, it was shown that PPARs could directly affect circadian transcription of clock genes (Figure 1, (9)) [26, 29, 30]. The expanding regulatory network between PPARs and CLOCK proteins opens new perspectives for understanding the interdependence of PPARs and the core clock in peripheral tissues.

2.1. The Master Clock Gene Products Regulate Transcription of PPARs. PPARs' circadian expression is controlled by the

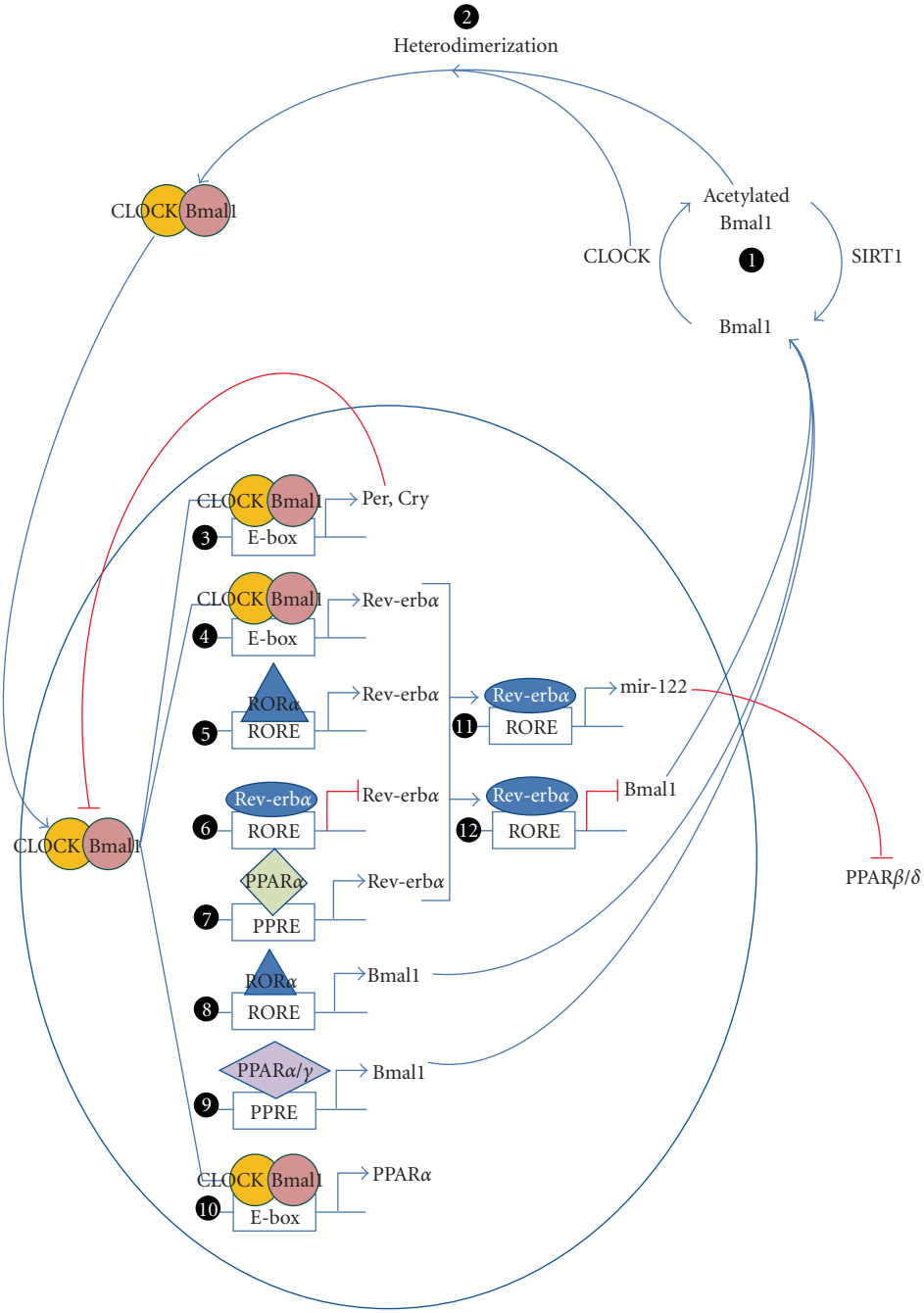

Figure 1: Regulatory networks of the core clock components and PPARs. (1) Bmal1 is acetylated by CLOCK which possesses acetyltransferase activity. This process can be reversed by SIRT1 deacetylase activity. Acetylated Bmal1 and CLOCK proteins heterodimerize (2) translocate into nucleus and activate transcription of Per, Cry (3), and Rev-erbα (4). In turn, Per and Cry form a repression complex which, upon translocation from the cytoplasm into the nucleus, inhibits transcription driven by CLOCK/Bmal1, including its own, constituting the main feedback loop. Bmal1 expression is controlled by Rev-erbα and RORα, the two primary players in the secondary loop, in an opposing manner. Upon binding to a common RORE, Rev-erbα suppresses transcription (12) while RORα exerts transcriptional activation of Bmal1 (8). The expression of Rev-erbα is driven by CLOCK/Bmal1 (4) and RORα (5) and suppressed by itself (6). PPARs and the core clock proteins reciprocally regulate each other. PPARs regulate the transcription of some clock genes, for example, PPARα activates Rev-erbα (7) and Bmal1 (9) while PPARγ only activates Bmal1 transcription (9). On the other hand, clock genes regulate expression levels of PPARs. CLOCK/Bmal1 drives PPARα expression (10) and Rev-erbα activates transcription of mir-122 (11), a microRNA which downregulates expression of PPARβ/δ posttranscriptionally.

core clock gene products in peripheral tissues. CLOCK, one of the key components in the circadian regulatory network, directly controls the circadian expression of PPARα. In wild-type mice, PPARα expression displays diurnal variation in the liver [25, 31–34]. However, this rhythmic expression of PPARα is abolished in the liver of CLOCK-mutant mice [25]. Furthermore, in contrast to the fibroblasts obtained from normal mice, fibroblasts from CLOCK-mutant mice exhibited decreased expression level and oscillation amplitude of PPARα in response to oscillation inducer Endothelin-1 (ET-1) [35]. These findings indicate that CLOCK controls the circadian expression of PPARα at the peripheral oscillator level. Sequence analysis revealed an E-box-rich region in exon 2 of the mouse PPARα gene [25]. Chromatin immunoprecipitation (ChIP) of CLOCK in NIH3T3 cells indicates that CLOCK interacts with this E-box-rich region [25], suggesting that CLOCK might activate PPARα transcription by interacting with E-Box in the exon 2 of PPARα. Using a reporter construct constituting of E-box-rich region of PPARα fused to luciferase, CLOCK and Bmal1 were shown to increase transcription by more than 25 folds [25]. E-Boxes are also present in human PPARα gene (Figure 1, (10)) [25]. Moreover, Bmal1, another component of biological clock, was also shown to affect PPARα circadian variation [26]. Furthermore, PPARα mRNA expression was severely downregulated in the liver of *Bmal1*$^{-/-}$ mice. Collectively, these results suggest that PPARα is a direct target gene of core clock proteins CLOCK/Bmal1.

In addition to directly controlling the level of PPARs, CLOCK/Bmal1 can potentiate PPARs-mediated transcription activation. CLOCK/Bmal1 heterodimers have been shown to increase the transcriptional activity of genes whose promoter contains PPAR response elements (PPREs) [27]. Increased transcriptional activity was further enhanced by treatment with PPAR agonists (i.e., fenofibrate for PPARα and troglitazone for PPARγ) [27]. When PPRE was removed from the promoter, CLOCK/Bmal1 exhibited no effects on transcription [27], suggesting that the potentiation of transcription by CLOCK/Bmal1 is dependent on PPAR. However, the mechanism of CLOCK/Bmal1 and PPARs' synergistic regulation of gene expression remains to be explored.

A recent paper provides a novel link between PPARβ/δ and Rev-erbα, an orphan nuclear receptor and central regulator of the clock gene. Rev-erbα was shown to maintain the circadian expression of mir-122, a microRNA abundantly found in hepatocytes, possibly via two ROREs in its promoter (Figure 1) [28]. mir-122 subsequently downregulates target genes by binding to a complementary sequence located in 3′ untranslated region (3′ UTR) [28]. Using reporter assays, mir-122 was shown to down-regulate PPARβ/δ [28]. Further investigation is needed to firmly establish the miRNA mediated regulation of PPARβ/δ by Rev-erbα.

2.2. PPARs Regulate the Peripheral Clock: A Role of Food Entrainment. PPARs are not only metabolic sensors but also circadian clock regulators. In the SCN of the PPARα-null mice, clock genes (Bmal1, Per2, Per3, Cry2, and Rev-erbα) exhibited normal diurnal variation [26]. Moreover, bezafibrate, a PPARα agonist, did not affect circadian expression of Per2, a core clock gene, in the SCN [29]. These findings indicate that PPARα is not essential for maintaining the normal central clock oscillation. The peripheral clock, on the other hand, was affected by PPARα expression. Changes in Bmal1 and Per3 expression was observed in the livers of PPARα-null mice. Although the overall oscillation phase remained unperturbed, the amplitude of Bmal1 was decreased while Per3 amplitude was increased significantly [26]. Circadian rhythm in peripheral tissues such as liver can be reprogrammed, also termed entrainment, by feeding cycle alteration [36, 37]. Like other nocturnal animals, mice normally feed at night. Phases of the mouse liver PPARα circadian expression could be inversed by daytime feeding [26]. In wild-type mice, clock genes (Bmal1, Per1, Per3, and Rev-erbα) showed inversed phase in response to daytime feeding corresponding to altered expression of PPARα [26]. However, in PPARα-null mice, although other clock genes can still be reset, Bmal1 was irresponsive to daytime feeding triggered by circadian reprogramming, suggesting that Bmal1 expression is controlled by PPARα [26]. Furthermore, PPARα-agonist fenofibrate could reset rhythmic expression and increase transcription of Bmal1, Per1, Per3, and Rev-erbα in livers of wild-type mice but failed to induce Bmal1 and Rev-erbα in livers of PPARα-null mice, indicating that upregulation of Bmal1 and Rev-erbα by fenofibrate was mediated through PPARα [26]. Another PPARα agonist, bezafibrate, was shown to stimulate phase advancement of Per2 circadian expression in peripheral tissues [29]. ChIP analysis reveals that PPARα directly binds to PPREs located at 1519 and 45 base pairs upstream of the transcription initiation site in Bmal1 and Rev-erbα promoters, respectively, [26]. Therefore, PPARα could directly regulate transcription of Bmal1 and Rev-erbα via binding to PPREs in their respective promoter regions (Figure 1, (7)). Moreover, Per2 was shown to further enhance PPARα-mediated activation of reporter gene fused to PPRE in a dose-dependent manner, suggesting that Per2 might function as a coactivator of PPARα [17]. However, further investigations are necessary to validate this novel relationship.

Similar to its family members, PPARγ can also modulate expression of biological clock components. PPARγ rhythmic expression has been shown to precede that of Bmal1 in mice blood vessels [30]. Knocking down of PPARγ abolishes rhythmicity of Bmal1, Cry1, Cry2, and Per2 in mice aorta [30]. The PPARγ-agonist rosiglitazone induces Bmal1 expression (Figure 1, (9)) [30]. ChIP analysis reveals that PPARγ also interacts with PPRE in Bmal1 promoter [30]. In reporter assays employing RORE-containing Bmal1 promoter segment, rosiglitazone treatment resulted in an increase in promoter activity, which was abolished when PPRE is mutated [30]. Taken together, PPARγ appears to be a major regulator of Bmal1 expression in blood vessels.

In summary, the central circadian clock components and PPARs exhibit a reciprocal regulation. Circadian clock proteins control PPARs expression by at least two mechanisms: (1) activation of PPARα transcription by CLOCK/Bmal1 heterodimer upon binding to the E-box in the PPARα

promoter and (2) downregulation of PPARβ/δ by microRNA stimulated by Rev-erbα. On the other hand, PPARs regulate the expression of clock genes via two different mechanisms as well: (1) Rev-erbα is positively regulated by PPARα; (2) Bmal1 is positively regulated by both PPARα and PPARγ. The multivariable regulatory loops of the core clock proteins and PPARs are diagrammatically summarized in Figure 1.

2.3. PPAR Cofactors in Circadian Rhythm

2.3.1. NcoR1 and Hdac3. In the absence of ligand, PPARs form a multicomponent complex with corepressors, such as nuclear receptor corepressor 1 (NcoR1) [38]. Histone deacetylase 3 (Hdac3), a histone modification enzyme, is recruited and stably bound to the repressor complex through a conserved deacetylase activation domain (DAD) in NcoR1 [39]. Hdac3 remodels chromatin in a way less favorable for access by basal transcriptional machinery, leading to the repression of PPAR-mediated transcription activation [38]. NcoR1 is directly involved in the biological clock system as a cofactor for Rev-erbα. Upon binding to RORE in the Bmal1 promoter, Rev-erbα recruits NcoR1/Hdac3 to suppress expression of Bmal1 [40]. NcoR1 and Hdac3 are critical for the regulation of clock genes and energy metabolism homeostasis. A mouse line was generated to harbor a mutation in NcoR1 DAD domain that abolished the ability of NcoR1 to interact with or activate Hdac3 [39]. In NcoR1 mutant knock-in mice, uncoupling of NcoR1 from binding to Hdac3 led to altered circadian rhythmicity [39]. Bmal1 expression level was higher in the mutant mice than that of wild-type [39]. This is consistent with the established corepressor function of NcoR1/Hdac3 for Rev-erbα which downregulates Bmal1. Circadian expression abnormality of Bmal1 and Rev-erbα was also observed in these mutant mice [39]. Moreover, the mutant mice exhibited altered expression patterns of liver genes involved in lipid metabolism. Phase shifts were observed with genes harboring fat catabolism function such as carnitine palmitotransferase 1a (Cpt1a), median chain aryl-CoA dehydrogenase (MCAD), and their regulator PPARα [39]. ATP citrate lyase (Acly) and acetyl CoA carboxylase2 (Acc2) displayed phased reversion [39]. Expression level of elongation of long-chain fatty acids family member 6 (Elovl6) was decreased by fourfold in mutant mice as compared with wild-type mice [39], which might explain the increased leanness and insulin sensitivity. The mutant mice demonstrated distinct, seemingly desirable phenotype including increased leanness, decreased body weight, reduced body fat, increased O_2 consumption, increased body heat generation, and improved insulin sensitivity [39]. Thus, uncoupling NcoR1/Hdac3 interaction could be beneficial for metabolic syndrome management.

2.3.2. PPARγ Coactivator 1α (PGC-1α). It is believed that ligand binding to PPAR triggers a conformational change which allows the dissociation of corepressors and recruitment of coactivators that many of which are known to be histone acetyltransferases (HATs) [41], resulting in transcription activation of target genes. PPARγ coactivator-1α (PGC-1α), as the name implies, is a known coactivator of PPARs [20]. In addition to PPARs, PGC-1α is capable of activating other nuclear receptors such as thyroid receptor b, estrogen receptor and glucocorticoid receptor TRβ, ER, and GR [42].

PGC-1α is well characterized to be a part of the biological clock. PCG-1α positively regulates the expression of clock components, including CLOCK, Bmal1, and Rev-erbα [43]. PGC-1α can physically interact with RORα and RORγ through the LXXLL motif and enhance RORα transcription activity [43]. When PGC-1α is recruited to the ROR-bound promoter, it could recruit p300 and GCN5 histone acetyltransferase to modify local chromatin structure to be permissive to transcriptional machinery [43]. On the Bmal1 promoter, PGC-1α binding is accompanied by an increase in histone H3 acetylation and histone 3 lysine 4 trimethylation (H3K4me3), two markers of transcriptional activation, while histone 3 lysine 9 dimethylation (H3K9me2), a marker that signifies transcription silencing, decreased [43]. Taken together, PGC-1α is a coactivator of RORα which converts chromatin from a quiescent to transcriptionally permeable state thus enhancing transcription. Disruption of PGC-1α leads to alteration in locomotor behavior, O_2 consumption, and expression pattern of metabolic genes and clock genes circuitry [43]. Thus, PGC-1α serves as a potential factor which couples circadian rhythm to energy status.

3. Medical Implications: Metabolic Syndrome and Other Diseases Potentially Linked to the Aberrant PPAR Circadian Rhythm

3.1. Circadian Oscillation of PPARs Displays a Strong Association with the Energy and Metabolism Homeostasis. PPARs play essential functions in energy homeostasis. Circadian oscillation of PPARs is essential for the temporal coordination of genes involved in energy and metabolic processes. PPARα exerts a strong impact on lipid metabolism. Disruption of PPARα resulted in alteration of the circadian expression of these metabolism-related genes [44]. Interestingly, the expression patterns of the lipolytic genes appear to oscillate in-phase with those of PPARα, while those of the lipogenic genes oppose the expression pattern of PPARα. In mouse, the mRNA level of cytosolic acyl-CoA thioesterase (CTE-I), an enzyme catalyzing lipid hydrolysis, exhibits diurnal rhythm parallel to the circadian PPARα expression. The expression of CTE-I can be induced by fasting; that is the expression is increased during the light phase and declined during the dark phase when feeding activity is abundant [44]. The fasting-induced CTE-I mRNA level is lower in PPARα-null mice than that in the normal mice [44], suggesting that CTE-I diurnal rhythm is regulated through PPARα. In wild-type but not in PPARα-mutant mice, the administration of the PPARα-agonist bezafibrate could induce circadian expression of fibroblast growth factor 21 (FGF21) [34], a hormone involved in lipolysis and hepatic ketogenesis [45], suggesting that bezafibrate-induced circadian effect is strictly PPARα-dependent.

On the contrary, the expression of enzymes involved in lipid synthesis such as fatty acid synthase (FAS) and

acetyl Co-A decarboxylase (ACC) in the fatty acid synthesis pathway and 3-hydroxyl-3-methylglutaryl-CoA reductase (HMG-CoAR) in cholesterol synthesis pathway oppose the expression patterns of PPARα. These enzymes exhibited increased expression during dark phase in mice [32]. Again, the circadian expression of these enzymes is abrogated in PPARα-null mice [32], indicating that the diurnal variation of these enzymes requires PPARα.

There is evidence suggesting that the feeding behavior is mediated, in part, by PPARα. Oleoylethanolamide (OEA), a naturally-produced lipid compound found in mammals including humans, is a satiety stimulator [46]. Later, OEA was shown to be a PPARα agonist and displayed circadian expression [47]. Administration of OEA analogues can suppress feeding and decrease weight gain in wild-type mice [47]. However, this response was not observed in PPARα-null mice [47], indicating that OEA regulates satiety through activation of PPARα. Nonetheless, the detailed mechanism by which OEA regulates circadian oscillation remains to be elucidated.

PPARβ/δ has been linked to regulation of body temperature and lipid profile. PPARβ/δ activates genes involved in fatty acid oxidation, resulting in increased lipolysis in adipocyte and skeletal muscle cells [48]. Expression of PPARβ/δ triggers lipolysis in brown adipose tissue, a body compartment which is central to adaptive thermogenesis, during which processed energy is dissipated as heat via uncoupling proteins (UCPs) [48]. PPARβ/δ expression cycle has been shown to oscillate in-phase with that of uncoupling protein 1(UCP1) [22], suggesting that PPARβ/δ could promote energy dissipation. However, this relationship needs to be validated experimentally.

In conclusion, PPARs serve as sensors which integrate energy and metabolic homeostasis to circadian clock. Therefore, the aberration of clock genes could result in altered expression of metabolic genes, leading to disturbance of energy status in affected organisms. This imbalance is known to attribute to metabolic syndrome, a complex disease with distinct hallmarks including obesity, dyslipidemia, hypertension, and elevated plasma glucose level [49]. Further experiments investigating this relationship could deepen our understanding of pathogenesis, which may pave ways for new strategies to fight against metabolic syndrome.

3.2. Effect of Gender on the Biological Clock and PPARs Expression. Clinically, patients with comparable health are usually administered the same treatment regimen, regardless of gender. However, with the emerging idea of individualized medication, gender might need to be taken into account. Experiments in mice revealed distinct expression patterns of clock genes in the liver and hepatic lipid homeostasis differences between males and females [50]. The majority of hepatic clock genes including CLOCK, Bmal1, Per1, Per2, Cry1, and Rev-erbα reached mRNA peaks 30 minutes earlier in female as compared to male mice, while Cry2 and Per3 peaked earlier in male than female mice [50]. Statistically calculated mean expression levels of Per1, Per3, and RORα were also higher in female mice [50] as well as the amplitude of Cry1 and Per3 [50]. Similarly, PPARs and their coactivators showed differential expression between the two genders. PPARα, PPARβ/δ, and PGC-1β expression peaked at least 30 minutes earlier in female mice [50]. Statistically calculated mean expression levels of PPARα, PGC-1α and PGC-1β were higher in female mice than the male counterparts [50]. Moreover, the expression pattern of genes involved in lipid metabolism, triglyceride and cholesterol profiles in liver and serum of male and female mice fluctuated differently throughout the day [50]. Taken together, these findings implicate gender associated disparity of clock gene rhythmicity, expression patterns of genes involved in energy homeostasis, and serum hepatic lipid profile. Since these differences could potentially affect the efficacy of drugs that target circadian clock and lipid metabolism, several issues are worth noting. Should drug administration be tailored specifically for males and females based on these differences? Can this intervention increase efficacy or reduce untoward effects of mediation? Further studies would be necessary to provide concrete solutions.

3.3. Other Circadian Physiology with PPARs Involvement: Stress, Sleep-Awake Cycle, and Blood Pressure. Circadian clocks essentially regulate rhythmic cellular and physiological processes, providing a platform for communications among different physiological processes. It is worthy of noting that PPARs are among a number of nuclear receptors that mediate the connections between circadian clocks and physiological processes. The functions of nuclear receptors in regulating circadian clock and physiology have been extensively discussed in several recent papers [51, 52] thus this paper will focus on PPARs. Besides their major roles in regulating lipid, glucose, and energy metabolisms, PPARs are also involved in regulating behavior rhythm and other physiological rhythms such as body temperature, blood pressure, and sleep phase.

As the endocrine system is also involved in clock entrainment by metabolic cues, there is functional crosstalk between PPARs and endocrine system. Aside from the nutrient status, PPARα level is regulated by oscillation of steroid hormones. In mice, PPARα gene expression is positively controlled by glucocorticoids [31, 53, 54], stress hormones whose secretion displays diurnal rhythm [31]. Mice PPARα mRNA and protein levels were also shown to oscillate in-phase with plasma corticosterone [31], suggesting that PPARα might play a role in the stress response.

PPARα has been implicated in regulating behavior rhythm such as sleep-wake cycle. Locomotor activity of mice with delayed sleep phase syndrome (DSPS), a disease described by persistent delayed sleep onset, can be restored by bezafibrate administration, implicating that bezafibrate may be a putative drug for DSPS treatment [29].

PPARs are also sensors of environmental cues to orchestrate distinct physiological rhythms such as body temperature, heart rate, and blood pressure. Mice with PPARγ deletion exhibits abnormal blood pressure and heart rate circadian rhythm, in accordance with decreased diurnal variation in sympathetic activity [30]. Alteration in circadian expression pattern of vascular Bmal1 has been observed in

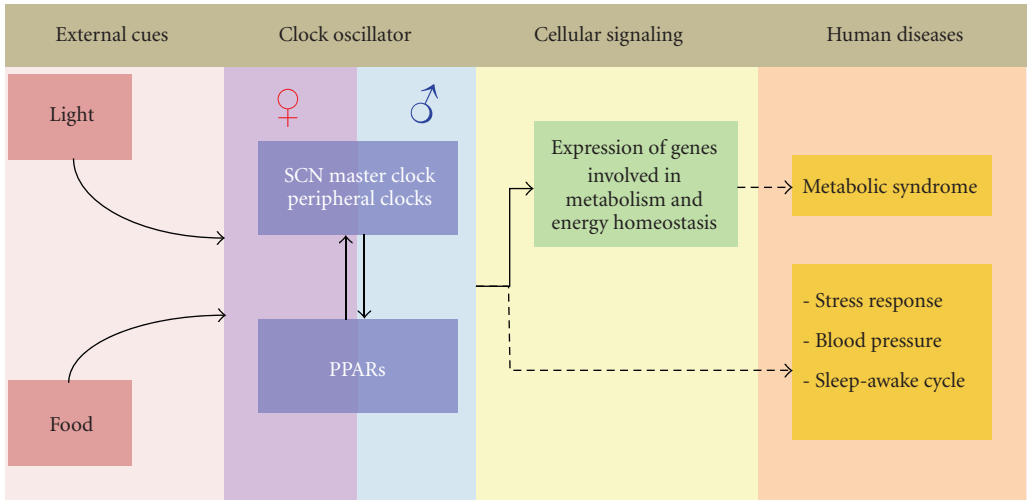

Figure 2: Overview of the functional link between the biological clocks and PPARs in health and disease. The external cues such as food and light can program SCN and peripheral clocks, leading to corresponding orchestrated expression of genes involved in metabolism and energy homeostasis. The gene products of clock components and PPARs reciprocally regulate each other, while both exhibit gender difference. Aberration in the biological clock-PPARs network is causative of metabolic syndrome and may also be responsible for human disorders including stress response, blood pressure, and sleep-awake cycle.

these mice [30], implicating the involvement of Bmal1 in cardiovascular physiology.

In summary, the biological clock is a self-sustained regulatory system which, under normal circumstance, allows appropriate acclimation of body physiology such as metabolic rate, blood pressure, and alertness to the immediate environment. In order to maintain homeostasis, environmental factors, by large light and food, trigger signal transduction to synchronize the circadian clock resulting in appropriate expression of downstream genes. Our paper exemplifies the bidirectional regulatory loop between PPARs and circadian clock. Together, PPARs and the biological clock play roles in maintenance of the expression of metabolic genes in response to the surrounding environment. Due to its strong association to energy status, deregulation of the PPARs-circadian clock system is believed to contribute, at least in part, to the development of metabolic syndrome. Furthermore, emerging evidence also suggests that disturbance in the PPAR-circadian clock system could affect various aspects of physiology including stress response, blood pressure, and sleep-awake cycle (Figure 2). Thus the disease link between PPARs and the circadian clock has become an exploratory area of PPARs research.

4. Conclusion

Circadian rhythm is essential for coordination of physiology and behavior in living organisms to respond to the immediate environment in a timely manner. Specialized proteins including CLOCK, Bmal1, Per, Cry, RORα, and Rev-erbα are responsible for the assembly of a complex regulatory system that possesses self-sustained circadian oscillation capacity in the circuit. PPARs in peripheral tissues exhibit a strong interplay with the central circadian clock components, serving both as affecters and effecters of the clock system. We diagrammatically summarize the biological clock transcriptional regulatory networks (Figure 1) and the functional roles of PPARs (Figure 2) in the context of circadian regulation. Since the major function of PPARs is to regulate metabolism and energy homeostasis, it seems plausible that PPARs are critical players to coordinate energy status of an organism to the central biological clock. Thus, dissecting the functional roles of PPARs in circadian rhythm could potentially advance our understanding on mechanisms of disorders in energy homeostasis and metabolism.

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Review Article

PPARs: Nuclear Receptors Controlled by, and Controlling, Nutrient Handling through Nuclear and Cytosolic Signaling

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Peroxisome proliferator-activated receptors (PPARs), which are known to regulate lipid homeostasis, are tightly controlled by nutrient availability, and they control nutrient handling. In this paper, we focus on how nutrients control the expression and action of PPARs and how cellular signaling events regulate the action of PPARs in metabolically active tissues (e.g., liver, skeletal muscle, heart, and white adipose tissue). We address the structure and function of the PPARs, and their interaction with other nuclear receptors, including PPAR cross-talk. We further discuss the roles played by different kinase pathways, including the extracellular signal-regulated kinases/mitogen-activated protein kinase (ERK MAPK), AMP-activated protein kinase (AMPK), Akt/protein kinase B (Akt/PKB), and the NAD+-regulated protein deacetylase SIRT1, serving to control the activity of the PPARs themselves as well as that of a key nutrient-related PPAR coactivator, PPARγ coactivator-1α (PGC-1α). We also highlight how currently applied nutrigenomic strategies will increase our understanding on how nutrients regulate metabolic homeostasis through PPAR signaling.

1. Introduction

1.1. PPARs: Nuclear Receptors Functioning as Metabolic Sensors. Energy homeostasis is mostly achieved by hormonal and nutrient-mediated control of the expression of genes encoding metabolic enzymes. Nuclear receptors are responsible for the transcriptional regulation of the vast majority of the aforementioned genes. These receptors are transcription factors that respond to small lipophilic hormones, vitamins, and metabolites. Among the nuclear receptors, the glucocorticoid, thyroid hormone, and estrogen receptors (GR, TR, and ER, resp.) are important regulators of genes involved in metabolic fuel homeostasis both during development and in response to metabolic stress, as well as in the regulation of cellular energy metabolism. Estrogen-related receptors (ERRs) also play critical roles in the regulation of cellular energy metabolism. Other nuclear receptors include the peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), the farnesoid X receptor (FXR), retinoid X receptor (RXR), and hepatocyte nuclear factor-4α (HNF-4α), all of which are activated by molecules of metabolic pathways, such as lipids and fatty acids (FAs), and thereby function as metabolic sensors. The PPARs take part in the genetic regulation of the complex pathways involved in mammalian metabolism, including fatty acid oxidation and lipogenesis that occur in response to nutritional and physiological stimuli. Taken together, PPARα or NR1C, PPARδ (also known as PPARβ) or NR1C2, and PPARγ or NR1C3 constitute group C in subfamily 1 of the superfamily of nuclear receptors [1].

1.2. PPARs: Structural Features and Interaction with Cofactors. Although the PPARs share high degrees of functional and structural similarities, they are encoded by distinct single-copy genes located on different chromosomes. Human PPARα is located on chromosome 22 [2, 3], PPARδ on chromosome 6 [4], and PPARγ on chromosome 3 [5].

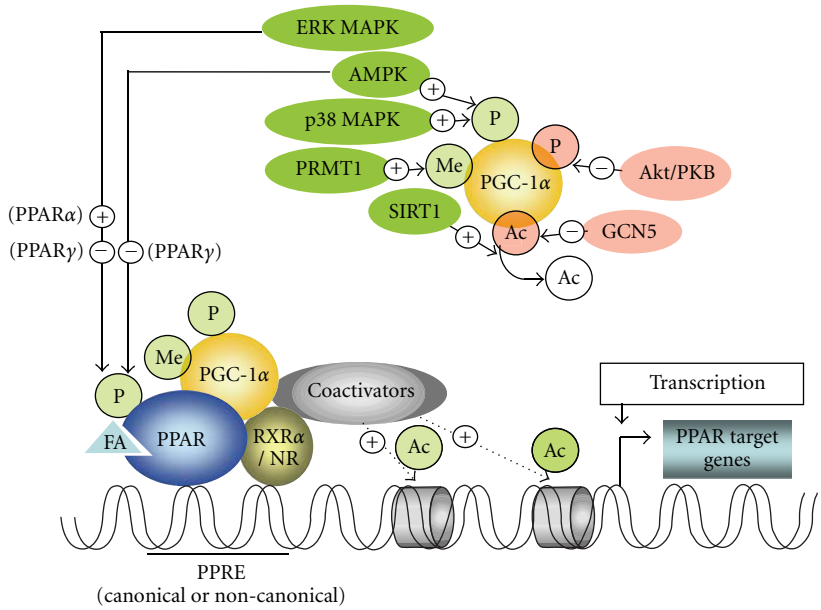

Figure 1: Modulation of the actions of PPAR through phosphorylation by ERK MAPK or AMPK or through regulation of PGC-1α activity by various signaling events. Phosphorylation of the PPAR receptors can either increase or decrease their activity. SIRT1-mediated deacetylation activates PGC-1α, while acetylation by GCN5 inhibits PGC-1α-directed gene expression. Phosphorylation by AMPK or p38 MAPK increases the stabilization of PGC-1α, whereas Akt/PKB-mediated phosphorylation facilitates its degradation. PRMT1 activates PGC-1α through methylation at several arginine residues. Activation of PGC-1α that is recruited to ligand-bound PPAR, the latter being complexed with RXR and/or other nuclear receptors, allows the recruitment of coactivators that acetylate the chromatin, allowing the DNA encoding a particular PPAR target gene to be transcribed. Ac, acetyl group; ERK MAPK, extracellular signal-regulated kinases/mitogen-activated protein kinase; AMPK, AMP-dependent protein kinase; Akt/PKB, Akt/protein kinase B; p38 MAPK, p38 mitogen-activated protein kinase; FA, fatty acid or metabolite from nutrients binding to and activating PPAR; Me, methyl group; P, phosphate group; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; PRMT1, protein arginine methyltransferase 1; RXR, retinoid X receptor; NR, nuclear receptor; SIRT1, sirtuin 2 ortholog 1; +, activation; −, inhibition.

The canonical PPAR response element (PPRE) is a DR1 motif (a direct repeat of the sequence separated by a single nucleotide, preferentially, and adenine [6]) formed by a 5′- and 3′-AGGTCA half-site [7]. This motif typically is present in the promoters of PPAR target genes, including those involved not only in nutrient handling but also in inflammation, cell growth, and differentiation [8, 9]. The formation of complexes between PPARs and other factors is required for the full transcriptional induction of PPAR targets in a variety of tissues. PPARs are predominantly located within the nucleus, where they generally heterodimerize with RXR. In order to achieve their nuclear activity, the PPARs, like all nuclear receptors, have distinct functional domains: an N-terminal domain involved in transcriptional activation, a DNA-binding domain consisting of two zinc-finger motifs, the second finger being involved in binding to the 5′-DR1 half-site as well as dimerization with RXR [6], a hinge region which allows for adequate rotation of the C-terminal domain for interaction with other proteins, and a C-terminal ligand-binding domain including the ligand-dependent activation function- (AF-2), important for RXR heterodimerization and interaction with cofactors [10, 11]. Again, like all nuclear receptors, PPARs act not alone but in association with cofactors that remodel the structure of chromatin in order to either permit or prevent transcription. In the absence of ligand (fatty acids or their derivatives), PPARs form complexes with corepressors such as NCoR, RIP140, or SMRT, which repress transcription through the recruitment of histone deacetylases [12–14]. In the presence of ligand, on the other hand, coactivators, such as p300, CBP, or SRC-1 (all being coactivators related to PPAR function but not primarily associated to nutrient regulation, indicated as coactivators in Figure 1), become bound to the amino terminal of PPARγ coactivator-1 (PGC-1, a key nutrient-modulated coactivator of the PPARs, discussed in Section 3), then acetylate and remodel chromatin, thus enhancing gene transcription via the relief of chromatin condensation (see Figure 1) [15–17]. Depending on the cell type, the ligand-induced conformational changes, and the sequence of the DNA-binding element, a specific complex is formed between the receptor and the coactivators or corepressors, thus allowing fine-tuning of the physiological response. This also explains the variety of changes in gene expression that occur when a nuclear receptor is activated by different ligands.

1.3. Interaction between PPARs and Other Nuclear Receptors. PPARs can associate with other nuclear receptors, and these interactions can involve noncanonical PPREs. Although PPARs and TRs generally compete for interaction with RXR and thus inhibit their respective activity [18], synergism

between PPAR and TR can also occur; an example of this is given in the context of nutrient handling, involving the regulation of the expression of the gene encoding uncoupling protein 3, playing a role in the handling of fatty acids within the mitochondria [19]. This interplay has been shown in rat skeletal muscle [20] as well as in cotransfection experiments in rat L6 myoblasts containing a reporter construct driven by the rat UCP3 promoter [20]. Activation of UCP3 gene transcription in vivo by thyroid hormone (T3) requires the presence of fatty acids (the natural ligands of PPARs; see Section 3), while in the absence of fatty acids, transcription can be restored by the PPARδ agonist L165041 [20]. The UCP3 gene promoter has been shown to contain a noncanonical thyroid hormone response element (TRE) termed TRE1 that is conserved from rodents to humans [20, 21], and this response element is also recognized by PPARs [21].

1.4. PPARs: Different Genes, Different Roles? It was previously assumed that the three known isoforms, PPARα, δ, and γ—which display tissue-specific expression (see Table 1) and possess different gene-regulatory profiles—had clearly distinct roles. For instance, PPARγ, expressed predominantly in adipose tissue and the immune system, existing as two distinct proteins $\gamma 1$ and $\gamma 2$, which arise by differential transcription start sites and alternative splicing [5], was assigned the key role as regulator of adipose development, lipid mobilization, and adipose insulin sensitivity [22], whereas PPARα-regulated genes were considered to be associated with lipid oxidation in muscle and liver [23]. The PPARα target genes include carnitine palmitoyltransferase I (CPT I), which is involved in the transport of long-chain fatty acyl groups into the mitochondria, medium-chain acyl-CoA dehydrogenase (involved in β-oxidation), and (specifically in liver) mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (the rate-limiting enzyme of ketogenesis), as well as peroxisomal acyl-CoA oxidase (peroxisomal β-oxidation) and microsomal cytochrome P450 (CYP) FA hydroxylases. Thus, PPARα would be expected to play a critical role in the maintenance of lipid homeostasis (oxidation and production). Although it seems clear that it is primarily involved in lipid metabolism, PPARα may also provide a link between dyslipidemia and diabetes. Exposure of insulin-sensitive tissues (in particular liver and skeletal muscle) to excess nonesterified FA and circulating triglycerides (triacylglycerol, TAG) induces insulin resistance [24], and this can be corrected by the administration of PPARα activators, the actions of which promote the removal of intracellular lipid through FA oxidation [25]. The PPARδ isoform is predominantly expressed in skeletal muscle (where it induces fatty acid oxidation and the expression of largely the same genes as does PPARα), but it is also expressed in brain, heart, liver, adipose tissue, and small intestine [26]. This receptor subtype, which is still under active study, is perhaps the most versatile of the three subtypes, to judge from its wide tissue distribution. PPARδ has been allocated a central role in the direction of fuel usage between different organs (for review, see [27]).

1.5. PPAR Cross-Talk and Fine-Tuning of Nutrient Handling. Because of the overlap in expression profiles between the PPARs [26, 28] (see Table 1), it is perhaps not surprising that there exists cross-talk among PPARs. Indeed, it has been shown that nonliganded PPARδ represses the transcriptional activity of PPARα and PPARγ [29, 30]. Contrasting evidence exists on a nongenomic action of PPARδ on regulation of PPARγ signaling. Using transient transfection studies, it has been shown that the ligand-binding domain of PPARδ, without binding to DNA, exerts ligand-dependent dominant-negative activity on PPAR$\gamma 1$ signaling [30], although in a previous study a non-DNA-binding PPARδ derivative failed to exert such an effect [29]. Since the PPARs act as RXR heterodimers, it is conceivable that, in analogy to ligand-dependent RXR competition between PPAR and liver X receptor (LXR) [31, 32], PPARδ and LXRα [33], PPARα and TR [34], as well as PPARγ and TR [35], competition for RXR could occur between the PPAR isoforms. Indeed, the nongenomic dominant-negative effect of PPARδ on PPARγ is likely to involve RXR sequestration, thus preventing PPARγ-RXR heterodimerization [30]. Interaction between PPARs and other nuclear receptors and PPAR cross-talk together allow for fine-tuning of interorgan nutrient handling, in concert with the effects of the various signaling molecules that are common to the nuclear receptors and that direct their actions.

2. Nutritional Control of Expression and Activation of PPARs

2.1. Nutrients: Natural PPAR Ligands. PPARs have been shown to be under nutritional control. Dietary nutrients and their derivatives (or adipose-derived fatty acids during food deprivation) directly control PPAR activity since they are the natural ligands of PPARs. PPARs display the greatest preference for monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs, resp.), as demonstrated by means of various ligand-binding assays [36, 37]. The fact that each PPAR activates a different gene program, despite their overlapping expressions, would seem to suggest that PPARs display ligand specificity. Indeed, the structure of the ligand-binding pocket differs considerably among the various PPARs, as revealed by X-ray crystal-structure analysis [37, 38]. Despite this, natural fatty acids can be ligands of all three PPAR isoforms. Using comparative nutrigenomics analysis, it has been recently shown that, in response to high-fat diet, the diet-induced target genes of PPARα are conserved between yeast, mouse, rat, and man, underlining the importance of nutritional control of PPAR function [39]. A different nutrigenomic approach consisted of the use of synthetic triglycerides composed of one single fatty acid in combination with gene expression profiling to examine the effects of various individual dietary fatty acids on hepatic gene expression in mice. Results revealed that (i) increased fatty acid chain length and degree of unsaturation increased the number of genes being upregulated and that (ii) genes regulated by dietary unsaturated fatty acids remained unaltered in PPARα knockout mice, identifying PPARα as their

Table 1: Tissue distributions of the various PPARs (RNA and protein) in adult rodents and humans. Abbreviations: GI, gastrointestinal; WAT, white adipose tissue; BAT, brown adipose tissue; CNS, central nervous system. Symbols: −, absent; ±, barely detectable; +, weak; ++, moderate; +++, high; ++++, very high (Taken from [26, 28, 45]).

Tissue	Protein/mRNA	PPARα	PPARβ/δ	PPARγ
GI tract				
Mouse	Protein		++++	
Human	mRNA	+ or ++	+ to ++++	++ to ++++
Mouse	mRNA	+ or +++		
Rat	mRNA	++ to ++++	++ to ++++	+ to ++
Liver				
Mouse	Protein		+++	
Rat	Protein	++++		
Human	mRNA	+ to ++++	+ or ++	+ to ++
Mouse	mRNA	++++		
Rat	mRNA	++++	++	−
Kidney				
Mouse	Protein		+++	
Human	mRNA	++ to ++++	+ to +++	+ to +++
Mouse	mRNA	+++		
Rat	mRNA	+++ to ++++	+++	±
Heart				
Mouse	Protein		++	
Human	mRNA	+++ to ++++	+ or +++	++ to +++
Mouse	mRNA	++		
Rat	mRNA	+ or +++	+	±
WAT				
Human	mRNA	+	++	++++
Rat	mRNA	+	++	+++
BAT				
Mouse	mRNA	+++		
Rat	mRNA	++++	++	++++
CNS				
Rat	mRNA	+	+ or +++	±
Brain				
Mouse	Protein		++	
Human	mRNA	++	+ or +++	
Rat	mRNA	+	+++	
Skeletal muscle				
Mouse	Protein		+	
Human	mRNA	++ to ++++	++ or ++++	+ or +++
Mouse	mRNA	+++	+++	±
Rat	mRNA	++	++++	±
Skin				
Mouse	Protein		+++	
Rat	mRNA	+	±	
Lung				
Mouse	Protein		++	
Human	mRNA	+	+ or +++	++++
Rat	mRNA	+	++	+

target, and the same genes were upregulated in mice treated with the PPARα agonist WY14643 [40]. Since the binding of a ligand promotes a conformational change that is permissive for interactions with tissue-specific coactivator proteins (see Section 3), allowing nucleosome remodeling and activation of the transcription of cell type-specific target genes [37, 41], it is conceivable that upon binding to a ligand-binding pocket a given fatty acid induces conformational changes, which differ among the various PPAR subtypes. Given that the transcriptional activity induced by each PPAR subtype is cell type specific [42], the different conformations induced following ligand binding might confer cell specificity on the various PPARs (through heterodimerization with different receptors and binding to cell type-specific cofactors).

2.2. Role of PPARs in the Adaptation to Nutrient Deprivation. One widely employed way of studying how PPAR expression and function is controlled by nutrients in different tissues is by imposing nutrient deprivation. The fasting state influences the actions of all known PPARs. For instance, fasting is known to increase PPARα signaling in the liver, through increased mRNA levels of the coactivator PGC-1α [43], and thereby tightly to regulate hepatic gluconeogenesis and FA oxidation. Interestingly, and perhaps paradoxically, it has recently been shown that during fasting upregulation of gene expression by PPARδ, not PPARα, is sensitive to adipose-derived plasma FA, thus assigning a clear role for PPARδ as a plasma FA sensor in liver [44]. Given the central role of PPARδ in controlling skeletal muscle lipid utilization, fasting (which results in a greater reliance on fatty acids) would be expected to increase PPARδ expression and/or activity. In line with this, starvation has been shown to result in a dramatic but transient upregulation of PPARδ mRNA in rat gastrocnemius muscle [45]. This correlated with rapid nuclear accumulations of PPARδ and the coactivator PGC-1α after food deprivation [45]. It has also been shown that, in skeletal muscle, PPARδ and PGC-1α physically interact with each other within the nucleus [46]. This interaction would then lead both to increased fatty acid levels and to increased expression of genes such as those for myosin heavy chain I (MHC I), thioesterase I (MTE I) [47], and carnitine palmitoyl transferase 1 (CPT1), as well as uncoupling protein 3 (UCP3) [48, 49], thereby underlining the role of PPARδ as a key regulator of muscle-fiber switching and fatty acid metabolism. The transient nature of the increased PPAR expression that occurs during starvation (both the mRNA levels and the nuclear accumulation decreasing once serum FA levels increase [45]) would imply that excessive intracellular fat accumulation inhibits PPAR expression. Indeed, rat PPARα and PPARδ mRNA are each shown to be downregulated after a 48-hour fast [45] and conversely suppression of free fatty acids (using the antilipolytic drug nicotinic acid) was found to increase the mRNA levels of skeletal muscle PPARδ [50]. Both the early fasting state and nicotinic acid treatment cause increased phosphorylation of AMP-activated protein kinase [45], which is known to interact functionally with, and stimulate, PPAR (see Section 3). Once serum fatty acid levels become elevated during food deprivation, the raised muscle AMPK phosphorylation level falls. Taken together, these data indicate that the initial absence of burnable fatty acids during food deprivation triggers a process in which the myocyte is rapidly "converted" to a cell type dedicated to the uptake and burning of fat. Once the intracellular FA levels reach a certain value, the myocyte switches off AMPK and reduces PPAR expression throughout the remainder of the starvation period. Similarly, a downregulation of PPARδ mRNA levels in human skeletal muscle expression has been reported in healthy human subjects after a 48-hour fast [51]. Although data from shorter food-deprivation periods in humans are lacking, it seems likely that, after food deprivation, there is a rapid but transient increase in skeletal muscle PPAR expression in humans too. Activation of the white adipose tissue PPARγ promotes lipid synthesis and storage [52]. Therefore, during fasting, the action of PPARγ has to be inhibited. Indeed, in mature adipocytes, during fasting, its action is inhibited by a physical interaction with the protein sirtuin 1 (SIRT1) (the mammalian Sir2 ortholog), the result being lipolysis [53]. In contrast, the action of PPARγ is enhanced by direct binding to lipin-1, a protein which is known to promote triacylglycerol storage within adipocytes, and to be less expressed during fasting, resulting in lipolysis [54]. There exists cross-talk between PPARγ and PPARδ in this context: PPARδ inhibits the control of PPARγ expression [55], enhancing lipolysis.

2.3. Influence of the Nutritional State on PPAR Action. It is well known that in the liver PPARα controls fatty acid oxidation [23], but the role of PPARδ in the liver has up to now scarcely been assessed. A recent transcriptional profiling analysis using PPARα- versus PPARδ-depleted mice has revealed that PPARδ exerts a distinct role in the liver, namely in the control of glucose utilization and lipoprotein metabolism, as well as the suppression of inflammation [56], whereas PPARα mainly controls hepatic lipid homeostasis, which was especially revealed during fasting, causing drastic changes in the hepatic gene expression profile of the PPARα-depleted mice but not in that of the PPARδ-depleted mice [56]. It should, however, be noted that, as described above, the same group assigned a predominant role for PPARδ as FA sensor in liver during fasting, being activated in response to a rise in plasma FA levels, in contrast to PPARα [44]. It is conceivable that dietary FAs and adipose-derived FAs may activate different PPAR-related pathways, and more research is clearly necessary to gain more insight into the respective roles of PPARα and PPARδ in nutrient handling in liver and other organs. In Figure 2, an overview of PPAR action on nutrient handling in the different metabolically active organs, on the basis of the above data, is depicted.

3. Nutrient Availability-Related Cytosolic Signaling Pathways Directly and Indirectly Modulating PPAR Activity

3.1. Control of PPAR Phosphorylation by Upstream Signaling Related to Fuel Use. Activation of cytosolic and nuclear

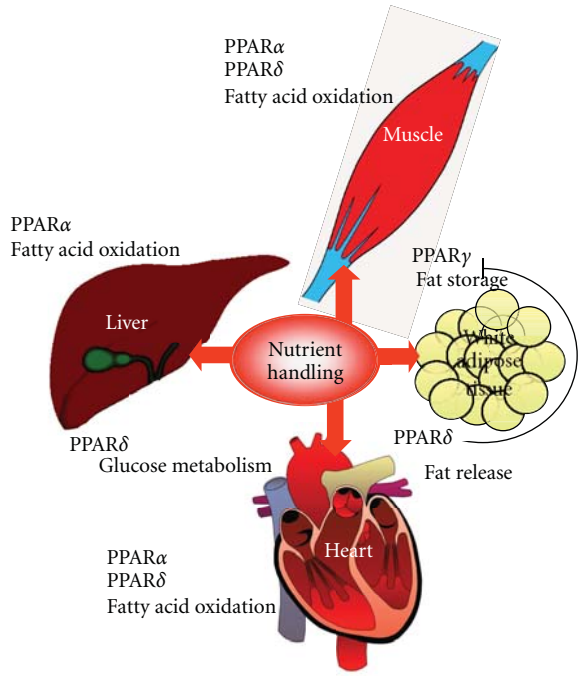

Figure 2: PPAR action in relation to nutrient handling in metabolically active tissues.

signaling, modulating the activity of PPARs, is nutrient regulated. Recent data have revealed that kinases whose activity is modulated by the nutritional state can directly act on phosphorylation of PPARs. Insulin increases phosphorylation and transcriptional activity of PPARα, due to increased activity of the extracellular signal-regulated kinases/mitogen-activated protein kinase (ERK MAPK) pathway [57]. This same pathway inhibits adipogenesis through phosphorylation and inactivation of PPARγ [58]. Both actions are in favor of an increase in insulin sensitivity. Adenosine monophosphate-activated protein kinase (AMPK) is an energy sensor that is activated when the cell-energy level is low [59]. Once activated, AMPK stimulates both glucose uptake and lipid oxidation to produce energy, while turning off energy-consuming processes. Direct proof for phosphorylation of PPARα by AMPK has not been provided thus far, but it has been shown that phosphorylation of PPARγ by AMPK represses both the ligand-dependent and -independent transcriptional activation function of the receptor [60], thus counteracting adipogenesis and favoring lipid oxidation. PPARδ phosphorylation and activity are also likely to be directly controlled by kinase pathways, but to our knowledge evidence for this has not been obtained yet.

3.2. Nutrient-Related Control of PPAR Activity through Activation of PGC-1. The aforementioned kinases as well as other kinases and proteins, functionally associated with nutrient availability, do not only act directly on the PPARs but also modulate the activity of important regulators of the activity of PPARs, namely the PGC-1s, well known as key transcriptional coactivators involved in the control of nutrient and energy metabolism. Three isoforms of PGC-1 are known [namely, PGC-1α, PGC-1β, and PGC-1-related coactivator (PRC)]. They each control mitochondrial physiology and FA oxidation, while exerting an isoform-specific regulation of different metabolic pathways [61]. PGC-1α and PGC-1β are highly expressed in heart, skeletal muscle, and brown adipose tissue, where, via nuclear respiratory factors (NRFs), they induce expression of genes involved in the regulation of mitochondrial biogenesis [61]. PGC-1 family members can interact not only with PPARs but also with other members of the nuclear receptor superfamily, such as ERR, LXR, and HNF-4α [62–64], and with distinct transcription factors and regulatory elements, including cAMP response element-binding protein (CREB), the lipogenic transcription factor sterol regulatory element-binding protein-1c (SREBP-1c), and forkhead box O1 (FOXO1) [43, 65–68]. PPARs, ERR, HNF-4α, and GR commonly bind to LXXLL motifs present in the N-terminal domain of PGC-1, while other transcription factors bind to different regions of the protein, hence allowing a coordinated transcriptional response to nutrient and physiological signals. Although at least two clinical studies have identified a correlation between mutations of the gene encoding PGC-1α and either insulin resistance or diabetes [69, 70], basic research has produced contrasting results. For instance, overexpression of PGC-1α, leading to increased PPARα expression in primary cultures of rat skeletal muscle cells, induces increased expression of the mammalian tribbles homolog TRB3, an inhibitor of Akt signaling [71], a result that implies that PGC-1α has the potential to cause insulin resistance through PPARα signaling. Moreover, PGC-$1^{-/-}$ mice are protected against the insulin resistance induced by a high-fat diet [72].

4. Nutrient-Related Factors Controlling PGC-1 Activity

In response to nutrient signaling, (e.g., in the fasting/fed state, discussed in Section 2), PGC-1 isoforms regulate their own transcription, but a posttranslational regulation also occurs. In particular, (i) phosphorylation, (ii) reversible acetylation, and (iii) methylation are key mechanisms by which the function of PGC-1α function is maintained. (i) Three protein kinases directly phosphorylate PGC-1α. P38 mitogen-activated kinase (P38 MAPK) phosphorylates PGC-1α [73], leading to a more active and stable protein that is unable to bind the p160 corepressor. Also, AMPK phosphorylates and activates PGC-1 [59] (Figure 1). Activation of PGC-1α by phosphorylation through p38 MAPK and AMPK occurs in muscle [74, 75] whereas, in contrast, phosphorylation of PGC-1α in the liver by Akt/protein kinase B (Akt/PKB, which is a central kinase in the insulin-signaling cascade) leads to decreased stability and activity (see [76]; Figure 1). It is noteworthy that AMPK is constitutively activated in the muscles of transgenic mice harboring an activated form of PPARδ [77], while skeletal muscle cells exposed to a pharmacological PPARδ activator show increased AMPK activity [78, 79]. (ii) By analogy with the antagonistic

phosphorylation induced by AMPK and Akt/PKB, the protein deacetylase SIRT1 opposes the action of GCN5, an acetyl transferase. GCN5 inhibits PGC-1 transcriptional activity by acetylating PGC-1 at several lysine residues [80, 81] while SIRT1 activates PGC-1 by deacetylating it and induces expression of PGC-1 gene targets (see [82, 83]; Figure 1). SIRT1, located within the cell nucleus, modulates gene expression in ways that depend on the cellular energy state, which it senses through the cell's NAD+ levels. SIRT1 is an important regulator of those metabolic processes that are initiated by a rise in the intracellular NAD+/NADH ratio when the energy supply is low. Recently, SIRT1-mediated deacetylation of PGC-1α was reported to play a critical role in the regulation of hepatic FA oxidation: nutrient signaling involving SIRT1 and PGC-1α activated gluconeogenic and fatty acid oxidation genes in the fasting liver [82]. Such nutrient signaling gives rise to increases in pyruvate and NAD+ levels, resulting in increases in the amount and enzymatic activity of SIRT1. Loss of SIRT1 from hepatocytes impairs PPARα signaling, resulting in decreased fatty acid oxidation and leading to the development of hepatic steatosis on a high-fat diet, whereas overexpression of SIRT1 induces expression of PPARα gene targets [84]. SIRT1 induces PPARα signaling through deacetylation of PGC-1α without affecting the formation of the PPARα-PGC-1α complex: in SIRT-knockdown hepatocytes, PGC-1α is still recruited to the PPAR response element (PPRE) of FA oxidation genes [84], but it remains acetylated and thus unable to induce transcription of PPARα gene targets. SIRT1 has been identified as a functional regulator of PGC-1α that induces a metabolic gene transcription program of muscle mitochondrial function and fatty acid oxidation during fasting [82]. Thus, consistent with a switch from glucose to fatty acid oxidation that occurs in nutrient-deprivation states, SIRT1 is required for induction and maintenance of fatty acid oxidation in response to low glucose concentrations. Importantly, the action of SIRT1 on PGC-1 differs among stimuli, deacetylating PGC-1α only in response to nutrient signaling, not to glucagon [82, 85]. In addition, whereas SIRT1 overexpression protects against both the hepatic steatosis and glucose intolerance induced by high-fat feeding [86, 87], oligonucleotide knockdown of hepatic SIRT1 in a rat model of T2DM has been reported to reduce hyperglycemia by normalizing basal hepatic glucose production and increasing hepatic insulin sensitivity, leading to the suggestion that novel SIRT1 inhibitors targeted at the liver might prove beneficial in the treatment of T2DM [88]. (iii) Protein arginine methyltransferase I (PRMT1; Figure 1) coactivates nuclear receptors [89] and has been reported to induce the PGC-1α function through methylation at several arginine residues in the C-terminal region [89]. Further, it has recently been demonstrated that impaired PRMT1 activity may be implicated in glucose intolerance in nonobese diabetic Goto-Kakizaki rats through disturbed hepatic glucose metabolism and insulin secretion [90]. An overview of the above discussed nutrition-related kinases and enzymes modulating PPAR activity directly or via PGC-1 is given in Figure 1.

5. Conclusions

The beginning of this century saw a rapid expansion of research on the obesity-counteracting potential of PPARs. It is important to consider that dietary changes can drastically interfere with interorgan signaling, and that PPARs can play an important role in this context. Excess fat supply and nutrient deprivation, followed by analysis utilizing comparative nutrigenomics and transcriptional profiling, have rapidly increased our knowledge on nutrient-mediated regulation of PPAR action, and has revealed that nutrient-based regulation of PPAR action is conserved from yeast to man. PPAR action is under tight control of upstream signaling through factors such as SIRT1 and AMPK, having PGC-1 as a target, and this signaling is also influenced by the nutritional state. The further application of potent transcriptomic profiling techniques under different dietary conditions will help to establish which natural ligands might activate each PPAR in a given cellular context. This research will be important since it will allow us to assess how even subtle changes in our daily diet can cause drastic variations in PPAR-mediated metabolic homeostasis. Normalization of lipid and glucose metabolism may be achieved via pharmacological modulation of PPAR activity but the obtained results are not always beneficial (not discussed in this review), which indicates that our knowledge on the intricate network controlling PPAR action is still far from complete. The challenge for future research is to unravel the complex nutrient-influenced metabolic signaling involving the aforementioned factors, in order to be able to "safely" interfere with these processes, as an important step to relieve the burden of obesity and its related disorders.

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Review Article

PPAR-γ Signaling Crosstalk in Mesenchymal Stem Cells

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Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a member of the nuclear receptor (NR) superfamily of ligand-activated transcriptional factors. Among other functions, PPAR-γ acts as a key regulator of the adipogenesis. Since several cytokines (IL-1, TNF-α, TGF-β) had been known to inhibit adipocyte differentiation in mesenchymal stem cells (MSCs), we examined the effect of these cytokines on the transactivation function of PPAR-γ. We found that the TNF-α/IL-1-activated TAK1/TAB1/NIK (NFκB-inducible kinase) signaling cascade inhibited both the adipogenesis and Tro-induced transactivation by PPAR-γ by blocking the receptor binding to the cognate DNA response elements. Furthermore, it has been shown that the noncanonical Wnts are expressed in MSCs and that Wnt-5a was capable to inhibit transactivation by PPAR-γ. Treatment with Wnt5a-activated NLK (nemo-like kinase) induced physical association of the endogenous NLK and H3K9 histone methyltransferase (SETDB1) protein complexes with PPAR-γ. This resulted in histoneH3K9 tri-methylation at PPAR-γ target gene promoters. Overall, our data show that cytokines and noncanonical Wnts play a crucial role in modulation of PPAR-γ regulatory function in its target cells and tissues.

1. Introduction

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) belongs to the nuclear receptor (NR) superfamily and regulates target gene mRNA expression in the ligand-dependent manner [1]. Similar to most known NRs, PPAR-γ contains distinct domains for binding the DNA (DBD), ligand (LBD), and various cofactor complexes. The structure of PPAR-γ LBD consists of 12 α-helices and 4 β-sheets [2].

For ligand-dependent transcriptional control by PPAR-γ, several distinct classes of transcriptional coregulators/coregulator complexes are indispensable in addition to basic transcription machinery to reorganize chromatin state at the genomic target loci [1, 3]. Transcriptional coregulators for NRs can be divided into two classes in regard to the mechanisms of chromatin reorganization. One class consists of histone modifying enzymes that reversibly modify the N-terminal tails of nucleosomal histone proteins [4, 5]. For example, acetylation and methylation at histone H3K4 and H3K36 are chromatin activating modifications and support transcriptional up-regulation by NRs [6, 7]. In contrast, transcriptional repression by NRs is coupled with inactivating modifications like deacetylation and methylation at histone H3K9 and H3K27 [8]. Accordingly, cognate histone modifying enzymes serve as NR coregulators.

The other class of transcriptional coregulators includes chromatin remodeling factors that directly reorganize nucleosomal arrays using ATP hydrolysis as a source of energy [9, 10]. Chromatin remodelers function as multi-subunit complexes and include ATPase catalytic subunits. Four distinct types of chromatin remodeling complexes (SWI/SNF, ISWF, WINAC, and NURD) have been so far identified as transcriptional coregulators of NRs [11, 12].

Besides ligand dependency, various signaling pathways modulate the ligand-dependent transactivation function of NRs. For example, phosphorylation in the N-terminal region of estrogen receptor alpha (ER-α) by certain pathway-activated protein kinases enhances the transactivation function of ER-α [13]. The transcriptional activity of PPAR-γ is also modulated through positive and negative crosstalk with

other signaling pathways [14]. The molecular mechanisms of the crosstalk include direct and indirect associations of PPAR-γ with intracellular signal transducers or transcriptional factors as well as covalent modifications of PPAR-γ protein, such as phosphorylation by signal-dependent protein kinases [15] or sumoylation by UBC9 [16]. Phosphorylation of PPAR-γ in the N-terminal domain suppresses the transactivation function of PPAR-γ by reducing affinity for PPAR-γ ligands [17], whereas ligand-dependent sumoylation of PPAR-γ represses the NF-κB activation and antagonizes inflammatory responses [16]. These clearly indicate that modifications in the PPAR-γ molecule play a pivotal role in modulation of its physiological action (Figure 1).

2. Signaling Crosstalk between PPAR-γ and Cytokines in MSCs

Mesenchymal stem cells (MSCs) derived from various adult tissues have the potential to differentiate into different lineages, including osteoblasts, chondrocytes, adipocytes, or myocytes [19–21]. Reflecting such pluripotency, a number of regulators involved in the control of MSC differentiation have been identified and characterized [19]. Bone morphogenetic protein (BMP) signaling molecules (particularly BMP-2, -4, -6, and -7) act as major osteogenic inducers and may also influence adipocyte differentiation [22] through induction of PPAR-γ corepressor, TAZ [23]. Recently, the hedgehog signaling has been shown to inhibit adipogenesis and induce osteoblastogenesis [24].

Since several cytokines (IL-1, TNF-α, TGF-β) inhibit adipocyte differentiation in MSC, we examined the effect of their signaling on the transactivation function of PPAR-γ. Treatment with TNF-α or IL-1 inhibited Tro-induced transcriptional activity of PPAR-γ. Interestingly, treatment with both Tro and cytokine (IL-1 or TNF-α) induced osteoblastogenesis in ST2 cells. Thus, cytokines and activated PPAR-γ appeared to stimulate cytodifferentiation of bone marrow progenitor cells into osteoblasts, in addition to cytokine-dependent interference with adipocyte differentiation. Since TNF-α and IL-1 are known to activate the NF-κB in the nucleus, and the nuclear NF-κB is indispensable for osteoclastogenesis from heamatopoetic stem cells, these cytokines appear to be physiologically important for the mesenchymal stem cell fate decision. We therefore studied effects of downstream mediators of the TNFα/IL-1 signaling on the MSC differentiation [14].

In ST2 cells, the TNF-α/IL-1-activated TAK1/TAB1/NIK (NFκB-inducible kinase) signaling cascade inhibited both the adipogenesis and Tro-induced transactivation by PPAR-γ. Though it was previously reported that phosphorylation of PPAR-γ by MAP kinase resulted in repression of the PPAR-γ function [15], we showed that TNF-α/IL-1-induced inhibition of PPAR-γ did not involve its phosphorylation by the NIK.

Consistent with suppression of the PPAR-γ-dependent luciferase reporter gene activity, the activated TAK1/TAB1/NIK was found to suppress the Tro-induced expression of endogenous PPAR-γ target genes. We found that treatment with these cytokines or ectopic expression of some of their downstream mediators blocked binding of PPAR-γ to its response element DNA sequences (PPRE) in the target gene promoters (Cbl-associated protein, CAP). CAP is a signaling protein that interacts with both c-Cbl and the insulin receptor that may be involved in the specific insulin-stimulated tyrosine phosphorylation of c-Cbl [25, 26]. Next, we have shown that the TAK1/TAB1/NIK pathway-activated NF-κB blocks the DNA binding of PPAR-γ at the PPRE. Together with the previous reports that agonist-activated PPAR-γ inhibits DNA binding by NF-κB [27], it appears that an association of ligand-activated PPAR-γ with nuclear NF-κB results in a complex incapable to interact with DNA at either corresponding binding sites (Figure 2).

Thus, we presume that TNF-α/IL-1 triggers activation of NF-κB through the TAK1/TAB1/NIK axis, leading to a physical association between PPAR-γ and NF-κB thereby inhibiting the ligand-dependent PPAR-γ transactivation. Since PPAR-γ is a prime regulator of adipogenesis, suppression of the PPAR-γ function may inhibit adipogenesis and consequently, shift the bone marrow cell fate decision towards the osteoblastogenesis [14].

3. Noncanonical Wnt Signaling Induces Osteoblastogenesis through Transrepression of PPAR-γ by Histone Methyltransferase Complex

Our recent studies of the effects of Wnts on the osteoblastogenesis and adipogenesis have shown that Wnt signaling may directly regulate the transactivation function of PPAR-γ in the MSCs [28]. Several frizzled receptors and Wnt ligands have been found expressed at significant levels in the ST2 cells and in mouse bone marrow cell primary culture. Interestingly, noncannonical Wnt ligand (Wnt-5a) and receptors (Frizzled-2 and -5) were found to be expressed in these cells at particular high levels [28]. While Wnt-3a, a canonical Wnt ligand, did not affect transactivation function of Tro-induced PPAR-γ, noncanonical Wnt-5a was capable to repress activation by PPAR-γ recombinant and endogenous PPAR-γ target gene promoters. We then explored an ability of downstream mediators of the Wnt-5a signaling to repress PPAR-γ and determined that CaMKII-TAK1/TAB2-NLK axis members were potent inhibitors of the receptor. This was consistent with reports that NLK-deficient mice exhibited increased adipocyte concentration in the bone marrow [29].

As the NLK acts as a downstream mediator in the Wnt-5a signaling pathway, we explored molecular basis of the transrepressive effects of NLK on the PPAR-γ transcriptional function. Since tricostatine A, an inhibitor of a wide range of HDACs, was unable to reverse the NLK-mediated suppression of PPAR-γ function, this opened a question about possible involvement of other inactivating histone modifying enzymes. NLK-containing protein complexes were biochemically purified from nuclear extracts of KCl-treated HeLa cells expressing FLAG-tagged NLK [9, 30] and

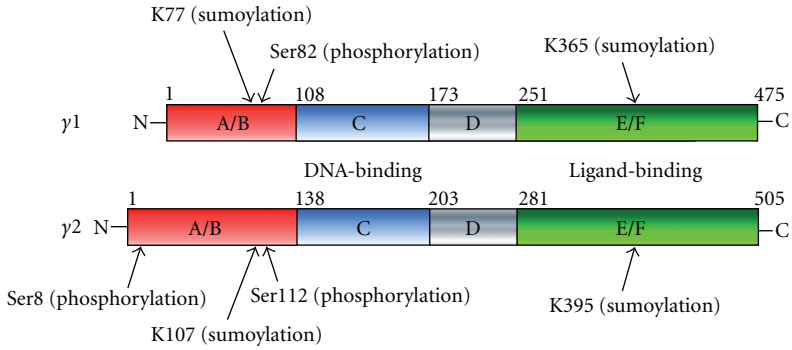

Figure 1: Structure and posttranslational modifications of PPAR-γ1, -γ2 proteins. Although PPAR-γ was ubiquitinated, lysine residues are not determined [18].

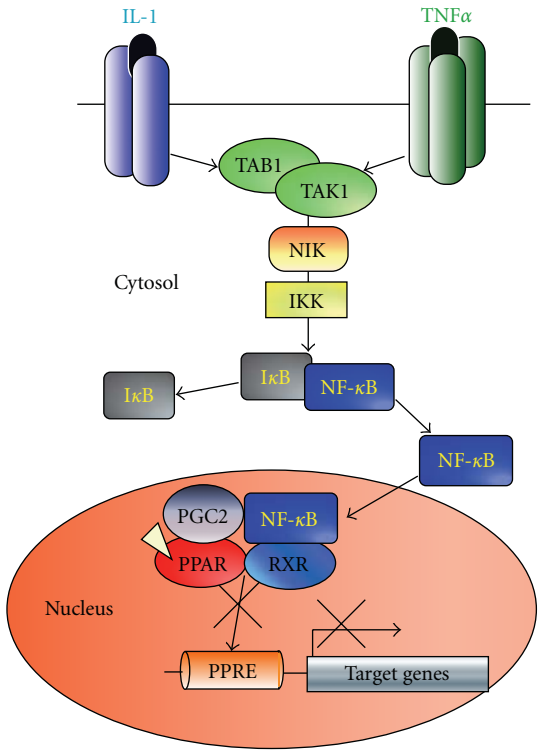

Figure 2: Schema of the proposed molecular mechanism of adipogenesis inhibition by TNF-α and IL-1 through suppression of PPAR-γ function by NF-κB activated via the NIK-TAK1/TAB1-mediated cascade.

a distinct NLK-nuclear protein complex with a molecular weight of around 400–500 kDa was isolated and analysed [28, 31]. In this complex, a 170 kDa component was identified as a SETDB1, a transcription inhibiting histone lysine-methyltransferase (HKMT) that methylates histone H3 at K9 [32, 33]. Importantly, in ST2 cells, treatment with Wnt5a induced a physical association of endogenous NLK-SETDB1 protein complexes with PPAR-γ.

ChIP analysis of endogenous transcriptional factors and histone modifications at the PPAR-γ response element (PPRE) in the aP2 gene promoter [34] has shown that treatment with Tro induced recruitment of known PPAR-γ coactivator SRC-1. However, simultaneous treatment with Wnt-5a and Tro induced recruitment of NLK and SETDB1 at the PPRE region. Consistently, an increase in histone H3 di- and tri-methylation at K9 was observed together with histone hypoacetylation. Such coordinated chromatin silencing histone modifications at the PPAR-γ target genes were more prominent after a 7-day treatment with Wnt-5a that was long enough to induce the osteoblastogenesis. Furthermore, an ectopic expression of either NLK or SETDB1 in the presence of Tro was potent to induce the osteoblastogenesis and inhibit the adipogenesis, whereas a knockdown of either NLK or SETDB1 potentiated the Tro-induced adipogenesis even in the presence of Wnt-5a. Thus, we have shown that Wnt-5a induces the osteoblastogenesis through attenuating the PPAR-γ-induced adipogenesis in the bone marrow MSC (Figure 3).

Upon Wnt-5a-induced activation of the noncanonical Wnt signaling, the SETDB1 HKMT forms a complex with phosphorylated NLK. This NLK/SETDB1 complex associates with PPAR-γ and methylates H3-K9 at the PPAR-γ target gene promoters leading to their transcriptional silencing. Interestingly, the NLK also suppresses the transactivation function of the A-Myb through histone methylation [35], suggesting that the NLK might control gene expression by histone modification through recruitment of SETDB1.

The noncanonical Wnt-5a ligand regulates MSC differentiation through the CaMKII-TAK1/TAB2-NLK signaling cascade that is distinct from the canonical Wnt pathway, which is mediated by the β-catenin/TCF signal transduction. Several recent reports have demonstrated that the canonical Wnt pathway mediated by LRP5/β-catenin is also indispensable for the osteoblastogenesis [36–38]. Hence, both the canonical and noncanonical Wnt pathways are considered to support the osteoblastogenesis in the bone marrow mesenchymal cells. However, only the noncanonical Wnt signaling appears to impair the PPAR-γ-inducible adipogenesis and switch the MSC differentiation into the osteoblastic lineage.

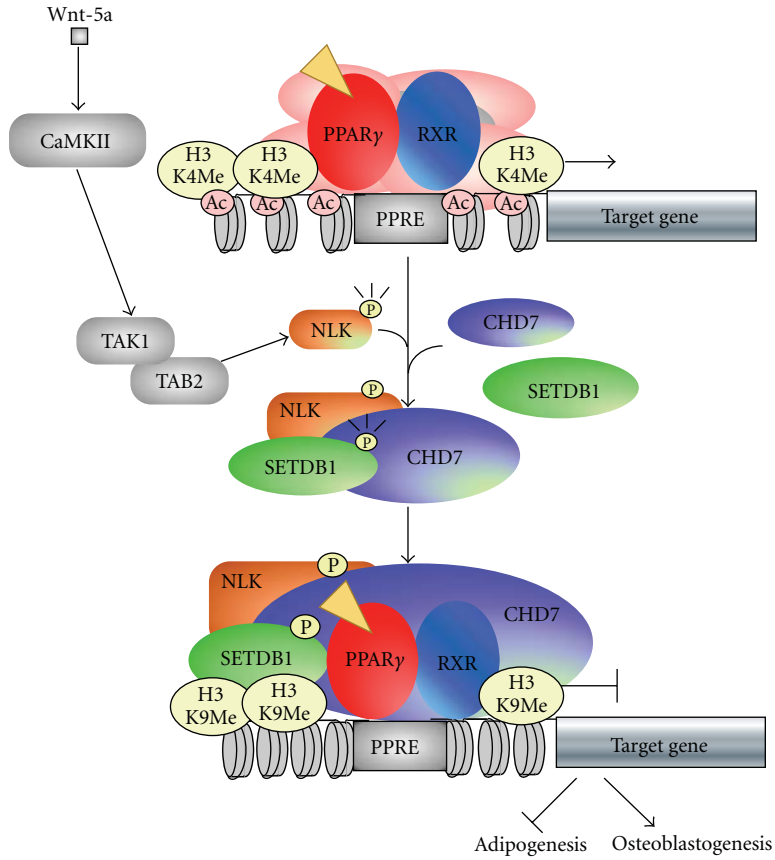

Figure 3: Schematic model of crosstalk between PPAR-γ and Wnt-5a signaling in MSC. NLK activated by the Wnt5a signaling pathway phosphorylates SETDB1 and forms a complex with PPAR-γ/RXR and chromodomain containing protein 7 (CHD7).

4. Conclusion

In summary, IL-1, TNF-α, and noncanonical Wnt signaling pathways suppress the PPAR-γ function in MSCs and thus, are capable to influence stem cell fate [39]. Interestingly, molecular mechanism of suppression of the PPAR-γ transcriptional activity by the IL-1 and TNF-α is different from that induced by the noncanonical Wnt ligands. IL-1 or TNF-α-activated NF-κB inhibits the DNA binding capacity of the receptor, while Wnt5a-activated NLK promotes PPAR-γ/SETDB1 complex formation leading to silencing epigenetic chromatin modifications at the PPRE. Recent studies show that PPAR-γ also plays pivotal roles in other cells and tissues, such as osteoclasts [40], kidney cells [41], and macrophages [27]. This opens questions about the existence of other mechanisms of modulations of the PPAR-γ physiological activity specific for these types of differentiated cells that may be different from those in stem cells.

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