PPARα: Master regulator of lipid metabolism in mouse and human

Identification of hepatic PPARα target genes by expression profiling

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درخت تو گر بار دانش بگیرد

به زیر آوری چرخ نیلوفری را

Once your tree bears fruits of knowledge
you will master the universe

Naser Khosrow, *Persian Poet, (1004 - 1088 AD)*

Abstract

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 large number of target genes. Although the role of PPARα as a gene regulator in liver has been well established, a comprehensive overview of its target genes has been missing so far. Additionally, it is not very clear whether PPARα has a similar role in mice and humans and to what extent target genes are shared between the two species.

The aim of the research presented in this thesis was to identify PPARα-regulated genes in mouse and human liver and thereby further elucidate hepatic PPARα function. The applied nutrigenomics approaches are mainly expression microarrays combined with knockout mouse models and in vitro cell culture systems.

By combining several independent nutrigenomics studies, we generated a comprehensive overview of PPARα-regulated genes in liver with the focus on lipid metabolism. We identified a large number of PPARα target genes involved in different aspects of lipid metabolism. Furthermore, a major role of PPARα in lipogenesis was detected. Our data pointed to several novel putative PPARα target genes. Next, we compared PPARα-regulated genes in primary mouse and human hepatocytes treated with the PPARα agonist Wy14643 and generated an overview of overlapping and species specific PPARα target genes. A large number of genes were found to be regulated by PPARα activation in human primary hepatocytes, which identified a major role for PPARα in human liver. Interestingly, we could characterize mannose binding lectin 2 (Mbl2) as a novel human specific PPARα target gene. Plasma Mbl2 levels were found to be changed in subjects receiving fenofibrate treatment or upon fasting. Regulation of Mbl2 by PPARα suggests that it may play a role in regulation of energy metabolism, although additional research is needed.

We also compared the PPARα-induced transcriptome in HepG2 cells versus primary human hepatocytes to investigate the suitability of HepG2 cells in PPARα research. The results revealed that the HepG2 cell line poorly reflects the established PPARα target genes and function, specifically with respect to lipid metabolism. Finally, we characterized the transcription factors Klf10 and Klf11 as novel PPARα target genes. Our preliminary findings using in

vitro transfection assays and in vivo tail vein injection of plasmid DNA suggested a potential metabolic role of Klf10 and Klf11 in liver.

In conclusion, this thesis has extended our understanding of PPARα-regulated genes and function in liver, and has specifically highlightened a major role of PPARα in human hepatocytes. This research has also given birth to a possible biomarker of hepatic PPARα activity which is of great interest for future studies. Considering the need for proper biomarkers in the field of nutrigenomics and beyond, the properties of Mbl2 as a biomarker should be further investigated. The identification of other novel putative PPARα target genes offers ample opportunities for continued research.

Chapter 1

General introduction

Nutrition

Nutritional science has been through a major evolution in recent decades. Starting out as a scientific discipline focused on establishing nutrient requirements and the prevention of nutrient deficiencies, in modern times nutritional science has gradually placed increasing emphasis on the prevention of chronic diseases. Chronic diseases that are presently in the spotlight are obesity and the related metabolic syndrome, both of which have experienced a major surge in prevalence in the past decades. Metabolic syndrome is defined by a number of characteristics including visceral obesity, insulin resistance, hypertension and dyslipidemia and is associated longitudinally with increased risk for cardiovascular disease and diabetes [1-3]. As a consequence, obesity and the metabolic syndrome predispose individuals towards a reduced quality of life and increased healthcare associated costs.

Although the first specific guidelines on the identification, evaluation and treatment of overweight and obesity were released in 1998, since then the trend towards increased global obesity rates has continued unabated [4, 5]. In the meantime, the realization has grown that traditional nutritional science focused mainly on physiological and epidemiological aspects of nutrition may fall short of providing all the answers necessary for an effective strategy towards combating obesity and its complications. In response, interest has grown into understanding the molecular mechanisms underlying the beneficial or adverse effects of food components, which became a basis for the introduction of the science of nutrigenomics.

Nutrigenomics

Nutritional genomics or nutrigenomics investigates the interaction between nutrients and genes at the molecular level by using genomics tools [6, 7]. As is the case for nutritional science in general, nutrigenomics research is mainly focused on disease prevention rather than to yield a specific cure. The main objective of nutrigenomics is to provide a solid mechanistic framework for evidence based nutrition aimed at reducing risk for chronic diseases such as cardiovascular disease, diabetes and cancer. Within this field of research, dietary nutrients and their metabolites are considered as signaling molecules that target cellular sensing systems, leading to changes in cellular and tissue function. The growing interest in understanding how nutrition acts at the molecular level has been accompanied by impressive technological advancements, leading to the emergence of a novel field generally referred to as genomics, which include transcriptomics, proteomics and metabolomics. Large scale gene expression profiling or transcriptomics is extensively used to measure global changes in mRNA level (the transcriptome) of a cell or tissue in response to external stimuli such as

nutrients or pharmacological reagents, or in response to certain types of diets and diseases. Application of these genomics tools in nutritional research gave rise to the birth of the field of nutrigenomics.

One of the main goals of nutrigenomics is to try to distinguish healthy individuals from individuals that are in a pre-diseased or diseased state by utilizing large scale gene expression profiling. In this way, by establishing biomarker profiles, nutritional interventions in the early diseased state may be guided towards restoring health, thereby preventing the need for intensive pharmacological therapy. Changes at the level of the transcriptome form the basis for changes at the level of corresponding proteome and metabolome, which thus are the linkage between the gene expression profile and a specific phenotype. Although the most extensive phenotypical characterization would be achieved by combining several genomics techniques, at the moment transcriptomics is the most developed and feasible tool applied in nutrigenomics.

In the recent years, microarray technology has emerged as a powerful tool to study whole genome gene expression. High density oligonucleotide arrays, such as the Affymetrix GeneChip® Arrays, are able to measure the expression of the entire genome of an organism in a single hybridization assay [8, 9]. Microarrays utilize gene specific probes representing individual genes which are attached to a glass surface. The experimental process starts with RNA isolation from the biological samples, labeling with a detectable marker followed by hybridization to the array. After subsequent washing, an image of the array is acquired by determining the extent of hybridization to each gene-specific probe. The data then need to be normalized to facilitate the comparison between the experimental samples [10, 11]. A good quality control of the arrays and proper statistical tests are critical for precise and reliable outcomes measurements.

Liver, the central player in metabolic homeostasis

The liver is the major site for the metabolism of nutrients including fatty acids, cholesterol, glucose, and amino acids, and plays a key role in the biotransformation of xenobiotics. The liver also has an immunologic function via the expression of specific pro- and anti-inflammatory cytokines acting either locally or systemically, and via production of an array of acute phase proteins. This variety of functions is due to the fact that the liver contains numerous cell types including paranchymal cells, stellate cells, sinusoidal endothelial cells, cholangiocytes and Kupffer cells. While paranchymal cells are the principal site for metabolic regulatory pathways, Kupffer cells are mainly responsible for the generation of inflammatory

reagents which can further influence the phenotypes of neighboring cells [12]. The liver can thus be considered to function at the crossroads of metabolic and inflammatory signaling.

The liver plays a central role in metabolic handling of lipids. Depending on nutritional status, fatty acids predominantly enter the liver as free fatty acids, as triglycerides within remnant lipoprotein particles, or are generated via de novo lipogenesis. Incoming fatty acids can be metabolized by the liver as fuel or can be stored in the from of triglycerides within lipid droplets. Additionally, fatty acids can be exported as triglycerides within very low density lipoproteins. Impaired balance between these pathways might promote hepatic triglyceride accumulation and lead to the development of hepatic steatosis, which may progress to chronic hepatic inflammation, insulin resistance and liver damage [13].

As a central metabolic organ, liver has the capacity to respond to numerous nutritional and hormonal signals [14]. In the fed state, dietary glucose stimulates insulin secretion from the pancreas, which travels directly to the liver via the portal vein and activates lipogenesis. In contrast, in the fasted state release of glucagon and adrenal cortisol, in combination with high plasma free fatty acid levels, leads to enhanced fatty acid oxidation. Liver can also contribute to the regulation of energy metabolism by secreting proteins that have systematic effects, thus acting as a part of the endocrine system [15, 16]. The capacity of the liver to coordinate metabolism is coupled to a very dynamic transcriptional regulatory network. Key transcription factors include the farnesoid X receptor (FXR), liver X receptor (LXR) and, peroxisome proliferator activated receptor alpha (PPARα).

PPARα, pharmacological, physiological and nutritional sensor

PPARα is a ligand activated transcription factor that belongs to the superfamily of nuclear hormone receptors and plays a major role in nutrient homeostasis [17-20]. Other known PPAR isoforms are PPARβ/δ and PPARγ. PPARα and PPARβ/δ are ubiquitously expressed, whereas PPARγ is mainly expressed in adipose tissue, macrophages and colon [21, 22]. Endogenous PPAR ligands are comprised of fatty acids and their derivatives such as acyl-CoAs, oxidized fatty acids, eicosanoids, endocannabinoids, and phytanic acid [20, 23-29]. Upon ligand binding PPARs form a heterodimer with the nuclear hormone receptor RXR and bind to specific DNA response elements (PPRE) in target genes to initiate gene transcription [20, 30].

At the functional level, PPARα is known as the master regulator of lipid metabolism in liver. Clinically, PPARα has been the target of fibrate class of drugs and is prescribed to improve

dyslipidemia by lowering fasting plasma triglycerides (TG) and raising plasma HDL levels [31-34]. With respect to its role in physiology, PPARα is needed for the adaptive response to fasting. The absence of PPARα elicits a complex phenotype characterized by fatty liver, hypoketonemia, hypoglycemia, and elevated plasma free fatty acids levels [35-37]. While the initial belief was that plasma free fatty acids can ligand-activate PPARα in liver, more recently it was demonstrated that hepatic PPARα can not become activated by plasma free fatty acids [38, 39]. Instead, PPARα serves as sensor for dietary fatty acids [40-42] and fatty acids generated via de novo lipogenesis [39]. It has been recently 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 [43]. The target genes and function of PPARα are described in more detail in chapter 2.

Outline of this thesis

The aim of the research presented in this thesis is to identify PPARα-regulated genes in mouse and human liver and thereby further elucidate hepatic PPARα function. The applied nutrigenomics approaches are mainly expression microarrays combined with knockout mouse models and in vitro cell culture systems.

In chapter 2, we provide a review of current knowledge on PPARα-regulated genes related to different biological processes in liver. In chapter 3, using microarray technology we generate a comprehensive overview of PPARα-regulated genes in liver with the focus on lipid metabolism. We identify a large number of PPARα target genes involved in different aspects of lipid metabolism. Furthermore, a major role of PPARα in lipogenesis was detected. Our data pointed to several novel putative PPARα target genes. Chapter 4 compares PPARα-regulated genes in primary mouse and human hepatocytes treated with PPARα agonist Wy14643 and generates an overview of overlapping and species specific PPARα target genes. In this chapter, a large number of genes was found to be regulated by PPARα activation in human primary hepatocytes, identifying a major role for PPARα in human liver. In chapter 5, we characterize mannose binding lectin 2 (Mbl2) as a novel human specific PPARα target gene and demonstrate changes in plasma Mbl2 levels in subjects receiving fenofibrate treatment or upon fasting. Regulation of Mbl2 by PPARα suggests that it may play a role in regulation of energy metabolism, although additional research is needed. Chapter 6 compares PPARα-induced transcriptome in HepG2 cells versus primary human hepatocytes to investigate the suitability of HepG2 cells in PPARα research. The results reveal that the HepG2 cell line poorly reflects the established PPARα target genes and function, specifically with respect to lipid metabolism. In chapter 7, we characterize transcription factors Klf10 and Klf11 as novel

PPARα target genes and perform preliminary experiments to identify their physiological role in liver using in vitro transfection assays and in vivo tail vein injection of plasmid DNA. Finally, general discussion and conclusions are presented in chapter 8.

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Chapter 2

Peroxisome Proliferator Activated Receptor alpha target genes

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Abstract

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.

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, PPARß/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 PPARß/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 co-repressors and recruitment of co-activators, remodeling of the chromatin structure, and finally facilitation of gene transcription [7]. This review 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 review 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 tran-

scriptional 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 criteria 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.

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 non-parenchymal 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 an 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.

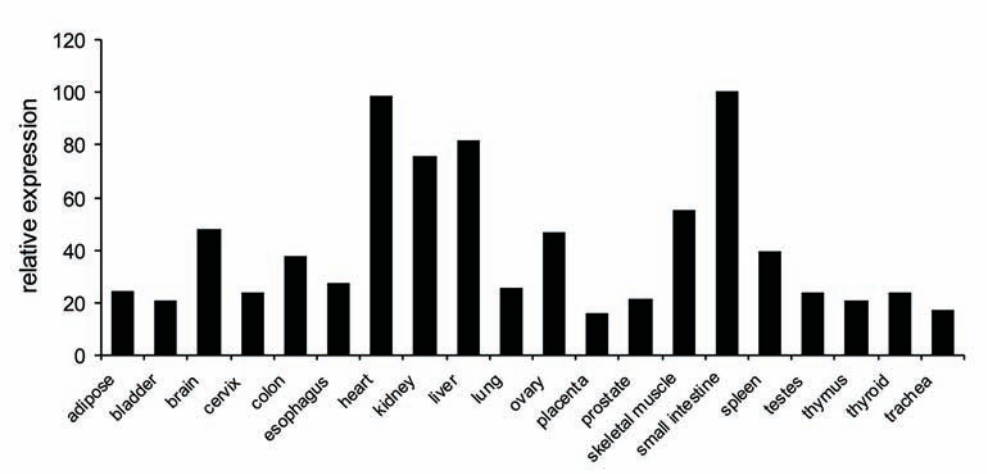

Figure 1. Expression profile of PPARα in human tissues. The FirstChoice Human Total RNA Survey Panel (Ambion) was reverse transcribed and used for qPCR using primers specific for human PPARα. Expression levels are expressed relative to small intestine, which showed the highest expression level (100%).

PPARα structure in mouse and human

Analogous to other nuclear receptor superfamily members, PPARα has a domain structure consisting of a 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, is crucial for dimerization with RXR, and contains the activating function-2 involved in physical interactions with co-regulatory 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 translate 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 ef-

fect 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 non-alcoholic 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].

PPARα ligands

PPARα serves as a receptor for a structurally diverse set of compounds. The most important class of synthetic PPARα ligands are 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].

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 (VLDL) 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 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 [62-64]. A detailed discussion of the specific genes within the various lipid metabolic pathways that are targeted by PPARα is provided on the right page (Table 1).

Lipid metabolism	Lipid/hormone transport	Adipor2 [24,69], Cd36 [24,69,91,122], Lepr [24,69], *Slc27a1* [69,94,121-123], Slc27a2 [24,69,70,91] Slc27a4 [24,69]
	Acyl-CoA formation/ hydrolysis/binding	Acot1 [24,69,239], Acot7 [69], Acot12 [24,69], *Acsl1* [24,67,69,81,124], Acsl3 [24,69], Acsl4 [24,69,91,125], Acsl5 [24,69,91], Acsm3 [24,69], Acss2 [69], *Fabp1* [24,69,77,129], Fabp2 [24,69,91], Fabp3 [24,69], Fabp4 [69,70], Fabp5 [69]
	Mitochondrial ß -oxidation/ oxidative phosphorylation	Acaa2 [24,69,70], Acadl [24,67,69,75,77], *Acadm* [24,55,67,69], Acads [24,54,67,69], Acadvl [24,67,69,91], Acad8 [69], Acad9 [69], Acad10 [69],Acot2 [24,69,239], Acot9 [69], Acot10 [69],*Cpt1a* [24,54,55,69,84-86], Cpt1b [24,69], *Cpt2* [24,67,69,87], Crat [24,69,77], Dci [24,69,70,75,94], Decr1 [24,69,75,95], **Etfa** [24], Etfb [24,69], Etfdh [24,69], Hadha [24,69,70,91,92], Hadhb [24,69,70,91], *Hadh* [24,69,70,75,77,93], Hadh2 [69], Hibch [24,69], *Slc25a20* [24,69,88], Slc22a5 [24,69,89,90], Txnip [24,69], Ucp2 [24,69,101-103], Ucp3 [69]
	Ketogenesis/ketolysis	Acat1 [24,69,70], Bdh [69], *FGF21* [24,62-64,69], Hmgcl [69], *Hmgcs2* [24,69,96-98]
	Peroxisomal ß-oxidation	Abcd2 [24,69,76], Abcd3 [24,69,76], Acaa1a [24,69,70,75], Acaa1b [24,69], Acot3 [24,69], Acot4 [24,69], Acot5 [24,69], Acot8 [69,78], *Acox1* [24,55,66-70], Crot [24,69,77], Decr2 [24,69,70,240] Ech1 [24,69,70,91], *Ehhadh* [24,69,71,72], **Hacl1** [24], Hsd17b4 [24,69,73,74], Peci [24,69,94,240], Pex11a [24]
	Microsomal (ω-hydroxylation)	**Aldh3a1** [24], *Aldh3a2* [24,69,241], Aldh9a1 [24], *Cyp4a1* [77,104,108-110], Cyp4a3 [55,77,109] *Cyp4a10* [24,69,70,88,104,106] , Cyp4a12a [24,69,94], Cyp4a14 [24,69,70,106,107], Cyp4f15 [24], **Cyp4x1** [24]
	Lipogenesis	Acaca [69], Acacb [69], Agpat2 [69], Agpat3 [24,69], Agpat5 [69], Agpat6 [69], Dgat1 [69,118], Elovl5 [69,242,243], Elovl6 [24,69,242,243], Elovl7 [69], Fads1 [24,69,242], *Fads2* [69,114,242], Fasn [69,118], Gpam [24,69,94], *Hsd17b12* [69], *Lpin2* [24,56,69], Mlycd [24,69], Mogat1 [24,69], *Mod1* [24,67,69,115], *Scd1* [24,69,116,244], Scd2 [69,70], Slc25a10 [69,240], Srebf1 [24,69,245]
	Lipases/lipid droplet proteins	*Adfp* [24,69,144], Ces1 [69], Ces3 [24,69], Cidea [24,69,153], Cidec [24,69,153], *GOs2* [24,69,154,155], Lipa [24,69], Lipe [24,69,70], Mgll [24,69,70,91], Oxpat/Lsdp5 [24,69,145,246] , **Plin1** [24], Pnpla2 [24,69], S3-12 [24,69]
	Lipoprotein uptake and metabolism	*Angptl4* [24,69,171,172], ApoA1 [177,178,180,247-249], ***Apoa2*** [24,250], ***Apoa5*** [24,169,170], ApoCIII [167,251,252], Lipc [24,69], Lipg [69], *Lpl* [69,94,122,162], Lrp4 [24,69], Pctp [24,69], *Pltp* [69,94,253,254], Mttp [24,69,255], Vldlr [24,69]
	Cholesterol/Bile transport and metabolism	Abca1 [24,69,197], Abcb4 [24,69,97,197], Abcb11 [69], Abcg5 [69,197], Abcg8 [69,197], Cav1 [24], *Cyp7a1* [24,69,201,261,262] , *Cyp8b1* [69,205], Cyp27a1 [201], FXR [69], *LXR* [197,263], Npc1 [69], Rab9 [24,69], Scarb2 [69], Slc10a1 [97], *Slc10a2* [69,264]
Other pathways	Glucose/Glycerol transport and metabolism	Aqp3 [24,69,188], Aqp7 [69], Aqp9 [69,188], Fbp2 [24,69], G6pc [24], *Gpd1* [24,69,188], Gpd2 [69,188], Gyk [24,69,188], Gys-2 [195], Ldha [69], Pcx [69], **Pck1** [24], Pdk1 [24], Pdk4 [24,69,193,194]
	Biotransformation	Akr1b10 [24], **Akr1c3** [24], **Cyp1a2** [24], Cyp2a5 [106], **Cyp2b6** [24], **Cyp2c8** [24], **Cyp2c9** [24], Cyp2c11 [234], Cyp2c12 [234], Cyp2c29 [106], Cyp2j2[24], **Cyp3a5** [24], Cyp3a7 [24], Cyp3a11 [24,106] Cyp3a43 [24], Ephx2 [24,265], Gsta3 [237], **Mgst3** [24], *Ugt1A9* [238]
	Amino Acid metabolism	Abat [24,208], Acmsd [208], Agxt2 [24,207], Arg1 [207], Asl [24,207], Ass1 [207], Cbs [24,208], Cps-1 [24,207], Cth [208], Got1 [207], Got2 [69,122,207,211], Gls [207], Gls2 [208], Gpt [24,208], Hal [208], Hpd [208], Oat [24,208], Odc1 [24,69,208], Otc [24,69,207], Pah [24,208], Psat1 [24,69,208], Tat [208,209]
	Inflammation	Apcs [217], Birc3 [217], Cebpb [256], Cd68 [24,230], Crp [24,217], CXCL10/IP10 [230], Fgb [24,216,234,257], F4/80+ [230], Icam-1 [24,230], Ifi47 [24,217], Igtp [217], Nfkbia [215,258,259], Il-1ß [230], Il-1r1 [107], Il1rn [217], Il1rap [217], Il-6 [107,260], Il-6ra [107,217], Il18 [217], Lcn2 [217,230], Lifr [217], Ccl2 [230], Ccl3 [230], Mt1 [24,217], Mt2 [217,230], Orm2 [217], Orm3 [24,217], Nfkb1 [24,256], Pla1a [24,217], Saa2 [217], Saa4 [24,217], Stat1 [230], Stat2 [217], Stat3 [217], Steap4 [24,217], Stress induced protein [217], Tnfα [230], Traf2 [217], Vcam-1 [24,230]

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.

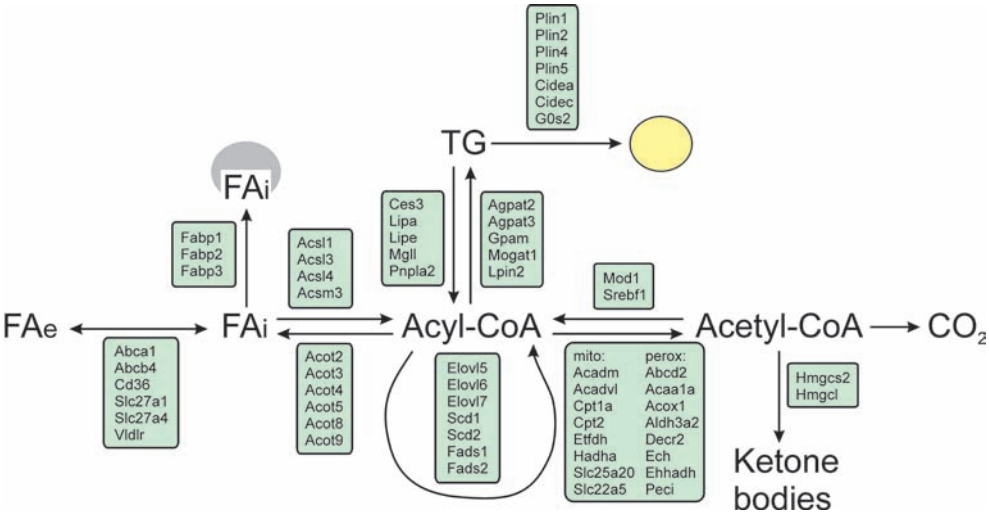

Figure 2. Schematic representation of PPARα target genes in different aspects of hepatic lipid metabolism.

Peroxisomal fatty acid ß-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 [65, 66]. 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 H2O2 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, 66-75]. Additionally, genes involved in peroxisomal fatty acid uptake (Abcd2 and Abcd3), conversion of fatty acid 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, 69, 76-78]. 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 [79]. In contrast, acti-

vation 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 [80-82]. 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].

Mitochondrial fatty acid β-oxidation

The crucial role of PPARα in mitochondrial fatty acid oxidation is illustrated by the phenotype of fasted PPARα-/- mice, which exhibit hypoketonemia, hepatic steatosis, and elevated plasma free fatty acid levels [54, 55, 83]. It is now evident that virtually every enzymatic step along 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, 67, 69, 70, 75, 77, 84-95].

Additionally, synthesis of ketone bodies via mitochondrial HMG-CoA synthase (Hmgcs2) and HMG-CoA lyase (Hmgcl) is governed by PPARα [24, 69, 96-98], as is the expression of genes encoding electron transferring flavoprotein and the corresponding dehydrogenase (Etfa, Etfb, Etfdh) [24, 69]. 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 [99, 100]. Finally, PPARα induces uncoupling proteins Ucp2 and Ucp3, which have been proposed to function as an outward transporter of non-esterified fatty acid anions from the mitochondrial matrix [24, 69, 101-103].

Microsomal fatty acid ω-hydroxylation

Cyp4A enzymes are members of the cytochrome P450 monoxygenase superfamily and catalyze microsomal ω-hydroxylation of fatty acids [104, 105]. Studies using PPARα-/- 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,

69, 77, 88, 104, 106-110]. Furthermore, expression is extremely sensitive to PPARα ligand-activation, indicating 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, 70, 111, 112]. 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 [113]. 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].

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, 114-116]. 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 [69]. 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 [117]. The effect of PPARα agonists on SREBP targets has been attributed to increased activation of SREBP1c via enhanced proteolytic cleavage [118]. Such a mechanism may also lead to increased SREBP1 mRNA via an autoloop regulatory circuit [119]. 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 [120]. 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 this pathways are upregulated by PPARα activation, including Gpam, various Agpat genes, Mogat1, Dgat1, and Lpin2 [24, 69, 94, 118]. 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.

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, 69, 70, 71, 94, 121-123].

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, 69, 91, 122]. Additionally, expression of numerous acyl-CoA synthetases is induced by PPARα [24, 67, 69, 81, 91, 124, 125]. Currently, limited information is available about the cellular localization and the structure/function relationship of Acyl-CoA synthetase enzyme [126].

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 [77, 127-129]. Recent studies indicate that Fabp1 may be involved in partitioning of FA to specific lipid metabolic pathways [130]. Other Fabp genes induced by PPARα activation in mouse liver include Fabp2, Fabp3, Fabp4, and Fabp5 [24, 69, 91]. Induction of Fabp4 (A-FABP, aP2) upon PPARα activation is likely occurring via its expression in Kupffer cells. Fabp4 expression in hepatocytes is correlated with acquisition of a steatotic phenotype concurrent with upregulation of PPARγ mRNA [131].

Lipases and lipid droplet proteins

PPARα-/- mice exhibit elevated hepatic TG accumulation, especially under fasting conditions [54, 132, 133]. Conversely, treatment with PPARα agonists lowers hepatic triglyceride levels in models of hepatic steatosis and can prevent the fasting-induced increase in liver TG [134, 135]. The anti-steatotic 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 [136-141]. 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 the genes are direct target of PPARα as well [142, 143]. 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 [144, 145]. 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 [146]. 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, 147]. Perilipin expression in human liver is correlated with development of steatotic liver [148].

Two recently identified lipid droplet-associated proteins that are not part of the perilipin family are Cidec (FSp27) and Cidea [149, 150]. Both proteins promote TG accumulation and are targets of PPARγ in adipocytes [151, 152]. In addition, they are regulated by PPARα in mouse liver, although the kinetics of induction of the two genes seems to be quite different [153]. 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 [154]. Previously, G0s2 was shown to be a direct PPARα target gene in mouse liver and PPARγ target in adipocytes [155]. 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 reflect 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.

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 [156-159]. 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 [135]. 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 review 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 [160]. 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 [135]. Clearance of TG-rich lipoproteins VLDL and chylomicrons is mediated by the enzyme lipoprotein lipase (LPL), which is attached to the capillary endothelium in of muscle and adipose tissue. Expression of Lpl in liver is restricted to Kupffer cells and upregulated by PPARα agonists [161, 162]. 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 [162, 163]. LPL activity is mostly regulated post-translationally via altered secretion from liver of LPL-modulating factors, including apolipoprotein C-III (Apoc3), apolipoprotein A-V (Apoa4), Angiopoietin-like protein 3 (Angptl3) and Angiopoietin-like protein 4 (Angptl4). Firstly, PPARα agonists down-regulate the expression of LPL inhibitor APOC3, supposedly via mechanisms involving the transcription factors REV-ERBα, HNF4α, or FOXO1 [164-167]. Secondly, PPARα agonists increase hepatic expression and plasma levels of APOA5, which is a positive regulator of LPL [168]. A functional PPAR responsive element has been identified in the promoter of the human Apoa5 gene, classifying Apoa5 as a direct PPARα target gene [169, 170]. 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 [171]. The DNA response element conferring PPAR regulation was located to intron 3 of the Angptl4 gene [172]. Finally, PPARα stimulates hepatic expression of the VLDL receptor (Vldlr) [24, 69]. 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 [173]. Thus, it appears that both pro- and anti-lipolytic pathways are activated by PPARα. Under conditions of pharmacological PPARα activation, the pro-lipolytic 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) [82,174-177]. Apoa1 gene expression is not induced by PPARα in rodents due to the presence of disabling mutations within the PPAR-response element [178]. 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 [178-180].

The impact of PPARα in HDL metabolism likely extends beyond regulation of apolipoproteins. Evidence suggests that both PPARα and PPARß/δ stimulate expression of endothelial lipase (Lipg) in liver [69, 181]. Endothelial lipase mainly carries phospholipase activity and its overexpression was shown to significantly reduce plasma HDL cholesterol levels [182-184]. Since Lipg is expressed in endothelial cells, macrophages and hepatocytes, regulation of hepatic Lipg by PPARα and PPARß/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 [185]. 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 density to phosphatidylcholines. In a recent publication, a role for Pctp in the metabolic response to PPARα was proposed [186]. Overall, it is evident that PPARα governs multiple aspects of plasma lipoprotein metabolism.

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 PPARα-/- mice display severe hypoglycemia [54, 55, 83]. 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), lactate dehydrogenase A [69]. Interestingly, regulation of Pck1 by PPARα was only observed in human hepatocytes [24]. Pyruvate carboxylase was identified as direct target of PPARγ in adipocytes [187].

PPARα was shown to have a specific role in the metabolic conversion of glycerol in liver by directly upregulating the expression of genes such as Gpd1, Gpd2, Gyk, Aqp3 and Aqp9 [188]. Besides governing glucose production, PPARα may also alter glucose utilization in numerous tissues via induction of pyruvate dehydrogenase kinase isoform 4 (Pdk4) [189-194]. Pdk4 phosphorylates and inactivates pyruvate dehydrogenase, thereby limiting carbon flux through glycolysis. Synthesis of glycogen is also affected in PPARα-/- mice, which may be mediated in part via defective regulation of Gys2 [195]. 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].

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, 196]. 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, 69, 197].

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 PPARα-/- mice in fasting condition [69]. Paradoxically, synthetic PPARα agonists reduce Cyp7a1 expression in both mice and human [198-201]. In agreement with the latter observation, fibrate treatment led 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 the 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 [202-204].

Other genes involved in bile acid synthesis that are regulated by PPARα include Cyp27a1 which is downregulated by PPARα agonists in PPARα dependent manner [201], and Cyp8b1 which is upregulated by PPARα [69, 205]. 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 [206]. Finally, PPARα stimulates expression of the hepatobiliary phospholipid transporter Abcb4 [24, 69, 97, 197].

PPARα and amino acid metabolism

Accumulating evidence supports a role for PPARα in regulation of amino acid and urea metabolism [207-210]. 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) [207, 210, 211]. In agreement with these data, PPARα-/- mice exhibit increased plasma urea levels [207]. 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 [207]. 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 [211, 212]. 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.

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 an anti-inflammatory effects of PPARα came from the observation that PPARα-/- 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 pro-inflammatory transcription factors including signal transducer and activator of transcription (Stat), Activator protein-1 (AP-1), and NF-κB [213]. 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 [214]. Additionally, PPARα induces the inhibitory protein IκBα, which normally retains NF-κB in a non-active form, leading to suppression of NF-κB DNA binding activity [215]. 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) [216]. 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 PPARα-/- mice [217]. 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 [216, 218, 219]. 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 [217].

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 [220]. 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 [221]. Epithelial Vanin-1 was also found to regulate inflammation-driven cancer development in a colitis-associated colon cancer model [222]. 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 PPARα-/- mice (Figure 3A). Negligible Vnn1 expression was detected in PPARα-/- mouse liver. Moreover, hepatic Vnn1 expression was significantly induced by 6h treatment with dietary fatty acids and by the synthetic PPARα agonists Wy14643 and fenofibrate (Figure 3B). Additional data lend strong support to the importance of PPARα in Vnn1 gene regulation in small and large intestine (Figure 3C, 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 down-stream of the transcription start site of the mouse Vnn1 gene were functional in a luciferase reporter assay in HepG2 cells (Figure 3E). 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 non-alcohol fatty liver disease and specifically non-alcoholic steatohepatitis (NASH). Several studies in mice have shown that PPARα activation can reduce or even reverse the progression of steatohepatitis [134, 223-228]. 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 [229]. In the absence of PPARα, liver steatosis and inflammation are enhanced in mice chronically fed a HFD [230]. 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.

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 [105, 111, 231]. 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 is carried out by sulfotransferases, UDP-glucuronosyltransferases (UGT), glutathione- S-transferases (GST) and N-acetyltransferases [231]. 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 [232, 233]. Studies have shown that peroxisome proliferators modulate exclusively Cyp4a class of monooxygenases (involved in the metabolism of biologically important compounds such as fatty acids, see Microsomal fatty acid

ω-hydroxylation) 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 [106, 234].

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 [235-237]. 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 [238]. 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 human.

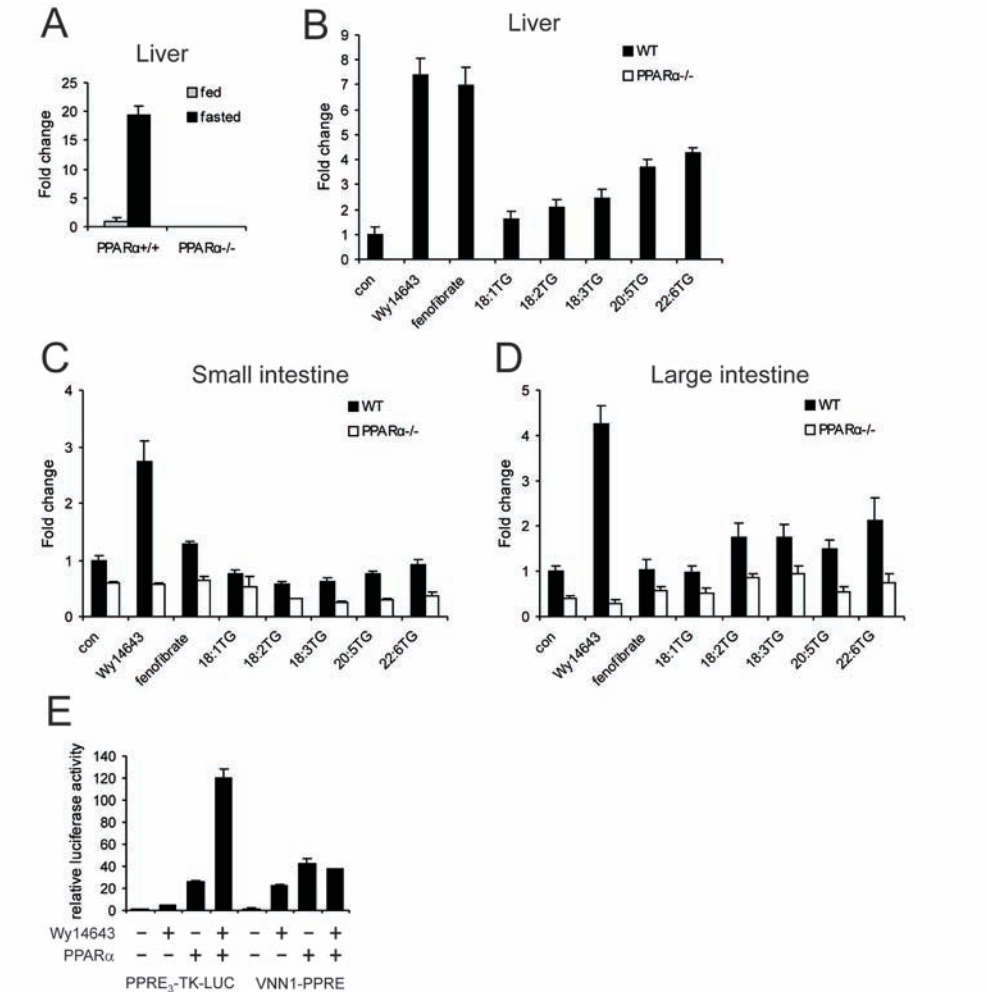

Figure 3. Vanin-1 likely represents a direct PPARα target gene. A) Vnn1 expression in livers of ad libitum fed and 24h fasted wildtype and PPARα-/- mice. B) Vnn1 expression in liver (B), small intestine (C) and large intestine (D) of wildtype and PPARα-/- mice 6h after administration of a single oral dose of Wy14643 (4mg), 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 h 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.

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 down-regulation 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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Chapter 3

Comprehensive analysis of PPARα-dependent regulation of hepatic lipid metabolism by expression profiling

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Abstract

PPARα is a ligand-activated transcription factor involved in the regulation of nutrient metabolism and inflammation. Although much is already known about the function of PPARα in hepatic lipid metabolism, many PPARα-dependent pathways and genes have yet to be discovered. In order to obtain an overview of PPARα-regulated genes relevant to lipid metabolism, and to probe for novel candidate PPARα target genes, livers from several animal studies in which PPARα was activated and/or disabled were analyzed by Affymetrix GeneChips. Numerous novel PPARα-regulated genes relevant to lipid metabolism were identified. Out of this set of genes, eight genes were singled out for study of PPARα-dependent regulation in mouse liver and in mouse, rat, and human primary hepatocytes, including thioredoxin interacting protein (Txnip), electron-transferring-flavoprotein ß polypeptide (Etfb), electron-transferring-flavoprotein dehydrogenase (Etfdh), phosphatidylcholine transfer protein (Pctp), endothelial lipase (EL, Lipg), adipose triglyceride lipase (Pnpla2), hormone-sensitive lipase (HSL, Lipe), and monoglyceride lipase (Mgll). Using an in silico screening approach, one or more PPAR response elements (PPREs) were identified in each of these genes. Regulation of Pnpla2, Lipe, and Mgll, which are involved in triglyceride hydrolysis, was studied under conditions of elevated hepatic lipids. In wild-type mice fed a high fat diet, the decrease in hepatic lipids following treatment with the PPARα agonist Wy14643 was paralleled by significant up-regulation of Pnpla2, Lipe, and Mgll, suggesting that induction of triglyceride hydrolysis may contribute to the anti-steatotic role of PPARα. Our study illustrates the power of transcriptional profiling to uncover novel PPARα-regulated genes and pathways in liver.

Introduction

The Peroxisome-Proliferator-Activated Receptors (PPARs) play a pivotal role in the regulation of nutrient metabolism. PPARs are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors [1-3]. They share a common mode of action that involves formation of heterodimers with the nuclear receptor RXR, followed by binding to specific DNA-response elements in the promoter of target genes. The genomic sequence recognized by PPARs, referred to as PPAR response element or PPRE, consists of a direct repeat of the consensus hexameric motif AGGTCA interspaced by a single nucleotide. Binding of ligands to PPARs leads to recruitment of co-activators and causes chromatin remodeling, resulting in initiation of DNA transcription and upregulation of specific PPAR target genes [4, 5]. Ligands for PPARs include both endogenous compounds, such as fatty acids and their eicosanoid derivatives, and synthetic agonists. Three different PPAR subtypes have been identified: PPARα, PPARβ/δ, and PPARγ. The latter isotype, which is most highly expressed in adipose tissue, is known to play an important role in adipocyte differentiation and lipid storage [6-8]. It is a target for an important class of antidiabetic drugs, the insulin-sensitizing thiazolidinediones. Expression of PPARβ/δ is ubiquitous and has been connected to wound healing, cholesterol metabolism, and fatty acid oxidation in adipose tissue and muscle [9-12]. Finally, PPARα is highly expressed in liver where it stimulates fatty acid uptake and activation, mitochondrial β–oxidation, peroxisomal fatty acid oxidation, ketogenesis, and fatty acid elongation and desaturation. In addition, it has a major role in glucose metabolism [13] and the hepatic acute phase response [14, 15]. Importantly, PPARα is the molecular target for the hypolipidemic fibrate class of drugs that lower plasma triglycerides and elevate plasma HDL (high density lipoprotein) levels.

In recent years, microarray technology has emerged as a powerful technique to study global gene expression. In theory, microarray analysis is a terrific tool to map PPARα-dependent genes and further characterize PPARα function. In practice, microarray yields a huge amount of data, the analysis and interpretation of which can be very difficult. Numerous studies have examined the effect of synthetic PPARα agonists on global gene expression using microarrays. While these studies uncovered many possible PPARα target genes, the manner in which the data were presented often rendered interpretation difficult. Part of the complexity is due to the size of the PPARα-dependent transcriptome in liver, which easily exceeds one thousand genes.

The aim of the present study was two-fold 1) to generate a comprehensive overview of PPARα-regulated genes relevant to hepatic lipid metabolism, and 2) to identify possible novel target genes and target pathways of PPARα connected with lipid metabolism. To that

end we 1) combined microarray data from several independent animal experiments involving PPARα-null mice. In these experiments, mice were either given Wy14643 or fasted for 24 hours; 2) focused on up-regulation of genes by PPARα in conformity with the general paradigm of transcriptional regulation by nuclear hormone receptors; 3) reduced complexity by progressively moving from the complete PPARα-dependent transcriptome towards genes relevant to lipid metabolism, and finally to the identification of possible PPARα target genes involved in lipid metabolism.

Methods and materials

Materials. Wy14643 was obtained from ChemSyn Laboratories (Lenexa, KS). Recombinant human insulin (Actrapid) was from Novo Nordisk (Copenhagen, Denmark). SYBR Green was from Eurogentec (Seraing, Belgium). DMEM, fetal calf serum, calf serum, and penicillin/streptomycin/fungizone were from Lonza Bioscience (Verviers, Belgium). Otherwise, chemicals were from Sigma (Zwijndrecht, The Netherlands).

Animals. Male pure-bred Sv129 and PPARα-null mice on a Sv129 background were used at 3-5 months of age (Jackson Laboratories, Bar Harbor, ME). Animals were fed normal laboratory chow (RMH-B diet, Arie Blok animal feed, Woerden, the Netherlands). Study 1: Fed mice were killed at the end of the dark cycle. Fasting was started at the onset of the light cycle for 24 hours (n=5 per group). Study 2 and 4: wild-type and PPARα-null mice were fed with Wy14643 for 5 days by mixing it in their food (0.1%, n=5 per group). Study 2 and 4 were carried out independently and 2 years apart. Study 3: wild-type and PPARα-null mice fasted for 4 hours received a single dose of Wy14643 (400 µl of 10 mg/ml Wy14643 dissolved in 0.5% carboxymethylcellulose) and were killed 6 hours later (n=5 per group).

Study 5: wild-type and PPARα-null mice at 2-3 months of age were given a high fat diet (D12451, Research Diets, New Brunswick, NJ) for 20 weeks (composition available at http://www.researchdiets.com/pdf/Data%20Sheets/DIO%20Series.pdf). During the last week, half of the mice were given Wy14643 for 7 days by mixing it in their food (0.1%, n=5 per group). Livers were dissected and immediately frozen in liquid nitrogen.

All animal experiments were approved by the animal experimentation committee of Wageningen University and were carried out in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals.

Primary hepatocytes. Rat (Wistar) and mouse (Sv129) hepatocytes were isolated by two-step collagenase perfusion as described previously [16]. Cells were plated on collagen-coated six-well plates. Viability was determined by Trypan Blue exclusion, and was at least 75%. Hepatocytes were suspended in William's E medium (Lonza Bioscience, Verviers, Belgium) supplemented with 10% (v/v) foetal calf serum, 20 m-units/ml insulin, 50 nM dexamethasone, 100 U/mL penicillin, 100 µg/mL of streptomycin, 0.25 µg/ml fungizone and 50 µg/ml gentamycin. The next day, cells were incubated in fresh medium in the presence or absence of Wy14643 (10 µM) dissolved in DMSO for 24 hours, followed by RNA isolation.

Human hepatocytes and Hepatocyte Culture Medium Bulletkit were purchased from Lonza Bioscience (Verviers, Belgium). Human hepatocytes were isolated from a single donor. Cells were plated on collagen-coated six-well plates. Upon arrival of the cells, the medium was discarded and was replaced by Hepatocyte Culture Medium. The next day, cells were incubated in fresh medium in the presence or absence of Wy14643 (50 µM) dissolved in DMSO for 12 hours, followed by RNA isolation.

Affymetrix microarray. Total RNA was prepared from mouse livers and primary hepatocytes using TRIzol reagent (Invitrogen, Breda, The Netherlands). RNA was either pooled per group or treatment (study 1 and 2, primary hepatocytes), or used individually (study 3 and 4), and further purified using RNeasy micro columns (Qiagen, Venlo, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) using 6000 Nano Chips according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number > 8.0). Ten micrograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing and scanning of Affymetrix Genechip MOE430 (study 1 and 2) or mouse genome 430 2.0 arrays (study 3 and 4) was according to standard Affymetrix protocols.

Scans of the Affymetrix arrays were processed using packages from the Bioconductor project [17]. Expression levels of probe sets were calculated using GCRMA [18], followed by identification of differentially expressed probe sets using Limma [19]. Comparison was between fasted wild-type and fasted PPARα-null mice (study 1) or between Wy14643-treated wild-type and Wy14643-treated PPARα-null mice (study 2-4). P-values were corrected for multiple testing using a false discovery rate method [20]. Probe sets that satisfied the criterion of FDR < 1% (q-value < 0.01) and fold-change >1.5 were considered to be significantly regulated. Functional clustering of the array data was performed by a method based on over-representation of Gene Ontology (GO) terms [21].

For the primary hepatocytes, expression levels were calculated applying the multi-chip modified gamma model for oligonucleotide signal (multi-mgMOS) [22] and a remapped chip description file [23].

All microarray datasets were deposited to Gene Expression Omnibus. The GEO series accession numbers are as follows: study 1: GSE8290, study 2: GSE8291, study 3: GES 8292, study 4: GSE8295, primary hepatocytes: GSE8302.

RNA isolation and Q-PCR. Total RNA was extracted from tissues with TRIzol reagent (Invitrogen, Breda, the Netherlands). 1 µg of total RNA was reverse-transcribed with iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was PCR-amplified with Platinum Taq DNA polymerase (Invitrogen) on a Bio-Rad iCycler or MyIQ PCR machine. Primers were designed to generate a PCR amplification product of 100-200 bp and were taken from Primerbank (http://pga.mgh.harvard.edu/primerbank). Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. The sequence of primers used is available upon request. The mRNA expression of all genes reported was normalized to 36B4 or cyclophilin gene expression.

In silico screening of putative PPREs using a PPRE classifier. Genomic sequences for mouse genes spanning 20 kB centered at the transcriptional start site (TSS) were extracted from the Ensembl database (NBCI36) and screened for DR1-type REs with predicted binding strength of at least 1%. The binding strength prediction was based on a PPRE classifier that uses a database of in vitro binding data for PPARs to assign predicted binding strength according to a classification scheme [24]. The conservation of the putative PPREs between mouse, human, dog and rat were evaluated using the Vertebrate Multiz Alignment and Conservation track available from UCSC genome browser (NCBI releases for human and mouse genomes, hg18 and mm8, February 2006).

Histological examination of liver. 5µ sections were cut from frozen liver pieces. For Oil Red O staining, sections were air dried for 30 minutes, followed by fixation in formal calcium (4% formaldehyde, 1% CaCl2). Oil Red O stock solution was prepared by dissolving 0.5 g Oil Red O in 500 mL isopropanol. A Oil Red O working solution was prepared by mixing 30 mL Oil Red O stock with 20 mL dH2O. Sections were immersed on working solution for 10 minutes followed by extensive washes in H2O. Haematoxylin and Eosin staining of frozen liver sections were carried out as described (http://www.ihcworld.com/histology.htm).

Results

Global analysis of PPARα-dependent gene regulation

We analyzed the data from 4 independent microarray studies to obtain a comprehensive picture of PPARα-dependent up-regulation of gene expression in mouse liver. In the first study, mRNA was compared between livers of 24-hour fasted wild-type and PPARα-null mice. In the second study, mRNA was compared between liver of wild-type mice and PPARα-null mice fed the PPARα agonist Wy14643 for 5 days. In these two studies, RNA was pooled from 4-5 mice and hybridized to Affymetrix MOE430A GeneChip arrays. Since no biological replicates were analyzed, only a fold-change threshold criteria could be applied. Using a cut-off of 1.5-fold, expression of a total of 1847 probesets was lower in 24-hour fasted PPARα-null mice compared with 24-hour fasted wild-type mice (Figure 1A) (http://nutrigene.4t.com/microarray/ppar2007/).

Using the same cut-off, 2234 probesets were at least 1.5-fold lower in the livers of PPARα-null mice fed Wy14643 compared to wild-type mice fed Wy14643 (http://nutrigene.4t.com/microarray/ppar2007/). The number of probesets that overlapped between the two groups was 569. A large proportion of these genes, which are thus under control of PPARα under pharmacological and physiological conditions, may represent target genes of PPARα.

In the third study, mRNA was compared between livers of wild-type mice and PPARα-null mice treated with Wy14643 for 6 hours, while in the fourth study mRNA was compared between livers of wild-type mice and PPARα-null mice fed Wy14643 for 5 days. Study 4 was carried out independent of study 2 in a different set of mice. For these two studies, biological replicates (4-5 mice per group) were run using Affymetrix mouse genome 430 2.0 GeneChip array, enabling statistical analysis of the data which was not possible for study 1 and 2. Applying a false discovery rate of 0.01 and a 1.5-fold cut-off, 1679 probesets were lower in the livers of PPARα-null mice compared to wild-type mice 6 hours after treatment with Wy14643, and 2207 probesets after 5 days of feeding Wy14643 (Figure 1B) (http://nutrigene.4t.com/microarray/ppar2007/). While the majority of genes regulated by PPARα after 6 hours of Wy14643 treatment were also, and generally more significantly, regulated after 5 days of Wy14643 treatment (overlap of 1001 probesets), many genes were specifically or more significantly regulated after 6 hours, including the direct PPAR target G0S2 and the EL gene, respectively. The complete set of data from study 2 and 4, which includes up- and down-regulated genes, has been submitted to the Peroxisome Proliferators compendium assembled by Dr. J.C. Corton (US EPA, Research Triangle Park, USA). They will be analyzed in conjunction with numerous other microarray experiments involving per-

oxisome proliferators to obtain the "peroxisome proliferator transcriptome". In addition, the datasets have been submitted to Gene Expression Omnibus.

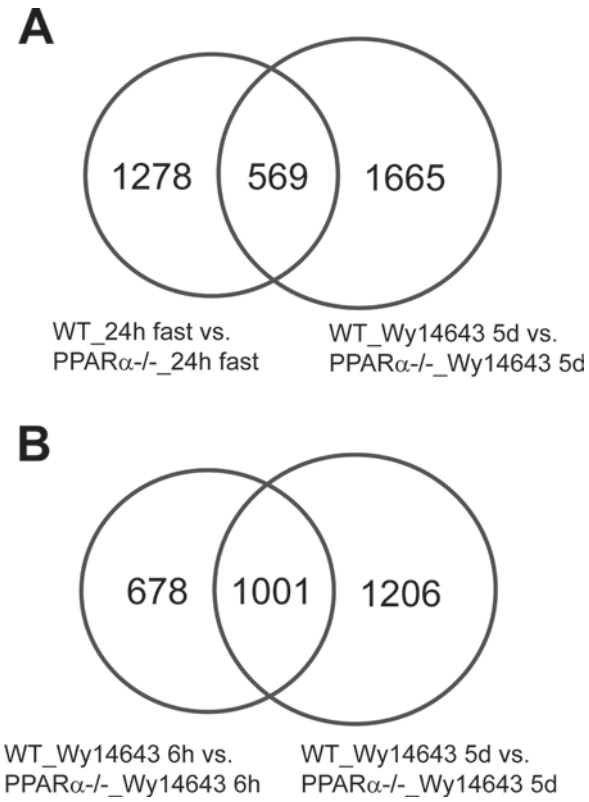

Figure 1: Microarray analysis of PPARα-dependent gene regulation in mouse liver. A. Venn diagram showing the number of differentially expressed probesets between livers of 24-hour fasted wild-type and PPARα-null mice, and between wild-type and PPARα-null mice treated with the PPARα agonist Wy14643 for 5 days. Pooled RNA was hybridized to Affymetrix MOE430A arrays. A fold-change of > 1.5 was used as cut-off. B. Venn diagram showing the number of differentially expressed probesets between livers of wild-type and PPARα-null mice treated with the PPARα agonist Wy14643 for 6 hours, and between wild-type and PPARα-null mice treated with the PPARα agonist Wy14643 for 5 days. RNA from individual mice was hybridized to mouse 430 2.0 arrays. Probesets that satisfied the criteria of fold-change > 1.5 and FDR < 0.01 were considered to be significantly regulated.

Pathway analysis of PPARα-dependent gene regulation

Functional clustering analysis of the microarray data by Gene Ontology classification indicated that numerous Gene Ontology classes were over-represented among the genes that were >1.5-fold up-regulated in 24-hour fasted wild-type compared to 24-hour fasted PPARα-null mice. The same was true for the comparison between wild-type and PPARα-null mice treated with Wy14643 for 5 days. Among the over-represented Gene Ontology classes we found many classes that are known to be governed by PPARα, including fatty acid beta-oxidation, acyl-CoA metabolism, leukotriene metabolism and peroxisome organization and biogenesis (http://nutrigene.4t.com/microarray/ppar2007/). Interestingly, we also noticed that numerous Gene Ontology classes were specifically up-regulated by PPARα under fasting conditions or by Wy14643 feeding. The data suggest, for example, that pyruvate metabolism and posttranslational protein targeting to membrane are specifically regulated in a PPARα-dependent manner by Wy14643 but not by fasting. Indeed, it is clear that some genes (for example Acot2 and Cd36) are PPARα-dependently regulated by Wy14643 and much less so by fasting, whereas others (for example Gpam, Hmgcs2) are PPARα-dependently regulated by fasting and much less so by Wy14643. However, it is important to emphasize that the ErmineJ Gene Ontology classification, as any functional clustering analysis, needs to be interpreted carefully.
The Gene Ontology classification analysis of the comparison wild-type vs. PPARα-null mice treated with Wy14643 for 6 hours (study 3) was almost identical to the analysis for mice treated with Wy14643 for 5 days (study 4), suggesting that most of the gene expression changes elicited by Wy14643 treatment are fast transcriptional responses in correspondence with direct regulation of gene expression by PPARα. One notable exception was the class representing the acute phase response, which was regulated by 5-day but not 6-hour treatment with Wy14643.

Comprehensive list of PPARα-targets involved in lipid metabolism

Using these lists of genes that are up-regulated by PPARα in mouse liver we were able to create a comprehensive picture of PPARα-regulated genes connected with lipid metabolism. Genes in bold are PPARα-dependently regulated by Wy14643 and during fasting, representing a conservative list of PPARα targets (Figure 2). Genes in normal font are PPARα-dependently regulated in any of the four studies included. From this picture it is evident that rather than merely regulating the rate limiting enzyme in fatty acid oxidation, PPARα appears to regulate virtually every single step in the peroxisomal and mitochondrial fatty acid oxidation pathway. Furthermore, many genes involved in fatty acid binding and activation, lipid transport, and glycerol metabolism, were controlled by PPARα. What is remarkable is

that PPARα also governs the expression of numerous genes involved in the synthesis of fats, which runs counter to the idea that PPARα mainly regulates fat catabolism. Several genes belonging to the lipogenic pathway have previously been recognized as PPARα targets, including Mod1 and Scd1, yet the extent of regulation by PPARα is unexpected [25]. Regulation of lipogenesis by PPARα was mainly observed after Wy14643 treatment, and to a much lesser extent after fasting.

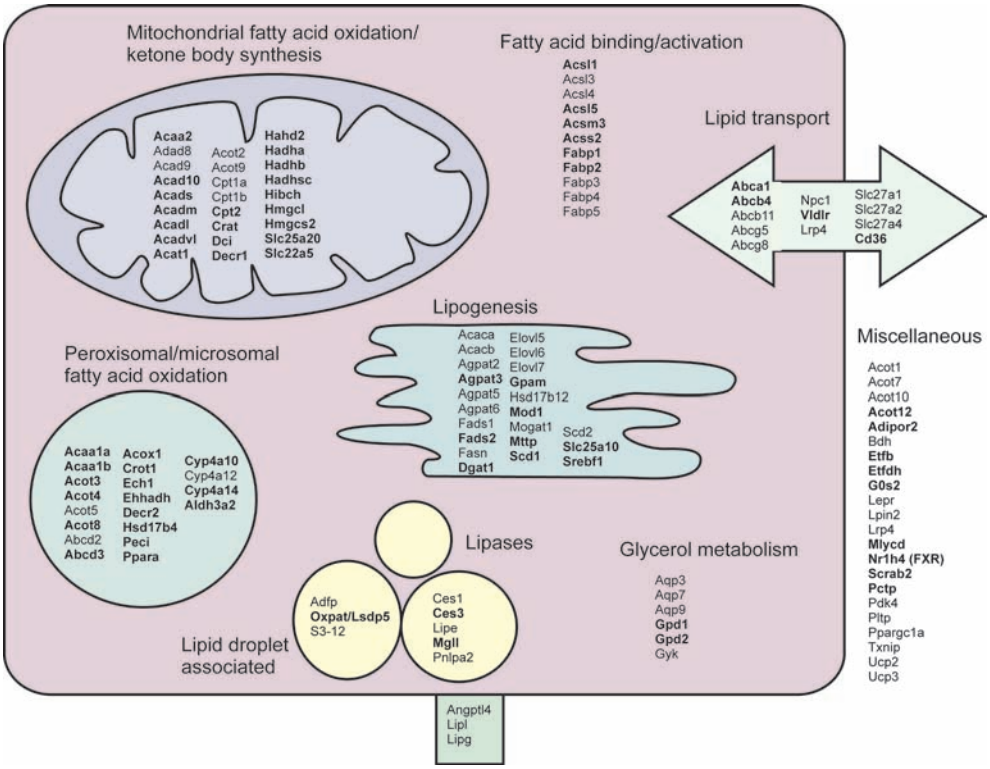

Figure 2: Overview of PPARα-regulated genes involved in hepatic lipid metabolism. Genes in bold are PPARα-dependently regulated during fasting and by Wy14643, representing a conservative list of PPARα targets. Genes in normal font are PPARα-dependently regulated in any of the four studies included. Functional classification is based on a self-made functional annotation system of genes involved in lipid metabolism (http://nutrigene.4t.com/microarray/ppar2007).

Novel putative targets of PPARα involved in lipid metabolism

In addition to providing an overview of PPARα-dependent gene regulation, we were interested in identifying novel PPARα-regulated genes that are implicated in lipid metabolism. To that end, we went through the array data from study 1 and 2 on the one hand, and study 3 and 4 on the other hand, and selected a number of genes to generate a heat map showing their PPARα-dependent up-regulation by fasting and/or Wy14643 (Figure 3). To our knowledge none of the genes shown, all of which are involved in hepatic lipid metabolism, has yet been reported to be regulated by PPARα. This includes phosphatidylcholine transfer protein (lipoprotein metabolism), glycerol-3-phosphate acyltransferase (triglyceride synthesis), very low-density lipoprotein receptor, choline phosphotransferase (phosphatidylcholine synthesis), and leptin receptor. Since all of these genes, except Abcg5, Abcg8 and Lipe, were up-regulated 6 hours after Wy14643 treatment, they possibly represent novel direct target genes of PPARα in liver, although PPREs have yet to be identified in their respective gene promoters.

Eight genes (shown in bold, Figure 3) were selected for more detailed investigation of PPARα-dependent gene regulation. Three of these genes are expected to be involved in the breakdown of hepatic triglycerides towards fatty acids: adipose triglyceride lipase (Pnpla2), hormone sensitive lipase (Lipe), and monoglyceride lipase (Mgll). Recent studies suggest that this threesome of genes is responsible for adipose tissue lipolysis [26-28]. In addition, we selected endothelial lipase (EL, Lipg), a recently identified member of triglyceride lipase gene family that is a major determinant of plasma HDL cholesterol [29-31], and electron transferring flavoprotein dehydrogenase (Etfdh) and electron transferring flavoprotein ß polypeptide (Etfb), which are components of the electron transport chain and accept electrons from at least nine mitochondrial matrix flavoprotein dehydrogenases [32, 33]. Finally, we selected phosphatidylcholine transfer protein (Pctp), which is involved in lipoprotein metabolism, and thioredoxin interacting protein (Txnip), which was recently identified as a major regulator of the hepatic response to fasting, similar to PPARα. The selection of these genes was based entirely on perceived novelty and potential functional importance of the observed regulation. Using real-time quantitative PCR (Q-PCR) we confirmed that the expression of all 8 genes in liver was increased by Wy14643 feeding in a PPARα-dependent manner (Figure 4A). In addition, we measured regulation of expression of this set of genes by PPARα during the course of fasting (Figure 4B). Expression of all 8 genes went up during fasting which, except for Pnpla2, was PPARα-dependent. However, the pattern of expression was remarkably different between the various genes, suggesting for each gene a complex and unique interplay between several fasting-dependent transcription factors, including PPARα.

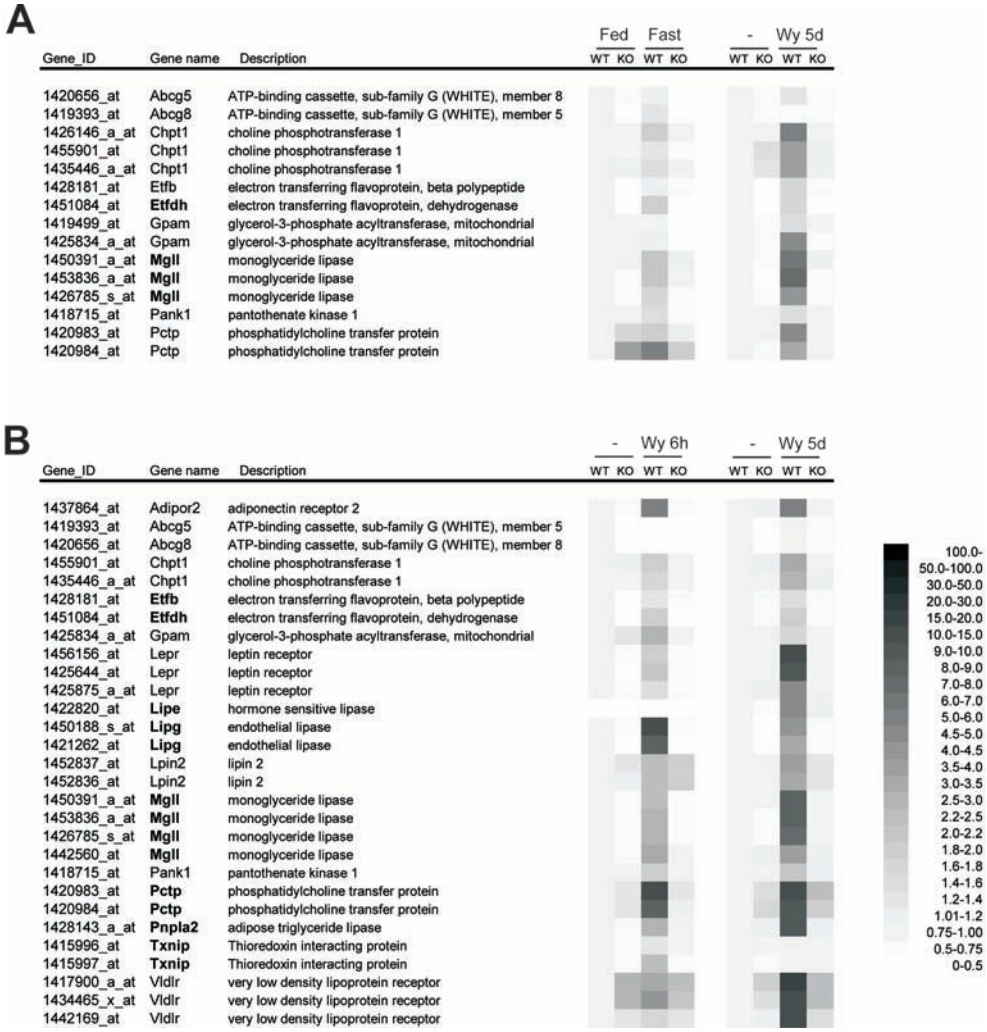

Figure 3: PPARα-dependent regulation in mouse liver of selected genes involved in lipid metabolism as shown by heat map. The (GCRMA normalized) expression data were derived from 4 separate microarray studies. Expression levels in wild-type mice without treatment were set at 1. A. Expression data derived from study 1 and 2. B. Expression data derived from study 3 and 4. Genes in bold were selected for expression analysis by Q-PCR and in silico screening for putative PPREs.

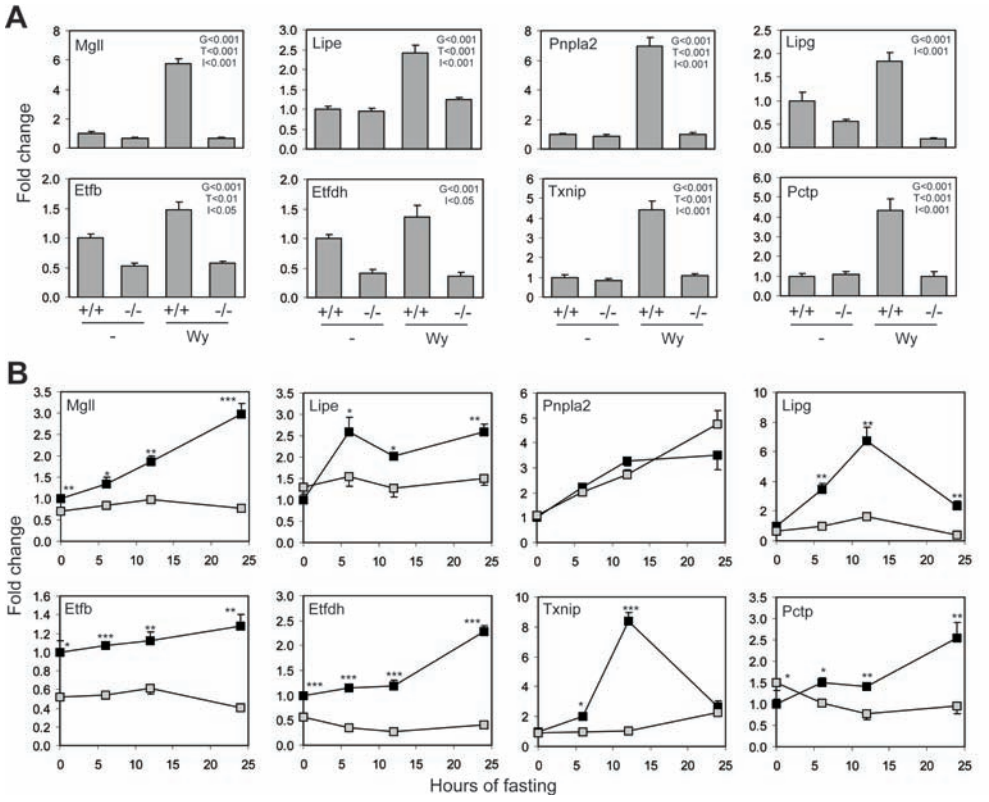

Figure 4: PPARα governs expression of selected genes in mouse liver.

A. Regulation of expression of selected genes by Wy14643-feeding (5 days) in liver of wild-type (+/+) and PPARα-null mice (-/-), as determined by Q-PCR. Error bars represent SEM. Differences were evaluated statistically using two-way ANOVA. Significance (p-value) of effect of genotype (G), treatment (T) and interaction (I) between genotype and treatment is indicated in each figure. B. Regulation of expression of selected genes by fasting in liver of wild-type (■) and PPARα-null mice (□), as determined by Q-PCR. Error bars represent SEM. Differences in expression between wild-type and PPARα-null mice at each time point were evaluated by Student t-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

PPARα-dependent regulation in primary hepatocytes

To examine whether the PPARα-dependent regulation of the set of genes shown in Figure 3 was not an indirect consequence of metabolic perturbations elicited by the experimental challenge, we studied the effect of PPARα activation in primary mouse, rat and human hepatocytes. Gene expression was first analyzed by microarray (Figure 5A), followed by targeted analysis of the selected 8 genes by Q-PCR (Figure 5B). Expression levels were calculated by applying a multi-chip modified gamma model for oligonucleotide signal (multi-mgMOS) [22] and a remapped chip description file [23] to allow for parallel analysis of the same gene within different species. Expression of almost every gene studied was highly up-regulated by Wy14643 in mouse and rat hepatocytes, compared to a more modest or no induction in human hepatocytes. For reasons that are not completely clear, in human hepatocytes, data from Q-PCR and microarray did not always perfectly align. Overall, the data indicate that the PPARα-dependent regulation observed in vivo can be reproduced in primary hepatocytes. Furthermore, the data suggest that expression of 6 genes is governed by PPARα in human as well.

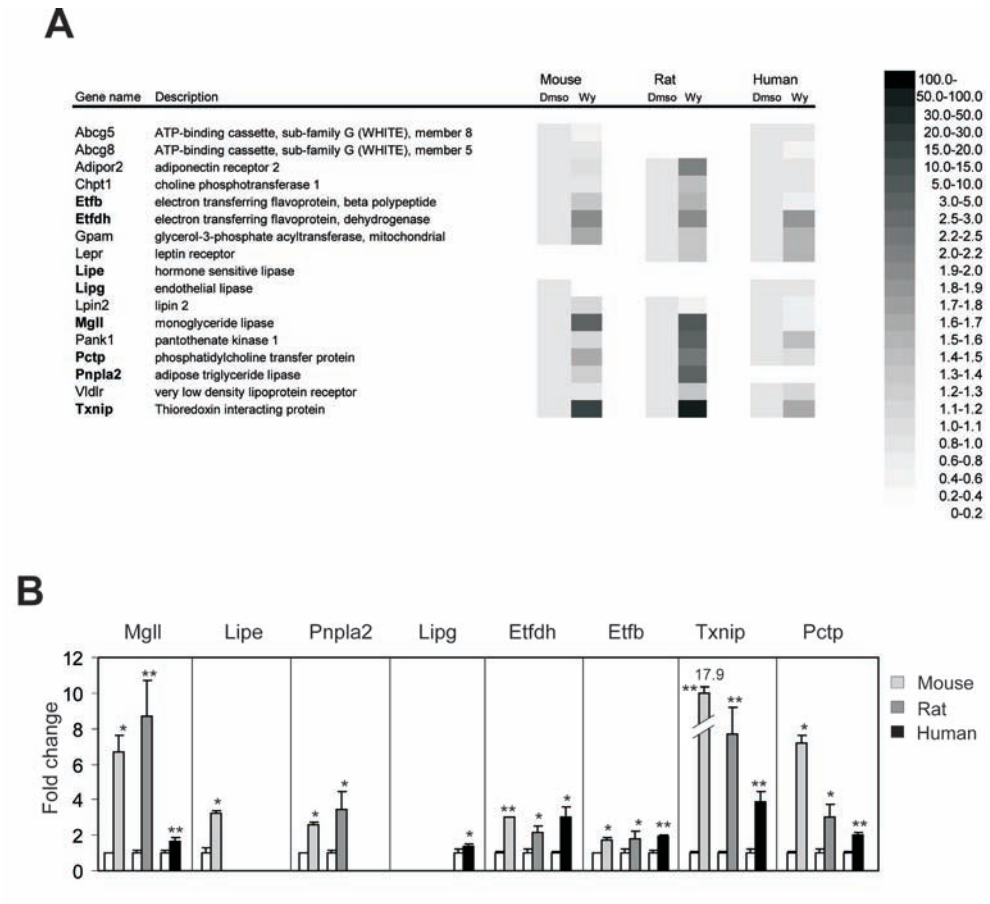

Figure 5: Regulation of selected genes involved in lipid metabolism in primary hepatocytes by Wy14643. A. Microarray-based heat map showing relative expression levels of genes calculated using a multi-chip modified gamma model for oligonucleotide signal (multi-mgMOS) and a remapped chip description file. Expression levels in the absence of ligand were set at 1. B. Relative induction of expression of selected genes in primary hepatocytes by Wy14643, as determined by Q-PCR. The primary hepatocytes used for Q-PCR and microarray analysis were from independent experiments. Genes were not included when expression was extremely low (Ct>30). Error bars represent SD. The effect of Wy14643 on gene expression was evaluated by Student t-test. * $p<0.05$; ** $p<0.01$.

In silico screening of putative PPREs

To evaluate whether the selected eight genes represent possible direct PPAR target genes, the (mouse) genes were analyzed for the presence of putative PPREs using an in silico screening method (Figure 6). Ten kb up- and downstream of the TSS were examined. For each putative PPRE identified, the predicted PPAR subtype specific binding strength was determined. For each gene, at least one PPRE was identified that was conserved among rat, dog and human. The Etfdh and Txnip genes were characterized by the presence of two very strong putative PPREs that were conserved in human. Up to six putative PPREs were identified in the Mgll gene, only one of which was conserved in human. A similar picture was found for Pnpla2. The putative PPREs located in the EL gene were weak and generally not conserved. Interestingly, a strong putative PPRE was identified in the Pctp gene, which however was not conserved in human. Conversely, the human Pctp gene contained several putative PPREs that were not conserved in mouse (data not shown).

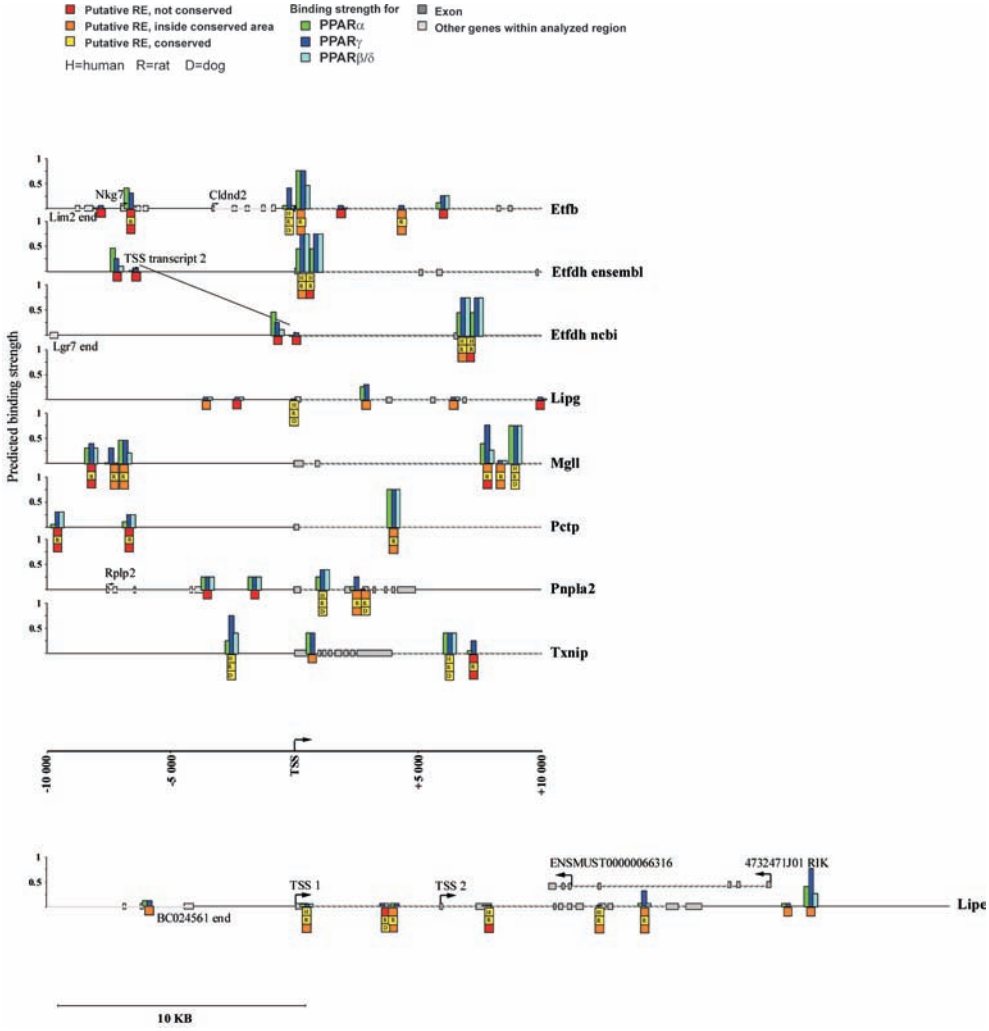

Figure 6: In silico screening for putative PPREs for the selected 8 genes, 10 kb up- and downstream of the transcriptional start site were examined for the presence of putative PPREs. For each putative PPRE identified, the predicted PPAR subtype specific binding strength was determined, as reflected by the height of the bar. The sequence conservation of the PPRE among various species is indicated.

PPARα activation prevents hepatic lipid storage after fasting

Our data extend the role of PPARα in hepatic lipid metabolism and suggest that PPARα may govern triglyceride hydrolysis. To find out whether activation of the triglyceride hydrolysis pathway by PPARα is associated with a decrease in hepatic triglyceride stores, we compared wild-type and PPARα-null mice fed a HFD for 20 weeks, followed by treatment for one week with Wy14643. Numerous studies, including ours [34], have shown that chronic HFD increases hepatic triglyceride stores. In wild-type mice fed the HFD, treatment with Wy14643 markedly decreased hepatic lipids (Figure 7A,B), as shown by smaller lipid droplets, which was paralleled by significant induction of expression of Pnpla2, Lipe, and Mgll (Figure 7C). These data suggest that induction of the triglyceride hydrolysis pathway may contribute to the overall reduction in liver triglycerides elicited by PPARα activation.

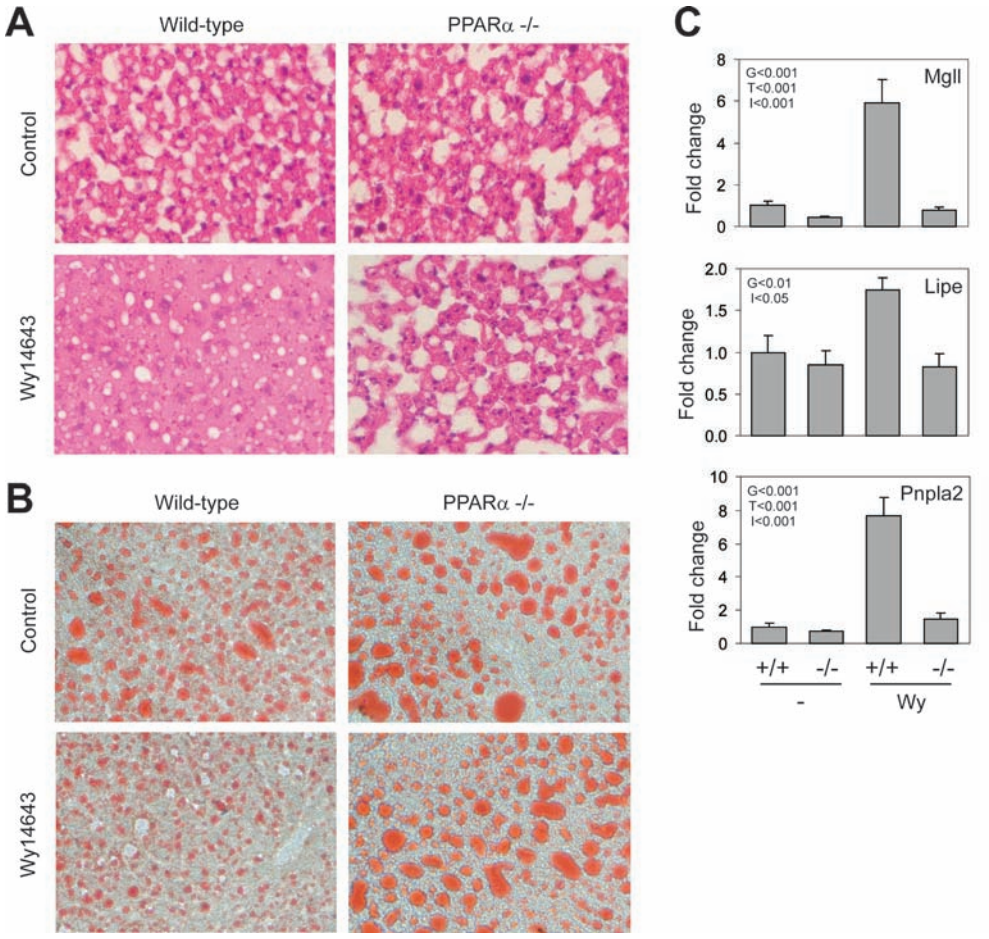

Figure 7: Induction of the triglyceride hydrolysis pathway by Wy14643 is paralleled by a decrease in hepatic lipid stores. Hematoxilin and eosin staining (A) and oil red O staining (B) of representative liver sections of wild-type and PPARα-null mice treated or not with Wy14643 for 7 days (magnification 200X). All mice were given a HFD for 20 weeks prior to Wy14643 treatment. (C) Hepatic expression of Mgll, Lipe, and Pnpla2 in the 4 experimental groups as determined by Q-PCR. Error bars represent SEM. Differences were evaluated statistically using two-way ANOVA. Significance (p-value) of effect of genotype (G), treatment (T) and interaction (I) between genotype and treatment is indicated in each figure.

Discussion

The aim of our study was two-fold 1) to generate a comprehensive overview of PPARα-regulated genes relevant to hepatic lipid metabolism, and 2) to identify possible novel target genes and target pathways of PPARα connected with lipid metabolism.

It can be argued that to identify possible novel PPARα targets the proper comparison should have been between wild-type and wild-type treated with Wy14643, as opposed to wild-type treated with Wy14643 and PPARα-null treated with Wy14643, in order to avoid inclusion of genes that are differentially expressed between wild-type and PPARα-null mice under basal conditions (and could represent genes indirectly regulated by PPARα). The rationale behind our decision was that we wanted to be open-minded about the PPARα dependent transcriptome and not exclude genes that are solely regulated by PPARα under basal conditions. For example, opting for the comparison wild-type vs. wild-type treated with Wy14643 would have led to the exclusion of Etfdh, which according to our data represents a prime candidate PPARα target gene in mouse and human. Furthermore, to enable comparison between the effects of fasting and Wy14643 it was essential to include the PPARα dependency, since the majority of genes regulated by fasting are regulated in a PPARα-independent manner.

Gene Ontology classification analysis showed that numerous pathways and biological processes beyond lipid metabolism were regulated by PPARα. We observed that the expression of almost 1700 probesets was significantly increased 6 hours after a single oral dose of Wy14643. Although not all genes regulated may represent direct PPARα targets, and even though the functional consequences of the observed regulation still needs to be demonstrated, these data at least suggest a major role for PPARα in hepatic gene expression and overall liver homeostasis.

In agreement with the first aim, we created a comprehensive overview of hepatic PPARα-regulated genes connected to lipid metabolism (Figure 2). A functional PPRE has been found in the promoter of several of these genes, classifying them as direct PPARα target genes, and many more genes have been shown to be up-regulated by PPARα without a functional PPRE having been identified [25]. It can be presumed that the far majority of genes presented in Figure 2 (as well as the other genes that were shown to be regulated by PPARα) are actually direct target genes of PPARα, but it is beyond the scope and capacity of the present study to address this issue in more detail. Our hope is that by combination of expression arrays with global analysis of promoter occupancy by PPARα using chromatin immunoprecipitation and tiling or promoter arrays (so called ChIP-on Chip analysis), the complete picture of direct PPARα target genes will be available in the future.

The second aim of our study was to identify possible novel target genes of PPARα representing specific steps in lipid metabolism unknown to be governed by PPARα. As part of this effort, we identified several genes for which a link with PPARα has not yet been reported, including VLDL receptor, leptin receptor, and choline phosphotransferase. We focused our energy on 8 genes for which regulation by PPARα was deemed most novel and functionally interesting. All 8 genes, except for Lipe, were significantly upregulated 6 hours after treatment with Wy14643.

Using an in silico method to screen for PPREs, for each gene several putative PPREs could be located within 10 kb of the transcriptional start site. Within this region, at least one PPRE was identified that was conserved among rat, dog and human. The presence of multiple strong putative PPREs within the mouse Mgll gene is in correspondence with the marked regulation of Mgll expression in mouse liver and isolated hepatocytes. To a lesser extent, this is also true for the Pnpla2 and Pctp genes. Furthermore, the predicted presence of 2 strong, well conserved putative PPREs in the Etfdh and Txnip genes is in agreement with the highest fold-induction of these genes by Wy14643 in primary human hepatocytes. Although in silico screening may not be able to substitute for analysis of direct promoter binding by ChIP, the predictive power of the method explored has been shown to be remarkably robust [24]. Our results also substantiate the developing notion that PPAR-dependent gene regulation is generally mediated by multiple PPREs, rather than a single PPRE.

One remarkable outcome of the global analysis of gene regulation by PPARα is that PPARα appears to play a major role in governing lipogenesis. While several genes involved in lipogenesis were already known as PPARα targets, including Δ5 and Δ6 desaturase (Fads), stearoyl-CoA desaturase (Scd), microsomal triglyceride transfer protein (Mttp), and malic enzyme (Mod1) [25], the extent of regulation of lipogenesis is somewhat surprising, especially since PPARα is generally considered to stimulate fat catabolism rather than fat synthesis. It can be speculated that upregulation of fatty acid desaturation and elongation enzymes by PPARα might serve to stimulate production of PPARα ligands, and is part of a feed-forward action of PPARα that also includes auto-regulation of gene expression.

Although the triglyceride hydrolysis pathway in liver still has to be fully elucidated, it may very well be similar to the pathway operating in adipose tissue [28]. Adipose tissue triglycerides are likely hydrolyzed in a three-step process catalyzed by adipose triglyceride lipase (Pnpla2), hormone sensitive lipase (Lipe), and monoglyceride lipase (Mgll) [26-28, 35]. Remarkably, deletion of the Pnpla2 gene in mice not only results in more adipose mass but also causes a marked increase in lipid storage in a variety of organs, including liver and heart, suggesting that the triglyceride hydrolysis pathway is conserved between various organs [28].

Disabling the PPARα gene is known to increase hepatic triglyceride accumulation, especially under conditions of fasting [34, 36, 37]. Conversely, treatment with PPARα agonists lowers hepatic triglyceride levels in various models of hepatic steatosis [38-41]. The anti-steatotic effect of PPARα has generally been ascribed to stimulation of fatty acid oxidation, which, by decreasing intracellular fatty acid levels, will act as a drain on intracellular triglyceride stores. However, our data suggest that PPARα may directly govern the triglyceride hydrolysis pathway in liver via up-regulation of lipases Pnpla2, Lipe, Mgll, and possibly Ces1 and Ces3 (Figure 2). Although it is impossible to provide definite experimental proof that induction of the triglyceride hydrolysis pathway by PPARα, or induction of fatty acid oxidation for that matter, is necessary and sufficient for its hepatic triglyceride-lowering effect, it likely contributes to the overall reduction in liver triglycerides elicited by PPARα agonists.

Our data suggest that expression of EL is under control of PPARα. EL, synthesized in endothelial cells, plays an important role in governing plasma lipoprotein concentrations and is a major determinant of plasma HDL cholesterol and apoAI concentrations. Indeed, over- expression of EL in the liver results in a significant decrease in HDL cholesterol and apoAI [29-31]. EL has been shown to have some triglyceride lipase but mainly phospolipase activity [42]. Although in silico screening failed to detect a strong PPREs in this gene, in our study EL expression was highly increased by 6 hours Wy14643 treatment and by fasting in a PPARα-dependent manner, suggesting that EL may be a direct PPARα target gene. As EL expression was minimal in primary hepatocytes, EL transcripts likely originated from liver epithelial cells rather than liver parenchymal cells. Although further work is necessary, we suspect that EL may be a direct PPARα target in endothelial cells. Considering that, in contrast to EL, PPARα agonists raise plasma HDL, the functional importance of regulation of EL by PPARα needs to be further validated.

Another novel PPARα-regulated gene of relevance to lipoprotein metabolism is Pctp. Pctp is a steroidogenic acute regulatory-related transfer domain protein that binds phosphatidylcholines with high specificity. Studies with Pctp null mice suggest that it may modulate HDL particle size and rates of hepatic clearance [43]. According to our data, expression of Pctp increases during fasting, which is abolished in PPARα-null mice. Wy14643 markedly up-regulated Pctp mRNA in mouse liver as well as in mouse, rat and human hepatocytes, suggesting it may represent a novel PPARα target gene.

Etfdh and Etfb are essential components of the oxidative phosphorylation pathway. They are responsible for the electron transfer from at least 9 mitochondrial flavin-containing dehydrogenases to the main respiratory chain [32, 33]. According to our data, expression of Etfdh and Etfb is governed by PPARα, suggesting that besides the ß-oxidation pathway, PPARα also

regulates components of the respiratory chain involved in the transfer of electrons from fatty acids and other molecules.

The last gene that we studied in more detail was Txnip, which is also known as Hyplip1. A spontaneous mutation within the Txnip gene gives rise to a complex phenotype that resembles familial combined hyperlipidemia, including hypercholesterolemia and hypertriglyceridemia [44]. Recent studies suggest that Txnip plays an important metabolic role in the fasting-feeding transition by altering the redox status of the cell, which results in stimulation of the tricarboxylic acid cycle at the expense of ketone body or fatty acid synthesis [45]. Indeed, Txnip-deficient mice show elevated plasma ketones, elevated free fatty acids, hypercholesterolemia and hypertriglyceridemia, yet decreased glucose levels [44, 46]. The phenotype is very similar to that of PPARα-null mice, with the exception of the elevated plasma ketones. Since hepatic expression of Txnip is decreased in PPARα-null mice, it can be hypothesized that part of the effect of PPARα deletion on lipid and glucose metabolism is mediated by down-regulation of Txnip in liver, which subsequently might affect redox status. It is unclear to what extent Txnip expression is affected by PPARα deletion in tissues other than liver.
In conclusion, our data indicate that the role of PPARα in hepatic lipid metabolism is much more extensive than previously envisioned. By generating a schematic overview of PPARα-dependent gene regulation in mouse liver, and, for a selected set of genes, by providing evidence for direct regulation by PPARα in rodents and human, we have extended the role of PPARα in the control of hepatic lipid metabolism.

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Chapter 4

Comparative analysis of gene regulation by the transcription factor PPARα between mouse and human

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Abstract

Studies in mice have shown that PPARα is an important regulator of hepatic lipid metabolism and the acute phase response. However, little information is available on the role of PPARα in human liver. Here we set out to compare the function of PPARα in mouse and human hepatocytes via analysis of target gene regulation.

Primary hepatocytes from 6 human and 6 mouse donors were treated with PPARα agonist Wy14643 and gene expression profiling was performed using Affymetrix GeneChips followed by a systems biology analysis. Baseline PPARα expression was similar in human and mouse hepatocytes. Depending on species and time of exposure, Wy14643 significantly induced the expression of 362-672 genes. Surprisingly minor overlap was observed between the Wy14643-regulated genes from mouse and human, although more substantial overlap was observed at the pathway level. Xenobiotics metabolism and apolipoprotein synthesis were specifically regulated by PPARα in human hepatocytes, whereas glycolysis-gluconeogenesis was regulated specifically in mouse hepatocytes. Most of the genes commonly regulated in mouse and human were involved in lipid metabolism and many represented known PPARα targets, including CPT1A, HMGCS2, FABP1, ACSL, and ADFP. Several genes were identified that were specifically induced by PPARα in human (MBL2, ALAS1, CYP1A1, TSKU) or mouse (Fbp2, lgals4, Cd36, Ucp2, Pxmp4). Furthermore, several putative novel PPARα targets were identified that were commonly regulated in both species, including CREB3L3, KLF10, KLF11 and MAP3K8.

Our results suggest that PPARα activation has a major impact on gene regulation in human hepatocytes. Importantly, the role of PPARα as master regulator of hepatic lipid metabolism is generally well-conserved between mouse and human. Overall, however, PPARα regulates a mostly divergent set of genes in mouse and human hepatocytes.

Introduction

The liver plays a major role in the coordination of lipid metabolism. It actively metabolizes fatty acids as fuel and is responsible for triglyceride export via synthesis of very low density lipoproteins. An imbalance between these pathways may lead to triglyceride accumulation and thus hepatic steatosis. Studies in mice have indicated that many aspects of hepatic lipid metabolism are under transcriptional control of the Peroxisome Proliferator Activated Receptor α (PPARα), a transcription factor belonging to the nuclear receptor superfamily. It is well established that impaired PPARα function is associated with hepatic lipid accumulation [1-3]. Consequently, synthetic agonists for PPARα are explored for the treatment of steatosis [4].

Besides PPARα, two other PPARs isotypes are known to exist: PPARβ/δ and PPARγ. The PPARs share a common mode of action that involves heterodimerization with the nuclear receptor RXR, followed by binding to PPAR response elements (PPREs) in target genes [5]. Activation of transcription is induced by binding of ligand, leading to recruitment of specific coactivator proteins and dissociation of corepressors. Expression of PPARα and PPARβ/δ is relatively ubiquitous, whereas PPARγ is mainly expressed in adipose tissue, macrophages and colon [6,7].

PPARα can be ligand-activated by endogenous agonists, which include fatty acids and fatty acid derivatives such as eicosanoids and oxidized fatty acids, as well as by various synthetic compounds [5,8,9]. The latter group induces proliferation of peroxisomes in rodents and are thus referred to as peroxisome proliferators. Peroxisome proliferators encompass a diverse group of chemicals ranging from herbicides and insecticides to industrial plasticisers, halogenated hydrocarbons, and fibrate drugs [10,11].

Most of the research concerning PPARα has focused on its role in the liver. A wealth of studies performed almost exclusively in mice has revealed that PPARα serves as a key regulator of hepatic fatty acid catabolism (reviewed in [12]). Using PPARα null mice, it has been shown that PPARα is especially important for the adaptive response to fasting by stimulating hepatic fatty acid oxidation and ketogenesis [2,13,14]. In addition, PPARα has been shown to govern liver inflammation, lipoprotein metabolism, glucose metabolism, and hepatocyte proliferation [12,15,16]. The latter response is known to be specific for rodents [17]. The species-specific effects of PPARα agonists on hepatocyte proliferation and associated hepatocarcinogenesis were ascribed to a number of factors including properties intrinsic to the PPARα protein, conservation and functionality of PPREs in the promoter of target genes, and presence or absence of co-regulators depending on the cellular environment [18].

However, apart from the differential effect on hepatocyte and peroxisome proliferation, it is not very clear whether PPARα has a similar role in mice and humans and to what extent target genes are shared between the two species. Based on the lower expression level of PPARα in human liver compared to mouse liver [19], the functionality of PPARα in human liver has been questioned [20]. This notion has been further reinforced by the limited impact of PPARα agonists on lipid metabolism genes in HepG2 cells [21], which represent the most widely used liver cell culture model.

However, a careful and comprehensive comparative analysis of gene regulation by PPARα between mouse and human hepatocytes has yet to be performed. To fill this gap we systematically compared the effect of activation of the transcription factor PPARα in primary mouse and human hepatocytes using a whole genome transcriptomics approach. Overall, the results reveal that PPARα regulates a mostly divergent set of genes in mouse and human hepatocytes and suggest that caution should be exercised when extrapolating the function of a transcription factor from mouse to human.

Materials and methods

Materials. Wy14643 was obtained from ChemSyn Laboratories (Lenexa, KS). Recombinant human insulin (Actrapid) was from Novo Nordisk (Copenhagen, Denmark). SYBR Green was from Eurogentec (Seraing, Belgium). Fetal calf serum, penicillin/streptomycin/fungizone were from Lonza Bioscience (Verviers, Belgium). Otherwise, chemicals were from Sigma (Zwijndrecht, The Netherlands).

Human primary hepatocytes. Human hepatocytes and Hepatocyte Culture Medium Bulletkit were purchased from Lonza Bioscience (Verviers, Belgium). Primary hepatocytes were isolated from surgical liver biopsies obtained from six individual donors who underwent surgery after informed consent was obtained for surgery with subsequent use of samples in experiments. Lonza utilizes the hospital's Institutional Review Board (IRB) to obtain approval before obtaining these tissues. The characteristics of the donors are presented in Table 1. Hepatocytes were isolated with two-step collagenase perfusion method and the viability of the cells was over 80%.

Cells were plated on collagen-coated six-well plates and filled with maintenance medium. Upon arrival of the cells, the medium was discarded and was replaced by Hepatocyte Culture Medium (HCM) with additives. The additives included Gentamicin sulphate/Amphotercin-B, Bovine serum albumin (Fatty acid free), Transferrin, Ascorbic acid, Insulin, Epidermal

growth factor, Hydrocortisone hemisuccinate. The next day, cells were incubated in fresh medium in the presence or absence of Wy14643 (50 μM) dissolved in DMSO for 6 and 24 hours, followed by RNA isolation.

Mouse primary hepatocytes. Mouse hepatocytes were isolated by two-step collagenase perfusion as described previously [51] from 6 different strains of mouse; NMRI, SV129, FVB, DBA, BALB/C and C57BL/6J. The characteristics of the mice used are presented in Table 1.

Cells were plated on collagen-coated six-well plates. Viability was determined by Trypan Blue exclusion, and was at least 75%. Hepatocytes were suspended in William's E medium (Lonza Bioscience, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum, 20 m-units/mL insulin, 10 nM dexamethasone, 100 U/mL penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL fungizone and 50 μg/mL gentamycin. After four hours the medium was discarded and replaced with fresh medium. The next day, cells were incubated in fresh medium in the presence or absence of Wy14643 (10 μM) dissolved in DMSO for 6 and 24 hours, followed by RNA isolation. Isolation of mouse primary hepatocytes was approved by the animal ethics committee of Wageningen University. A 5-fold lower concentration of Wy14643 was used in mouse primary hepatocytes to take into account the higher affinity of Wy14643 for mouse PPARα compared to human PPARα [52].

Characteristics of the human liver donors

	Age (years)	Sex	Pathology
Donor 1	63	Male	Colo-rectal Metastasis
Donor 2	44	Male	Metastasis
Donor 3	70	Female	Hepatic metachrone lesion
Donor 4	54	Female	Metastasis
Donor 5	73	Male	Carcinome hepatocellular
Donor 6	73	Male	Endocrine carcinoma

Characteristics of the different mouse strains

Mouse strain	Age (months)	Sex
NMRI	4	Male
BL/6	2	Male
Sv129	4	Male
FVB	5	Female
Balb/c	6	Male
DBA	3	Female

Table1. Characteristics of human liver donors and mouse different strains.

Affymetrix microarray. Total RNA was prepared from human and mouse primary hepatocytes using TRIzol reagent (Invitrogen, Breda, The Netherlands). RNA was used individually and further purified using RNeasy micro columns (Qiagen, Venlo, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) using 6000 Nano Chips according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number > 8.0). Five hundred nanograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing and scanning of Affymetrix Gene chip mouse genome 430 2.0 arrays (mouse primary hepatocytes) and human genome U133 2.0 plus was according to standard Affymetrix protocols.

Scans of the Affymetrix arrays were processed using packages from the Bioconductor project [53]. Expression levels of probe sets were calculated using GCRMA, followed by identification of differentially expressed probe sets using Limma. Comparison was made between treated and untreated (control) human primary hepatocyte, the same was compared for mouse primary hepatocyte. Probe sets that satisfied the criterion of Raw $P < 0.05$ and a mean fold-change $> \pm 1.1$ were considered to be significantly regulated. These selection criteria were based on careful inspection of the fold-changes in expression and their statistical significance of some known PPARα target genes, including Acadvl, Fatp4, and Acox1, which barely exceeded these thresholds. Functional analysis of the array data was performed by a method based on overrepresentation of Gene Ontology (GO) terms [54-56] and Gene Set Enrichment analysis [57]. Orthologs were retrieved via Homologene (NCBI). HomoloGene is a system for automated detection of homologs among the annotated genes of several completely sequenced eukaryotic genomes.

All Microarray data reported in the manuscript is described in accordance with MIAME guidelines.

Q-PCR. 1 μg of total RNA was reverse-transcribed with iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was PCR-amplified with Platinum Taq DNA polymerase (Invitrogen) on a Bio-Rad iCycler or MyIQ PCR machine.
Primers were designed to generate a PCR amplification product of 100-200 bp and were taken from Primerbank (http://pga.mgh.harvard.edu/primerbank). Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. The sequence of primers used are provided in Supplementary Table 4. The mRNA expression of genes reported was normalized to universal 18S gene expression. To compare PPARα expression in mouse and human hepatocytes, primers were used that yielded amplicons of equal length. A standard curve was included to confirm amplification efficiency of 100%±2 for PPARα and for the 18S control gene. PPARα expression was calculated as 1/(2^(CtPPARα a-Ct18S)), allowing for direct comparison between the two species.
Human liver RNA was obtained via Ambion and represented a mixture of RNA from 3 individuals without liver disease. Mouse liver RNA was obtained from 5 male mice on mixed genetic background (C57Bl/6-Sv129, fed state).

Results

PPARα plays an important role in primary human hepatocytes

We first determined PPARα expression in mouse and human liver by quantitative real-time PCR. PPARα mRNA was only slightly lower in human liver compared to mouse liver (Figure 1A). In order to study the effect of PPARα activation on gene expression in human and mouse liver, primary human and mouse hepatocytes were incubated with the PPARα agonist Wy14643 for 6 or 24h. To minimize potential statistical bias, the diversity of the six human donors was mimicked by performing the equivalent mouse experiment in primary hepatocytes from six different mice varying in age, sex and genetic background (Table 1). The choice of using Wy14643 as PPARα agonist was based on a pilot experiment in which primary human hepatocytes were treated with equal concentrations of either Wy14643 or fenofibrate (50 μM). In general, we found that established PPARα target genes were more strongly induced by Wy14643 compared to fenofibrate (data not shown).

The expression of PPARα itself was similar between mouse and human hepatocytes (Figure 1B). While in mouse hepatocytes PPARα mRNA decreased during the course of the incubation, the opposite was the case in human hepatocytes. Treatment with Wy14643 consistently increased the expression of the established PPARα targets Cpt1a and Pdk4 in mouse and human hepatocytes, indicating activation of PPARα (Figure 1C).

To study the effect of PPARα activation on global gene expression, microarray analysis was performed using Affymetrix GeneChips. We first performed principal component analysis (PCA) to sort out the major sources of variation in our microarray data. The PCA plot for 6h Wy14643 treatment clearly shows that the principal source of variation is between the species (Figure S1). Additionally, the results indicate that: 1) there is large variation between the various mice at basal level (untreated cells), whereas the variation between the human donors is small; 2) the effect of PPARα activation is more pronounced in mice than in humans; 3) the effect of PPARα activation is consistent between the various mice.

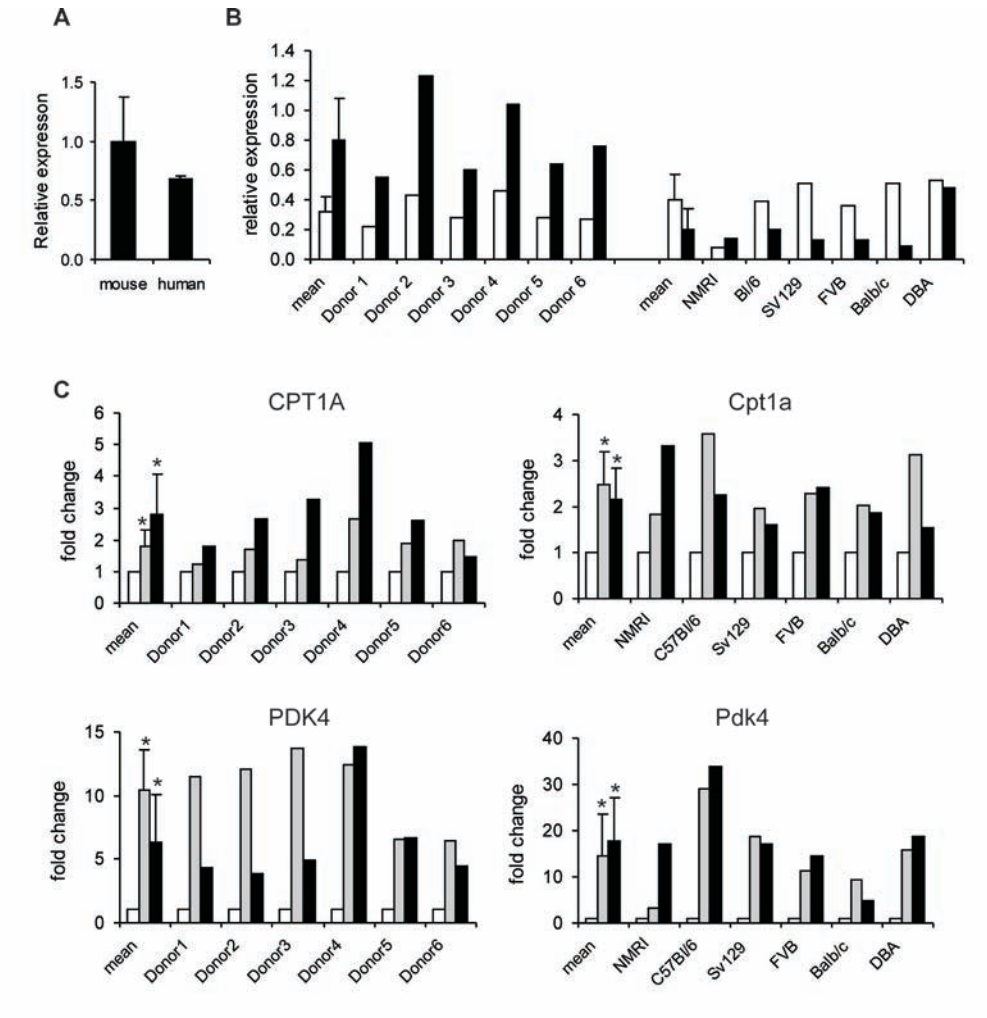

Figure 1. Activation of PPARα in mouse and human hepatocytes. (A) PPARα mRNA expression levels in human versus mouse liver as expressed relative to universal 18S. (B) PPARα mRNA expression levels in human versus mouse primary hepatocytes as expressed relative to universal 18S. Expression was determined at 6h (open bars) and 24h (black bars) in control-treated cells (DMSO). (C) Relative induction of expression of carnitine palmitoyltransferase 1A (Cpt1a) and pyruvate dehydrogenase kinase 4 (Pdk4) was determined in human and mouse primary hepatocytes treated with Wy14643 for 6h (gray bars) and 24h (black bars). Expression of cells treated with DMSO was set at 1 (white bars). Error bars represent SD. *P < 0.05 according to Student's T-test.

We found that in human hepatocytes Wy14643 treatment for 6h significantly altered the expression of 705 genes. A considerably larger number of genes were regulated by Wy14643 in mouse hepatocytes (Figure 2). More stringent selection dramatically reduced the number of significantly regulated genes in human hepatocytes, while it had much less of an effect in mouse hepatocytes (data not shown). Surprisingly, more prolonged Wy14643 treatment augmented the number of significantly regulated genes in human hepatocytes, but not in mouse hepatocytes. The latter result may be related to the lower expression of PPARα in mouse hepatocytes after prolonged incubation (Figure 1B). Overall, these data demonstrate a major impact of PPARα activation in human hepatocytes.

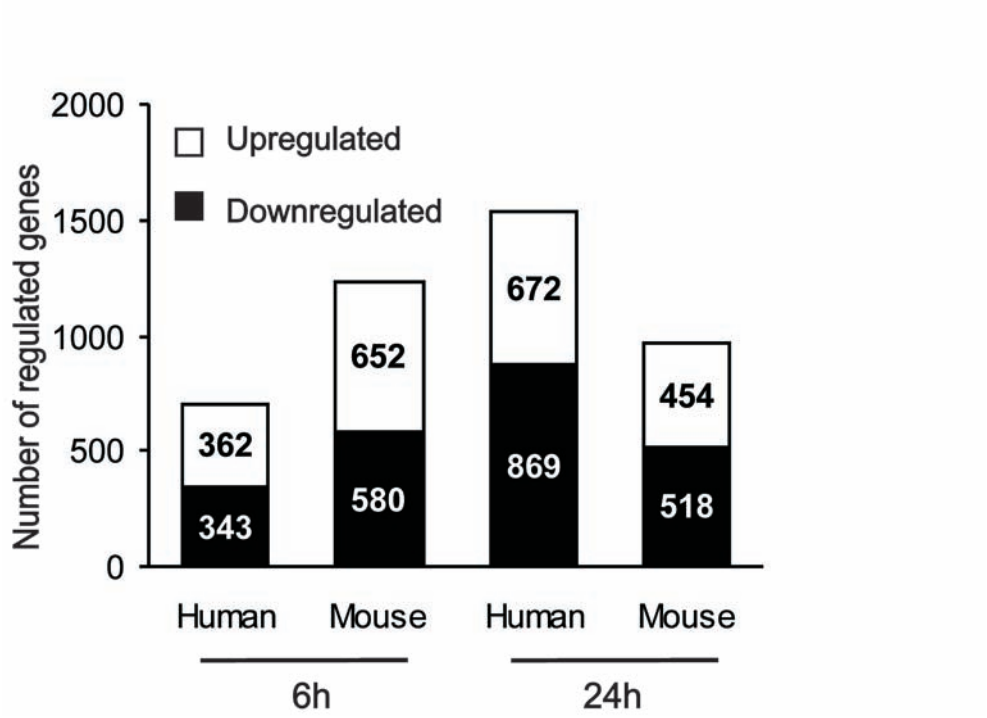

Figure 2. Wy14643 treatment causes major changes in gene expression in human and mouse hepatocytes. Bars show number of up- and down-regulated genes in primary human and mouse hepatocytes treated with Wy14643 for 6h or 24h. Genes were considered significantly regulated if mean fold change (MFC) > 1.1 and $P < 0.05$.

PPARα regulates a mostly divergent set of genes in mouse and human primary hepatocytes

We next determined the overlap in genes regulated by Wy14643 in mouse and human hepatocytes. Data from 6h and 24h Wy14643 treatment were combined to prevent creating a bias from possible differences in kinetics of gene regulation between mouse and human and separate analyses were carried out for up- and down-regulated genes. A total of 125 genes were found to be induced by Wy14643 in both species, many of which were involved in various aspects of lipid metabolism (Figure 3A). A smaller number of genes was found to be downregulated by Wy14643 in both species (Figure 3B). However, the far majority of genes were regulated specifically in one of the species, which would suggest that in general PPARα -dependent gene regulation is poorly conserved between mouse and human. A complete list of regulated genes in the various categories is available in Supplementary Table 1.

To explore the possible functional impact of PPARα activation in mouse and human hepatocytes, we analyzed for overrepresented Gene Ontology classes in response to Wy14643 treatment using ErmineJ. Again, data from 6h and 24h Wy14643 treatment were combined. Out of 115 GO classes overrepresented after Wy14643-treatment of mouse hepatocytes, 48 showed overlap with human hepatocytes (Figure 3C). The overlapping GO classes generally represented various aspects of hepatic fatty acid metabolism including peroxisomal metabolism (Supplementary Table 2). The GO classes specific for the mouse hepatocytes also mostly corresponded to lipid metabolic pathways, suggesting that regulation of lipid metabolism is the dominant function of PPARα in mouse hepatocytes. In contrast, the GO classes specific for human hepatocytes included alternative metabolic processes including bile acid metabolic process, and various aspects of amino acid metabolism.

A similar type of analysis focusing on upregulated genes was carried out using gene set enrichment analysis (GSEA). Results from Ingenuity were generally concordant with GSEA and will not further be elaborated on here. Out of 33 pathways induced by PPARα activation in mouse hepatocytes, 20 were also induced in human hepatocytes (Figure 3D), Similar to GO analysis, pathways commonly regulated in mouse and human were mostly related to lipid metabolism (Supplementary Table 3). Interestingly, the glycolysis-gluconeogenesis pathway was specifically upregulated by Wy14643 in mouse (Figure 4A), while xenobiotic metabolism was specifically upregulated in human (Figure 4B). Overall, these data show that PPARα governs a mostly divergent set of genes in mouse and human hepatocytes, although more significant overlap was observed at the pathway level.

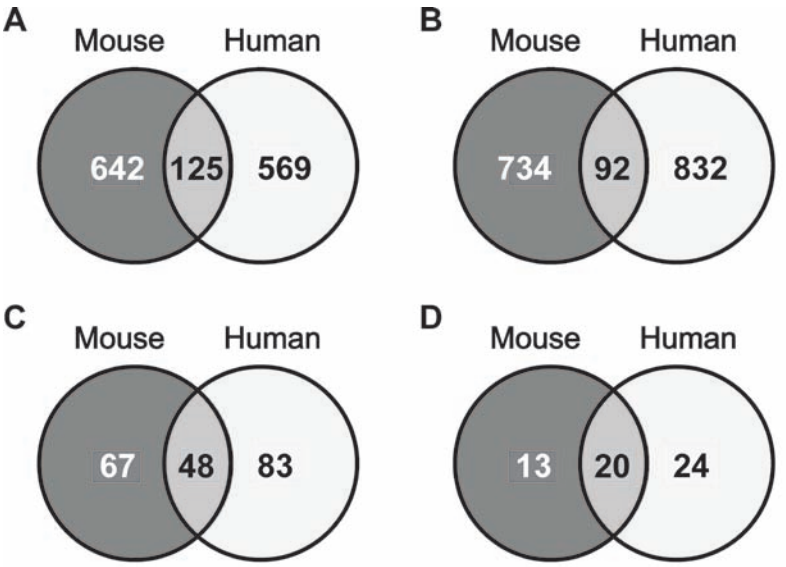

Figure 3. Limited overlap at individual gene level but major overlap at pathway level. Venn diagrams showing overlap in significantly upregulated (A) and (B) downregulated genes after treatment with Wy14643 in mouse versus human hepatocytes. Genes were included if they were significantly regulated by Wy14643 at 6h and/or 24h. Criteria for significance was mean fold-change (MFC) > 1.1 and $P < 0.05$. Genes without orthologs in the other species and/or not present on the array of the other species were excluded. (C) Venn diagram showing overlap in overrepresented Gene Ontology classes upon Wy14643 treatment in mouse and human hepatocytes based on a functional class score method. Data from 6h and 24h Wy14643 treatment were combined in a single analysis. Only GO classes containing 8 to 125 genes and FDR < 0.0001 were included in the Venn diagram. (D) Venn diagram showing overlap in upregulated processes analyzed by GSEA. Only gene sets having a size between 15 and 250 genes were included in the analysis. To account for multiple hypothesis tasting, gene sets having a FDR < 0.25 were selected. Sources of the gene sets: BIOCARTA, GENMAPP, KEGG, SIGNALING ALLIANCE, SIGNALING TRANSDUCTION, GEARRAY and SK manual.

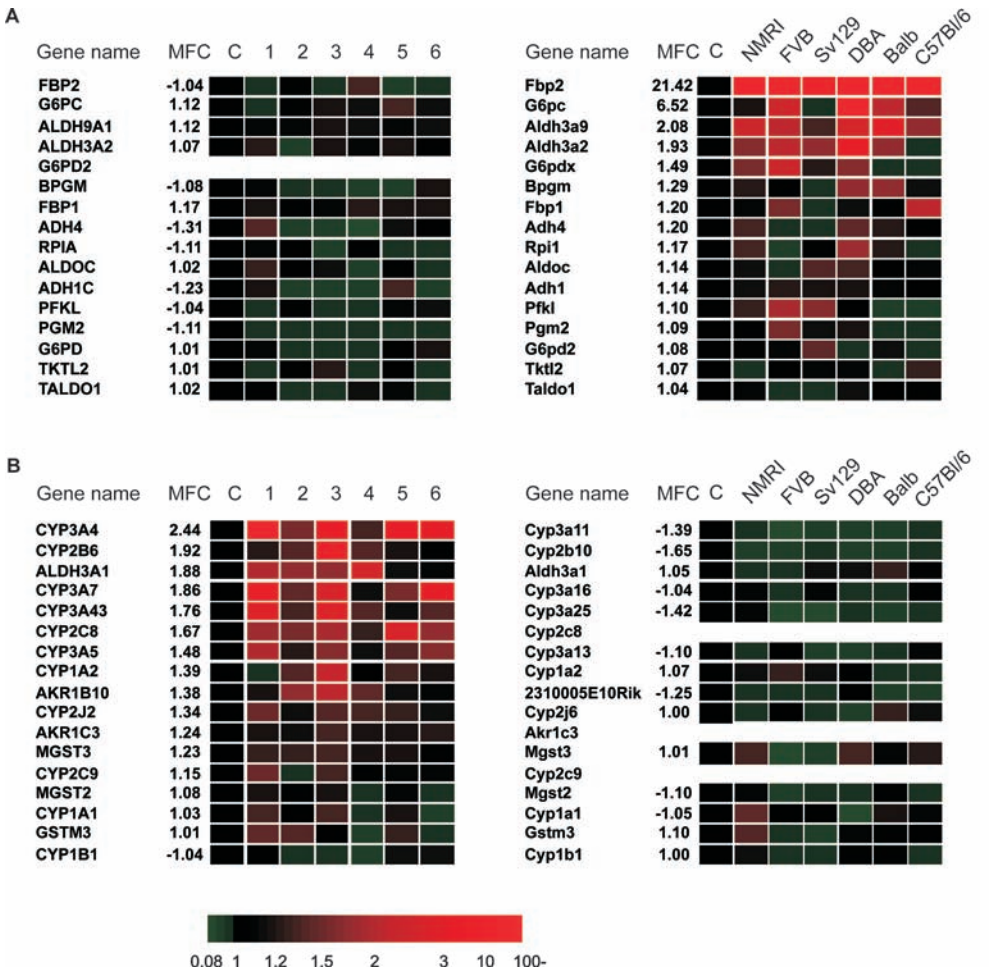

Figure 4. Heat map illustrating the species-specific regulation of two gene sets originating from Gene set enrichment analysis (GSEA). (A) Glycolysis-gluconeogenesis as a mouse-specific upregulated gene set. (B) Xenobiotic metabolism as a human-specific upregulated gene set. Genes are ranked based on the mean fold change (MFC). Expression levels in the DMSO-treated cells were set at 1.

Identification of human and mouse-specific novel putative PPARα target genes

In order to identify additional genes that are specifically regulated by PPARα in one particular species, we performed scatter plot analysis comparing the effect of 6h PPARα activation between mouse and human (Figure 5A). A similar plot was created for 24h PPARα activation (Figure 5B). A number of genes could be identified that were induced by Wy14643 specifically in human (MBL2, CYP1A1, HMOX1 and TSKU) or mouse (FBP2, LGALS4, PXMP4 and UCP2). To directly compare the effect of Wy14643 on specific genes between mouse and human, genes that were upregulated by 6h or 24h Wy14643 in human hepatocytes were ranked according to their mean fold-change and the changes in expression compared between the individual donors (Figure 6 and Figure S2, respectively). The changes in expression of their mouse orthologs are presented in parallel. The picture clearly illustrates the human-specific induction of MBL2, ALAS1, TSKU, and many other genes. The specific induction of TSKU was confirmed by qPCR (Figure S3A). Interestingly, the top 50 of most highly induced genes contain a remarkably high number of established PPARα targets, regulation of which was conserved in mouse hepatocytes. This includes genes involved in mitochondrial fatty acid oxidation and ketogenesis (HMGCS2, CPT1A, CPT2, SLC25A20), peroxisomal/microsomal fatty acid oxidation (ECH1, CYP4A11), fatty acid binding and activation (FABP1, ACSL1, ACSL3), and lipid droplet associated proteins (ADFP). Wy14643 also stimulated expression of a number of secreted PPARα targets including FGF21 and ANGPTL4. These data support an important role for PPARα in the regulation of lipid metabolism in human hepatocytes. Besides numerous known PPARα target genes, several putative novel PPARα targets were found to be commonly regulated by Wy14643 in mouse and human, including the transcription factors CREB3L3, KLF10 and KLF11, and MAP3K8. Induction of KLF10 was confirmed by qPCR (Figure S3B).

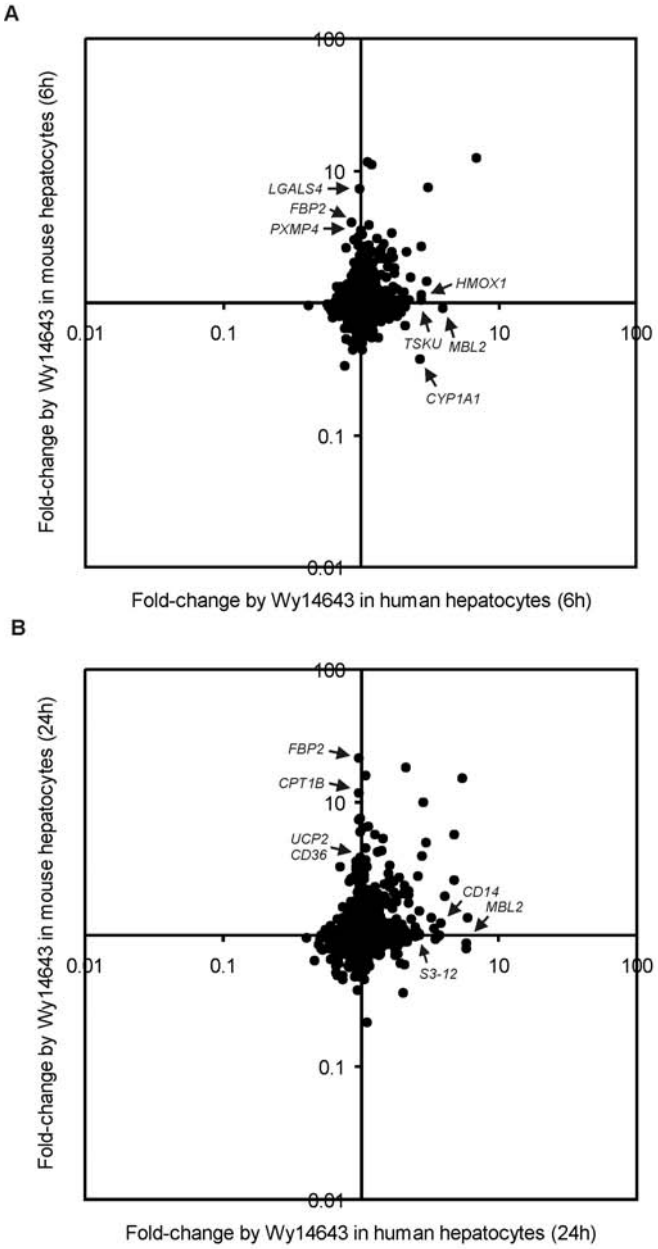

Figure 5. Limited similarity in Wy14643-induced gene regulation between mouse and human hepatocytes. Scatter plots demonstrating similarities and differences in gene regulation by 6h (A) and 24h (B) PPARα activation between human and mouse hepatocytes. Graphs show fold-changes in gene expression after treatment with Wy4643 in human hepatocytes (x-axis) and mouse hepatocytes (y-axis). Selected genes that are upregulated specifically by Wy4643 in human or mouse are indicated.

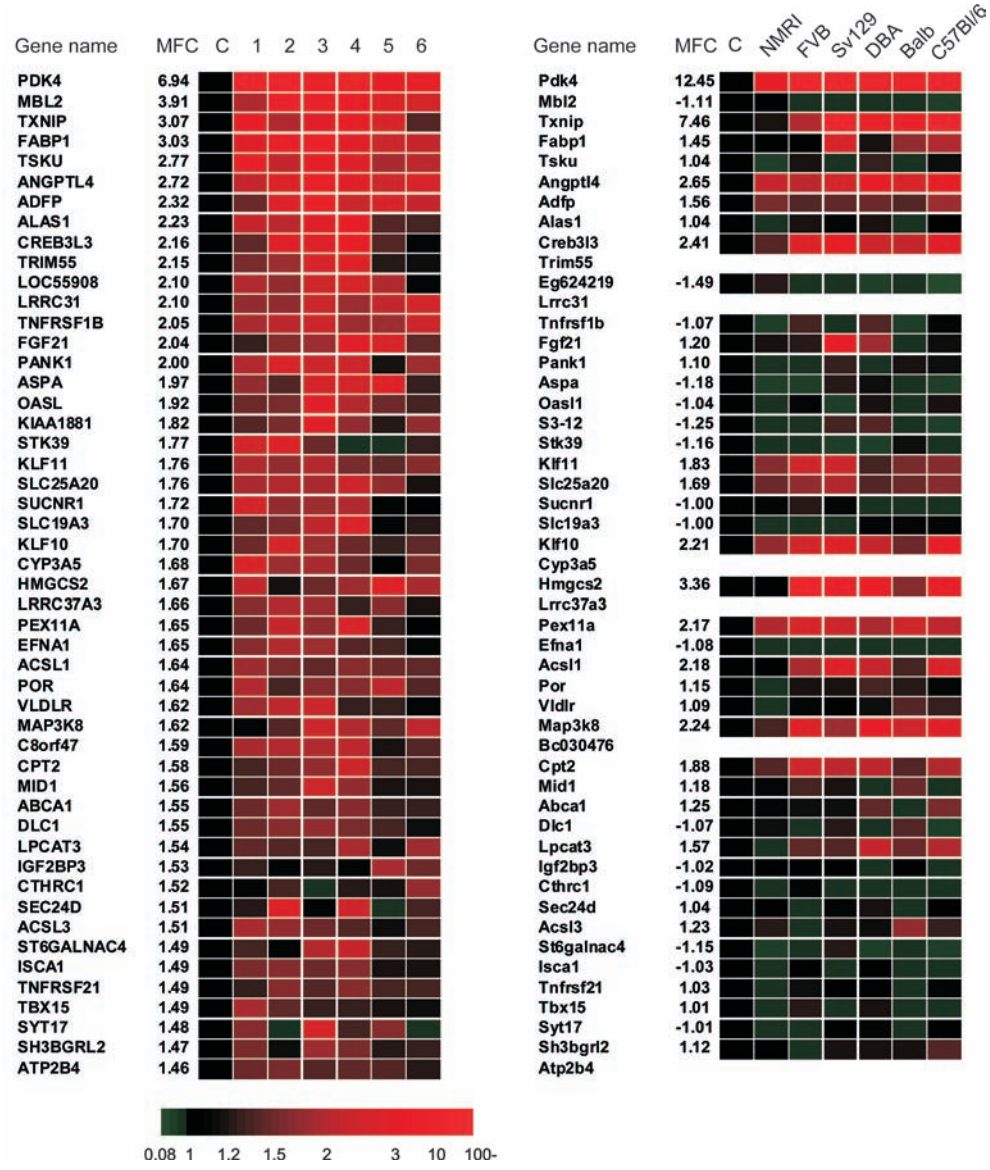

Figure 6. Partial conservation of Wy14643-induced gene regulation between human and mouse hepatocytes. Heat map illustrating the relative induction of the top 50 of upregulated genes in response to 6h Wy14643 treatment in human hepatocytes. All genes were significantly changed ($P < 0.05$) and were ranked based on mean fold-change (MFC). Expression levels in the DMSO-treated cells were set at 1. Relative changes in expression of the corresponding mouse orthologs in mouse hepatocytes are shown in parallel.

Conversely, the scatter plots and ranking of genes also clearly revealed numerous genes that were specifically induced by Wy14643 in mouse, including Fbp2, Lgals4, and Pxmp4, as well as known PPARα target genes such as Cd36, Cpt1b and Ucp2 (Figures 5B and 7; Figure S4). The mouse specific induction of Fbp2 was confirmed by qPCR (Figure S3C). These data suggest that in general the effect of PPARα activation is remarkably dissimilar between mouse and human hepatocytes. Nevertheless, many established PPARα targets representing key genes in lipid metabolism are commonly regulated in mouse and human.

The role of PPARα in hepatic lipid metabolism is well conserved between mouse and human

We showed that a large proportion of the genes commonly regulated by PPARα in mouse and human were involved in some aspect of lipid metabolism. To better appreciate the conservation of PPARα's role as master regulator of hepatic lipid metabolism, we classified genes according to specific lipid metabolic pathways to create a comprehensive picture of PPARα-dependent gene regulation (Figures 8A,B). The picture reveals that in human hepatocytes PPARα activation induces the expression of many genes involved in different aspects of lipid metabolism, including fat oxidation, fat synthesis, intracellular TG storage and hydrolysis, membrane transport, intracellular activation and trafficking of fatty acids and lipoprotein metabolism. Comparison with the corresponding picture for mouse reveals a remarkable conservation at the pathway level, indicating that the role of PPARα in hepatic lipid metabolism is highly similar between mice and human. The sole exception is lipoprotein metabolism, represented by APOA2 and APOA5, which was exclusively regulated in human hepatocytes. It is also evident that fewer peroxisomal genes are induced by Wy14643 in human vs. mouse hepatocytes. Interestingly, within a particular metabolic pathway the specific genes upregulated by Wy14643 to some extent differ between mouse and human. Taken together, the results suggest that in human and mouse hepatocytes PPARα has an equally important role in governing lipid metabolism with the exception of lipoprotein metabolism and to a lesser extent peroxisomal metabolism. However, the specific genes under control of PPARα in mouse and human are partially different.

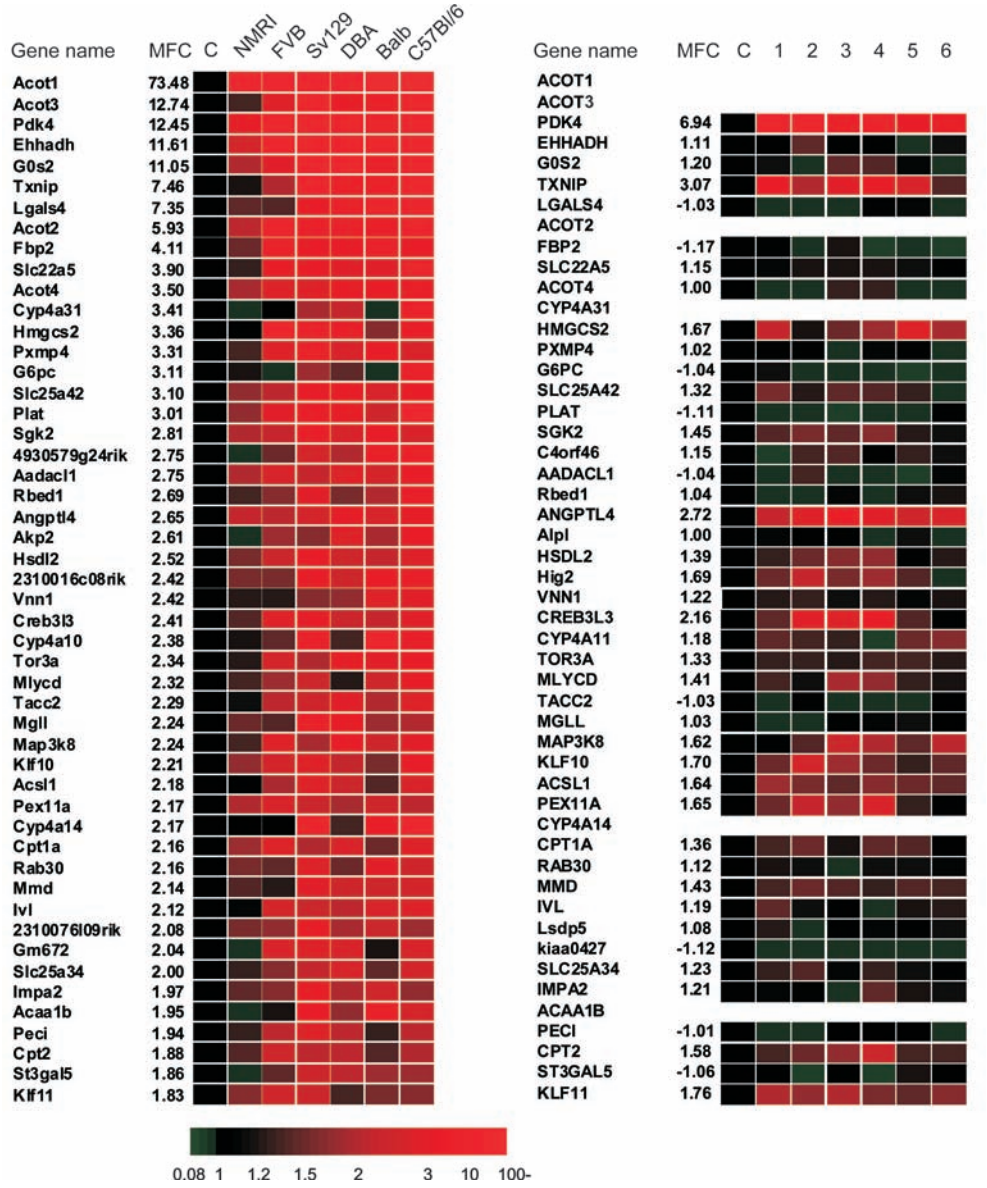

Figure 7. Partial conservation of Wy14643-induced gene regulation between mouse and human hepatocytes. Heat map illustrating the relative induction of the top 50 of upregulated genes in response to 6h Wy14643 treatment in mouse hepatocytes. All genes were significantly changed ($P < 0.05$) and were ranked based on mean fold-change (MFC). Expression levels in the DMSO-treated cells were set at 1. Relative changes in expression of the corresponding human orthologs in human hepatocytes are shown in parallel.

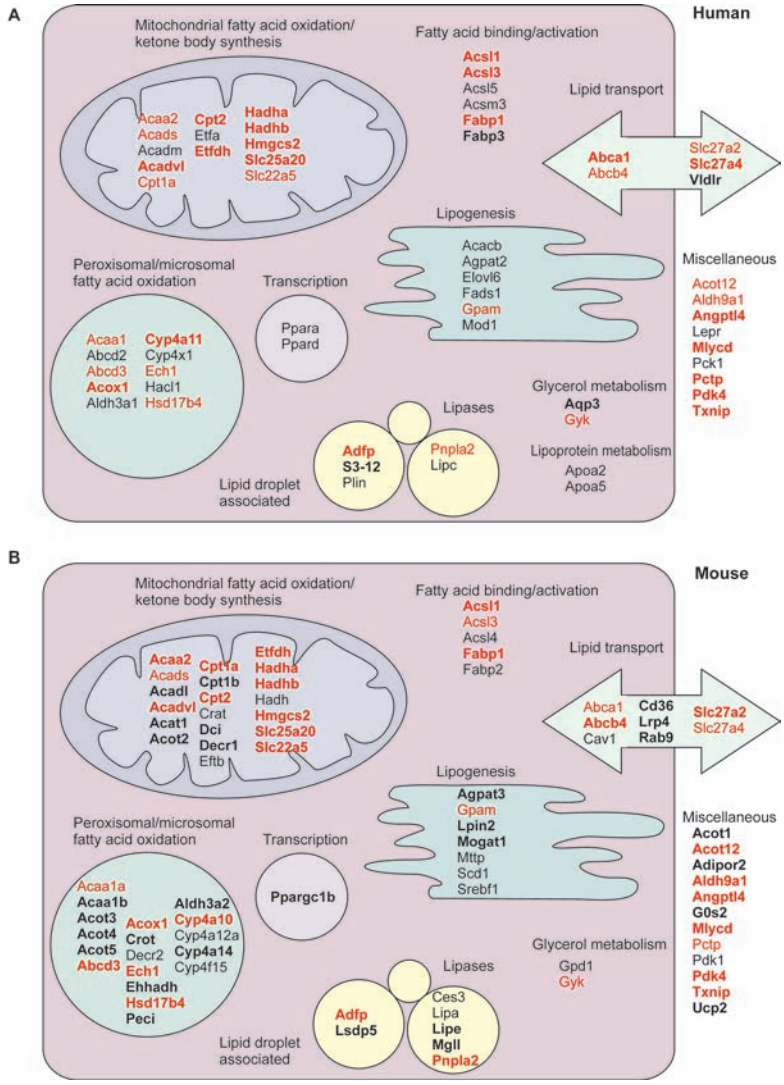

Figure 8. PPARα serves as a global transcriptional regulator of lipid metabolism in mouse and human hepatocytes. Genes significantly upregulated by Wy14643 and that function in lipid metabolism were classified into specific metabolic pathways. Separate pictures were created for human hepatocytes (A) and mouse hepatocytes (B). Genes significantly upregulated by Wy14643 at both time points of 6h and 24h are shown in bold. Genes significantly upregulated by Wy14643 in human and mouse hepatocytes are shown in red. Genes significantly upregulated by Wy14643 at one time point only are shown in normal font. Functional classification is based on a self-made functional annotation system of genes involved in lipid metabolism.

Discussion

Numerous studies have examined the effect of PPARα activation or deletion on hepatic gene regulation using transcriptomics. In general, these studies indicate that unlike many other nuclear receptors, PPARα governs the expression of a large set of genes, many of which are involved in fatty acid metabolism [22-27]. However, there has been no systematic comparison of the whole genome effects of PPARα activation in human versus mouse hepatocytes [28]. Accordingly, in the present paper we systematically compared the effect of PPARα activation in primary mouse and human hepatocytes using a whole genome transcriptomics approach. A number of important general conclusions can be drawn from our work. First, perhaps contrary to common conception, our data support a major role for PPARα in human liver, as evidenced by the large number of genes altered upon PPARα activation in primary human hepatocytes. Second, even though the human and mouse hepatocytes were not cultured under identical conditions, we feel comfortable to conclude that PPARα regulates a mostly divergent set of genes in mouse and human liver. For example, we found that metabolism of xenobiotics is specifically regulated by PPARα in human liver. Third, the role of PPARα as a master regulator of hepatic lipid metabolism is well conserved between mouse and human. However, within each lipid metabolic pathway the specific genes under control of PPARα in mouse and human differ to some extent.

In recent years, the role of PPARα in human liver has been questioned based on RNase protection data showing 10-fold lower levels of PPARα mRNA in human liver compared with mouse liver [19]. Additionally, human hepatoma HepG2 cells were shown to respond poorly to PPARα activation [21]. In contrast, we show by quantitative realtime PCR that in liver tissue and primary hepatocytes PPARα expression levels are similar between mouse and human. It is inherently difficult to compare hepatic PPARα expression between species as PPARα mRNA fluctuations throughout the day [29], is increased by fasting [30], and is reduced under conditions of inflammation [31]. Changes in PPARα expression will likely influence the transcriptional response to PPARα activation. Our comparative analysis of hepatic gene regulation by human PPARα vs. mouse PPARα should thus be considered an approximation. Despite the limitations, our analysis represents a major advancement in our understanding of PPARα function in human liver.

Consistent with a major role of PPARα in human hepatocytes, the number of genes significantly regulated by Wy14643 was very high and was similar to the number in mouse hepatocytes. Although induction of gene expression by Wy14643 was generally less robust in human hepatocytes, these cells likely lost some sensitivity due to the extended time between isolation and harvesting. We were able to exclude differences in cultured medium as an explanation for the lower fold-inductions in human hepatocytes (data not shown).

While the number of genes commonly regulated by PPARα in mouse and human hepatocytes may seem relatively small, which would suggest minor overlap in PPARα function between the two species, the overlap is more impressive when studied at the level of gene ontology. Many of the overlapping gene ontology classes represent pathways of lipid metabolism. Supporting these data, many of the 125 genes co-regulated by PPARα in mouse and human are involved in various aspects of hepatic lipid handling, including peroxisomal and mitochondrial fatty acid oxidation (ACOX1, ECH1), ketogenesis (HMGCS2), fatty acid binding and activation (FABP1, ACSL3), and fatty acid uptake (SLC27A2). Our analysis demonstrates that in human liver, analogous to the situation in mouse liver [26], PPARα serves as a global transcriptional regulator of lipid metabolism.

In addition to numerous established PPARα target genes, several genes were found to be co-regulated by Wy14643 in mouse and human that have not yet been linked to PPARα, including the liver specific transcription factor CREB3L3. CREB3L3 was recently shown to be involved in the hepatic acute phase response, suggesting that it may partially mediate the effects of PPARα on acute phase response [32]. Other conserved novel putative targets include MAP3K8, SGK2, and the transcription factor KLF10 and KLF11. KLF10 and KLF11 encode three zinc-finger Krüppel-like transcription factors that binds GCrich/Sp1-like sequences and influence cell proliferation [33].

The inability of PPARα agonists to induce peroxisome proliferation in human is well acknowledged, although the precise mechanism remains to be fully elucidated. Using humanized PPARα mice, it has been shown that the human PPARα receptor has the ability to induce peroxisome proliferation and peroxisomal fatty acid oxidation in the context of a mouse liver [34,35]. However, in a previous study using HepG2 cells engineered to express PPARα at levels similar to mouse liver, ACOX1 and other peroxisomal genes were not induced by PPARα [36]. Similar results were obtained in primary human hepatocytes treated with fenofibrate [37]. In contrast, we find that a number of genes involved in peroxisomal fatty acid oxidation, including the prototypical PPARα targets ACOX1, ECH1, PEX11A, and ACAA1, is commonly induced by PPARα in mouse and human. Simultaneously, we find that induction by PPARα of numerous other peroxisomal genes, including Ehhadh, Pxmp4, Acot4, and Peci, is specific for mouse. Our data argue against a general mechanism and suggest that any lack of conservation of PPARα-dependent gene regulation between mouse and human must be determined at the level of individual target genes.

Previously, it was shown that APOA1, APOA2 and APOA5 are upregulated by PPARα agonists, which was found to be specific for humans [38-41]. While we confirm the human-specific upregulation of APO2 and APOA5 by Wy14643, we could not confirm the upregulation

of APOA1 by Wy14643. Rather, we found a minor but statistically significant decrease in APOA1 expression after 6h of Wy14643 treatment. The reason for this discrepancy is unclear but may be related to the type of PPARα agonist used. Overall, our data indicate that regulation of apolipoproteins A by PPARα is specific for humans, which very likely accounts for the human specific induction of plasma HDL levels by fibrates [42].

Several individual genes were identified that were also specifically regulated by Wy14643 in human hepatocytes. This includes the secreted mannose-binding lectin MBL2, which is an important protein of the humoral innate immune system [43], and TSKU, which encodes a secreted protein involved in development [44]. Regulation of CYP1A1 by PPARα in human hepatocytes has been previously observed [45], and was shown here to be part of a more comprehensive regulation of biotransformation enzymes by PPARα that was specific for human hepatocytes. Importantly, while genes belonging to the Cyp4a class are exclusively regulated by PPARα in mouse, genes belonging to CYP classes 1-3 are specifically regulated by PPARα in human, which confirms previous analyses [46,47].

A number of pathways was found to be specifically induced by Wy14643 in mouse hepatocytes, including glycolysis/gluconeogenesis, pentose phosphate pathway, and glycerolipid metabolism, as were several specific lipid metabolic pathways. A similar mouse-specific response was observed at the level of individual genes. Most notable examples were FBP2 (fructose-1,6-bisphosphatase 2), LGALS4 (lectin, galactoside-binding, soluble, 4), and several ACOTs (Acyl-CoA thioesterases).

Studies in mice have yielded considerable evidence for a direct role of PPARα in hepatic glucose metabolism. Importantly, fasted PPARα -/- mice exhibit markedly reduced plasma glucose levels [30]. Other studies have suggested a direct link between PPARα and hepatic gluconeogenesis [48-50]. In contrast, human trials generally do not support an effect of PPARα activation on plasma glucose levels. Accordingly, it is tempting to relate these seemingly discrepant results to the observed mouse-specific regulation of glucose metabolic pathways. In conclusion, PPARα activation has a major impact on gene regulation in human liver cells. Importantly, the role of PPARα as a master regulator of hepatic lipid metabolism is generally well conserved between mouse and human. Overall, however, PPARα regulates a mostly divergent set of genes in mouse and human hepatocytes suggesting that caution should be exercised when extrapolating the function of a transcription factor from mouse to human.

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Appendix Chapter 4

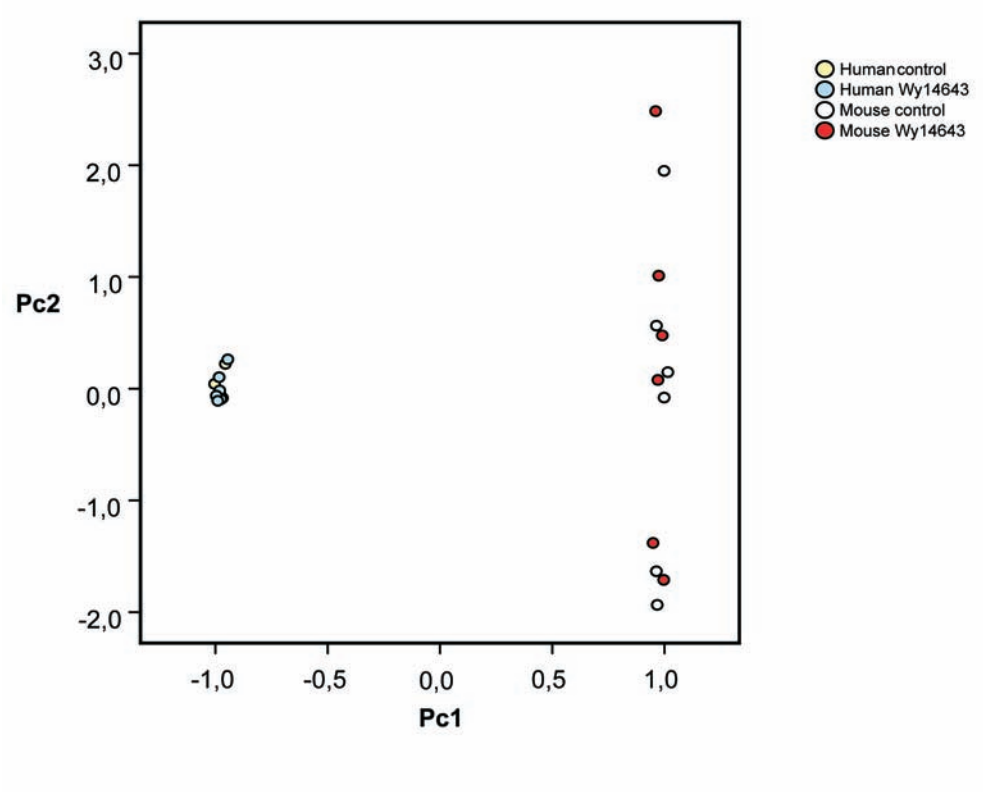

Figure S1 Principal component analysis illustrating the major sources of variation in our microarray dataset. In the first dimension, data separate based on species. The second dimension illustrates the effect of Wy14643 treatment.

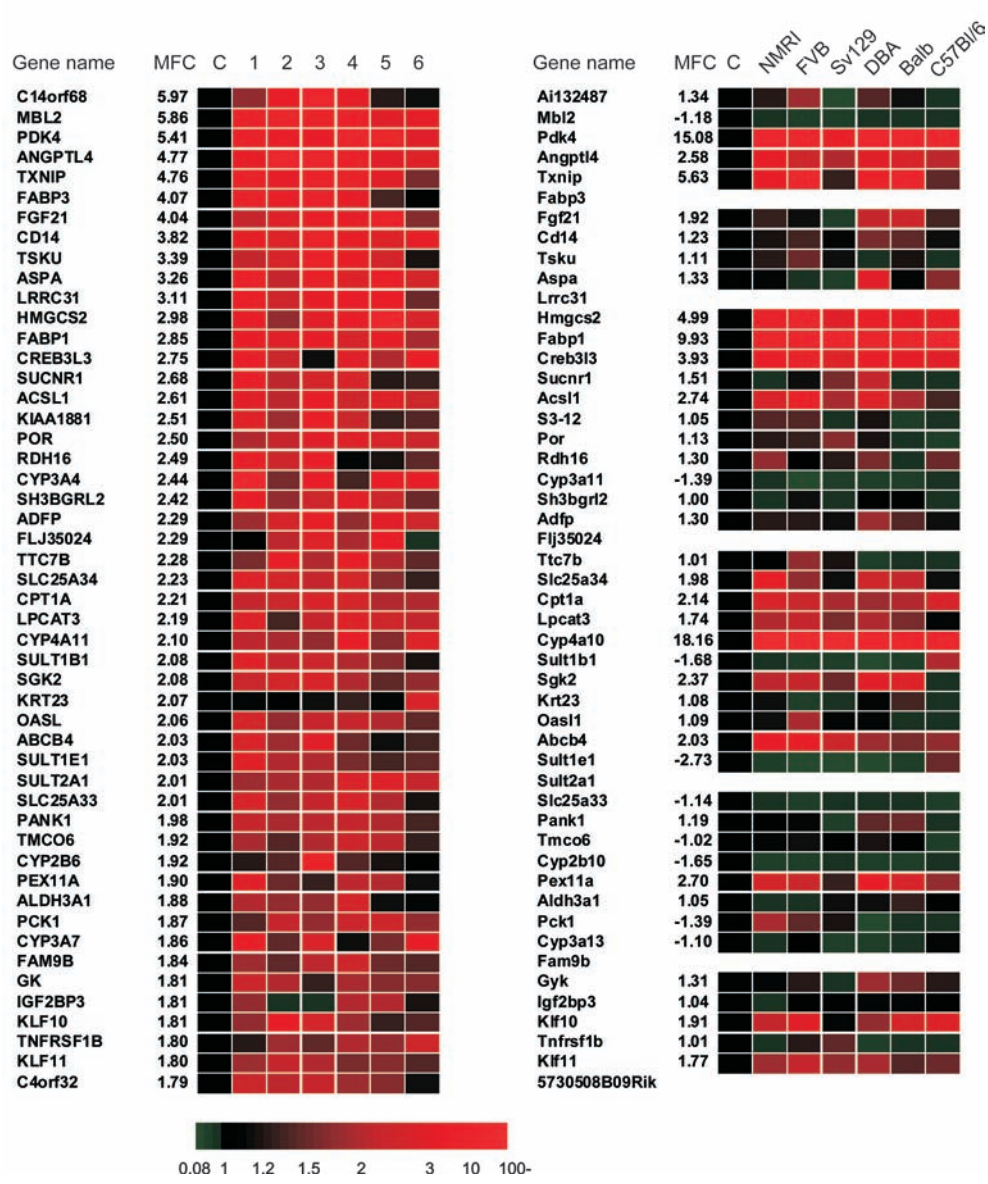

Figure S2 Heat map illustrating the relative induction of the top 50 of upregulated genes in response to 24h Wy14643 treatment in human hepatocytes. All genes were significantly changed ($P < 0.05$) and were ranked based on mean fold-change (MFC). Expression levels in the DMSO-treated cells were set at 1. Relative changes in expression of the corresponding mouse orthologs in mouse hepatocytes are shown in parallel.

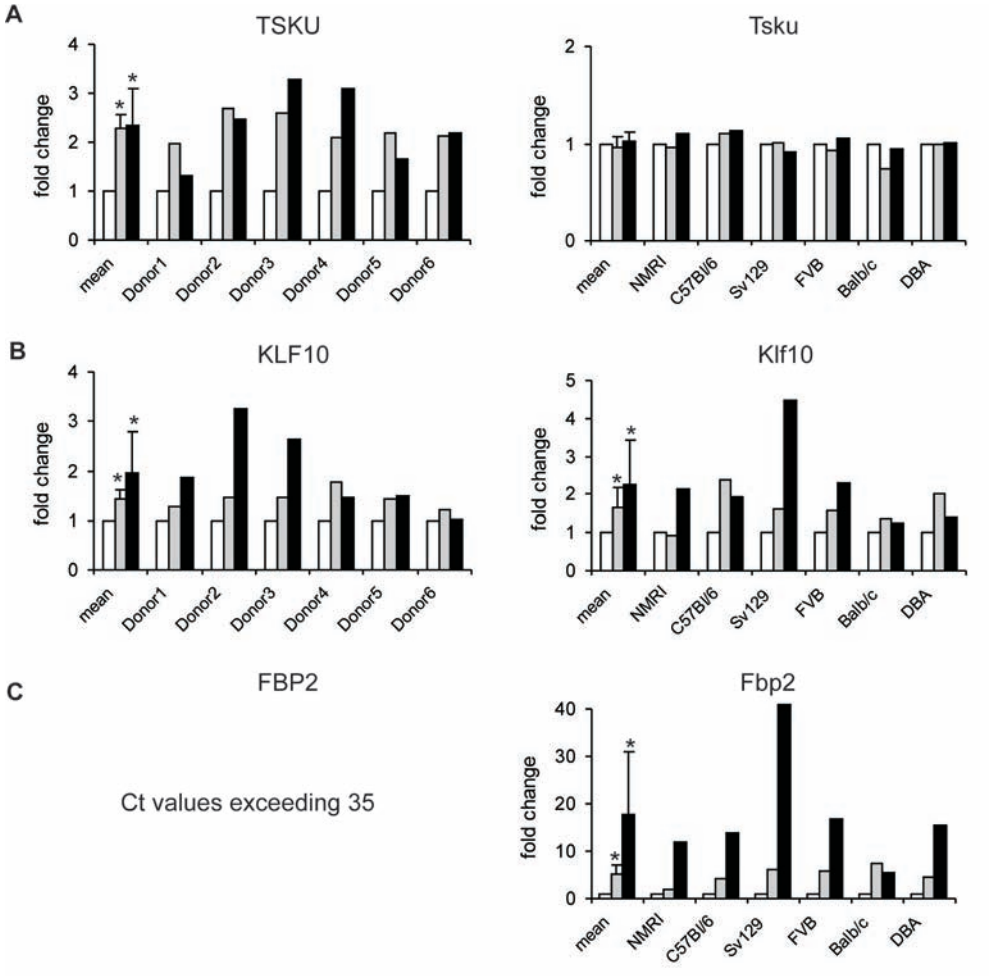

Figure S3 Species-specific induction of novel putative PPARα genes by Wy14643. (A) Relative induction of Tukushin (TSKU) by Wy14643 in human and mouse hepatocytes. (B) Relative induction of Kruppel-like factor 10 (KLF10) by Wy14643 in human and mouse hepatocytes. (C) Relative induction of fructose bisphosphatase 2 (Fbp2) by Wy14643 in mouse hepatocytes. Inductions for 6h (grey bars) and 24h (black bars) Wy14643 treatments are shown. Expression of cells treated with DMSO was set at 1 (white bars). Gene expression was determined by qPCR. Error bars represent SD. *$P < 0.05$ according to Student's T-test.

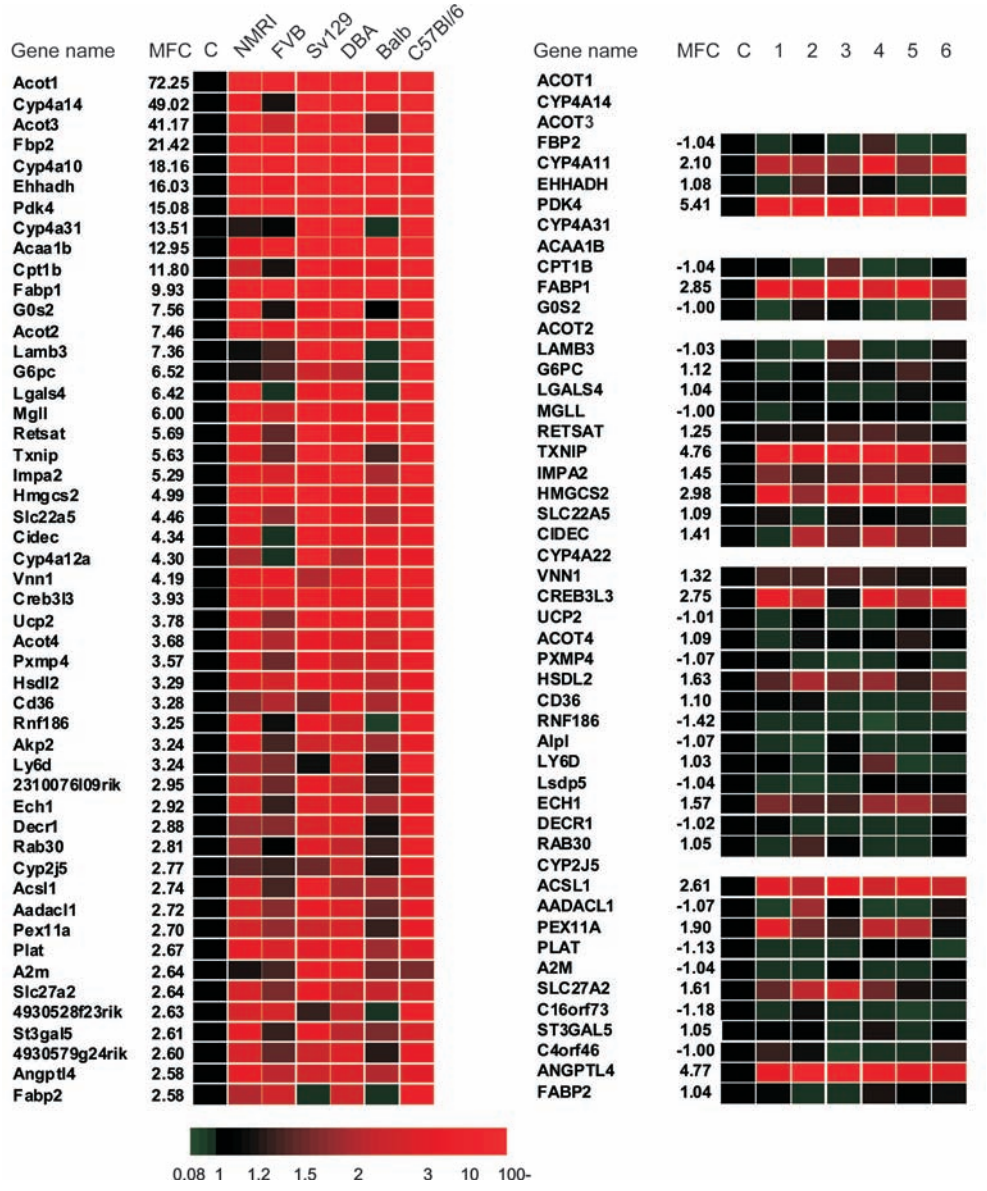

Figure S4 Heat map illustrating the relative induction of the top 50 of upregulated genes in response to 24h Wy14643 treatment in mouse hepatocytes. All genes were significantly changed ($P < 0.05$) and were ranked based on mean fold-change (MFC). Expression levels in the DMSO-treated cells were set at 1. Relative changes in expression of the corresponding human orthologs in human hepatocytes are shown in parallel.

Additional files can be found online:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2729378

Chapter 5

Mannose binding lectin is a circulating mediator of hepatic PPARα activity in human

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Abstract

PPARα is a major transcriptional regulator of hepatic lipid metabolism. Here, we aimed to find novel circulating mediators of PPARα activity in human. Microarray analysis was performed on primary human hepatocytes treated with Wy14643 and data selected for secreted proteins. Expression of liver-specific mannose-binding lectin (MBL2), a soluble mediator of innate immunity, was markedly upregulated by PPARα activation. Induction of MBL2 mRNA and protein was confirmed in HepG2 cells but not in mouse hepatocytes.
In human subjects, fasting increased plasma MBL2 levels. Importantly, in two independent clinical trials, treatment with PPARα-agonist fenofibrate markedly increased plasma MBL2 levels. The relative induction of plasma MBL2 by fenofibrate was not correlated with the relative induction of plasma ANGPTL4 or FGF21. These results identify MBL2 as circulating target of PPARα in humans and suggest that MBL2 may mediate effects of PPARα on innate immunity.

Introduction

The liver is a key organ in the control of lipid metabolism and whole energy homeostasis. Although not generally appreciated as an important contributor to endocrine regulation of energy metabolism, recent studies point to the liver as a source of secreted factors that have profound metabolic effects elsewhere in the body [1, 2]. Studies in mice have demonstrated that many aspects of hepatic lipid metabolism are under transcriptional control of the Peroxisome Proliferator Activated Receptor α (PPARα), a transcription factor belonging to the nuclear receptor superfamily. Lack of PPARα in mice leads to acute energy shortage in liver upon fasting and is characterized by defective ketone body formation, hypoglycemia, elevated plasma free fatty acids, and severe hepatic steatosis [3, 4]. Recently, it has been shown that PPARα also governs the hepatic production of secreted proteins FGF21 and ANGPTL4 [1, 5]. While FGF21 has been shown to serve as a mediator of the PPARα-induced starvation response in mice [6, 7], ANGPTL4 is now well established as a potent regulator of plasma triglyceride levels via inhibition of lipoprotein lipase [8]. The present study was undertaken to identify potential novel circulating mediators of PPARα activity in human. Our results indicate that MBL2, a soluble effector of innate immunity and putative co-receptor for Toll-like receptors [9], is a circulating target of PPARα in human liver and may mediate effects of PPARα on innate immunity.

Materials and methods

Materials. Wy14643 and GW7647 were obtained from ChemSyn Laboratories (Lenexa, KS). SYBR Green was from Eurogentec (Seraing, Belgium). Fetal calf serum, penicillin/streptomycin/fungizone were from Lonza Bioscience (Verviers, Belgium). Otherwise, chemicals were from Sigma (Zwijndrecht, The Netherlands).

Primary hepatocytes isolation. Primary human hepatocytes from 6 donors were purchased from Lonza Bioscience (Verviers, Belgium). Details of isolation and procedure are described in a previous publication [10]. Briefly cells were isolated from surgical liver biopsies by two-step collagenase perfusion method and incubated in the presence or absence of Wy14643 (50 μM) dissolved in DMSO for 6 and 24 hours, followed by RNA isolation.
Mouse hepatocytes were isolated as described previously from 6 different strains of mouse: NMRI, SV129, FVB, DBA, BALB/C and C57BL/6J [10, 11].
Cells were incubated in fresh medium in the presence or absence of Wy14643 (10 μM) dissolved in DMSO for 6 and 24 hours, followed by RNA isolation. Isolation of mouse primary hepatocytes was approved by the animal ethics committee of Wageningen University.

Affymetrix microarray analysis. RNA isolation and subsequent processing for microarray were carried out as previously described [10]. Hybridization, washing and scanning of Affymetrix Gene chip human genome U133 2.0 plus and mouse genome 430 2.0 arrays was according to standard Affymetrix protocols.
Analysis of the microarray data was as previously described [10]. Genes encoding secreted proteins were selected using Gene Ontology Classification, SignalP and ngLOC (n-gram-based Bayesian classifier) predicting tools.

Real time quantitative PCR. 1 µg of total RNA was used for reverse-transcription with iScript (Bio-Rad, Veenendaal, the Netherlands). PCR was performed with Platinum Taq DNA polymerase (Invitrogen) on a Bio-Rad iCycler or MyIQ PCR machine. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. The mRNA expression reported was normalized to universal 18S gene expression.
Primer sequences used: hMBL2, forward: GCAAACAGAAATGGCACGTATC, reverse: CTGGAACTTGACACACAAGGC; mMbl1, forward: CTGTGGCTATCCCCAGGAAT, reverse:TCACGTACATGAACTGCCCTT;mMbl2,forward:TGACAGTGGTTTATGCAGAGAC, reverse: CGTCACGTCCATCTTTGCC.
For determination of tissue expression of MBL2, cDNA was prepared from FirstChoice® Human Total RNA Survey Panel (Ambion).

HepG2 Cell Culture. Human hepatoma HepG2 cells were grown in DMEM containing 10% FCS and 1% penicillin-streptomycin (20,000 units/ml potassium penicillin, 20,000µg/ml streptomycin sulfate) at 37ºC/5% CO2. PPARα ligands (Wy14643 10µM, GW7647 10 µM) or vehicle (DMSO) was added to the cells for 6h. Cells were harvested using TRIzol (Invitrogen, Breda, The Netherlands).
For protein measurement, HepG2 cells were incubated in DMEM without FCS followed by addition of GW7647 (10 µM) and Wy14643 (10 µM) for 24h. The medium was collected and protein analysis was performed using commercially available MBL2 Oligomer ELISA kit (Bioporto Diagnostics, Copenhagen, Denmark).

Human subjects. In the fasting study, blood was taken from 4 healthy young males (age 19-22). The full details of this study can be found elsewhere [13]. Briefly volunteers received an identical meal at 17.00 before the start of a 48h fasting period. During the fasting period, the subjects were not allowed to eat or drink anything except water. Blood samples were taken at baseline and after 48h of fasting.

For the first fenofibrate study, serum was sampled from eleven obese females with type 2 diabetes mellitus and serum triglyceride concentrations above 2.0 mmol/l at baseline and

after a 3-month treatment with micronized fenofibrate (200 mg/d, Lipanthyl). Full details of this study can be found elsewhere [14].

For the second fenofibrate study, fasted blood samples were taken from 19 male and female subjects (age 30-70 yrs) with a BMI of at least 27 kg/m2 before and after a 6-week treatment with micronized fenofibrate (200 mg/d, Lipanthyl). The full details of this study can be found in supplemental text.

All human experiments were approved by the medial ethics committee of Wageningen University or Maastricht University, the Netherlands; or of the First Faculty of Medicine and General University Hospital, Prague, Czech Republic. Subjects were informed about the design and purpose of the study and provided fully informed written consent.

Plasma/serum MBL2 analysis. Plasma MBL2 levels were determined using a commercially available MBL2 Oligomer Elisa kit (Bioporto Diagnostics, Copenhagen, Denmark) using biotinylated monoclonal detection antibody following the instructions of the manufacturer.

Statistical analysis. Statistical significant differences were calculated using Student's T-test. The cut-off for statistical significance was set at a P-value of 0.05 or below. Plasma MBL2 levels were log-transformed before analysis.

Results

MBL2 expression in liver is regulated by PPARα in human specific manner

The aim of the present paper was to screen for novel circulating mediators of PPARα activity in human. To that end, we treated primary human hepatocytes with the PPARα agonist Wy14643 for 24 hours and performed Affymetrix microarray analysis. Differentially expressed genes encoding secreted proteins were selected using Gene Ontology Classification. In addition to established secreted targets of PPARα such as ANGPTL4 and FGF21, expression of the gene encoding mannose-binding lectin (MBL2) was significantly upregulated by PPARα activation in all donors (Figure 1A). In fact, MBL2 represented the most highly induced gene encoding a secreted protein after Wy14643 incubation. MBL2 represents a soluble mediator of innate immunity that plays a critical role in innate immune protection against pathogens [15]. Other inflammation-related genes encoding secreted proteins that were induced by PPARα activation in primary human hepatocytes included CC chemokines CCL14, CCL15, and CCL16. Induction of MBL2 was confirmed by PCR and was already

observed after 6h of PPARα activation (Figure 1C). In contrast to human hepatocytes, incubation of mouse hepatocytes with Wy14643 did not result in significant induction of MBL2 (Figure 1B), which was confirmed by qPCR (Figure 1D). It should be emphasized that mouse expresses two MBL2 isomers: Mbl1 and Mbl2.

Induction of MBL2 by PPARα activation was reproduced in HepG2 cells. Treatment of the cells with Wy14643 and GW7647 significantly increased MBL2 gene expression levels (Figure 1E) as well secretion into the medium (Figure 1F). To investigate whether MBL2 expression may be regulated by PPARs in other tissues, we first screened a panel of organs for the presence of MBL2 mRNA. MBL2 expression was exclusive to liver among a panel of 20 human tissues (Figure S1). Overall, these data demonstrate that MBL2 expression and secretion are induced by PPARα in human liver.

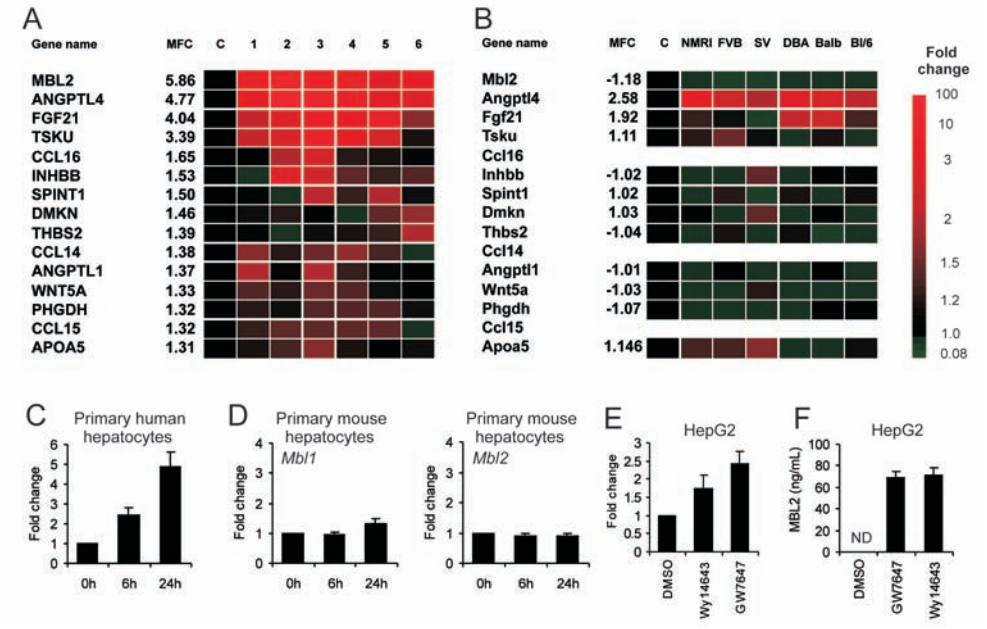

Figure 1. PPARα regulates MBL2 expression in human hepatocytes. A) Heat map illustrating the relative induction of genes encoding secreted proteins in response to 24h Wy14643 treatment in human hepatocytes. B) Relative changes in expression of the corresponding mouse orthologs in mouse hepatocytes. All genes were significantly changed ($P<0.05$) and were ranked based on mean fold-change (MFC). Expression levels in the DMSO-treated cells were set at 1. C) Relative induction of Mannose binding lectin (MBL2) by Wy14643 (50 μM) in human primary hepatocytes. D) Relative induction of Mannose binding lectin 1 (Mbl1) and Mannose binding lectin 2 (Mbl2) by Wy14643 (10 μM) in mouse primary hepatocytes. E) Relative induction of MBL2 by 6h Wy14643 (10 μM) and GW7647 (10 μM) in HepG2 cells. Expression of cells treated with DMSO was set at 1. Error bars represent SEM. F) MBL2 protein concentration in the serum free medium of HepG2 cells incubated 24h in the presence or absence of Wy14643 and GW7647 as assessed by ELISA. Error bars represent SEM. ND (Not detected).

Human MBL2 plasma levels are increased by fenofibrate

Our aim was to find novel circulating mediators of PPARα activity in human. Accordingly, we studied the effect of fasting, which leads to activation of hepatic PPARα [3], on plasma MBL2 levels. In line with published data, the inter-individual variation in baseline plasma MBL2 levels was very high. Although the number of subjects was limited, MBL2 consistently went up upon fasting in all 4 subjects studied (Figure 2A) ($P=0.05$).

Next, we wanted to study the effect of PPARα activation by synthetic agonists. To that end, we measured serum levels of MBL2 protein in 9 obese females with type 2 diabetes before and after three months of treatment with PPARα agonist fenofibrate (200 mg/day). In spite of the large inter-individual variation, fenofibrate raised serum MBL2 in all subjects, with a mean increase of 86% ($P<0.05$) (Figure 2B).

To further substantiate this finding, we measured plasma MBL2 in 19 overweight subjects before and after 6 weeks of treatment with the PPARα agonist fenofibrate (200 mg/day). Again, fenofibrate raised plasma MBL2 in all subjects (Figure 3). The mean increase in plasma MBL2 was identical to the above mentioned study at 86% ($P<0.001$). The data show that circulating MBL2 levels are increased by PPARα activation in human.

Other circulating targets of PPARα that are produced in liver and that exhibit an increase in plasma levels upon fibrate treatment are FGF21 and ANGPTL4 [18-21]. Indeed, we found a significant increase in plasma FGF21 (413%, $P<0.001$) and ANGPTL4 (69%, $P<0.01$) by 6 week fenofibrate treatment in the 19 overweight subjects (Figure 3). To examine whether some individuals are generally more responsive to PPARα activation regardless of the target gene studied, we tested the correlation between the relative increase in plasma MBL2 and the relative increase in plasma FGF21 or ANGPTL4 upon fenofibrate treatment. No significant correlations were observed (data not shown), arguing against the notion that individuals could be classified according to general PPARα responsiveness, at least on the basis of changes in plasma levels of PPARα targets.

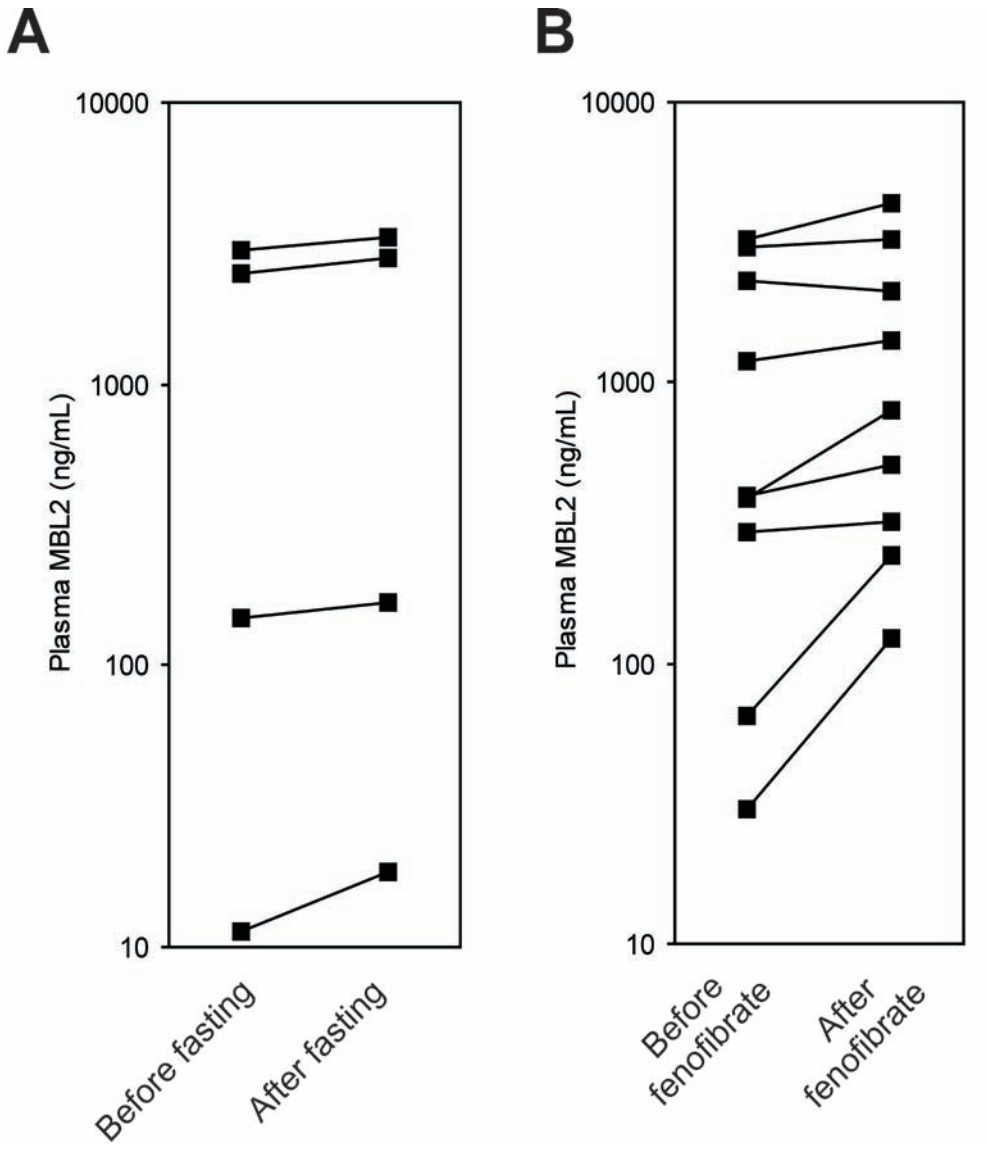

Figure 2. Plasma levels of MBL2 are increased by fasting and fenofibrate treatment. (A) 48h of fasting increased plasma MBL2 concentrations in 4 healthy males (P=0.05). (B) three months of fenofibrate treatment (200 mg/day micronized; Lipanthyl) significantly increased plasma MBL2 concentrations in 9 female subjects with type 2 diabetes mellitus (mean increase 86%, P<0.02).

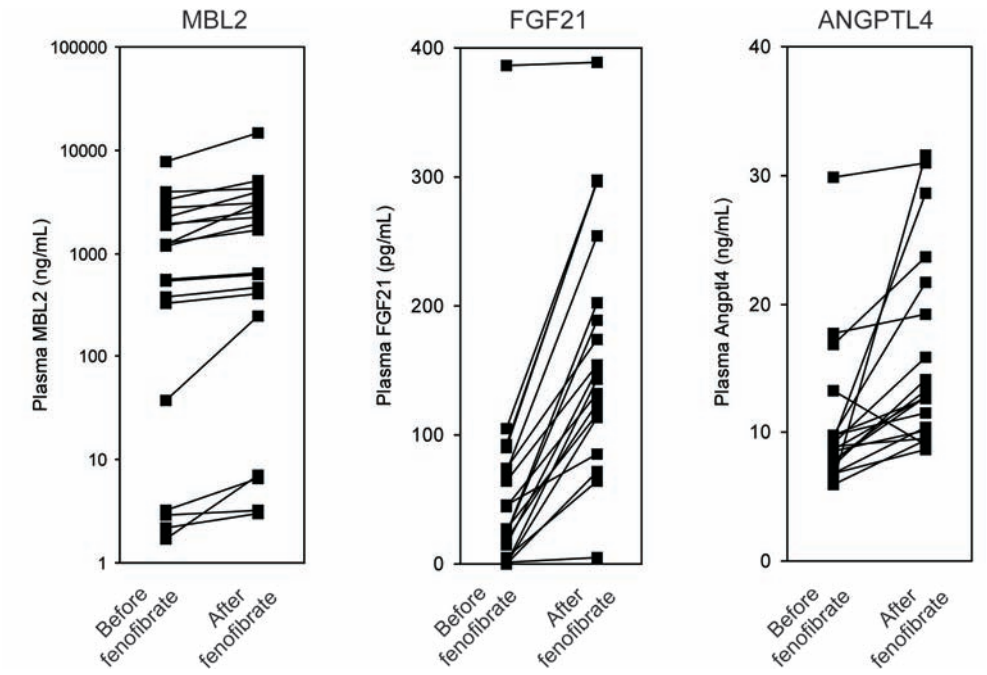

Figure 3. Fenofibrate treatment increases plasma levels of MBL2, FGF21 and ANGPTL4. Six weeks of fenofibrate treatment (200 mg/day micronized; Lipanthyl) significantly increased plasma MBL2 (86%, P<0.001), FGF21 (413%, P<0.001) and ANGPTL4 (69%, P<0.01) concentrations in 19 overweight subjects.

Discussion

MBL2 is an important player in complement cascade activation as part of the first line host defense. In the present paper we show that: 1) MBL2 is a target gene of PPARα in human hepatocytes and 2) plasma MBL2 levels are increased by chronic PPARα activation via fibrate drugs. MBL2 may thus represent a novel circulating mediator of PPARα action.

MBL2 recognizes and binds to conserved carbohydrate structures present on the surface of microorganisms [9, 15]. MBL2 binding results in activation of the lectin pathway of the complement system by the action of MBL2-associated serine proteases (MASPs), which associate with circulating MBL2 in their inactive proenzymatic forms [22]. Alternatively, MBL2 acts as an opsonin, leading to stimulation of phagocytosis by binding to cell-surface receptors present on phagocytic cells. A wealth of data published in the past decade show that in addition to being a crucial regulator of hepatic lipid metabolism, PPARα also has a major impact on inflammatory pathway [23]. The pronounced induction of MBL2 by PPARα in human liver fits within the role of PPARα as important regulator of inflammation and innate immunity [24].

Currently, little is known about factors controlling plasma MBL2 levels. While MBL2 levels in serum are known to be largely determined by polymorphisms in the MBL2 gene, differences in plasma MBL2 of up to 10-fold can be found between individuals despite identical genotypes [25]. Also, little is known about regulation of MBL2 gene expression. The specific expression of MBL2 in liver has been suggested to be mediated by HNF3 based on the presence of specific response element in the MBL2 promoter and its ability to bind HNF3 in vitro [26]. However, extensive evidence for regulation of MBL2 by HNF3 is currently lacking. Clearly, regulation of MBL2 by PPARα does not exclude regulation by HNF3.

Recently, evidence was provided that MBL2 may also be expressed in extra-hepatic tissues [27]. However, similar to our study, expression was so low that the functional relevance of extra-hepatically produced MBL2 may be questioned.

In our study we surprisingly found that every subject that received fenofibrate exhibited an increase in plasma MBL2. Although baseline difference in plasma MBL2 are largely related to polymorphisms in the MBL2 gene, our data suggest part of the variation may be due to differences in PPARα activity and/or expression level. In this context, it is interesting to note that two other secreted proteins that were highly induced by PPARα activation in primary human hepatocytes, ANGPTL4 and FGF21, also show large inter-individual variations in their plasma level. Similar to MBL2, plasma levels of ANGPTL4 and FGF21 are increased

by treatment with PPARα agonist [18-21]. In the present study, no significant correlations were found between the relative increase in plasma MBL2 and the relative increase in plasma FGF21 or ANGPTL4 upon fenofibrate treatment. The data argue against the notion that individuals could be classified according to general PPARα responsiveness, at least on the basis of changes in plasma levels of PPARα targets.

Our data indicate that PPARα is unable to induce MBL2 expression in mouse liver. Previously, several genes have been reported to be specifically regulated by PPARα in human, including ApoAI, ApoAII and ApoAIV [28]. For each of these genes the loss of regulation in mouse was related to lack of conservation of the functional PPREs. Since the PPRE(s) responsible for induction of MBL2 by PPARα remains elusive, it is impossible to determine whether a similar mechanism applies here.

MBL2 is known to be under a tight physiological regulatory system, which is evident by its stable circulating profiles in healthy individuals, independent of age, gender, time, physical exercise [29]. Alterations in MBL2 levels in disease states have been suggested to be partly caused by hormonal changes. A limited number of clinical trials have shown an stimulatory effect of growth hormone and thyroid hormones on MBL2 levels [30, 31], which was reproduced in hepatocytes cell lines [32]. Preliminary data from our group show that plasma MBL2 levels are reduced by insulin in healthy subjects, but not in patients with type 2 diabetes. The inhibitory effect of insulin on plasma MBL2 is in agreement with a previous study that reported higher MBL2 levels in patients with insulin-dependent type 1 diabetes [33]. Regulation of MBL2 by PPARα and by various metabolically active hormones suggests that MBL2 may play a role in regulation of energy metabolism, although additional research is needed.

In conclusion, our data point to MBL2 as potential circulating mediator of PPARα activity in human. Future studies should investigate a possible role for MBL2 in regulation of energy metabolism.

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Appendix Chapter 5

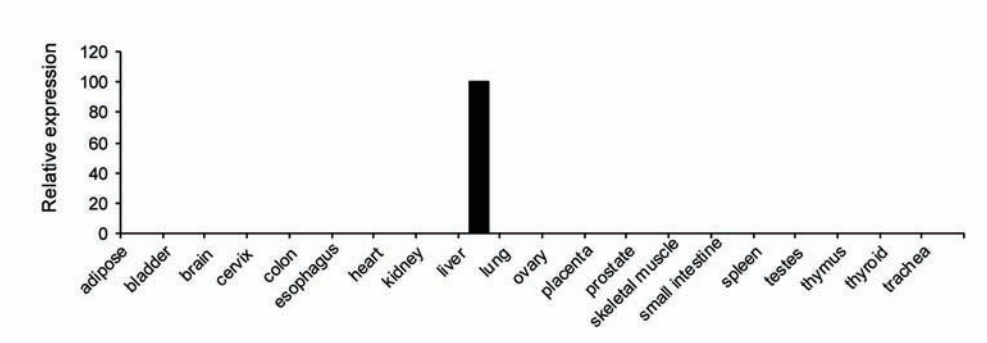

Figure S1. MBL2 is expressed specifically in human liver. mRNA expression of MBL2 was determined in human tissues by Q-PCR. Human RNA represented a mix from several individuals (AMBION, First choice human total RNA). Expression levels were related to the liver which was the tissue showing highest expression.

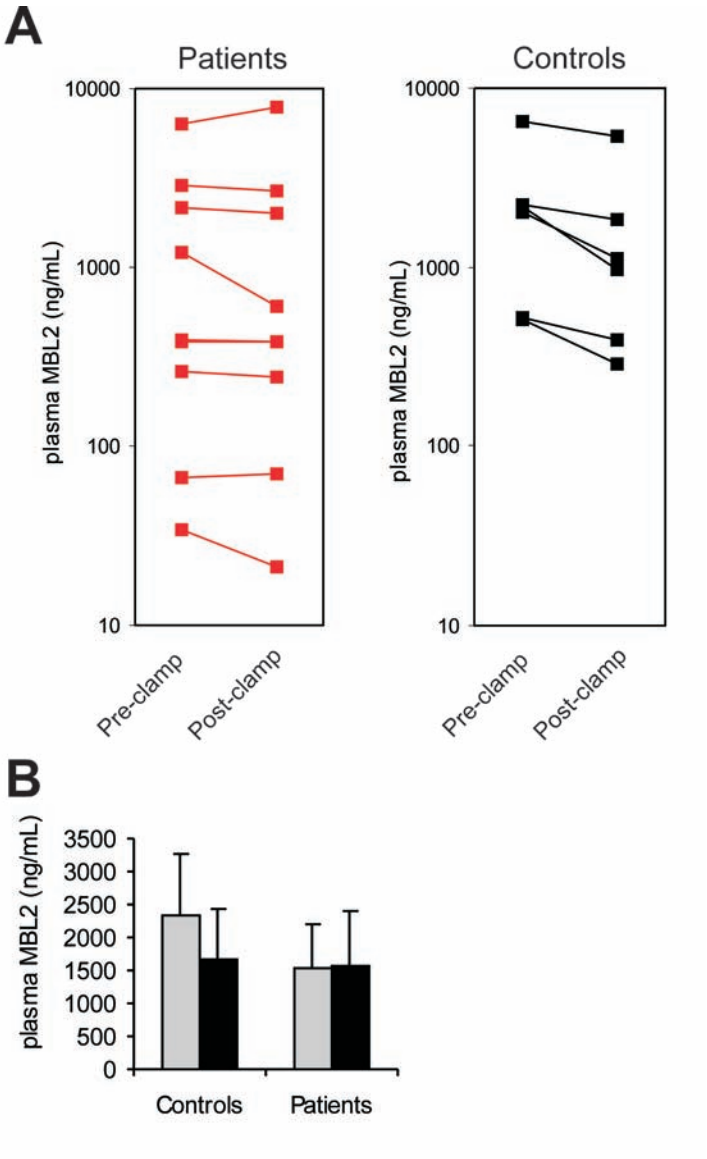

Figure S2. Insulin suppresses MBL2 plasma concentration.

Changes of MBL2 concentrations before and after 180 minutes of isoglycemic-hyperinsulinemic clamp in a group of 6 healthy controls and nine T2DM patients (A). Mean concentration of plasma MB2 levels of the controls and patients before and after 180 minutes of isoglycemic-hyperinsulinemic clamp (B). Error bars represent SEM. Quantification assessed by using MBL Oligomer ELISA kit.

Supplemental methods

Subjects

Subjects with a BMI of at least 27 kg/m2 were recruited via posters in the university and hospital buildings and via advertisements in local newspapers. Subjects came to the university for a screening visit. On this visit, fasting blood was sampled for analyses of serum lipids and lipoproteins. In addition, height and body weight were determined. Furthermore, subjects had to complete a medical and general questionnaire. Exclusion criteria were BMI below 27 kg/m2, impairment of kidney and liver function, serum total cholesterol above 8 mmol/L, serum triglycerides above 4 mmol/L, taking medication that could influence the study outcome or could interfere with fenofibrate treatment, use of fish oil supplements, consumption of plant sterol or stanol-enriched food products, having donated blood within 1 month prior to the start of the study, having a diagnosis of any long-term medical condition (e.g. diabetes, cardiovascular diseases, epilepsy) or experiencing strong symptoms of allergy. After screening, twenty-six subjects met all our inclusion criteria and started this study. Subjects received oral and written information about the nature and risk of the experimental procedures before their written informed consent before the start of the study. The study was approved by the Medical Ethical Committee of Maastricht University.

After inclusion of 26 subjects, 6 subjects dropped out and were not included in the analysis (1 man underwent surgery for an aneurism, 1 woman had complained about vapours during the placebo period, 1 man and 1 woman did not regularly attend appointments and were excluded, 1 man had a work-related reason, and 1 man had personal reasons). Ten men and ten women completed the trial. Subjects were 52 ± 12 y (mean ± SD), with a bodyweight of 98 ± 19 kg for men and 95 ± 20 kg for women, a BMI of 31 ± 5 kg/m2 for men and 34 ± 5 kg/m2 for women, and a waist circumference of 118 ± 13 cm for men and 116 ± 11 cm for women. Serum concentrations of total cholesterol were 6.23 ± 1.18 mmol/L, of LDL cholesterol 3.97 ± 1.09 mmol/L, of HDL cholesterol 1.52 ± 0.44 mmol/L, of triglycerides 1.63 ± 0.59 mmol/L, and of glucose 5.34 ± 0.73 mmol/L. Systolic blood pressure was 131 ± 14 mmHg and diastolic pressure was 91 ± 8 mmHg. Four subjects smoked. According to the National Cholesterol Education Program Adult Treatment Panel III guidelines to diagnose the metabolic syndrome, subjects had on average 2.2 ± 1 criteria of the metabolic syndrome and 7 out of 20 subjects could be diagnosed as having the metabolic syndrome.

Study design

The study had a randomized, double-blind, placebo-controlled, crossover design. Each subject enrolled in random order in a fish oil, a fenofibrate and a placebo period for 6 weeks with a wash-out period of at least 2 weeks between the intervention periods. During the fish oil intervention, subjects had to consume 8 fish oil capsules (Marinol C-38™, Lipid Nutrition, Wormerveer, the Netherlands), providing approximately 3.7 g/d n-3 LCPUFA (1.7 g/d EPA and 1.2 g/d DHA, corresponding to 160-240 g fatty fish/d), together with 2 capsules placebo-matching fenofibrate (200 mg/d cellulose). During the fenofibrate period, subjects consumed 2 capsules providing 200 mg/d micronized fenofibrate (Lipanthyl®, Fournier Laboratories, Dijon, France), together with 8 placebo-matching fish oil capsules (containing 80% High Oleic Sunflower Oil (HOSO)). During the placebo period, subjects received 8 HOSO capsules together with 2 cellulose capsules. Subjects were instructed to ingest half of the capsules before breakfast and the other half before dinner with a glass of water. Subjects were restricted in their fish consumption to a maximum of one portion a week. During the study, subjects recorded any symptom of illness, visits to physician, medication used, alcohol consumption, and any deviations from the protocol in diaries. Body weight was recorded at weeks 0, 5 and 6 of each intervention period and blood pressure was monitored using a sphygmomanometer (Omron M7, CEMEX Medische Techniek BV, Nieuwegein, the Netherlands). At the end of the three intervention periods, energy and nutrient intakes were estimated for the previous 4 weeks using a food frequency questionnaire (FFQ).

Blood sampling

In week 5 and week 6 of each intervention period, subjects arrived in the morning after an overnight fast and after abstinence from drinking alcohol the preceding day. Venous blood samples were collected in BD vacutainer® tubes (Becton Dickinson Company, NJ, USA). Serum was obtained by clotting the blood for 30 minutes, followed by 30 min centrifugation at 2000xg. EDTA, NaF and heparin plasma were obtained by centrifugation at 2000xg for 30 minutes at 4°C, directly after sampling. Serum and plasma aliquots were snap frozen and stored at -80 °C until analysis.

Clinical safety parameters

Serum concentrations of markers of liver and kidney function (total bilirubin, asparagine aminotransferase (ASAT), alanine-aminotransferase (ALAT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GT), ureum, and creatinine) from week 6 of each intervention period were determined at the department of Clinical Chemistry, University Hospital Maastricht (Beckman Synchron CX7 Clinical systems, Beckman).

Chapter 6

Comparative microarray analysis of PPARα induced gene expression in the human hepatoma cell line HepG2 and primary human hepatocytes

David L.M. van der Meer, Maryam Rakhshandehroo, Mohammad O. Ullah, Philip J. de Groot, Sacco C. de Vries, Michael Müller, Sander Kersten

Manuscript in preparation

Abstract

PPARα is an important transcriptional regulator of hepatic lipid metabolism. Most of the research on PPARα in liver has been carried out in mice or using hepatocyte cell lines. However, to what extent results from cell lines properly reflect the function of PPARα remains to be established. The aim of the present study was to compare the transcriptional response to PPARα activation between HepG2 and primary human hepatocytes, the latter being considered as the gold standard. Our transcriptomics analysis reveals that the response to PPARα agonist is remarkably dissimilar between HepG2 cells and primary human hepatocytes. While expression of many established PPARα targets shows significant induction by PPARα activation in primary hepatocytes, this is much less the case in HepG2 cells which are characterized by marginal induction of a limited number of PPARα targets. This set of PPARα targets include CYP1A1, CPT1A, ADFP, and TRIB3. Instead, PPARα activation in HepG2 cells leads to induction of stress response pathways. In conclusion, our results show that HepG2 cells relatively poorly reflect the established function of PPARα in lipid metabolism, in contrast to primary human hepatocytes. Accordingly, with respect to PPARα function, caution should be exercised when extrapolating data from HepG2 cells to human liver.

Introduction

Peroxisome proliferators activated receptors (PPARs) are ligand inducible nuclear receptors that play a major role in the regulation of cellular energy homeostasis. Three PPARs have been identified: PPARα (NR1C1), PPARb/d (NR1C2) and PPARg (NR1C3). PPARs are activated by fatty acids and fatty acid derivates, as well as by a diverse group of synthetic compounds [1-3]. PPARα is well expressed in liver and other tissues with a high rate of fatty acid catabolism such as heart and skeletal muscle [4] and regulates the transcription of numerous genes involved in fatty acid oxidation, fatty acid uptake and transport, ketogenesis, gluconeogenesis, amino acid metabolism and inflammation. Compared to PPARα, PPARβ/δ is expressed in a broader rage of tissue including brain, small intestine, heart, skeletal muscle, adipose and skin tissue [5-7]. Recent studies have linked PPARβ/δ to regulation of glucose homeostasis, fatty acid metabolism, wound healing, and inflammation [8-11]. The third isotype, PPARγ, is highly expressed in adipose tissue where it is involved in adipose cell differentiation and lipid storage [12]. In contrast to the classical steroid receptors, PPARs form heterodimers with another nuclear receptor named Retinoid X Receptor (RXR). According to the traditional view, target genes of PPARs are characterized by a PPAR responsive element (PPRE) in their promoter region that is capable of binding to PPAR/RXR heterodimers. However, recent studies suggest that many PPAR binding sites are located distant from the transcription start site and that regulation may be conferred by DNA looping [13, 14]. Upon ligand binding to PPAR, a conformational change of the receptor results in the dissociation of corepressor proteins and the binding of several coactivator proteins which ultimately results in initiation of transcription of a target gene [15].

Because PPARα is expressed at high level in liver, lots of research has been carried out on the role of PPARα in the liver. Many of these studies have made use of mouse models due to the relative ease to obtain whole liver tissue directly from mice as well as the possibility to perform in vivo gene targeting. However the obvious drawback of rodent models are differences in the regulation of biological processes between rodents and human. Consequently, extrapolation of data from rodent models to the human situation can be problematic. For example, the hepatomegaly and peroxisome proliferation observed in response to PPARα activation in mouse and rat is not observed in human cells [16]. However, in contrast to mouse tissue, whole human liver tissue and primary human hepatocytes are not widely available and are expensive, thereby limiting their use. Instead, much research is performed using liver derived and immortalized cell-lines such as the hepatoma derived cell-line HepG2. Several studies have used this cell line to investigate the role of PPARα in human liver cells [17-21]. HepG2 cells are derived from a hepatoma in 15 year old Caucasian male [22]. The advantage of this immortalized cell line is the almost unlimited availability and reduced variability compared

to donor-derived primary hepatocytes. However, differences in this cell line in comparison to primary hepatocytes would be expected due to its transformation from a differentiated and low proliferating hepatocyte to a highly proliferating cancer cell line. Previous studies comparing the two cell systems have mainly been focused on the difference in responsiveness to toxicological stimuli and biotransformation in the two cell types [23-30]. Furthermore, previous studies addressed differences in basal gene expression profiles between HepG2 cells and primary hepatocytes using microarray technology [31, 32]. These studies show alterations in gene expression in HepG2 cells that are related to its transformation to a cancer cell line, which includes genes involved in cell cycle and check-point control as well as genes involved in cell death, lipid metabolism, transport and xenobiotic metabolism. Surprisingly, Harris et al. also reported loss of gene expression of several genes in primary hepatocytes that were actually expressed in whole liver and HepG2 cells, suggesting that is some cases HepG2 cells would be a preferred human liver cell model. To date, no systematic effort has been performed comparing the transcriptional response to PPARα activation in HepG2 cells and human primary hepatocytes.

Material and methods

Cell culture. HepG2 cells were grown in phenol red free Dulbecco's modified medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamate and antibiotics. Cells were kept at 37 °C and 5% CO2. Cells were split the day before experiments. Human hepatocytes and Hepatocyte Culture Medium Bulletkit were purchased from Lonza Bioscience (Verviers, Belgium). Primary hepatocytes were isolated from surgical liver biopsies obtained from six individual donors who underwent surgery after informed consent was obtained. Hepatocytes were isolated with two-step collagenase perfusion method and the viability of the cells was over 80%. Cells were plated on collagen-coated six-well plates and filled with maintenance medium. Upon arrival of the cells, the medium was discarded and was replaced by Hepatocyte Culture Medium (HCM) with additives. Additives included Gentamicin sulphate/Amphotercin-B, Bovine serum albumin (Fatty acid free), Transferrin, Ascorbic acid, Insulin, Epidermal growth factor, Hydrocortisone hemisuccinate..

Transcriptomics. Total RNA was extracted from either HepG2 cells or primary hepatocytes with TRIzol reagent (Invitrogen) and subsequently purified using the SV Total RNA Isolation System (Promega). RNA quality was measured on an Agilent 2100 bioanalyzer (Agilent Technologies) using 6000 Nano Chips according to manufacturer's instructions. RNA was judged as suitable for array hybridization only when samples showed intact bands corresponding to the 18S and 28S rRNA subunits, displayed no chromosomal peaks or RNA

degradation products and had a RNA integrity number (RIN) above 8.0. Five micrograms of RNA were used for one cycle cRNA synthesis (Affymetrix). Hybridization, washing and scanning of Affymetrix human genome 133 2.0 plus arrays was carried out according to standard Affymetrix protocols. Scans of the Affymetrix arrays were processed using packages from the R/Bioconductor project. Arrays were normalized with quantile normalization and expression levels of probe sets were calculated using the Robust Multichip Average (RMA) method. Differentially expressed probe sets were identified using IBMT and genes were considered to be significantly changed when raw q-values were smaller the 0.05 and fold-change was above 1.2 or below -1.2.

Comparative analysis. To compare the number of genes regulated in HepG2 cells as well as the number of cells in primary hepatocytes a venn diagram was created with venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Scatterplots were made by plotting all expression values of both cell types with the use of the SPSS statistical program. For comparison of the top 50 upregulated genes between HepG2 cells and primary hepatocytes, heatmaps were created based on fold changes with the use of Microsoft Excel. PCA scores were obtained from all expression values taken from the arrays of both HepG2 cells and primary hepatocytes with the use of R package FectoMiner. Obtained PCA scores were plotted using SPSS. To define significant difference between cell groups a one-way ANOVA combined with a Tukey post hoc analysis was performed.

Biological characterization. To characterize genes according to their biological function a Gene Set Enrichment Analysis (GSEA) was performed. As a cut off for enriched gene sets the FDR-q value was set at <0.25. Enriched gene sets were ordered according to their normalized enrichment score (NES).

Results

To compare PPARα induced gene expression in HepG2 cells and primary human hepatocytes, cells were treated with PPARα agonist for 6 or 24 hours and changes in gene expression analyzed by microarray. A fold-change threshold of 1.2 and a minimal q-value of 0.05 was used. The lower-fold change threshold was chosen due to the limited magnitude of gene induction by PPARα agonists in human hepatocytes and HepG2 cells. After 6 hours of agonist treatment, 339 genes were upregulated and 282 genes downregulated in HepG2 cells, compared to 144 genes upregulated and 73 genes downregulated in primary human hepatocytes (Figure 1). The difference in number of genes regulated between the two cell systems was even more pronounced after 24 hour agonist treatment.

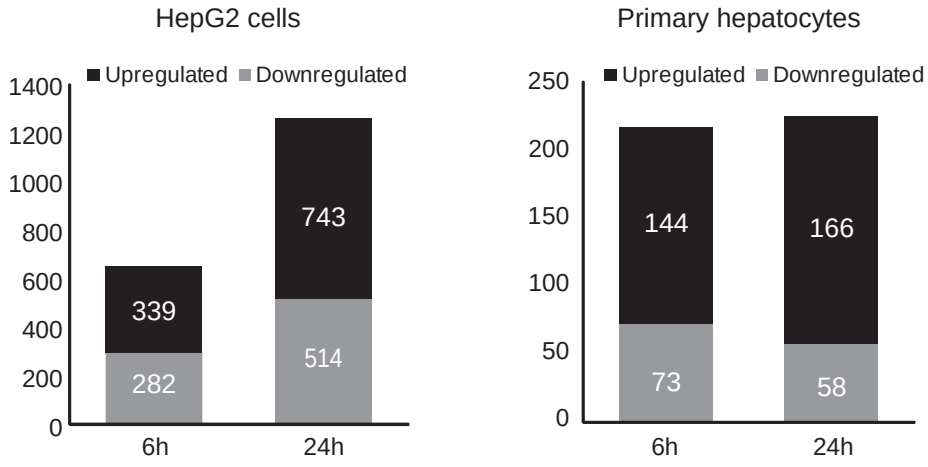

Figure 1: Expressional changes in HepG2 cells and primary hepatocytes upon PPARα activation. Bars indicate total genes regulated at either 6 hours or 24 hours of PPARα agonist treatment with the upper part representing the number of upregulated genes and the bottom part the number of downregulated genes. Genes were considered significantly changed when fold changes were >1.2 for upregulated genes and <-1.2 for downregulated genes as well as a q-value <0.05.

To further study the response to PPARα activation in HepG2 cells and primary hepatocytes, the overlap in gene regulation between the two cell systems was analyzed. As shown in Figure 2, 20 and 34 genes were commonly upregulated in both cell types after 6 hours and 24 hour of agonist treatment, respectively. These numbers represent 13.8% and 20.5% of the total number of genes upregulated in primary hepatocytes, but only 5.9% and 4.6% of the total number of genes upregulated in HepG2 cells after 6 hours and 24 hours of PPARα activation, respectively. Even less overlap was observed when examining the genes downregulated upon agonist treatment.

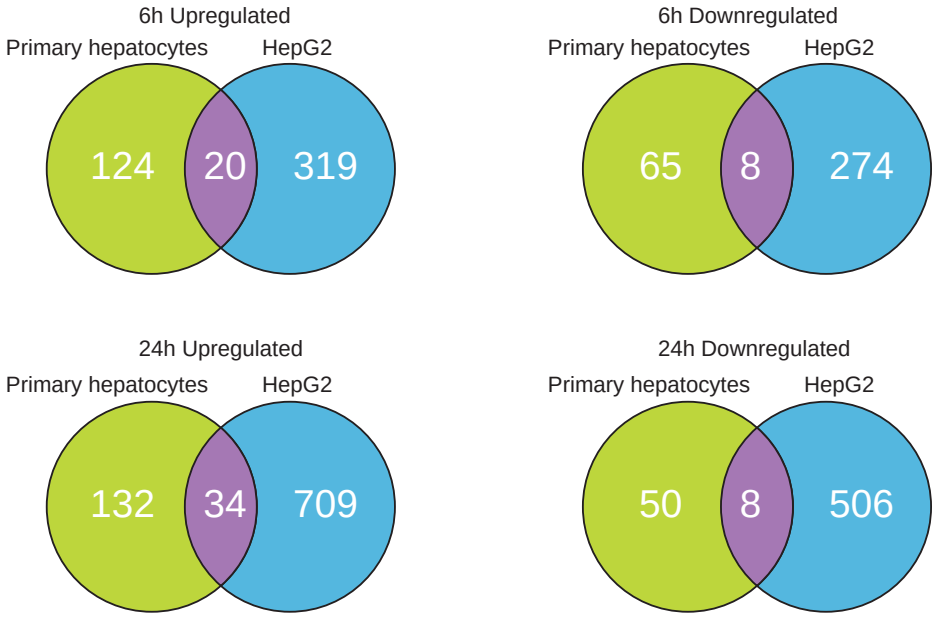

Figure 2: Overlap between differential regulated genes in HepG2 cells and primary hepatocytes. Venn diagrams represent overlap between either the upregulated genes or downregulated genes in HepG2 cells and primary hepatocytes.

To globally compare the gene expression profiles in both cell types, we performed principal component analysis (PCA) on the expression values of all genes after 6 or 24 hours agonist treatment. As shown in Figure 3, principal component 1 (PC1) explains 79.8% and 78.5% of the variation in gene expression at 6 and 24 hours, respectively. PC1 scores for all primary hepatocyte samples are not significantly different. Similarly we observed grouping of PC1 scores for all HepG2 samples without any significant changes between the HepG2 samples. However, comparing the PC1 scores of the two cell types shows a significant difference between the cell types, indicating that PC1 explains significant variation between the general expression profiles of the two cell types. Furthermore, we observed significant separation of donor 5 and 6 in principal component 2 (PC2), indicating a general expression profile that is weakly different from the other four donors explained by PC2. Interestingly, these two donors are the two oldest male donors. PC1 and PC2 did not explain any significant variation caused by PPARα agonist treatment in either cell type when analyzing all expression data of both cell types together.

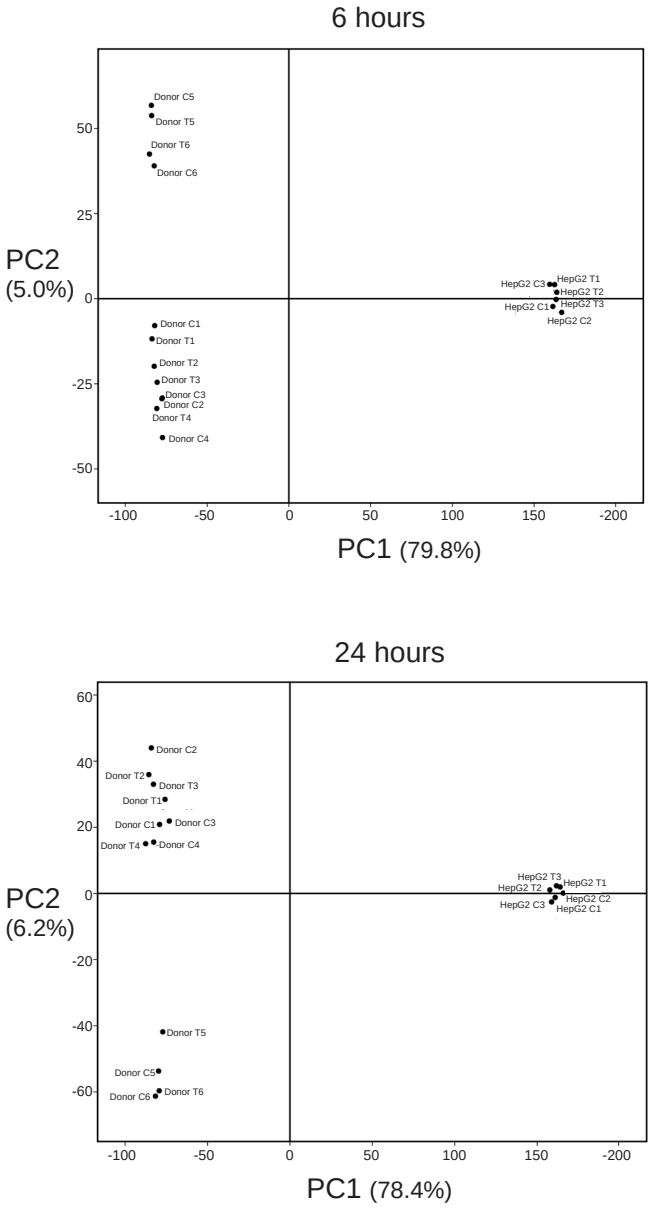

Figure 3: Principal component analysis of genes expressed in HepG2 cells and primary hepatocytes. Principal component (PC) 1 and 2 represent the variation found between the samples after either 6 hours or 24 hours PPARα activation.

To further compare changes in gene expression upon PPARα activation between HepG2 cells and primary hepatocytes, changes in gene expression for all genes on the array were plotted in a scatter diagram (Figure 4). The results show that with the exception of a few genes (e.g. KLF10, CYP1A1, ADFP), the majority of genes regulated were regulated specifically in one of the cell types. Genes encoding for metallothioneins were specifically regulated in HepG2 cells. In contrast, many well described PPARα target genes, including PDK4, ANGPTL4 and FABP1 [33-36], were regulated specifically in human primary hepatocytes.

To further analyze similarities in gene regulation between the two cell types, we selected the top 50 upregulated genes in HepG2 cells and show the corresponding changes in gene expression in primary hepatocytes. Conversely, we selected the top 50 upregulated genes in primary hepatocytes and show the corresponding changes in gene expression in HepG2 cells (Figure 5). The top 50 genes induced in HepG2 cells show a consistent response between the three replicates. Among the genes responding most strongly are several genes coding for metallothioneins, which do not show any changes in primary hepatocytes. Similar to the scatter diagram, the results show that very few genes induced by PPARα agonist treatment in primary hepatocytes are also regulated in HepG2. Furthermore, the top 50 upregulated genes in HepG2 very poorly reflects the known role of PPARα in lipid metabolism and instead points towards induction of cellular stress response. In contrast, in primary hepatocytes a large number of genes within the top 50 of upregulated genes are involved in lipid metabolism and represent established PPARα targets. These include ANGPTL4, FABP1, PCK1, HMGCS2, S25A20, ACSL3, CPT2 and several others. These various types of analyses indicate a poor match between the effect of PPARα activation between primary hepatocytes and HepG2 cells. Overall, the observed changes in gene expression in HepG2 very poorly resemble the role of PPARα in lipid metabolism, and mostly points towards induction of cellular stress response. The set of genes robustly induced in both cell types was limited to VLDLR, ADFP, CYP1A1, CPT1A, KLF10 and LOC55908.

These data suggest that HepG2 cells are an inferior model to study PPARα dependent gene regulation, especially in relation to its role in lipid metabolism.

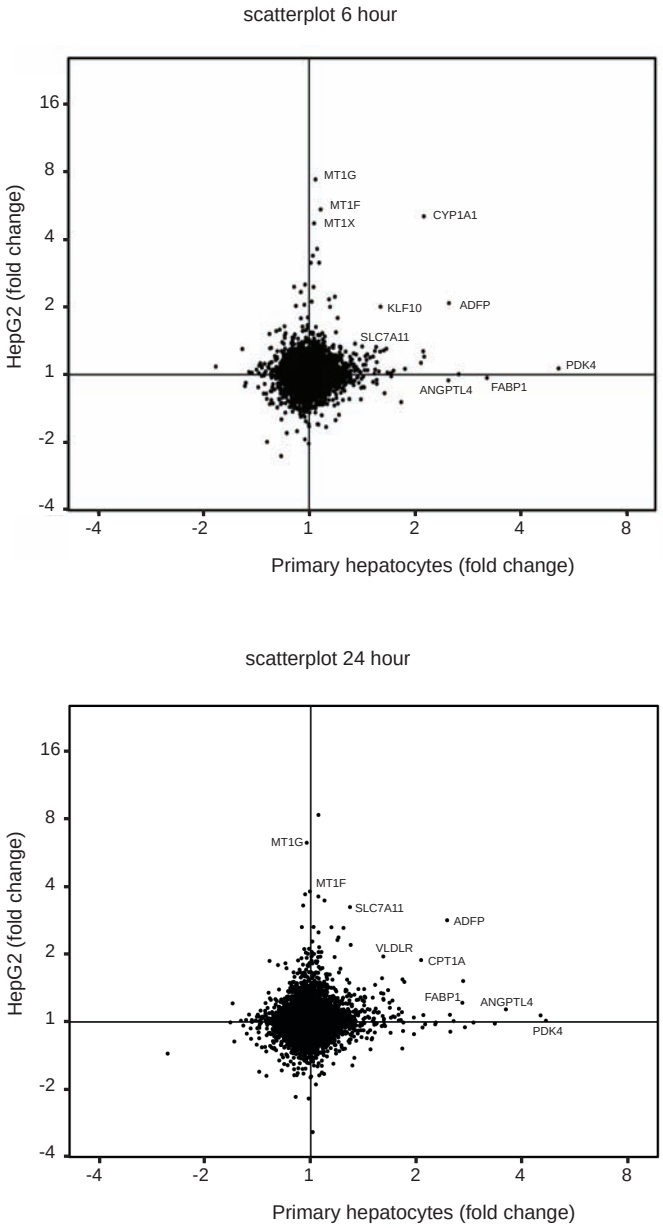

Figure 4: Global gene expression comparison in HepG2 cells and primary hepatocytes upon PPARα activation. All expression values of all genes were plotted in scatter diagram based on fold change after PPARα activation. Selected PPARα target genes are indicated as well as some metallothionins specifically regulated in HepG2 cells.

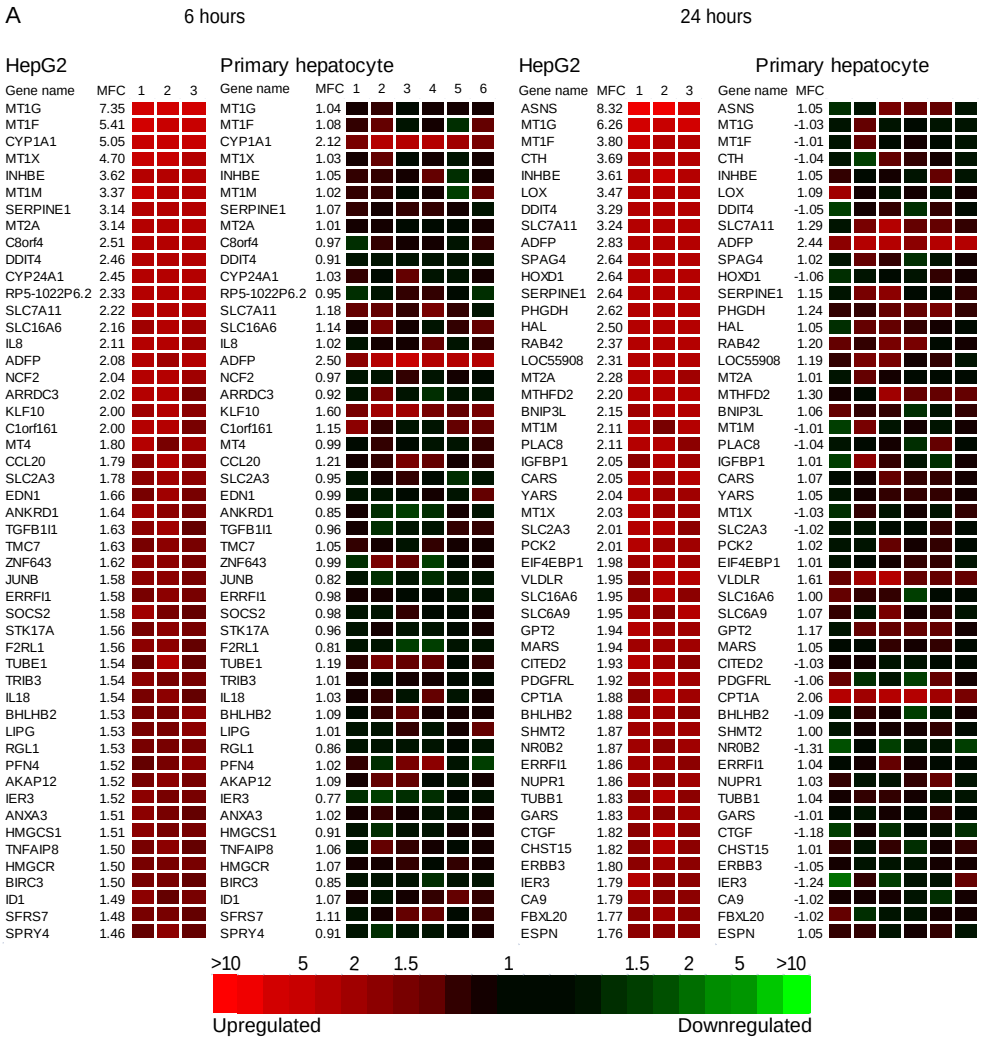

Figure 5A: Heatmaps representing top 50 regulated genes in HepG2 cells and primary hepatocytes after PPARα agonist treatment. (A) Top 50 significantly upregulated genes in HepG2 cells were compared with expressional change of the corresponding gene in primary hepatocytes. Genes were considered upregulated when fold change >1.2 and q-value was <0.05.

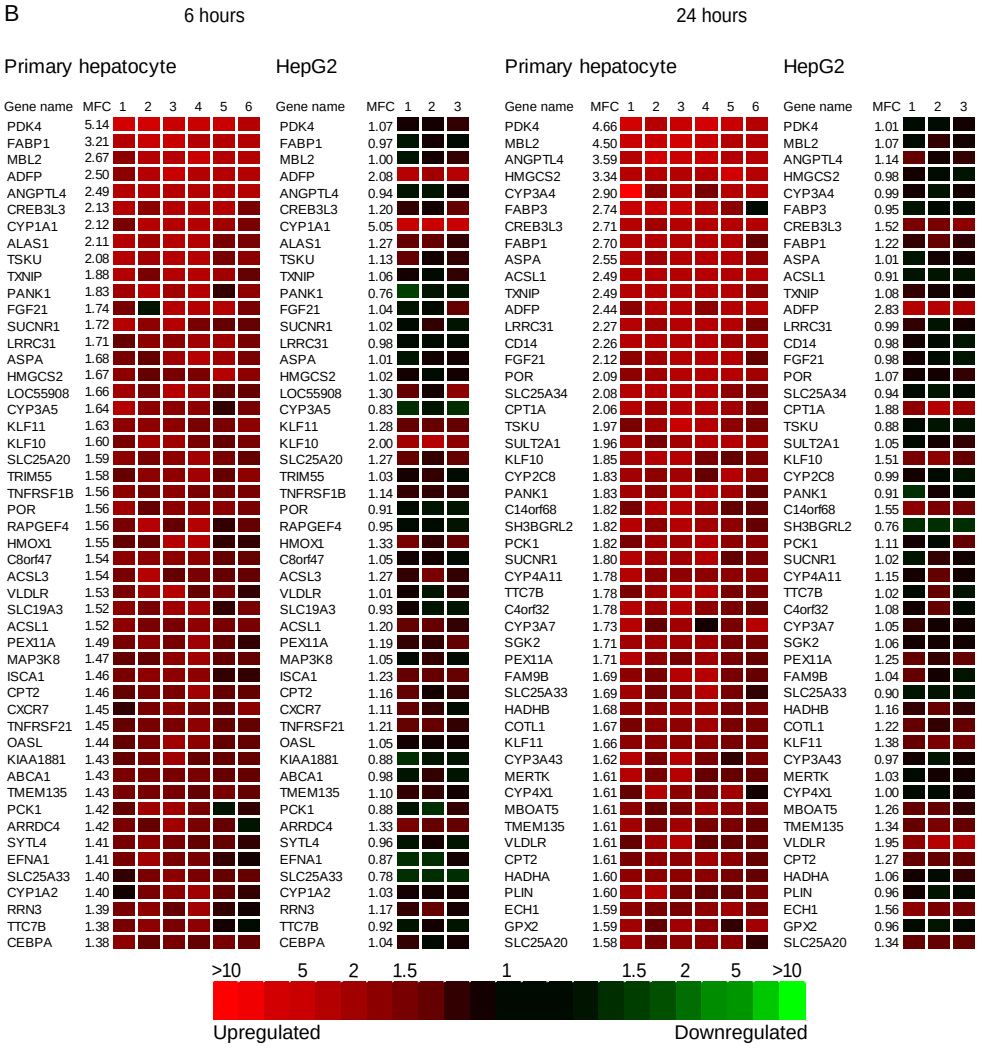

Figure 5B: Heatmaps representing top 50 regulated genes in HepG2 cells and primary hepatocytes after PPARα agonist treatment. (B) Top 50 genes changed in primary hepatocytes were compared to the corresponding gene change in HepG2 cells. Genes were considered upregulated when fold change >1.2 and q-value was <0.05.

Biological classification

The biological classification of genes specifically upregulated or downregulated in either HepG2 cells or primary hepatocytes, or commonly regulated in both cell types was investigated using Gene Set Enrichment Analysis (GSEA). A global comparison of biological processes regulated in HepG2 cells and primary hepatocytes showed a rather low number of commonly up regulated pathways in the two cell types after 6 hours agonist but slightly a better overlap was found after 24 hours agonist (Figure 6A). The biological process enriched by PPARα agonist in both cell types belonged to the classical PPARα-dependent pathway involved in fatty acid beta oxidation. Additional enrichment of PPARα related pathways was found among the biological processes specifically enriched in primary hepatocytes (Figure 6B). In contrast, pathways exclusively upregulated by PPARα agonist in HepG2 cells were related to steroid/sterol metabolism (6 hours). Interestingly, the amino acid degradation pathway of valine and (iso)leucine was upregulated in both cell types after 24 hours PPARα agonist treatment. Previously, amino acid metabolism was shown to be downregulated by PPARα in mouse liver [37]. A similar analysis was performed on genes downregulated after 6 and 24 hours of PPARα stimulation. After 24 hours of agonist treatment no pathways were commonly downregulated in both cell types (Figure 6A), which is consistent with the minimal overlap observed at the individual gene level (Figure 2). Consistent with the known suppressive effect of PPARα on inflammation, inflammatory pathways, such as the chemokine and interleukine pathways were downregulated by PPARα agonist in both primary hepatocytes and HepG2 cells after 6 hours PPARα activation (data not shown).

To further explore differences in gene regulation between HepG2 and primary hepatocytes, we compared the expression of several nuclear receptors and co-activator proteins under basal condition. As shown in Figure 7, the expression of most nuclear receptors was not very different between the two cell types. However, the nuclear receptors HNF4A, PPARα and RXRα were more highly expressed in HepG2 cells compared to primary hepatocytes. These data suggest that the minor effect of PPARα agonist in HepG2 cells on established PPARα targets and pathways is not related to low PPARα expression. Additionally, we compared the expression of several coactivators known to be involved in the PPARα dependent gene regulation (Figure 7). Whereas MED1, SRC3 and PRIP were much more highly expressed in HepG2 cells, CITED2 and PCAF were much more highly expression in primary hepatocytes.

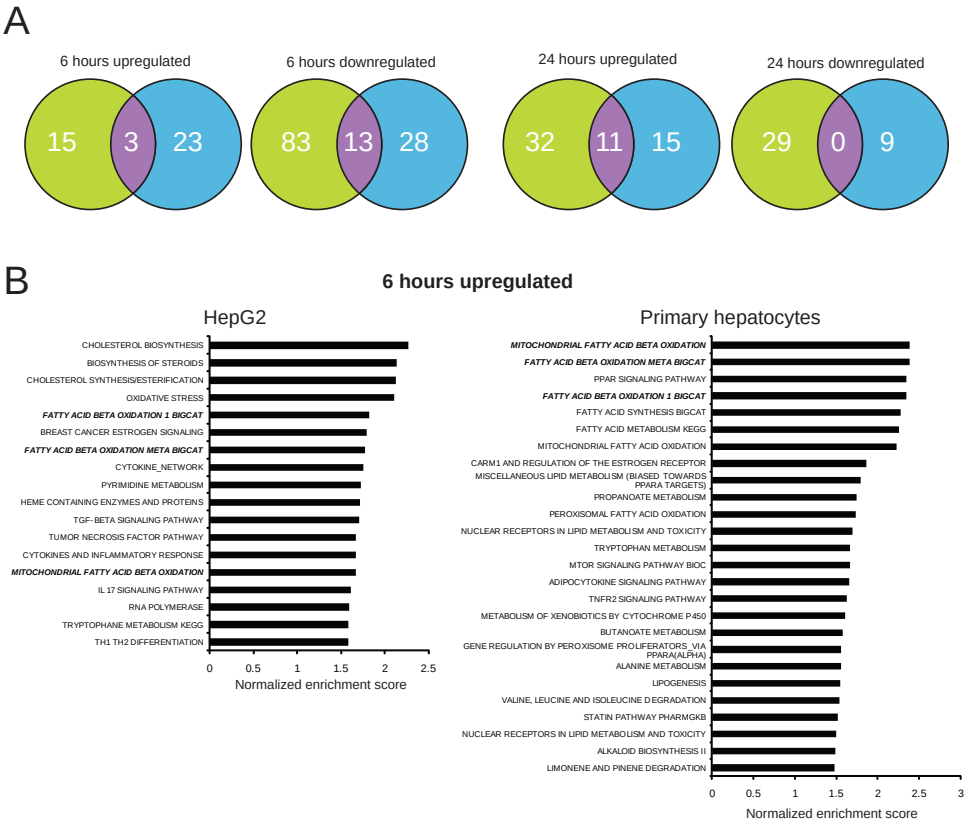

Figure 6: Biological classification of genes regulated in HepG2 cells and primary hepatocytes upon PPARα induction. (A) Regulated genes in primary hepatocytes and HepG2 cells were classified based on biological function using Geneset Enrichment Analysis (GSEA) and compared. Pathways with a FDR-q value below 0.25 were considered enriched. (B) All biological pathways enriched after 6 hours PPARα agonist treatment in HepG2 cells and primary hepatocytes. Pathways are ordered by normalized enrichment score (NES). Pathways enriched in both cell types are indicated (bold).

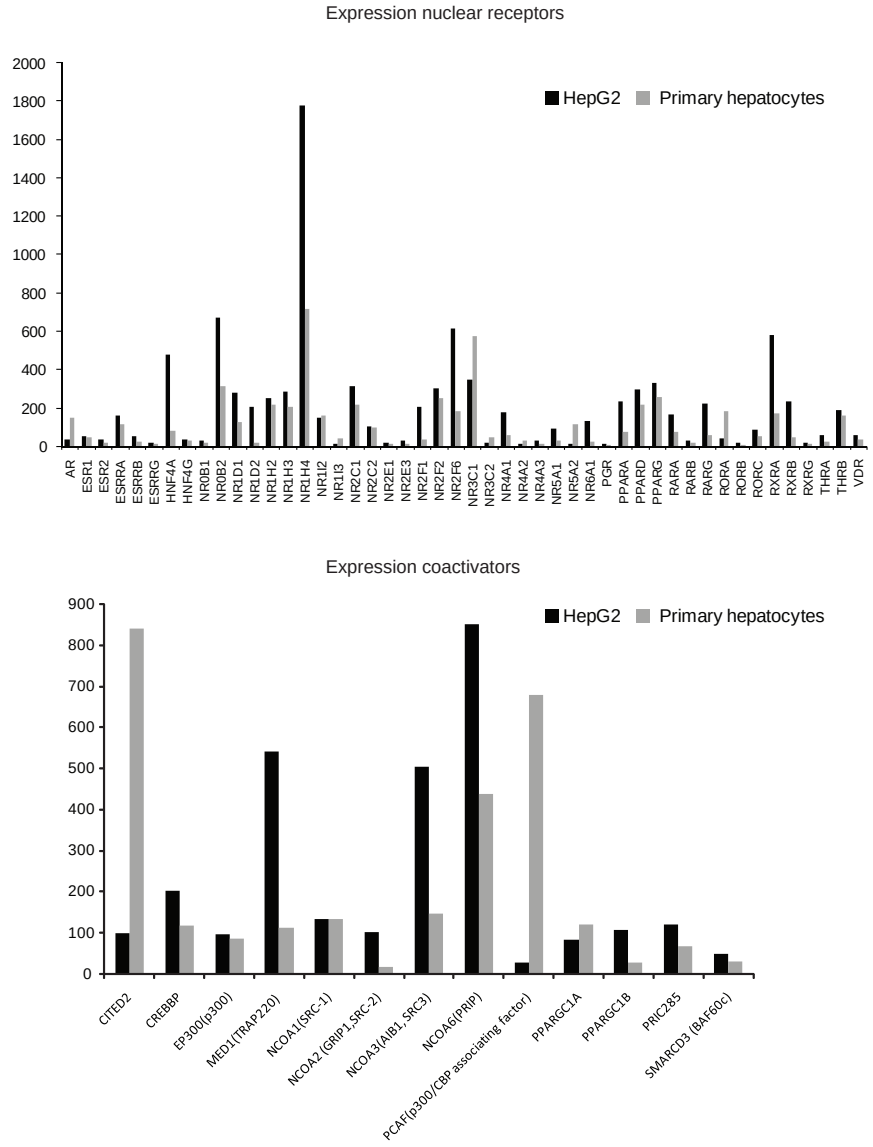

Figure 7: Basal expression of selected nuclear receptors and coactivators.

(A) Basal expression of nuclear receptors in HepG2 cells an primary hepatocytes are compared. (B) Expression values of selected coactivators known to be involved in PPARα signaling were compared at basal conditions in HepG2 cells and primary hepatocytes.

Discussion

Most of the research on PPARα in liver has been carried out in mice or using hepatocyte cell lines. Previously, we addressed the similarities in response to PPARα activation between human and mouse primary hepatocytes [38]. Here, we concentrate our analysis on the most widely used liver cell line, which is the hepatocarcinoma-derived cell line HepG2. Since cell lines often lose functional properties compared to the tissue from which they were derived from, it is important to investigate the suitability of these cell lines to study PPARα-dependent gene regulation. Overall, our data indicate that care should be taken in the use of HepG2 as a model to study PPARα function in human liver.

Basal differences in gene expression between HepG2 cells and primary hepatocytes have been previously studied using microarrays [31, 32]. It was found that 31% of the genes expressed in HepG2 cells are specific for this cell type, which included genes involved in pathways that are expected to be activated in an immortalized cell line such as cell cycle control, oncogenes and tumor suppressor genes. Similarily, Ligoru et al. found 4306 genes to be differentially expressed between HepG2 cells and primary hepatocytes under basal conditions. Again, several of these genes are involved in cell cycle regulation and checkpoint control. Surprisingly, Harris et al. found several genes to be similarly expressed in HepG2 and whole liver, but diminished in primary hepatocytes, indicating that primary hepatocytes may not always reflect the in vivo situation better compared to HepG2 cells.

One previous report compared the transcriptional changes upon PPARα stimulation in HepG2 cells to a rat cell line (FAO) using microarray technology [39]. Very minor overlap in PPARα-dependent gene regulation was observed between the two cell lines. Furthermore, very few genes involved in lipid metabolism were induced by PPARα activation in HepG2 cells. Remarkably, we could discern little overlap between genes regulated by PPARα in HepG2 cells in our study and in the abovementioned report. One possible reason may be differences in the properties of the HepG2 cells used as well as differences in culture conditions.

Our data show that the total number of genes regulated by PPARα agonist in HepG2 cells markedly exceeded the number in primary hepatocytes. One likely reason is that HepG2 cells are a lot more homogenous and consequently the response to PPARα activation is less variable and thus more likely to be statistically significant. Indeed, the overall magnitude of fold changes in expression were not noticeably different between the two cell types. However, the changes in gene expression upon PPARα activation in HepG2 cells poorly reflect the established function of PPARα in lipid metabolism. In contrast to primary hepatocytes, only a limited number of known PPARα targets were induced by PPARα agonist in HepG2

cells, which included CPT1A, ADFP, and TRIB3 [40]. Instead, treatment of HepG2 cells with PPARα agonist induced the expression of numerous genes involved in stress response pathways including various metallothioneins and DDIT4.

Numerous explanations may account for the differential response to PPARα activation between HepG2 cells and primary hepatocytes. Although HepG2 cells and primary hepatocytes were grown in different culture medium, it is unlikely that this would account for the vast difference in response to PPARα activation. One possibility is that genome accessibility is altered in cancer derived cell lines such as HepG2, resulting in a differential response to PPARα activation. Another explanation relates to differences in overall coactivator expression between the cell types. For instance, expression of CITED2 and PCAF was significantly higher in primary hepatocytes compared to HepG2 cells. Contrary to our expectation, expression of PPARα itself was higher in HepG2 cells. Finally, individual genes may respond less in HepG2 cells because of mutations in the promoter region. For instance, the PPARα target gene PDK4 shows decreased basal expression in HepG2 cells because of a mutation in either a SP1 or CBF binding site within its promotor [41], which may account for the lack of induction of PDK4 upon PPARα activation.

With respect to downregulation of gene expression by PPARα, which has been much less explored mechanistically but likely accounts for a major portion of PPARα action, we found downregulation of several genes involved in acute inflammation pathways in HepG2 cells and primary hepatocytes after 6 hours of PPARα activation. Previously, studies in intact mouse models have indicated a major role for PPARα in governing hepatic and vascular inflammation [4, 42].

In conclusion, our results show that HepG2 cells relatively poorly reflect the established function of PPARα in lipid metabolism, in contrast to primary human hepatocytes. Accordingly, with respect to PPARα function, caution should be exercised when extrapolating data from HepG2 cells to human liver.

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Chapter 7

The Krüppel like factors KLF11 and KLF10 are putative novel PPARα target genes in liver with a potential metabolic role

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Manuscript in preparation

Abstract

The liver is the site of expression of a very dynamic transcriptional regulatory network that plays a key role in the regulation of lipid metabolism. One of the important transcription factors in liver is PPARα, which has been identified as a master regulator of hepatic lipid metabolism. The aim of the present study was to better understand the regulatory role of PPARα in liver by identifying potential novel target genes of PPARα. To this aim, we treated primary mouse and human hepatocytes with the PPARα agonist Wy14643 and screened the top differentially expressed genes for novel PPARα target genes.

We found KLF11 and KLF10 to be significantly upregulated by PPARα activation in both mouse and human hepatocytes, suggesting they may be novel PPARα target genes. We could further confirm their PPARα-dependent induction in the liver of mice treated with PPARα agonists. Next, we set up a preliminary in vitro transfection study in which we tried to characterize KLF11 and KLF10 target genes in mouse primary hepatocytes.

Our data suggest that KLF11 overexpression may have an inhibitory effect on PPARα gene expression. We also observed the downregulation of genes related to lipogenesis. Despite successfully overexpressing KLF11 and KLF10 in mouse liver using hyperdynamic tail vein injection of naked plasmids, we did not find any significant effects on PPARα gene expression level. The data suggest interaction between PPARα, KLF11 and KLF10. Additional experiments need to be carried to investigate this interaction in more detail.

Introduction

The liver is a central regulator of nutrient homeostasis. This regulatory effect is largely achieved via a very dynamic transcriptional regulatory network that modulates genes involved in different biological pathways. Thus, the liver is as an interesting therapeutic target for the prevention of chronic diseases such as diabetes and cardiovascular disease. A key characterized transcription factor in liver is the peroxisome proliferator activated receptor α (PPARα). PPARα belongs to the nuclear hormone receptor superfamily and has been identified as a master regulator of lipid metabolism in liver [1-4]. Lack of PPARα in mice leads to acute energy shortage in liver upon fasting and is characterized by defective ketone body formation, hypoglycemia, elevated plasma free fatty acids, and severe hepatic steatosis [5, 6]. In addition, PPARα has been shown to govern glucose metabolism, lipoprotein metabolism, amino acid metabolism, liver inflammation and hepatocyte proliferation (rodent specific).

In clinical practice PPARα is the target of hypolipidemic fibrate class of drugs that lower plasma triglycerides and elevates plasma HDL (high-density lipoprotein) levels [1, 3, 7-9]. Therefore, PPARα target genes has been extensively studied in past years and there is still a lot interest in characterizing potential new PPARα-regulated genes.

Krüppel like factors (KLFs) are another family of transcription factors expressed in liver and many other tissues [10-13]. KLFs are members of Sp1-like transcription factor family with three conserved DNA binding zinc finger domains in their C-terminal region and variant N-terminal domains. KLF proteins bind to GC box or CACCC boxes of genes involved in key biological cellular functions including cell proliferation, differentiation and apoptosis. KLF10 [14, 15] and KLF11 [16] are characterized by the existence of three repressor domains (R1, R2, R3) [17] as a common structural feature which can interact with corepressors such as SID or SID/R1 [18]. They are alternatively called TIEG1 (KLF10) and TIEG2 (KLF11) since they are induced early in response to TGF-ß [14]. Studies in pancreas acinar cell-specific KLF11 transgenic mice have shown that KLF11 overproduction negatively regulates exocrine pancreas cell proliferation [19]. The role of KLF11 in endocrine pancreas has been established by Neve et al. who showed that KLF11 expression in a pancreatic beta cell line is increased in response to high glucose levels and plays a role in insulin secretion [20]. Moreover, KLF11 and KLF10 gene variants have been shown to be involved in genetic susceptibility to type 2 diabetes [20, 21]. Despite their significant expression in liver, not much its known about their regulatory mechanisms and metabolic role in liver.

The aim of the present study was to better understand the regulatory role of PPARα in liver by identifying its potential novel target genes that can further characterize PPARα function.

Here we show that KLF11 and KLF10 are possibly novel PPARα-regulated genes in liver. We further tried to characterize their physiological role in liver by utilization of in vitro transfection assays and in vivo delivery of naked plasmids. Our preliminary in vitro data suggest that KLF11 could have inhibitory effect on PPARα expression. We also observed the downregulation of lipogenesis-related genes by KLF11 overexpression. These interesting findings needs to be further evaluated in complementary experiments.

Methods and materials

Materials. Wy14643 and Fenofibrate were obtained from ChemSyn Laboratories (Lenexa, KS). SYBR Green was from Eurogentec (Seraing, Belgium).
Fetal calf serum, penicillin/streptomycin/fungizone were from Lonza Bioscience (Verviers, Belgium). KLF11 and KLF10 transfection ready full length cDNA clones were obtained from Sanbio, BV (Uden, The Netherlands). pEGFP-N2 expression vector was in the stock. Effectene® reagents and Maxi Prep kit were from Qiagen (Hilden, Germany). Otherwise, chemicals were from Sigma (Zwijndrecht, The Netherlands).

Primary hepatocytes isolation. Mouse hepatocytes were isolated as described previously from 6 different strains of mouse: NMRI, SV129, FVB, DBA, BALB/C and C57BL/6J [22]. Cells were incubated in fresh medium in the presence or absence of Wy14643 (10 μM) dissolved in DMSO for 6 hours, followed by RNA isolation. Isolation of mouse primary hepatocytes was approved by the animal ethics committee of Wageningen University.

Human primary hepatocytes from 6 donors were purchased from Lonza Bioscience (Verviers, Belgium). Details of isolation and procedure are described in a previous publication [22]. Cells were isolated from surgical liver biopsies by two-step collagenase perfusion method and incubated in the presence or absence of Wy14643 (50 μM) dissolved in DMSO for 6 hours, followed by RNA isolation.

Affymetrix microarray analysis. RNA isolation and subsequent processing for microarray were carried out as previously described [22]. Hybridization, washing and scanning of Affymetrix mouse genome 430 2.0 arrays and Gene chip human genome U133 2.0 plus was according to standard Affymetrix protocols. Analysis of the microarray data was as previously described [22].

Real time quantitative PCR. 1 μg of total RNA was used for reverse-transcription with iScript (Bio-Rad, Veenendaal, the Netherlands). PCR was performed with Platinum Taq DNA poly-

merase (Invitrogen) on a Bio-Rad iCycler or MyIQ PCR machine. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. The sequence of primers used are listed in table 1. For determination of tissue expression of mKLF11 and mKLF10, RNA came from one healthy female adult mouse (strain FVB). Human RNA represented a mix from several individuals (AMBION, First choice human total RNA).

Animal experiments. Male SV129 PPARα-/- mice and corresponding Wt mice (2-6 months of age) were purchased at the Jackson Laboratory (Bar Harbor, Maine, USA). The animals were switched to a run-in diet consisting of a modified AIN76A diet (corn oil was replaced by olive oil) (Research Diet Services, Wijk bij Duurstede, the Netherlands), two weeks before start of the experiment. The animals were fasted 4 hours (starting at 5 a.m.) before receiving an oral gavage of WY14643 and Fenofibrate (400 µl of 10 mg/ml WY14643 or Fenofibrate dissolved in 0.5% carboxymethylcellulose). Six hours after the gavage, mice (4 to 5 mice per group) were sacrificed. For the fasting experiment, animals (3-5 months of age) were rather fed a normal laboratory chow diet (RMH-B diet, Arie Blok animal feed, Woerden, the Netherlands) or fasted for 24 hours starting at the onset of the light cycle (n = 4-5 per group). Livers were removed and directly frozen into liquid nitrogen and stored at -80ºC.
The animal experiments were approved by the Local Committee for Care and Use of laboratory Animals at Wageningen University.

Cell culture and transfections. Primary mouse hepatocytes were isolated from SV129 male mice (4-5 mice per group) as described previously [23]. Briefly, after cannulation of the portal vein, the liver was perfused with calcium free Hank's I, calcium containing Hank's II and collagenase (Sigma-Aldrich, Zwijndrecht, the Netherlands) solution respectively. All the solutions was pregassed with carbogen (95% O2 and 5% CO2). Next the liver cells are released and filtered followed by several washing using Krebs buffer.

The cell viability was assessed by using trypan blue (Sigma-Aldrich) and was around 80%. Hepatocytes were suspended in William's E medium (Lonza Bioscience, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum, 20 m-units/mL insulin, 10 nM dexamethasone, 100 U/mL penicillin, 100 µg/mL of streptomycin, 0.25 µg/mL fungizone and 50 µg/mL gentamycin. Cells were plated on collagen (Serva Feinbiochemica, Heidelberg, Germany) coated wells with a density of 500,000 cells/ml and seeded at a density of 140,000 cells per well in a 12-well plate format. After 2 hours the medium was discarded and replaced with fresh medium. After a total 4 hours of incubation, cells were transfected using 1µg DNA plasmid per well and Effectene® reagents. Cells were transfected for 7 hours and then the medium was replaced by fresh medium without the transfection reagents.

The transfection efficiency was evaluated by pEGFP-N2 uptake using fluorescence microscopy 24 hours after adding the Effectene® reagents followed by cells harvest and RNA isolation using TRIzol. RNA was purified using RNeasy micro columns (Qiagen, Venlo, the Netherlands) and further was pooled per group. Total RNA (100ng) was labeled using GeneChip® whole transcript sense target assay. The corresponding labelled RNA samples were hybridized on GeneChip Mouse Gene Exon 1.0 ST Arrays, washed, stained and scanned on Affymetrix GeneChip 3000 7G scanner. Scans of the Affymetrix arrays were processed using packages from the Bioconductor project [24]. Comparison was made between pEGFP-N2 transfected primary hepatocytes (control) and KLF11/KLF10 transfected hepatocytes. Genes that satisfied the criterion fold-change > ±1.5 were considered to be regulated.

Hydrordynamic tail vein injection. The hydrodynamic tail vein injection of naked plasmid DNA is an effective in vivo gene delivery method into the hepatocytes and is an important tool to elucidate the function of novel genes in vivo [25]. In this method, a relative large volume containing the plasmid DNA is rapidly delivered into the tail vein. The tail vein drains into the vena cava. A large bolus results in large liquid volume in vena cava which can not be handled by heart and goes back (predominantly) in to the liver, resulting in gene transfer [26, 27]. Previous studies reported the survival outcome of this method to be 99% and they did not observed ill effects [28-30]. Plasma level of liver enzymes such as alanine aminotransaminase (ALT) was increased transiently 24h after the injection and went back to normal level after few days. Liver histology showed minimal damage that resolved within a week. This method enhance gene transfer to hepatocytes by opening transiently the hepatic endothelial barrier. The increased pressure is needed for movement of the DNA out of sinusoids and the transfer to the hepatocytes.

We injected male NMRI mice with PBS or Ringer solution containing 50 µg KLF11 (n = 4), KLF10 (n = 3) and as control pEGFP-N2 (n = 4) expression vectors (total volume of the injection was 10% of the total body weight). The injection time was less than 10 seconds. A 3ml syringe with a 27G 0.5 needle was used. Mice were under anesthesia with isoflurane during the injections. 24h after the injection time mice were sacrificed and different livers lobes were removed and directly frozen into liquid nitrogen (stored at -80ºC) or fixed by immersion in 4% PBS-buffered formaldehyde, processed in an automatic tissue processor, embedded in paraffin and sectioned at 5µM for GFP fluorescence microscopy.

Immunostaining of GFP protein was performed on paraffin-embedded liver sections using a primary antibody against yellow fluorescent protein (YFP) and a secondary anti rabbit polyclonal antibody. Sections were dewaxed in xylene and rehydrated in a series of graded alcohols. Antigen retrieval was performed by placing the slides in citrate buffer and heat them in a

microwave oven 10 min (70% power) without lid and 30 min (50% power) with lid. Sections were incubated one hour with the primary antibody diluted 1: 500 in PBS (24ºC) followed by one hour incubation with secondary antibody diluted 1:100 in PBS. Visualization of the complex was done using AEC Substrate Chromogen for 10 minutes at room temperature. After counterstaining with Meyer's hematoxylin sections were mounted with Imsol or Kaiser's. Negative controls were used omitting

The animal experiments were approved by the animal experimentation committee of Wageningen University.

Statistical analysis. Statistical significant differences were calculated using Student's T-test. The cut-off for statistical significance was set at a P-value of 0.05 or below.

Results

KLF11 and KLF10 expression in mouse and human liver is regulated by PPARα

In order to find novel putative PPARα-regulated genes in liver, we treated primary mouse and human hepatocytes with the PPARα agonist Wy14643 for 6 hours and performed Affymetrix microarray analysis. Top differentially expressed genes were identified. In addition to well-known PPARα target genes such as PDK4, FABP1, ADFP and ANGPTL4, we found KLF11 and KLF10 to be significantly upregulated by PPARα activation in both mouse and human hepatocytes, suggesting they may be putative novel PPARα target genes. Induction of KLF11 and KLF10 was confirmed by PCR (Figure 1A). In addition, a comparison between liver RNA from Wt and PPARα-/- mice treated or not with the synthetic PPARα ligands Wy14643 and fenofibrate for 6 hours revealed significant PPARα-dependent induction of KLF11 and KLF10 (Figure 1B). To assess if physiological activation of PPARα can also regulate KLFs, we compared liver RNA from Wt and PPARα-/- mice subjected to 24h fasting (Figure 1C). Baseline KLF10 and KLF11 expression were significantly decreased in PPARα-/- mice compared to the corresponding wildtype mice. Interestingly, fasting did not affect KLF10 and KLF11 expression in Wt mice, whereas expression went up significantly in PPARα-/- mice. Overall, these data suggest that KLF11 and KLF10 may be novel PPARα target genes in mouse and human liver.

We identified the ubiquitous expression of KLFs mRNA in a panel of mouse and human tissues, data shown only for KLF11 (Figure 2). This suggests that KLF11 and KLF10 expression may be also regulated by PPARα in other tissues. The mRNA levels of KLF11 and KLF10 were slightly higher in primary hepatocytes compared to total liver (Figure S1).

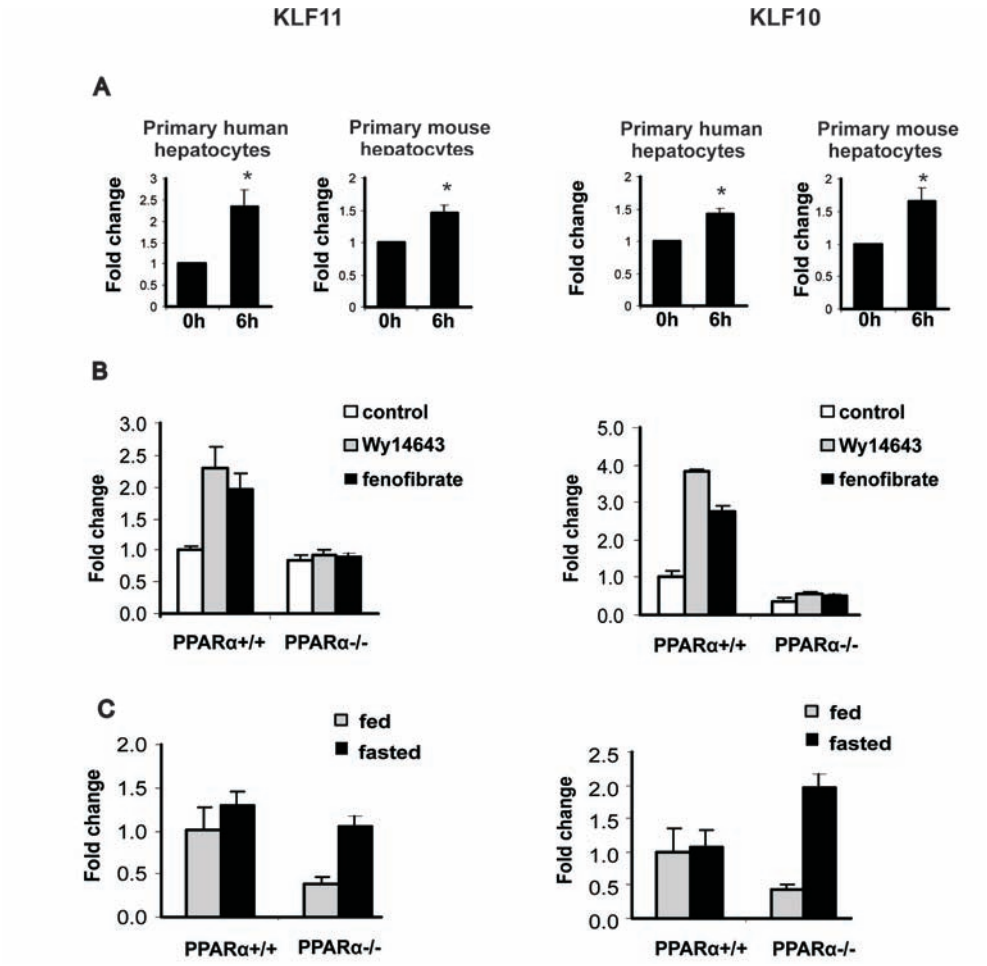


Figure 1. KLF10 and KLF11 are putative novel PPARα target in mouse and human liver. (A) Human and mouse primary hepatocytes were treated with Wy14643 (50μM and 10μM respectively) or DMSO as control for 6h. Relative induction of KLF11 and KLF10 by Wy14643 was determined by qPCR. Expression levels in the DMSO-treated cells were set at 1. Error bars represent SEM. *P < 0.05 according to Student's T-test. (B) Microarray gene expression of KLF11 and KLF10 in livers of wildtype and PPARα-/- mice 6 hours after receiving an oral gavage of the PPARα agonists Wy14643 or fenofibrate (4mg) (n= 4-5 mice per group). Gene expression levels from wildtype animals that received only vehicle were set at 1. Error bars represent SEM. (C) Microarray gene expression of KLF11 and KLF10 in livers of fed and 24 hour fasted wildtype and PPARα-/- mice (n = 4-5 mice per group). Gene expression levels from wildtype fed animals were set at 1. Error bars represent SEM.

Identifying KLF11 and KLF10 target genes in liver

In order to identify the physiological role of KLF11 and KLF10 in liver, we started with a preliminary in vitro study in which we transfected primary mouse hepatocytes with KLF11 and KLF10 plasmids using Effectene® transfection reagents. We managed to overexpress KLF11 and KLF10 genes by approximately 30 and 40-fold (Figure S2). The effect of KLF11 and KLF10 overexpression on whole genome gene expression was explored using microarray.

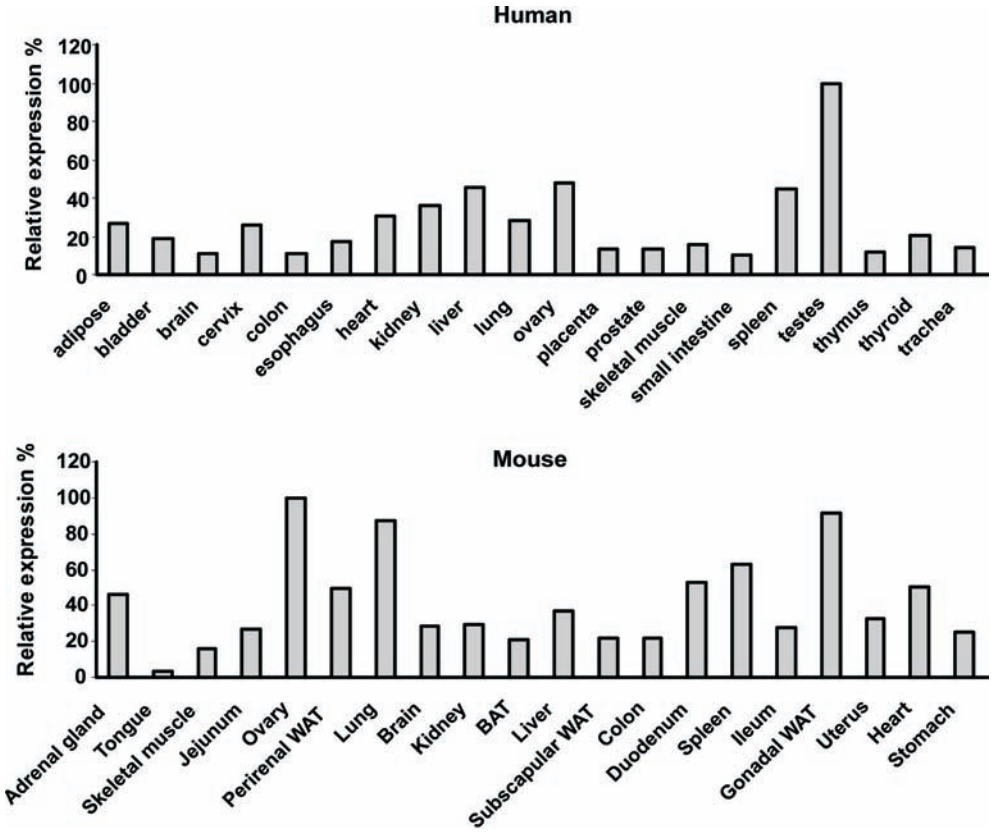
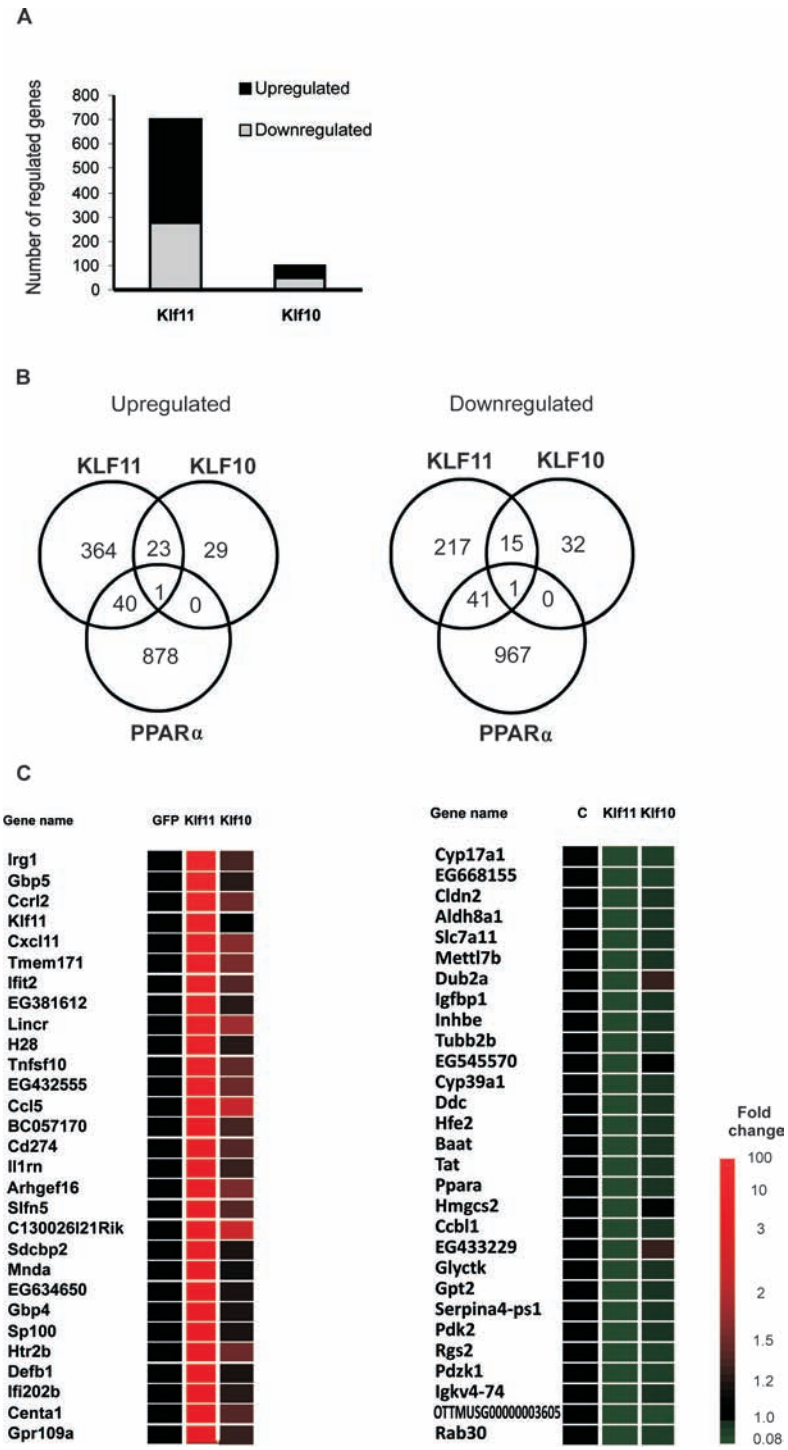


Figure 2. KLF11 is expressed ubiquitously in human and mouse tissues. mRNA expression of KLF11 was determined in human and mouse tissues by Q-PCR. Human RNA represented a mix from several individuals (AMBION, First choice human total RNA). Mouse RNA was obtained from one healthy female adult mouse (strain FVB). Expression levels were relative to the tissue with the highest expression. BAT: Brown adipose tissue; WAT: White adipose tissue.

Overexpression of KLF11 altered expression of 702 genes with more genes being upregulated compared to downregulated. In comparison, KLF10 overexpression had a much smaller effect on gene regulation with 53 genes upregulated and 48 genes downregulated (Figure 3A). Next we determined the overlap in genes regulated by the KLF11 or KLF10 with known PPARα target genes originating from our previous publication [22]. Separate analysis was carried out for up- and down-regulated genes (Figure 3B).

Only a total of 41 and 42 genes were found to be up- and down-regulated by both KLF11 overexpression and PPARα activity, respectively. However, the majority of the regulated genes were regulated rather specifically by KLF11 or PPARα. KLF10 regulated genes did not overlap with PPARα target genes except for one upregulated gene (Tmem171) and one downregulated gene (Gnpnat1). KLF10 and KLF11 showed also a minor overlap. The considerable overlap between KLF11 regulated genes and known targets of PPARα became the focus of the rest of the study. A complete list of regulated genes in the various parts of the Venn diagrams is available in Supplementary table 1.

In order to zoom in and identify the top up- or down-regulated genes upon KLF11 overexpression, we created a array based heat map representing genes which are ranked according to their fold change compared to the control (Figure 3C). The changes in the expression of the same genes upon KLF10 overexpression are presented in parallel. The top upregulated genes by KLF11 mainly represent inflammation related genes such as immune related gene 1 (IRG1) and chemokines (CXCL11, CCL5). Interestingly, the list of top downregulated genes included PPARα and its well-known target gene HMGCS2, which was specific for KLF11. To further explore the possible functional impact of KLF11 overexpression in hepatocytes, ingenuity pathway analysis was performed with the focus on downregulated processes. Remarkably, the top downregulated metabolic pathway by KLF11 overexpression was lipid metabolism. Some other top downregulated pathways included molecular transport, small molecule biochemistry and cellular development (data not shown). So from these preliminary data we speculated that KLF11 activity could have a functional impact on lipid metabolism possibly via the inhibitory effect on PPARα expression.


A
Number of regulated genes
800
700
600
500
400
300
200
100
0
Upregulated
Downregulated
Klf11
Klf10
B
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Downregulated
KLF11
KLF10
364
23
29
40
1
0
878
PPARα
KLF11
KLF10
217
15
32
41
1
0
967
PPARα
C
Gene name
GFP Klf11 Klf10
Irg1
Gbp5
Ccrl2
Klf11
Cxcl11
Tmem171
Ifit2
EG381612
Lincr
H28
Tnfsf10
EG432555
Ccl5
BC057170
Cd274
Il1rn
Arhgef16
Slfn5
C130026I21Rik
Sdcbp2
Mnda
EG634650
Gbp4
Sp100
Htr2b
Defb1
Ifi202b
Centa1
Gpr109a
Gene name
C Klf11 Klf10
Cyp17a1
EG668155
Cldn2
Aldh8a1
Slc7a11
Mettl7b
Dub2a
Igfbp1
Inhbe
Tubb2b
EG545570
Cyp39a1
Ddc
Hfe2
Baat
Tat
Ppara
Hmgcs2
Ccbl1
EG433229
Glyctk
Gpt2
Serpina4-ps1
Pdk2
Rgs2
Pdzk1
Igkv4-74
OTTMUSG00000003605
Rab30
Fold change
100
10
3
2
1.5
1.2
1.0
0.08

Figure 3. KLF11 and KLF10 overexpression can mediate hepatic gene regulation with minor overlap with PPARα target genes. (A) Mouse primary hepatocytes were transfected with expression vectors for KLF11 and KLF10 using Effectene® reagents. 24 hours after transfection, RNA was isolated and hybridized to GeneChip Mouse Exon 1.0 ST Arrays. Bars show number of up- and down-regulated genes. Genes were considered regulated if fold change > ±1.5. (B) Venn diagrams showing overlap in upregulated and downregulated genes by KLF11, KLF10 and PPARα in mouse primary hepatocytes. PPARα target genes were recruited from our previous publication [22]. (C) Heat maps illustrating the relative up- and down regulation of the top regulated genes in response to KLF11 overexpression in mouse hepatocytes. Genes were ranked based on their fold-change. Expression levels in the pEGFP-N2 transfected cells were set at 1. Relative changes in response to KLF10 overexpression are shown in parallel.

KLF11 overexpression significantly downregulates PPARα

In order to evaluate the reproducibility of the negative regulatory effect of KLF11 activity on PPARα gene expression, we repeated the in vitro transfection of KLF11 and KLF10 expression vectors in primary mouse hepatocytes isolated from four SV129 male mice. Q-PCR data confirmed the significant downreguation of PPARα mRNA by KLF11 overexpression. The inhibitory effect on PPARα mRNA expression was specific for KLF11 overexpression and was not observed for KLF10 (Figure 4A).

Next we checked the expression of a number of genes related to different biological processes (Figure 4B). KLF11 overexpression reduced the expression of genes involved in lipogenesis (SCD1 and SCD2). We also observed a trend towards a reduction for a ketogenesis related gene (HMGCS2). On the other hand, KLF11 overexpression stimulated the expression of CXCL10, which is an inflammatory chemokine. No effect was detected on CPT1a and bile and cholesterol transporter related genes ABCB11 and ABCG5.

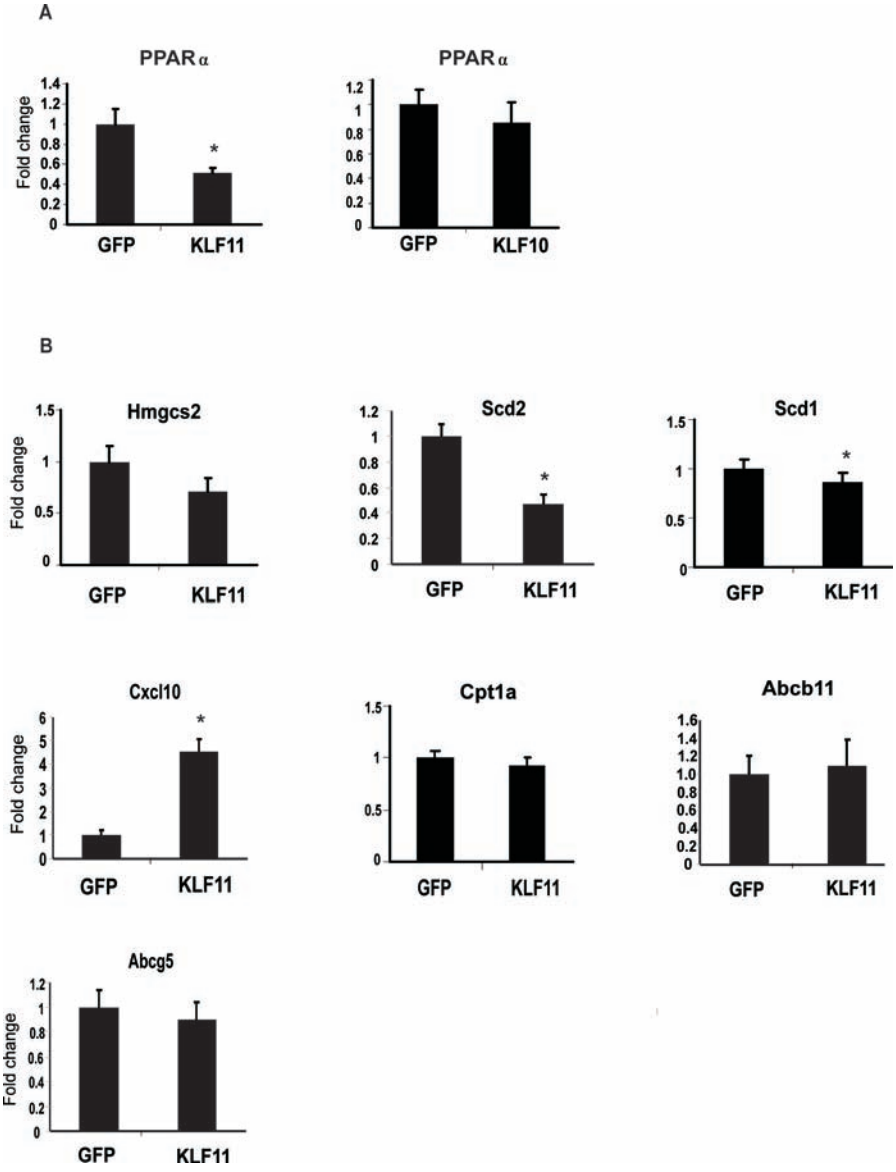

Figure 4. KLF11 overexpression downregulates hepatic PPARα gene expression and influences SCD1, SCD2 and CXCL10. (A) PPARα expression in mouse primary hepatocytes (n=4-5 per group) transfected with KLF11, KLF10 and pEGFP-N2 DNA plasmids. (B) mRNA expression of a few selected genes in KLF11 transfected mouse primary hepatocytes was determined by qPCR (n=4 per group). Expression levels in the pEGFP-N2 transfected cells were set at 1. Error bars represent SEM. *P < 0.05 according to Student's T-test.

In vivo effect of KLF11 and KLF10 overexpression on PPARα gene expression

In order to evaluate the inhibitory effect of KLF11 activity on PPARα gene expression in vivo, we performed hydro-dynamic tail vein injection (HTV) using expression vectors for KLF11 and KLF10. As control we injected mice with pEGFP-N2 expression vectors. Firstly, to evaluate the efficiency of HTV method we detected the presence of green fluorescent protein (GFP) by fluorescence microscopy in different liver lobes. We could observe clear fluorescence in all different liver lobes. The highest uptake was mainly observed in the cells surrounding hepatic portal and central veins (Figure 5A). Staining of the liver tissue with an antibody against green fluorescent protein confirmed the microscopy findings (Figure 5A). As identified by q-PCR analysis, we could successfully overexpress KLF11 (16 fold) and KLF10 (35 fold) genes by HTV method (Figure 5B). In contrast to our expectation, no difference in PPARα gene expression, could be detected upon KLF11 nor KLF10 overexpression (Figure 5C). There was also a large variation in PPARα gene expression levels among different mice within each group and thus creating large standard error bars.

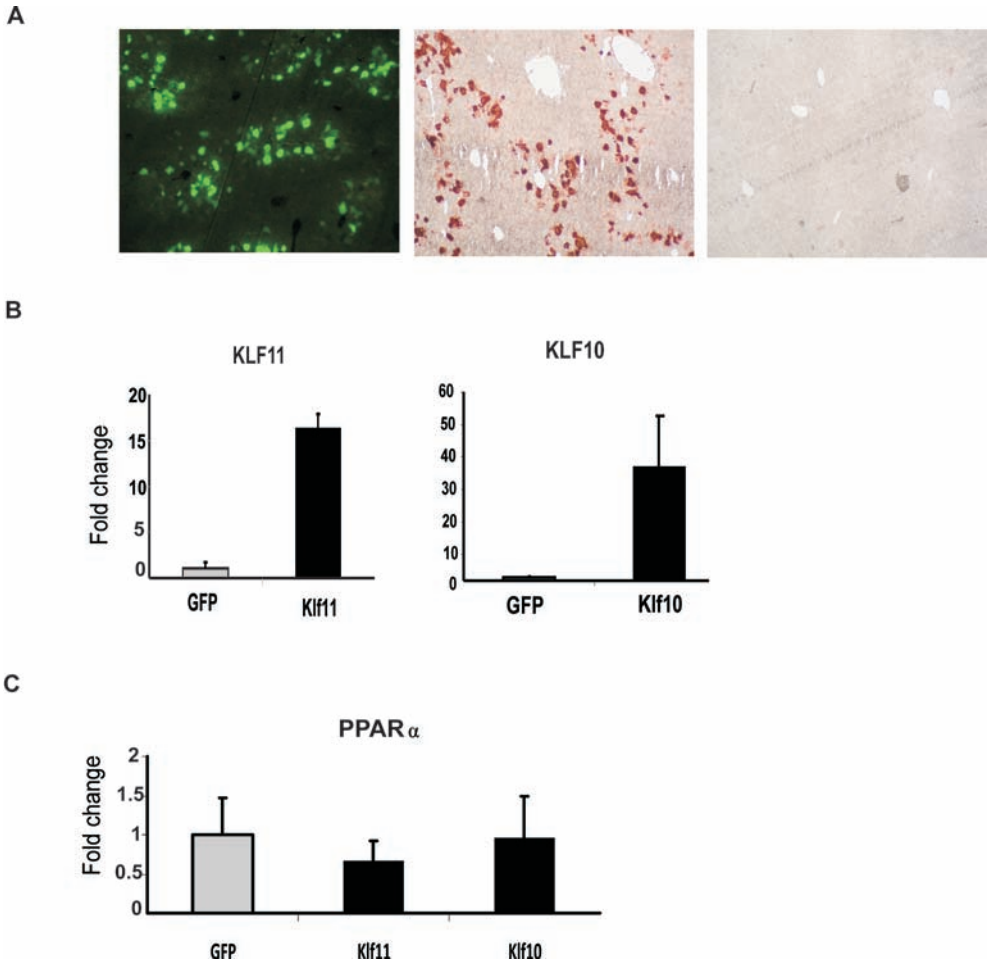

Figure 5. KLF11-KLF10 overexpression in liver via hydrodynamic tail vein injection does not influence PPARα gene expression. (A) 10x magnification image of mouse left lobe of liver tissue by fluorescence microscopy (left). Liver was fixed in formaldehyde for GFP fluorescence microscopy. 10x magnification image of mouse left lobe of liver tissue stained with an antibody against GFP (middle), or negative control. (B) KLF11 and KLF10 gene expression in the liver of mice injected with DNA plasmids (n=3-4 per group) determined by qPCR. (C) PPARα gene expression in the liver of mice injected with DNA plasmids (n=3-4 per group) determined by qPCR. Expression levels in the pEGFP-N2 injected mice were set at 1.Error bars represent SEM.

Discussion

PPARα controls many aspects of hepatic lipid metabolism by modulating the expression of numerous genes [4, 31]. PPARα deletion has been linked to triglyceride storage in liver [5, 32, 33], while its activation has been shown to reverse hepatic steatosis [34]. Thus, characterizing novel PPARα target genes has gained a lot importance to further identify its regulatory role and mechanism of action.

Besides PPARα, liver is also a site of expression for other transcription factors including Krüppel-like factors. Members of this protein family have been previously implicated in the regulation of metabolism in liver [35], skeletal muscle [36] and adipocytes [37]. However not much is known about the metabolic role of KLF11 and KLF10 in liver and factors controlling their regulation.

In the present study, we identify transcription factors KLF11 and KLF10 as putative novel PPARα target genes. Few lines of evidence suggest a link between KLF10-KLF11 and PPARα. As it was recently shown by Guillaumond et al. both PPARα and KLF10 are circadian clock controlled metabolic sensors and more interestingly they share a few clock controlled target genes suggesting the coordinated action of these regulators [38]. Here we show that activation of PPARα by synthetic ligands significantly upregulates KLF10 and KLF11 gene expression in mouse liver, which was conserved in primary human hepatocytes. PPARα-independent regulation of KLFs by fasting suggests the involvement of another transcription factor in their regulation. Previously, we have characterized PPARß/δ as a free fatty acid sensor in liver [39]. By knowing that PPARα-/- mice have increased flux of plasma free fatty acids, it is tempting to speculate the involvement of PPARß in the hepatic regulation of KLF10-KLF11 in fasted state.

An interaction between PPARß and another KLF protein family member (KLF5) has been demonstrated by Oishi et al. They showed that under basal condition, SUMOylated KLF5 is in a transcriptionally repressive regulatory complex containing unliganded PPARß. A ligand dependent activation of PPARß caused deSUMOylation of KLF5 which promotes its interaction with PPARß, recruitment of coregulators and PPARß activity followed by regulation of metabolic genes [36].

Interestingly, our in vitro preliminary data suggests that KLF11 overexpression can downregulate PPARα gene expression and also suppress lipid metabolism related genes. This suggests a potential negative feedback mechanism whereby KLF11 down-regulates its regulator. The need for this feedback inhibition could be related to the known KLF11 effects of mediating

TGF-ß signaling and inflammation in liver, which may implies that its levels must be tightly controlled. Clearly, additional research is needed.

Although transfection of primary hepatocytes is a challenging task, we could optimize a protocol to obtain reasonably high transfection efficiencies and could successfully overexpress the targeted genes in vitro as measured by Q-PCR and evaluated by GFP fluorescence microscopy. However, confirmation of the changes at the protein level will be worthwhile.
In order to evaluate the in vivo effect of KLF10 or KLF11 overexpression on PPARα gene expression, we utilized the method of hydro-dynamic tail vein injection. By the means of this method, we managed to significantly overexpress KLF10 and KLF11 in liver. Unfortunately, we observed a large variation in PPARα gene expression levels between the mice within each injection group. The large variation may be related to a number of issues: site of DNA overexpression, paranchymal cells versus non- paranchymal cells, the variable amount of endoplasmic reticulum (ER) stress caused by enhanced protein metabolism and inflammatory consequences, the involvement of other regulatory factors in the interaction between KLF11 and PPARα in vivo. Efficient uptake of the DNA plasmids by liver paranchymal cells has been previously reported for smaller plasmid structures [26]. The size of the DNA plasmid can influence its transport from sinusoids to hepatocytes. The KLF10 and KLF11 expression plasmids used in our study were approximately 9kb, which may have impacted their transport. Moreover, we do not know if this variation only applies to PPARα gene expression or if it is more general phenomenon observed with hydro-dynamic tail vein injections. Hydrodynamic tail vein injection have been previously used in order to overexpress genes encoding liver secreted proteins. However, to our knowledge it has not been used to overexpress transcription factors and study target gene regulation.

Despite the limitations of the present study, which needs follow up experiments, we could optimize hydro-dynamic tail vein injections, which is rather a simple way of DNA delivery to the liver and is less labor intensive compared to other transduction methods using viral vectors. Our primary findings suggest KLF10 and KLF11 are PPARα target genes in liver and point at a possible interaction between KLF11 and PPARα. Considering the importance of PPARα signaling in fatty acid metabolism, we can speculate about a role of KLF11 in hepatic lipid metabolism.

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Appendix Chapter 7

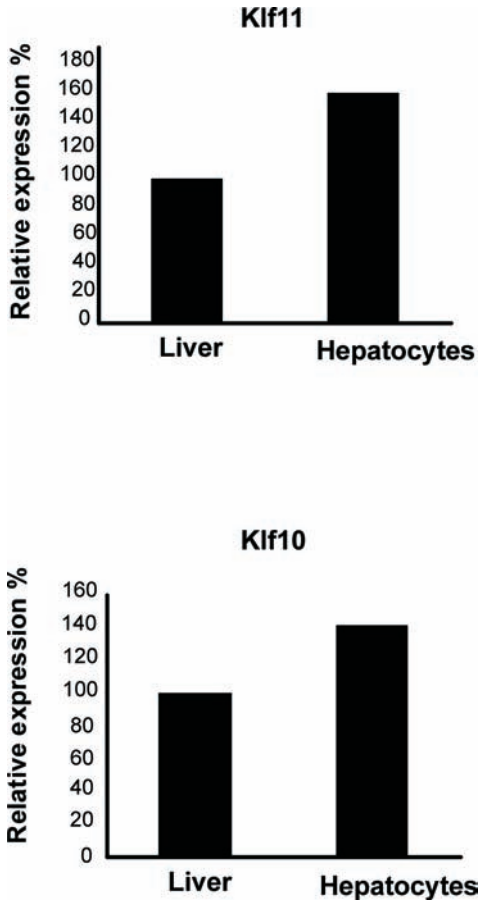

Figure S1. KLF11 and KLF10 gene expression in mouse liver and primary hepatocytes. mRNA expression of KLF11 and KLF10 was determined by qPCR in mouse liver and primary hepatocytes isolated from two C57BJ6 mice. Expression level in the liver is set at 100.

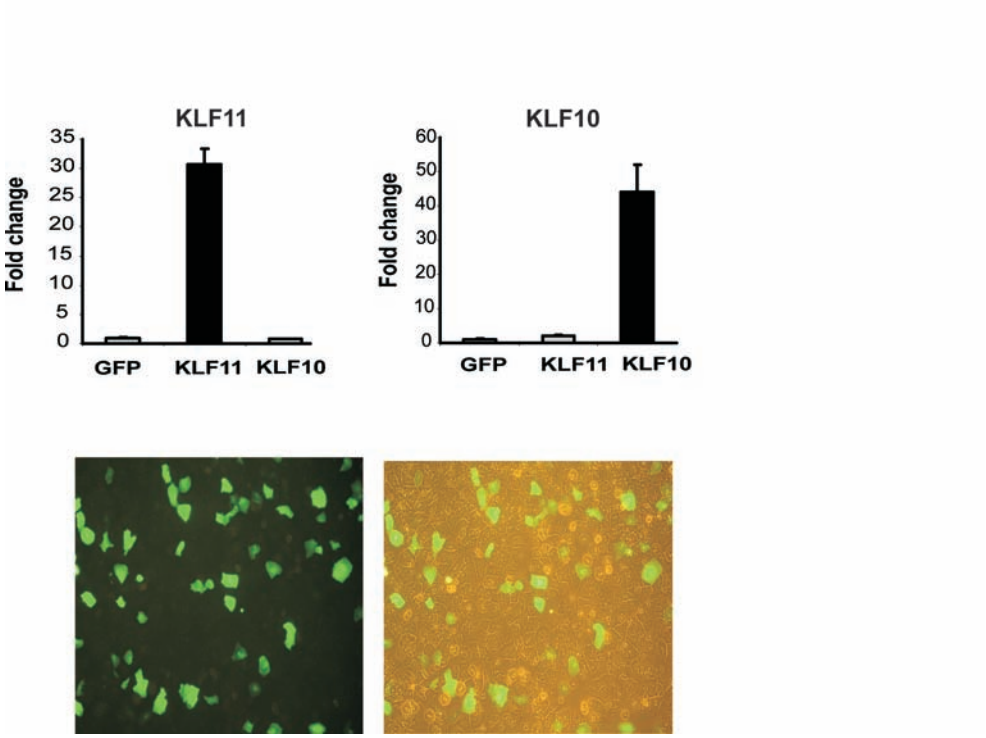

Figure S2. KLF11-KLF10 overexpression in mouse primary hepatocytes transfected with DNA plasmids. KLF11 and KLF10 mRNA expression determined by qPCR in mouse primary hepatocytes isolated from a Sv129 mouse, and transfected with pEGFP-N2 (control), KLF11 and KLF10 DNA plasmids. The expression of the cells transfected with pEGFP-N2 is set at one. Error bars represent SEM (top). 10x magnification image of pEGFP-N2 transfected primary hepatocytes under fluorescence microscope (bottom).

Additional files can be found online:
http://nutrigene.4t.com/data/MRphd/index.html

Chapter 8

General discussion

Since the discovery of PPARα in 1990 [1], a wealth of studies have been performed to expand our knowledge about its role in nutrient and energy metabolism. Synthetic agonists of PPARα lower plasma triglycerides and raise plasma high-density lipoprotein (HDL) levels and are thus used clinically for the treatment of dyslipidemia linked with obesity, diabetes, and cardiovascular diseases [2-6]. Since the biological function of a transcription factor is mainly coupled to the function of its target genes, many PPARα-regulated genes and processes have been so far screened by applying microarray techniques combined with other functional genomics tools such as adenoviral gene delivery and siRNA-mediated gene silencing. However, the large size of the PPARα-dependent transcriptome, which is especially true in liver, makes this approach challenging. Furthermore, microarray technology yields a huge amount of data, which makes the analysis and interpretation of the findings very difficult. Thus, there is still of great interest to make sense out of the large PPARα-dependent transcriptome and to identify novel PPARα controlled genes and processes.

Therefore, the first objective of the research presented in this thesis was to better characterize and understand PPARα function in liver by identifying PPARα-regulated genes and metabolic pathways. To this aim we applied a comprehensive genome analysis using microarray technology combined with knockout mouse models. We analyzed the data from independent microarray studies in which comparisons were made between mRNA from livers of 24-hour fasted wild-type and PPARα-null mice or mRNA from livers of wild-type mice and PPARα-null mice fed PPARα agonist Wy14643 for 6 hours and for 5 days. This comprehensive approach enabled us to generate a schematic overview of PPARα-regulated genes relevant to hepatic lipid metabolism. Compared to previously gained knowledge, our data indicate the extensive role of PPARα in hepatic lipid metabolism, identifying a large number of PPARα target genes involved in different aspects of lipid metabolism, starting from genes involved in fatty acid uptake through membranes, followed by genes linked to fatty acid activation, intracellular fatty acid trafficking, mitochondrial, peroxisomal and microsomal fatty acid oxidation, fatty acid synthesis and storage, and glycerol metabolism.

Remarkably, while a few genes involved in lipogenesis were already known as PPARα targets including Δ5 and Δ6 desaturases (Fads), stearoyl-CoA desaturase (Scd), microsomal triglyceride transfer protein (Mttp), and malic enzyme (Mod1) [7], we found many other PPARα-regulated genes to be implicated in lipogenesis, suggesting a major role of PPARα in governing hepatic lipogenesis. The involvement of PPARα in upregulation of fatty acid desaturation and elongation enzymes might be part of a feed-forward action that includes auto-regulation of gene expression by stimulating the production of PPARα ligands.
Interestingly, the observed regulation of many lipogenic genes by PPARα was not conserved in primary hepatocytes treated with PPARα agonist Wy14643 as shown in chapter 4. This

observation suggests that PPARα mostly regulates lipogenesis via indirect mechanisms. One of the candidates for mediation of this indirect effect is Srebf1, an essential transcriptional regulator of fatty acid synthesis in liver. Consistent with this notion, induction of lipogenic genes by chronic PPARα activation was completely abolished in Srebf1-/- mice [8]. A cross-talk between PPARα and Srebf signaling has been suggested via direct physical interaction with Srebf. Thus, an alternative mechanism may be that PPARα is recruited to promoters of Srebf targets and stimulates Srebf activity [9]. Our data showed upregulation of Srebf1 gene expression by PPARα activation in liver and mouse primary hepatocytes. Possibly, this effect occurred via PPARα-induced Srebf1 proteolytic cleavage and Srebf1 activation, thereby generating an autoloop regulatory circuit that may also lead to increased Srebf1 mRNA [10, 11]. The relatively modest induction of Srebf1 mRNA level by PPARα in primary hepatocytes may have been too insignificant to cause appreciable induction of lipogenic genes. Moreover, there is a possibility for the involvement of other coregulatory factors. The transcriptional activity of Srebfs often requires cooperation with other DNA binding transcription factors such as Sp1 and Creb as well as coactivators [12].

Our comprehensive analysis also led to the identification of novel PPARα target genes including Etfdh, Etfb, El, Pctp, Txnip, Pnpla2, Lipe, Mgll. The last three genes are known to be involved in the 3 steps of triglyceride hydrolysis in adipose tissue [13-16]. Their regulation by PPARα suggests that the anti-steatotic effect of PPARα activity, which has been mainly ascribed to induction of fatty acid oxidation, may also be directly mediated via induction of the triglyceride hydrolysis pathway. Although the triglyceride hydrolysis pathway in liver has yet to be fully elucidated, there is a possible similarity to the pathway operating in adipose tissue [15].

To provide evidence for the direct regulation of a few novel putative target genes of PPARα, we took advantage of in silico screening of the potential PPREs. However, it should be noted that more recent studies reveal relatively poor overlap between the detection of a PPRE in the 1-2 kb region upstream of the transcriptional start site of a gene and actual binding of the transcription factor and gene regulation [9, 17, 18]. However, we tried to limit the list of putative PPARα target genes by employing a conservative set of criteria that takes into account the role of PPARα during fasting, by zooming in on upregulated genes rather than downregulated genes, and by addressing the timing of gene induction upon PPARα activation. Nevertheless, more detailed evaluation of direct regulation of each gene by PPARα demands complementary assays that include examination of DNA binding and identifying functional PPREs, which was beyond the scope and aim of the research presented in this thesis.

Most of the research on the role of PPARα in liver has been performed in mice, revealing that PPARα serves as a key regulator of hepatic lipid metabolism [7]. Interestingly, the role of PPARα in human liver has been questioned based on its detected lower expression levels compared to mouse liver [19]. Furthermore, PPARα has clear species-specific effects: for example, PPARα agonists cause hepatocyte proliferation and hepatocarcinogenesis only in rodents [20]. Another example is the effect of PPARα activity on lipoprotein metabolism and the human specific upregulation of Apoa1, Apoa2 and Apoa5 by PPARα, leading to the observed human specific induction of plasma HDL levels by fibrates [21].

The species specific function of PPARα has been ascribed to a number of molecular mechanisms including intrinsic properties of PPARα protein, conservation and functionality of PPREs, and the cellular environment in relation to the presence and absence of co-regulators [22]. Apart from the differential effects on hepatocytes proliferation, hepatocarcinogenesis and lipoprotein metabolism, it is not clear whether PPARα has a similar role in mice and human and to what extent target genes can be classified as species-specific or commonly regulated genes.

Accordingly, in our second aim we set out to perform a comprehensive comparative analysis of gene regulation by PPARα between mouse and human hepatocytes. Contrary to common conception, we found similar PPARα expression levels in liver tissue and primary hepatocytes between mouse and human. Our data identified a large number of genes regulated by PPARα activation in human primary hepatocytes, identifying a major role for PPARα in human liver.

The reason for the discrepancy with the previous study showing an approximately 10-fold lower PPARα expression in human liver compared to mouse liver may be related to the methodology applied to measure gene expression (RNAse protection versus qPCR) [19]. Ideally, it would be of great interest to measure basal PPARα expression in freshly isolated liver tissue from the donors but this is practically impossible since we don't have access to these donors. Instead we measured PPARα in human liver RNA obtained via Ambion, which represents a mixture of RNA from 3 individuals without liver disease, as well as in mouse liver RNA obtained from 5 mice on mixed genetic background (fed state). The results showed that expression of PPARα in human liver is only slightly lower compared to mouse liver, supporting our data obtained in primary hepatocytes. In general, comparative analyses of PPARα gene expression are complicated by the observation that PPARα in mouse liver fluctuates throughout the day [23], is increased by fasting [24], and is reduced under conditions of inflammation [25]. Recently, we were also able to confirm the latter observation in liver sections obtained from human subjects with steatohepatitis (our unpublished data).

Besides genes involved in mitochondrial and microsomal fatty acid oxidation, we also found a considerable number of genes involved in peroxisomal fatty acid oxidation to be induced by PPARα in human primary hepatocytes. Our finding argues against the common notion that PPARα does not regulate fatty acid oxidation in human liver. It is well acknowledged that PPARα agonists do not induce peroxisome proliferation in human although the molecular basis of this species difference is not known.

It was suggested that in human, decreased expression levels or the activity of PPARα contributes to the resistance to peroxisome proliferation upon treatment with fibrate drugs. To examine the mechanism behind these species differences, several approaches have been applied. In this regard, overexpression of human PPARα in HepG2 cells did not lead to induction of Acox1 and other peroxisomal genes [26]. Data obtained with primary cultures of human hepatocytes yielded similar results [27]. A different approach to study the role of human PPARα is by using so called PPARα-humanized mice. Upon fenofibrate treatment, these mice exhibited decreased serum triglycerides and marked increases in known PPARα target genes encoding peroxisomal, mitochondrial, and microsomal fatty acid oxidation enzymes, indicating that human PPARα is a functional receptor. Strikingly, despite the induced peroxisome proliferation and peroxisomal fatty acid oxidation genes within the context of mouse liver, unlike wild-type mice, the PPARα-humanized mice did not display increases in carcinogenic responses [28, 29]. In line with the humanized PPARα mice findings, our data also identified the conserved induction of many PPARα target genes specifically related to fatty acid oxidation in mouse and human. We also found the induction of a few peroxisomal genes by PPARα in both species. It should be mentioned, however, that numerous other peroxisomal genes showed mouse specific regulation.

Interestingly, we found that at the individual gene level PPARα mostly governs different set of genes in mouse and human liver, which was evident by the relative small number of genes commonly regulated by PPARα in mouse and human hepatocytes. In contrast, the overlap became more impressive when studied at the level of gene ontology. Many of the overlapping gene ontology classes represented pathways of lipid metabolism and accordingly, we could define a conserved role of PPARα as a master regulator of lipid metabolism between two species.

One of the strong points of this research is the use of human primary hepatocytes rather than the most widely used liver cell line, HepG2. As discussed in chapter 6, the HepG2 cell line poorly reflects the established PPARα target genes and function, specifically with regards to lipid metabolism. It can be argued that human primary hepatocytes are isolated from liver biopsies of patients undergoing surgery, so it is not clear to what extent their basal transcrip-

tome can reflect the gene expression profile of a healthy hepatocyte. Knowing that a more ideal experimental setup is practically and ethically unfeasible, and is unlikely to yield vastly different results, we believe that we utilized the most suitable model for studying PPARα function in human liver. Despite the limitations, our analysis represents a major advancement in our understanding of PPARα function in human liver.

Interestingly, our study also identified novel human and/or mouse PPARα target genes, including Klf10, Klf11 (commonly regulated), CYP classes 1-3 (human specifically regulated), and Fbp2 (mouse specifically regulated) which provides further clues towards the function of PPARα in mouse and human liver. One major concern related to species specific target genes is that cultured hepatocytes are extremely sensitive to culture conditions. Since these conditions were not exactly the same for mouse and human hepatocytes with respect to the culture medium and its ingredients, the question arises if the detected target genes are really species specific. We tested this aspect by culturing mouse hepatocytes in both types of medium and addressed, by qPCR, the response to Wy14643 for few selected target genes, including those which were species specific. We obtained marked induction of Cpt1a and Fbp2 by Wy14643 in mouse hepatocytes cultured in the medium for human hepatocytes and we did not observe any induction of Tsku, one of the identified human specific PPARα target genes, in mouse hepatocytes regardless of which medium was used. Our data also did not reveal a systematically lower induction of PPARα targets by Wy14643 in mouse hepatocytes cultured in medium for human hepatocytes. Thus, we believe that the species specific regulation of identified target genes is not due to the differences in culturing medium.

We also found a number of pathways to be specifically induced by PPARα in mouse or human hepatocytes. An example is mouse specific regulation of glucose metabolic pathways by PPARα. This finding is relevant to the studies in mice showing a direct role of PPARα in hepatic glucose metabolism and gluconeogenesis [24, 30-32], while human trials generally do not support an effect of PPARα activation on plasma glucose levels [33]. Our analysis also pointed towards human-specific regulation of certain xenobiotic-metabolizing enzymes by PPARα, which confirms previous analyses showing genes belonging to Cyp classes 1-3 are specifically regulated by PPARα in human [34].

The comprehensive analysis of PPARα-regulated genes in human primary hepatocytes also led us to identify a novel PPARα target gene, called Mbl2, with human specific regulation. MBL2 is mainly known to play a role in innate immunity. Considering the role of Mbl2 in immune system and knowing that PPARα mainly downregulates immune-related genes [35, 36], makes it interesting to speculate about a metabolic function for Mbl2 besides its immune related effects, which is of great interest for future research.

A precedent for a linkage between a component of the complement pathway and lipid metabolism is set by a protein called acylation stimulating protein (ASP).
Upon Mbl binding to pathogens followed by conformational changes in the Mbl multimer, the lectin complement pathway is initiated. Activation of the lectin pathway is followed by an enzymatic cascade generating C3 convertase, which in turn produces C3b and C3a [37]. Arginine removal from activated C3 (C3a) by carboxypeptidase results in C3a-desArg, also known as ASP. Thus, Mbl2 might lead to ASP production. ASP is a circulating adipokine which acts as an anabolic stimulator of TG storage in adipocytes. Different studies have demonstrated that ASP influences fat storage by stimulating diacylglycerol acyltransferase (Dgat) activity, the rate limiting step in triglyceride (TG) synthesis [38], increases glucose transporter Glut4 translocation [39], indirectly stimulates LPL activity in adipose tissue [40], and inhibits lipolysis [41]. This effects are mediated via the ASP receptor, C5L2, a seven transmembrane G protein coupled receptor [42, 43]. Since both the ASP precursor, and its receptor C5L2 are also expressed in liver, unidentified effects mediated by ASP on liver metabolism are expected [44]. Additional experiments need to be carried to investigate this hypothesis in more detail.

Remarkably, we could also detect changes at plasma Mbl2 levels in patients received fenofibrate treatment or in healthy subjects upon fasting. These findings suggest Mbl2 as a putative novel mediator of hepatic PPARα activity. Compared to other liver secreted proteins including Angptl4 and Fgf21, which are also expressed in other tissues than liver, Mbl2 exclusive expression in liver makes it an interesting tissue specific biomarker. Thus, measurement of Mbl2 plasma levels may be an indicator of hepatic PPARα activity as far as it is measured within the same individual before and after a challenge such as fenofibrate treatment. However, the Mbl2 gene harbors polymorphisms in its promoter region and it is yet not known if these polymorphisms could influence the PPARα binding properties. Despite the possibility of existing mutations, we observed that fenofibrate treatment increased Mbl2 plasma levels in all subjects irrespective of variant basal plasma Mbl2 levels. The question remains if individuals can be classified into different categories of PPARα responsiveness based on the magnitude of observed changes in plasma levels of specific PPARα target genes. We could not find a correlation between the relative increase in plasma levels of several PPARα secreted target genes upon fenofibrate treatment, including Mbl2, Angptl4 and Fgf21, suggesting that individuals cannot easily be classified based on PPARα responsiveness.

The human specific regulation of Mbl2 by PPARα can involve a number of different mechanisms. One is the loss of functional PPREs, which has been previously found for other human specific PPARα target genes including those encoding apolipoproteins [45]. In case of Mbl2, the functional PPREs that mediate regulation by PPARα remain to be elucidated, and

thus it is unclear whether the functional PPRE is conserved between species. Other possible mechanisms are the intrinsic properties of human PPARα protein, and the cellular context, including presence of specific co-activators.

Previously, many studies have been performed to identify genes regulated by PPARα. In general, these studies indicate that unlike many other nuclear receptors, PPARα governs the expression of a large set of genes, many of which are involved in fatty acid metabolism. A brief overview of some of the studies that applied microarray technology and that were aimed at identifying hepatic PPARα target genes clearly shows some major limitations and gaps in the analysis and findings which we tried to remedy in our research.

One of the first studies using microarray technology discovered that PPARα influences amino acid metabolism and urea synthesis. The integrated strategy was the activation of PPARα by synthetic agonist Wy14643 combined with using PPARα knockout mouse model [46]. In another study aiming at finding novel PPARα target genes, mice were treated with the PPARα agonists Wy14643 and fenofibrate for 2-3 days. The authors could confirm the previously reported PPARα target genes in ß-oxidation and lipid metabolism plus discovering a few novel PPARα regulated genes [47]. In another similar study, using cDNA arrays, changes in hepatic gene expression in mice exposed to Wy14643 for two weeks were measured. Besides finding increased expression of genes involved in lipid and glucose metabolism and genes associated with peroxisome biogenesis, a large number of genes were found to be repressed, which were not studied further by the authors [48]. In most of the studies evaluating the effect of PPARα agonists on liver gene expression profiles, a long term activation of PPARα is targeted and not always a knock out model is applied [49, 50]. As a consequence, it is unclear whether the observed changes in gene expression reflect direct gene regulation or are due to indirect mechanisms.

There are also examples of in vitro studies applying microarray technique for a large scale profiling of gene expression changes elicited by PPARα activation. In one study, mouse primary hepatocytes were exposed to multiple concentrations of several PPARα agonists for 24h hours followed by global genes expression profiling. The aim of the study was to understand the molecular mechanisms responsible for the pleiotropic effects of PPARα agonists. The authors found regulation of many genes in lipid metabolism plus a few genes involved in oxidative reactions [51]. In the only study aimed at comparing expressional responses to PPARα activation in human and rodents, primary human, rat and mouse hepatocytes were exposed to PPARα synthetic agonist for 72 hours and gene expression analysis performed using Affymetrix GeneChips. The authors categorized the differentially expressed genes into three main groups: fatty acid transport and metabolism, xenobiotic metabolism and cell pro-

liferation and death. They found genes in the peroxisomal pathway that were specifically regulated in mice, while specific target genes in the xenobiotic pathway were only regulated in human. Genes involved in fatty acid metabolism and transport were regulated across all the species [52].

Considering the importance of PPARα as a lipid sensor, which can become activated by dietary fatty acids [53-55], it is of great importance to study the effect of natural PPARα ligands on hepatic gene expression profile. Therefore, it was recently 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 [56]. The considerable large overlap between the genes regulated by fatty acids and by the PPARα agonist Wy14643 demonstrates the usefulness of transcriptomics studies using synthetic PPARα agonists to augment understanding of nutrient mediated gene regulation.

In the research presented in this thesis, we have mainly studied the PPARα mediated gene regulation by synthetic agonists, due to the lack of transcriptional changes in primary hepatocytes in response to fatty acids. The reason behind this lack of responsiveness is not clear, but several possible mechanisms can be proposed. One could be the low expression of intracellular fatty acid binding proteins, which have been proposed to transport fatty acids to the nucleus. We could detect a significant decline in Fabp1 and Fabp2 gene expression levels upon culturing hepatocytes. This was also the case for PPARα expression itself. Fabp1 has been shown to interact with PPARα [57] and therefore appears to be needed for shuttling of fatty acids to PPARα [58-60]. Fabp2 is also able to bind fatty acids [61-64] and may interact with PPARs. Accordingly, we tried to overcome these unfavorable changes by transfecting mouse primary hepatocytes with Fabp1, Fabp2, PPARα and PPARß DNA plasmids. Although we successfully overexpressed these genes, we could still not restore the in vivo observed effects of fatty acids on PPARα activation. It could be argued that the lack of response to fatty acids in hepatocytes may be related to the form in which fatty acids are delivered to the cells [65], the mechanisms of internalization, and presence of distinct fatty acid pools within hepatocytes [66, 67]. We also tried to augment the response of HepG2 cell line to fatty acids by using histone deacetylase inhibitors including butyrate [68, 69] and Trichostatin A [70, 71] in order to make the chromatin less dense and increase accessibility for transcription [72-74], but this methods also failed to cause PPARα activation by fatty acids.

Compared with other microarray studies published, the strength of the research approach presented in this thesis is multi-fold; first, activation of PPARα by specific agonist Wy14632 for both shorter and longer duration of exposure. Second, a systematic comprehensive approach involving whole mouse and human genome analysis combined with using proper pathway

analysis tools in order to visualize the changes at the pathway level. Third, application of knockout mouse models to find PPARα dependent regulations. Fourth, combination of physiological and pharmacological PPARα stimuli, and in vivo versus in vitro approaches.

Nevertheless, transcriptomics studies carry several limitations; one is that besides the ability to screen for the changes at the expression level of a very large number of genes and thus generating gene expression profile pictures, they do not provide information on the precise molecular mechanisms that underlie the observed regulations. They also do not provide us with information on protein-DNA interactions which can be assessed by other strategies such as ChIP-on-chip or ChIP-Seq techniques. Another critical issue is the statistical approach used to identify differences in gene expression. Statistical significance is not necessarily representing biological significance and often statistical criteria preclude the detection of small changes in gene expression as observed in nutrients mediated signaling or human studies. In such cases, applying a improper statistical tool can result in a major loss of information. The best approach to fully understand the functional relevance and mechanisms of PPAR dependent gene regulation is to combine transcriptomics methods with other functional genomics tools that cause alterations in the expression of potential molecular mediators, such as siRNA based gene silencing or knock-out technology.

Overall this thesis represents a good example of the combination of microarray technology with a knockout mouse model in order to characterize the functional role of a transcription factor in gene regulation via identifation of its target genes and pathways. This research has truly extended our understanding of PPARα-regulated genes and function in liver, and has specifically high-lightened a major role of PPARα in human hepatocytes. This research has also given birth to a possible biomarker of hepatic PPARα activity which is of great interest for upcoming studies. Considering the need for proper biomarkers in the field of nutrigenomics and beyond, further evaluation of Mbl2 as a biomarker is of huge importance. The identification of other novel putative PPARα target genes offers ample opportunities for continued research.

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Summary

Metabolic syndrome is defined by a number of metabolic disorders including visceral obesity, insulin resistance, hypertension and dyslipidemia and it is associated longitudinally with increased risk for cardiovascular disease and diabetes. The number of people with metabolic syndrome is increasing and the prevalence of type 2 diabetes shows an upward trend. Nutrigenomics can contribute to the prevention of numerous chronic diseases including the metabolics yndromebypr ovidinga s olidm echanisticf rameworkf ore videnceba sednut rition.
Nutrigenomics investigates the interaction between nutrients and genes at the molecular level by using genomics tools and is mainly focused on disease prevention rather than disease cures. Within the field of nutrigenomics, dietary nutrients and their metabolites are considered as signaling molecules that target cellular sensing systems. Members of the nuclear receptor superfamily play a major role in sensing nutrients and mediating their effects on gene expression. One important group of these nuclear receptors are PPARs, which encompass PPARα, PPARß/δ and PPARγ.

PPARα is a ligand activated transcription factor that plays a major role in nutrient homeostasis. At the functional level, PPARα is known as the master regulator of lipid metabolism in liver. Clinically, it serves as the molecular target of the fibrate class of drugs which lower fasting plasma triglycerides and raise plasma HDL levels and are thus prescribed for the treatment of dyslipidemia. Thus, there is large interest to identify PPARα novel controlled genes and processes.

The first aim of the research presented in this thesis was to better characterize and understand PPARα function in liver by identifying PPARα-regulated genes and metabolic pathways. With the help of independent microarray studies we generated a schematic overview of PPARα-regulated genes relevant to hepatic lipid metabolism, leading to the identification of a large number of PPARα target genes involved in different aspects of lipid metabolism. Furthermore, we identified novel PPARα target genes and characterized a major role of PPARα in lipogenesis.

Since the role of PPARα in liver has mostly been studied in mice, as a second aim we set out to perform a comprehensive comparative analysis of gene regulation by PPARα between mouse and human hepatocytes. We were able to find a large number of genes regulated by PPARα activation in human primary hepatocytes, identifying a major role for PPARα in human liver. While we found minor overlap at the individual gene level, PPARα mostly governed many overlapping gene ontology classes representing pathways of lipid metabolism. Most of the genes commonly regulated in mouse and human were involved in lipid

metabolism and many represented known PPARα targets. Accordingly, the role of PPARα as master regulator of hepatic lipid metabolism is well conserved between mouse and human. One of the strong points of this research was the use of primary human hepatocytes rather than the most widely used liver cell line, HepG2. As discussed in this thesis, the HepG2 cell line poorly reflects the established PPARα target genes and function, specifically in relation to lipid metabolism.

One of the additional aims of this research was to identify novel PPARα target genes and the comprehensive analysis of PPARα-regulated genes in human primary hepatocytes led to the characterization of a novel PPARα target gene, called mannose binding lectin (Mbl2), with human specific regulation. Regulation of Mbl2 by PPARα suggests that it may play a role in regulation of energy metabolism, although additional research is needed. We could also detect changes in plasma Mbl2 levels in subjects receiving fenofibrate treatment or upon fasting. These findings suggest Mbl2 as a potential circulating mediator of hepatic PPARα activity in human.

Finally, we found transcription factors Klf11 and Klf10 to be significantly upregulated by PPARα activation in both mouse and human hepatocytes, suggesting they may be novel PPARα target genes. We could further confirm their PPARα dependent induction in the liver of mice treated with PPARα agonist. Interestingly, our preliminary in vitro data suggest that Klf11 overexpression in primary hepatocytes can downregulate PPARα gene expression. We utilized the method of hydro-dynamic tail vein injection of naked plasmid to study the in vivo effects of Klf11 and Klf10 overexpression in liver. Although we could successfully induce hepatic Klf11 and Klf10 gene expression, we failed to reproduce the in vitro data. Overall, the data suggest interaction between PPARα, Klf11 and Klf10. Additional experiments need to be carried to investigate this interaction in more detail.

In this thesis, microarray technology and transcriptomics are applied to characterize the role of PPARα via identification of its target genes and pathways. This research has truly extended our understanding of PPARα-regulated genes and function in liver, and has specifically highlighted a major role of PPARα in human hepatocytes. This research has also given birth to a possible biomarker of hepatic PPARα activity which is of great interest for upcoming studies. The identification of novel putative PPARα target genes offers ample opportunities for continued research.

Samenvatting (Summary in Dutch)

Metabool syndroom wordt gedefinieerd door de aanwezigheid van een aantal metabole afwijkingen waaronder viscerale obesitas, insuline resistentie, hypertensie en dyslipidemie, en gaat gepaard met een verhoogd risico voor hart en vaatziekten en diabetes. Het aantal mensen met metabool syndroom is stijgende waardoor ook de prevalentie van type 2 diabetes een opwaartse tendens laat zien. Nutrigenomics kan bijdragen aan de preventie van talrijke chronische ziekten waaronder het metabool syndroom door mechanistische inzichten aan te reiken die de basis vormen voor zgn. evidence-based nutrition.

Binnen het gebied van nutrigenomics bestudeert men de interactie tussen voedingsstoffen en genen op moleculair niveau door gebruik te maken van genomics technieken. Het onderzoek richt zich vooral op de preventie van ziekten en beschouwt voedingsstoffen en daarvan afgeleide metabolieten als signaal stoffen die inwerken op cellulaire sensor systemen. Een belangrijk voorbeeld van zo'n sensor systeem vormen de nucleaire receptoren. Nucleaire receptoren, waaronder de zogenaamde PPARs, zijn als groep verantwoordelijk voor de regulatie van gentranscriptie door voedingsstoffen.

PPARα is een door ligand geactiveerde transcriptiefactor die een belangrijke rol speelt bij nutrient homeostase en vooral bekend staat als algemene regulator van de vetstofwisseling in de lever. De receptor fungeert als moleculaire target voor een specifieke groep medicijnen, de zogenaamde fibraten, die gebruikt worden bij de behandeling van dyslipidemie en het plasma triglyceriden en HDL gehalte respectievelijk verlagen en verhogen. Er is om die reden grote interesse om een beter beeld te krijgen van de door PPARα gereguleerde processen en genen.

Het eerste doel van het in dit proefschrift beschreven onderzoek was om de functie van PPARα in lever beter in kaart te brengen door het identificeren van door PPARα-gereguleerde genen en metabole paden. Door middel van diverse microarray studies is een uitgebreid schematisch overzicht geconstrueerd van door PPARα gereguleerde genen die betrokken zijn bij het vetmetabolisme. Daarnaast zijn nieuwe PPARα target gene geïdentificeerd en is een betrokkenheid van PPARα in lipogenese aangetoond.

Een tweede doel van het onderzoek was om een uitgebreide vergelijking te maken tussen levercellen van muis en mens met betrekking tot de effecten van PPARα op genexpressie. Hiervoor werkt gekozen omdat de kennis over PPARα vooral afkomstig is uit studies in muizen en er relatief weinig bekend is over de rol van PPARα in de mens. Een groot aantal genen werd door PPARα gereguleerd in primaire hepatocyten van de mens, waarbij er op

het niveau van individuele genen relatief weinig overlap was met door PPARα gereguleerde genen in primaire hepatocyten van de muis. Daarentegen was er relatief wel veel overlap op het niveau van gen ontologie klassen die specifieke paden in het vetmetabolisme vertegenwoordigen. Het merendeel van de genen die door PPARα werd gereguleerd in zowel muis en mens was betrokken bij het vetmetabolisme. De rol van PPARα als algemene regulator van het vetmetabolisme is aldus goed geconserveerd tussen muis en mens. In het onderzoek is gebruik gemaakt van primaire levercellen van de mens in plaats van de veel gebruikte HepG2 cellen. Zoals in dit proefschrift beschreven geven HepG2 een zeer beperkte weergave van de rol van PPARα in het vetmetabolisme.

Een verder doel van het proefschrift was het identificeren van nieuwe target genen van PPARα. Een uitgebreide analyse van door PPARα gereguleerde genen in primaire hepatocyten van de mens leidde tot de vondst van een nieuw humaan specifiek PPARα target gen met als naam mannose binding lectin (Mbl2). Regulatie van Mbl2 door PPARα impliceert een mogelijke rol in het vetmetabolisme en werd ondersteund door de effecten van fibraten en vasten op het plasma Mbl2 gehalte, al is verder onderzoek noodzakelijk. De bevindingen wijzen op een mogelijke rol van Mbl2 als circulerende effector van PPARα activiteit in de lever.

Naast Mbl2 werden ook de transcriptie factoren Klf10 en Klf11 geinduceerd door PPARα activatie in hepatocyten van zowel muis als mens, daarmee suggererend dat Klf10 en Klf11 mogelijke nieuwe target genen van PPARα zijn. Regulatie door PPARα in de lever kon worden bevestigd in muizen behandeld met PPARα agonist. Een opvallende bevinding was dat overexpressie van Klf11 in levercellen de expressie van PPARα onderdrukte. Met behulp van een speciale techniek waarbij plasmiden in de staart van de muis geïnjecteerd worden is geprobeerd deze resultaten te bevestigen. Alhoewel significante overexpressie van Klf10 en Klf11 bereikt werd, konden de in vitro data niet worden gereproduceerd. De data wijzen op een interactie tussen PPARα en Klf10 en KLF11 maar verder onderzoek is noodzakelijk om deze interactie beter uit te werken.

In dit proefschrift werden microarray en transcriptomics technieken toegepast om de rol van PPARα beter in kaart te brengen door middel van het identificeren van nieuwe target genen en pathways. Het onderzoek heeft geleid tot een beter inzicht in de functie van PPARα bij regulatie van genexpressie in de lever en heeft vooral het belang van PPARα in de lever bij de mens blootgelegd. Het onderzoek heeft ook een mogelijk nieuwe biomarker opgeleverd die interessant is voor toekomstige studies. Tevens geeft de identificatie van talloze nieuwe mogelijke PPARα target genen voldoende aanleiding voor verder onderzoek.

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Curriculum Vitae

Maryam Rakhshandehroo was born in April 7th, 1979 in Tehran, Iran. After completing high school in 1997, she started her Bachelor studies in the filed of Nutrition at Shahid Beheshti university of Medical Science and Health Services in Tehran, Iran. In the last year of her Bachelor studies, she worked as a trainee in a few hospitals, focusing on disease specific diets. In 2004, she moved to Netherlands to get a Master's degree in Nutrition and Health at Wageningen University. The MSc courses she took was a combination of clinical and molecular nutrition. For her Master's thesis, she chose health and disease specialization and her thesis subject was about Maternal lipid status and congenital heart defect which was in cooperation with Erasmus medical centre in Rotterdam. After finalizing her thesis she decided to do her internship in Nutrition, Metabolism and Genomics group (NMG) of Wageningen University, and to learn more about molecular nutrition. During her internship period she started with identifying target genes of PPARα in liver. In 2006 she began her PHD project within NMG of Wageningen University. This project was supervised by Prof. Dr. Michael Müller and Dr. Sander Kersten. The PHD research main goal was to characterize the role of transcription factor PPARα in mouse and human liver and the results are described in this thesis.

List of publications

Rakhshandehroo M, Hooiveld GJ, Müller M, Kersten S. Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. PLoS One. 2009 Aug 27;4(8):e6796.

Rakhshandehroo M, Sanderson LM, Matilainen M, Stienstra R, Carlberg C, de Groot PJ, Müller M, Kersten S. Comprehensive Analysis of PPARalpha-Dependent Regulation of Hepatic Lipid Metabolism by Expression Profiling. PPAR Res. 2007:26839.
-Rakhshandehroo and Sanderson are joint first authors.

Smedts HP, Rakhshandehroo M, Verkleij-Hagoort AC, de Vries JH, Ottenkamp J, Steegers EA, Steegers-Theunissen RP. Maternal intake of fat, riboflavin and nicotinamide and the risk of having offspring with congenital heart defects. Eur J Nutr. 2008 Oct;47(7):357-65.

Smedts HP, de Vries JH, Rakhshandehroo M, Wildhagen MF, Verkleij-Hagoort AC, Steegers EA, Steegers-Theunissen RP. High maternal vitamin E intake by diet or supplements is associated with congenital heart defects in the offspring. BJOG. 2009 Feb;116(3):416-23.

Rakhshandehroo M, Knoch B, Müller M, Kersten S (2010) Peroxisome Proliferator Activated Receptor alpha target genes. Manuscript in press.

Rakhshandehroo M, de Wit NJ, Bragt MC, Afman L, Haluzik M, Mensink RP, Müller M, Kersten S (2010) Mannose binding lectin is a circulating mediator of hepatic PPARα activity in human. Manuscript Submitted.

Van der Meer DL, Rakhshandehroo M, Ullah OM, de Groot PJ, de Vries SC, Müller M, Kersten S. Comparative analysis of PPARα induced gene expression in human primary hepatocytes and the human hepatoma cell line HepG2. Manuscript in preparation.

Rakhshandehroo M, Müller M, Kersten S. The Krüppel like factors KLF11 and KLF10 are putative novel PPARα target genes in liver with a potential metabolic role. Manuscript in preparation.

Overview of completed training activities

Discipline specific activities

Systems biology: Statistical analysis of omics data, Wageningen, 2006
NuGo week, Oslo, 2007
7th International Masterclass Nutrigenomics, Wageningen, 2007
International Conference on the Bioscience of the lipids, Maastricht, 2008
FEDERA Symposium on Obesity, Leiden, 2008
First Benelux nuclear receptor meeting, Utrecht, 2008
Centre for Integrative Genomics (CIG) Symposium, Lausanne, Switzerland, 2008
NWO Nutrition Meetings, Papendal/Deurne, 2006-2009
Netherlands Lipoprotein club, Leiden, 2008-2009
EASL-AASLD Monothematic Conference: Nuclear Receptors and liver disease, Austria, 2009
8th Masterclass Nutrigenomics, Wageningen, 2009
Summer school on Nuclear Receptor Signalling, Greece, 2009
Symposium Nuclear Receptors; 2nd Benelux Nuclear Receptor Meeting, Oegstgeest, 2009
The Marius Tausk Professorship Symposium in honour of Prof.dr.John Cidlowski, Oegstgeest, 2009
8th Dutch Endo-Neuro-Psycho Meeting, Doorwerth, 2009
EASL Monothematic conference on signalling in the liver, Amsterdam, 2010
World Pharma, Copenhagen, 2010
NuGo week, Glasgow, 2010

General Courses

International course on Laboratory Animal Science, Utrecht University, 2007
NuGO Introduction course, Wageningen, 2007
Networking workshop, TI Food and Nutrition, Wageningen, 2008
Career Orientation,Wageningen, 2009
Nutritional Sciences Forum, Arnhem, 2009

Optional Activities

Organization and participation in Journal club, Division of Human Nutrition, Wageningen University (every four weeks)
NMG group Journal club, Wageningen university (every two weeks)
NMG Scientific meetings, Wageningen university (every week)
Nutrigenomics Consortium (NGC) Scientific meetings, Utrecht (every two months)
PhD retreat, Wageningen University, 2007
Human Nutrition PhD Tour, Scandinavian countries, 2009

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